Food-borne viruses in Europe

Rapid detection of transnational foodborne viral infections and elucidation of transmission routes through molecular tracing and development of a common database.

Final report

June 2004
Food-borne viruses in Europe

Rapid detection of transnational foodborne viral infections and elucidation of transmission routes through molecular tracing and development of a common database.

Final report

June 2004

Quality of Life and Management of Living Resources

KEY ACTION 1: Food, Nutrition and Health.

1.2 Development of tests to detect and processes to eliminate infectious and toxic agents throughout the food chain, activities:
   1.2.1 Improved understanding and control of contamination conditions
   1.2.2 Rapid detection tests for pathogens, xenobiotics and hormones

Proposal full title:
Rapid detection of transnational foodborne viral infections and elucidation of transmission routes through molecular tracing and development of a common database.

Proposal acronym:
Foodborne viruses in Europe

Contract number: QLK1-1999-00594
Food-borne viruses in Europe
Foodborne viruses in Europe Network

Principal contract partners

**Netherlands**: National Institute for Public Health and The Environment, RIVM.
**Finland**: Helsinki University Central Hospital
**Denmark**: Statens Serum Institute
**Sweden**: Swedish Institute for Infectious Disease Control
**UK**: Health Protection Agency
**Germany**: Robert Koch-Institut Berlin
**Spain**: Instituto de Salud Carlos III, Universitat de Barcelona & Universitat de Valencia.
**France**: IFREMER & Université de Bourgogne, Dijon
**Italy**: Istituto Superiore di Sanità

Additional partners

**Hungary**: State Public Health Service, Pecs
**Slovenia**: University of Ljubljana
Bilthoven, June 2004

I am proud to present the final report of the ‘Food-borne viruses in Europe network’, a research project funded by the European Commission under the 5th framework program (contract QLK1-1999-00594). As you can see leafing through this document, the project has been quite successful, and has led to a stream of publications unraveling the importance of viruses as food-borne pathogens in Europe. The work is by no means completed, and we hope to continue our activities in the near future, following negotiations on new contracts.

A major conclusion for me, as coordinator of this network, is that the work was timely and that our ‘hunch’ was correct: the food- and waterborne mode of transmission has a clear impact on the epidemiology of enteric viruses in Europe. We found evidence of widespread, international dissemination of noroviruses via food, and have provided the building blocks for a surveillance network. In a pilot phase, we monitored over 1500 outbreaks, 10% of which were obviously related to food-borne transmission. In addition to that, there is a ripple effect that is difficult to quantify, because the noroviruses that we studied are very contagious, and secondary and tertiary waves of infection occur.

The central data collection was not the easiest part of the work, but has taken great steps forward. We have worked through the hurdles of trying to bring together epidemiological data and laboratory data from a highly diverse group of researchers, all with their personal and national preferences. Again, the system is by no means perfect, but does provide some experience that should help to build the networks that are envisioned in the plans for a European CDC.

Our academic interest has been triggered and satisfied by the molecular virological findings, which have shown that caliciviruses evolve rapidly by recombination and mutation (shift and drift), and that these changes may have a great impact on the epidemiology of these viruses and the burden of disease associated with them. An important observation was that contaminated foods might contain multiple lineages of norovirus. Infecting people with several related but distinct lineages of virus sets the stage for the generation of recombinants, which essentially are novel viruses. This added risk of the food-borne outbreaks is an important one, and should not be neglected, even if food-borne outbreaks are greatly outnumbered by person-to-person outbreaks.

Last but not least, we tried to tackle the issue of virus detection in foods. This has turned out to be the most challenging aspect of our work. Clearly, shellfish stand alone in the success of methods aimed at detecting viral contamination. Despite serious efforts, we still are nowhere near having reliable methods for virus detection in other matrices. In fact, one may have to resort to alternative approaches for dealing with virus contamination, such as the use of model viruses to study critical processing steps, and outbreak surveillance and data linking to detect common source outbreaks.
In this book, you will find the output of the consortium described in published and unpublished manuscripts. This is preceded by a ‘readers guide’ which serves as an executive summary of the approach and summarizes the main results, with references to the manuscripts for those readers who are interested in detailed descriptions of the work.

I hope you will find this overview as exciting as we do. We will continue our work and are entering a new contract with the European Commission to try to continue our work. Please do not hesitate to contact me if you have additional questions.

Marion Koopmans, co-ordinator

On behalf of the Foodborne Viruses in Europe Network
Diagnostic Laboratory for Infectious Diseases and Perinatal Screening

P.O. Box 1, 3720 BA Bilthoven, The Netherlands
(Antonie van Leeuwenhoeklaan 9)

tel: 31.30.274 39 45
fax: 31.30.274 44 18

Marion.koopmans@rivm.nl
Contents

1. Background 9
2. Aims and Objectives 9
3. Participants 12
4. Setting the stage: state of the art at the start of the project period in 2000. 13
5. Working towards harmonized surveillance 14
6. Diagnostic aspects of food-borne virus infections 15
7. Lessons from molecular virological analysis 17
8. Virus detection in food items: can it be achieved? 19
9. Early detection of common source outbreaks by networking 20
10. Results from enhanced surveillance 22
11. Conclusions and future research needs 25
12. Output from the project 27
Food-borne viruses in Europe
1. **Background**

The work presented in this book are the aggregated results of a three-year combined research and surveillance project, which was funded by the European Commission under the 5th framework program within DG Research. The EC provided funding for a network of 11 groups in 9 countries to work on rapid detection of transnational food-borne viral infections and elucidation of transmission routes through molecular tracing and development of a common database. The grant was provided under the program for Quality of Life and Management of Living Resources, KEY ACTION 1: Food, Nutrition and Health. Priority 1.2. Development of tests to detect and processes to eliminate infectious and toxic agents throughout the food chain, activities. In addition to the principal contract partners, groups in Slovenia and Hungary were actively involved (headed by Dr. Poljsak-Prijatelj, and Dr. Szücs, respectively).

2. **Aims and Objectives**

**Objectives and expected achievements:**

In this project proposal we set out to join forces in Europe to allow more rapid and internationally standardized assessment of the spread of food-borne viral pathogens. Mapping these pathways will allow identification of high risk foods or processing methods, as well as high risk import/transport routes, which subsequently can be targeted by prevention programs. In this project we will generate data which are essential for well-funded assessment of the risks associated with consumption of certain food items. This will be done by combining complementary expertise from the fields of diagnostic virology, molecular virology, epidemiology, food-borne infections, food-processing, and molecular biology.

**Overall objectives**

The project team has set the following overall objectives:

1. To study the importance of enteric viruses as causes of illness across Europe, with a special focus on multinational outbreaks of infection with Norwalk-like viruses and hepatitis A virus.
2. To develop novel, standardized, rapid methods for virus detection and typing to be used in all participating laboratories.
3. To establish the framework for a rapid, prepublication exchange of epidemiological, virological and molecular diagnostic data.
4. To determine which are the high-risk foods and major transmission routes of food-borne viral infections in the different countries and between countries.
5. To describe the pattern of diversity within and between countries, and identify potential pandemic strains at the onset.
6. To investigate the mechanisms of emergence of these strains, including the possibility of spill-over from animal reservoirs.

**Research objectives:**
The project has four lines of research each with objectives as listed:

1. **Objectives of molecular and diagnostic virology program**
   1.1. Compare existing methods for detection and genotyping of viruses using fecal specimens and vomit from humans.
   1.2. Select consensus assays (genomic regions) for use throughout the study and for third parties that are starting up work on enteric viruses.
   1.3. Develop one or more rapid, user-friendly tests for detection and typing of known food-borne pathogens, notably Norwalk-like viruses and hepatitis A virus, based on the consensus regions chosen sub 1.2.
   1.4. Validate the assay(s) using a panel of coded specimens containing food-borne viruses as well as other viruses and negative control specimens.
   1.5. Implement the assay(s) in diagnostic / research centers, which participate in the study.
   1.6. Do sequence analysis of newly identified or emerging genotypes, and add the appropriate diagnostic reagents (probes) to the assay(s) developed sub 1.3.
   1.7. Analyze sequences of emerging strains at the onset and after several rounds of replication in humans to determine the composition of the quasispecies cloud as an indicator of a recent bottleneeking event such as crossing of a species barrier.

2. **Database for rapid exchange**
   2.1. Agree on a minimum dataset to enter in the joint database.
   2.2. Contact Enternet to establish possible overlaps and joint activities
   2.3. Develop a limited-access reporting network which enables rapid, pre-publication exchange and phylogenetic analysis of sequence or genotyping information from virus strains implicated in outbreaks of food-borne illness, along with a minimal dataset of epidemiological data.
   2.4. Analyze the European dataset for patterns of distribution of virus genotypes across countries, for emergence of novel genetic variants, and for matching strains from animal and human samples.

3. **Objectives of the Epidemiology program**
   3.1. Perform a comparative analysis of the existing surveillance systems against more definitive epidemiological studies in the participating countries, establishing their sensitivities and shortcomings to allow better comparison of data from different countries. Estimate the number of affected persons, and the proportion of food-borne viral infections from these studies.
   3.2. Develop standard criteria (based e.g. on Kaplan) for identifying food-borne viral outbreaks, appropriate investigation guidelines and minimum datasets for the network for use in a 2-year enhanced surveillance project.
3.3. Link available molecular and epidemiological data and follow-up on outbreaks that are suspected of being multinational common-source outbreaks (based on comparison of outbreak strains through the network) to establish mechanisms of emergence of such epidemics.

3.4. Review evidence for different transmission pathways e.g. animal - human, multinational, identify important food types implicated.

3.5. Produce report of 2 years enhanced surveillance of human calicivirus across Europe, including transmission patterns, and the burden of illness, including food-borne.

3.6. Identify further important questions.

4. Objectives of the Food technology programme

4.1. Compare existing methods for extraction of viral nucleic acid from different foods (matrices) with special emphasis on methods that are aimed at concentration of intact viral particles as an indicator of viable virus.

4.2. Develop quantitative PCR-assays based on the recommendations developed sub 1.1.

4.3. Study the detection limit and sensitivity of virus detection (and typing) from food using artificially contaminated foods as well as implicated food items collected during confirmed viral outbreaks.

4.4. Establish which are the high risk foods for different viruses and in different countries

4.5. Determine the levels of contamination of the implicated food items to provide basic information for future risk assessment.

4.6. Identify multinational outbreaks of food-borne illness as soon as possible to alert other potential consumers.
3. Participants

Principal contract partners

Partner 1. Netherlands: Dr. M. Koopmans, Diagnostic Laboratory for Infectious Diseases and Perinatal Screening, National Institute for Public Health and The Environment, Antonie van Leeuwenhoeklaan 9, pobox 1, 3720BA Bilthoven

Partner 2. Finland: Dr. K-H von Bonsdorff, Helsinki University Central Hospital, Division of Virology, Haartmaninkatu 3, 00290 Helsinki

Partner 3. Denmark: Dr. B. Böttiger, Virus Diagnostics Laboratory, Statens Serum Institute, Department of Virology, Artillerivej 5, DK-2300, Copenhagen

Partner 4. Sweden: Dr. L. Svensson, Swedish Institute for Infectious Disease Control, Department of Virology, Karolinska Institute, SE171 82 Solna

Partner 5. UK: Dr. D. Brown, Virus Reference Laboratory, Central Public Health Laboratory, 61 Colindale Avenue, London, NW9 5EQ

Partner 6. Germany: Dr. E. Schreier, Robert Koch-Institut Berlin, Molecular Virology, 13353 Berlin

Partner 7. Spain:
7.1. Dr. Alicia Sanchez, Centro Nacional de Microbiologia, Instituto de Salud Carlos III, Virologia Immunologia Sanitarias, Madrid

7.2. Dr. A. Bosch, Departament de Microbiologia, School of Biology, Universitat de Barcelona, Diagonal 645, 08028 Barcelona; collaborating with Dr J Buesa, University of Valencia.

Partner 8. France:
8.1. Dr. F. LeGuyader, Microbiology Laboratory, IFREMER, Rue de L’ile D’Yeu, BP1049-4400, Nantes

8.2. Dr. Pierre Pothier, Laboratoire de Virologie, CHU du Bocage, 21034 Dijon CEDEX

Partner 9. Italy: Dr. L. Toti, Food Technology Department, Food Laboratory, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Roma

Additional partners

Partner 10: Hungary: Dr. G. Szucs Regional Laboratory of Virology, State Public Health Service, Szabadsag u. 7., Pecs, Hungary

Partner 11: Dr. M. Poljsak-Prijatelj, Medical Faculty, Institute of Microbiology and Immunology, Laboratory for Electron Microscopy, Zaloska 4, 1105 Ljubljana, Slovenia
4. Setting the stage: state of the art at the start of the project period in 2000

In order to provide a starting point for the studies, we reviewed available literature in Europe for studies investigating the role of viruses as causes of (food-borne) gastroenteritis (Koopmans et al., 2002a; Lopman et al., 2002a). Caliciviruses from two genera, the noroviruses (NoV) and sapoviruses (SaV), are a common cause of acute, nonbacterial gastroenteritis in humans. Although the first human calicivirus was discovered nearly 30 years ago, much of the epidemiological and biological character of these viruses is only now beginning to unfold. Investigation has been difficult due to a number of factors: the viruses cannot be amplified by in vitro cell culture or animal models and electron microscopy (EM; Figure 1) is often not sensitive enough to detect the viruses in stool samples. Recent advances in molecular diagnostic techniques and the advent of a baculovirus expression system have highlighted the clinical and public health importance of calicivirus in all age groups, their ability to cause infection via a number of transmission routes as well as their considerable genetic diversity. These characteristics, in conjunction with the inability of humans to develop long-term immunity make caliciviruses an important public health issue in Europe and worldwide. Both papers highlighted the need for a more structured approach to studying food-borne illness in Europe.

![Figure 1: Electron micrograph showing sapoviruses (left) and norovirus particles in stool samples from humans. Bar indicates 50 nm.](image)

5. Working towards harmonized surveillance

In order to obtain baseline data, we started by reviewing currently available laboratory capability across Europe, and a review of the existing surveillance systems and/or specialized data collections (Lopman et al., 2002b). Our aim for this effort was to study how data on possible viral gastroenteritis outbreaks from the different countries were collected, which case definitions were used, what minimal dataset was available, how the etiology of outbreaks was established, and how data were aggregated to a National dataset. All the countries had laboratories that can test for hepatitis A virus (HAV) antibody in human serum. Eight of the ten surveyed European countries maintained a national database of HAV cases. Food could be tested for the presence of HAV in Finland, Italy, Spain, France and Denmark. All surveyed countries had at least one laboratory that tests for NoV by reverse transcriptase-polymerase chain reaction and all also had the capability to use electron microscopy. Five countries maintained a national database of NoV cases and nine maintained a national database of NoV outbreaks. Almost all participant countries had laboratories that can test for NoV in food items including shellfish.

To gain understanding of surveillance and epidemiology of viral gastroenteritis outbreaks in Europe, we compiled data from 10 surveillance systems in the network. Established surveillance systems found NoV to be responsible for >85% (N =3,714) of all nonbacterial outbreaks of gastroenteritis reported from 1995 to 2000. However, the absolute number and population-based rates of viral gastroenteritis outbreaks differed markedly among European surveillance systems. A wide range of estimates of the importance of food-borne transmission was also found. We reviewed these differences to identify a minimum dataset that could be collected by all participants (Lopman et al., 2003a).


6. Diagnostic aspects of food-borne virus infections

In the survey mentioned in section 5 of this report, we assessed which laboratories had diagnostic tests for the two priority viral diseases available, i.e. NoV and HAV. All countries reported having at least one laboratory capable of testing for NoV and HAV in humans (Lopman et al., 2002b). Virus detection in shellfish was available in all but 2 countries. Detection in water and food animals was less commonly available across Europe. A subsequent activity was comparing the performance of the diagnostics used across the network by a collaborative evaluation using a wide range of NoV collected throughout Europe (Vinjé et al., 2003).

Conclusion was that sensitivity and specificity of the different molecular diagnostic tests, even though targeting the same region of the genome, varied greatly. However, in subsequent discussions, it became clear that attempts at standardization the approach are futile. Current practice remains that laboratories use their favorite RT-PCR tests, and that new starting laboratories tend to (re) develop them based on strains available locally. This remains to be a handicap when comparing data internationally. A way forward would be to start this process top down: international exchange of data should be done based on an agreed algorithm, whereas the throughput for routine diagnostic methods can be done with the locally favorite tests.

At the start of the project, limited sequence data were available for NoV. With the increasing number of outbreak strains that were analyzed through this collaborative research, the lack of standardization for diagnostic RT-PCR proved to have its positive spin-off: because overlapping genomic fragments were analyzed, we were able to aggregate a large sequence database around the primer binding sites of the assay which ranked as number one in the round robin exercise. Therefore, we were able to predict best primer fits based on the currently identified sequence diversity across Europe. This approach was used for design of an optimal PCR (Vennema et al., 2002).

With increased use of molecular detection methods, the risk of false positive test results through contamination of new tests with PCR products from previously tested samples increases. This risk is especially large when nested PCR assays are used, in which a double round of amplifications is performed. Therefore, steps taken to reduce the likelihood of contamination in these assay formats should be encouraged. Rule number one is based on Good Laboratory Practice specifically for molecular diagnostic labs, with RNA extraction, preparation of PCR mixtures and addition of RNA done in designated (preferably positive pressure) laboratories with dedicated equipment. In addition, modified procedures can be developed to decrease the risks of carry-over contamination as described by Höhne et al. (2003).


7. Lessons from molecular virological analysis

Molecular epidemiological studies are increasingly popular in the field of clinical calicivirus research. Based on an (arbitrary) genetic typing system, caliciviruses are divided in genera, genogroups and genotypes. Most illness in humans appears to be associated with NoV, although data on community-based studies are sparse. Therefore, the role of SaV, the second genus within the family Caliciviridae remains to be studied with similar approaches. The NoV genogroup II (GGII) viruses dominate in all parts of the world (Lopman et al., 2002b; Koopmans et al., 2002b). Several genotypes co-circulate, but the majority of infections is associated with only a few genotypes. The epidemiology of these genotypes is only beginning to be addressed. Future studies are needed in which systematic surveillance of outbreaks of gastroenteritis is done for prolonged periods of time, and with harmonised methods in order to find explanations for the apparent emergence of calicivirus variants in populations, the fluctuations in their presence, virulence differences and modes of transmission including the possibility of zoonotic transmission. This data was reviewed in reference 8, and the application of molecular typing resulted in the discoveries described in chapter 10.

Like NoV, HAV can be subtyped by using sequence analysis of selected genomic regions. A clear difference between HAV and NoV, however, is that HAV variability seems to be limited to genetic variability, and is not clearly translated into changes in the antigenic properties of the viral particles. Understanding the basis for this (limited) diversity will eventually increase our understanding of HAV evolution. This in turn is important for interpretation of data in HAV diversity assembled across the world. The studies by Sánchez et al. (2003 a and b) showed that- although seemingly restricted- HAV does accumulate mutations upon replication, similar to what has been described for other RNA viruses. The fact that these mutations are not translated into antigenic changes may be a factor reflecting adaptation of HAV to its human host.

Preliminary steps into studies of NoV evolution were taken by Nilsson et al. (2003) in their analysis of the viral changes during chronic infection of an immuno-suppressed heart transplant recipient. The observation that NoV may result in persistent infection and diarrhoea was novel and shows that additional studies are needed to map the burden of illness due to NoV. Sequence analysis of the viruses found showed that most changes occurred in a region of the viral surface, the so-called protruding domain. This was taken as suggestive evidence for a role in the immune response as a driving force for NoV evolution. It remains to be seen if this mechanism is reserved for chronically infected individuals and therefore exceptional.


8. Virus detection in food items: can it be achieved?

One of the work packages in this project was dedicated to developing methods for virus detection in high risk foods. Previously, methods for virus detection in shellfish have been developed with moderate success, although standardization is lacking, and full validation of these methods for food screening purposes needs to be done. From outbreak investigations, lettuce and fresh fruits such as berries have been implicated as the highest risk food items. Therefore, we focused on developing assays for screening such products. Different extraction methods were designed by the participating partners, and then compared during a workshop hosted by one of the institutes. Food samples were artificially contaminated with different enteric viruses, including an animal calicivirus that might serve as a model. The advantage was that this virus can be grown in cultured cells, and therefore can be used to assay loss of infectivity. The comparison showed promising results for some of the methods. Further work needs to be done to put these results into practical use, especially the application of food virology to samples that are naturally contaminated. In a theoretical approach we reviewed what might be contributing factors to emergence of food-borne viruses. We compared viral characteristics for the known high-risk food-borne viruses and reviewed the literature for data on virus survival and stability (Koopmans and Duizer, 2004). From this, we learnt that many of the commonly used food processing methods would not result in complete inactivation of viruses if they were present in the product prior to processing. Therefore, emphasis should be on primary prevention.

An example of source contamination, and the associated problems, came from the work involving a large oyster-related problem across Europe in 2000-2001, which we detected through the network. A difficulty was that multiple variants of NoV were detected in the food and in patients in outbreaks following consumption. A different sequence may therefore be found at first examination between cases and implicated food. Only after refined analysis the link can be made using molecular techniques. The study also showed that there are few studies that have looked at a broad range of inactivation procedures for NoV. This was addressed in a study by Duizer et al. (2004) for caliciviruses and by Groci et al (2002) for HAV. Both studies confirmed the high stability of these viruses under adverse conditions. Interestingly, inactivation kinetics were different for different food items.


9. Early detection of common source outbreaks by networking

An import aspect of studying diseases internationally is having a platform in place for exchange and sharing of data. In order to be able to study the objectives set out in section 2, we needed a system that potentially could serve as an early-warning system. Specifically, we set out to understand modes of transmission of virus lineages by combining molecular virological data with epidemiological data. The intent was that timely detection of a new variant would allow us to go back into the data from the outbreak investigation to help understand the relevance of these findings. Therefore, we developed two linked databases (Figures 2 and 3). One was used to aggregate epidemiological data that were entered in an outbreak investigation form via the internet. Access was password controlled. We made great efforts to design a user-friendly system. A minimum dataset was identified for collection during outbreaks of (viral) gastro-enteritis, and a web-based questionnaire was designed to collect this data [Koopmans et al., 2003]. Several fields in the epidemiological database can be searched to look for possible similarities in the descriptive epidemiology of the outbreaks that have been reported. The second database was collecting laboratory data, mostly sequence information on the viruses found in cases, food or environmental samples. This database can be searched by the participants to look for matching sequences in other countries. Our aim is to have links between the databases, so that possible indications of common source events can rapidly studied by comparing sequences or by comparing descriptive epidemiology.

Notes: CORPORATE NAME: European Consortium on Foodborne Viruses.
Food-borne viruses in Europe

Figure 2: Outline of the setup used by the Food-borne virus Network to identify potential common-source outbreaks.

Figure 3: Websites of the two linked central databases developed and used by the Food-borne virus project, with web-based outbreak reporting (top; http://www.eufoodbornevirologies.net) and sequence matching (bottom; https://hypocrates.rivm.nl/bnwww/).
10. Results from enhanced surveillance: enteric viruses as emerging disease

One of the aspects of the project was a 2-year enhanced surveillance, in which we worked with the predefined outbreak reporting form to aggregate data from outbreak surveillance systems in the different countries (Figure 4). During this period which ran from 11/2001 to 4/2003, a total of 1528 outbreaks were reported in the database. The rate of outbreaks reported per million of the population ranged considerably, from < 1 % in Italy and France to over 18 in Finland. At this stage, it remains to be seen if these differences are reflective of the functioning of the surveillance system or reflect true differences. An interesting difference was noted for the data from Sweden, which in a previous retrospective outbreak report had the highest rates of outbreaks per million. The partners from Sweden have indicated that this may be related to the dissemination of NoV detection methods to laboratories across the country, which results in reduced coverage of the lab-based surveillance system.

Figure 4: Rate of outbreaks per million inhabitants per country during the enhanced surveillance period.

Ninety-two percent of the outbreaks were associated with NoV. Other enteric viruses detected were astrovirus, HAV, rotavirus, and SaV. Overall, 10% of outbreaks were reported to be food- or waterborne. These outbreaks were significantly larger than outbreaks attributed to person-to-person transmission. A vehicle was reported in 37% of outbreaks only, a broad range of food items was found to be involved.

In total, 69% of the NoV outbreaks were associated with Genogroup II.4 viruses (GGII4). The proportion of GGII.4 was highest in the winter of 2001/2002, when a novel variant GGII4 was detected across Europe. The GGII4 viruses were significantly more frequently
detected in outbreaks labeled as person-to-person outbreaks and in healthcare settings (Fisher’s exact, P < 0.01). In multivariate case-case analysis, in which GGII.4 outbreaks were compared with non-GGII.4 outbreaks this association was confirmed. This was not entirely due to the unusual variant GGII4 that emerged in 2002, because seasonality was independently associated, with an increased odds ratio for GGII.4 outbreaks in the last winter episode of the enhanced surveillance.

**Outbreaks with possible international consequences**

During the study period, two intriguing observations were made. In the winter of 2000/2001, a novel variant NoV, designated GGIIb was identified which was observed first in August 2000 in association with a large drinking-water related outbreak of gastro-enteritis in France, and in the subsequent winter season in 6 other countries in Europe. The initial outbreaks attributed to infection with the IIb variant in 3 countries were traced back to oysters that had been imported from France and met all microbiological quality assurance parameters. The GGIIb variant caused between 7 and 71% of all recognised NoV outbreaks detected during that season in different countries. Further characterisation of the new lineage of viruses showed that this is a particularly promiscuous lineage, and that the novel variant parent virus has successfully recombined with at least 4 existing capsids. This example clearly demonstrates that food-borne and waterborne transmission may have serious impact on the spread – and possibly even the generation – of emerging viral infections across countries. It also illustrates the need for virus-specific quality control criteria for food.

The second winter, 2001/2002 was also remarkable in that we were alerted to unusual outbreak activity. Throughout Europe an increase in NoV outbreaks was noted. Sequence data of the detected viruses showed that the increase coincided with the emergence of a new GGII.4 strain. The new virus had a distinct sequence in the gene coding for the RNA dependent RNA polymerase, which was not seen before January 2002, emerged in January and was the dominant cause of NoV outbreaks by mid summer in all but one of the participating countries in the FBVE network (Lopman et al., 2004). Further research is needed to understand if and how the observed changes translate to distinct biological properties such as infectivity, antigenicity or (environmental) stability of the new strain. The example does show that the network approach used is suited to detect and investigate the emergence of a new strain. Interestingly, the strain correlated to the increase in NoV outbreaks in 2002 is related to the strain that emerged in the winter of 1995-1996, and caused a global epidemic of NoV outbreaks. This suggests that viruses of the GGII.4 genotype have properties facilitating transmission, and thereby have the propensity to cause epidemics.

The two examples given, recombination and genetic drift, are examples of the two ways by which a single stranded RNA virus with an unsegmented genome can evolve. This property is an important explanation for the observation that many of the emerging pathogens are RNA viruses. In addition to this, several local outbreaks were analyzed and reported (section 12).
11. Conclusions and future Research needs

The work described in this overview shows that food- and waterborne transmission play an important role in the spread of norovirus (NoV, previously named Norwalk-like viruses) throughout Europe. NoV are ubiquitous, highly contagious, cause large international outbreaks of gastro-enteritis. This is of concern: current quality control for food and water in Europe measures bacterial contamination, and does not monitor viral contamination. Therefore, food can pass microbiological quality control, but still contain viruses. Matched with the virtual absence of a surveillance system for detection of common-source outbreaks of illness due to enteric viruses, this highlights a weak spot in European infectious disease control. The NoV in fact serve as sentinels: when present, common-source outbreaks will relatively easily be detected due to the high attack rate and short incubation period. More insidious, however, are the enteroviruses and fecally-transmitted hepatitis viruses with a high proportion of asymptomatic infection after an incubation period of up to 2 months. These viruses cause illnesses like hepatitis (hepatitis A and E viruses) and infections of the central nervous system (enteroviruses). Signaling common source outbreaks with these viruses is virtually impossible without a strong (molecular) laboratory component to underpin the epidemiological investigations, aided by international exchange of laboratory data. This should be developed to better be prepared at the European level.

The data sharing across Europe has revealed numerous examples of seemingly localized viral disease outbreaks, which may impact on the epidemiology of these viruses in other countries. Outbreaks of water-borne or food-borne diseases in popular tourist resorts have been detected. Often, the true extend of such outbreaks remains a mystery, but is likely to be far greater than what has been detected through routine surveillance. We have seen examples of virus shift and drift, both mechanisms that are known to enhance the diversity of viruses. These examples are worrisome, in that the conditions are favorable for their generation: some of the foods that were examined in our project were contaminated with multiple variants. The experimental design to create a novel virus would be to simultaneously infect a susceptible host with 2 or more related viruses. While we can not exactly pinpoint the moment of generation of the variants that we detected, it is a very likely hypothesis that they are a result of food-borne or water-borne exposure.

A way out of this dilemma might be to develop guidelines for microbial food safety that include viral contaminants. Sofar, that is not the case, and there is increasing evidence that viruses slip through the mazes of the control web for food quality. That implies that – in the HACCP analysis, knowledge on behavior of viruses needs to be included, that food handlers should specifically be educated to reduce the likelihood of contamination for all pathogens, and that guidelines should be drafted that reliably indicate the presence of absence of viruses. That is easier said than done: the screening of food samples – with the exception of oysters – is in its infancy stages. Given the nature of viral contamination (very low dose, periodic) it will be difficult if not impossible to develop methods that are both reliable AND practical. Therefore, bridging the gap between food quality control and public
health surveillance may be needed, and research to provide some data on the predictive value of outbreak investigations for detection of food contamination. This should include the role of virus typing and data sharing. Data are also needed on effective prevention strategies for food-borne contamination, and – as part of the missing data – stability of different food-borne viruses in different environmental circumstances, products, and processing methods.
12. Output from the project

In addition to the above results and publications, participants have described national aspects of the work funded through this contract in various types of publications. In the last section of this book, the publications are listed.


Notes: CORPORATE NAME: European Consortium on Foodborne Viruses.


Food-borne viruses in Europe
Foodborne viruses

Marion Koopmans a,*, Carl-Henrik von Bonsdorff b, Jan Vinje a, Dario de Medici c, Steve Monroe d

a National Institute of Public Health and the Environment, Research Laboratory for Infectious Diseases, Antonie van Leeuwenhoeklaan 9, 3720 BA Bilthoven, The Netherlands
b Helsinki University Central Hospital, Division of Virology, Helsinki, Finland
c Food Technology Department, Istituto Superiore di Sanità, Rome, Italy
d Viral Gastroenteritis Unit, Respiratory and Enteric Virus Branch, Division of Viral and Rickettsial Diseases, Centers for Disease Control, Atlanta, GA, USA

Received 23 November 2001; received in revised form 18 March 2002; accepted 22 March 2002
First published online 24 April 2002

Abstract

Foodborne and waterborne viral infections are increasingly recognized as causes of illness in humans. This increase is partly explained by changes in food processing and consumption patterns that lead to the worldwide availability of high-risk food. As a result, vast outbreaks may occur due to contamination of food by a single foodhandler or at a single source. Although there are numerous fecal- or orally transmitted viruses, most reports of foodborne transmission describe infections with Norwalk-like caliciviruses (NLV) and hepatitis A virus (HAV), suggesting that these viruses are associated with the greatest risk of foodborne transmission. NLV and HAV can be transmitted from person to person, or indirectly via food, water, or fomites contaminated with virus-containing feces or vomit. People can be infected without showing symptoms. The high frequency of secondary cases of NLV illness and – to a lesser extent – of hepatitis A following a foodborne outbreak results in amplification of the problem. The burden of illness is highest in the elderly, and therefore is likely to increase due to the aging population. For HAV, the burden of illness may increase following hygienic control measures, due to a decreasing population of naturally immune individuals and a concurrent increase in the population at risk. Recent advances in the research of NLV and HAV have led to the development of molecular methods which can be used for molecular tracing of virus strains. These methods can be and have been used for the detection of common source outbreaks. While traditionally certain foods have been implicated in virus outbreaks, it is clear that almost any food item can be involved, provided it has been handled by an infected person. There are no established methods for detection of viruses in foods other than shellfish. Little information is available on disinfection and preventive measures specifically for these viruses. Studies addressing this issue are hampered by the lack of culture systems. As currently available routine monitoring systems exclusively focus on bacterial pathogens, efforts should be made to combine epidemiological and virological information for a combined laboratory-based rapid detection system for foodborne viruses. With better surveillance, including typing information, outbreaks of foodborne infections could be reported faster to prevent further spread. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Norwalk-like virus; Foodborne virus

Contents

1. General introduction ........................................................................................................... 188
   1.1. Introduction .................................................................................................................. 188
   1.2. Cost of illness ............................................................................................................... 188
2. Food- and waterborne gastroenteritis with a focus on caliciviruses .................................. 189
   2.1. Introduction .................................................................................................................. 189
   2.2. Background ................................................................................................................... 190

* Corresponding author. Tel.: +31 (30) 2742391; Fax: +31 (30) 2744449. E-mail address: marion.koopmans@rivm.nl (M. Koopmans).

1 A concise version of this paper was discussed by the Codex Alimentarius, committee on food hygiene, 1999. FAO/WHO document CX/FH 99/11, Rome.
1. General introduction

1.1. Introduction

The importance of foodborne transmission of viruses is increasingly recognized [1], and the World Health Organization has signaled an upward trend in their incidence [2]. It is also understood that the burden of infection is grossly underestimated by routine surveillance [3,4]. The aging population (with increasing numbers of people at risk for complications of enteric infections) and the globalization of infectious diseases due to rapid international travel and (food) trade add to the notion that the burden of illness is likely to increase in the years to come [5]. Vast outbreaks may occur due to contamination of food by a single foodhandler or at a single source, as has been documented on several occasions.

Numerous viruses can be found in the human intestinal tract (Table 1). The food- and waterborne viruses can be divided into three disease categories:

1. viruses that cause gastroenteritis (e.g. astrovirus, rotavirus, the enteric adenoviruses, and the two genera of enteric caliciviruses, i.e. the small round structured viruses or ‘Norwalk-like viruses’ (NLV), and typical caliciviruses or ‘Sapporo-like viruses’ (SLV));
2. fecal–orally transmitted hepatitis viruses: hepatitis A virus (HAV), hepatitis E virus (HEV);
3. viruses which cause other illness, e.g. enteroviruses.

In addition, several viruses are listed that also replicate in the intestinal tract, but are not implicated in foodborne transmission, or whose role is unknown.

Viruses, unlike bacteria, are strict intracellular parasites and cannot replicate in food or water. Therefore, viral contamination of food will not increase during processing, and may actually decrease. This implies that viral infection via contaminated food depends on viral stability, amounts of virus shed by an infected individual, method of processing of food or water, dose needed to produce infection, and susceptibility of the host. Most food- or waterborne viruses are non-enveloped and are relatively resistant to heat, disinfection and pH changes. Problems in the detection of viral contamination of food or water are that generally the contaminated products will look, smell, and taste normal, and that (molecular) diagnostic methods for most of these viruses are not routinely available in food microbiology laboratories. In this paper, the major viral causes of foodborne infections will be reviewed. We have focussed on those viruses that are most commonly transmitted by food, namely NLV and HAV.

1.2. Cost of illness

The cost of illness due to viral foodborne infections is not known exactly, but it is likely to be high. In the USA,
Mead et al. [6] recently estimated that foodborne diseases cause approximately 79 million illnesses, 325,000 hospitalizations, and 5,000 deaths each year. For just the few foodborne pathogens for which cost estimates have been made, medical charges and lost productivity already cost society US$ 5–6 billion annually in the USA [7]. The total costs of salmonellosis are estimated to be US$ 1.2–1.5 billion.

Although viral infections until recently were not commonly diagnosed, it is becoming clear from epidemiological studies that caliciviruses alone may be as frequent causes of foodborne illness as Salmonella [6,8]. Costs of illness can be high due to their frequent occurrence and high transmissibility [9,10]. In addition, there are studies that suggest that viral enteric infections cause deaths in the elderly, deaths that are largely preventable [8,11–13].

In the USA, some 84,000 cases of hepatitis A are reported annually, of which an estimated 5% are foodborne or waterborne [6]. Common source foodborne hepatitis A outbreaks attract a great deal of public attention and concern, and require considerable public health control efforts. The estimated total cost of a single common source outbreak involving 43 persons, associated with an HAV-infected foodhandler, was approximately US$ 800,000 (Centers for Disease Control and Prevention (CDC), unpublished). Outbreaks associated with contaminated foodstuffs have resulted in nationwide recalls.

2. Food- and waterborne gastroenteritis with a focus on caliciviruses

2.1. Introduction

Epidemic and sporadic gastroenteritis is an important public health problem in both developed and developing countries [14,15]. In the last 27 years, several viruses have been identified as etiological agents of viral gastroenteritis in humans [16]. In most studies of food- and waterborne viruses, samples have been screened for viruses by tissue culture isolation techniques or by electron microscopy (EM). Some enteric viruses, however, cannot be grown in tissue culture, and EM is rather insensitive with a detection limit of around 10^5–10^6 particles per ml of stool suspension. Broadly reactive and user-friendly diagnostic tests, such as enzyme-linked immunosorbent assays (ELISA), have routinely been used for group A rotavirus and adenovirus in clinical specimens only. Recently, ELISA-based assays have been developed for detection of astroviruses and NLV [17–19], but the latter lack the broadness that is required for generic detection. No similar assays exist for testing food samples. As a result of these limitations, foodborne viral gastroenteritis is usually not diagnosed.

In the absence of virus detection assays, a tentative diagnosis of viral gastroenteritis can be made based on epidemiological criteria described by Kaplan et al. [20]. Characteristic features are: acute onset after a 24–36-h incubation period, vomiting and/or diarrhea lasting a few days, a high attack rate (average 45%), and a high number of secondary cases [20,21]. Using this definition, an estimated 32–42% of foodborne enteric infections in the USA are caused by viruses. Outbreaks of gastroenteritis may be caused by rotaviruses, astroviruses, adenoviruses, and the human enteric caliciviruses. The human caliciviruses are assigned to two genera, which are currently described as ‘Norwalk-like viruses’ (NLV), also known as small round structured viruses, and ‘Sapporo-like viruses’ (SLV), also known as typical caliciviruses [22–25]. The NLV cause illness in people of all age groups, whereas the SLV predominantly cause illness in children [26].

The relative importance of the different viruses as causes of food- and waterborne infections is not exactly known, but clearly NLV are the main cause of viral outbreaks [5,20,21,27], and their incidence reportedly has been in-

---

Table 1

<table>
<thead>
<tr>
<th>Gastroenteritis</th>
<th>Possibly gastroenteritis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotavirus group A, B, C</td>
<td>Picobirnavirus</td>
</tr>
<tr>
<td>Adenovirus types 40, 41</td>
<td>Torovirus</td>
</tr>
<tr>
<td>Astrovirus serotypes 1–8</td>
<td>Coronaviruses</td>
</tr>
<tr>
<td>Norwalk-like caliciviruses</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>Sapporo-like caliciviruses</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td></td>
<td>Aichivirus</td>
</tr>
<tr>
<td></td>
<td>Parvo-like viruses, Small round featureless viruses</td>
</tr>
</tbody>
</table>

Hepatitis

<table>
<thead>
<tr>
<th>Hepatitis A virus</th>
<th>Enteroviruses:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatitis E virus</td>
<td>- polio 1–3</td>
</tr>
<tr>
<td></td>
<td>- Coxsackie A 1–22, 24</td>
</tr>
<tr>
<td></td>
<td>- Coxsackie B 1–6</td>
</tr>
<tr>
<td></td>
<td>- echo 1–9, 11–27, 29–34</td>
</tr>
<tr>
<td></td>
<td>- entero 68–71</td>
</tr>
<tr>
<td></td>
<td>- Aichi virus</td>
</tr>
</tbody>
</table>

The name Aichivirus has been given to (different) viruses in the calicivirus family and in the picornavirus family.
2.2. Background

Epidemic non-bacterial gastroenteritis or ‘winter vomiting disease’ was described as early as 1929 but the numerous attempts to propagate the presumed viral etiologic agent in vitro have met with little success [29]. A major breakthrough was the discovery of the Norwalk virus (NV) using immune EM (IEM) in fecal samples collected during an outbreak of gastroenteritis, which occurred in 1968 in an elementary school in Norwalk, OH, USA [30]. These viruses were non-enveloped and the particles had a ‘fuzzy’ amorphous appearance and measured 34–38 nm in diameter. Volunteer studies established NV as an enteric pathogen and, except for the unsuccessful cultivation of the virus, fulfilled Koch’s postulates [31,32]. Since then, additional studies have been carried out using fecal samples from other outbreaks of gastroenteritis, containing particles which were morphologically indistinguishable from NV [33,34]. These viruses have often been named after the geographic setting in which the outbreak occurred, for example, Hawaii virus, Snow Mountain virus, Montgomery county virus, Toronto virus. This practice became a routine procedure and persists to the present day.

In 1976, viruses with the typical calicivirus morphology were observed in the stools of infants suffering from diarrhea [35,36]. Typical calicivirus particles measure 30–34 nm in diameter; they can be identified by their characteristic cup-shaped depressions. Astroviruses may have the five/six-pointed surface star on up to 20% of the particles, which makes it possible to distinguish them from typical caliciviruses [36]. However, because these viruses were detected as frequently in asymptomatic as in sick children, no clinical significance could be attached to this finding. The first convincing association with disease came from a study of ‘winter vomiting disease’ which occurred in a school in London in January 1978 [37], and since then, typical caliciviruses have been linked with cases of mild diarrhea in both infants and children and shown to induce illness in volunteers [38].

2.3. The viruses

Human caliciviruses are small, non-enveloped spherical viruses, measuring between 28 and 35 nm in size that contain a single-stranded RNA genome of 7.3–7.6 kb. The genome is of positive polarity, and contains coding information for a set of non-structural proteins, located at the 3′-end of the genome, and one major structural protein at the 3′-end [22]. The distinguishing feature between the genome organization of NLV and SLV is the arrangement of the open reading frames (ORFs). In both genera, ORF1 encodes a large polyprotein (1789 amino acids for NV) with conserved regions of amino acid similarity with the picornavirus 2C helicase, 3C protease, and 3D polymerase [22,24]. For NLV, the ORF2 region encodes the single major capsid protein (≈56 kDa) [24]. A third ORF encodes a minor structural protein with a predicted molecular mass of 22.5 kDa [39]. In SLV, the region encoding the capsid protein (60 kDa for the prototype of this group, Manchester virus) is found in the same reading frame as ORF1, and is contiguous with the non-structural proteins. Together, the non-structural genes and the major capsid gene form one long polyprotein, which occupies over 90% of the total genome [25]. Similar to NLV, SLV possess a small ORF at the 3′-end of the genome encoding a protein of 17.5 kDa.

2.4. Clinical symptoms

Following a 1–3-day incubation period, infected persons may develop (low-grade) fever and vomiting, diarrhea, and headache as prominent symptoms. The illness generally is considered mild and self-limiting, with symptoms lasting 2–3 days [20,26]. Data from a recently completed community-based cohort study in The Netherlands were surprising in that 20% of NLV-infected persons reported symptoms for more than 2 weeks, suggesting that NLV infections may be less innocuous (Rockx et al., in preparation). In adults, projectile vomiting occurs frequently. Sometimes parenteral fluid therapy or even hospitalization is required, with up to 12% of cases hospitalized in a recent outbreak in military recruits [27,33,40,41]. Deaths associated with NLV outbreaks have been reported, but the etiologic association needs to be confirmed [8,12]. The average attack rate is high (typically 45% or more) [10]. Virus is shed via stools and vomit, starting during the incubation period, and lasting up to 10 days, and possibly longer [20,26,42,43]. NLV infections are highly contagious, resulting in a high rate of transmission to contacts. Mixtures of symptoms may occur, since contaminated foods may contain multiple agents [44].

2.5. Pathogenesis

Little is known about the mechanisms by which NLV cause diarrhea. In duodenal biopsies taken from infected volunteers, lesions were seen in the intestinal epithelium at 1 day post infection with the Norwalk virus or Hawaii virus as inoculum. The changes were villous broadening, abnormal epithelial cells, loss of and an inflammatory response in the lamina propria with infiltration of polymorphonuclear leukocytes and lymphocytes. At 5–6 days after ingestion villous shortening and crypt hypertrophy were observed. D-Xylose absorption was significantly reduced.
2.6. Diagnosis in humans

Despite numerous attempts by several groups of investigators, NLV have never been isolated in cell or tissue culture, and diagnosis has never been made historically by visualization of virus particles by EM [30,47]. However, EM is a relatively insensitive technique, requiring the presence of a minimum of around $10^5-10^6$ particles per ml of stool sample, and – unlike some other enteric viruses – NLV are not shed to very high maximum titers. This may not be a problem in outbreak investigations, when similar results may be obtained when stool samples have been collected promptly [48]. For community-based studies, however, this was an impediment until the complete genome of the NLV prototype, the Norwalk virus, was sequenced from cDNA clones derived from RNA that had been extracted from a bulk stool specimen [23,24]. From early work with IEM, and later sequence analysis of genomes of different NLV strains, it became evident that the NLV are in fact an antigenically and genetically diverse group of viruses [49–53]. At present, genome-based detection methods are available, in which fragments of the viral RNA are amplified directly from stool samples by reverse-transcriptase polymerase chain reaction (RT-PCR) ([54,55], reviewed in [47]). Initial studies using these methods to detect viral RNA in outbreak specimens confirmed the unusual level of divergence, even when a highly conserved region of the viral genome was selected as a target for the RT-PCR [51,56–58]. Since then, second generation assays have been developed, which have been optimized for detection of a broad range of NLV, by targeting conserved motifs in the non-structural protein genes [9,57,59,60]. Following detection of NLV by RT-PCR, the PCR products can be characterized further by sequencing (described in Section 2.9).

Although NLV cannot be grown in cell culture, efforts have been made to develop antigen-based detection methods. For this purpose, recombinant NLV capsids have been developed for use as control antigens [18,19,60–65]. However, the current problem is that (hyper)immune responses are predominantly type-specific, and that assays based on these reagents are narrow in their applicability [19,60,65]. NLV are a diverse group of viruses, and can be divided into two, possibly three broad genogroups, based on antigenic and genetic criteria (see Section 2.9). Recently, a NV-specific monoclonal antibody was characterized with reactivity to strains from four out of five other variants within genogroup I NLV that were tested [66]. Similarly, for genogroup II NLV, a common epitope has been identified [67]. When tested in an ELISA, this monoclonal appeared to detect GGI viruses as well, suggesting that it detects a genus-specific epitope. These monoclonal antibodies offer the first hope for development of a more broadly reactive detection assay. For a complete overview of NLV diagnostics see Atmar and Estes [47].

2.7. Epidemiology

Following the development of molecular detection methods, it has become clear that NLV infections are among the most important causes of gastroenteritis in adults and often occur as outbreaks which may be foodborne. However, an important message is also that the estimate of the proportion of foodborne NLV outbreaks varies greatly from one country to the other, due to differences in case definition, surveillance systems, and methods used. In The Netherlands, approximately 80% of outbreaks of gastroenteritis that were reported in the past seven years to RIVM through health services NLV [9,10,68,69]. More than half of these outbreaks occurred in nursing homes [10,68]. The proportion of foodborne outbreaks was 14% in 1994–2000, with 83% of these attributed to NLV. This clearly is an underestimate as foodborne outbreaks are usually reported through the regional food inspection services, rather than municipal health services. In a survey of all outbreaks of infectious intestinal disease in England and Wales between 1992 and 1994, 27% outbreaks were caused by NLV (for comparison: 32% of the outbreaks were due to Salmonella spp.) [8]. NLV were the cause of 6% of foodborne outbreaks. Since outbreak specimens were mostly examined by EM, the actual number of NLV outbreaks may be higher [8,19]. In the US, 86 of 90 (96%) outbreaks of non-bacterial acute gastroenteritis reported to CDC between January 1997 and June 1998 were caused by NLV infection. Of those outbreaks for which a mode of transmission was reported, 24 of 51 (47%) were considered foodborne. Several large outbreaks with a serious impact on troops on military aircraft carriers have been described [70]. Nosocomial outbreaks are common [10,71,72]. In Finland, hospital outbreaks (mostly on geriatric wards) are almost exclusively caused by NLV, but there is serious underreporting. Also in Finland, 56% of the epidemics reported as foodborne, from which stool samples (and foodstuffs, in some instances) were submitted for virological screening, were NLV-positive [73]. Of water epidemics 12/15 have been NLV-positive. Since 1998 15 berry-related epidemics have been identified [67]. When tested in an ELISA, this monoclonal appeared to detect GGI viruses as well, suggesting that it detects a genus-specific epitope. These monoclonal antibodies offer the first hope for development of a more broadly reactive detection assay. For a complete overview of NLV diagnostics see Atmar and Estes [47].
illustrates that contamination of these foods was not linked to a single common source (Fig. 1). In addition to outbreaks, recent publications suggest that caliciviruses are among the most common causes of sporadic gastroenteritis [3,4,68,75,76]. In The Netherlands, a set of population-based case-control studies showed that overall the incidence of gastroenteritis was quite high, at 280 cases/1000 persons per year for community cases of gastroenteritis, of which 8 cases/1000 persons per year seek treatment by a physician (1:35) [3,77,78]. Five percent of the patients who visit their physician for gastroenteritis were infected with NLV (compared with 4% for Salmonella), as well as 17% of persons in a sentinel population who developed diarrhea during the course of a 1-year cohort study [3,68,79,80]. People from all age groups were affected, with a slightly higher incidence in very young children. Similar studies in the UK suggested a slightly lower incidence of community cases of gastroenteritis (190 cases/1000 persons per year) but with a higher rate of referral to the physician [4]. Again, NLV were surprisingly common causes of illness in people of all age groups. The NLV are the second most common cause of gastroenteritis in young children [3,75,81,82]. The course of illness appears to be somewhat milder than rotavirus gastroenteritis [75]. This may explain the lower percentage of hospitalized children with NLV that has been reported [83,84]. Asymptomatic infections are common [3,82].

2.8. Risk groups

Outbreaks of NLV gastroenteritis (not only foodborne) are common in institutions such as nursing homes and hospitals. The attack rates typically are high (40–50% on average, but up to 100%) in both residents and personnel of such institutions, which in turn leads to major understaffing problems during outbreaks [10,85]. Sporadic cases of viral gastroenteritis also occur frequently in these settings. The risk factors for these infections are currently under investigation in the UK and in The Netherlands.

According to Gerba et al. [86] the groups of individuals who would be at the greatest risk of serious illness and mortality from water- and foodborne enteric microorganisms include young children, the elderly, pregnant women, and the immunocompromised. This segment of the population currently represents almost 20% of the population (in the USA) and is expected to increase significantly in the years to come, due to increases in life-span and the number of immunocompromised individuals. Worldwide, diarrheal diseases account for millions of deaths annually, mostly in developing countries [15]. In developed countries, mortality due to diarrhea is low, but does occur in young children [14,87,88] and in the elderly (where >50% of all mortality occurs [11,13,86]). While specific mortality data on NLV are not available, given the high incidence of calicivirus infections in the elderly, it is likely that deaths resulting from calicivirus infection do occur [8,12].

2.9. Molecular epidemiology

The early studies demonstrating the great variability of NLV soon led to the notion that it was important to be able to distinguish between strains in order to better understand their epidemiology. Typically, variant viruses would be characterized by neutralization assays using hyperimmune sera or panels of monoclonal antibodies in a tissue culture infectivity assay. However, because no one has succeeded in culturing these viruses in vitro, their antigenic relationships have been evaluated primarily by cross-challenge studies and IEM or solid phase IEM with viruses purified from stool samples [20,30,33,34,52,53]. Since this could not be done with every new variant that is identified, genome characterization by sequence analysis

Fig. 1. Phylogenetic tree showing NLV lineages, based on a 125-bp fragment within a conserved region of the viral polymerase gene, that were found in outbreaks of gastroenteritis in Finland (identified by number), including 12 outbreaks in which raspberries were implicated as the most likely source of infection (indicated by arrows). GG = genotype. MV = Mexico virus, TV = Toronto virus, HV = Hawaii virus, SOV = Southampton virus, NV = Norwalk virus and DSV = Desert Shield virus, all reference strains of NLV.
has been used to provide an interim system of genotyping. As the genotypes ideally would correlate with serotypes, the sequence of the major structural protein gene was used as the basis for phylogenetic analysis. [89,90]. Sequence analyses of viruses from different outbreaks and different geographical locations have confirmed that NLV can be divided into two major genetic groups (termed genogroups) based on capsid sequence and polymerase sequence data [51,56–58]. NV, Southampton virus, and Desert Shield virus are members of genogroup I. Snow Mountain virus, Hawaii virus, Toronto virus are members of genogroup II. In addition, a highly divergent virus (Alphatron) was assigned to a putative third genogroup. Within genogroups I and II stable lineages have been identified, based on phylogenetic analysis based on the complete capsid gene of at least two representatives per cluster [85,91,92]. To date, 15 distinct genotypes have been recognized, but as more strains are characterized, this number is likely to increase [89,90].

It is well established that many different genotypes of NLV cocirculate in the general population, causing sporadic cases and outbreaks [59,68,72,75,81,82,91–93]. Typically, strain sequences are (almost) identical within outbreaks, and different when specimens from different outbreaks are analyzed. Thus, when identical sequences are found in different patients or different clusters of illness, a common source for the infection should be suspected [74,94–110]. Conversely, finding different sequences in people with a supposedly common source infection suggests independent contamination, unless there is an association with sewage-contaminated water: in epidemics due to sewage contamination, often more than one NLV genotype is encountered [73,100,110]. Molecular epidemiology has been used on several occasions to confirm (e.g. [94–96,104–106]) or disprove links between outbreaks (e.g. [108]). Occasionally, epidemics occur in which the majority of outbreaks are caused by a highly related group of viruses within a genotype, with only minimal differences in the conserved polymerase gene fragment that is used for genotyping [10,111–113]. These epidemics may be widespread and even global [113]. The mechanisms behind emergence of epidemic types or fluctuations in the prevalent genotypes of NLV are unknown. Hypotheses include large-scale foodborne transmission of a single strain, and the possibility of spillover from a non-human reservoir.

2.10. Immunity

Little is known about immunity to NLV infections. Antibody ELISA assays have been developed by using recombinant capsids as antigen, and preliminary studies suggest that – in outbreaks and in volunteer studies – people develop antibodies mostly restricted to the infecting genotype with some cross-reactivity. From experimental infections in volunteers it is known that infected persons may become protected from reinfection, but only for a short period, and again only when the challenge virus is closely related to the genotype of the strain that was used for the infection [60,62]. Seroprevalence studies with the recombinant antigens have shown that antibodies to NLV are very common in the population, even when the recombinant NV capsids are used in populations where viruses from the Norwalk cluster have not been identified for a long time. Volunteers with antibodies to the infecting genotype reportedly may have a higher risk of illness and a steeper dose–response curve [42,114]. It is unclear what this means. Hinkula et al. [115] have shown that the seroprevalence may differ markedly for different antibody isotypes, suggesting that the lack of protection in part may be explained because a different type of antibody was the better correlate of protection. The lack of broadly reactive, long-lived immunity to natural infection suggests that development of a protective vaccine may be problematic.

2.11. Modes of transmission

NLV are transmitted by direct person-to-person contact or indirectly via contaminated water [94,96,100,102,105,106], food [74,95,98,103,108–110] or contaminated environments [97,101,107,116]. Clearly, person-to-person transmission is by far the most common route of infection. The infectious dose can be probably as low as 10–100 virus particles [114,117]. However, many foodborne NLV outbreaks have been described, often resulting from contamination by an infected foodhandler. In addition, several waterborne outbreaks of NLV have been described, both directly (e.g. consumption of tainted water) or indirectly (e.g. via washed fruits, by swimming or canoeing in recreational waters) [94,96,100,102,105,106]. Of special interest is the finding that a substantial proportion of bottled mineral waters contained caliciviral RNA [118], although these findings need to be confirmed by others.

Since projectile vomiting is a common feature following NLV infection and viruses can be present in vomit [43,119], aerosolized vomit is recognized as an important vehicle for transmission, both by mechanical transmission from the vomit-contaminated environment and even by airborne transmission [97,116,117,119–122]. The most compelling evidence for airborne transmission came from a study by Marks et al. [107]: they described an outbreak of gastroenteritis following a meal in a large hotel during which one of the guests vomited, and found an inverse relationship between attack rate per table and the distance from the person who got sick.

It is important to note that contamination may occur not only at the end of the food distribution chain, but at almost any step from farm to table. Foodborne illness associated with consumption of oysters has been traced back to a crewmember of a harvesting boat [104]. From that same outbreak investigation, it was reported that 85% of oyster-harvesting boats in the area routinely disposed of
sewage overboard. Little is known about the hygienic conditions in harvesting areas in other parts of the world, not only for shellfish, but also for products such as fresh fruits. Infected foodhandlers may transmit infectious viruses during the incubation period and after recovery from illness [99,109,123]. Another aspect of NLV epidemiology is that foodhandlers may unknowingly transmit viruses, e.g. when they have a sick child at home [98].

Besides person-to-person transmission via food vehicles, zoonotic transmission has been reported for some enteric viruses, although this appears to be of no significance for foodborne infections [124]. This may change, however, based on new data for NLV. Until recently, the NLV were considered to be pathogens with humans as the sole host. Recently, however, NLV were found in healthy pigs in Japan and in historic calf stool specimens from the UK and from Germany [125–128]. The calf viruses, named Newbury agent and Jena virus, are pathogenic for young calves. The two bovine enteric caliciviruses and the pig enteric calcivirus are genetically distinct from human strains, but cluster within the NLV genus. In a pilot study in The Netherlands, pooled stool samples from calves, fattening pigs, and adult cows were tested for the presence of NLV [119]. Thirty-three (45%) of the calf herds tested positive for a NLV strains belonging to one cluster, with highest homology with the Newbury genotype. The Netherlands calf viruses were sufficiently distinct to suggest that they may be a separate lineage (based on capsid sequencing). If confirmed, that implies that in calves also several lineages or genotypes of NLV cocirculate, and that zoonotic transmission cannot be excluded by finding distinct strains in animals and humans. In addition, one pig herd was found positive for a virus, which was very similar to the pig calcivirus from Japan [119]. These findings raise important questions on the host range of the NLV. At this stage the animal NLV appear to form genetically distinct stable lineages, but are sufficiently related to the human NLV to suggest that under the right conditions interspecies transmission could occur. Animal calciviruses in another genus within the family Caliciviridae (the genus *vesivirus*) have a wide host range, and interspecies transmissions have repeatedly been documented [129].

3. Foodborne hepatitis

3.1. Introduction

The viruses which cause hepatitis can be divided into enterically transmitted viruses (HAV, HEV), and parenterally transmitted hepatitis viruses (hepatitis B, C, D, G). For food- or waterborne transmission, only the enterically transmitted viruses are relevant. HAV is a virus in the family Picornaviridae, to which the enteroviruses also belong (including poliovirus) [130]. HEV shows some resemblance with viruses from the family Caliciviridae (to which the NLV belong), but has not (yet) been included in a virus family because of unique characteristics [22].

Hepatitis E has only relatively recently been established as a cause of hepatitis, when large waterborne outbreaks occurred in India and Pakistan. The virus is endemic over a wide geographic area, primarily in countries with inadequate sanitation where HAV is endemic as well (south-east Asia, Indian subcontinent, Africa), but not as widespread as HAV. In industrialized countries HEV infections are rare, and are usually travel-related [131–133]. HEV outbreaks can be distinguished based on the higher attack rate of clinically evident disease in persons 15–40 years of age compared with other groups, higher overall case fatality rates (0.5–3%), and the unusually high death toll in pregnant women (15–20%). In younger age groups, the majority of people with symptoms due to HEV infection may present without jaundice, unlike those infected with HAV [134–136]. Since HEV can cause illness with high mortality in pregnant women, a study of foodborne virus transmission in our opinion should include HEV. The recent discovery that HEV is common in pigs and rats and that pig HEV have been found in humans is reason for a careful evaluation of HEV cases in countries where the virus is not endemic in the human population [137,138]. However, the primary source for HEV infection appears to be fecally contaminated water, and few human cases have been reported in regions where pig HEV is endemic, suggesting that interspecies transmissions of HEV are not common in countries with high standards of living. The subsequent discussion will focus on HAV [139–141]. Since excellent reviews are available for HAV in general, the focus of this section will be mostly on the possibility of foodborne transmission [135].

3.2. The virus

HAV are small, non-enveloped spherical viruses, measuring between 27 and 32 nm in diameter. They contain a single (positive-) stranded RNA genome of approximately 7.5 kb in length that encodes a large polyprotein [142]. The genome organization differs from that of the Caliciviridae, in that the genes encoding the non-structural proteins are located at the 3′-portion of the genome, and the genes encoding the structural proteins at the 5′-end [143]. The polyprotein is processed to four structural and seven non-structural proteins by proteinases encoded in and around the 3C region [144]. Replication efficiency seems to be controlled by amino acid substitutions in the 2B and 2C regions [145].

3.3. Clinical symptoms

Infection with HAV can produce asymptomatic or symptomatic infection after a median incubation period of 30 days (range 15–50 days). The illness caused by HAV infection is characterized by non-specific symptoms
that can include fever, headache, fatigue, nausea and abdominal discomfort, followed by symptoms and signs of hepatitis 1–2 weeks later [134]. The likelihood of having symptoms with HAV infection is related to the age of the infected individual. Among children younger than 6 years of age, most infections are asymptomatic, and children with symptoms rarely develop jaundice. Among older children and adults, infection is usually symptomatic, and jaundice occurs in the majority of patients [136,146]. The illness is generally self-limited, lasting up to several months, and infrequently causes fulminant disease. All patients with hepatitis A in a 4-year hospital- and physician-based surveillance regained normal liver function within 20 months of the acute illness [134]. However, the case fatality rate among persons > 50 years old with hepatitis A reported to the CDC in the USA is 1.8% [136]. Persons with chronic liver disease are at increased risk of fulminant hepatitis with hepatitis A [147–149]. HAV has not been shown to cause a persistent infection, and has not been associated with chronic liver disease. Prolonged or relapsing disease lasting up to 6 months occurs in 10–15% of patients [150–153].

Peak virus shedding of HAV in feces occurs during the 2 weeks before the onset of jaundice or normal liver enzyme elevation. The concentration of virus in stool declines after jaundice appears, although prolonged shedding may occur, particularly among infants and children (reportedly up to 5 months post infection [134,136]). Robertson et al. [154] found low levels of HAV RNA in stools from children for up to 10 weeks after the onset of symptoms. Reactivation of viral shedding can occur during relapsing illness. Viremia occurs soon after infection and persists through liver enzyme elevation. In a recent study, HAV RNA in serum was detected an average of 17 days before the alanine aminotransferase peak, and viremia persisted for an average of 79 days after the liver enzyme peak. The average duration of viremia was 95 days (range, 36–391 days) [150].

3.4. Pathogenesis

The exact pathogenesis of hepatitis A is not understood. Virus enters via the intestinal tract, and is transported to the liver following a viremic stage, in which virus can be detected in the blood stream. Hepatocytes are the site of replication, and virus is thought to be shed via the bile. In experimental infections in non-human primates, HAV viral antigen and/or genomic material has been found in the spleen, kidney, tonsils and saliva, suggesting that other sites of replication may exist. In vitro, cells are generally not destroyed by the virus, and the damage to liver epithelial cells in vivo often is limited [155].

3.5. Diagnosis in humans

Hepatitis A cannot be distinguished from other types of viral hepatitis on the basis of clinical features [136]. Diagnosis of acute hepatitis A is made by detection in serum of IgM antibody to the capsid proteins of HAV (IgM anti-HAV). In most persons with acute HAV infection, IgM anti-HAV becomes detectable 5–10 days before the onset of symptoms and can persist for up to 6 months [156]. Commercial diagnostic tests are available for the detection of IgM and total (IgM and IgG) anti-HAV in serum. Virus (up to 10^9 particles per ml) can be detected by molecular methods in stool and serum samples of infected individuals, but these methods are not generally used for diagnostic purposes [155]. Detection of sporadic cases or small clusters of foodborne hepatitis A is problematic because of difficulties in recalling exposures during the long incubation period, the simultaneous occurrence of cases from person-to-person transmission, and the lack of routinely collected data about foodborne exposures. For this, the use of molecular strain typing offers new possibilities (as described in Section 3.8).

3.6. Epidemiology

Worldwide, differing patterns of HAV endemicity can be identified, each characterized by distinct patterns of the prevalence of antibody to HAV (anti-HAV) and hepatitis A incidence, and associated with different levels of prevailing environmental (sanitary and hygienic) and socioeconomic conditions. In much of the developing world, HAV infection is endemic, and the majority of persons are infected in early childhood; virtually all adults are immune [136]. In these areas, HAV transmission is primarily from person to person. Outbreaks are rare because most infections occur among young children who generally remain asymptomatic. Paradoxically, as socioeconomic and environmental conditions improve and HAV decreases in endemicity (Fig. 2), the overall incidence and average age of reported cases often increase because older individuals are susceptible and develop symptoms with infection [157]. An illustration of this can been seen from national surveillance data: for instance in England and Wales, the annual notification rate of HAV infection has risen fourfold between 1987 and 1991 from 3.6 to 14.6 per 100000 population [165]. In Italy, data collected from

![Fig. 2. Seroprevalence of antibodies to HAV in The Netherlands in different age groups in 1979 and 1995. Data adapted from [210,211].](image-url)
a surveillance system for type-specific acute viral hepatitis (SEIEVA) showed that the incidence of HAV declined from 10/100,000 in 1985 to 2/100,000 during the period 1987–1990, while an increase was observed after 1991. The highest attack rate was observed in the 15–24-year age group [158].

Thus, in the industrialized parts of the world where HAV endemicity is relatively low, hepatitis A occurs sporadically and in the context of community-wide epidemics that recur periodically [157]. Transmission remains primarily from person to person, but large common source outbreaks also can occur. Outbreaks of hepatitis A are common in crowded situations such as institutions, schools, prisons, and in military forces. The increased number of susceptible individuals allows common source epidemics to evolve rapidly, and the likelihood of such epidemics is increasing [136]. Foodborne outbreaks have been reported in most parts of the world except those with the highest and lowest HAV endemicity [159–171]. An outbreak in Shanghai in 1988 involving 250,000 cases occurred in association with consumption of contaminated clams [161]. In a recent outbreak in the USA, 213 persons developed hepatitis A after eating contaminated frozen strawberries that were distributed in school lunch programs [163]. In 1996 and 1997, a large HAV epidemic occurred in southern Italy, Puglia region, with 11,000 notifications especially among young adults. The main risk factor in this epidemic outbreak was consumption of mussels [166]. A large HAV epidemic occurred in Finland among drug abusers (around 300 cases) due to contaminated amphetamine [172,173]. In outbreak situations, up to 20% of cases are due to secondary transmission. Waterborne outbreaks are unusual, but have been reported in association with drinking fecally contaminated water and swimming in contaminated swimming pools and lakes [136,160].

3.7 Risk groups

In the developed world, groups at increased risk of HAV can be identified. In case-control studies in England and Italy, factors associated with increased risk of HAV included international travel to areas of intermediate or high HAV endemicity, a household contact with hepatitis A, sharing a household with a child aged 3–10 years, consumption of bivalve mollusks, and consumption of untreated water [165]. In Italy, shellfish consumption was the most frequently reported source of infection over the period considered [158]. In the USA, 25,000–35,000 cases are reported each year, of which approximately 12–25% can be attributed to household or sexual contact with a case. Other identified potential sources of infection include association with child care centers (11–16%) and international travel (4–6%). Although several food- and waterborne outbreaks of HAV have been described [159,160,163,167], in the USA only 5% of reported cases occur in association with a recognized food- or waterborne outbreak [6]. However, approximately 50% of reported cases do not have a recognized source of infection. In many countries in the developed world, cyclic outbreaks have occurred among users of injection drugs and men who have sex with men [173,174]. In The Netherlands, around 20% of HAV cases are related to international travel to high-risk areas; in areas of very low endemicity such as Scandinavia, in many years international travel accounts for the majority of reported cases.

Since the case fatality rate of HAV infection increases with age, risks of more serious illness are higher for older age groups, provided they have not encountered HAV throughout their life [157]. Persons with hepatitis C infection and possibly those with chronic hepatitis B are at increased risk of fulminant hepatitis following superinfection with HAV [147–149].

3.8 Molecular epidemiology

Molecular detection and typing assays have been developed for HAV [163,175,177]. These assays can detect HAV in stool and serum specimens from patients with hepatitis [150,177,178]. Seven genotypes of HAV have been recognized, four of which occur in humans. The other three genotypes have been found in captive Old World monkeys [179]. Genetically distinct lineages are found in different geographic regions and among patients in particular risk groups [154,173,174,176,180,181]. The genetic diversity of HAV has been used to verify the occurrence of outbreaks including foodborne [142,153,159,163,167,169] and waterborne outbreaks [160], and to link apparently sporadic cases to recognized foodborne and waterborne outbreaks [167,182].

3.9 Immunity

Only one serotype of HAV has been found so far, of which the antigenicity is determined by an immunodominant antigenic site [183]. A single HAV infection appears to induce lifelong immunity to the strains containing this epitope, but it remains to be seen if antigenic variants exist. IgM appears rapidly, with the majority of cases positive at 3 days post onset of symptoms [156]. Levels of IgM readily decline, and after 6 months 75% of patients have no detectable IgM antibodies left. IgG anti-HAV begins to rise early in the course of infection and remains detectable for life, conferring protection against repeated HAV infection. Susceptibility to HAV infection can be determined using commercially available assays that detect IgM and total (IgG and IgM) antibody to HAV in serum.

3.10 Modes of transmission

HAV is transmitted by the fecal–oral route, most often
from person to person. This is exemplified by the high transmission rates among young children in developing countries, in areas where crowding is common and sanitation is poor, and in households and child care settings [136,155]. Transmission also occurs from ingestion of contaminated food [159,161–166,168–171] and water [136, 160]. One report suggests the possibility of HAV via contaminated drinking glasses in a bar [184]. On rare occasions, HAV infection has been transmitted by transfusion of blood or blood products collected from donors during the viremic phase of infection, which appears to be much longer than was previously assumed [150,159,177, 180,185,186]. Although HAV has been detected in saliva of experimentally infected non-human primates, transmission by saliva has not been demonstrated. A large outbreak among intravenous drug users in Finland was attributed to the possibility of fecal contamination of amphetamine associated with transportation of drugs in the gastrointestinal tract, which of course will be difficult to prove [172].

4. General aspects

4.1. High risk foodstuffs

Bivalve molluscan shellfish are notorious as a source of foodborne viral infections, because filter-feeding shellfish can concentrate HAV up to 100-fold from large volumes of water, allowing accumulation of virus from fecally contaminated water ([95,104,110,164,166,169,187]; for review see [188]). Depuration, a practice that may reduce bacterial contamination, is far less effective in reducing viral contamination. Quality control of food and water on the basis of the detection of indicator organisms for fecal contamination has proven to be an unreliable predictor of viral contamination [187,189]. For shellfish, screening both of growing waters and of shellfish could be done, but the relative sensitivities of these approaches need to be evaluated.

Several other foods, however, have also been implicated as vehicles of transmission (desserts, fruits, vegetables, salads, sandwiches): the bottomline message is that any food that has been handled manually and not (sufficiently) heated subsequently is a possible source of infection [8]. It is important to note, however, that contamination may occur not only at the end of the food chain, but at almost every step in the path from farm to table.

Outbreaks associated with food, particularly raw produce, contaminated before reaching the food service establishment have been recognized increasingly in recent years [74,99,159,163,168]. This produce appears to have been contaminated during harvest, which could occur from handling by virus-infected individuals. However, a better understanding of the precise mechanisms whereby viral contamination of raw produce occurs is needed to better focus prevention efforts in this area. Widespread transmission can occur when commercial facilities prepare food that is distributed to geographically distant locations. It is clear that some currently used industrial food processing methods will not sufficiently inactivate viruses if present in the foods before processing [190].

Recent studies with novel techniques show that infected foodhandlers may shed virus for longer periods of time [42,178] and therefore may remain infectious even after full recovery [109]. Enteric viruses may persist for extended periods on materials that are commonly found in institutions and domestic environments (such as paper, cotton cloth, aluminum, china, glazed tiles, latex, and polystyrene [191]).

4.2. Virus detection in food and water

Although diagnostic methods have been developed for the detection of virus or viral RNA in food and water, they have not found their way to routine laboratories in most parts of the world [192–201]. Most studies of virus detection in food have focussed on shellfish, for which several groups have developed slightly different protocols, and comparative studies are needed to determine which assays should be recommended (reviewed in [188]). Recently, some methods were reported for virus detection in other foods, but their application in the field remains anecdotal [202,203]. It remains unclear what the predictive value is of a negative test. This information is needed before screening of such specimens can be done to monitor contamination.

A special problem is that NLV cannot be grown in tissue culture, and HAV only with moderate success. As a result, data on the correlation between the presence of viral genes (as tested by RT-PCR) and viable virus are lacking. Arnal et al. [204] assayed the stability of HAV in artificial sterile seawater by RT-PCR and by cell culture. The HAV genome was detectable by RT-PCR for 232 days while virus particles were detectable in cell culture for only 35 days, suggesting that detection of the HAV genome by RT-PCR is not a reliable indicator of the presence of viable virus. Polish et al. [205] found that only stool specimens that were positive for HAV by ELISA were infectious for tamarins, suggesting that the viral load may be a determining factor of infectivity.

For outbreak diagnosis, the current approach is the screening of stool specimens from cases and controls, combined with an epidemiological investigation to assess food-specific attack rates. Foods with a significant odds ratio may then be examined by molecular methods, although no information is available about the sensitivity of these methods for outbreak diagnosis and – in the case of HAV – implicated foods usually have been consumed or discarded by the time the outbreak is recognized due to the long incubation period. Therefore, the combination of epidemiological outbreak investigations and molecular
4.3. Prevention and disinfection

Increasing the awareness of all foodhandlers about transmission of enteric viruses is needed, with special emphasis on the risk of ‘silent’ transmission by asymptomatically infected persons and those continuing to shed virus following resolution of symptoms. While it may be unclear what proportion of foodborne infections can be attributed to workers in different parts of the food chain, it is important that viruses become part of science-based hazard analysis and critical control point (HACCP) systems to identify risks and to help identify gaps in knowledge. At present, insufficient data are available to determine which steps are going to be critical for all foods. Preventive measures differ for the different transmission routes.

(i) Shellfish: for shellfish, strict control of the quality of growing waters can prevent contamination of shellfish. This includes control of waste disposal by commercial and recreational boats. Guidelines specifically aimed at reduction of viral contamination are needed, as it has become clear that the current indicators for water and shellfish quality are insufficient as predictors of viral contamination [189,199–201].

(ii) Food items contaminated by infected foodhandlers: personal hygiene is most important in preventing foodborne viral infection, and includes frequent handwashing and wearing gloves. This should apply for all points in the food chain where foodstuffs are handled manually. The infectious dose of NLV appears to be extremely low [114]. As a result, even with strict sanitary measures, infection may not always be prevented. Foodborne outbreaks have occurred due to contaminated food sources that passed all microbiological assays. A common-sense guideline is to remove people with symptoms consistent with viral gastroenteritis from the production chain until at least 2 days after remission of the symptoms. A practical problem with this guideline is that an unknown proportion of viral infections will be subclinical, viral shedding may last longer, and – even in the incubation period – infected persons may shed sufficient amounts of virus to cause food contamination [42,123,171,177,178]. The kinetics of viral shedding have only been studied in a few infected volunteers, and may not reflect the real-life situation when people may have been infected with a low dose of infectious virus. Given the highly infectious nature of NLV and HAV, and the documented risk of virus transmission to food during the incubation period, it is envisioned that guidelines should be developed that consider the occurrence of gastroenteritis in contacts (e.g. children) of people working in critical points in the food chain. This should be based on data on the kinetics of viral shedding following natural infection. In addition to encouraging handwashing and other hygienic measures, policies involving ‘no bare hands contact’ by handlers of food that will be eaten without further cooking have been implemented in many areas. It is important to note that contamination can be particularly widespread after vomiting, due to aerosol formation and subsequent transport of virus particles by air.

The globalization of the food market has hampered the implementation of control measures to assure safe food. It is not clear whether routine monitoring of food specimens for viral contamination will be feasible. However, for prevention of foodborne transmission, it is also essential that food items are not grown or washed in fecally contaminated water.

Highly effective inactivated hepatitis A vaccines are available for use before exposure. To reduce the frequency with which foodhandlers with hepatitis A are identified, vaccination of foodhandlers has been advocated and implemented in some cities in the USA [185]. However, such policies have not been shown to be cost-effective and generally are not recommended in the USA or other developed countries. Whether HAV vaccination is feasible for preventing foodborne transmission for specific countries or regions depends on many local factors (e.g. level of endemicity, hygienic conditions) and needs to be evaluated for these specific situations, based on HACCP analysis. Ig reportedly is more than 85% effective in preventing hepatitis A when given within 2 weeks of exposure [206]. In the USA, when a foodhandler is identified with hepatitis A, it is recommended that Ig be given to other foodhandlers at the establishment, and, under limited circumstances, to patrons. Once cases are identified that are associated with a food service establishment, it is generally too late to administer Ig to patrons, since the 2-week period during which Ig is effective will have passed. This factor may explain some of the lack of success of Ig treatment as outbreak interventions [207].

HAV is resistant to low pH (up to pH 1) and to heating, surviving 1 h at 60°C [179]. It appears to be extremely stable in the environment, with only a 100-fold decline in infectivity over 4 weeks at room temperature, and 3–10 months in water [155,190]. HAV appears to be relatively resistant to free chlorine, especially when the virus is associated with organic matter. Heating foods (such as shellfish) to temperatures > 85°C for 1 min and disinfecting surfaces with a 1:100 solution of sodium hypochlorite in tap water will inactivate HAV. Little is known about the stability of NLV outside the host, and infectivity can hardly be measured due to the absence of a cell culture system. From experiments with adult volunteers in the 1980s it has been suggested that NV is resistant to low pH (2.7), ether extraction, and heat treatment (30 min at 60°C) [26]. Steaming oysters may not prevent NLV gastroenteritis [208]. The virus reportedly is quite resistant to chlorine as remains infectious after 30 min in the presence of 0.5–1 mg free chlorine per liter. At higher concentrations, the virus is inactivated (>2 mg per liter free chlo-
rime [26]). These findings have to be interpreted with caution, as data from recent dose–response studies makes it clear that very high doses of virus were used in earlier volunteer challenge experiments. Therefore, reduction of infectivity due to various treatments may not have been detected. Based on semiquantitative detection by using PCR units, drinking water treatment processes using coagulation–floculation–sedimentation, filtration, and disinfection with free chlorine, monochloramine, ozone, chlorine dioxide or UV irradiation all reduce the amount of NV more than four log steps [209].

5. Legislation, rules and regulations

Statutory sanitary control for shellfish relies on microbiological criteria to define the suitability of these products, as for instance in EU Council Directive 91/492/EC. This European directive establishes that microbial quality of shellfish should be monitored by measuring counts of total fecal coliform bacteria, *Escherichia coli*, and *Salmonella*. The Directive establishes no specific microbiological criteria concerning the presence of enteric viruses, even though it has clearly been shown that there is no correlation between the presence of viruses and the presence of coliform bacteria and/or *E. coli*; in fact HAV, enteroviruses and NLV have been detected in mussels that otherwise meet bacteriological standards [187–189,199,200]. Similarly, it is not clear if and how depuration will reduce the levels of viral contaminants, especially for NLV, since quantitative methods for their detection are not yet available.

6. Recommendations

To provide baseline data for future intervention and prevention programs, studies are needed to estimate the burden of illness and cost of illness due to foodborne viral infections with special emphasis on determining the burden of illness in the elderly. To enable this, better surveillance for illness is needed, as well as tools for molecular tracing of viruses throughout the food chain and through populations. Rapid methods for detection and typing of foodborne viruses should be developed and rapid exchange of typing information between laboratories and between countries should be encouraged. To enable this, current and newly developed methods need to be evaluated for comparability and need to be standardized. The feasibility of using these methods for food screening should be studied. The mechanism of emergence of epidemic strains should be studied, including the possible link with animal calicivirus infections.

Preventive measures directed at reduction of bacterial infections and general hygienic measures not always suffice to reduce viral infections and contamination. Studies are needed to evaluate if public campaigns directed at prevention of viral foodborne infections are likely to be successful. To reduce the risk of shellfish-related foodborne outbreaks efforts should be made to maintain/improve the quality of growing waters. In order to achieve this, studies are needed that address the detection of viral contaminants, the effects of wastewater treatment on viral load, and the study of environmental factors that contribute to bioaccumulation and depuration of viruses. The use of sludge waste as fertilizer and of wastewater for irrigation should be evaluated for risks of viral contamination. Hepatitis A vaccination should be considered as part of the HACCP approach to reduce the risk of foodborne hepatitis A.

Given the high incidence of foodborne viral infections, it is time for a conscious effort to raise the awareness about the risk of foodborne transmission of viruses. Viruses should be included in all steps of the HACCP process. While the role of virus-infected foodhandlers in transmission of NLV and HAV is well established, the risk of virus contamination is not limited to the final stages of the production process, and the potential role of infected harvesters or workers anywhere in the food chain should be considered. The food industry and the scientific community should work together in a joint effort to develop an integrated plan of action to address foodborne viral infections. This plan should identify both research priorities and strategies for implementation of the findings in HACCP systems.

7. Conclusions

Although there are numerous fecal–orally transmitted viruses, the risk of foodborne transmission is highest for NLV and HAV. The ease of foodborne transmission can in part be attributed to the extreme stability of the viruses outside their host, and to the highly infectious nature. NLV and HAV can be transmitted from person to person, or indirectly via food, water, or fomites contaminated with virus-containing feces or vomit. People can be infected without showing symptoms. The high frequency of secondary cases of NLV illness and – to a lesser extent – of hepatitis A following a foodborne outbreak results in amplification of the problem. The burden of illness is highest in the elderly, and is therefore likely to increase in the years to come due to the aging population. For HAV, the burden of illness may increase following hygienic control measures, due to a decreasing population of naturally immune individuals and a concurrent increase in the population at risk.

Recent advances in the research of NLV and HAV have led to the development of molecular methods which can be used for molecular tracing of virus strains. These methods can be and have been used for the detection of common source outbreaks. While traditionally certain foods have
been implicated in virus outbreaks, it is clear that almost any food item can be involved, provided it has been handled by an infected person. There are no established methods for detection of viruses in foods other than shellfish, and current microbiological quality control relies on bacterial counts, which are not correlated with the presence of viruses. Little information is available on disinfection and preventive measures specifically for these viruses. Studies addressing this issue are hampered by the lack of culture systems. For HAV a vaccine is available, which confers full protection from illness.

Where does this leave us? Let’s all face it: international foodborne viral outbreaks are an event waiting to happen, and may very well go unnoticed with the existing surveillance systems that focus almost exclusively on bacterial pathogens. Well-standardized surveillance networks are needed that combine epidemiological and virological information for a combined laboratory-based rapid detection system for foodborne viral outbreaks. With better surveillance, documented outbreaks of foodborne infections could be reported faster, in time to take preventive measures to stop further spread.

References


204 M. Koopmans et al./FEMS Microbiology Reviews 26 (2002) 187–205


Human caliciviruses in Europe

B.A. Lopman a,b,*, D.W. Brown b, M. Koopmans c

a Gastrointestinal Diseases Division, PHLS Communicable Disease Surveillance Centre, 61 Colindale Avenue, Colindale, London NW 9 SEQ, UK
b Enteric, Respiratory and Neurological Virus Laboratory, Central Public Health Laboratory, Colindale, London NW 9 5HT, UK
c Research Laboratory for Infectious Diseases, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands

Accepted 23 October 2001

Abstract

Caliciviruses are single-stranded RNA viruses, which are divided into four genera based on their morphology and genomic structure. Viruses from two genera, the Norwalk like viruses and Sapporo like viruses, are a common cause of acute, nonbacterial gastroenteritis in humans. Although the first human calicivirus discovered nearly 30 years ago, much of the epidemiological and biological character of these viruses is only now beginning to unfold. Investigation has been difficult due to a number of factors, the viruses cannot be amplified by in vitro cell culture or animal models and electron microscopy (EM) is often not sensitive enough to detect the viruses in stool samples. Recent advances in molecular diagnostic techniques and the advent of a baculovirus expression system have highlighted the clinical and public health importance of calicivirus in all age groups, their ability to cause infection via a number of transmission routes as well as their considerable genetic diversity. These characteristics, in conjunction with the inability of humans to develop long-term immunity make HuCV an important public health issue in Europe and worldwide. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Human calicivirus; Norwalk-like virus; Sapporo-like virus; Viral gastroenteritis; Virology; Epidemiology

1. Public health importance of viral gastroenteritis

Gastroenteritis is one of the most common and, in public health terms, most important diseases in man. During the first 5 years of life, every child will contract diarrhoeal disease, and with it comes the risk of dehydration and nutritional deficiency (Bern and Glass, 1994). Children in developing countries suffer from an average of 2.2–3.9 episodes of gastroenteritis annually (Bern and Glass, 1994; Snyder and Merson, 1982). Particularly in developing countries, diarrhoeal disease can be severe. In children under 5 years of age, it is responsible for 25–30% of all deaths (Bern and Glass, 1994; Martines et al., 1993).

In more developed countries, mortality is rare, but it is nonetheless an important cause of morbidity and economic cost (Monroe et al., 2000). In England and Wales, one out of every five people has a case of Infectious Intestinal Disease (IID) annually (Wheeler et al., 1999) and there are over
300 deaths and 35,000 hospital admissions every year (Djuretic et al., 1996). In The Netherlands, the incidence of gastrointestinal diseases was also found to be very high, with 283 episodes per 1000 person-years (de Wit et al., 2001b). The burden of illness is highest in the young and elderly (Hedlund et al., 2000; Wheeler et al., 1999).

The contribution of human caliciviruses (HuCVs) (‘Norwalk-like virus’ [NLV] and ‘Sapporo-like virus’ [SLV]), to the totality of IID has only recently begun to be studied in a meaningful way. The IID study in England and the Sensor study in The Netherlands found these viruses to be the cause of 13 and 17%, respectively, of all community cases that could be attributed to an agent (bacterial, parasitic or viral) (de Wit et al., 2001a; Wheeler et al., 1999). Of all gastroenteritis outbreaks reported to the surveillance unit for England and Wales, nearly 50% are due to NLVs (Dedman et al., 1998) which is similar to figures in the US (Mead et al., 1999), Finland (Lew et al., 1994), Sweden (Hedlund et al., 2000), The Netherlands (Koopmans, 2001), Germany (Schreier et al., 2000), Japan (Inouye et al., 2000) and others.

Modern methods of food distribution provide a mechanism for large and diffuse, even trans-national, outbreaks (Chadwick and McCann, 1994; Deneen et al., 2000; Dowell et al., 1995; Gaulin et al., 1999a; Ponka et al., 1999). The same is true of outbreaks caused by contaminated drinking waters (Beuret et al., 2000; Brugha et al., 1999; Cannon et al., 1991; Kaplan et al., 1982c; Kukkula et al., 1999). However, outbreaks in healthcare setting may have the greatest public health impact (Dedman et al., 1998; Hedlund et al., 2000). Not only can outbreaks in nursing homes and hospitals be prolonged and affect large numbers of already vulnerable people, but they are difficult to control and often result in staff illness and, occasionally, in the closure of wards.

2. Taxonomy and genetic classification

HuCVs contain one molecule of linear positive-sense RNA and the virion is 27–40 nm in diameter when viewed by negative-stain electron microscopy (EM). Their taxonomy, which was originally based on morphology, has been refined due to recent data based on genetic sequencing and phylogenetic analysis (Dingle et al., 1995; Jiang et al., 1993; Lambden et al., 1993; Liu et al., 1995). The family Caliciviridae was recently divided into four designated genus: Lagovirus, Vesivirus, NLV and SLV (Virus Taxonomy, 2000). Small round structured virus (SRSV) was the name used to describe the genera now called NLV. NLV and SLV are interim names and are likely to change again in the future. Unlike NLVs and SLVs, members of the Lagovirus and, Vesivirus genera are principally veterinary infections. Based on both on morphology (NLVs lack the cup-like appearance of SLV) and genome sequence and organisation, NLVs and SLVs are grouped as separate genera (Caul, 1996; Chiba et al., 2000).

The NLV genus branches into at least two distinct genogroups (GGI and GGII) based on genetic divergence in the polymerase and capsid regions (Ando et al., 2000). However, there is as yet no consensus as to how the genogroups should be subdivided since considerable strain diversity occurs within each genogroup (Fankhauser et al., 1998; Green et al., 2000). A number of researchers have proposed schemes for sub-grouping strains into genotypes (or genetic clusters). Recent suggestions contain approximately seven GGI clusters which include the prototype Norwalk, Southampton and Desert Shield viruses reference strains and approximately ten GGII genotypes which include the Snow Mountain, Toronto, Bristol and Hawaii viruses reference strains (Ando et al., 2000; Hale et al., 2000; Koopmans, 2001; Vinjé et al., 2000; Vinjé and Koopmans, 2000). Researchers at the CDC have proposed a third genogroup (GGIII) containing the animal-infecting Jena and Newbury agent-2 viruses (Ando et al., 2000), but their taxonomic status is yet to be agreed.

The SLVs are also split into two genogroups. The prototype Sapporo virus belongs to genogroup I and the London virus belongs to genogroup II (Koopmans, 2001).
3. A history of human caliciviruses

The syndrome associated with caliciviral gastroenteritis was described in the medical literature over 70 years ago. However, it would be many years later that a causative agent could be linked with the condition Zahorsky described in 1929 as ‘winter vomiting disease’ (Zahorsky, 1929).

Studies conducted in Cleveland, Ohio in the late 1940s demonstrated just how much gastroenteritis could not be attributed to known bacterial or parasitic pathogens. Of the 1466 cases ascertained over a 30-year period, 1104 (75%) had no ‘adequate explanation’ (Hodges et al., 1956). These were said to represent (for the most part) acute, infectious, nonbacterial gastroenteritis (Dingle et al., 1956). Clinical studies, where volunteers were exposed to faecal extracts that had been filtered to remove all bacteria, confirmed the hypothesis that a viral agent was the likely cause (Gordon et al., 1947). In fact, it was already becoming clear that more than one viral agent could cause infection of the intestine (Jordan et al., 1953). Nonetheless, more than 20 years later, the pathogenesis of this common illness remained uncertain.

In the autumn of 1968, 50% of students and teachers at an elementary school in the town of Norwalk, Ohio were struck with an illness ‘characterised principally by nausea, vomiting, and abdominal cramps’ (Adler and Zickl, 1969). Nearly a third of the family contacts of primary cases fell ill (with an average incubation time of 48 h). Since no bacterial agent was found, a viral cause was suspected. However, because these viruses did not grow in tissue culture, no causative agent could be recovered from ill patients, as had been the case in previous outbreaks (Adler and Zickl, 1969). Characterisation studies, using passage of agent through volunteers, later revealed that the ‘Norwalk agent’ was a small particle (<36 nm) and was resistant to ether, acid and moderate heating (Dolin et al., 1971, 1972). However, all attempts to culture the agent in organ cells or to transmit it to an animal were unsuccessful (Dolin et al., 1971).

In 1972, after extensive attempts by a number of researchers (Dolin et al., 1972; Jordan et al., 1953), Kapikian et al. found a 27 nm virus particle by immune electron microscopy (IEM) (Kapikian et al., 1972). Several other viral causes of gastroenteritis, most notably rotavirus and adenoviruses, were elucidated in the 1970s by the same technique. EM has since been the primary diagnostic method of enteric virus detection. As other, morphologically indistinguishable agents were found, they were arbitrarily named after their place of discovery (e.g. Montgomery County, Hawaii and Mexico) (Caul, 1996).

In 1976, the calicivirus with classical morphology, now called SLV, was first suggested to be an infection of humans (Madeley and Cosgrove, 1976). In the subsequent years, a number of outbreaks were definitively associated with SLV, thus confirming it as a pathogen of the human intestine (Chiba et al., 1979, 1980; Cubitt et al., 1979). SLVs were initially distinguished from NLVs by their ‘Star of David’ morphological appearance when viewed with the electron microscope (Caul and Appleton, 1982).

The biological similarity of NLVs to SLVs was substantiated by Greenberg et al.’s elucidation of the virion makeup of NLV in 1981 (Greenberg et al., 1981). The single structural protein with a molecular weight of 59,000 and a soluble protein of molecular weight 30,000 made it clear that ‘the protein structure of the virion is similar to that of the Caliciviridae family’ (Greenberg et al., 1981). Understanding of the taxonomy of these viruses, as well as improvements in diagnostics were furthered by the publication of the complete genome sequence of the Norwalk and Southampton viruses (SV) in 1993 (Jiang et al., 1993; Lambden et al., 1993). This genomic information, along with the subsequent characterisation of an SLV (Matson et al., 1995; Numata et al., 1997) confirmed the findings of Greenberg et al. (1981) that NLV and SLV are distinct, but related viruses (Cubitt et al., 1994; Liu et al., 1995; Matson et al., 1995).
4. Clinical picture

In the absence of other factors, infections with NLVs and SLVs are typically mild and self-limiting. The onset of illness is abrupt, usually within 24–28 h after exposure and the duration of illness is relatively short (12–60 h) (Adler and Zickl, 1969; Kaplan et al., 1982a). A high percentage of patients present with diarrhoea, abdominal cramps and nausea (Adler and Zickl, 1969; Hedberg and Osterholm, 1993), but it is the high frequency of projectile vomiting in adults that distinguishes NLV from other viral and common bacterial pathogens such as *Salmonella*, *Shigella*, and *S. aureus* (Adler and Zickl, 1969; Caul, 1996). In fact, recognition of an NLV outbreak can reliably be based on clinical symptoms and epidemiological characteristics alone (Kaplan et al., 1982a). It is important to note that the description of clinical symptoms comes from a handful of outbreak reports and not much is known about the range of symptoms in the community.

4.1. Pathogenesis

NLVs and SLVs are contracted by humans via the oral route. As acid-stable viruses they pass through the stomach; replication is thought to occur in the small intestine (Caul, 1996). Most of our knowledge concerning the pathogenesis of HuCVs comes from the human volunteer studies performed in the US. Individuals gave intestinal biopsy samples before and after exposure to the viral agents (Agus et al., 1973; Dolin et al., 1975; Schreiber et al., 1973, 1974). Light and EM showed that individuals with clinical illness exhibit lesions on the small intestinal mucosa. The mucosa lining becomes inflamed and absorptive epithelial cells develop an abnormal appearance. Blunting of the villi, shortening of the microvilli, dilation of the endoplasmic reticulum, swollen mitochondria, and intracellular edema are also observed microscopically. Within 2 weeks, however, the small intestine returns to a normal histological appearance in these healthy volunteers (Agus et al., 1973; Dolin et al., 1975; Schreiber et al., 1973, 1974). However, the range of pathology which may occur in more susceptible people (children and elderly) remains largely unknown.

4.2. Immunity

In some studies, mucosal lesions were found in volunteers who had ingested infectious stool filtrates, but showed no clinical illness (Schreiber et al., 1973, 1974). Still other exposed volunteers showed no signs at all, clinical or pathological, of infection. If these volunteers were later re-challenged with the same inoculum, some developed illness and others did not (Parrino et al., 1977; Wyatt et al., 1974). Thus, some degree of short-term immunity appears to be conferred, at least among some people.

The issue of long-term immunity has also been tested by the volunteer study. Parrino et al. challenged 12 volunteers with the Norwalk agent (Parrino et al., 1977). Six of them fell ill, and when challenged 27–42 months later, they all became ill again. Four of these volunteers were exposed a third time 4–6 weeks later; only one of them fell ill. While these findings support the theory that there is some short-term immunity to HuCVs, long-term immunity does not appear to be conferred by a single infection. Like in other volunteer studies, some of those exposed did not develop any signs of illness. In this study, the same individuals who did not develop illness in the first exposure did not develop illness when re-challenged either. This could not be explained by initial circulating antibody levels. Possibly, differences in a local immune response of the intestinal mucosa or a genetic trait (a specific receptor, for example) may explain why some individuals can develop NLV-related gastroenteritis and others cannot. In 1998, a foodborne NLV outbreak occurred in Australia among a group of tourists visiting an Aboriginal community (Ewald et al., 2000). Though they consumed the same meal, none of the Aboriginal residents fell ill. If it is assumed there is no long-term immunity to NLV, one plausible explanation of this community immunity is that frequent exposure might stimulate sustained immunity. Another possibility is differences in genetic susceptibility.
The cross-challenge of volunteers with Norwalk, Hawaii and Montgomery County agents first began to depict the complicated immunological relationship among different NLV strains (Wyatt et al., 1974). The Norwalk and Hawaii agents (now known to belong to different genogroups) were determined to be antigenically distinct since disease caused from one agent failed to confer immunity to the other. However, those previously infected with the Norwalk agent, did not develop disease when challenged with Montgomery County agent. Years later, serological tests of antibody response to Norwalk, Hawaii and Montgomery County agents confirmed the complicated antigenic relatedness (Madore et al., 1990). Similar to the earlier cross-challenge studies, Madore et al. exposed volunteers to one of three agents. Then after ingestion of one of the other agents, they found that serum antibody titer rose in most volunteers. However, a recent study of NLV in children did find that circulating antibody was protective against infection (Lew et al., 1994). Work by Noel et al. indicated that antibody (AB) is broadly cross-reactive across GGI stains (Noel et al., 1997). However, genetically similar GGII strains were shown to be antigenically distinct. These findings again highlight the complexities of short-term HuCV immunity.

Contrary to rotavirus, high incidence and attack rates of NLV are seen in healthy adults even though most would have been previously infected in childhood. It appears that only a limited short-term immunity is conferred by NLV infection, but our understanding about human immunity to infection and disease due to NLVs remains limited.

5. Diagnostics for calicivirus infection

5.1. Electron microscopy

Since a cell culture system for HuCV has not been developed, EM has been a fundamental tool used by investigators. In the UK, a reporting network of the EM units of the diagnostic public health laboratories has been the basis of the national surveillance system (Caul, 1996). Highly skilled microcopists are required to reliably detect HuCVs from prepared stool samples (Kapikian, 1994), though NLVs can pose a particular challenge since they do not have the distinct calyx (or cup-like) surface morphology of the SLVs (Fig. 1) (Caul and Appleton, 1982).

Specimens are prepared for EM by a fairly simple and inexpensive negative staining technique (Doane, 1994). But, direct detection of HuCVs by EM is only possible in samples with a high viral load. EM is too insensitive to detect HuCVs in samples with less than 10⁶ particles per ml (Doane, 1994). Thus, these enteric viruses can only be detected for approximately 48 h after disease symptoms cease (Caul and Appleton, 1982).

In IEM, stool samples are visualised after reaction with antibody derived from convalescent-phase sera from infected individuals with gastroenteritis (Atmar and Estes, 2001). Antigen and antibody form immune complexes, which can be negatively stained (Doane, 1994). IEM was used by Kapikian et al. in their discovery of the Norwalk

![Fig. 1. Negatively stained EM images of [NLV] particles at \( \times 250,000 \) magnification. Courtesy of Dr H. Appleton, Enteric, Respiratory and Neurological Viruses Laboratory, Central Public Health Laboratory.](image)
agent (Kapikian et al., 1972) and, since then it has been used to characterise other HuCVs (Dolin et al., 1982; Thornhill et al., 1977; Vial et al., 1990), though its use as a diagnostic tool has been limited by the lack of defined antisera.

5.2. Immunoassays

Jiang et al.’s design of a baculovirus expression system for Norwalk viral capsid proteins provided a means of harvesting large amounts of virus particles (Jiang et al., 1992b) that are morphologically and antigenically similar to the native viruses (Green et al., 1993). Hyperimmune serum for use in an antigen-detecting EIA was generated by exposure of animals to the engineered Norwalk virus proteins (rNV) (Jiang et al., 1992b). Recombinant antigens and EIAs have since been designed for numerous other NLV variants including Mexico, Snow Mountain, Hawaii, Desert Shield, Toronto, Grimsby, Sapporo, Southampton and Lordsdale viruses (Atmar and Estes, 2001). Antigen detecting EIAs using monoclonal antibodies (native baculovirus-expressed proteins) have also been prepared for a number of NLVs (Hardy et al., 1996; Herrmann et al., 1995). While these assays are highly sensitive compared with the above-mentioned techniques, their use in diagnostic laboratories has been limited by their narrow specificity (Jiang et al., 2000). In other words, antigen-detecting EIAs will only detect a narrow range of related HuCVs and fail to detect the majority of these genetically diverse viruses.

5.3. Molecular techniques

Amplification of the Norwalk virus by reverse transcription-polymerase chain reaction (RT-PCR) was first achieved by Jiang et al. (1992a) and De Leon et al. (1992) in 1992 and has since become a common diagnostic and research tool worldwide. The complete sequencing of a range of HuCVs (Dingle et al., 1995; Jiang et al., 1993; Lambden et al., 1993) has led to the development of many primer pairs for use in RT-PCR. In comparison to EM, RT-PCR is a far more sensitive diagnostic tool, able to detect virus 2 weeks after infection and possibly longer (Parashar et al., 1998; Yamazaki et al., 1996). Due to the genetic diversity among Caliciviridae, it has been difficult to find an appropriately sensitive and specific primer pair to detect all NLVs. A number of primer pairs for the most conserved region of the genome (the RNA polymerase gene) have been designed and the ones described by Ando et al. (1995b), Green et al. (1995), Vinjé and Koopmans (1996), Le Guyader et al. (1996a) are among those most commonly used (Atmar and Estes, 2001). The helicase, capsid and open reading frame three regions of the virus genome have also been used as a target for RT-PCR (De Leon et al., 1992; Green et al., 2000; Vinjé et al., 2000; Yamazaki et al., 1996). Analysis of more than one region may be important in the detection of unique or recombinant strains (Vinjé et al., 2000). Though there are no reports of a full evaluation of the RT-PCR assays against the full range of currently circulating genotypes, it is likely that the sensitivity of detection varies substantially for different genotypes.

A variation on the PCR, the nested RT-PCR, has been used to further increase sensitivity. Green et al. showed that by using two rounds of PCR, with the second set of primers ‘nested’ within the region captured in the first round, sensitivity can be increased 10 to 1000 times and can have a greater specificity (Green et al., 1998a). Thus, nested RT-PCR may be most applicable to environmental and foodstuff surveys, where virus is found in smaller quantities (Green et al., 1998a). However, the risk of contamination, and, thus, false positive results, is of concern.

A number of techniques are used to confirm and further analyse RT-PCR products. Running products on electrophoresis gel is a simple means of visualising whether a DNA product of predicted length was amplified (Green et al., 1998a). But, non-specific DNA amplification will produce other bands on the gel that can make results difficult to interpret. This is particularly important in environmental samples where virus may be present at low numbers.

There are a number of hybridisation assays (including dot, slot, liquid and Southern hybridisation) where a labelled, virus-specific probe is hybridised with the PCR product. Again, the genetic diversity of NLVs means that there is no single ‘catch-all’ probe but, a small panel can
usually capture the majority of circulating strains (Ando et al., 1995b; Green et al., 1995; Le Guyader et al., 1996a; Vinje and Koopmans, 1996). An enhanced application of this hybridisation principle, the reverse line blot (RLB), makes use of a set of genotype-specific probes simultaneously and hence, provides both confirmation of nucleic acid amplification and genotyping information (Vinje and Koopmans, 2000). The heteroduplex mobility assay (HMA) is another method of typing PCR amplicons. Without sequencing, the HMA can be used to screen for commonly circulating strains (Grimsby and Mexico) and discern them from other (non-Grimsby, non-Mexico) strains (Mattick et al., 2000). The HMA is a useful screening device but in its current format runs the risk of not detecting virus if there is a change in the prevalent genotype. The HMA and RLB techniques are useful in processing multiple samples simultaneously and, therefore, are useful in large-scale epidemiological studies. Introducing such strain typing in the routine diagnostic laboratory could lead to the detection of common source outbreaks.

DNA sequencing of the amplicons, while expensive and labour-intensive, offers the most virological information (Fankhauser et al., 1998; Kukkula et al., 1999; Maunula et al., 1999; Vinje et al., 2000; Yamazaki et al., 1996). Sequences can be compared with those from other samples, and may provide an indication about the common source of an outbreak. Since relatively few complete NLV genomes have been sequenced, and genetic recombination may occur, much remains to be learned about the use of genomic sequence information for molecular epidemiology (Atmar and Estes, 2001; Vinje et al., 2000). Nonetheless, the conserved pol region has the great advantage that it can be amplified for nearly all variants in a single assay.

5.4. Epidemiologic methods

Historically, laboratory confirmation has not been possible for the majority of outbreaks. Of course, this was the situation before Kapikian et al.’s discovery of the Norwalk agent, but, because of relative insensitivity of EM, the aetiology of most outbreaks could still not be confirmed (Kapikian et al., 1972; Kapikian et al., 1982a). Even today, the aetiology of many outbreaks go unconfirmed because the sensitive immuno- and molecular diagnostics are not widely available outside reference laboratories and because appropriate samples are not always collected. HuCV spread can be rapid and the implementation of infection control measures often cannot wait for laboratory diagnosis.

In the absence of laboratory confirmation, epidemiologic traits of an outbreak can be used to identify a viral cause. A set of criteria proposed by Kaplan et al. stipulates that an outbreak can be attributed to a viral cause if: (1) stool cultures are negative for bacterial pathogens; (2) mean incubation time is 24–48 h; (3) mean duration is 12–60 h; (4) and there is vomiting in ≥ 50% of cases (Kaplan et al., 1982a). It has been proposed that the criteria be extended to include increased frequency of vomiting relative to fever as an alternative to criteria (4) (Hedberg and Osterholm, 1993). This method of assessment cannot, however, be used for determining a particular viral aetiology (NLV or SLV, for example), but rather is useful as a public health tool and for assessing the overall burden of viral gastroenteritis.

6. Prevalence and incidence of calicivirus in Europe

6.1. Community-based studies

While a number of longitudinal studies have estimated the incidence of infectious gastrointestinal disease in the community (Hodges et al., 1956; Monto and Koopman, 1980), only a few, recent studies have employed a diagnostic algorithm capable of HuCV detection. The Sensor study followed two cohorts of the Dutch population for 6 months each, from December 1998 to December 1999 (de Wit et al., 2001b). After standardisation, the incidence of infectious gastroenteritis was determined to be 283 cases per 1000 person-years. In the case-control component of the study, viral agents accounted for 34% of all cases, with NLV the most common viral pathogen, accounting for 11% of cases. SLVs were found in 6% of all cases.
Similarly, a community cohort of nearly a half-million was followed-up from 1993 to 1996 in England’s IID study (Sethi et al., 1999). The overall rate of 194 cases per 1000 person-years was lower than the overall rate in the Netherlands (Wheeler et al., 1999). The rate of NLV infection was 13 cases per 1000 person-years (6% of all cases) and the rate of SLV was 2.2 cases per 1000 person-years (0.01% of all cases) (IID Study Team, 2000; Wheeler et al., 1999). These lower rates in England may be because EM was the diagnostic tool for HuCVs as opposed to RT-PCR in the Dutch Sensor study (de Wit et al., 2001b). This may also have contributed to the large proportion of community cases of unknown aetiology (61%) in the IID study. Nevertheless, NLV was still the most common organism detected.

In Finland, a 2-year follow-up of approximately 2400 children (2 months to 2 years of age) in a randomised, double-blind, placebo controlled rotavirus vaccine trial confirmed the importance of HuCV (Pang et al., 2000). Using RT-PCR to examine stool samples, HuCVs were found to be as common as rotavirus among the cohort of children. NLVs were responsible for 20% of cases and SLV for 9%. By scoring the severity of disease episodes among the children in the cohort, this study analytically confirmed that NLV infection, with its high frequency of vomiting, is a more severe disease than is caused by SLV, but is less severe than rotavirus disease. Therefore, surveys which include a community (sub-clinical) component would be expected to ascertain a larger burden of NLV than GP or hospital-based studies.

6.2. General practices

The UK’s IID study also showed that HuCV rates, when measured by presentation to a general practitioner (GP), were approximately one-sixth of those in the community. However, there may be a substantial under-ascertainment of community cases since institutions where outbreaks may be disproportionately frequent (such as hospitals, residential homes, universities and prisons) were excluded from the study population (Wheeler et al., 1999). Nonetheless, 6.5% of the cases presenting to a GP tested positive for NLV and 1.5% tested positive for SLV (Tompkins et al., 1999).

A similar GP-based study in the Netherlands found NLVs slightly less frequently (5.0% of cases) (de Wit et al., 2001a). SLVs were detected in 2.0% of cases. Since the Dutch study used RT-PCR to detect HuCVs and the English study used the less-sensitive EM, it is somewhat surprising that rates were lower in the Netherlands. This may be due to a real difference in NLV incidence between the two countries, but there is also evidence that consultation rates for gastroenteritis may be lower in the Netherlands due to an active deferral policy (de Wit et al., 2001a).

6.3. Surveillance

A system of general outbreak surveillance for IID in England and Wales has been operated from the Communicable Disease Surveillance Centre (CDSC) since 1992. From its origin until 1995, information on 2154 general outbreaks had been collected (Dedman et al., 1998). Laboratory testing (primarily EM) confirmed 709 (33%) of these outbreaks were caused by NLV. SLV outbreak reports are far less common and account for only a few outbreaks a year (Evans et al., 1998).

Surveillance conducted in Sweden from 1994 to 1998 also points out the high fraction of gastroenteritis outbreaks attributable to NLVs (Hedlund et al., 2000). Of 676 outbreaks analysed, 407 (60%) were attributed to NLV and nine were attributed to SLVs (by EM). HuCVs were the predominant aetiology of outbreaks in every investigated setting and thus, Hedlund et al. concluded that NLVs cause most outbreaks in both hospital and community outbreaks in Sweden (Hedlund et al., 2000). Similarly, HuCVs were found as the primary cause of outbreaks in Finland (Maunula et al., 1999), Germany (Schreier et al., 2000) and The Netherlands (Koopmans et al., 2000; Vinje et al., 1997).

In a French survey, Bon et al. detected NLVs by RT-PCR in 14% of stools of children consulting their GP. None of the detected agents were SLVs (Bon et al., 1999).
Although detailed community based studies have only be done in a few European countries, data from the outbreak surveillance systems suggest that NLV is a common infection around the world.

6.4. Seasonality

Since Zahorsky’s original description of ‘winter vomiting disease’, it has been clear that viral gastroenteritis has a distinct seasonality (Zahorsky, 1929). Mounts et al.’s review of 12 studies showed that NLV is predominantly seen during the colder months of the year (Mounts et al., 2000). This was the case regardless of patient’s age, methods of detection or whether case or outbreak was the studied event. In European settings, rates begin increasing in October or November, peak around January, and tail-off by May or June (Dedman et al., 1998; Hedlund et al., 1999; Koopmans et al., 2000; Maunula et al., 2000; Pang et al., 2000; Schreier et al., 2000; Vinje et al., 1997).

6.5. Age distribution

NLV and SLV infections can occur at any age. Age-specific estimates depend heavily on whether surveillance is conducted at the doctor’s office or in the community, and what population is covered by the surveillance. The highest incidence of HuCV infection is in children under 5 years and, among children, the most common cause of gastroenteritis is viral, with NLV being at least as frequent as rotavirus (de Wit et al., 2001b; Tomkins et al., 1999; Pang et al., 2000). Dutch GP data suggests the odds of seeing a doctor because of NLV infection generally decreases with increasing age (de Wit et al., 2001a). English GP data suggests the same, with rates among under 2 year olds and 2–4-year-olds much higher (＞1200 and ＞300 cases per 100,000 person-years, respectively) than those of the other age groups (5–89 years, approximately 100 cases per 100,000) (IID Study Team 2000). Seroprevalence studies (discussed below) demonstrate that the majority of children have an infection by the age of 5 years. Still, NLV, unlike SLV, is a common pathogen among adults (de Wit et al., 2001b; IID Study Team 2000).

It should be remembered that GP-based studies essentially measure consultation rates, not infection rates. Since HuCVs typically cause a fairly mild infection, rates derived from GP settings may be inaccurate and biased towards children, who may be more likely to consult and may have a more severe disease. Also, the population-based studies may have under-ascertained NLV and SLV in the elderly because institutions, including nursing home and hospitals were excluded (Sethi et al., 1999).

Thus, even though rates derived from routine surveillance suffer from under-ascertainment and are biased towards institutional settings, they may provide insight into the importance of HuCV (Dedman et al., 1998). A large number of HuCV outbreaks are reported from nursing homes with elderly residents (Dedman et al., 1998; Hedlund et al., 2000; Koopmans et al., 2000; Maunula et al., 1999; Schreier et al., 2000; Vinje et al., 1997). However, attack rates are only slightly lower among staff than among elderly residents (Dedman et al., 1998; Vinje et al., 1997).

6.6. Seroprevalence

Several methods for detecting Ab to HuCV have been developed to allow for the screening of large numbers of specimens (Atmar and Estes, 2001). In the first generation of these, the immune adherence hemagglutination assay (IAHA), purified viral particles from stool specimens are used as antigen (Ag), and form antigen–antibody–complement interactions that are detected on a microtiter plate (Greenberg and Kapikian, 1978). However, this technique was quickly replaced by the blocking radio-immunoassay (RIA) (Brandt et al., 1981; Nakata et al., 1983) which requires less antigen and is more sensitive (Greenberg and Kapikian, 1978). Nakata et al. then converted the RIA into a more widely applicable enzyme immunoassay (EIA) with no loss in sensitivity (Nakata et al., 1988). However, these techniques were limited in use by diagnostic and public health laboratories since the required reagents could not be derived from any non-hu-
man source (Jiang et al., 2000). Also, considering what we know now about the considerable diversity of NLV, it is unlikely that these tests were broadly reactive.

However, EIAs designed to detect human antibody to HuCVs are broadly reactive. Thus, a number of sero-epidemiology studies have been conducted in Europe and worldwide using antibody-detecting EIAs. Serologically-based assays are unlikely to be used as diagnostic tool since it is difficult to discern if antibody presence is from a current or previous infection (Caul, 1996; Jiang et al., 2000).

The difficulties of studying the sero-epidemiology of HuCVs has largely been overcome by the advent of NLV baculovirus capsid protein expression systems (Jiang et al., 1992b). EIAs have been designed to detect Abs to the prototype Norwalk virus (NV) as well as eight other HuCV strains (Jiang et al., 2000). These Ab assays, which are based on recombinant virus-like protein (VLP), are more widely reactive than Ag tests (Gray et al., 1994; Jiang et al., 1992b; Okhuysen et al., 1995; Treanor et al., 1988). Ab assays have, therefore, been used in a number of large seroprevalence studies that have concluded that HuCVs are extremely common worldwide (Black et al., 1982; Chiba et al., 1980; Cubitt et al., 1998; Greenberg et al., 1979; Jing et al., 2000; O’Ryan et al., 1998; Parker et al., 1994; Smit et al., 1999).

European seroprevalence surveys have concluded the same (Fig. 2). In a survey (N = 3250) in England, Gray et al. found that nearly three-quarters of those tested had rNV antibody (Gray et al., 1993). Ab prevalence was highest among the middle age and elderly; at every group 30 years or older, Ab prevalence was near 90%. Prevalence was also fairly high among infants (< 6 months old) at 75%. This is likely a measure of maternal antibody, reflecting the high seroprevalence among adults. In the 6–11 months age, antibody prevalence was 25%, then rising through adolescence and young adulthood. A very similar pattern was found in Sweden (Ab to NV) with overall prevalence at approximately 80% (Hinkula et al., 1995). In Norway, a survey of military recruits detected NV Ab in 30% which is considerably less than the same age group in other European countries (Myrmel et al., 1996).

Viruses closely related to the original NV are rarely found in molecular studies, yet seroprevalence of Ab to NV is high (but varying from country to country). Ab to Southampton virus (SV), another GGI virus, which is more commonly recognised than NV was found to have a much lower seroprevalence in Italy (only 30%) (Pelosi et al., 1999). There are several explanations for this difference, but parallel studies using harmonised assays are needed to compare seroprevalence rates across countries.

The issue of Ab acquisition at young ages was further addressed in a survey of 400 children (and 100 adults) in London (Parker et al., 1994). Sixty one percent of neonates (younger than 2 months) had Ab to NV. Prevalence fell to 3% during the first year of life, then consistently rose in every sequential age group through childhood. Parker et al. suggest that Ab prevalence quickly rises at around 2 years of age when children begin to come in frequent contact with other children (Parker et al., 1994).

Lew et al.’s 2-year serologic follow-up of Finnish infants provided insight into NV infection in the first 2 years of life (Lew et al., 1994). Serum was taken four times throughout follow-up. Half of all children exhibited a serologic response (as defined by a ≥ 4-fold increase in IgG titre) between consecutive testing. The majority of these seroresponses occurred between 4 and 14 months of age (31%) as opposed to between 14 and 23 months (19%). And, therefore, by (mean age) 23 months, 73% had NV IgG. This is markedly higher than was found at this age in other prevalence studies, Europe and worldwide.

7. Transmission of human caliciviruses

The HuCVs cause outbreaks through a number of well-documented transmission routes including person-to-person, foodborne and waterborne routes. In recent years the role of environmental contamination and contamination of raw fruit and vegetables have been demonstrated. The possibility of zoonotic spread remains plausible. Person-to-person transmission has been documented by two routes, faecal–oral and aerosol formation.
following projectile vomiting. In fact, outbreaks are often, if not always, the result of more than one mode of transmission and it may be difficult to attribute the outbreak to a single mode of transmission. For example, a food-handler may become infected by a person in his or her home. The food-handler then can contaminate a food product, which can lead to an outbreak, which is then perpetuated by person-to-person transmission and/or environmental contamination (Pether and Caul, 1983). Thus, an outbreak may be seeded by one mode, yet the majority of infections can be caused by a different mode (see Fig. 3).

7.1. Person-to-person transmission

Overall, person-to-person spread is the most commonly recognised mode of transmission in
outbreaks. From 1992 to 1995, person-to-person transmission was reported as the primary mode in 72% NLV outbreaks in England and Wales (Dedman et al., 1998). The characteristically high attack rate (≈ 50%) of NLVs occurs as a result of their low infectious dose and by being excreted before (~ 12 h) and after (2 weeks or more) illness (Cliver, 1997; Kaplan et al., 1982a). It has long been known that infectious particles can be transmitted by the faecal–oral route (Dolin et al., 1971; Jordan et al., 1953) but evidence is mounting that airborne transmission is also important. The high frequency of vomiting associated with HuCV, and the resultant aerosolisation of particles makes such a mode feasible. A number of outbreaks in closed settings or institutions have likely been due to extensive vomiting (Caul, 1994; Chadwick and McCann, 1994; Chadwick et al., 1994; Noah, 1994). A recent example occurred when a diner vomited at a restaurant. Sequence analysis showed that diners who subsequently fell ill (mean 36 h) had identical NLV strains in their stool. No food items were implicated, but people sitting closer to the diner who vomited had higher risk of infection (Marks et al., 2000). Since respiratory infection by HuCV has not been found, aerosolised virus must presumably be swallowed after inhalation for infection of the intestine to occur.

7.2. Foodborne transmission

Estimates of the relative importance of foodborne transmission of HuCV vary from country to country. In Sweden, 16% of HuCV outbreaks from 1994 to 1998 were associated with food- or water-borne transmission; in the UK food was implicated in 5% of outbreaks from 1992 to 1999 as were 17% of outbreaks reported through municipal health services in The Netherlands (Koopmans et al., 2000). In the US, food vehicles were estimated to play a more important role (40% of NLV infections) (Mead et al., 1999). Regardless of whether these differences are real or an artefact of methodology, foodborne infection appears to be an important mode of transmission since modern foodstuff distribution allows viruses to reach a wide population. Foodborne vehicles of HuCV infection are typically one of three forms: contaminated bivalve shellfish, items contaminated by

Fig. 3. Transmission routes of human caliciviruses The relatively large size of the person-to-person symbol represents current understanding of its importance as the dominant mode of transmission. However, other routes, like environmentally contaminated shellfish, may seed wide epidemics, introduce new strains to an area or cause infection with multiple strains—thus providing the right circumstances for genetic recombination to occur. Often, outbreaks are not exclusively spread by one route and attributing an outbreak to a single mode of transmission is somewhat arbitrary.
infected food handlers or fruits/vegetables contaminated through irrigation or washing.

7.2.1. Bivalve molluscs

Bivalve shellfish are filter-feeders. Although HuCVs do not replicate in shellfish gut, the shellfish accumulate and concentrate viruses from sewage-contaminated waters. Since oysters are often consumed raw or slightly cooked, they are particularly efficient at causing infection (Advisory committee on the microbiological safety of food, 1998; Ministry of Agriculture Food and Fisheries 1996). In Europe, Council Directive 91/42/EEC determines commercial standards for oysters. Oyster samples are tested monthly by bacterial analysis of the shellfish flesh, but not for viruses (Green et al., 1998a; Le Guyader et al., 1996b). While bacterial monitoring is a good indicator of faecal contamination, it is limited in that bacterial counts may fluctuate more rapidly than viral counts and viruses can survive longer in marine environment than bacterial pathogens (Ministry of Agriculture Food and Fisheries 1996). Thus, samples that satisfy bacterial standards may still be contaminated with viruses (Lees, 2000). In addition, depuration procedures that successfully remove bacteria are inadequate at removing viruses.

Oyster outbreaks are frequently reported, though people who eat oysters often accept the illness that comes with them (Ang, 1998; Chalmers and McMillan, 1995; Dowell et al., 1995; Godoy et al., 2000; Gunn et al., 1998a; Le Guyader et al., 1996b). While bacterial analysis of the shellfish flesh, but not for viruses (Green et al., 1998a; Le Guyader et al., 1996b). While bacterial monitoring is a good indicator of faecal contamination, it is limited in that bacterial counts may fluctuate more rapidly than viral counts and viruses can survive longer in marine environment than bacterial pathogens (Ministry of Agriculture Food and Fisheries 1996). Thus, samples that satisfy bacterial standards may still be contaminated with viruses (Lees, 2000). In addition, depuration procedures that successfully remove bacteria are inadequate at removing viruses.

Oyster outbreaks are frequently reported, though people who eat oysters often accept the illness that comes with them (Ang, 1998; Chalmers and McMillan, 1995; Dowell et al., 1995; Godoy et al., 2000; Gunn et al., 1982; Stafford et al., 1997). Outbreaks are under-recognised since cases who ate the same infectious shellfish are often not identified as part of the same outbreak due to their geographical separation (Dowell et al., 1995). Nonetheless, shellfish outbreaks may represent a particularly important mode of transmission. Since shellfish typically become contaminated by sewage, they may harbour multiple HuCV strains that are circulating in the community (Lees, 2000). This is supported by outbreak investigations which have found mixed infections (from NLV genogroup I and genogroup II) in patients after consumption of raw shellfish (Ando et al., 1995a; Sugieda et al., 1996). Since genetic recombination of HuCVs does in fact occur (Vinjé et al., 2000), multi-strain infections seeded by shellfish would provide a plausible setting for this to happen.

7.2.2. Products contaminated by food-handlers

Since HuCVs can probably be destroyed by adequate cooking (> 1 min at 90 °C), vehicles contaminated by infected food handlers are typically products eaten raw (such as salads) or not cooked after handling (such as sandwich fillings) (Kilgore et al., 1996; Viral gastroenteritis sub-committee of the PHLS virology committee, 1993). In many non-shellfish foodborne outbreaks, a food-handler who was ill prior to or during preparation of the implicated food can be identified (Hedberg and Osterholm, 1993). There is potential for large-scale outbreaks. In an exceptional example, a bakery employee infected over 3000 people (Kuritsky et al., 1984). The baker had multiple episodes of vomiting and diarrhoea on his way to work and during his shift. As part of the preparation of some 10 000 frosted food items, he submerged his arms in 76 l of cream frosting, thus contaminating the batch.

The potential of foodborne contamination from vomitus was proven by the investigation of an outbreak at a hotel in North Yorkshire, England where a kitchen employee suddenly vomited in a sink (Patterson et al., 1997). The sink was cleaned with a chlorine disinfectant, but still had sufficient NLV load to contaminate a potato salad prepared in the sink the following day.

Using molecular typing Daniels et al. proved an outbreak was a result of contamination by a food-handler (Daniels et al., 2000). By RT-PCR, they detected the sample sequence in: (1) stool of university students who ate from a sandwich bar; (2) ham served at the bar; and (3) from the child of a food-handler infected before the university outbreak.

Typical guidelines usually suggest that food-handlers do not return to work for 48–72 h after their symptoms cease, and that none of their family members or close contacts are ill (Hedberg and Osterholm, 1993; Viral gastroenteritis sub-committee of the PHLS virology committee, 1993). However, outbreak investigations have found NLV in the stool of food-handlers after 10
symptom-free days (Parashar et al., 1998). Further complicating the issue is the potential of food-handlers to shed virus before symptoms commence (Stolle and Sperner, 1997). In a number of outbreaks, food-handlers, who were probably incubating the disease fell ill soon after they prepared an implicated food vehicle (Guest et al., 1987; Lo et al., 1994; Pether and Caul, 1983). It would be difficult to recommend control measures, aside from general hygiene, in situations of the pre-symptomatic food-handler. In most situations, it is recommended that food-handlers do not return to work until 48 h after symptoms cease. Longer absences are typically impractical even though viral shedding may continue much longer than this period.

7.2.3. **Fruit and vegetable contamination**

Molecular typing also helped to elucidate an international foodborne outbreak. The detection of matching sequences from incidents in Europe and Canada provided evidence that cases were linked (Gaulin et al., 1999b; Glass et al., 2000; Ponka et al., 1999). Further epidemiologic investigation determined that the international outbreak could be traced to raspberries from Slovenia. Fruit and vegetables can become contaminated by their irrigation waters, by washing or spraying prior to freezing or by infected food handlers involved in harvesting.

7.3. **Waterborne transmission**

Drinking water can provide a source for outbreaks of viral gastroenteritis in nearly any setting. Outbreaks have occurred as a result of contamination of private wells, public wells as well as small and large-scale community water systems (Brugha et al., 1999; Kaplan et al., 1982c; Kjeldsberg et al., 1989; Kukkula et al., 1997; Lawson et al., 1991; Wilson et al., 1982). Supplies can become infiltrated with contaminated water in times of heavy rainfall when floods occur (Cannon et al., 1991). In one water system, a drop in pressure due to heavy demand allowed the mixing of potable and nonpotable waters (Kaplan et al., 1982c). Outbreaks have occurred due to municipal sewage and septic tank leaks as well as runoff contamination into springs and streams (Hedberg and Osterholm, 1993). In France, investigators of a hospital outbreak successfully detected identical sequence NLV in both tap water and stool samples from patients (Schvoerer et al., 1999). Another large outbreak affecting 1500–3000 people occurred in Finland after a groundwater well was contaminated by polluted river water during a flood (Kukkula et al., 1997).

Commercial distribution and production of ice facilitated a geographically wide outbreak of NLV (Cannon et al., 1991). Ice from a manufacturer whose facilities were contaminated by flooding was implicated in outbreak involving cases from Pennsylvania to Delaware. Bottled waters may also be contaminated. A Swiss investigation detected NLVs by RT-PCR in 21 of 63 bottles of commercially available mineral water, though no cases were linked (Beuret et al., 2000) and so far the results could not be confirmed by others.

7.4. **Other environmental contamination**

In part, the importance of waterborne outbreaks is due to the resilience of HuCVs. When compared with a panel of other waterborne pathogens (including poliovirus type 1, human rotavirus, simian rotavirus and f2 bacteriophage) NLVs are more resistant to chlorine inactivation (Keswick et al., 1985). Thus it is not surprising that NLVs can be found in a wide range of environmental settings. RT-PCR methods have been used to detect NLVs in bathing and recreational waters (Schvoerer et al., 2000), sewage (Lodder et al., 1999) and on surfaces of different materials (Green et al., 1998b; Wyn-Jones et al., 2000). However, due to the high sensitivity of RT-PCR, a positive result does not always signify presence of infectious virus. In hospital outbreaks, surfaces including lockers, curtains and commodities become contaminated with virus (Green et al., 1998b). A wide range of objects was shown to be contaminated during an outbreak in a North-West England hotel (Cheesbrough et al., 2000). Apart from more predictable surfaces such as carpets and toilet seats, other surfaces such as tables, mantle pieces and light fittings had detectable levels of virus on their surfaces.
Nonetheless, reports of outbreaks attributed to an environmental exposure have been largely descriptive and reliant on circumstantial evidence (Cheesbrough et al., 1997; Gray et al., 1997). For example, the NLV infection of two men who removed carpet after a hospital outbreak was attributed to this environmental exposure after other exposures were ruled out (Cheesbrough et al., 1997).

7.5. Zoonoses

Caliciviruses are important pathogens in animals as well as humans. However, numerous unsuccessful attempts to infect animals (aside from chimpanzees) (Wyatt et al., 1978) as well as cell lines with HuCVs suggests that NLVs and SLVs are highly species-specific pathogens (Clarke and Lambden, 1997). Interspecies transmission, if it does occur, is likely to be a very rare event.

However, recent discoveries have piqued interest in the possibility of zoonotic transmission. In Japan NLV genes were detected by RT-PCR in the caecum of pigs (Sugieda et al., 1998). In the UK, the molecular characterisation of bovine caliciviruses have proven that they are more similar to human NLV than to animal caliciviruses (Dastjerdi et al., 1999; Liu et al., 1999). The detection of NLV sequences in 33 of 75 (44%) pooled faecal samples from veal farms in different geographic areas in the Netherlands suggested that this NLV infection is naturally occurring (van Der Poel et al., 2000). Thus, the sequence similarity of swine and bovine strains to human strains suggests that animal reservoirs of calicivirus infection are plausible.

Additionally, these viruses have been shown to cause diarrhoeal outbreaks in calves under experimental conditions (Dastjerdi et al., 1999; Liu et al., 1999). Animal (sea lion) caliciviruses have been observed to infect different species (Smith et al., 1983), such interspecies transmission is probably very uncommon. Aside from the extreme scenario of a lab worker injected with a San Miguel sea lion calicivirus (Smith et al., 1998), zoonotic transmission has never been observed. It is also important to remember that genetically similar viruses (e.g. influenza) can exist in different species without resulting in substantial zoonotic transmission (van Der Poel et al., 2000).

7.6. Transmission in hospitals and nursing homes

For a number of reasons, NLVs pose a particular risk to health in hospitals and residential/nursing homes. Virus can be introduced into institutions by food, water or a visitor from the community (Chadwick et al., 2000; Lo et al., 1994; Pether and Caul, 1983; Schvoerer et al., 1999). And since living quarters in both nursing homes and hospitals are tight and personal hygiene may be reduced as a result of poor health or incontinence, conditions are prime for person-to-person transmission. Since illness will strike the elderly or those already ill, HuCV is more likely to cause severe illness or death in these settings (Centers for Disease Control and Prevention, 2001). For all these reasons, very large and protracted outbreaks, which are difficult to control, can occur in these institutional settings (Gellert et al., 1990; Mayoral et al., 2000).

The high attack rates typical of HuCV outbreaks are not confined to residents and patients. Attack rates among both staff can be in the range of 30–50% (Caceres et al., 1998; Gellert et al., 1990; Green et al., 1998b; Kaplan et al., 1982d; Marx et al., 1999; Mayoral et al., 2000; Vinje et al., 1997). Infection among staff poses a problem in two respects: (1) their movements facilitate transmission to new areas; and (2) their resulting absence from work causes staffing shortages and economic loss (Caceres et al., 1998; Pether and Caul, 1983).

From 1992 to 1995, 277 hospital and 263 residential home outbreaks (39 and 37% of total, respectively) were detected by routine surveillance in England and Wales (Dedman et al., 1998). While this surveillance system may be more sensitive at detecting institutional outbreaks compared with food-mediated or community outbreaks, the sheer numbers illustrate the importance of institutional NLV outbreaks (Dedman et al., 1998). The same was found during the 1997–1998 winter season in Germany where one third of all outbreaks occurred in hospitals and one-third occurred in nursing homes (Schreier et al., 2000).
Table 1
The frequency of [NLV] genogroup I and II across Europe

<table>
<thead>
<tr>
<th>Setting</th>
<th>Year</th>
<th>% Genogroup I</th>
<th>% Genogroup II</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Outbreak-based studies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>West and North Yorkshire and Humberside, England (Hale et al., 2000)</td>
<td>1992-1998</td>
<td>&lt;1</td>
<td>68</td>
</tr>
<tr>
<td>The Netherlands (Koopmans, 2001)</td>
<td>1996-1999</td>
<td>10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>90&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Germany (Schreier et al., 2000)</td>
<td>1997-1998</td>
<td>6</td>
<td>94</td>
</tr>
<tr>
<td>East Anglia, UK (Maguire et al., 1999)</td>
<td>1996-1997</td>
<td>2</td>
<td>98</td>
</tr>
<tr>
<td><strong>Case-based studies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>French hospital (Traore et al., 2000)</td>
<td>1996-1998</td>
<td>8</td>
<td>79</td>
</tr>
<tr>
<td>Irish hospital (Foley et al., 2000)</td>
<td>1996-1998</td>
<td>14</td>
<td>86</td>
</tr>
<tr>
<td>Dijon, France (Bon et al., 1999)</td>
<td>1995-1998</td>
<td>11</td>
<td>89</td>
</tr>
<tr>
<td>The Netherlands (Koopmans, 2001)</td>
<td>1996-1999</td>
<td>16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>84&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

NB: Genogroup I plus II does not always equal 100% as some specimens are not captured by characterization method used in investigation.

<sup>a</sup> Yearly average.

Surveillance of cases of gastroenteritis in hospital settings has demonstrated the considerable amount of diarrhoea attributable to NLV (Foley et al., 2000) and the importance of nosocomial transmission (Traore et al., 2000). The true frequency of outbreaks in institutional settings remains to be determined partially because such settings have been excluded from longitudinal study (de Wit et al., 2001a; Sethi et al., 1999).

8. Molecular epidemiology of calicivirus in Europe

8.1. Molecular epidemiology

As well as improving diagnostics, molecular techniques have been applied epidemiologically to further understanding of the modes of transmission, the endemicity of HuCV and the occurrence of epidemics. As is demonstrated in Table 1, GGII NLVs are predominant in Europe just as they are worldwide (Fankhauser et al., 1998; Smit et al., 1999). Sporadic case or outbreak surveillance schemes, regardless of whether they are community-based or set in hospitals, routinely confirm that GGII strains are responsible 75% or more of the time (Bon et al., 1999; Foley et al., 2000; Hale et al., 2000; Koopmans, 2001; Maguire et al., 1999; Schreier et al., 2000; Traore et al., 2000).

However, the distribution of genotypes differs between countries and within countries from year to year. In West and North Yorkshire and Humberside, UK, three antigenic types were monitored over a 6-year period (from 1992 to 1998) (Hale et al., 2000). The GGII strains (represented by Grimsby-like and Mexico-like viruses) were predominant for the entire study period. In the first year of the study, Grimsby predominated. In the second year, the Mexico virus emerged as the most common strain, only to again be replaced by the Grimsby strain the following year. A similar pattern was seen in outbreaks in the Netherlands (Koopmans et al., 2000; Koopmans, 2001). The Mexico-like virus emerged as the cause of many outbreaks in the 1994 season. From 1995 to mid-1996, an ‘epidemic’ of outbreaks was caused by the Grimsby-like virus (in the Netherlands and worldwide) (Noel et al., 1999) and was followed by a small ‘epidemic’ of a Leeds-like strain. Certain strains were more common in the community cases compared with outbreaks suggesting that these strains, which fail to cause outbreaks, may be less virulent (Koopmans, 2001).

These examples of the emergence of a single strain raise fundamental questions about the biological and epidemiological events that allow such events to occur. Since NLV infection only seems to confer short-term immunity, changes in herd immunity could permit the emergence of a new
endemic strain (Hale et al., 2000). Perhaps animal reservoirs exist or genetic variants can develop altered tissue tropism (and can spread more easily by the respiratory route, for example) (Koopmans et al., 2000). Widely distributed water and foodborne outbreaks also provide a means of introducing a new genotype to a new area. Use of molecular tracking, as exemplified in an international NLV outbreak transported by raspberries (Ponka et al., 1999), will be key in identifying seeding events where a virus may eventually cause an epidemic or become endemic in a population.

9. Calicivirus worldwide

In Europe and worldwide, antibody prevalence to HuCVs is high (Jing et al., 2000; Nakata et al., 1998; Ryder et al., 1982). An international study of NV antibody found seroprevalence across all ages to be ≥ 70% in Nepal, Bangladesh, Switzerland, Belgium, Ecuador and the US (Greenberg et al., 1979). Among relatively isolated peoples, markedly lower prevalence has been found in the Gabaro villagers in Ecuador (0/16 positive) (Greenberg et al., 1979) or the Amazonian Maiogong villagers (39% positive) (Gabbay et al., 1994). But, these findings as well as those in Europe, Italy (Pelosi et al., 1999) and Norway (Myrmel et al., 1996) of low seroprevalence may be due to the testing strategy. Furthermore, even in some areas where antibody prevalence is high, it has not been possible to establish a clear link between seroconversion and diarrhoeal disease (Black et al., 1982; Smit et al., 1997).

The relative burden of particular modes of transmission may also differ internationally. If nursing practices or hospital design varies from country to country, perhaps person-to-person transmission will be less burdensome in some places. Likewise, food hygiene practices or water quality control measures would affect HuCV transmission. However, comparison of international figures must take into account the differences in surveillance schemes. For example, it is difficult to tell if the discrepancy of the reported 40% foodborne transmission of NLV in the US (Mead et al., 1999) and approximately 10% foodborne in the UK (Dedman et al., 1998) is a real difference or is artefactual.

10. Conclusions

The public health impact of HuCV infections are increasingly being recognised. The NLVs are the commonest cause of outbreaks of non-bacterial gastroenteritis, the most commonly recognised foodborne viral infection and second only to rotavirus as a cause of severe diarrhoea in children. Key factors underpinning this high burden of infection are their low infectious dose, their stability in the environment, the wide diversity of strains and the lack of any long-term immunity to infection or illness.

The recent development of sensitive molecular tests for diagnosis, quantification and characterisation of these agents has led to the recognition of the importance of NLV infection. Future application of these approaches to understanding routes of transmission and the effectiveness of control measures holds real promise for improved understanding of the epidemiology of HuCVs and improved control measures for foodborne transmission. Early studies of candidate HuCV vaccines have shown good immune responses and further study of these vaccine candidates and of the immune correlates against HuCV disease is likely to provide important insights on intestinal mucosal immunity.

References


Gray JJ, Cunliffe C, Ball J, Graham DY, Desselberger U, Estes MK. Detection of immunoglobulin M (IgM), IgA,


Liu BL, Clarke IN, Caud EO, Lambden PR. Human enteric caliciviruses have a unique genome structure and are distinct from the Norwalk-like viruses. Arch Virol 1995;140:1345–56.
Matson DO, Zhong WM, Nakata S, Numata K, Jiang X, Pickering LK, Chiba S, Estes MK. Molecular characterization of a human calicivirus with sequence relationships...


Wyn-Jones AP, Pullin R, Dedoussis C, Shore J, Sellwood J.


La rougeole en France : impact épidémiologique d'une couverture vaccinale sub-optimale

I. Bonmarin, D. Levy-Bruhl
Institut de Veille Sanitaire, Saint-Maurice, France

La promotion de la vaccination en France depuis 1983 a abouti à une réduction de 97% de la morbidité et de 60% de la mortalité de la rougeole. Cependant, le taux de couverture stagnant et sub-optimal à 84% entraîne un déplacement de l'âge des cas dans des tranches où les complications sont plus fréquentes et sévères. Ainsi, la proportion des plus de 10 ans est passée de 13% en 1985 à 48% en 1997, la transmission de la rougeole se maintenant en France. Pour éliminer la maladie, une couverture vaccinale à 2 doses et à plus de 95% serait nécessaire.

L'introduction de la vaccination a fait chuter l'incidence de la rougeole dans beaucoup de pays du monde.

En 1994, le continent américain s'est engagé dans une politique d'élimination de la maladie qui a abouti à l'interruption de la transmission indigène du virus en Amérique du Nord et à une baisse considérable de l'incidence dans le reste du continent (1,2). Les pays d'Europe dont la France se sont à leur tour engagés dans une politique d'élimination de la rougeole qui doit aboutir en 2007 (3). La Finlande n'enregistre déjà plus de cas autochtone depuis 1996 (4).

La France est-elle sur le même chemin ? Pour répondre à la question, voici un état des lieux de l'épidémiologie de la rougeole en France.

Évolution de la politique vaccinale

Le vaccin a été mis sur le marché en France en 1966 et introduit dans le calendrier vaccinal en 1983, à l'âge de 12-15 mois. Trois ans plus tard, la ➤

SURVEILLANCE REPORT

Measles in France: the epidemiological impact of suboptimal immunisation coverage

I. Bonmarin, D. Levy-Bruhl
Institut de Veille Sanitaire, Saint-Maurice, France

The promotion of immunisation in France since 1983 has resulted in a 97% reduction in morbidity and a reduction of 60% of mortality. However, the stable and sub-optimal coverage around 84% leads to a shift in higher age groups, where complications are more frequent and serious. The proportion of those aged over 10 years was 13% in 1985 and reached 48% in 1997, the transmission of measles being maintained in France. To eliminate the disease, vaccine coverage with 2 doses and over 95% would be necessary.

The incidence of measles has decreased in many countries around the world since the introduction of immunisation.

In 1994, a policy was initiated in the Americas to eliminate the disease. This has resulted in the interruption of the virus’ autochthonous transmission in North America, and a substantial decrease of its incidence in the rest of the Americas (1,2). European countries, including France, have set themselves the goal of eradicating measles by 2007 (3). In Finland, no autochthonous cases of measles have been recorded since 1996 (4).

Is France on the way to achieving this goal? To answer this question, this article presents an overview of the epidemiology of measles in France.

Evolution of the immunisation policy

The vaccine came on the market in 1966 in France and was introduced into the vaccination schedule in 1983, for children ➤
vaccination contre les oreillons et la rubéole y était associée.

En 1996, une seconde dose, justifiée par la perspective d’élimination de la maladie, était introduite pour les enfants âgés de 11 à 13 ans. Cette seconde dose permet de protéger les enfants qui ont échappé à la vaccination et ceux qui n’y ont pas répondu (5 à 10 % d’échecs après une première dose). Elle évite l’accumulation de sujets non-protégés et empêche ainsi l’élosion de foyers épidémiques. Des travaux de modélisation (5) ont montré que l’élimination de la rubéole serait plus rapide en avançant l’âge de la seconde dose. Elle est donc maintenant recommandée entre 3 et 6 ans (6).

Jusqu’à 6 ans, un rattrapage est organisé pour que les enfants reçoivent 2 doses. Passé cet âge et jusqu’à 13 ans, une seule dose est proposée à ceux qui auraient échappé complètement à la vaccination. Les vaccinations sont réalisées principalement par les médecins libéraux. Des campagnes de promotion ciblées sur le triple vaccin Rubéole-Rubéole-Oreillons sont organisées chaque année. En 1999, l’élément phare de la campagne a été la gratuité du vaccin. En le rendant plus accessible, cette action a conféré au vaccin un statut prioritaire.

Surveillance de la couverture vaccinale


Par ailleurs, des enquêtes transversales bisannuelles sont menées en milieu scolaire sur un échantillon représentatif de la population française âgée de 6 ans.

Figure 1
Vaccination coverage surveillance

En 1996, a second dose, justified by the elimination prospect of the disease was introduced for children aged 11-13 years. This second dose enables us to protect children who have missed vaccination, and also those for whom it was not effective (there are failure rates of between 5 and 10% after the first dose). This prevents the accumulation of unprotected subjects, thereby hampering the emergence of clusters. Modelling studies (5) have shown that the disease could be eliminated faster if the second dose were given to children at a younger age, and recommendations are now to give it to children between 3 and 6 years of age (6).

Up to the age of 6, two doses are administered. After 6 and until 13, only one dose is offered to those who have never been vaccinated. Vaccination is carried out mostly by doctors in private practice. Campaigns are carried out each year to promote the triple vaccine for measles, mumps and rubella (MMR). The focus of the 1999 campaign was that the vaccine was available free of charge. This action gave the vaccine a priority status by making it more accessible.

Vaccination coverage surveillance

Since 1985, data on vaccination coverage have been collected and managed by the Direction de la Recherche des Etudes de l’Evaluation et des Statistiques (DREES) at the Ministry of Health.

National and departmental evaluations rely on the yearly analyses of health certificates filled by the doctors at a child’s twenty fourth month. Although these are mandatory, the certificates of only 60% of children aged 24 months are sent and analysed by the DREES. Coverage for the first dose alone is evaluated. Moreover, twice-yearly transversal surveys are carried out in schools using a representative sample of the French population aged 6 years.
La couverture nationale des enfants de 24 mois est passée de 32 % en 1985 à 80 % en 1994 et stagnée depuis à cette valeur (figure 1). Elle était de 82,7 % en 1999 et s’échelonnait entre 60,5 % et 92,3 % selon les départements, le Nord de la France enregistrant les couvertures les plus hautes (7) (figure 2). Les données provisoires de 2000 montraient une couverture à 84,2 %.

Lors de la dernière enquête en 2000-01, la couverture à l’âge de 6 ans était de 90 %, témoignant du caractère incomplet du rattrapage après l’âge de 2 ans (8).

Surveillance de la morbidité

En 1945, la rougeole a été inscrite sur la liste des maladies à déclaration obligatoire mais les médecins notifiant peu, les déclarations ont été arrêtées en 1986.

Depuis 1985, le réseau Sentinelles, créé par l’INSERM U.444, surveille la rougeole (9). Il associe par voie téléinformatique environ 300 médecins libéraux bénévoles qui déclarent chaque semaine le nombre de cas de rougeole vus en consultation. La définition de cas, comparable à celle utilisée par le CDC (10) comporte une éruption généralisée de plus de 3 jours et une fièvre supérieure à 38,5 °C associées à une toux, un coryza ou une conjonctivite.

Depuis sa création, les médecins du réseau ont rapporté plus de 8000 cas de rougeole. L’incidence nationale extrapolée à partir de ces données a beaucoup diminué, passant de près de 300 000 cas par an en 1985 à environ 10 000 cas en 2000 (figure 3).

Parallèlement, le réseau Sentinelles a observé une augmentation de l’âge des cas. La proportion des patients âgés de plus de 10 ans est passée de 13 % en 1985 à 48 % en 1997. La proportion de cas vaccinés a également augmenté de 3 % à 58 % pendant la même période. Cette augmentation reflète avant tout l’amélioration de la couverture vaccinale.

Jusqu’en 1998, ces proportions étaient calculées à partir de plus de 200 cas décrits par les médecins du réseau. Par la suite, ce nombre s’est réduit à une cinquantaine rendant l’interprétation des données très difficile.

La surveillance repose uniquement sur des données cliniques et il n’existe actuellement aucun recueil des cas confirmés au laboratoire et aucune modalité de typage des souches.

En parallèle de ce système de surveillance, des données de morbidité ont été rapportées lors d’investigations de cas groupés. De 1995 à 2000, 7 investigations ont été effectuées ou raportées à l’Institut de Veille Sanitaire. L’efficacité vaccinale, calculée dans 2 écoles primaires et un lycée sur des cas cliniques et des cas confirmés biologiquement, a toujours été supérieure à 92 % (11,12).

Surveillance de mortalité

Les données de mortalité sont recueillies par le Centre d’Épidémiologie sur les causes médicales de décès (CépiDc) et disponibles à partir de 1979.

Seules les causes principales de décès ont été analysées. En effet, une étude détaillée incluant toutes les causes de décès (principales, associées et immédiates) montrait que seuls 2 décès n’étaient pas inclus aux 35 causes principales répertoriées entre 1995 et 1998, soit une sous-estimation de 5 % du nombre de décès déclarés.

Figure 4
Nombre et causes de décès annuels par rougeole, et proportion d’enfants de moins de 5 ans (moyenne sur 3 ans) 1979-1998, France / Number and causes of yearly deaths due to measles, and rate of children under 5 years of age (median over 3 years), 1979-1998, France

Source : CépiDc

National coverage of 24 month old children was 32% in 1985 and reached 80% in 1994. It has been stable since then (figure 1). It was 82.7% in 1999 and varied from 60.5% and 92.3% depending on the departments. Northern France attained the highest coverage rates (7) (figure 2). Provisional data for 2000 showed an 84.2% coverage rate.

During the last survey conducted in 2000-01, coverage for six year olds was 90%. This shows that catch up vaccination was incomplete for children over the age of 2 years (8).

Surveillance of mortality

In 1945, measles was made a notifiable disease, but this notifiable status was suspended in 1986 because of the low level of notifications being made by doctors.

The Sentinel network, created by the INSERM U444 has been monitoring measles since 1985 (9). It is composed of around 300 volunteer general practitioners, who report electronically the number of measles cases observed during their consultations each week. The case definition, comparable to the one used by the Centers for Disease Control and Prevention (CDC) (10) includes generalised rash of more than three days, and a fever above 38.5 °C, associated with coughing, coryza or conjunctivitis.

Since its creation, the network doctors have reported more than 8000 cases of measles. National incidence, extrapolated from this data, has decreased considerably, from nearly 300 000 cases per year in 1985 to around 10 000 cases in 2000 (figure 3).

At the same time, the Sentinel network observed an increase in the age of the cases. The proportion of patients over 10 years old rose from 13% in 1985 to 48% in 1997. The proportion of vaccinated cases also increased from 3% to 58% over the same period. This increase primarily reveals the improvement of vaccination coverage. Until 1998, these rates were calculated from more than 200 cases described by the network doctors. In 1998, this number was reduced to around fifty cases, which has made data interpretation very difficult.

Surveillance relies on clinical data alone, and presently no laboratory confirmation of cases or procedure for the typing of strains exist.

Alongside this surveillance system, data on morbidity has been reported during the investigation of clusters or small outbreaks. From 1995 to 2000, seven investigations were led by or reported to the Institut de Veille Sanitaire. Vaccine efficacy, calculated in two primary schools and a high school on clinical and biologically confirmed cases has always been over 92% (11,12).
La mortalité a baissé régulièrement, passant d’une trentaine de décès par an dans les années 80 à moins de 10 maintenant. La proportion de décès survenant chez les enfants de moins de 5 ans a chuté de près de plus de 50 % en 1979 à moins de 5 % ces dernières années (figure 4).

Dans la même période, les causes principales de décès se sont également modifiées. De 1979 à 1987, les causes autres qu’encéphalitaires représentaient la majorité des décès, les broncho-pneumonies étant responsables de 34 % de ces autres causes. Depuis 1987, les encéphalites sont les premières causes de décès reportées que ce soit les leucoencéphalites sclérosantes subaiguës (ou panencéphalites) survenant quelques années après la rougeole ou les encéphalites morbillieuses, survenant quelques jours (ou quelques mois pour les encéphalites aiguës retardées) après la rougeole.

**Etude sérologique**

Une étude séro-épidémiologique européenne (13) (ESEN) a été menée dans sept pays. Les immunoglobulines G de près de 29 000 sérums ont été analysées par des méthodes de titrage standardisées (14). En France métropolitaine, les sera provenaient de sujets ayant eu un prélèvement sanguin dans des laboratoires d’analyse médicale. Le choix des sera se faisait selon des quotas par groupe d’âge, sexe et région préalablement définis pour garantir une représentativité satisfaisante mais sans échantillonnage aléatoire (15).

En France, environ 3 500 cas, essentiellement prélevés en 1998, ont été analysés (13) (figure 5). Les résultats pour la rougeole ont montré une proportion importante d’enfants séro-négatifs (7 % chez les enfants âgés de 5 à 19 ans). Les proportions de séronégatifs observées dans toutes les tranches d’âge en dessous de 20 ans sont supérieures aux seuils de séronégativité proposés par le bureau européen de l’OMS pour atteindre l’objectif d’élimination de la rougeole. Les données sérologiques ont également confirmé que la moitié Sud de la France métropolitaine, définie comme précédemment dans la figure 2, était moins bien protégée que la moitié Nord.

**Autres données**

Actuellement, seules les données de l’Enquête de Prescription Permanente Médicale (16) permettent d’apprécier la mise en place en 1998 du rattrapage jusqu’à 11-

**Figure 5**

Suscceptibilité à la rougeole en fonction de l’âge et de la région géographique, 1998, France (n=3500) / Measles susceptibility depending on age and geographic location, 1998, France (n=3500)

En France, environ 3 500 cas, essentiellement prélevés en 1998, ont été analysés (13). Les résultats pour la rougeole ont montré une proportion importante d’enfants séro-négatifs (7 % chez les enfants âgés de 5 à 19 ans). Les proportions de séronégatifs observées dans toutes les tranches d’âge en dessous de 20 ans sont supérieures aux seuils de séronégativité proposés par le bureau européen de l’OMS pour atteindre l’objectif d’élimination de la rougeole. Les données sérologiques ont également confirmé que la moitié Sud de la France métropolitaine, définie comme précédemment dans la figure 2, était moins bien protégée que la moitié Nord.

**Figure 6**


Figure 6


---

This represents a 5% underestimation of the number of deaths reported.

Mortality has decreased steadily, from around 30 deaths per year in the 1980s to less than 10 per year at the present time. The proportion of deaths in children under five years of age decreased from over 50% in 1979 to less than 5% in recent years (figure 4).

In the same period, the main causes of death also changed. From 1979 to 1987, causes other than encephalitic ones represented the majority of deaths, bronchopneumonias being responsible for 34% of the other causes. Since 1987, encephalitis is the first reported cause of deaths, whether it is late encephalitis occurring a few years after measles (subacute sclerosing panencephalitis) or acute immediate or delayed postmeasles encephalitis occurring a few days or months after measles.

**Serological survey**

A European seroepidemiological survey (13) (ESEN) was carried out in seven countries. Immunoglobulins G of nearly 29 000 serum samples were analysed with standardised titration methods (14). In metropolitan France, serum samples came from subjects whose blood samples were drawn in medical laboratories. The choice of serum samples was made according to predefined quotas by age group, sex, and location to ensure a satisfactory representativeness without random sampling (15).

In France, about 3500 serum samples mainly in 1998 were analysed (13) (figure 5). Results for measles showed an important rate of seronegative children (7% in those aged between 5 and 19 years). Rates of seronegatives observed in all age groups under 20 are higher than seronegativity thresholds proposed by the WHO European region to reach the objective of measles elimination. Serological data have also confirmed that the half southern metropolitan France, as defined in figure 2 was less protected than the northern half.

**Other data**

Only the data from the Permanent Medical Prescription Survey (16) currently allows us to appreciate the implementation of catch up doses for children up to the ages of 11-13, and the introduction of the second dose in 1998, through the analysis of prescriptions made by private practitioners.
13 ans et l’introduction de la seconde dose par l’analyse des prescriptions en milieu libéral.

Cette enquête se fait grâce à un fichier de médecins libéraux, généralistes et spécialistes, à partir duquel est tiré un échantillon représentatif stratifié sur la région et le niveau d’activité du médecin. Pendant une période de sept jours, le praticien relève une série d’informations sur l’ensemble des patients vus, ainsi qu’une copie de l’ensemble de ses prescriptions. Les résultats sont exprimés en valeurs extrapoliées, ce qui permet de redresser l’échantillon observé à l’ensemble des médecins français.

Chez les enfants âgés de moins de 2 ans et demi, le taux de prescription du triple vaccin est resté identique depuis 1996, données confirmées par la stagnation de la couverture vaccinale au 24e mois. Le taux de prescription a augmenté entre 2 ans et demi et 7 ans depuis 1997, lors de l’abaissement de l’âge de la seconde dose, aboutissant à une couverture pour la seconde dose d’environ 50 %. Enfin, chez les enfants âgés de plus de 7 ans, les prescriptions ont très largement augmenté sans qu’il soit possible de faire la part entre les vaccinations effectuées comme des secondes doses tardives et celles correspondant à des premières doses chez des enfants non vaccinés. La baisse des cas enregistrés par le réseau Sentinelles ces dernières années est en faveur de la seconde hypothèse (figure 6).

Discussion

La promotion de la vaccination depuis 1983 a été accompagnée d’une réduction de 97 % de la morbidité et de 60 % de la mortalité de la rougeole. Ces bons résultats ne sont pas synonymes d’élimination. La couverture stagnante et sub-optimale à 84 % entraîne un déplacement de l’âge des cas et le maintien de la transmission de la maladie sur le territoire.

En effet, du fait de la réduction de la circulation du virus, les cas de rougeole sont maintenant plus âgés. Or les complications sont plus fréquentes et la léthalité plus élevée chez l’adulte avec comme première cause de décès, l’encéphalite aiguë (17). Ce déplacement de l’âge des cas pourrait ainsi expliquer partiellement la baisse moins rapide de la mortalité comparée à celle de la morbidité, un nombre stable des décès par encéphalite morbilleuse depuis 1988 et l’augmentation de l’âge des patients décédés. Les décès par encéphalite surviennent une dizaine d’années après la contamination par le virus de la rougeole et ne reflètent pas l’incidence actuelle de la maladie. Par contre, ils illustrent le potentiel sévère de la rougeole considérée trop souvent comme totalement bénigne.

Ces effets paradoxaux de la vaccination liés au déplacement de l’âge ne peuvent être contrecarrés que par l’élimination de la maladie. Elle requiert une couverture vaccinale de plus de 95 % et l’administration de 2 doses d’un vaccin efficace.

Le vaccin utilisé en France est de bonne qualité puisque les mesures d’efficacité vaccinale faites lors d’investigations de cas groupés ont toujours été au-delà de 90 %. Par contre, la couverture vaccinale n’est pas assez élevée pour éviter l’accumulation de sujets susceptibles comme le confirment les études sérologiques et l’écloration de foyers épidémiques. La France est encore dans la phase « lune de miel », nom donné quand la baisse des cas fait croire à une maladie en voie de disparition alors que se forment, sans aucune manifestation, des poches d’individus non-protégés, source des prochains foyers épidémiques. Les efforts de vaccination ont permis une forte baisse de l’incidence sans pour autant éviter la formation à bas bruit de ces poches. Les activités de rattrapage importantes de ces dernières années ont probablement réduit ces poches de susceptibles sans pour autant les faire disparaître. Les différences géographiques sont importantes et en parallèle des campagnes de promotion nationale, chaque département doit s’attacher à identifier les populations les moins vaccinées pour pouvoir y remédier, recherche qui est rendue possible par la mesure des couvertures vaccinales, l’identification des cas groupés et la notification de tous les cas.

L’amélioration des couvertures doit s’accompagner d’un changement du système de surveillance. En effet, la baisse d’incidence rend les estimations du réseau Sentinelles peu précises. En 2001, l’incidence a été extrapolée ➤

This survey is carried out by a list of GPs and specialists, from which is selected a representative sample stratified according to the doctors’ region and activity level. During a period of seven days, the doctor reports a set of information on all his or her patients, as well as copies of all prescriptions. The results are then extrapolated to all French practitioners, yielding national incidence estimates.

In children under the age of two and a half years, the rate of prescription of the triple vaccine has remained stable since 1996, data confirmed by the stagnation of vaccine coverage at 24 months. The rate of prescription has increased in children aged between two and a half and seven years of age since 1997, when the age for the second dose was lowered, yielding a coverage for the second dose of about 50%. Finally, in children over seven years of age, prescriptions have increased widely, although it has been impossible to dissociate vaccines administered as late second doses from those corresponding to first doses in non-vaccinated children. The decrease of cases registered by the Sentinel network in recent years favours the second hypothesis (figure 6).

Discussion

Since 1983, promotion of measles vaccination has been accompanied by a 97% reduction in morbidity and a 60% reduction in mortality. These encouraging results are not equivalent to elimination. Stable and suboptimal coverage around 84% causes a shift in the age of patient, and the continuation of disease transmission within the country.

Because of the reduced circulation of the virus, measles cases now occur in older patients. But complications are more frequent and case fatality ratio is higher in adults, with acute encephalitis being the major cause of death (17). This age shift in cases could therefore partly explain the slower decrease of mortality compared to morbidity, a stable number of deaths caused by post-measles encephalitis since 1988, and the increase in the age of the deceased patients. Deaths caused by subacute sclerosing panencephalitis occur about ten years after infection by the measles virus, and do not reflect the current incidence of the disease, although they illustrate the severe potential of a disease that is too often considered to be totally benign.

These paradoxical effects of vaccination linked to the age shift can be fought only by eliminating the disease, and this requires a vaccine coverage of more than 95% and the administration of two doses of an efficient vaccine.

The vaccine used in France is of good quality considering that the measures of vaccine efficacy carried out in investigations of clustered cases have always been over 90%. However, the coverage reached is not high enough to avoid the accumulation of susceptible subjects, as confirmed by serological surveys and the occurrence of epidemic clusters. France is still in the ‘honeymoon’ period, the name given when the decrease of cases suggests that a disease is disappearing, yet without any sign, groups of unprotected individuals are forming the source of future outbreaks. Vaccination efforts have allowed an important decrease of incidence without preventing the silent formation of these groups. Important catch up activities in recent years have probably reduced these groups of susceptible subjects without causing them to disappear. Geographical differences are important, and alongside national promotional campaigns, each department must strive to identify the populations with the lowest vaccination coverage in order to find solutions. This search is made possible by the measurement of local vaccine coverage, the identification of clusters, and notification of all cases.

Improving vaccine coverage must be accompanied by a change in the surveillance system. The decrease of incidence makes the estimates from the Sentinel network very imprecise. In 2001, incidence was extrapolated from 22 reported cases; the 95% confidence interval was 2870-14050 (18). The decrease in the number of cases also ➤

EUROSURVEILLANCE VOL. 7 - N° 4 AVRIL - APRIL 2002 59
À partir de 22 cas rapportés, l'intervalle de confiance à 95% était 2870-14050 (18). La baisse des cas diminue également la valeur prédictive positive de la définition clinique. Ainsi, en Angleterre et au Pays de Galles, en 2000, 2466 rougeoles ont été notifiées à partir de la définition clinique, 1751 (71%) ont eu un échantillon salivaire prélevé et 74 (4 %) d’entre eux étaient positifs (19).

A terme, les outils de surveillance devront être ceux utilisés par les pays plus avancés dans le processus d’élimination (20), à savoir notification exhaustive, définition clinique large pour avoir une sensibilité élevée et détecter tous les cas suspects, confirmation biologique pour améliorer la spécificité et ne retenir que les vrais cas, typage des souches pour retracer leur origine, mesure des couvertures vaccinales pour chacune des doses, estimation de la proportion de population susceptible par modélisation ou étude sérologique.

Les foyers épidémiques touchant les collectivités, les médecins scolaires doivent être impliqués dans la surveillance de la maladie. Des protocoles de conduite à tenir autour d’un ou plusieurs cas de rougeole vont être développés pour inciter à la notification et à l’investigation de cas.

Des projets de recherche sont menés par le réseau Sentinelles en collaboration avec le Public Health Laboratory Service (PHLS) à Londres, et portent sur l’évaluation du diagnostic salivaire de détection des IgM et le typage virologique des souches. Les résultats contribueront à établir l’appui biologique nécessaire à l’élimination de la maladie.

En conclusion, la France a amélioré sa couverture vaccinale mais le taux atteint permet la transmission de la maladie et déplace l’âge des cas, à un âge où complications et létalité sont plus élevées. Pour éliminer la maladie une couverture vaccinale à 2 doses à plus de 95 % devra être obtenue.

Tous les outils peuvent se mettre peu à peu en place pour atteindre cet objectif mais l’obstacle majeur reste de convaincre décideurs, professionnels de santé et public de la nécessité d’éliminer la maladie.

Remerciements / Acknowledgements

Je tiens à remercier Pierre Formenty, Cathy Roth, Roberta Andraghetti et Guenael Rodier pour leur relecture attentive du manuscrit et leurs précieuses commentaires.

I thank Pierre Formenty, Cathy Roth, Roberta Andraghetti and Guenael Rodier for reviewing the manuscript and providing helpful comments.

References

8. Gaugnon N. La santé des enfants de 6 ans à travers les bilans de santé scolaire, Etudes et Résultats, 155, DREES 2002
Cet article décrit une enquête sur les capacités nationales des laboratoires de diagnostic et sur les bases de données de surveillance des virus d’origine alimentaire, auprès du consortium européen sur les « Foodborne viruses in Europe ».

Tous les pays ont des laboratoires pouvant rechercher les anticorps anti-VHA dans le sérum humain. Huit des dix pays européens étudiés entretiennent une base de données nationale sur les cas de VHA. La nourriture peut être analysée pour la présence du VHA en Finlande, en Italie, en Espagne, en France et au Danemark.

Tous les pays étudiés ont au moins un laboratoire pour analyser le virus de Norwalk (VDN) par PCR inverse et microscopie électronique. Cinq pays maintiennent une base de données nationale sur les cas de VDN, et neuf autres ont une base de données nationale sur les épidémies de VDN. Presque tous les participants ont des laboratoires capables de détecter la présence du VDN dans les aliments tels que les fruits de mer.

### Tableau / Table

<table>
<thead>
<tr>
<th>Cas humains</th>
<th>Surveillance</th>
<th>Analyses environnementales</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nombre de laboratoires analysant</td>
<td>Nbre total de bases de données recueillant des informations sur</td>
<td>Nombre de laboratoires analysant</td>
</tr>
<tr>
<td>Human cases</td>
<td>Total number of databases collecting information on</td>
<td>Number of laboratories testing for</td>
</tr>
<tr>
<td>VHA par EIA</td>
<td>VDN par RT-PCR</td>
<td>VDN dans les fruits de mer NLV in shellfish</td>
</tr>
<tr>
<td>VDN par ME</td>
<td>VDN par RT-PCR</td>
<td>VDN dans l'eau et les boissons NLV in water and in drinks</td>
</tr>
<tr>
<td>VDN NLV by EM</td>
<td>VDN NLV by RT-PCR</td>
<td></td>
</tr>
<tr>
<td>Cas de VHA</td>
<td>Cas de VDN</td>
<td>Epidémies de VDN</td>
</tr>
<tr>
<td>Cases of HAV</td>
<td>Cases of NLV</td>
<td>Outbreaks of NLV</td>
</tr>
<tr>
<td>VDN dans les fruits de mer</td>
<td>VDN dans l'eau et les boissons</td>
<td></td>
</tr>
<tr>
<td>NLV in shellfish</td>
<td>NLV in water and in drinks</td>
<td></td>
</tr>
</tbody>
</table>

**Cas humains**

- **Angleterre et Pays de Galles / England and Wales**: >100 laboratoires analysant pour VHA par EIA, VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **Danemark / Denmark**: >10 laboratoires analysant pour VHA par EIA, VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **Finnland / Finland**: 10 laboratoires analysant pour VHA par EIA, VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **France**: >100 laboratoires analysant pour VHA par EIA, VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **Allemagne / Germany**: 20 laboratoires analysant pour VHA par EIA, VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **Italie / Italy**: >100 laboratoires analysant pour VHA par EIA, VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **Slovénie / Slovenia**: 2 laboratoires analysant pour VHA par EIA, VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **Espagne / Spain**: >100 laboratoires analysant pour VHA par EIA, VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **Suede / Sweden**: 10 laboratoires analysant pour VHA par EIA, VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **Pays-Bas / The Netherlands**: >20 laboratoires analysant pour VHA par EIA, VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.

**Surveillance**

- **VHA par EIA**: 2 laboratoires analysant pour VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **VDN par RT-PCR**: 1 laboratoire analysant pour VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **Cas de VHA**: 1 laboratoire analysant pour VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **Cas de VDN**: 1 laboratoire analysant pour VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **Épidémies de VDN**: 1 laboratoire analysant pour VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **VDN dans les fruits de mer**: 1 laboratoire analysant pour VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **VDN dans l'eau et les boissons**: 1 laboratoire analysant pour VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **VDN chez les animaux destinés à la consommation**: 1 laboratoire analysant pour VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.

**Analyses environnementales**

- **VHA par EIA**: 2 laboratoires analysant pour VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **VDN par RT-PCR**: 1 laboratoire analysant pour VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **Cas de VHA**: 1 laboratoire analysant pour VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **Cas de VDN**: 1 laboratoire analysant pour VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **Épidémies de VDN**: 1 laboratoire analysant pour VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **VDN dans les fruits de mer**: 1 laboratoire analysant pour VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **VDN dans l'eau et les boissons**: 1 laboratoire analysant pour VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
- **VDN chez les animaux destinés à la consommation**: 1 laboratoire analysant pour VDN par ME, NLV par EM, VDN par RT-PCR, VDN NLV by EM, VDN NLV by RT-PCR.
Le rôle prépondérant des aliments et de l'eau dans la transmission des virus de Norwalk (VDN) et dans celui de l'hépatite A (VHA) est de plus en plus reconnu. Les épidémies qui se répandent ainsi ont le potentiel de toucher un grand nombre de personnes, de se répandre géographiquement de manière importante, et peut-être même d'introduire de nouveaux variants dans une région. Le typage des virus par des méthodes moléculaires modernes a montré comment un aliment contaminé dans un pays peut causer des épidémies dans un autre, suivant les filières d'importation de produits alimentaires. La population européenne présente une faible immunité vis à vis du VHA, car les conditions sanitaires et la qualité de l'eau sont assurées. Le risque d'une épidémie d'hépatite A est donc important.

Les virus de Norwalk (VDN) ne peuvent pas être cultivés en laboratoire. Le diagnostic est donc basé sur la microscopie électronique (ME) ou de plus en plus, sur les techniques moléculaires. Le VHA est diagnostiqué en routine par la détection des anticorps spécifiques au virus.

Il y a de nombreuses déclarations d'épidémies de VDN et VHA d'origine alimentaire, mais l'incidence réelle de ces infections et la contribution des épidémies au poids de ces maladies restent mal connues.

Le projet de recherche « Foodborne viruses in Europe » (Virus d'origine alimentaire en Europe) a été récemment financé par l'Union européenne afin d'obtenir de meilleures informations sur l'impact des virus d'origine alimentaire au sein de l'UE. Les groupes ont été inclus dans le réseau d'après leurs motivations et leurs expériences dans le domaine des gastro-entérites virales. Les pays participants ont relié leurs réseaux de surveillance virologique et épidémiologique pour détecter les épidémies transnationales et élucider les voies de transmission. Afin de faciliter la création d'une base de données européenne, nous avons demandé à tous les pays participants au Programme « Foodborne Viruses in Europe » des informations sur les capacités techniques de leurs laboratoires et sur les bases de données recensant les cas et les épidémies de gastro-entérites virales.

Méthodes

Un questionnaire a été envoyé par courriel aux douze instituts participants (de 10 pays) au projet (voir liste des participants) et également à l'institut de microbiologie et d'immunologie de Slovénie qui n'était pas un membre officiel du groupe. Les groupes ont été inclus dans le projet parce qu'ils étaient intéressés par la biologie moléculaire du VDN ou du VHA, l'épidémiologie et/ou la sécurité alimentaire. Les informations ont été recueillies selon : 1) les techniques diagnostiques utilisées en routine dans le laboratoire et/ou les laboratoires à la fois publics et privés; 2) les types de cas de gastro-entérites virales; 3) les réseaux de diagnostic, d'épidémiologie et de surveillance de ces infections, 4) la contribution des épidémies au poids des maladies; 5) les types de bases de données nationales utilisées; 6) le rôle du laboratoire dans l'impact de ces maladies et le découpage de ces types de bases de données nationales, et 7) l'importance de chaque variable dans le réseau d'après les motivations du laboratoire et ses expériences dans le domaine.

Figure

Bases de données nationales pour les infections virales d'origine alimentaire / National database of foodborne viral infections

A. Bases de données nationales pour les cas d'hépatite A / National hepatitis A case databases

B. Bases de données nationales pour les cas de VDN / National NLV case databases

C. Bases de données nationales pour les épidémies de VDN / National NLV outbreak databases

Methods

A questionnaire was sent by email to twelve participant institutions (from ten countries) in the “Foodborne viruses in Europe” project (see participants list) as well as the Institute of Microbiology and Immunology (Slovenia), which was not officially a member of the group. Groups were included in the project based on their interest in NLV and HAV molecular biology, epidemiology and/or food safety. Information was collected on 1) the diagnostic techniques used in routine laboratories 2) the number and types of databases used that store information on laboratory capabilities as well as the databases that record cases and outbreaks of viral gastro-enteritis.

T
toire, 2) le nombre et le type de bases de données utilisées pour le stockage des informations sur les infections à VDN et VHA, et 3) les capacités d’investigation environnementale pour ces virus.

Une base de données a été définie comme : toute série d’informations électroniques ou imprimées concernant des cas individualisés humains ou des synthèses d’épidémies. Une base de données nationale est destinée à recueillir des informations sur toutes les zones géographiques d’un pays, mais il n’y avait aucune précision quant au niveau national ou régional de la déclaration.

Résultats
Le questionnaire de l’étude complété a été renvoyé par les 10 pays.

Hépatite A

Diagnostics humains

Surveillance
Tous les pays, à l’exception de la France et de la Suède, ont une base de données nationale recensant tous les cas de VHA (une étude de surveillance pilote a démarré en France) (figure A). Parmi ceux qui ont des bases de données sur les cas de VHA, tous recensent des informations issues des déclarations de laboratoires, sauf l’Allemagne. L’Angleterre, le Pays de Galles, l’Italie, la Finlande et les Pays-Bas disposent d’autres bases de données incluant des rapports de médecins en soins primaires et d’autres établissements de santé. De plus, il existe également des bases de données sur les cas de VHA provenant d’autres études spécifiques : un rapport d’investigation de médecins en santé publique en 1998 (Angleterre et Pays de Galles), une étude pilote de surveillance de l’infection à VHA (France), et une autre sur les voyageurs avant la vaccination contre cette hépatite (Slovénie).

Analyse environnementale

Virus de Norwalk

Capacité diagnostique chez l’homme
Tous les pays utilisent la RT-PCR et la microscopie électronique (ME) pour détecter les virus de Norwalk, bien que, dans certains pays, la microscopie électronique ne soit utilisée qu’en dernier recours (tableau). Deux laboratoires en Angleterre et au Pays de Galles réalisent le test EIA (enzyme immuno assay) basé sur un recombinant du VDN.

Surveillance

Des bases de données sur les épidémies de VDN sont maintenues dans chaque pays. Elles sont toutes basées sur les laboratoires, à part celle NLV and HAV infection ; and 3) the capacity for environmental testing for these viruses.

A database was defined as: any organised set of electronic or paper-based information on individual human cases or summaries of outbreaks. A national database was considered one designed to collect information from all geographic regions of a country, but no stipulations were made concerning the overall or regional levels of reporting.

Results
A completed survey questionnaire was returned from all 10 countries.

Hepatitis A

Human diagnostics
All countries have the capability to test for HAV immunoglobulin (Ig) in human serum but, the number of laboratories performing tests in each country varies widely (table). Laboratories in Germany, Spain, France, The Netherlands and Finland test for HAV RNA by reverse-transcription polymerase chain reaction (RT-PCR).

Surveillance
All countries except France and Sweden have a national database of HAV cases (though a pilot surveillance has begun in France) (figure A). Of those countries that do maintain HAV case databases, all contain information from laboratory reports except for Germany. England and Wales, Italy, Finland, and the Netherlands maintain additional databases that receive reports from primary care doctors and other health care institutions. In addition, HAV databases derived from a range of special studies also exist: an outbreak survey of public health physicians in 1998 (England and Wales), a pilot surveillance of HAV infection (France), and a survey on travellers before vaccination (Slovenia).

Environmental testing
Food can be tested for the presence of HAV genetic material by RT-PCR by laboratories in Finland, Italy, Spain, France, and Denmark. Laboratories in these five countries as well as England and Wales can test for HAV in water and drinks.

Norwalk-like virus

Human diagnostic capability
All countries use RT-PCR and EM to detect NLVs, though in some countries EM is now used as a last line of detection (Table). Two laboratories in England and Wales use an enzyme immuno assay (EIA) based on recombinant NLV.

Surveillance
All countries, except Spain, maintain at least one (laboratory-based) database of cases of NLV. In the Netherlands databases also hold information from primary care and other healthcare providers. In addition, there are historical databases from surveys such as the Dutch NIVEL case-control study, and SENSOR cohort study as well as a study of outbreaks in the western regions of France. Also in France, historical databases exist from a study of children (1995-98). England and Wales, Germany, Finland, Slovenia and Denmark have national databases of NLV cases (figure B).

Databases of NLV outbreaks are kept in every country. These databases are all laboratory-based apart from one based on primary care data (France), and two others based on health care facility data in Denmark and The Netherlands. There are also databases from special studies of structured outbreak surveillance in England, and Wales and The Netherlands. Except for Italy, outbreak databases in every country are designed to collect national data (figure C),
qui repose sur les données en soins primaires (France), et deux autres sur les organismes de santé au Danemark et aux Pays-Bas. Il existe également des bases de données sur des études spécifiques de surveillance d'épidémies en Angleterre, au Pays de Galles et aux Pays-Bas. Hormis l'Italie, les bases de données sur les épidémies sont destinées à recueillir des données nationales (figure C), mais cela ne signifie pas que toutes les épidémies soient nécessairement investiguées et déclarées. En France et au Danemark, seules les épidémies d'origine alimentaire ou hydrique sont déclarées.

Análises environnementales

Les laboratoires qui peuvent analyser les selles humaines ont également les moyens d'analyser les selles animales, bien que seuls la Finlande, l'Angleterre, le Pays de Galles, la France, le Danemark et les Pays-Bas réalisent ces tests. La plupart des pays disposent également de laboratoires pouvant analyser la nourriture (notamment les fruits de mer), l'eau et les boissons (tableau).

Discussion

L'inventaire des capacités techniques est le premier pas vers l'harmonisation des laboratoires et de la surveillance des virus d'origine alimentaire dans les pays participants. Les dispositifs et l'infrastructure des laboratoires d'analyses pour dépister des virus pathogènes d'origine alimentaire varient considérablement en Europe. Cependant, le partage des prélèvements et des expériences au sein du consortium européen rendront ces diverses capacités complémentaires. Certains pays ont un système de diagnostic plus centralisé, presque toutes les analyses étant assurées par un ou deux laboratoires. Dans d'autres, comme l'Angleterre, le Pays de Galles, l'Allemagne, les Pays-Bas et l'Italie, les diagnostics sont fournis par de nombreux laboratoires. Bien que, dans cette étude, la majorité des pays disposent de laboratoires pouvant déceler une infection à VHA chez l'homme, beaucoup ne recherchent pas la présence du VHA dans la nourriture, l'eau ou les boissons. Ainsi, les infections d'origine alimentaire ou hydrique ne peuvent être confirmées biologiquement sans une assistance internationale. Tous les pays sont capables de rechercher la présence du virus de Norwalk dans les selles humaines par RT-PCR et microscopie électronique. Par rapport à la microscopie électronique, la RT-PCR est un outil de diagnostic beaucoup plus sensible, capable de détecter le virus jusqu'à deux semaines après l'infection. Ainsi, l'utilisation de la RT-PCR pourrait accélérer la confirmation biologique des cas et des épidémies de VDN. En raison de la diversité génétique des VDN, il a été difficile de développer un test suffisamment spécifique et sensible. Afin d’harmoniser les méthodes diagnostiques utilisées par le réseau, les laboratoires ont analysé un lot représentatif de prélèvements de selles en utilisant les différents tests disponibles dans les pays participants au réseau. Les résultats de cette évaluation seront présentés ailleurs.

L'analyse des fruits de mer peut être réalisée dans la plupart des pays, bien qu'il n'existe pas de méthodes standardisées satisfaisantes. Il n'y a aucun test établi pour analyser les aliments, et l'un des objectifs du projet « Virus d'origine alimentaire en Europe » est de développer de telles techniques. Les laboratoires en Finlande, aux Pays-Bas, en Angleterre et au Pays de Galles, au Danemark et en France, ont utilisé leurs équipements pour rechercher chez des animaux destinés à la consommation la présence de VDN, évaluant ainsi la possibilité d'infections zoonotiques. Bien que la transmission du VDN de l'animal à l'homme n'ait jamais été démontrée, les peaux de cette éventualité n'ont fait qu'augmenter ces dernières années. Des séquences géniques du VDN ont été détectées chez des porcs et dans du bétail, le matériel génétique des calicivirus bovins étant très proche de celui du VDN humain. Tous les pays ont au moins une base de données sur les épidémies de VDN, et tous, hormis l'Italie, ont une base de données nationale. La confirmation des gastro-entérites à VDN par la surveillance en routine s'est révélée infructueuse, car la maladie est généralement bénigne – dans le sens où les personnes affectées n'ont pas besoin de consulter un médecin. Bien que le niveau de confirmation varie considérablement, nous pensons qu'une base de données internationale qui regroupe toutes les épidémies serait la plus adaptée pour le projet.

- although this does not necessarily mean that all outbreaks that are investigated are then reported. In France and Denmark, only food- or waterborne outbreaks were reported.

- Environmental testing

Laboratories that can test human stools also have the ability to test food animal stools, although only Finland, England and Wales, France, Denmark, and The Netherlands perform such tests. Most countries also have laboratories that test foods (namely shellfish) as well as water and drinks (see table).

Discussion

This inventory of laboratory capacity is the first step towards harmonisation of laboratory and surveillance of foodborne viruses in participating countries. Both the capability and the infrastructure of laboratories testing for foodborne viral pathogens in Europe vary widely. The sharing of samples and experience through the Foodborne Viruses in Europe consortium will however make these diverse capabilities complementary. Some countries have a more centralised diagnostic service with nearly all testing of foodborne viruses concentrated in one or two laboratories. In other countries, such as England and Wales, Germany, The Netherlands, and Italy, diagnostics are provided in many laboratories.

Although the majority of countries in this survey have laboratories that can test for HAV infection in humans, many do not test for HAV in food, water or drinks and therefore, food and waterborne infections cannot be microbiologically proven without international assistance. All countries can test human faeces for NLV by RT-PCR and EM. In comparison to EM, the RT-PCR is a substantially more sensitive diagnostic tool, able to detect virus up to two weeks after infection. Thus, the use of RT-PCR could increase the laboratory ascertainment of cases and outbreaks of NLV. Due to the genetic diversity of NLVs it has been difficult to develop a sufficiently sensitive and specific assay. In order to harmonise diagnostic methods used across the network, laboratories have tested a representative panel of stool samples using the different assays used among the network participants. The results of this evaluation will be presented elsewhere.

Testing of shellfish can be performed in most countries though satisfactory methods have not been standardised. There are no established tests for assaying other foods, and one of the aims of the “Foodborne Viruses in Europe” is to develop such techniques. Laboratories in Finland, The Netherlands, England and Wales, Denmark, and France have used their facilities to test food animals for NLV, thus capturing the possibility of zoonotic infection. Though transmission of NLV from animal to humans has not been demonstrated, evidence of the potential has been mounting in recent years. NLV genes have been detected in pigs and cattle, and the genetic material of bovine caliciviruses were shown to be very similar to human NLV.

All countries have at least one database of NLV outbreaks and all but Italy have a database with national coverage. Ascertainment of NLV gastroenteritis by routine surveillance has been shown to be poor since the condition is typically mild in that it does not cause the affected individuals to seek medical attention. Though the level of ascertainment may vary widely, we believe that an international database that captures outbreaks would be most appropriate for the “Foodborne Viruses in Europe” network.

Diagnostics and, therefore, surveillance of foodborne viruses are rapidly evolving fields. Molecular techniques used to detect viral RNA (by RT-PCR assay) and virus particles (EIA) are increasingly used in diagnostic laboratories. And, many of the national databases and surveillance networks that are referred to in these reports have been in existence for a short period of time. For example, reporting of NLV cases in Germany to a central database began as recently as January 2001.
Les diagnostics et donc, la surveillance des virus d'origine alimentaire sont des domaines en pleine expansion. Les techniques moléculaires pour détecter l'ARN viral (par RT-PCR) et les particules virales (EIA) sont de plus en plus utilisées par les laboratoires de diagnostic. De nombreuses bases de données nationales et les réseaux de surveillance mentionnés dans ces rapports n’existent que depuis peu. Par exemple, la déclaration des cas de VDN à une base de données centrale en Allemagne n’a commencé qu’en janvier 2001.

Grâce au typage moléculaire, une épidémie transnationale de VDN d'origine alimentaire a été décrite. Elle concernait des framboises contaminées, en provenance de Slovénie, à l'origine de toxifiinctions alimentaires en Europe et au Canada. Des épidémies d'origine alimentaire et hydrique de grande ampleur ont été documentées à plusieurs reprises, mais cette épidémie transnationale démontre l'impact potentiel que la grande distribution de produits alimentaires industriels peut avoir sur la santé des populations. En instaurant une base de données épidémiologiques et moléculaires, le projet « Foodborne viruses in Europe » étudiera la fréquence de telles épidémies et des voies de transmission associées. Cette base de données permettra également la détection des épidémies et des variants des virus variants à un stade précoce.

Le projet « Foodborne virus in Europe » est financé par la Commission européenne, Direction générale de la Recherche, « Qualité de vie et Gestion des ressources vivantes » QLK1-CT-1999-00594. « Foodborne Viruses in Europe” is funded by the European Commission, Directorate General Research under the “Quality of Life and Management of Living Resources”: QLK1-CT-1999-00594.

Remerciements / Acknowledgements
Le projet “Foodborne virus in Europe” est financé par la Commission européenne, Direction générale de la Recherche. « Qualité de vie et Gestion des ressources vivantes » QLK1-CT-1999-00594. « Foodborne Viruses in Europe” is funded by the European Commission, Directorate General Research under the “Quality of Life and Management of Living Resources”: QLK1-CT-1999-00594.

References

RAPPORT D’INVESTIGATION
Salmonella enterica sérotype Dublin en Autriche

En Austria, el sérotype Dublín de Salmonella enterica, adaptado a bovins, es raramente o a épidemias humanas. En 2000, en Austria, el sérotype Dublín estaba en la mayoría europea, con una incidencia de 0,1 caso por millón de habitantes. Nos damos cuenta que la gran mayoría de los casos humanos son de sérotype Dublín, chez les voies de transmission associées. Cette base de données permettra également la détection des épidémies et des variants virales à un stade précoce. En Austria, el sérotype Dublín de Salmonella enterica, adaptado a bovins, es raramente o a épidemias humanas. En 2000, la incidencia de 0,1 caso por millón de habitantes. Nos damos cuenta que la gran mayoría de los casos humanos son de sérotype Dublín, chez...
l’homme ou l’animal, sont épidémiologiquement liées à deux districts du Tyrol. Cette concentration de cas peut s’expliquer par un mode d’élevage du bétail typique à cette région, les pâturages alpins. Le risque de contaminations croisées est accru par le regroupement d’animaux provenant de plusieurs fermes. Le bétail infecté est une source de contamination pour les hommes, généralement par la consommation de viande de bœuf et de lait de vache. L’électrophorèse en champ pulsé et le ribotypage automatisé ont permis de retrouver des clones caractéristiques de Dublin au Tyrol dans trois des cinq isolats à l’origine d’infections humaines. Pour empêcher les contaminations croisées dans les grands troupeaux et la contamination des pâturages par du bétail infecté de façon latente, il faudrait éventuellement commencer par procéder à un dépistage bactériologique du portage fécal avant la transhumance des troupeaux à risque vers les parcages de montagne, et avant le retour des animaux des pâturages à risque vers les fermes. Une recherche appropriée est nécessaire.

D e par sa large dissémination dans l’environnement, sa prévalence dans la chaine alimentaire globale, sa virulence et son adaptabilité, Salmonella enterica a un impact considérable en médecine, en santé publique et sur l’économie mondiale (1). Les infections à S. enterica non-typhique se manifestent le plus souvent par une gastro-entérite aiguë spontanément résolutive. S. enterica est identifiée dans les hémocultures chez 1 à 4 % des personnes immunocompétentes présentant une gastro-entérite à salmonelles (2).


Étude épidémiologique


Épidémiological review

The database of the national reference laboratory for salmonella in Austria was used to find all initial S. Dublin strains isolated in Austria between 1990 and June 2001. A total of 77 salmonella isolates were of serovar Dublin. Six strains originated from humans (two blood cultures, four stool specimens) and 64 strains from cattle (23 faecal specimens, 36 organs from deceased animals, five meat samples from slaughtered animals culled due to illness). Four isolates were from chamois (organs), one isolate from a water sample and one from clay, both taken from water ponds epidemiologically involved in zoonotic outbreaks. One strain was isolated from pooled faecal material at a small farm during routine screening before the slaughter of a chicken flock (approximately 20 birds).

Figure 1 presents the S. Dublin isolates in chronological order stratified into “human”, “cattle”, and “other” isolates. Figure 2 gives the geographical distribution (and place of infection if different from place of diagnosis).

The six human S. Dublin strains originated from three Austrian provinces (three from the Tyrol, two from Upper Austria, and one from Carinthia). Both patients from Upper Austria acquired S. Dublin abroad (Vietnam and India). The patient from Vienna was born in Nigeria; she was positive for antibodies to HIV and denied any travel outside Vienna during her three years in Austria. The Tyrolean cases all came from the two eastern districts of Kufstein and Kitzbuehel, and could be linked to the consumption of unpasteurised cows’ milk (two) and veal from domestic butchering (one).
Tyrol venaient tous des deux districts à l’est de la région, Kufstein et Kitzbuehel. Leur contamination était sans doute liée à la consommation de lait de vache non pasteurisé, pour deux d’entre eux, et à celle de viande de veau tué à la ferme, pour le troisième.

Les isolats non humains de S. Dublin venaient de cinq provinces autrichiennes. Tous les cas survenus à Vienne et en Basse-Autriche étaient liés à des épidémies dans les pâturages d’été avoisinants, au Tyrol et à Salzbourg. Le bétail malade a été transporté par camion vers des installations vétérinaires à Vienne et Mödling pour établir un diagnostic.

Il s’est avéré que l’infection à S. Dublin correspond à une zoonose endémique au Tyrol et à Salzbourg. L’isolat de Haute-Autriche, prélevé sur un animal abattu pour cause de maladie aux abattoirs de Linz, provenait également d’un cas endémique. On a pu remonter jusqu’à une petite ferme dans le district de Ried im Innkreis, qui a perdu trois animaux malades en peu de semaines. Tous les animaux étaient nés et élevés dans cette ferme, qui avait acquis deux vaches dans le district tyrolien de Kitzbuehl 15 ans auparavant.

Nous avons comparé l’incidence des infections humaines à S. Dublin en Autriche et dans 15 autres pays d’Europe occidentale, en utilisant la base de données d’Enter-net sur les salmonelles. En 2000, il y avait 181 cas dans les 16 pays européens étudiés dans la base de données, avec un taux européen de 0,4864 cas par million d’habitants. Les taux par pays varient de 0 à 3,3333 cas : en Autriche l’incidence est de 0,1250 cas par million d’habitants. Les données d’Enter-net ne permettent pas une comparaison fiable des taux entre pays, qui sont biaisés par de grandes différences dans la sensibilité et la définition de cas dans ces pays.

Étude biologique

Les 21 isolats de S. Dublin d’origines humaine et non-humaine étaient disponibles pour procéder au sous-typage biologique afin de déterminer leur appartenance clonale et donc épidémiologique. Le soustypage a été effectué en utilisant un ribotypage automatisé (RiboPrinter®) après digestion par l’enzyme de restriction EcoRI (tableau), et l’analyse de la PFGE en utilisant les enzymes de restriction XbaI, BlnI et SpeI, selon la méthode de Liesegang et al. (4) (figure 3 et tableau). Le typage moléculaire des isolats de S. Dublin sélectionnés et représentatifs montrent que les isolats de S. Dublin à partir des cas humains et des abattoirs de Linz sont clonal identiques et indistinguishables.

The non-human S. Dublin isolates originated from five Austrian provinces. All the cases that occurred in Vienna and Lower Austria were connected to outbreaks in neighbouring summer pastures in Tyrol and Salzburg; for diagnostic purposes, sick cattle were transported in lorries to veterinary facilities in Vienna and Mödling.

In Tyrol and Salzburg, S. Dublin was found to be an endemic zoonosis. The isolate from Upper Austria, from an animal slaughtered because of illness at the abattoir in Linz was also found to be from an endemic case. It could be traced back to a small farm in the district of Ried im Innkreis, which lost three animals to disease within a few weeks. All animals were bred and reared at this farm, which had acquired two cows from the Tyrolean district of Kitzbuehl 15 years before.

The Enter-net salmonella database was used to compare incidences of S. Dublin infections in humans in Austria and 15 other Western European countries. In 2000 there were only 181 cases in the 16 European countries analysed in the database with a European rate of 0.4864 cases per million population. The national rates range from 0–3.3333 cases depending on the country: the rate for Austria is 0.1250 cases per million population. Enter-net data do not allow uncritical comparison of rates between countries because of the large differences in sensitivity and ascertainment of cases within these countries.

Laboratory review

Twenty-one S. Dublin isolates of human and non-human origin were available for further laboratory based subtyping to determine their clonal and therefore their epidemiological relatedness. Subtyping was performed using automated ribotyping (RiboPrinter®) and EcoRI as restriction enzyme (table) and PFGE pattern analysis (figure 3 and table) using the restriction enzymes XbaI, BlnI and SpeI according to Liesegang et al. (4) The clonal analysis of the selected and representative isolates of S. Dublin reveals the “Austrian” human isolates to be of heterogeneous nature; only two of the isolates were found to be clonal identical and indistinguishable from the isolates from calves, chicken, and...
salmonelles à la source la plus courante d’infection chez le bétail, mais clairement différents des isolats allemands (données non communiquées ici, voir 5, 6). Les souches de S. Dublin portant les numéros 18 et 20 dans le tableau, prélevées chez un patient de Linz contaminé au Vietnam, et un patient de Vienne, originaire du Nigéria séropositif pour le VIH, sont de types différents de celles du Tyrol, prouvant ainsi l’existence d’une autre association épidémiologique. La souche S. Dublin de 1993 (running number 21) originée d’un clone différent issu de bétail du Tyrol, non retrouvé parmi les isolats de 2000 (5, 6).

Discussion

Salmonella enterica sérotype Dublin, adaptée aux bovins, est considérée comme la cause la plus courante d’infection à salmonelles chez le bétail, avec S. Typhimurium (5-7). Le bétail infecté est une source de contamination pour les humains, généralement par la consommation de viande de bœuf et de lait de vache (8, 9). Les infections humaines à S. Dublin sont rares en Autriche. En 2000, l’Autriche était dans la moyenne européenne, avec une incidence de 0,1 cas par million d’habitants. Il est à noter que trois des isolats humains d’Autriche sont hétérogènes : seuls deux isolats avaient pour les humains, généralement par la consommation de viande de bœuf avec reée comme la cause la plus courante d’infection à salmonelles chez le bétail, non retrouvé parmi les isolats de 2000 (5, 6). Les souches de S. Dublin portant les numéros 18 et 20 dans le tableau, prélevées chez un patient de Linz contaminé au Vietnam, et un patient de Vienne, originaire du Nigéria séropositif pour le VIH, sont de types différents de celles du Tyrol, prouvant ainsi l’existence d’une autre association épidémiologique. La souche humaine de S. Dublin de 1993 (n° 21), émanant d’un clone différent issu de bétail du Tyrol, non retrouvé parmi les isolats de 2000 (5, 6).

Tableau / Table

Caractérisation des isolats de S. Dublin selon leurs propriétés moléculaires et clonales / Subdifferentiation of S. Dublin isolates by their molecular and clonal properties

<table>
<thead>
<tr>
<th>Numéro Running number</th>
<th>Origine Origin</th>
<th>Résistance aux antibiotiques Antibiotic resistance</th>
<th>Ribotype Ribotype</th>
<th>Profil plasmidique en Md Plasmid profile in Md</th>
<th>PFGE</th>
<th>Type clonal / Clonal type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>humaine / human, 2000</td>
<td>SMZ</td>
<td>A</td>
<td>50</td>
<td>1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>bétail / cattle, 2000</td>
<td>SMZ</td>
<td>A</td>
<td>50</td>
<td>1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>bétail / cattle, 2000</td>
<td>SMZ</td>
<td>A</td>
<td>50</td>
<td>1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>bétail / cattle, 2000</td>
<td>SMZ</td>
<td>A</td>
<td>50</td>
<td>1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>terre / clay, 2000</td>
<td>SMZ</td>
<td>A</td>
<td>50</td>
<td>1 1a 1 1</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>bétail / cattle, 2000</td>
<td>SMZ</td>
<td>A</td>
<td>sans plasmide plasmid free</td>
<td>1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>bétail / cattle, 2000</td>
<td>SMZ</td>
<td>A</td>
<td>50</td>
<td>1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>bétail / cattle, 2000</td>
<td>OTE,SMZ, STR, SXT</td>
<td>A</td>
<td>50</td>
<td>1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>bétail / cattle, 2000</td>
<td>SMZ</td>
<td>A</td>
<td>50</td>
<td>1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>poulet / chicken, 2000</td>
<td>SMZ, STR</td>
<td>A</td>
<td>50</td>
<td>1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>bétail / cattle, 2000</td>
<td>SMZ</td>
<td>A</td>
<td>50</td>
<td>1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>bétail / cattle, 2000</td>
<td>SMZ</td>
<td>A</td>
<td>50</td>
<td>1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>bétail / cattle, 2000</td>
<td>SMZ</td>
<td>A</td>
<td>50</td>
<td>1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>poulet / chicken, 2000</td>
<td>SMZ</td>
<td>A</td>
<td>50</td>
<td>1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>essai multicentrique / Ring trial, Bilthoven</td>
<td>SMZ</td>
<td>B</td>
<td>50</td>
<td>5 1 4 5</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>humaine / human, 1998</td>
<td>SMZ</td>
<td>A</td>
<td>50</td>
<td>1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>bétail / cattle, 2000</td>
<td>SMZ</td>
<td>A</td>
<td>50</td>
<td>1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>humaine / human, 1998</td>
<td>SMZ</td>
<td>C</td>
<td>sans plasmide plasmid free</td>
<td>2 2 2 2</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>humaine / human, 1996</td>
<td>SMZ</td>
<td>A</td>
<td>50</td>
<td>1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>humaine / human, 1993</td>
<td>SMZ</td>
<td>A</td>
<td>50</td>
<td>3 3 3 3</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>humaine / human, 1993</td>
<td>SMZ</td>
<td>E</td>
<td>50</td>
<td>4 1b 1a 4</td>
<td></td>
</tr>
</tbody>
</table>

SMZ = sulfonamide, OTE = tetracycline, STR = streptomycin, SXT = cotrimoxazole
haute montagne pendant les mois d’été. Cette garde commune du bétail génère un risque accru d’infections croisées. L’infection enzootique à salmonelle du bétai adulte est, de ce fait, une maladie transmissible typique des alpages. S. Dublin peut survivre pendant plus d’un an dans l’environnement avec des conditions climatiques variées (10,11). Les signes cliniques se manifestent généralement par une diarrhée aiguë responsable de la déshydratation, avec un taux de mortalité élevé. Un avortement spontané, habituellement dans le septième ou huitième mois, peut être le seul symptôme de l’infection (12). Un cas d’infection à S. Dublin n’est pas facile à identifier rétrospectivement, car les excrétions de salmonelles sont généralement transitoires et les titres des anticorps sériques agglutinants tombent souvent à un niveau très faible peu après l’avortement (12). L’infection à S. Dublin chez le bétai se caractérise aussi par le nombre important de porteurs (13-15).

Dans les districts de Kufstein et de Kitzbuehel, les vétérinaires connaissent bien le problème de l’infection à S. Dublin chez le bétai (5-7). Comme la loi autrichienne concernant les contagions animales, écrite en 1909, ne précise aucune réglementation sur les infections à salmonelles dans le bétai, les éleveurs doivent supporter les conséquences et les coûts liés à la maladie. En Autriche, la déclaration des cas animaux de salmonellose n’est même pas obligatoire ; ce n’est que dans le cadre de l’inspection alimentaire que les résultats positifs doivent être rapportés au vétérinaire responsable du district. Il serait tout à fait souhaitable d’instaurer une législation, comme la loi allemande sur les infections à salmonelles chez le bétai, pour favoriser l’instauration de mesures médicales préventives (16).

Pour la protection du consommateur, il serait nécessaire de rédiger des lois pour indemniser les éleveurs de bétai de toutes leurs dépenses et pertes (animaux infectés et rejetés) dues à S. Dublin, dans le but d’obtenir une collaboration optimale entre les propriétaires des cheptels et les vétérinaires officiels. Le minimum consisterait au moins à couvrir les frais d’analyses biologiques (recherche de salmonelles dans un prélèvement par examen). Le plus souvent, actuellement, la recherche des sources d’infection n’est pas effectuée en raison de son coût. Le dépistage bactériologique des troupeaux ayant un antécédent connu d’infections à S. Dublin serait un bon point de départ pour éviter la contamination éventuelle des pâturages alpins par du bétai infecté de façon latente et produisant des fécès potentiellement infectieuses. Quant au dépistage bactériologique du portage fécal avant le retour du bétai des pâturages associés à des infections, il pourrait éviter la contamination croisée de grands troupeaux de différentes exploitations.

Plusieurs épidémies européennes des années précédentes, associées à des produits laitiers, montrent que les infections à S. Dublin chez le bétai représentent sans aucun doute un risque is increased risk of cross infection due to the communal keeping of animals from various farms. The enzootic salmonella infection of adult cattle is therefore a typical contagious disease in grazing pastures. S. Dublin can survive for more than one year in the environment under various climatic conditions (10, 11). Clinical manifestation is usually characterised by severe diarrhoea and resulting dehydration with a high rate of mortality. Spontaneous abortion, usually in the seventh or eighth month, can be the only symptom of infection (12). Retrospective identification of a case of S. Dublin may prove difficult as excretion of the organism is usually transient and the serum agglutinating antibodies frequently fall to low titres soon after the abortion (12). Another characteristic of S. Dublin infection of cattle is its high carrier rate (13-15).

The problem of S. Dublin infection in cattle is well known to veterinarians in the districts of Kufstein and Kitzbuehel (5-7). As Austrian law concerning animal contagions, written in 1909, does not specify any regulations on salmonella infection in cattle, the owners of the cattle are left to carry the consequences and the costs thereof. In Austria, cattle afflicted with salmonella infections do not even have to be reported to the authorities; only within the framework of meat inspection do positive results have to be reported to the veterinarian in charge of the district. Legal rulings, such as the existing German law concerning salmonella infection in cattle, would be most desirable to facilitate preventive medical measures (16).

For consumer protection it would be necessary to create regulations to compensate cattle farmers for their entire expenses and losses (spoiled and rejected animals) due to S. Dublin with the aim of achieving optimal co-operation between the owners of the animals and the official veterinarians in charge. At the very least, costs of laboratory examinations (faecal specimens examined for salmonella: 15 ATS or 1,1€ per specimen should be covered. For financial reasons, searches for sources of infection are generally being avoided at present. Bacteriological screening of herds with a known history of S. Dublin infection would be a start to prevent future contamination of alpine pastures through latently infected cattle excreting potentially infectious faeces. Bacteriological screening for faecal carriage before the return of cattle from pastures known to be connected with infections might be able to prevent cross-contamination of large mixed herds.

Various European outbreaks connected to dairy products that occurred during the previous years demonstrate that S. Dublin in cattle certainly poses a preventable health risk for humans (8,9,17). The exact economic loss as a result of S. Dublin infections in humans and animals is not known. The official cost of the 14 days hospitalisation of the sole Austrian patient diagnosed with S. Dublin infection in the year 2000 came to a total of ➤

Figure 3
Analyse des souches de S. Dublin par PFGE (restriction par XbaI) / PFGE pattern analysis (XbaI restriction) of strains

Piste 1, origine humaine n°1 ;
Piste 2, origine humaine n°19 ;
Piste 3, bétai n°2 ;
Piste 4, terre n°5 ;
Piste 5, poulet n°10 ;
Piste 6, origine humaine n°18 ;
Piste 7, origine humaine n°20 ;
Piste 8, origine humaine n°21 ;
Piste 9, essai multicentrique n°16 ;
Piste 10, pour comparaison ;
Piste 11, pour comparaison ;
S. = Salmonella Typhimurium LT 2./
Lane 1, human origin, running no. 1;
Lane 2, human origin, no. 19;
Lane 3, cattle, no. 2;
Lane 4, clay, no. 5;
Lane 5, chicken, no. 10;
Lane 6, human origin, no. 18;
Lane 7, human origin, no. 20;
Lane 8, human origin, no. 21;
Lane 9, ring trial, no. 16;
Lane 10, for comparison;
Lane 11, for comparison;
S. = Salmonella Typhimurium LT 2.
L’unique patient autrichien chez qui une infection à S. dublin chez l’homme et chez les animaux. La question de savoir si une analyse du rapport avantages/coût peut garantir une éradication évitable pour l’homme (8,9,17). On ne connaît pas la liste des contacts nationaux est disponible dans les numéros précédents ou sur le site web

La liste des contacts nationaux est disponible dans les numéros précédents ou sur le site web
**INDEX**

**DANS LES BULLETINS NATIONAUX**
Une sélection dans les derniers numéros parus

**IN THE NATIONAL BULLETINS**
A selection from current issues

---

**BOTULISME / BOTULISM**

**CHOLERA**
- Two cases of imported cholera in the Czech Republic. Eurosurveillance Weekly 2002; 6: 020321.

**CRYPTOSPORIDIOSE / CRYPTOSPORIDIOSIS**

**GRIPPE / INFLUENZA**
  [http://www.ltikalmar.se/smitshyttet/NyInformation/epiaktuell.htm](http://www.ltikalmar.se/smitshyttet/NyInformation/epiaktuell.htm)

**HÉPATITE / HEPATITIS**
  [http://www.isis.nv.nl/epi-bul/](http://www.isis.nv.nl/epi-bul/)

**HÉPATITES VIRALES / HEPATITIS VIRAL**

**INFECTIONS RESPIRATOIRES / RESPIRATORY INFECTIONS**
  [http://www.isis.nv.nl/epi-bul/](http://www.isis.nv.nl/epi-bul/)

**LEGIONELLOSE / LEGIONELLOSIS**

---

**EUROSURVEILLANCE VOL. 7 - N° 4 AVRIL / APRIL 2002**
POULIDISME / MALARIA


SALMONELLA


TUBERCULOSIS / TUBERCULOSIS


Viral pathogens are the most common cause of gastroenteritis in industrialized countries (1,2). Mead et al. have estimated that of the 38.6 million annual cases of gastroenteritis in the United States, 30.8 million (80%) are the result of viral infections (3). Enteric viral pathogens include Rotavirus A, Astrovirus, adenovirus, and Sapovirus, but most viral gastroenteritis infections are caused by Norovirus (formerly Norwalk-like viruses) (1–3). The use of molecular diagnostics including reverse-transcriptase polymerase chain reaction (RT-PCR) and antigen detecting enzyme immunoassays (EIA) (4–20) have changed researchers’ understanding of the epidemiology of human Caliciviridae (including Norovirus and Sapovirus) (21). For example, using RT-PCR assays, Pang et al. showed that caliciviruses were as common a cause of infection as rotaviruses among children <2 years of age (22).

In addition, many reports have established the importance of noroviruses as a cause of outbreaks of food- and waterborne illness (23–28), though estimates of the proportion of infection spread by these modes vary widely: from 14% in England and Wales (29) to <40% in the United States (7). While person-to-person transmission is probably the mode of infection of most cases, food- and waterborne infections may be of particular importance since these outbreaks have the potential to involve large numbers of people and wide geographic areas and, perhaps, to introduce new variants to an area (30).

A research network to study foodborne viruses in Europe was recently funded by the European Union. Through this project, the participant institutes have networked their virologic and epidemiologic surveillance in order to detect transnational outbreaks, elucidate transmission routes, and make international comparisons of the epidemiology of viral gastroenteritis. We chose to study outbreaks rather than community cases because viral gastroenteritis is a very common infection (1); therefore, enumeration of epidemics (or outbreaks) may be more practical and useful since individual cases are poorly reported (31). International comparisons of surveillance data are difficult because criteria for effective surveillance customarily varies across borders (32).

The objective of this survey was to capture information on the structure of outbreak surveillance in each country (including sources of data and definitions employed) and to gain estimates of the frequency of outbreaks, as well as to compare the setting of outbreaks, the importance of foodborne transmission, and the use of characterization techniques. We present surveillance data from viral gastroenteritis outbreaks from 1995 to 2000 collected by participant European countries. These data provide baseline information for future harmonization and comparison efforts.

Methods

A questionnaire was sent by e-mail to the project leaders of the 13 participant institutions (from 10 countries) in the Foodborne Viruses in Europe group. The questionnaire, administered in English, was developed and completed in collaboration with research and medical virologists and epidemiologists working in viral gastroenteritis surveillance. General information on surveillance systems (including sources of data, estimate of national population under surveillance, definition of viral gastroenteritis outbreak, and number of such outbreaks investigated) was collected for the period 1995–2000. More detailed epidemiologic data (setting, mode of...
transmission, and implicated food vehicles) were collected from outbreaks that occurred in 2000. Contributors were sent a summary report and asked to confirm that the data presented accurately represented their surveillance.

Results

Data Sources of Surveillance Systems

One completed survey questionnaire was received from all 10 countries. A range of sources contributed data on viral gastroenteritis outbreaks for European surveillance systems (Table 1), including diagnostic reference laboratories, local public health staff, food inspectorates, and physicians. We derive our data from routine surveillance except for Germany, where systematic national surveillance was not operational during the survey period. German data were collected from laboratories that performed RT-PCR diagnostics in the surveyed period. The same applies to the Netherlands, Finland, and Sweden, although the collaborating centers in these countries run the sole reference laboratory service.

Outbreak Definition and Geographic Coverage of Surveillance Systems

All surveillance systems reported data collected on outbreaks from the whole population of their respective countries except for Italy, where a small geographically convenient sample of approximately 1% of the population was covered by surveillance (Table 2). Both the criteria and the use of outbreak definitions differed among the surveillance systems (Table 2). Some systems collected information only on incidents that met a specific definition; other systems collected information on all incidents and then applied definitions retrospectively for analysis. Some surveillance systems required laboratory confirmation to attribute an outbreak to an enteric viral pathogen. Among systems requiring laboratory confirmation, a range of stringency existed from at least one positive sample (England and Wales) to half of all stools positive for virus (Finland and the Netherlands).

Outbreaks Investigated

Outbreak reports were available from the entire surveyed period (1995–2000) from a few countries: England and Wales, Slovenia, Spain, the Netherlands, and Sweden. The overall numbers of outbreaks investigated ranged from 2 in Italy to 1,643 in England and Wales (Table 3).

National outbreak reporting rates for each country were calculated by dividing annual outbreaks by national population (Figure 1). Rates in Sweden (9–22 outbreaks/million in population) were markedly higher than in any other country. In most countries, approximately 3–7 outbreaks per million population were ascertained annually. Since 1997, outbreak reporting rates have been increasing in most countries.

Completeness of Basic Epidemiologic Data

Participants were asked how many of the outbreaks reports from the year 2000 included details on first date of onset, last date of onset, number of persons ill, number of persons hospitalized, number of related deaths, and setting of the outbreak. Completeness of these data differed substantially between countries: none of the data were available from Sweden, whereas data were almost 100% complete for all categories in England and Wales, Denmark, and Slovenia (Figure 2).

Setting of Outbreaks

The settings where reported outbreaks occurred differed substantially by country (Figure 3). In England and Wales, Spain, and the Netherlands, most reported outbreaks occurred in hospitals and residential homes (78%, 64%, and 66%,

![Table 1. Sources of information of viral gastroenteritis surveillance systems in the Foodborne Viruses in Europe network](image)

<table>
<thead>
<tr>
<th>Country</th>
<th>Diagnostic microbiology laboratory</th>
<th>Food safety inspectorate</th>
<th>Physician/ patient reports</th>
<th>Local/regional public health authority</th>
<th>Type of outbreaks reported</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denmark</td>
<td>Yes</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
<td>Food/waterborne</td>
</tr>
<tr>
<td>France</td>
<td></td>
<td></td>
<td></td>
<td>Yes</td>
<td>Food/waterborne</td>
</tr>
<tr>
<td>England and Wales</td>
<td></td>
<td></td>
<td>Yes</td>
<td></td>
<td>All</td>
</tr>
<tr>
<td>Italy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>All</td>
</tr>
<tr>
<td>Finland</td>
<td>Yes&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td>All</td>
</tr>
<tr>
<td>Sweden</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>All</td>
</tr>
<tr>
<td>Germany</td>
<td></td>
<td></td>
<td></td>
<td>Yes&lt;sup&gt;b&lt;/sup&gt;</td>
<td>All</td>
</tr>
<tr>
<td>Slovenia</td>
<td></td>
<td></td>
<td></td>
<td>Yes</td>
<td>All</td>
</tr>
<tr>
<td>Spain</td>
<td></td>
<td></td>
<td></td>
<td>Yes</td>
<td>All</td>
</tr>
<tr>
<td>the Netherlands&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td>Yes</td>
<td>All&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Participant is sole laboratory performing viral testing, and coordination is conducted at National Public Health Laboratory and National Food Administration.

<sup>b</sup>Norovirus became a reported disease in January 2000. From 1997 to 2000, reports from local health departments were collected unsystematically.

<sup>d</sup>Dutch national data were collected from three systems: notification system, food safety inspectorate, and laboratory-based system (from diagnostic microbiology laboratories, local/ regional public health authorities, physician/patient reports, and other institutions in which outbreaks occurred).

<sup>d</sup>Foodborne only for systems 1 and 2.
respectively), whereas in Denmark, 13 (76%) of 17 reported outbreaks occurred in food outlets. In Denmark, surveillance is done by the Food Safety Inspectorate, which collects reports of suspected foodborne outbreaks only. The Inspectorate is not informed of person-to-person spread outbreaks, which are more commonly seen in residential institutions and hospitals. In Slovenia, the majority of reported outbreaks occurred in day-care centers (10/14; 71%), and in France, most reported outbreaks occurred in private houses (7/9; 78%). In France, reporting was recommended only for large outbreaks or if oysters, an item commonly consumed in French households, were the suspected vehicle of infection.

Food and Water as Sources of Outbreaks

Among countries conducting broad-based outbreak surveillance, the following proportions of viral gastroenteritis outbreaks were reported to be associated with food- or waterborne transmission: Finland (24%), the Netherlands (17%), Slovenia (14%), Spain (7%), and England and Wales (7%) (Table 4). Very rarely was laboratory evidence (detection of the same organism in the vehicle and stool specimens) or statistical evidence (case-control or cohort) available that demonstrated the association of the vehicle with illness. During the survey period, Danish and French surveillance almost exclusively focused on outbreaks transmitted through food and water. Therefore, estimates of the proportion of food and water

Table 2. National coverage and use of clinical definitions for viral gastroenteritis by European surveillance systems

<table>
<thead>
<tr>
<th>Country</th>
<th>National coverage %a</th>
<th>Definition of viral gastroenteritis outbreakb</th>
<th>Laboratory confirmation required</th>
<th>Outbreak definition applied</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denmark</td>
<td>100</td>
<td>Kaplan’s, shellfish</td>
<td>Always</td>
<td>Always</td>
</tr>
<tr>
<td>England and Wales</td>
<td>100</td>
<td>General</td>
<td>Yes</td>
<td>Always</td>
</tr>
<tr>
<td>Finland</td>
<td>100</td>
<td>Clinical</td>
<td>Yes</td>
<td>Always</td>
</tr>
<tr>
<td>France</td>
<td>100</td>
<td>Clinical, shellfish</td>
<td>Yes</td>
<td>Always</td>
</tr>
<tr>
<td>Germany</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Italy</td>
<td>1</td>
<td>Clinical</td>
<td>Always</td>
<td>Always</td>
</tr>
<tr>
<td>Slovenia</td>
<td>100</td>
<td>Clinical</td>
<td>Sometimes</td>
<td>Sometimes</td>
</tr>
<tr>
<td>Spain</td>
<td>100</td>
<td>General</td>
<td>Yes</td>
<td>Always</td>
</tr>
<tr>
<td>Sweden</td>
<td>100</td>
<td>Kaplan’s, clinical</td>
<td>Yes</td>
<td>Always</td>
</tr>
<tr>
<td>Netherlands</td>
<td>100</td>
<td>System 1: clinical</td>
<td>Yes</td>
<td>Always</td>
</tr>
<tr>
<td></td>
<td></td>
<td>System 2: Kaplan’s</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>System 3: clinical</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.** Reported outbreaks of viral gastroenteritis, European surveillance, 1995–2000

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Denmark</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>England and Wales</td>
<td>392</td>
<td>352</td>
<td>151</td>
<td>219</td>
<td>239</td>
<td>290</td>
<td>13 (4)</td>
<td>273 (96)</td>
<td>1,643</td>
</tr>
<tr>
<td>Finland</td>
<td>5</td>
<td>27</td>
<td>35</td>
<td>58</td>
<td>1</td>
<td>1 (2)</td>
<td>56 (97)</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>France</td>
<td>4</td>
<td>9</td>
<td>7</td>
<td>8</td>
<td>19</td>
<td>28</td>
<td>1 (14)b</td>
<td>5 (71)b</td>
<td>43</td>
</tr>
<tr>
<td>Germany</td>
<td>1</td>
<td>53</td>
<td>145</td>
<td></td>
<td></td>
<td>227</td>
<td>0</td>
<td>227 (100)</td>
<td>426</td>
</tr>
<tr>
<td>Italy</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2 (100)</td>
<td>2</td>
</tr>
<tr>
<td>Slovenia</td>
<td>8</td>
<td>6</td>
<td>8</td>
<td>4</td>
<td>5</td>
<td>14</td>
<td>8 (57)</td>
<td>6 (43)</td>
<td>45</td>
</tr>
<tr>
<td>Spain</td>
<td>37</td>
<td>24</td>
<td>25</td>
<td>29</td>
<td>66</td>
<td>55</td>
<td>6 (43)c</td>
<td>8 (57)c</td>
<td>236</td>
</tr>
<tr>
<td>Sweden</td>
<td>81</td>
<td>130</td>
<td>130</td>
<td>130d</td>
<td>190d</td>
<td>195d</td>
<td></td>
<td></td>
<td>856</td>
</tr>
<tr>
<td>the Netherlands</td>
<td>25</td>
<td>69</td>
<td>54</td>
<td>36</td>
<td>58</td>
<td>59</td>
<td>5 (13)d</td>
<td>32 (84)d</td>
<td>301</td>
</tr>
</tbody>
</table>

**a**Number of outbreaks attributed to organism (percentage of year 2000 outbreaks).

**b**Based on seven laboratory-confirmed viral outbreaks.

**c**Based on 14 laboratory-confirmed viral outbreaks.

**d**Approximate figures.

**e**Based on 38 laboratory-confirmed viral outbreaks.
transmission from these countries cannot be compared to the general estimates in other countries.

Molecular Characterization Techniques

Different molecular techniques were used by participating institutes to characterize virus from outbreaks in 2000. Reverse line blot was used in the Netherlands and Spain, and the heteroduplex mobility assay was used in England and Wales. Sequence analysis was performed in England and Wales, Finland, France, Italy, Germany, Spain, and the Netherlands; EIA were used in England and Wales, and a microplate hybridization technique was used in Finland.

Discussion

Viral gastroenteritis infection, typically a self-limiting condition of short duration in humans, is extremely common and associated with relatively few deaths. Surveillance of outbreaks of this infection, rather than individual cases, may be more appropriate. In our review of the surveillance for this infection in Europe, we found variations in the organizations conducting surveillance, the surveillance definition of a viral gastroenteritis outbreak, the populations under surveillance, and the completeness of descriptive and analytical epidemiology and diagnostic information.

Researchers comparing surveillance information at an international level should consider the outputs of surveillance, as well as the influence of methodology and structure of surveillance on these outputs. Surveillance for viral gastroenteritis in Europe is poorly developed; systems vary in their sources of data, definitions, and use of diagnostic techniques. These differences are reflected in the wide range of numbers of outbreaks, population-based rates, and epidemiologic patterns observed across Europe. Nonetheless, our comparison of this surveillance data was an informative exercise because international epidemiologic databases of viral gastroenteritis infections have not been developed. In many of the countries included in the Foodborne Viruses in Europe network, viral gastroenteritis has not been considered a priority, and these countries do not have a well-developed surveillance system. This inventory of surveillance data will aid in the development of a more consistent and complete surveillance across Europe.

These data clearly show that both the absolute number and the population-based rates of viral gastroenteritis outbreaks differ substantially between European surveillance systems. From 1995 to 2000, 1,643 outbreaks of viral gastroenteritis were investigated by the Public Health Laboratory Service in England and Wales, but the outbreak rates (number of outbreaks/population) were highest in Sweden for every surveyed year. Some variation in these figures occurred because a number of the surveillance systems required laboratory confirmation while others did not (Table 2). However, the criteria suggested by Kaplan et al. to recognize an outbreak of viral etiology is widely used and is generally accepted as an effective clinical tool in the absence of diagnostic information (33). Interestingly, surveillance systems with the most stringent outbreak criteria, including laboratory confirmation of outbreaks (England and Wales, Finland, and Sweden) ascertained the most outbreaks, likely because surveillance in these countries is more developed and integrated better with reporting bodies.

However, even the surveillance systems with the highest figures greatly underascertain viral gastroenteritis. A study of infectious intestinal disease in England and Wales estimated that only 1/300–1,500 cases of Norovirus gastroenteritis are reported to national surveillance (34). For a case to be ascer-
tained by national surveillance, patients must be examined by their primary-care doctor, a specimen must be taken and submitted for laboratory testing, the test must be positive (the amount of false negatives will depend on the diagnostic technique), and the surveillance unit must be notified. Ascertaining outbreaks requires an additional step in which investigators must recognize epidemiologic links between cases. While this chain of events will differ from country to country, the principle of underascertainment affects all surveillance. However, outbreak recognition and investigation will, through case finding, lead to better ascertainment of persons affected in outbreaks.

Although most surveillance systems may be designed for national coverage, reports were incomplete to a varying degree. Ascertained outbreaks varied geographically and were incomplete, as demonstrated by the large variation in reported outbreaks (Table 3).

This survey found that the great majority of European viral outbreaks could be attributed to Norovirus. In Denmark, England and Wales, Finland, France, and Sweden, >95% of nonbacterial outbreaks were attributed to noroviruses as were 84% of outbreaks in the Netherlands. The relative number of infections from noroviruses was lower in Slovenia (43%) and Spain (57%), although these estimates are based on a small number of outbreaks (n=14 for both). These figures are consistent with previous reports that Norovirus could be detected in 91% of all nonbacterial infectious intestinal disease outbreaks in the Netherlands (9) and 89% of such outbreaks in Sweden (35). Similarly, Fankhauser et al. found Norovirus responsible for 96% of nonbacterial outbreaks in the United States (7).

Estimates of the importance of foodborne transmission also varied widely in this survey. Foods were implicated as the vehicle of transmission in 16 (94%) of 17 outbreaks in Denmark and 28 (100%) of 28 outbreaks in France because surveillance systems in these countries were designed to detect foodborne disease. In countries with more general outbreak data, estimates of foodborne transmission were lower: 7 (17%) of 41 in the Netherlands, 14 (24%) of 58 in Finland, and 20 (7%) of 290 in England and Wales, although laboratory and statistical evidence of association with food or water was scant.

The settings of outbreaks also reflected the proportion of reported outbreaks that were ascertained to be foodborne. For example, in Denmark, 75% of all reported outbreaks were set in food outlets. In Spain, the Netherlands, and England and Wales, most reported outbreaks occurred in residential homes and hospitals, with only a small fraction occurring in food outlets.

In Finland, the National Public Health Laboratory is the only facility in the country testing for Norovirus and, therefore, is aware of all such investigations. Most other surveil-

Table 4. Foodborne transmission and supporting evidence of implicated food vehicles, European surveillance, 2000

<table>
<thead>
<tr>
<th>Country</th>
<th>Total outbreaks</th>
<th>Food/waterborne outbreaks (%)</th>
<th>Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Laboratory</td>
</tr>
<tr>
<td>Denmark</td>
<td>17</td>
<td>16 (94)</td>
<td>1</td>
</tr>
<tr>
<td>England and Wales</td>
<td>290</td>
<td>20 (7)</td>
<td>1</td>
</tr>
<tr>
<td>Finland</td>
<td>58</td>
<td>14 (24)</td>
<td>0</td>
</tr>
<tr>
<td>France</td>
<td>28</td>
<td>28 (100)</td>
<td>2</td>
</tr>
<tr>
<td>Germany</td>
<td>227</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Italy</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Slovenia</td>
<td>14</td>
<td>2 (14)</td>
<td>0</td>
</tr>
<tr>
<td>Spain</td>
<td>14</td>
<td>1 (7)</td>
<td>0</td>
</tr>
<tr>
<td>Sweden</td>
<td>190</td>
<td></td>
<td></td>
</tr>
<tr>
<td>the Netherlands</td>
<td>41</td>
<td>7 (17)</td>
<td>0</td>
</tr>
</tbody>
</table>

*aSame organism found in stool specimen and food vehicle.
*bStatistically significant result from cohort or case-control study.
Timely feedback of surveillance data to participants is an essential step in the cycle of continued improvement of a surveillance system (41) that we have made possible through this European Union–funded network. In addition to describing the current state of viral gastroenteritis surveillance in Europe, this report will act as a baseline to interpret prospective outcomes of the Foodborne Viruses in Europe network.

Foodborne Viruses in Europe is funded by the European Commission, Directorate General Research under the Quality of Life and Management of Living Resources - QLK1-CT-1999-00594.

Mr. Lopman is an epidemiologist at the Gastrointestinal Diseases Division of the Public Health Laboratory Communicable Disease Surveillance Centre. He coordinates the epidemiologic surveillance for the Foodborne Viruses in Europe consortium.

References


Emerging Infectious Diseases • Vol. 9, No. 1, January 2003
RESEARCH


Address for correspondence: Ben Lopman, Gastrointestinal Diseases Division, Communicable Disease Surveillance Centre, 61 Colindale Avenue, London, England NW9 5EQ; fax: +44 208 200 7868; e-mail: blopman@phls.org.uk

Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

Emerging Infectious Diseases launches Web-based manuscript submission and peer review in 2003

Emerging Infectious Diseases has launched a Web-based system for manuscript submission and peer review. The system allows authors, reviewers, editors, and editorial office staff direct access to journal operations through the Web.

To submit a manuscript using Manuscript Central:
2. Click on Submit Manuscript (upper right hand side), which takes you to the Manuscript Central Web site.
International Collaborative Study To Compare Reverse Transcriptase PCR Assays for Detection and Genotyping of Noroviruses

Jan Vinjé,1† Harry Vennema,1 Leena Maunula,2 Carl-Henrik von Bonsdorff,2 Marina Hoehne,3 Eckart Schreier,3 Alison Richards,4 Jon Green,4 David Brown,4 Suzanne S. Beard,5 Stephan S. Monroe,3 Erwin de Bruin,1 Lennart Svensson,6 and Marion P. G. Koopmans1∗

Research Laboratory for Infectious Diseases, National Institute of Public Health and the Environment, Bilthoven, The Netherlands1; Haartman Institute, Department of Virology, Helsinki, Finland2; Robert Koch-Institute, Berlin, Germany3; Central Public Health Laboratory, London, United Kingdom4; Viral Gastroenteritis Section, Centers for Disease Control and Prevention, Atlanta, Georgia5; and Swedish Institute for Infectious Disease Control, Solna, Sweden6

Received 6 May 2002/Returned for modification 20 July 2002/Accepted 29 December 2002

Noroviruses (previously called Norwalk-like viruses [NLVs]) have emerged as the single most common cause of outbreaks as well as sporadic cases of acute gastroenteritis in children and adults (13, 20, 55). Epidemiological investigations of outbreaks have shown that the most important modes of transmission are person-to-person contacts and contaminated food and water (20, 31). Multistate and global outbreaks linked to the distribution of contaminated foods and water have been reported (4, 6, 14, 45). Moreover, the emergence of a common strain with global distribution has raised important questions on the likelihood of massive food-borne introduction of new NLV strains (43, 51).

Since human NLVs have not yet been cultivated in vitro, detection has traditionally relied on electron microscopy (EM) or immune-EM (11, 25). Although several reference centers still utilize EM for the routine investigation of outbreak samples, the successful cloning and sequencing of complete and partial NLV genomes has allowed the development of more sensitive reverse transcriptase PCR (RT-PCR) methods. First-generation RT-PCR assays that used primers based on the prototype Norwalk virus performed poorly since the genetic diversity of NLV strains was much greater than anticipated initially and therefore the primers selected needed to be optimized (12, 26, 41, 44). Using sequence information of an increasing number of NLV strains, several research groups successfully developed RT-PCR assays based on improved primers targeting a conserved region of open reading frame 1 (ORF1) coding for the viral RNA polymerase (POL) region (1, 17, 21, 30, 34, 40, 50, 56). Subsequently, these assays have been used successfully in epidemiological studies for the diagnosis of NLVs in fecal specimens from both outbreaks and sporadic cases (1, 7, 13, 17, 37, 40, 46, 50, 51). In addition, primers directed to other regions of the NLV genome have been developed, including the 2C helicase, the 5′ end of the capsid region, and ORF3 (22, 38, 42, 52, 54, 56, 57). In general, however, assays based on these regions are less broadly reactive. With few exceptions (46), most assays for the detection of NLVs in stool samples are based on a single-round format, whereas for detection of low copy numbers in environmental samples (hem)nested RT-PCR assays have successfully been developed and applied (23, 48).

Because NLVs cannot yet be cultured in vitro, antigenic typing has been limited to typing by solid-phase immune-EM and enzyme-linked immunosorbent assays based on recombinant capsid proteins (28, 35). Recently, the National Institute for Public Health and the Environment (RIVM; Bilthoven, The Netherlands) and the Centers for Disease Control proposed similar NLV genotyping schemes based on the diversity in the entire capsid gene (2, 33). Correlation between genotype and antigenic type was at least found for strains from nine
genotypes (19, 52). The current consensus is that the NLVs commonly found in humans can genetically be divided into two genogroups (GI and GII), which further segregate into genotypes. At least 15 genotypes exist with an arbitrary cutoff of a 20% amino acid difference across capsid sequences (19, 53). In addition, two new NLV genogroups have been proposed: NLVs detected in fecal specimens of calves with Jena virus as the prototype virus (GIII [2, 36]) and genogroup GIV, which consists of human strains that cluster equidistantly with both GI and GII viruses, with Alphatron virus as the prototype virus (33, 53). For the majority of NLV strains tested in a panel of 31 strains, there was agreement between clustering of strains based on the capsid gene and a small region of the POL gene used for diagnosis (52). Consequently, the current used POL-based assays can provide indications of the level of diversity in epidemiological studies (1, 15, 37, 40, 46, 51). For comparability and exchange of data, it is important to agree on a specific region for detection and subsequent typing.

For interpretation of RT-PCR, methods other than gel electrophoresis are essential to prevent false-positive results. Hybridization and DNA sequencing of the RT-PCR products are commonly used methods for confirmation of RT-PCR products (1, 17, 21, 34, 50). Recently, NLV genotyping methods for high throughput have been described that target an overlapping region in the POL region used for diagnosis (5, 39, 40, 53). The lack of international harmonization of detection and genotyping methods for food- and water-borne viral infections, including NLV, has precluded full use of the molecular information for tracking of outbreaks across borders and for elucidation of the major transmission routes. Therefore, we initiated a collaborative research project funded by the European Union (EU) to allow more rapid and internationally harmonized assessment of the spread of food-borne viral pathogens (QLK1-1999-00594 [EU 5th Framework Program]). To understand how data from different regions can be aggregated, we compared five different RT-PCR assays for detection of NLVs with a panel of coded stool samples.

**MATERIALS AND METHODS**

**European consortium.** The consortium consists of 12 partner laboratories in nine countries (p1 [RIVM], p2 [University Central Hospital, Helsinki, Finland], p3 [Statens Serum Institute, Copenhagen, Denmark], p4 [Swedish Institute for Infectious Disease Control, Solna, Sweden], p5 [Central Public Health Service, London, United Kingdom], p6 [Robert Koch Institut, Berlin, Germany], p7 [Instituto de Salud Carlos III, Majadahonda, Spain], p8 [IFREMER, Nantes, Invec], p9 [CHU du Bocage, Dijon, France], p10 [Istituto Superiore di Sanità, Rome, Italy], p11 [University of Barcelona, Barcelona, Spain], and p12 [University of Valencia, Valencia, Spain] across Europe. Among these, 10 laboratories are involved in the diagnosis of NLVs in humans on a routine basis or as a national or regional reference laboratory (Table 1). Coordination of the project was contracted out to RIVM (M. P. G. Koopmans [EU contract QLK1-1999-00594]). Overall, the aim of this consortium was to allow more rapid and internationally standardized assessment of the spread of food-borne viruses, including elucidation of the mechanisms of emergence of novel variants, by harmonization of methods, the use of a common database, and epidemiological follow-up of international food-borne viral infections. There are four work packages, namely, (molecular) virology (WP1), database (WP2), epidemiology (WP3), and food microbiology (WP4). The work described here was performed within WP1. The objectives of this work package were to compare the performance of existing methods for detection and (geno)typing of NLV and hepatitis A virus by using a coded panel of stool specimens, resulting in the selection of consensus assays for use in outbreak investigations across Europe.

**Inventory of methods.** One of the objectives of the present study was to compare methods and, if possible, to select a consensus assay for the molecular detection of NLVs to be used throughout Europe, with a special focus on multinational outbreaks. Information on the methods used by the different partner laboratories for RNA extraction, RT-PCR detection, and genotyping of NLVs was obtained by using a questionnaire.

**Assembling and coding of the stool panel.** A coded stool panel of 82 samples was compiled from stool specimen collections from five different partner laboratories (p1, p2, p4, p5, and p6). The specimens had been collected over a 4-year period of time (1997 to 2000), were derived from both outbreaks and sporadic cases of gastroenteritis, and had previously been tested for NLVs by RT-PCR (p1, p2, p4, p5, and p6) and EM (p2 and p4). The specimens had been stored either as original stool samples at 4°C (p1, p2, p4, and p5) or as 10% suspensions in phosphate-buffered saline at −20°C (p6). All samples were shipped frozen to the p1 laboratory (RIVM) and were coded by an epidemiologist who had no prior knowledge of contents or origin of the samples. In addition, nine stool samples representing nine different NLV genotypes were included as positive controls. The panel samples were frozen at −70°C and shipped on dry ice to four different participating laboratories. All laboratories were asked to assess the presence of NLVs in the specimens by using their routine diagnostic workup and, in addition, to determine the detection limit of the samples containing viruses from nine genotypes. Instead of a stool panel, viral-RNA extracts of a complete panel were shipped to the central reference laboratory for NLV detection in the United States (Viral Gastroenteritis Section, Centers for Disease Control), designated the p13 laboratory.

**RNA extraction and RT-PCR.** Viral RNA was extracted from the panel samples according to published standard procedures used in each partner laboratory (18, 34, 40, 46, 50). The essentials of the procedures are listed in Table 1. Laboratories p1, p5 and p6 used a silica-based method (8) and laboratory p2 used Tripure reagent (Roche) as described previously (40). For RT-PCR, each partner laboratory tested the RNA extracts by using published assay formats and primer pairs (Table 2). Laboratory p1 used a separate step for the RT reaction, followed by a PCR assay with the primer pair JV12-JV13 (50); laboratory p2 used a separate RT with NVp110, followed by PCR with the primers NVp110, Ni, and NVp69 (40); laboratory p5 used two RT-PCR assays with the E3-Ni and E3-
Ando primer pairs, respectively (18); and laboratory p6 used a recently published nested RT-PCR assay format (46), with some modifications. Briefly, for the first PCR an equimolar mixture of primers 32 and 32a and antisense primer 36 were used. In the second PCR a mixture of primers 33, 33a, 35, and 35a was used (Table 2). Laboratory p13 tested RNA extracted by p1 by using a single-tube RT-PCR assay targeting the 3' end of ORF1 (region B) (16). Detection of NLV RT-PCR products was confirmed by using Southern hybridization and reverse line blot hybridization (RLB) (50, 53) (p1), microplate hybridization (40) (p2), and sequencing (p1, p5, p6, and p13).

**Detection limits.** To determine the detection limit of each RT-PCR assay, participants were asked to prepare and test 10-fold serial dilutions of purified RNA prepared from undiluted fecal samples containing nine different NLV genotypes as determined by sequence analysis (50). The PCR titer was calculated as the lowest dilution giving a clear positive signal after RT-PCR.

**NLV genotyping by RLB.** For high-throughput screening, NLVs were genotyped at the p1 laboratory by RLB, which was performed as described previously (53). Briefly, all panel sample extracts were amplified by using the standard RT-PCR, including a biotin-labeled JV13 primer. The amplification products were hybridized to a set of 18 immobilized oligonucleotide probes, each corresponding to a genogroup (I or II) or genotype (53). Hybridization was performed in a miniblotter system (MN45, Immunetics, Cambridge, Mass.) that can analyze 40 samples simultaneously. After hybridization, bound PCR products were detected by chemiluminescence (53). Phylogenetic analysis of a 145-nucleotide (nt) region of the POL gene was used as the “gold standard” for genotype identification (53).

**DNA sequencing and phylogenetic analysis.** All extracts that resulted in NLV RT-PCR products of the appropriate size (237 bp for p1, 113 bp for p5, 338 bp for p6, and 213 bp for p13) were sequenced in both directions by using dye terminator chemistry. PCR products of the p2 laboratory were not sequenced. The NLV genogroups and genotypes were assigned according to a recently proposed classification system (2, 20, 33). Briefly, genotypes within genogroup I or II (GI or GII) are represented by a cryptogram of the prototype strain, followed by the genogroup and cluster number in parentheses as follows: Hu/NLV/GI/Norwalk/1968/US (GI.1), Hu/NLV/GI/Southampton/1991/UK (GI.2), Hu/NLV/GI/DesertShield395/1990/SA (GI.3), Hu/NLV/GI/Chiba407/1987/JP (GI.4), Hu/NLV/GI/Masgrove/1989/UK (GI.5), Hu/NLV/GI/Hesse3/1997/DE (GI.6), Hu/NLV/GI/Winchester/1994/UK (GI.7), Hu/NLV/GI/Hawaii/1971/US (GI.11), Hu/NLV/GI/Melksham/1994/UK (GI.2), Hu/NLV/GI/Toronto24/1991/CA (GI.3), Hu/NLV/GI/Bristol/1993/UK (GI.4), Hu/NLV/GI/Hillingdon/1990/UK (GI.5), Hu/NLV/GI/Scarocft/1990/UK (GI.6), Hu/NLV/GI/Leeds/1990/UK (GI.7), and Hu/NLV/GI/Amsterdam/1998/NL (GI.8). In addition, we included sequences of strains belonging to a distinct branch within a genotype (Hu/NLV/GI/Stavanger/1995/NO (GI.3b), Hu/NLV/GI/Wortley/1990/UK (GI.1b), a sequence that tentatively is assigned to a fourth genogroup (Hu/NLV/GI/Alphatron/1998/NL (GIV.1)), and viruses of which the grouping in ORF1 is different than in ORF2 in the phylogenetic analyses.

**Combining data and selection criteria for the assays.** After each laboratory returned its results for detection (p1, p2, p5, p6, and p13), genotyping (p1), and sequencing (p1, p5, p6, and p13) to the coordinator (p1), the code of the panel was broken. Assays were ranked based on an arbitrary scoring system for sensitivity and detection limit based on the actual data from the panel evaluation from lowest (score 1) to highest (score 5). In addition, having a single-round format was assigned two points, whereas having a nested format was assigned the score 0, since the latter was considered less desirable to use in a clinical diagnostic setting.
RESULTS

Inventory of methods. All partner laboratories used an assay targeting the POL gene in the NLV genome. The overlap of the amplified region between the assays, however, was 63 nt only. Based on the inventory (Table 1), we selected four assays (p1, p2, p5, and p6) for comparative evaluation (18, 40, 46, 50). The assays reflect the diversity of RT-PCR detection methods for NLVs used by different partner institutes. In addition, at the p13 laboratory, a recently developed RT-PCR assay was used that targets a different region within ORF1 with no sequence overlap at all (16).

Sensitivity of five different RT-PCR assays for the detection of NLVs. After we broke the code of the panel, we found that 82 of the 91 samples of the stool panel had previously tested positive for NLV by either RT-PCR (75 samples) or by EM (7 samples). The remaining nine samples had been included as negative controls and originally tested negative by RT-PCR. Therefore, all calculations of the sensitivity rate were performed by using the 82 NLV-positive samples as a 100% score (Table 3). In total, 69 (84%) of the samples tested positive in at least one of the assays. The overall sensitivity rate of the assays (considering any positive test result) ranged from 52 to 73% (73% for p6, 67% for p1, 60% for p13, 59% for p2, and 52% for p5). When we compared the different assays, we found that the p1 assay had the highest percentage of positive samples when three (91%) or four (100%) of the five assays yielded positive results (Table 3).

Sensitivity related to origin of samples. To identify whether there was a relationship between performance of an assay and the geographic origin of the samples or different sample stor-
age conditions, the results were summarized based on the origin of the panel samples (Table 3). From the 20 p6 samples that had previously tested positive the p6 assay scored 65%, whereas in the other assays a range of from 15 to 40% of the samples scored as positive. For the panel samples of other geographical origin, the sensitivity rates ranged from 55 to 70% for the p2 samples, 53 to 84% for the p4 samples, 57 to 86% for the p5 samples and 67 to 100% for the p1 samples. Of the 11 samples that had been submitted to the panel as positive samples but that tested negative upon retesting, seven (64%) were of p6 origin and had been stored as 10% fecal suspensions at −20°C. When p6 samples were excluded from analysis, the overall sensitivity rate of the assays increased significantly (65% for p5, 66% for p2, 77% for p13, 81% for p6, and 81% for p1 [Table 3]).

**Sensitivity related to genotype.** The nucleotide sequences of all RT-PCR products of the expected sizes for the p1, p5, p6, and p13 samples were determined. A stretch of 145 nt that overlapped sequences of both p1 and p6 and sequences from representative strains of 15 different genotypes (52) was used to create a multiple alignment from which a phylogenetic tree was constructed, and most panel strains could be assigned to one of the existing genotypes (20, 52) (Table 3). NLV sequences were obtained from 66 panel strains (13 GI, 52 GII, and 1 GIV strains; Fig. 1) in the POL region (p1, p5, and p6 together). For three strains, sequences of less than 145 nt were obtained and therefore were not included in the phylogenetic analysis. One of these strains was detected in a sample in which a completely different NLV sequence was detected by a different partner laboratory (sample 3, Table 4). Overall, the RT-PCR assays detected between 54 to 100% for the GI strains and between 58 to 85% for the GII and GIV strains (Table 3). The sensitivity of detection by genotype was lower for the p2 assay for GI strains (54%) and for the p5 assay for GII and GIV strains (58%). The performance results for each RT-PCR assay, including the nine samples that originally tested negative, are shown in Table 4.

**Detection limit.** To calculate the detection limit of each RT-PCR assay, each partner laboratory determined the RT-PCR endpoint titers of nine different NLV strains. The detection limit differed from 10^{3}-fold for the Southampton strain up to 10^{7}-fold for the Toronto strain (Fig. 2).

**Ranking of the assays.** After we compared the overall sensitivity, the detection limit, and the PCR format (single-round or nested) of each of the assays evaluated in the present study, it was clear that no single assay stands out as best based on all of these criteria. However, the p1 assay scored higher for sensitivity and for not having a nested format.

**Genotyping.** (i) **Sequencing.** In order to classify the NLV strains in the present study, we combined and extended three previously published NLV classification schemes (2, 20, 33) based on NLV strains for which complete ORF2 sequences were available. To assign genotypes by using POL sequences, we used our previously defined working criteria: >85% similarity based on the nucleotide sequence of the POL fragment for GI strains and 90% for GII strains (52). Phylogenetic analysis of 66 POL sequences obtained in the present study revealed that they could be grouped into 17 clusters; 5 within GI, 11 within GII, and 1 (Alphatron; GIV) clustering almost equidistant from GI and GII (Fig. 1). Of these, 16 correlate with known genotypes (Southampton [GI.2], Chiba [GI.4], Hesse [GI.6], Winchester [GI.7], Hawaii [GII.1], Melksham [GII.2], Toronto [GII.3], Bristol [GII.4], Hillingdon [GII.5], Seacroft [GII.6], Leeds [GII.7], Amsterdam [GIL.8], and Alphatron [GIV.1]) or subclusters (e.g., Stavanger [GI.3b], Worley [GII.Ib], and Rotterdam). One potentially new genotype could be recognized, strain “GII Finland,” with an −15% nucleotide difference from the currently known GII clusters (Fig. 1).

(ii) **RLB.** Genotyping of NLVs by RLB was performed with a membrane to which a set of 18 probes was described previously (Fig. 3) (53). In all, 50 of the 55 (91%) samples tested positive by RLB and, of these, 37 (74%) could be genotyped directly when compared with the sequence of the POL region. Of the 13 untypeable strains, 11 reacted only with a genogroup probe (GI or GII) and were negative with a genotype-specific probe (four Bristol strains, two Sindlesham strains, two Leeds strains, one Rotterdam strain, one Desert Shield strain, and one untypeable GII strain). Two strains that were determined to belong to the Worley subcluster (GII Ib) by sequencing were incorrectly genotyped by RLB as Bristol and Melksham, respectively.

**DISCUSSION**

To establish an international laboratory surveillance network for the detection of multinational outbreaks of NLV gastroenteritis across Europe, a harmonization of methods for NLV diagnosis is needed. Molecular detection of NLVs has found widespread use since the development of RT-PCR assays that allow successful amplification of viruses that are often shed in low numbers and are genetically extremely heterogeneous (1, 20). By using these assays as the diagnostic tool, it has been established that NLVs are the most common cause of outbreaks of gastroenteritis worldwide (15, 33, 37, 43, 51). Harmonized detection and genetic comparison of NLV strains ideally requires an assay that has high sensitivity and specificity, that is easy to standardize, that preferably (though not necessarily) uses a single-round format, and that results in a product that is sufficiently long for reliable phylogenetic typing.

In the present study, we selected five different RT-PCR assays and tested their performance with a panel of 91 stool specimens. To have the best representation of circulating NLV strains, this panel included specimens from five different countries, specimens that were EM positive but had previously tested as RT-PCR negative, and specimens representing at least nine different NLV genotypes. Overall, the sensitivity was acceptable (>84%) for all assays with the samples that were unambiguously positive (four of five or more tested positive). The sensitivity went down to as low as 62% (p5) when all positive results were included. This suggests differences in the detection limit for the assays with decreasing sensitivity, which was observed most prominently for the p2, p5, and p13 assays. Indeed, for the p5 test, detection limits were rather low, suggesting that this is the explanation for the lower sensitivity. The p2 assay detected viruses in highly diluted samples but appears to be more selective, since only 54% of the GI viruses were detected with this test. This may be explained by the limited number of GI probes that are used in the microplate hybridization assay which, given the high sequence diversity between
NLV strains, should be significantly increased to detect all currently known genotypes. This finding implies that the assays should be used with caution: while they may work well with outbreak specimens (multiple samples), problems may arise when the assays are used in community surveillance or environmental studies. A nested format was considered to be less favorable when used as a consensus assay in a laboratory surveillance network because of the higher risk of cross-contamination in clinical diagnostic laboratories. Copurification of RT-PCR inhibitors during RNA extraction, lack of primer specificity, or inadequate interpretation of EM data are possible explanations for samples that previously tested positive for NLVs by EM (p2 and p4) but could not be confirmed by RT-PCR.

Four of the five RT-PCR assays evaluated in the present study include primers that target the POL region involving GLPSG and YGDD amino acid motifs and are among the most frequently used in the field (1, 17, 34, 40) or have been used for screening NLVs in large-scale epidemiological studies (13, 51). To extend our comparative analysis, we included a
recently developed RT-PCR assay targeting a different region at the 3′ end of ORF1 (16). Other regions of the genome have been used as targets as well, including 2C helicase (54, 57), the 5′ end of the capsid region (21, 42), and ORF3 (52, 56). However, since these regions have a greater genetic diversity than the POL region, they are less frequently used. It remains to be seen whether data obtained for genotype classification by using the region B fragment (16) correlate with clustering based on the capsid gene, as has been done for the p1 assay, since there was no clear advantage of using this region over the existing assays. Furthermore, a strong disadvantage would be the loss of the current POL sequence database. Therefore, we favor the assays that target the overlapping region within ORF1. To promote further harmonization, we recommend for newly starting groups the p1 assay as the method of choice for the EU network. It is clear, however, that the overall sensitivity is less than optimal for all assays. This could be due to the loss of RNA upon storage and shipping. Of the panel samples that had previously tested positive for NLV, only 84% were found to be positive in at least one of the five RT-PCR assays, raising questions regarding the stability of the viruses in these false-negative samples. The preferred storage condition for diagnostic work is fresh stool specimens at 4°C, but samples were divided into aliquots and frozen for shipment. Since the majority of the false-negative samples originated from one laboratory from which 10% fecal suspensions in phosphate-buffered saline were included in the panel, instead of the original undiluted fecal sample, it is conceivable that the viruses in these samples may have deteriorated during storage, shipment, or freeze-thawing, as has been described previously (9). Therefore, we recommend the use of raw stool samples stored at −70°C or lower for future primer evaluation studies.

Several factors can affect the sensitivity and specificity of RT-PCR assays, including the viral RNA extraction method, the primers used in amplification, and the methods used for confirmation of test results. For extraction of viral RNA, all laboratories participating in the present study used a guanidine thiocyanate-based extraction procedure to release viral RNA and subsequent purification by either binding onto silica beads (8) or precipitation by ethanol (10). Although direct heat release of NLVs from fecal extracts has been described (47), the guanidine isothiocyanate-silica method has been reported to be the most successful approach to removing inhibitors from fecal specimens (24). Differing efficiencies of extraction and RT among the different assays might also explain discrepancies that could be addressed with viral cDNA as the starting material. Visual interpretation of gels after gel electrophoresis yielded bands in the right range for several samples in the p1, p5, and p13 assays, demonstrating the necessity to confirm the specificity of the amplicons by a second method such as DNA sequencing of the RT-PCR products, Southern hybridization, microplate hybridization (40), or RLB (53). Nonspecifically amplified DNA can easily be generated because low annealing temperatures (i.e., 37°C) are used during PCR to tolerate some mismatches between primers and NLV templates (3, 51, 54). This is considered essential for the successful generic detection of such a genetically diverse group of viruses.

All five RT-PCR assays detected the majority of the genetic clusters present in the panel. However, there were considerable differences among the performance of the assays because only 34% of the samples tested positive in all assays. Overall, the p2 assay performed relatively poorly in detecting GI strains, and the p5 assay performed poorly for the GII strains. The reason for the latter result is unclear since both assays use primer Ni, which detects primarily GII strains (17). Primer NV69 (p2) was developed based on the Norwalk virus se-
TABLE 4. Overall RT-PCR results for 91 Stool Samples

<table>
<thead>
<tr>
<th>Stool panel</th>
<th>RT-PCR result</th>
<th>Cluster</th>
<th>RLB type</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Origin</td>
<td>p1 p2 p5 p6 p13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>p6 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>p6 + + + +</td>
<td>GI 2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>p6 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>p2 + + + +</td>
<td>Rotterdam</td>
<td>Rotterdam</td>
</tr>
<tr>
<td>5</td>
<td>p4 + + + +</td>
<td>GL6</td>
<td>GI</td>
</tr>
<tr>
<td>6</td>
<td>p2 + + + +</td>
<td>GL6</td>
<td>GI</td>
</tr>
<tr>
<td>7</td>
<td>p1 + + + +</td>
<td>GL6</td>
<td>GI</td>
</tr>
<tr>
<td>8</td>
<td>p6 + + + +</td>
<td>GL6</td>
<td>GI</td>
</tr>
<tr>
<td>9</td>
<td>p6 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>p6 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>p6 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>p5 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>p1 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>p5 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>p4 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>p4 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>p6 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>p2 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>p5 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>p6 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>p2 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>p2 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>p6 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>p1 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>p5 + + + +</td>
<td>Rotterdam</td>
<td>Rotterdam</td>
</tr>
<tr>
<td>26</td>
<td>p4 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>p5 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>p5 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>p5 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>p5 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>p4 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>p2 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>p6 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>p2 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>p5 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>p1 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>p6 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>p4 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>p6 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>p4 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>p4 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>p5 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>p6 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>p2 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>p2 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stool panel</th>
<th>RT-PCR result</th>
<th>Cluster</th>
<th>RLB type</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Origin</td>
<td>p1 p2 p5 p6 p13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>p2 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>p6 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>p4 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>p4 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>p4 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>p6 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>p4 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>p2 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>p4 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>p5 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>p5 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>57</td>
<td>p4 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>58</td>
<td>p4 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>59</td>
<td>p5 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>p5 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>p5 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>62</td>
<td>p4 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>63</td>
<td>p4 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>p5 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>p5 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>p2 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>67</td>
<td>p2 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>68</td>
<td>p2 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>69</td>
<td>p4 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>p5 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>71</td>
<td>p5 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>p6 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>73</td>
<td>p6 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>p2 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>p2 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>76</td>
<td>p2 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>77</td>
<td>p4 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>78</td>
<td>p4 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>79</td>
<td>p1 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>p5 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>81</td>
<td>p2 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>82</td>
<td>p6 + + + +</td>
<td>GI 1</td>
<td></td>
</tr>
<tr>
<td>83</td>
<td>p1 + + + +</td>
<td>GI 2</td>
<td></td>
</tr>
<tr>
<td>84</td>
<td>p1 + + + +</td>
<td>GI 3b</td>
<td>Stavanger</td>
</tr>
<tr>
<td>85</td>
<td>p1 + + + +</td>
<td>GI 4</td>
<td></td>
</tr>
<tr>
<td>86</td>
<td>p1 + + + +</td>
<td>GI 4</td>
<td></td>
</tr>
<tr>
<td>87</td>
<td>p1 + + + +</td>
<td>GI 4</td>
<td></td>
</tr>
<tr>
<td>88</td>
<td>p1 + + + +</td>
<td>GI 4</td>
<td></td>
</tr>
<tr>
<td>89</td>
<td>p1 + + + +</td>
<td>GI 4</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>p1 + + + +</td>
<td>GI 7</td>
<td></td>
</tr>
<tr>
<td>91</td>
<td>p1 + + + +</td>
<td>GI 7</td>
<td></td>
</tr>
</tbody>
</table>

* Two different NLV sequences were detected by two different laboratories; only from GI 2 was a 145-nt sequence obtained.
* No sequence was obtained; genotyping was performed by RLB only.
* Strains cluster differently in the POL and capsid regions: Sindlesham groups with Hesse (GI 6) in the complete capsid region but forms a distinct group in the POL region. Similarly, Rotterdam forms a distinct group in the POL region and groups with Toronto (GI 3) in the complete capsid gene.
* Samples 83 to 91 were included as NLV-positive controls.
* Samples that originally tested negative by RT-PCR.
* Samples that originally tested positive by EM.

Although it is the most conserved region of the genome, the POL region, which is the target of most of the primers used in the present study, still has a high level of sequence diversity (1, 52). This template variability has a direct effect on the primer homology and thus for the detection limit for different primer pairs for different genetic clusters. Thus far, only for the p1 assay has the detection limit been estimated to be 3 to 30 RNA viral particles after a Hawaiian strain was mixed with known concentrations of 80-nm latex beads (33). In the present study, we compared the detection limit of the different assays by using RNAs from nine different NLV genotypes, and the results showed that there is significant variation in detectable quantities of different NLV strains with no consistent pattern.

Recently, two similar schemes for the genetic classification of NLVs have been proposed (2, 33) and were summarized by Green et al. (20). The current consensus is that the entire capsid gene sequence is needed to define genotypes. To date, at least 15 different genotypes have been recognized based on
>80% amino acid similarity in the complete capsid gene (19, 53). With few exceptions, NLV strains cluster similarly when different regions of the genome are analyzed (52). In our study, genotyping by RLB showed a high level of agreement with POL typing after sequencing and phylogenetic analyses. However, several genotype-specific probes missed corresponding strains and, therefore, need to be refined. Other high-throughput NLV genotyping systems have recently been described (5, 39) and require further evaluation. The elegance of RLB is its straightforwardness of standardization between laboratories, since probe-labeled membranes can be prepared at a central facility and used by others. In addition, new probes directed toward newly emerging strains can easily be added. The limitations of this typing method include the minimum size of the amplicon (160 bp) necessary to target the probes on the membrane (53) and the limited sequence variation in the POL region between some clusters (e.g., Hawaii and Wortley), which makes the design of cluster-specific probes challenging. The amplicon size limitation precludes the use of assays p2 and p5 in combination with this method. In addition, RLB clearly cannot be used to compare closely related strains for which sequence analysis will remain the method of choice. Genotyping by microplate hybridization is another approach that could be used for high-throughput typing (40). However, the temperature (50°C) of hybridization, as well as subsequent washing steps, prevent an easy transfer of probes used in RLB to an enzyme-linked immunosorbent assay format. Ideally, genotyping should be performed with capsid sequences as a template since these most reliably reflect antigenicity and possibly serotype epitopes. Thus far, the majority of strains show good agreement between polymerase and capsid clustering (52), but as more and more sequences become available and recombinants are likely to circulate (29, 52; H. Vennema, unpublished data), it remains to be seen if the POL region alone is the appropriate region for the genotyping of NLVs.

In conclusion, this is the first study that evaluates five different RT-PCR assays for the detection of NLVs. Although no single assay stands out as the best by all criteria, evaluation of sensitivity, detection limit, assay format, and successful implementation in several other laboratories prompts us to recommend the p1 assay to laboratories that want to newly initiate NLV diagnostics. However, it must be stressed that no single assay at present detects all variants of NLV and that followup testing of negative stool samples with additional primer sets should be considered for all outbreaks matching Kaplan’s criteria (32). Due to the complementarities of some of the assays the sequence data generated in the present study, together with the increasing database of NLV strains, make it possible to design assays that may result in higher overall sensitivity and broad reactivity. Finally, although the addition of probes could

FIG. 3. Detection and simultaneous typing of NLVs from 30 panel strains (strains 31 to 60). Probes specific for the detection of genogroup I (GIa and b) and genogroup II (GII) and for 15 ORF1 clusters are indicated (53). The name of each probe is shown with the representative full capsid number (e.g., GII.4) in parentheses. ❋ The Rotterdam probe detects strains that are GII.3 in the capsid region, but that group separately in the POL region; p, positive Bristol (GII.4) control; n, negative control (water). Note that probes specific for strains of the clusters Seacroft (GII.6), Amsterdam (GII.8), and Alphatron (GIV.1) were not included on the membrane.
further improve its performance, we feel that the RLB method may help to standardize methods for genotyping of NLV strains across Europe.

ACKNOWLEDGMENT
We thank Matty de Wit for coding the stool panel.

REFERENCES


My Favourite Reagent

Rational optimization of generic primers used for Norwalk-like virus detection by reverse transcriptase polymerase chain reaction

H. Vennema, E. de Bruin, M. Koopmans *

Diagnostic Laboratory for Infectious Diseases and Perinatal Screening, National Institute for Public Health and the Environment, P.O. Box 1, 3720 BA Bilthoven, The Netherlands

Keywords: Rational optimization; Generic primers; Norwalk-like virus

1. Introduction

Norwalk-like caliciviruses (NLV) are increasingly recognized as causes of gastro-enteritis world-wide. Because these viruses can not be grown in tissue culture, molecular detection by reverse-transcriptase PCR (RT-PCR) has been used since the genome sequence of the prototype virus named Norwalk virus was published (Jiang et al., 1993). With the recognition that NLV are a quite diverse genus of viruses, several protocols have been published for generic detection methods that differ substantially in sensitivity and specificity (Vinje and Koopmans, 1996). A key issue is that due to the great diversity of NLV, it is difficult to do a complete assay validation, using a broad range of genetically distinct viruses. To address this, we have recently completed the evaluation of a sequence-directed modification of a primer set for NLV RT-PCR detection.

2. Approach

From the NLV sequences available in GenBank we selected those containing binding sites for our current primers JV12 or JV13, which target the viral RNA-dependent RNA polymerase (Vinje and Koopmans, 1996). The selected sequences ($n = 131$ for the antisense primer JV13, $n = 122$ for the sense primer JV12; Fig. 1) represent a wide range of NLV genotypes. In Fig. 1 we listed the number of times the four different nucleotides were present in each position of the primer binding sites. The RNA sequence is listed for the RT primer (JV13) and the cDNA sequence is listed for the forward PCR primer (JV12). Most positions were conserved in all available sequences, and identical to the nucleotides in the original primers. Positions close to the 3’ end of the primer are most important for amplification so we concentrated on two positions in JV13 and one in JV12 for design of optimal primers. The hybridization properties of the reverse primer are special if this primer is used to prime the RT reaction as it is in our protocol. The RT priming involves DNA–RNA hybridization, which allows A–U as well as G–U base-pairing. In caliciviruses (and many other RNA viruses) C to U, and U to C transitions are
quite frequent, especially in the third base of a codon. This was clearly visible in sequence positions 1 (and also in position 7), where both C and U occur, and primer JV13 contains a G residue. In position 4 (also a 3rd base position) however, not only C and U bases occurred but also G and A. Therefore, the matching position in the primer was changed to inosine (I), which allows base-pairing with all four nucleotides (Fig. 1, JV13I). Inosine was chosen rather than N (a mixture of all four nucleotides) since the latter would mean a 4-fold dilution of the effective primer concentration. The binding site of JV12 contains a C/T/G transition in position 3 from the 3’ end. Primer JV12 binds to the cDNA. Therefore, base-pairing of G to T is not an option. For the replacement of the JV12 primer we changed the corresponding T to a Y (C/T) (Fig. 1, primer JV12Y).

## 3. RT-PCR protocol

The RT-PCR was carried out as described previously (Vinjé and Koopmans, 1996, 2000). Briefly, RNA was extracted from 100 μl of a 10–20% stool suspension by binding to silica beads in the presence of guanidinium isothiocyanate (Boom et al., 1990). Reverse transcription of one tenth of the extracted RNA was done for 60 min at 42 °C after annealing with JV13I at 0.3 μM in 15 μl of 10 mM Tris–HCl pH 8.3, 50 mM KCl, 3 mM MgCl2, 1 mM dNTP, 40 U/ml RNAGuard, and 5 U AMV-RT (Promega, Leiden, The Netherlands). Five microliter of the RT-mix was added to 45 μl of a PCR-mix containing 10 mM Tris–HCl pH 9.2, 50 mM KCl, 1.2 mM MgCl2 (final concentration 1.5 mM), 0.2 mM dNTPs, 2.5 units ampliTaq and 0.3 μM of JV12Y. Samples were denatured for 3 min at 94 °C and subjected to 40 cycles at 94 °C for 1 min, 37 °C for 1 min 30 s, and 74 °C for 1 min. The low annealing temperature is vital to allow binding to a wide range of viral sequences. Specificity of products was confirmed by Southern blot hybridization.

## 4. Results

The new primers were evaluated with RNA from a panel of stool samples previously used for comparison of the diagnostic assays employed in different European laboratories (Vinjé et al.,
submitted). The panel consisted of 20 stool samples of diverse genotypes from each of four European laboratories involved in a EU-sponsored project (QLK1-1999-00594), three negative controls and nine stool samples of selected genotypes from our own collection. The latter were used for a sensitivity estimation by dilution of RNR (Vinje et al., submitted). The modified primers JV12Y/JV13I detected NLV in 60 of 91 panel samples, seven more than detection with JV12/JV13. In particular additional viruses of the Leeds genotype were detected. To determine the detection limit for JV12Y/JV13I we tested several dilutions of isolated RNA dilution ranges previously used for sensitivity estimation. This showed that for seven of nine strains the modified primers allowed equally sensitive detection of NLV. For one strain detection was one log less sensitive and for another the detection limit was two logs higher. Recently, a large food-borne outbreak was reported in Sweden (Johansson et al., 2002). The NLV involved could not be amplified with JV12 and JV13. Instead the authors used Nvp110 and Nvp69 (Wang et al., 1994). Subsequently the primer-binding regions of JV12 and JV13 were sequenced (GenBank accession AF356599). This revealed six mismatches in the region of JV12 and two in the region of primer JV13. All these mismatches are represented in Fig. 1. At our request the authors also tried primers JV12Y and JV13I and reported that they worked equally well as Nvp110 and Nvp69. The latter primer is only suitable for genogroup I NLVs.

Acknowledgements

This work is part of the project Foodborne Viruses in Europe (QLK1-1999-00594) funded by the European Union.

References


Detection and Characterization of Norovirus Outbreaks in Germany: Application of a One-Tube RT-PCR Using a Fluorogenic Real-Time Detection System

M. Höhne* and E. Schreier
Robert Koch-Institute, Berlin, Germany

Outbreaks of gastroenteritis caused by Norovirus are an increasing public health problem worldwide. The virus is transmitted by contaminated food and by person-to-person. In Germany, a new health reporting system including Norovirus infections has been introduced in 2001. Norovirus outbreaks (73%) diagnosed in our lab occurred in the first half of the year. Phylogenetic analysis shows that 90% belonged to genogroup II (GGII) with the majority related to prototypes Lordsdale/93 and Tarragona/2001. To date, PCR is the most sensitive and specific method for the detection but several procedures are needed to evaluate the amplification product. To minimize the risk of product carryover, a fast and simple procedure with a minimal number of steps are required. A one-tube RT-PCR method is described using the real-time TaqMan-PCR system. Primer sets and probes for both genogroup I (GGI) and genogroup II (GGII) sequences were developed. The specificity and sensitivity of this method was evaluated on 70 Norovirus positive stool samples of 70 outbreaks in Germany and 34 European samples by comparing the detection rate to those of an in-house RT-nested PCR. Overall, 93% of the PCR positive samples have been detected by the TaqMan-PCR including isolates of four different GGI and seven different GGII genotypes. Using plasmid standards for quantitation, virus loads between $10^2$ and $10^{10}$ genomic equivalents/ml stool suspension were found. The advantages of the one-step RT/TaqMan-PCR system for detection and quantitation of Norovirus are the high throughput of clinical samples and a decrease of the risk of contamination. J. Med. Virol. 72:312–319, 2004.

© 2004 Wiley-Liss, Inc.

KEY WORDS: human calicivirus; Norovirus; real-time PCR; genotype distribution; viral load

INTRODUCTION

Norwalk-like viruses are one of the most common causes of gastroenteritis in all age groups and large outbreaks remain an important public health problem worldwide. The enteric virus is shed in the stool and transmitted by contaminated food and from person to person. Morphology and genome organization led to its classification as member of the family Caliciviridae which now comprises the two human-related genera Norovirus (previously “Norwalk-like virus,” NLV) and Sapovirus (previously “Sapporo-like virus,” SLV) according to the last update of the 7th ICTV Report on virus taxonomy [van Regenmortel et al., 2000]. In Germany, a new health reporting system has been introduced in January 2001 including reporting of Norovirus infections. Thus, overall 9,223 cases have been reported to the public health authorities for the year 2001, most of which occurred in 486 outbreaks (173 outbreaks of less than 5 cases and 313 outbreaks of more than five cases, Robert Koch-Institut, 2002).

To date, human caliciviruses have not been cultured in vitro. For a long time, electron microscopy was the method used most widely for detection of caliciviruses, restricted to only a few special laboratories and exhibiting a low sensitivity. Recently, commercially enzyme immunoassays have been developed but showing limited sensitivity [Richards et al., 2003]. For that reason, the development of molecular diagnostic methods plays an important role. After cloning and sequencing of the human prototype Norwalk/68/US (M87661) and some related strains, e.g., Hawaii/71/US (U07611), Lordsdale/93/UK (X86557) Desert Shield/90/SA (U04469) or Mexico/89/MX (U22498), the reverse
transcriptase-polymerase chain reaction (RT-PCR) became the method used commonly for detection and classification of calicivirus RNA. Molecular characterization of partial and full-length sequences revealed a large genomic diversity among clinical specimens [Green et al., 1993; Wang et al., 1994; Schreier et al., 2000, Vinje et al., 2000]. Phylogenetic analysis of Norovirus strains led to the classification of two genogroups including until now seven genotypes in the genogroup I (GGI) and eight genotypes in the genogroup II (GGII) with at least 20% amino acid differences [Vinje et al., 2000]. In order to increase the detection rate of the highly variable NLV to estimate their true role in gastroenteritis outbreaks, several PCR primer systems have been developed [Green et al., 1993; Ando et al., 1995; Brown and Atmar, 1996; Le Guyader et al., 1996; Jiang et al., 1999; Honma et al., 2000; Rockx et al., 2002]. To evaluate the specificity of the amplified genome region subsequent procedures are used, for example, a second round of PCR and agarose gel analysis [Green et al., 1998; Schreier et al., 2000], probe hybridization [Ando et al., 1995; Yamazaki et al., 1996], hybridization to multiple genotype-specific oligonucleotides (reverse line blot hybridization format) [Vinje and Koopmans, 2000] or light cycler PCR using SYBR green [Miller et al., 2002]. To minimize the risk of PCR product carryover, contamination during handling of large amounts of clinical outbreak samples and to ensure high reproducibility, simple and fast procedures with minimal numbers of steps are required.

We describe a rapid and reproducible one-tube RT-PCR method using the TaqMan fluorogenic real-time detection system. Using primer sets and dual-labeled fluorogenic probes specific for GGI and GGII sequences of Norovirus within the ORF2 gene (capsid region), the fluorescence intensity is detected in real-time and through the caps of closed tubes. After hybridization of the fluorogenic probe labeled with both a fluorescent reporter and a quencher dye, the 5′–3′ nuclease activity of Taq polymerase is cleaving the TaqMan probe during the amplification. Thus, the reporter is separated from the quencher dye resulting in increasing fluorescence intensity of the reporter proportional to the starting copy number of the target nucleic acid. The advantages of the one-tube TaqMan-PCR assay are the high throughput of clinical samples, the elimination of pre- and post-PCR processing steps (e.g., separate RT reaction and agarose gel analysis), and the decrease of contamination risks. Furthermore, the real-time RT-PCR protocol enables quantitation of Norovirus–RNA by comparing the results for the clinical samples to those generated from a standard curve.

**MATERIALS AND METHODS**

**Outbreaks**

Fecal specimens from 70 gastroenteritis outbreaks occurring in 2001 in Germany and sent to our lab for virological investigation were used for this study. A panel of 34 NV–PCR and/or EM-positive stool samples from other European countries collected over a 4-year period of time (1997–2000) deriving from both outbreaks and sporadic cases were obtained within a European collaborative study in 2001 [Vinje et al., 2003].

**RNA Extraction and RT-Nested PCR**

Viral RNA was extracted from a fecal suspension using a spin column technique (QIAGEN, Hilden, Germany). Briefly, 0.05–0.1 g of patient stool were mixed with 160 μl of phosphate-buffered saline (PBS), vortexed for about 1 min and centrifuged for 3 min at 10,000 rpm. Clarified supernatant (140 μl) were extracted by the QIAamp viral RNA kit according to manufacturer’s instruction and RNA was eluted in 50 μl 0.01 M Tris-HCl (pH 7.0). For genotyping and to compare the TaqMan-PCR results, all patient samples were subjected to RT-nested PCR using primer pairs located in the ORF1 region of the Norovirus genome as described previously [Oh et al., 2003]. Therefore, 5 μl of the extracted RNA were reverse transcribed using 100 U M-MLV-RT (Life Technologies, Gaithersburg, MD) and antisense primer NV7. Reverse transcriptase reaction was carried out at 42°C for 5 min and 37°C for 20 min with subsequent denaturation for 5 min at 94°C. For the first round of amplification 5 μl of the RT reaction, the antisense primer NV7 and a mixture of sense primers NV1a and NV1b were used. One microliter of the first-round product was subjected to a second round reaction containing the sense primer mixture NV6 and NV6a and the antisense primer mixture NV4 and NV4a. An equimolar mixture of primers were used to detect a broad range of sequence variants of Norovirus genomes. Amplifications were carried out for 35 cycles with 30 sec 94°C, 30 sec 42°C, 45 sec 72°C, followed by a last 5 min extension at 72°C. To avoid cross-contamination, all steps (nucleic acid preparation, pipetting of mixtures for first round and especially for second round amplification, and product analysis by agarose electrophoresis) were carried out in strictly separated labs using filter tips and including appropriate negative controls for preparation, reverse transcription and PCR.

**DNA Sequencing and Phylogenetic Analysis**

The second round PCR products of the ORF1 were sequenced directly with the Big dye terminator cycle sequencing kit (Applied Biosystems, Warrington, Great Britain) in both directions using an ABI Prism 377 automated sequencer. Multiple sequence alignments were undertaken with the CLUSTAL W program version 1.6 and phylogenetic trees were produced using the Phylogeny Interference Package (PHYLIP) version 3.57c [Felsenstein, 1989]. The nucleotide distances were estimated using the DNADIST program, and unrooted phylogenetic trees were produced using the neighbor-joining method (PHYLIP). Phylogenetic trees were drawn using the TREEVIEW program [Page, 1996].
Oligonucleotide Primers and Probes for TaqMan-PCR

For the creation of primer sets and TaqMan probes permitting the detection of a broad range of sample sequences following prototype strains of Noroviruses were used for comparative sequence analysis: Lordsdale/93/UK (X86557), Camberwell/93/UK (AF145896), Hawaii/71/US (U07611), Mexico/89/MX (U22498), Melksham/94/UK (X81879), Norwalk/68/US (M87661), Desert Shield/90/SA (U04469), Southampton/91/UK (L07418), and HSS/3/97/DE (AF093797). For the TaqMan-PCR conserved sequences within the ORF2 gene region were selected. Primers and probes are listed in Table I and were obtained from BioTez (Berlin, Germany). The TaqMan probes were designed so that the predicted melting temperature was at least 10°C higher than the predicted melting temperature of the primers.

**TaqMan-PCR**

A single-tube RT-PCR was optimized for the detection and quantitation of Norovirus GGI and GGII RNA in stool samples by using the TaqMan technology (Applied Biosystems). The single-tube RT-PCR was carried out in a 0.2 ml 96 tube plate (Thermo-Fast 96 from Abgene, Surrey, UK) covered with a heat sealing film (Clear Strong Seal from Abgene, Surrey, UK). The reaction mixture was made up to a volume of 25 μl containing 5 μl of the RNA-preparation, 4.5 mM MgCl₂ (for GGI) or 8.0 mM MgCl₂ (for GGII), 0.4 mM (each) dATP, dCTP, dGTP, and dUTP, 0.6 μM of both the sense and the antisense primer (see Table I), 0.2 μM of the specific fluorescence labeled TaqMan probe TM3 (GGII) or 0.4 μM of TM probe 6 (GGI), 0.1 μM ROX as a passive reference, 20 U RNasin, and 1 μl of OneStep RT-PCR Enzyme Mix (QIAGEN, Hilden, Germany) containing a mixture of Omniscript and Sensiscript Reverse Transcriptases and HotStar DNA Polymerase. The probes consisted of oligonucleotides dual-labeled with 5’-reporter dye FAM (6-carboxy-fluorescein) and 3’-quencher dye TAMRA. Norovirus–RNA was reverse transcribed into cDNA (30 min at 50°C) followed by heat inactivation of the transcriptases and the initial activation of the HotStar polymerase by incubation at 95°C for 15 min. Amplification was carried out for 45 cycles with 15 sec at 94°C and 1 min at 56°C. During amplification, the plate was automatically scanned at 518 nm (FAM) and 582 nm (TAMRA). ROX was used as passive internal reference to which the reporter dye signal was normalized (R₀). The increase of R₀ (ΔR₀) is plotted versus the cycle number. The threshold cycle (C铊) represents the cycle at which the fluorescence signal generated during amplification by cleavage of the probe passes above a threshold baseline. The starting copy number in clinical samples was calculated by comparison of their C铊 values to those of a standard curve (Fig. 1).

The DNA standard templates containing the viral sequence from nt 5007 to nt 5385 according to X86557 (Lordsdale/93/UK) for GGI and sequence nt 5321 to nt 5574 according to M87661 (Norwalk/68) for GGII sequences were generated from German samples by PCR and cloning into pGEM-T vector (Promega, Mannheim, Germany). Plasmid concentrations were determined by UV spectroscopy (260 nm/280 nm) and numbers of genomic equivalents were calculated using the relations 1 OD₉₀₀ₐₙ₉₉ = 50 μg/ml, 1 mol = 6.022 x 10²³ N. Serial dilutions (5 x 10⁻² to 5 x 10⁻¹) of the purified plasmids were used as standards. Aliquots of dilutions were frozen at −20°C and used only once for the TaqMan-PCR.

**RESULTS**

**Analysis of Norovirus Outbreaks in Germany**

During the year 2001, seventy Norovirus outbreaks from 11 out of 16 states of the Federal Republic of Germany were diagnosed by RT/nested PCR in our laboratory, 73% occurred in the first half of the year (January to June) whereas during the summer and autumn (July to November) a clear decline was observed (Fig. 2A). The majority of these outbreaks occurred in hospitals (46%) and in nursing homes (33%) (Fig. 2B).

To investigate the genetic variability of Norovirus circulating in Germany in 2001, the nucleotide sequences of a part of the RNA dependent RNA polymerase gene (ORF1) was determined and aligned to prototype Norovirus strains. Norovirus genotypes are assigned according to a classification scheme published recently [Vinje et al., 2003]. Thus, as GGII prototypes, strains Amsterdam/1998/NL (GII.8), Leeds/1990/UK (GII.7), Hillingdon/1990/UK (GII.5), Melksham/1994/UK (GII.2), Mexico/1989/MX (GII.3), Hawaii/1972/US

<table>
<thead>
<tr>
<th>Genogroup</th>
<th>Primer</th>
<th>Sequence</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGI</td>
<td>NV65a (sense)</td>
<td>5'-TGGACAGGGRATCGCRACTCT</td>
<td>5321–5340</td>
</tr>
<tr>
<td></td>
<td>NV120 (antisense)</td>
<td>5'-AYATUCACGGGGGTATTTTT</td>
<td>5593–5574</td>
</tr>
<tr>
<td></td>
<td>TM6 probe</td>
<td>5'-GGGTCTCTTAGACGGCATCATATTAC</td>
<td>55345–5380</td>
</tr>
<tr>
<td>GGII</td>
<td>NV107a (sense)</td>
<td>5'-AGGCAATTCAGATGGATG</td>
<td>5007–5026</td>
</tr>
<tr>
<td></td>
<td>NV117 (antisense)</td>
<td>5'-TCGACGCCATTCATTTAC</td>
<td>5100–5081</td>
</tr>
<tr>
<td></td>
<td>TM3 probe</td>
<td>5'-TGGGGGCGATGCACAATGCAC</td>
<td>5048–5070</td>
</tr>
</tbody>
</table>

a Genomic location of primers and probes are based for GGI on the sequence of Norwalk/68/US (M87661) and for GGII on the sequence of Lordsdale/93/UK (X86557).

b TM probes are labeled at 5' -end with 6-FAM and at 3' -end with TAMRA.
(GI.1), Lordsdale/1993/UK (GI.4), and Taragona/2001/SP, as well as subclusters Grimsby/1995/UK and Camberwell/1993/UK were used. For GGI strains HSS3/1997/DE, Southampton/1991/UK (GI.2), Chiba 407/1987/JP (GI.4), Norwalk/1968/US (GL.1), Desert Shield/1990/SA (GL.3), and Birmingham/1993/UK (GL.3a) were involved. Alphatron/1998/NL (GIV.1) is a novel genotype showing sequence diversity of more than 50% with GGI and GGII sequences. Comparisons of German sequences to prototype strains showed that 63 out of the 70 outbreaks analyzed belong to GGII (90%), 6 (8.6%) to GGI and in one outbreak a mixture of GGI and GGII sequences was found. Within GGII, the sequences shared similarity with six different genotypes (Leeds/90, Hillingdon/90, Melksham/94, Mexico/89, Tarragona/01, and Lordsdale/93); the majority was related to prototypes Lordsdale/93 and Tarragona/2001. Within GGI, German sequences were most related to the prototype Birmingham/93 and Chiba/87. Figure 3 shows a phylogenetic tree which includes a selection of sequences from German outbreaks.

One-Tube Real-Time PCR for Norovirus Detection

One-Tube RT/TaqMan-PCR—Linearity, Detection Limit, and Amplification Efficiency

The dilution curve of the plasmid standards showed linearity over 6 and 7 orders of magnitude (5 x 10^1 to 5 x 10^7 molecules) for the GGI and GGII TaqMan-PCR system, respectively (Fig. 4). Coefficients of variation of the between-run amplification were in the range of 2.36–10.06%.

To compare the detection limits of the one-tube TaqMan-PCR to our in-house RT/nested PCR, 10-fold serial dilution of Norovirus RNA representing four different genotypes (Southampton/91-like, GGI.2; Hawaii/72-like, GGII.1; Toronto/91-like, GGII.3; Lordsdale/93-like, GGII.4) were used in both systems. In two samples (Hawaii and Lordsdale), the detection limit of the TaqMan-PCR reached at least the same or a higher dilution positive for the nested PCR (10^-6, 10^-4). For the other two samples (Southampton, Toronto), the TaqMan-PCR differed in one and two orders of magnitude, respectively.

The comparability of amplification efficiencies of the DNA standard to the RNA samples for the TaqMan-PCR was determined by comparing dilution series of both nucleic acids. The slopes (ΔC_T) of the dilution curves of the standard plasmid and the viral RNAs
(sample 93 and 94) were approximately the same (Fig. 5). Thus, the amplification efficiencies of the DNA standard and the sample RNA can be considered as comparable.

Specificity of TaqMan-PCR

from Germany and 34 European samples tested previously as Norovirus positive were examined. From German outbreaks 66/70 (94.3%) samples were positive by the real-time TaqMan-PCR including samples of six different GGII genotypes and two different GGI genotypes.

From the collection of European samples, which were genotyped by the authors of the collaborative study [Vinje et al., 2003], 31/34 (91%) were TaqMan-PCR positive including samples of four different GGI genotypes and seven different GGII genotypes.

**Virus Load Determination in Stool Samples**

In addition to the rapid qualitative analysis of stool samples the real-time PCR permits the determination of virus loads within a Norovirus outbreak or the follow-up of viral release in patients after disappearance of clinical symptoms. As shown in Figure 6, virus loads between $10^2$ and $10^{10}$ genomic equivalents per ml stool suspension with a median of $1.14 \times 10^7$ have been determined by the TaqMan-PCR in German outbreak samples. In the panel of European samples, a median virus load of $7.14 \times 10^7$ genomic equivalents per ml stool suspension $(10^7$ to $3.2 \times 10^{10})$ were found. Thus, 5–10 genomic equivalents per TaqMan-PCR assay could be detected.

To follow up the duration of virus shedding, three patients infected during outbreaks were examined by nested PCR and real-time PCR. Two patients were PCR positive for 8 and 10 days, respectively (not shown). Interestingly, the third patient, a 2-year-old girl without other significant health problems known, was PCR positive for up to 60 days showing a mean virus load of $2.33 \times 10^6$ genomic equivalents/ml stool suspension (Fig. 7). The last specimen collected at day 87 was Norovirus–PCR negative.

**DISCUSSION**

During the last few years increasing attention has been paid to viral gastroenteritis outbreaks. Since the development of molecular methods for the detection of Norovirus infections and their widespread application by diagnostic laboratories, it has been clear that the Norovirus is one of the most common cause of gastroenteritis outbreaks in adults and in children [Dedman et al., 1998; Hedlund et al., 2000; Green et al., 2002;...
was also reported in over the half of Norovirus-infected individuals immune response. After the development of Norovirus load in stool samples may be due to 10–100 copies per reaction allowed the range of up to 7 orders of magnitude and the analytical load in each sample. The considerably wide dynamic range of up to 7 orders of magnitude and the analytical sensitivity of 10–100 copies per reaction allowed the detection of the wide range of virus loads found in stool samples, the amplification and detection of up to 96 samples can be obtained in less than 3 h with a minimized risk of carryover contamination.

In conclusion, the one-step RT/TaqMan-PCR system described above for detection and quantitation of Norovirus in stool samples is a fast, sensitive, and reproducible method which could be also adapted for automation. After preparation of the viral nucleic acid from stool samples, the amplification and detection of up to 96 samples can be obtained in less than 3 h with a minimized risk of carryover contamination.

REFERENCES


Molecular epidemiology of human caliciviruses

Marion Koopmans, Elisabeth van Strien and Harry Vennema

Diagnostic Laboratory for Infectious Diseases, National Institute of Public Health and the Environment, Bilthoven, The Netherlands

Abstract

Caliciviruses are among the most common infections in humans, and are a major cause of illness in the community, and of outbreaks of gastro-enteritis in institutions such as nursing homes and hospitals. While the course of illness is generally mild, the sheer numbers of infections, and the high attack rate result in a substantial burden of illness. In recent years, molecular detection techniques have been developed and used initially to study the incidence of caliciviruses. With increasing refinement, strain typing is now being used to further our understanding of the epidemiology of calicivirus infections. By the powerful combination of epidemiological investigations and molecular strain typing, novel information has emerged which adds to our knowledge of the modes of transmission of these viruses, and their evolutionary mechanisms.

Introduction

In recent years, several studies have addressed the importance of caliciviruses as causes of gastro-enteritis in different selections of the population (Fig. 1). There are data from community surveys in the UK, Sweden, and The Netherlands, from physician-based studies in the UK, The Netherlands and France, from special surveys in young children in Finland, Canada and South-Africa, and from outbreak surveillance in many other countries (Tables 1-3 and references therein). The results of these studies paint the picture that caliciviruses, and especially the Norwalk-like group of caliciviruses (NLV) are among the most common causes of illness in the community, with the highest incidence in young children, but also a substantial burden of illness in adults. Surveys of outbreaks almost all show that the majority of outbreaks investigated is attributed to NLV (Table 1). For community-based studies, the positivity rates range more widely (1-22%). These differences are likely to be related to differences in methods used for virus detection and to differences in selection of the study population. The highest community incidence was reported from Finland, where a group of children was monitored during a rotavirus vaccine trial. Although the incidence of NLV in the com-
munity survey in The Netherlands was significantly lower, the rates were much higher in young children (19% in the 1-4 year olds)(de Wit et al., 2001a). Overall, illness due to caliciviruses is relatively mild, which is reflected by the lower incidence of caliciviruses in patients that are consulting their physician. However, the few hospital-based studies that have been done indicate that NLV may lead to hospitalisation (Table 3). In special surveys of outbreaks, NLV are seen as the most common cause of outbreaks of gastro-enteritis in institutions such as nursing homes, hospitals, homes for the mentally handicapped, and as a prominent cause of food-related outbreaks. National differences in the selection of outbreaks that are reported and investigated make it difficult to compare rates between countries, and the proportion of outbreaks attributed to caliciviruses varies greatly (Lopman et al., submitted for publication).

Molecular detection and typing assays have been developed for caliciviruses. They are increasingly used in epidemiological studies of caliciviruses, to provide insight in the diversity of caliciviruses, trace the source of outbreaks, and study virulence traits. In this paper, we will review the state of the art of calicivirus molecular epidemiology.

**Taxonomic structure of the family *Caliciviridae***

The *Caliciviridae* are a family of positive-strand RNA viruses that infect a wide range of species including humans(Green et al., 2000). There are now 4 recognised genera within the family, with most animal caliciviruses belonging to two genera named *vesi*
virus and lagovirus, and the human pathogens along with some animal viruses grouped into the genera Norwalk-like virus (NLV) and Sapporo-like virus (SLV). The names for the last two genera are tentative, and will change in the near future (pending the adoption of a proposed nomenclature in March 2002 by the International Committee for Taxonomy of Viruses). Type strains for the 2 genera of calicivirus that infect humans are Norwalk virus (Hu/NLV/I/Norwalk/1968/US), and Sapporo virus (Hu/SLV/Sapporo/1982/JP). Well-known representatives of the animal caliciviruses are the vesicular exanthema of swine virus (type strain Po/VV/VESV/VESV-A48/1948/US), feline caliciviruses (both belonging to the genus vesivirus), and the rabbit hemorrhagic disease viruses (genus lagovirus; Ra/LV/RHDV/GH/1988/GE). In this chapter we will focus on the NLVs and SLVs.

Structure of the viruses

The calicivirus genome is approximately 7.5 kb long, with the polarity of messenger RNA (positive-strand RNA, polyadenylated 3'-end)(Jiang et al., 1993; Lambden et al., 1993). Across the NLV genome, 3 open reading frames (ORFs) are recognised, with the largest one (ORF1) located at the 5'-end of the genome, coding for the non-structural proteins (Fig. 2). The 2 ORFs (ORF 2 and 3) located at the 3'-end encode the virion-associated proteins. The genome is packaged into an icosahedral particle, built by assembly of 90 dimers of the major capsid protein expressed from ORF2(Jiang et al., 1992b). The Mr of the major capsid protein is approximately 60 kDa. In addition, a second minor capsid protein of 10kDa was identified, encoded by ORF3, which is probably involved in RNA binding and is located on the inner surface of the virus capsid. Additional coding assignments have been recognised for an RNA-dependent RNA polymerase (RdRp), a 2C-like nucleoside triphosphatase (NTPase), a VPg, and a proteinase, based on conserved amino-acid motifs located within ORF1 (Fig. 2). For NLV, the ORF overlaps by of few nucleotides with ORF1. For SLV, the genome organisation is similar to that of viruses in the genus lagovirus, in that the capsid protein is in frame and contiguous with the non-structural polyprotein. The capsid protein has been expressed in baculovirus expression systems, yielding virus-like particles (VLPs) by self-assembly of the capsid proteins(Jiang et al., 1992b). These VLPs have been used to study the structure of the Norwalk virus particle by cryo-electron microscopy and computer imaging (Prasad et al., 1994, 1996). Based on these observations, the capsid protein is thought to consist of a shell domain, involved in the basic structure of the icosahedral particle, and a protruding domain. A detailed overview of the genome organisation and structure of caliciviruses is given by (Green et al., 2001).

Molecular detection techniques

The start of the molecular era for caliciviruses with the cloning of the Norwalk virus genome has greatly facilitated epidemiological studies addressing their role as causes
Table 1
Summary of studies of outbreaks of (viral) gastroenteritis, listing methods used for detection, and results of typing (N = number of outbreaks; n = number of individual cases; % pos = % of outbreaks/cases attributed to calicivirus; GG = genogroup; GGII.4 = viruses belonging to the genotype of Bristol-like viruses, nPCR = nested PCR (after reverse transcription), HMA = hybrid-mobility assay; EM = electron microscopy; EIA = enzyme immunoassay; nr = not reported; * = proportion of strains belonging to this category.

<table>
<thead>
<tr>
<th>Study population</th>
<th>Country</th>
<th>Period</th>
<th>Design</th>
<th>N</th>
<th>% pos</th>
<th>Method</th>
<th>GG I*</th>
<th>GG II*</th>
<th>GGII.4*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>National</td>
<td>Japan</td>
<td>1989-98</td>
<td>Outbreaks</td>
<td>190</td>
<td>100</td>
<td>nPCR</td>
<td>14</td>
<td>87</td>
<td>&lt;32</td>
<td>Kawamoto et al., 2001</td>
</tr>
<tr>
<td>City</td>
<td>Japan</td>
<td>1995-98</td>
<td>non-bacterial outbreaks</td>
<td>13</td>
<td>100</td>
<td>nPCR</td>
<td>0</td>
<td>62</td>
<td>21</td>
<td>Ohyama et al., 1999</td>
</tr>
<tr>
<td>City</td>
<td>Japan</td>
<td>4/96-4/99</td>
<td>Outbreaks</td>
<td>64</td>
<td>73</td>
<td>PCR</td>
<td>11</td>
<td>79</td>
<td>7</td>
<td>Iriani et al., 2000</td>
</tr>
<tr>
<td>Infant home</td>
<td>Japan</td>
<td>76-95</td>
<td>infant home survey</td>
<td>36</td>
<td>42</td>
<td>PCR</td>
<td>nr</td>
<td>nr</td>
<td>nr</td>
<td>Nakata et al., 2000</td>
</tr>
<tr>
<td>State</td>
<td>Australia</td>
<td>1980-1996</td>
<td>reference laboratory, n = 6226</td>
<td>3,5</td>
<td>EM</td>
<td>5</td>
<td>95</td>
<td>68</td>
<td>Wright et al., 1998</td>
<td></td>
</tr>
<tr>
<td>National</td>
<td>New Zealand</td>
<td>8/95-7/96</td>
<td>outbreaks</td>
<td>83</td>
<td>100</td>
<td>PCR</td>
<td>22</td>
<td>63</td>
<td>31</td>
<td>Greening et al., 2001</td>
</tr>
<tr>
<td>State</td>
<td>US</td>
<td>88</td>
<td>Outbreaks</td>
<td>20</td>
<td>90</td>
<td>PCR</td>
<td>10</td>
<td>90</td>
<td>70</td>
<td>Green et al., 2002</td>
</tr>
<tr>
<td>National</td>
<td>US</td>
<td>1/96-6/97</td>
<td>non-bacterial outbreaks</td>
<td>90</td>
<td>100</td>
<td>PCR</td>
<td>6</td>
<td>93</td>
<td>50</td>
<td>Fankhauser et al., 1998</td>
</tr>
<tr>
<td>Regional</td>
<td>UK</td>
<td>7/92-6/98</td>
<td>Outbreaks</td>
<td>706</td>
<td>100</td>
<td>EM, EIA</td>
<td>nr</td>
<td>nr</td>
<td>nr</td>
<td>Hale et al., 2000</td>
</tr>
<tr>
<td>Regional</td>
<td>UK</td>
<td>92-95</td>
<td>Outbreaks</td>
<td>9</td>
<td>100</td>
<td>EM, PCR</td>
<td>33</td>
<td>67</td>
<td>33</td>
<td>Green et al., 1997</td>
</tr>
<tr>
<td>Regional</td>
<td>UK</td>
<td>97-98</td>
<td>Outbreaks</td>
<td>130</td>
<td></td>
<td>PCR, HMA</td>
<td>nr</td>
<td>nr</td>
<td>57</td>
<td>Mattick et al., 2000</td>
</tr>
<tr>
<td>Regional</td>
<td>UK</td>
<td>96-97</td>
<td>Outbreaks</td>
<td>94</td>
<td>76</td>
<td>EM, PCR</td>
<td>2</td>
<td>98</td>
<td>96</td>
<td>Maguire et al., 1999</td>
</tr>
<tr>
<td>Regional</td>
<td>Ireland</td>
<td>93-98</td>
<td>Outbreaks</td>
<td>nk</td>
<td>nk</td>
<td>PCR</td>
<td>12</td>
<td>15</td>
<td></td>
<td>Foley et al., 2001</td>
</tr>
</tbody>
</table>
Table 1
Continued

<table>
<thead>
<tr>
<th>Study population</th>
<th>Country</th>
<th>Period</th>
<th>Design</th>
<th>N</th>
<th>% pos</th>
<th>Method</th>
<th>GGII*</th>
<th>GGII*</th>
<th>GGII.4*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>National</td>
<td>Norway</td>
<td>11/98-5/99</td>
<td>Outbreaks</td>
<td>52</td>
<td>40</td>
<td>nPCR</td>
<td>13</td>
<td>87</td>
<td>91</td>
<td>Vainio et al., 2001</td>
</tr>
<tr>
<td>Regional</td>
<td>Finland</td>
<td>97-98</td>
<td>Outbreaks</td>
<td>16</td>
<td>100</td>
<td>PCR</td>
<td>31</td>
<td>70</td>
<td>50</td>
<td>Maunula et al., 1999</td>
</tr>
<tr>
<td>Regional</td>
<td>Germany</td>
<td>11/97-5/98</td>
<td>Outbreaks</td>
<td>16</td>
<td>100</td>
<td>nPCR</td>
<td>6</td>
<td>94</td>
<td>68</td>
<td>Schreier et al., 2000</td>
</tr>
<tr>
<td>National</td>
<td>Netherlands</td>
<td>96</td>
<td>Outbreaks</td>
<td>60</td>
<td>87</td>
<td>PCR</td>
<td>8</td>
<td>91</td>
<td>87</td>
<td>Vinje et al., 1997</td>
</tr>
<tr>
<td>National</td>
<td>Netherlands</td>
<td>94-95</td>
<td>Outbreaks</td>
<td>22</td>
<td>91</td>
<td>PCR</td>
<td>10</td>
<td>90</td>
<td>50</td>
<td>Vinje et al., 1996</td>
</tr>
<tr>
<td>National</td>
<td>Netherlands</td>
<td>96-01</td>
<td>Outbreaks</td>
<td>222</td>
<td>80</td>
<td>PCR</td>
<td>11</td>
<td>89</td>
<td></td>
<td>Koopmans et al., 2000, 2001 and unpublished data</td>
</tr>
</tbody>
</table>
Table 2
Summary table of community-based studies of (viral) gastroenteritis. (For abbreviations see legend of Table 1).

<table>
<thead>
<tr>
<th>Study population</th>
<th>Country</th>
<th>Year</th>
<th>Design</th>
<th>n</th>
<th>% pos</th>
<th>Method</th>
<th>GGI</th>
<th>GGII</th>
<th>GGII.4</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>National</td>
<td>Finland</td>
<td>9/93-9/94</td>
<td>rotavirus vaccine trial, community</td>
<td>1447</td>
<td>22</td>
<td>PCR</td>
<td>8</td>
<td>92</td>
<td>2b: 47%</td>
<td>Pang et al., 1999</td>
</tr>
<tr>
<td>City</td>
<td>Sweden</td>
<td>2/98-1/99</td>
<td>community survey</td>
<td>123</td>
<td>20</td>
<td>PCR</td>
<td>nr</td>
<td>nr</td>
<td>nr</td>
<td>Lindqvist et al., 2001</td>
</tr>
<tr>
<td>National</td>
<td>Netherlands</td>
<td>99</td>
<td>community cohort</td>
<td>709</td>
<td>11</td>
<td>PCR</td>
<td>22</td>
<td>80</td>
<td>32</td>
<td>de Wit et al., 2001a</td>
</tr>
<tr>
<td>National</td>
<td>Netherlands</td>
<td>96-99</td>
<td>physician-based study</td>
<td>857</td>
<td>5</td>
<td>PCR</td>
<td>14</td>
<td>86</td>
<td>29</td>
<td>de Wit et al., 2001b</td>
</tr>
<tr>
<td>Regional</td>
<td>UK</td>
<td>8/93-1/96</td>
<td>physician-based study</td>
<td>2893</td>
<td>1.5</td>
<td>EM</td>
<td>nr</td>
<td>nr</td>
<td>nr</td>
<td>Tompkins et al., 1999</td>
</tr>
<tr>
<td>Regional</td>
<td>UK</td>
<td>8/93-1/96</td>
<td>community cohort</td>
<td>715</td>
<td>8</td>
<td>EM</td>
<td>nr</td>
<td>nr</td>
<td>nr</td>
<td>Tompkins et al., 1999</td>
</tr>
<tr>
<td>Regional</td>
<td>Canada</td>
<td>91-95</td>
<td>survey pediatric patients</td>
<td>768</td>
<td>18</td>
<td>PCR</td>
<td>3</td>
<td>97</td>
<td>2b: 65%</td>
<td>Levett et al., 1996</td>
</tr>
<tr>
<td>Regional</td>
<td>Canada</td>
<td>11/97-7/98</td>
<td>pediatric patients</td>
<td>226</td>
<td>1</td>
<td>EM</td>
<td>nr</td>
<td>nr</td>
<td>nr</td>
<td>Waters et al., 2000</td>
</tr>
<tr>
<td>Regional</td>
<td>Canada</td>
<td>11/97-7/98</td>
<td>day-care centers</td>
<td>211</td>
<td>1</td>
<td>EM</td>
<td>nr</td>
<td>nr</td>
<td>nr</td>
<td>Waters et al., 2000</td>
</tr>
<tr>
<td>Regional</td>
<td>South Africa</td>
<td>10/91-10/95</td>
<td>survey pediatric patients</td>
<td>1296</td>
<td>2.5</td>
<td>EM, EIA, PCR</td>
<td>10</td>
<td>90</td>
<td>nr</td>
<td>Wolfaird et al., 1997</td>
</tr>
<tr>
<td>Regional</td>
<td>South Africa</td>
<td>89-91</td>
<td>community cases</td>
<td>299</td>
<td>1</td>
<td>EM, EIA, PCR</td>
<td>ins</td>
<td>ins</td>
<td>ins</td>
<td>Wolfaird et al., 1995</td>
</tr>
<tr>
<td>City</td>
<td>Brasil</td>
<td>8/88-91</td>
<td>community cohort, children</td>
<td>120</td>
<td>10</td>
<td>PCR</td>
<td>63</td>
<td>37</td>
<td>13</td>
<td>Parks et al., 1999</td>
</tr>
</tbody>
</table>
Table 3

Summary of hospital-based studies of (viral) gastroenteritis. (For abbreviations see legend of Table 1).

<table>
<thead>
<tr>
<th>Study population</th>
<th>Country</th>
<th>Year</th>
<th>Design</th>
<th>n</th>
<th>% pos</th>
<th>Method</th>
<th>GGI</th>
<th>GGIi</th>
<th>GGIi.4</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regional</td>
<td>Canada</td>
<td>11/97-7/98</td>
<td>hospitalised children</td>
<td>1638</td>
<td>&lt;1</td>
<td>EM</td>
<td>nr</td>
<td>nr</td>
<td>nr</td>
<td>Waters et al., 2000</td>
</tr>
<tr>
<td>City</td>
<td>France</td>
<td>12/95-2/98</td>
<td>hospitalised children</td>
<td>414</td>
<td>14</td>
<td>PCR</td>
<td>11</td>
<td>89</td>
<td>42</td>
<td>Bon et al., 1999</td>
</tr>
<tr>
<td>Regional</td>
<td>France</td>
<td>96-98</td>
<td></td>
<td></td>
<td></td>
<td>PCR</td>
<td>8</td>
<td>79</td>
<td></td>
<td>Tracore et al., 2000</td>
</tr>
<tr>
<td>City</td>
<td>Sweden</td>
<td>10/96-10/97</td>
<td>hospitalised patients,</td>
<td>851</td>
<td>3</td>
<td>EM</td>
<td>nr</td>
<td>nr</td>
<td>nr</td>
<td>Svenungsson et al., 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>adults, case-control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>City</td>
<td>Australia</td>
<td>95-96</td>
<td>children</td>
<td>360</td>
<td>2.5</td>
<td>PCR</td>
<td>89</td>
<td>11</td>
<td>0</td>
<td>Schmial et al., 2000</td>
</tr>
<tr>
<td>City</td>
<td>Australia</td>
<td>99</td>
<td>children</td>
<td>354</td>
<td>9</td>
<td>PCR</td>
<td>5</td>
<td>95</td>
<td>77</td>
<td>Kirkwood and Bishop, 2001</td>
</tr>
<tr>
<td>City</td>
<td>China</td>
<td>11/94-8/96</td>
<td>children</td>
<td>186</td>
<td>7.6</td>
<td>PCR</td>
<td>43</td>
<td>57</td>
<td>nr</td>
<td>Qiao et al., 1999</td>
</tr>
<tr>
<td>Regional</td>
<td>India</td>
<td>11/88-1/99</td>
<td>hospital, all ages</td>
<td>80</td>
<td>15</td>
<td>EIA, PCR</td>
<td>30</td>
<td>70</td>
<td>nr</td>
<td>Kang et al., 2000</td>
</tr>
</tbody>
</table>
of illness. Until then, the gold standard for calicivirus detection was visualisation of the particles directly in stools by electron microscopic examination (EM) of a preparation stained with electron-dense contrast fluids (Atmar and Estes 2001). However, only few countries have sufficient and sufficiently experienced electron microscopists to be able to run a national reference service for caliciviruses based on EM. As a result, the importance of caliciviruses as a cause of illness remained obscure. Another disadvantage is the rather low sensitivity of EM. This may not be a problem in outbreak situations, when detecting the pathogens in a fraction of all cases may be diagnostic for a calicivirus outbreak. In the investigation of sporadic cases, however, EM most likely will underestimate the true number of positives (Wolfardt et al., 1995). These disadvantages have been overcome with the development of RT-PCR detection methods, following the successful cloning of the genome of Norwalk virus. Initially, RT-PCR assays were optimised using a limited range of variants, which resulted in highly sensitive detection but with a narrow diagnostic range (Jiang et al., 1992a; Moe et al., 1994; Ando et al., 1995). With the finding that caliciviruses are genetically very diverse, several groups have tried successfully to develop broader, generic detection methods. At present, published protocols list a range of primer sets that have been developed and used for the detection of NLV and SLV (Ando et al., 1995; Atmar and Estes 2001; Green et al., 1993; Jiang et al., 1999a; LeGuyader et al., 1996a and b; Maunula et al., 1999; Schreier et al., 2000; Vinjé et al., 1996 and 2000; Wright et al., 1998). Most assays target conserved regions within the RNA polymerase gene, which has also allowed the use of the sequence of the PCR products for typing (Fig. 2).

What is molecular epidemiology?

Molecular epidemiology is NOT drawing a set of samples from a historic collection, run some typing methods, and interpret the findings. The real power of molecular epidemiology lies in the best of both approaches: a combination of sound epidemiological and molecular-diagnostic research. A range of strain characterisation methods can be used to provide possible explanations for the observations made through epidemiological studies. The laboratory methods used can be numerous, from biological assays (typing against a panel of neutralising monoclonal or polyclonal antibodies, looking at cytopathogenic effects in cell culture, effects on red blood cells) to molecular analysis (such as sequence analysis of selected regions of the viral genome, mutational analysis, phylogeny). An example from the field of virology is the use of molecular typing to monitor progress in the global WHO campaign that aims at the eradication of poliovirus and the paralytic illness that it causes. Poliovirus has a positive sense RNA genome, that is genetically quite flexible in that the genome accumulates mutations during replication of the virus in humans, at a rate of approximately 1% of nucleotides per year (Wimmer et al., 1993). In addition to this, polioviruses can be divided genetically into lineages or genotypes that tend to cluster geographically (Lipskaya et al., 1995). As a consequence, sequencing of a representative region of the poliovirus genome (e.g. the VP1-2a junction region) is used to map polioviruses imported into
Fig. 2. Genome organisation of caliciviruses and localisation of assigned genes coding for the major capsid protein, an RNA-dependent RNA polymerase (RdRp), a 2C-like nucleoside triphosphatase (NTPase), a Vpg, and a proteinase (Pro). Enlarged section represents target regions of commonly used RT-PCR assays for detection and strain typing of NLV all located within the RdRp. Conserved motifs are indicated (YGDD and GLPSG). Other abbreviations on the RdRp gene are primer codes. RLB is reverse line blot typing assay.

non-endemic countries to their probably region of origin (Mulders et al., 1995 a and b; van der Avoort et al., 1995). Such importations may then point to an area, which requires intensified vaccination campaigns.

Molecular analysis of caliciviruses

A first question in molecular epidemiological studies is which methods to use for the laboratory investigations. The answer to this question depends on the issue that is addressed by the researcher (Table 4). For caliciviruses, most studies have focussed on understanding modes of transmission of viruses through the community. For this specific purpose, any method that would reliably group identical or highly similar viruses
Table 4
Examples of questions for which molecular typing data can be used, and possible approaches to address these questions

<table>
<thead>
<tr>
<th>Question</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taxonomic grouping</td>
<td>Mapping of signature sequences for calicivirus in non-structural genes, overall genome organisation</td>
</tr>
<tr>
<td>Is this a calicivirus?</td>
<td>Mapping of signature sequences for calicivirus in non-structural genes</td>
</tr>
<tr>
<td>Does this virus belong to a new calicivirus genotype?</td>
<td>Phylogeny based on complete capsid sequence, indicative grouping based on RdRp sequence type</td>
</tr>
<tr>
<td>Are these viruses from a common-source?</td>
<td>Sequence comparison of a representative genome region, e.g. RdRp or capsid, preferably combined with epidemiological data</td>
</tr>
<tr>
<td>Are these viruses antigenically related?</td>
<td>Antigenic tests</td>
</tr>
<tr>
<td>Are there differences in virulence of viruses belonging to different genotypes?</td>
<td>Genotyping combined with clinical/epidemiological data using standardised methods and well-designed epidemiological studies or a model system</td>
</tr>
<tr>
<td>Are these emerging viruses?</td>
<td>Systematic molecular typing data combined with epidemiological information</td>
</tr>
</tbody>
</table>

together, and separate them from less closely related relatives, would suffice. At present, sequence analysis of the viral genome suits this purpose. An important dilemma is which areas of the genome should be analysed. Relatively few calicivirus genomes have been completely sequenced, so that currently "the best genome region" for virus typing does not exist. An attempt at getting some more information was done by Vinjé et al., (2000b) and Green J. et al. (2000), who compiled a panel with 31 different NLV from clinical specimens, and sequenced regions in the RdRp-gene, the complete capsid gene, and part of the 3' ORF. The panel was composed from a selection of stool samples that had been submitted for diagnostic tests to the two institutes over the past few years, and therefore were thought to represent the majority of the currently circulating strains in these countries (UK and The Netherlands). Based on their work, the viruses could be grouped into 15 distinct lineages, 7 belonging to genogroup I and 8 belonging to genogroup II (Table 5; Fig. 3) (Koopmans et al. 2001). Viruses belonging to one genotype had >80% amino-acid similarity across the capsid protein. By a similar approach, (Ando et al., 2000) proposed a numerical system for genotypes based on phylogenetic grouping according to genetic relatedness in the major capsid protein. NLVs have been found that are almost equidistant from GGI and GGII, but were arbitrarily assigned to GGII because they had highest similarity with the GGII viruses in a
Table 5
Classification of current genogroups and genotypes of NLV from humans (Adapted from Green et al. (2001)).

<table>
<thead>
<tr>
<th>GG</th>
<th>genotype</th>
<th>Reference virus</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>Norwalk/1968/US</td>
<td>KY/89/JPN</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Southampton/1991/UK</td>
<td>White Rose, Crawley</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Chiba 407/1987/JP</td>
<td>Thistle Hall, Valetta, Malta</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Masgrove/1989/UK</td>
<td>Butler</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Hesse 3/1997/GE</td>
<td>Sindlesham, Mikklei, Lord Harris</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Winchester/1994/UK</td>
<td>Lwymontley</td>
</tr>
<tr>
<td>II</td>
<td>1</td>
<td>Hawaii/1971/US</td>
<td>Wortley, Grlington</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Melksham/1994/UK</td>
<td>Snow Mountain, Melksham</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Toronto 24/1991/CA</td>
<td>Mexico, Auckland, Rotterdam</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Bristol/1993/UK</td>
<td>Lordsdale, Camberwell, Pilgrim, SymGreen, Grimsby</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Hillingdon/1990/UK</td>
<td>White river, Welterhof</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Seacroft/1990/UK</td>
<td>Gwynedd, Venlo, Creche</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Leeds/1990/UK</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Amsterdam/1998/NL</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>1</td>
<td>Alphatron/1998/NL</td>
<td></td>
</tr>
</tbody>
</table>

region at the start of the capsid gene (Fig. 3, strain designated GGNANA)(Vjinje and Koopmans 2000)). Ando et al. proposed to assign these viruses to a new genogroup. By analogy, the SLV have been divided into 4 genotypes, belonging to 2 genogroups (Vjinje et al., 2000b; Clarke and Lambden 2000; Jiang et al., 1997, 1999; Noel et al., 1997; Liu et al., 1995; Numata et al., 1997).

Besides humans, animals may also carry caliciviruses related to the NLV and SLV. In cattle and pigs, viral sequences were found which cluster with GGI NLV (Jena virus, NA), GGII (swine NLV) and SLV (swine SLV PEC)((Guo et al., 1999; Dastjerdi et al., 1999, 2000; Liu et al., 1999; Sugieda et al., 1998; van Der Poel et al., 2000). The genetic composition of the PEC virus suggest its placement in a separate GG within the genus SLV (39% aa identity with SLV). For the cattle NLVs, Ando et al. (2000) proposed to assign them to a distinct GG, although the reported sequences suggest placement within the GGI viruses is more justified (Dastjerdi et al. 2000).
Fig. 3. Phylogenetic tree showing relationships between currently recognised clusters of NLV found in animals and humans, based on currently available NLV partial capsid gene sequences from Genbank or from a European database of published and unpublished NLV sequences. This includes published and unpublished sequences from France, Spain, the UK, The Netherlands, Germany, Finland, Sweden, and Denmark. This database will be described elsewhere. The alignments are based on sequences from the following variants: bovine NLV (n = 3); swine NLV (n = 2); GGI.1 Hawaii-like viruses (n = 38); GGI.2, Melksham-like viruses (n = 11); GGI.3 Mexico-like viruses (n = 50); GGI.4, Bristol-like viruses (n = 85); GGI.5 Hillingdon-like viruses (n = 16); GGI.6 Seacroft-like viruses (n = 53); GGI.7 Leeds-like viruses (n = 7); GGI.8 Amsterdam-like viruses (n = 8); GGNANA Alphatron-like viruses (n = 3); Erfurt-like viruses (n = 9); GGI.1 Norwalk-like viruses (n = 26); GGI.2 Southampton-like viruses (n = 10); GGI.3 Desert Shield-like viruses (n = 11) and Birmingham-like viruses (n = 13); GGI.4 Queens Arms-like viruses (n = 49); GGI.5 Musgrove-like viruses (n = 6); GGI.6 Sindlesham-like viruses (n = 3); and GGI.7 Winchester-like viruses (n = 3).

What does this divergence mean for taxonomical grouping? Since caliciviruses have some similarities with viruses belonging to the family Picornaviridae, we could look at these viruses for comparison. The family Picornaviridae has been divided into 6 genera, with viruses as diverse as the poliovirus (genus enterovirus), and foot-and-mouth-disease virus (genus aphthovirus). Between genera, genomes differ > 55% in nts and 64% in aa (less than 36% aa identity), and have less than 33% aa homology across the VP1 protein sequence, which encodes the most external and immunodominant of the picornavirus proteins (Oberste et al., 1998, 1999, 2002). Within a genus, different species of viruses share 44-58% nt in the VP1 sequence (aa identity 34-55%). This is in the range of differences seen between caliciviruses belonging to different genogroups (37-44% aa identity across the capsid gene). We have described a variant NLV (Hu/NLV/Alphatron/98/NL) which had 52% maximum sequence identity across the capsid with known NLVs and clustered almost equidistantly to GGI and GGH viruses (Vinje
and Koopmans 2000). Picornavirus serotypes generally have >85% aa identity across the VP1 gene, which is in the range of the cut-off for calicivirus genotype (>80% aa identity) (Table 6) (Oberste et al., 1999).

Table 6

comparison of genetic similarities between viruses belonging to the family *Picornaviridae* and *Caliciviridae* in relation to taxonomic grouping. Indicated are % sequence similarities with other viruses in the family, and the ensuing taxonomic placement of a strain with that sequence.

<table>
<thead>
<tr>
<th>Sequence similarity</th>
<th>Picornavirus</th>
<th>Calicivirus</th>
</tr>
</thead>
<tbody>
<tr>
<td>45% nt, 34% aa</td>
<td>genus</td>
<td>genus</td>
</tr>
<tr>
<td>44-58% nt, 34-55% aa</td>
<td>species</td>
<td>genogroup</td>
</tr>
<tr>
<td>&gt;85% aa</td>
<td>serotype</td>
<td>genotype</td>
</tr>
</tbody>
</table>

An important step in the use of sequence data for molecular epidemiological studies is the comparison of sequences and the subsequent analysis of their evolutionary relationships. This is done by making the best possible alignments. Aligning highly diverse sequences may be a challenging task. For caliciviruses, we have used the conserved motifs within the amplified diagnostic RT-PCR fragment (in the RdRp gene; Fig. 2) to check for the correctness of the alignments. After alignment, sequences are imported into one of several possible programmes for estimating relationships between viruses. Most commonly used are distance-based methods, in which the differences between sequences as expressed in percentage of nucleotide divergence is translated into an evolutionary distance. These methods assume that all positions across a sequence evolve at a constant rate, which clearly is not the case for viral genomes (Oberste et al., 1999). Therefore, phylogenetic trees drawn need to be interpreted with caution. A modified distance based method designed by (Van de Peer et al., 2000) may offer some advantages as it allows the calculation of position-specific evolutionary rates for a specified reference sequence database. A problem for the NLV however remains the extreme diversity, even when focussing on the polymerase gene as one of the most conserved regions.

For the majority of caliciviruses studied by Vinjé et al. (2000) and Green et al. (2000), the placement in phylogenetic trees was consistent, regardless which region of the genome or which clustering method was used for the phylogenetic inference. So far, most molecular epidemiology has been done on the basis of sequence analysis of a small part of the RdRp-gene. A pragmatic reason for this is that most diagnostic RT-PCR assays target this region (Fig. 2). Somewhat surprisingly, this work has shown that there is an extreme diversity at the level of these conserved regions, with
as much as 40% nucleotide differences within the conserved RdRp fragment within a genogroup (Wang et al., 1994; Ando et al., 1995; Green et al., 1994; Norcott et al., 1994; Lew et al., 1994). Since little sequence information is available for the other parts of the genome, it remains to be seen whether other regions are more conserved and therefore may be better targets for generic detection assays.

**Antigenic diversity**

The most biologically relevant antigenic typing is serotyping, in which viruses are divided into serogroups or serotypes on the basis of their reactivity with non-neutralising or neutralising antibodies. Human caliciviruses have not been adapted to cell culture, however, and this handicap precludes the use of virus neutralisation assays. Despite this, there is some evidence of antigenic diversity between caliciviruses, although the precise relationships are not defined (Lewis et al., 1995). Sequence diversity across parts of the major capsid protein gene (ORF2) is informative, as the ORF2 product makes up most of the virion structure, and therefore is exposed to the host's immune system (Jiang et al., 1992c). Therefore, one could speculate that diversity across the capsid gene would be correlated with antigenic diversity. Prior to molecular typing, comparison of viruses from stools of different sources by solid phase immuno-electron microscopy with convalescent sera (SPIEM) had already provided evidence for the presence of antigenic differences between variants. The viruses analysed by Vinjé et al. (2000) and Green et al. (2000) belonged to 12 distinguishable SPIEM types that all segregated into different genetic clusters or genotypes based on sequence analysis of the complete capsid. Similarly, differential reactivity was observed of convalescent sera with recombinant baculovirus — expressed antigens assays (Green et al., 1993; Gray et al. 1994; Okhuysen et al., 1995, Monroe et al., 1993). Correlation between antigenic type and genotype can also be studied from work done with recombinant VLP ELISAs. Antigen detection ELISA assays have been developed by using hyper-immune sera from laboratory animals (rabbits, guinea pig, chicken) immunised with recombinant VLPs. The reported specificities vary: Kobayashi et al. (2000) tested all positive stool specimens detected by an ELISA using reagents specific for Chiba virus, a GGII virus, and found only viruses closely related to the Chiba strain by molecular typing. This suggested that rVLP-based ELISA assays based on a single antigen have a specificity that is too narrow for their use as a diagnostic method for virus in stool. In contrast, Marks et al., (2000) reported the use of an ELISA based on recombinant Lordsdale virus (GGII.4) that detected some viruses from GGI outbreaks, suggesting the existence of one or more cross-reacting epitopes between viruses belonging to different genogroups.

Several groups have studied reactivity of convalescent sera collected from persons with recent infections. Jiang et al. (1992c) reported limited cross-reactivity in serologic responses in volunteers infected with different viruses using the rNV ELISA. Similarly, Myrme and Rimstad (2000) cloned the capsid of a GGI virus, and found reactivities in sera from humans that had tested negative in the rNV ELISA for antibody detection.
In contrast, Noel et al. (1999) found extensive serological cross-reactivity in convalescent sera for GGI viruses belonging to different genotypes within GGI. Others found occasional cross-reactivity between GG, although the homologous response was far greater (Hale et al., 1998, Treanor et al., 1993).

Combined, the data suggest that viruses belonging to different genotypes are antigenically distinct, but induce antibodies that cross-react with viruses belonging to the same genogroup. Low level cross-reactivity occurs with viruses belonging to a different GG. It is conceivable that the cross-reactive antibodies become detectable after repeated infections only, and that the lesser reported serological cross-reactivity reported GGI results from the lower likelihood of repeated exposure to GGI viruses (Tables 1-3). Which epitopes are involved and how the observed antigenic differences correlate with serotypes remains unclear. The capsid gene of human caliciviruses can be divided in two domains: the N terminal, conserved, shell domain that encodes for a part of the capsid protein that constitutes the core of the particle, and the protruding domain, encoding parts of the protein that are surface exposed. Within the protruding domain, a highly variable region has been defined (P2 region, residues 281-404 for Norwalk virus), that probably plays a role in virus antigenicity (Green et al., 2001).

Consequences of molecular diversity for diagnostics

An issue that has been raised at the first Calicivirus Workshop in Atlanta in 1999, and that has not yet been addressed in comparative studies is how the data obtained with the molecular detection and typing assays can be compared. The most commonly used primers map to a region in the RdRp gene, which is slightly different for each of the RT-PCR assays used (Fig. 2). This raises some questions:

Can the data obtained with different assays be compared?

Comparative evaluation of assays is needed to study congruence between the tests. Since all published assays reportedly have a high sensitivity and specificity, this issue may seem trivial. However, the great diversity of caliciviruses makes it difficult to do a full evaluation of a diagnostic test. In fact, most assays have been optimised by use of RNA from the most commonly circulating variants as a reference template rather than specimens covering the full range of variants. Thus, the sensitivity of the assays in detecting less common variants is not known, and may differ greatly between assays. This information is crucial in order to be able to compare data from different studies.

In a comparative analysis of 4 currently used protocols, in which we compiled a panel of stool samples covering a wide range of variants, we found that the overall performance of the different assays was very similar. Overall sensitivity of the assays ranged from 82 to 100%. There were, however, significant differences when looking at sensitivity of detection of viruses belonging to different genogroups or genotypes, and assays were less than optimal for detecting the GGI viruses. The sensitivity of detection of GGI viruses ranged from 45 to 100%, and for GGI from 64 to 100% (Vinjé
et al., 2002). This stresses the importance of targeted evaluation of the currently used diagnostic tests. Special attention needs to be put into implementing generic tests in diagnostic laboratories. The optimised protocols result from a trade-off between broad-ness and sensitivity. For individual variants, lower detection limits may be reached with increased stringency of the assay (e.g. by raising the annealing temperature), and laboratory workers not familiar with generic tests often wish to redesign the protocols. That may, however, result in a decreased sensitivity of detection of other lineages of viruses.

**Do data obtained with a specific primer set change over time?**

RNA viruses have sloppy polymerases, which allow incorporation of mismatches during the replication of the genome, thereby introducing mutations. As a consequence, the progeny of one virus typically consists of a swarm of closely related sequences ("quasispecies"). Therefore, by analogy with other RNA viruses, the caliciviral genome may drift over time. Ando et al. (2000) have suggested that the positivity rate of PCR assays used for virus detection in outbreaks of gastroenteritis goes down over time as a result of accumulation of mutations in the primer binding sites. Clearly, this depends on the target region: the reverse primer of our standard RT-PCR for virus detection and typing anneals to the highly conserved YGDD motif of the RdRp. These amino acids are essential in the function of the RNA polymerase, and therefore mutants will have a disadvantage. Hence, the mutant viruses will not survive competition with the existing virus population.

**How informative is phylogeny based on a short RNA fragment?**

Currently, the overlap between the amplified fragments for the assays developed by Ando et al. (1995), Vinjé and Koopmans (1996), Norcott et al., (2004), LeGuyader et al. (1996), and Maunula et al. (1999) is a marginal 60 nucleotides. It goes without saying that this fragment is too small for reliable clustering. This is a problem in the case of suspected international common source outbreaks: viruses that are grouped as identical based on the short 60 nt fragment may in fact be quite different. To demonstrate this, we aligned a panel of 71 different sequences from our collection, and clustered them by our routine method, after deleting increasing amounts of nucleotides. Of the sequences that were different based on the complete sequence in our collection, 65-75% were grouped as identical when the sequences were truncated to the 60nt minimal fragment, depending on which segment of the amplicon was deleted (Fig. 4). This does not mean that these comparisons are useless. It does, however, imply that viruses cannot be labelled as identical based on the small overlap alone, and that additional analysis will have to be done to confirm or disprove possible relatedness.
Use of molecular techniques to study calicivirus virus epidemiology

In recent years, a number of studies have been done in which molecular typing of caliciviruses was used (Tables 1-3). The studies address different segments of the population (Fig. 1). Overall, GGI viruses are detected most commonly, with the proportion of strains belonging to GGI ranging from 11-100%. There seems to be a difference in developing countries, where GGI viruses were reported more commonly (average 37%, range 10-63) as compared with data from countries with higher standards of living (average proportion GGI 15%, range 0-89). There are no data to explain this difference, although GGI viruses are more commonly found in water- or sewage-related contamination. Therefore, the higher rates of GGI viruses found in the studies in developing countries may result from exposure to contaminated water. The apparent association of GGI viruses with a waterborne mode of transmission suggests possible differences in stability of viruses outside the host.

Molecular epidemiological studies of caliciviruses are still in their infancy. Very few community studies of gastroenteritis have been done, and even less have used molecular typing techniques. In our community studies, we found viruses belonging to 9 different genotypes circulating within the same year in 1999 (de Wit et al., 2001a and b; Koopmans et al., 2001). Viruses in genogroups II.4 (Bristol/Lordsdale-like viruses) and II.7 (Leeds-like viruses) were the most common. Outbreak surveys support the notion that some genotypes are more common than others, and especially the...
GGII.4 viruses have been seen as predominant. The proportion of outbreaks attributed to viruses of this genotype ranges from 7 to 91% (Table 1). Based on this observation, recombinant GGII.4 (rGGII.4) antigen detection ELISA for screening stool samples has been introduced in some laboratories (Marks et al., 2000). Similarly, typing methods selective for identification of viruses belonging to this genotype are used (Mattick et al., 2000). Unfortunately, however, these assays do not provide insight into the molecular epidemiology until the antigenic relationships between viruses and their effect on sensitivity of antigen detection is resolved. Until then, we need molecular typing methods.

The dominance of some genotypes over others is not a constant feature, and several groups have reported seasonal fluctuation. In Fig. 5 the annual proportion of GGII.4 viruses is plotted for the passive outbreak surveillance system in The Netherlands. This shows that in this country the GGII.4 variants are common, but that the proportion of outbreaks attributed to this genotype fluctuates significantly.

![Graph](image1)

**Fig. 5.** Distribution of NLV genotypes in outbreaks of gastro-enteritis in The Netherlands from 1994-2001 (top), and total number of outbreaks reported per year (bottom).

Emerging variants, mystery or misinterpretation?

In 1996, GGII.4 viruses were the dominant variant, as was observed in other countries. Based on this observation, (Noel et al., 1999) suggested that this variant spread epidemically across the globe and might be a novel variant. Other studies, however,
have shown that similar GGII.4 variants were present in the community long before the GGII.4 epidemic (Green et al., 2002; Wright et al., 1998) found significant amino-acid differences when comparing the genome of a common GGII.4 strain detected in 1988, with the 1996 “global strain”. Could it be that GGII.4 viruses are the most common worldwide, and that the so-called epidemic resulted from a simultaneous flurry of studies which brought this to light? Or was there really something peculiar about the 1996 common strain, which might explain the increased number of outbreaks overall in The Netherlands (Fig. 5) and in the US when this virus was circulating (Vinjé et al., 1997; Noel et al., 1999)? When comparing distribution of genotypes for different age groups in a community-based case-control study of gastro-enteritis in The Netherlands, NLV of the genotype GII.4 were found more frequently in young children than other NLV. In addition, GGII.4 viruses were not detected in persons without symptoms, whereas the other genotypes were (30% of GGII.3 positives; chi square, \( p = 0.03 \))(Koopmans et al., unpublished observations). This is where the lack of a cell culture system and of an animal model is painfully clear: it would be quite interesting to compare biological behaviour of GGII.4 viruses from outbreaks during the “epidemic years” with those from historic collections.

Interestingly, through a collaborative approach to outbreak investigations across Europe, we saw another “epidemic” rise in the winter of 2000/2001 when a novel variant emerged almost simultaneously in 7 countries in Europe. In this case, the epidemic could be traced back to introductions of the virus by contaminated food, and its subsequent spread (Koopmans et al., manuscript in preparation). International food- or waterborne outbreaks have been described, and may be much more common than we think. Numerous outbreak reports illustrate the lack of awareness of the possibility of virus transmission via this route. In The Netherlands, we estimated that approximately 100,000 cases of NLV infection can be traced back to consumption of contaminated food, which is more than the number of cases of Salmonellosis (de Wit et al., submitted for publication). The CDC in Atlanta has published even higher estimates, and suggest that 66% of all food-related illness in the US may be due to NLV infection (Mead et al., 1999). Current microbiological quality controls for food are not aimed at detection of viruses, and are known to be poor indicators of their presence.

**Do animals constitute a reservoir for enteric caliciviruses?**

As described above, viruses similar to the NLVs and SLVs in humans have been found in cattle and pigs. One of the popular hypotheses to explain the epidemic rise of a single variant was that these might be novel to the population and had recently been introduced from some animal reservoir(s). As attractive as it sounds, there are no data to support this hypothesis at present. Despite an increasing number of studies in which all viruses found are subjected to genotyping, no “animal” caliciviruses have so far been found in humans. Nevertheless, this merely shows that animal-human transmissions are not the most common mode of transmission. It may still happen. Recent data from work with hepatitis E virus may be leading the way: HEV was considered
to be an infection with a rather narrow host tropism, and a travel-associated diseases in developed countries. Recently, however, viruses highly related to HEV were found in pigs, and people with professional exposure to pigs (vets, farmers) were shown to have increased prevalence of antiHEV antibodies, suggesting possible zoonotic infections (Meng 2000; Meng et al., 1997, 1998). Indeed, a few human infections with viruses that group with the pig HEV variants now have been described, and there are indications of exposure in risk groups (Meng et al., 2002). This could be a scenario for the caliciviruses: there is no evidence of very efficient zoonotic transmission, yet occasional species jumps may take place (in both directions). If so, chances are that – given the high incidence of calicivirus infections - dual infections occur, and recombinant viruses are generated which subsequently can spread through the community. Not surprisingly, recombination has been documented for caliciviruses (Vinje et al., 2000b). Additional studies, both serological and molecular studies are needed to clarify this interesting and important issue.

Use of molecular epidemiology in outbreak tracing

A promising application is the use of molecular typing to help establish modes of transmission in outbreak investigations. This has been used to support evidence for foodborne-, waterborne-, and environmental transmission routes (Table 7 and references therein). More detailed review of the data in light of the issues described above shows that proving such transmission by molecular methods is not as easy as it may seem. The outbreaks in which the GGII.4–like viruses were detected may seem clear-cut. But given the high prevalence of these viruses in the community and in outbreak surveys, how can one disprove that related sequences are found by chance? Cheesbrough et al. (2000) reported that some sequence variation occurred in the cases in a protracted outbreak in a hotel in the UK that all had a GGII.4 virus. Isn’t that remarkable in view of the highly conserved structure of the GGII.4 cluster? In 1996, in The Netherlands 60 consecutive seemingly unrelated outbreaks were caused by a virus in the GGII.4 cluster, and viruses with identical partial sequences of the polymerase gene were found in several countries all over the world in the same period (Fig. 6; Sweden, Japan, France, Australia, US) (Vinje et al., 1997; Noel et al., 1999). The sequence variation described in the case strains by Cheesbrough et al. (2000) therefore may illustrate in fact that this was not a protracted outbreak, but a series of outbreaks resulting from repeated introductions. Clearly, the hygienic conditions in the hotel were less than optimal, since widespread environmental contamination was demonstrated elegantly in this study. Parashar et al. (1998) linked cases to a food-handler based on identical sequence in a 81 nt region. However, the data presented in Fig. 4 show that this may be misclassification, since only 30-40% of different sequences were clustered as different based on a 81 bp region of the polymerase sequence. In the studies listed in Table 1 that reported GII.4 viruses and had typing information, identical viruses were found in a proportion of 7 out of 9 studies that had sequenced a fragment of less than 200 base pairs. In contrast, none of the studies in which a longer sequence was used
Table 7

Outbreaks of gastroenteritis in which molecular typing has been used to build evidence (for abbreviations see legend of Table 1).

<table>
<thead>
<tr>
<th>Setting</th>
<th>Country</th>
<th>n (sick)</th>
<th>Probable source</th>
<th>Level of evidence</th>
<th>Proposed explanation</th>
<th>Typing</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>University</td>
<td>US</td>
<td>125</td>
<td>Deli bar</td>
<td>identical virus in stool and food</td>
<td>asymptomatic food handler, sick child</td>
<td>GGIL4</td>
<td>Daniels et al., 2000</td>
</tr>
<tr>
<td>Town</td>
<td>Finland</td>
<td>&gt;1655</td>
<td>Drinking water</td>
<td>identical virus in stool and water</td>
<td>breakdown in chlorination</td>
<td>GGIL4 (stool, water)</td>
<td>Kukkula et al., 1999</td>
</tr>
<tr>
<td>Hospital</td>
<td>France</td>
<td>6</td>
<td>Drinking water</td>
<td>identical virus in stool and water</td>
<td>nr</td>
<td>GGIL2</td>
<td>Schvoerer et al., 1999</td>
</tr>
<tr>
<td>Restaurant</td>
<td>UK</td>
<td>&gt;52</td>
<td>Sick person</td>
<td>identical virus in all cases</td>
<td>aerosols from vomit</td>
<td>GGII.7</td>
<td>Marks et al., 2000</td>
</tr>
<tr>
<td>Company</td>
<td>Finland</td>
<td>&gt;509</td>
<td>Frozen raspberries</td>
<td>food-specific odds ratio</td>
<td>spraying berries with surface water</td>
<td>GGII</td>
<td>Ponka et al., 1999</td>
</tr>
<tr>
<td>Hotel</td>
<td>UK</td>
<td>850</td>
<td>Environmental contamination</td>
<td>virus in environmental swabs</td>
<td>contamination following an initial outbreak</td>
<td>GGIL.4</td>
<td>Cheesbrough et al., 2000</td>
</tr>
<tr>
<td>Hotel</td>
<td>UK</td>
<td>94</td>
<td>Kitchen sink</td>
<td>identical virus in food handler and case</td>
<td>food preparation in contaminated sink</td>
<td>GGIL.4</td>
<td>Patterson et al., 1997</td>
</tr>
<tr>
<td>Restaurant</td>
<td>Canada</td>
<td>66</td>
<td>Raspberries</td>
<td>identical virus in food and cases</td>
<td>contaminated food imported from Bosnia</td>
<td>GGI</td>
<td>Gaulin et al., 1999</td>
</tr>
<tr>
<td>Company</td>
<td>US</td>
<td>&gt;85</td>
<td>Sandwich</td>
<td>identical virus in food and food handler</td>
<td>asymptomatic food handler</td>
<td>GGIL2</td>
<td>Parashar et al., 1998</td>
</tr>
</tbody>
</table>
reported identical viruses. These are examples to illustrate the importance of careful interpretation of the data: finding identical sequences is more convincing with a longer stretch of sequence, and if the viruses belong to clusters that are rare in the community. Background data on the diversity of data from viruses circulating in the community at the time of the outbreaks help support evidence. It remains essential that the findings are supported by epidemiological evidence, at least until sufficient outbreak reports have been done to know the reliability.

Special attention in outbreak tracing is needed for outbreaks in which the source of virus may be from sewage contamination. Typically, in such outbreaks mixtures of viruses may be detected within cases involved in a common-source outbreak. Some people may test positive for one virus, others for a different variant. It is clear from the outbreak surveys that this is a rule rather than an exception in the outbreaks in which sewage-contaminated oysters are involved (Ohyama et al., 1999; Iritani et al., 2000; 2002; Greening et al., 2001; Maunula et al., 1999). As a consequence, finding a virus in the food that differs from that in stools from cases does not necessarily disprove a causal link in these cases.

Surveys within a geographic region over several years

From the studies published so far, caliciviruses appear to have a worldwide distribution. In outbreak surveys, the GGII virus are pre-dominant. The most commonly detected variants overall are the GGII.4 viruses. An alignment of currently available partial GGII.4 sequences from Genbank, as well as unpublished sequences from a European database (Koopmans et al. manuscript in preparation) is shown in Fig. 6. Variants detected in Europe and outside of Europe appear on every branch of the dendrogram, suggesting that there is no geographic clustering. Similarly, from data available over a 17-year period, there is no apparent drift in the sequences of this particular region. These data are consistent with worldwide endemic circulation of viruses belonging to this genotype. However, closer inspection of sub-branches shows that identical sequences often originate during the same season and from the same country. This is somewhat contrasting with the first observation, and suggests events that favour the distribution of progeny of a particular virus. That could happen through a seeding event (e.g. large common-source outbreak) or by selection of variants with increased fitness. For the latter explanation, one would expect that such variants would persist as for instance the 1995/6 "common strain" (Fig. 6) which was found world-wide in a relatively short span of time (Noel et al., 1999). Only with prolonged, harmonised surveillance in which epidemiological and virological information is collected concurrently, and characterisation of carefully selected variants, we may learn whether these observations are correct. The answers to these questions are important, since they show us a lot about the modes of transmission of emerging enteric pathogens through the population.
Fig. 6. Alignment of viruses within the GGI4 cluster, based on nucleotide sequences of a fragment of the polymerase gene for 338 variants detected in association with outbreaks and sporadic cases of gastroenteritis worldwide. Sequences obtained were either downloaded from Genbank (all GGI4 sequences, n = 55), or drawn from the European database of calicivirus sequences (see legend for Fig. 3). Sequences from GGI4.1 (Hawaii-like viruses) were used as an outgroup (bottom of figure).
Concluding remarks

Molecular epidemiological studies are increasingly popular in the field of clinical calicivirus research. Based on an (arbitrary) genetic typing system, caliciviruses are divided in genera, genogroups and genotypes. Most illness in humans appears to be associated with NLVs, although data on community-based studies are sparse. Therefore, the role of SLVs remains to be studied with similar approaches. The NLV GGI virus dominates in all parts of the world. Several genotypes co-circulate, but the majority of infections is associated with only a few genotypes. The epidemiology of these genotypes is only beginning to be addressed. Future studies are needed in which systematic surveillance of outbreaks of gastroenteritis is done for prolonged periods of time, and with harmonised methods in order to find explanations for the apparent emergence of calicivirus variants in populations, the fluctuations in their presence, virulence differences and modes of transmission including the possibility of zoonotic transmission. International collaborations coupled with rapid exchange of information on outbreak data are crucial if we want to look further than the horizons of our own countries. After all, with foodborne transmission being a major source of calicivirus infections, what use are borders?

References


185:133-46.


48. Lipskaya, G. Y., Chervonskaya, E. A., Belova, G. I., Maslova, S. V., Kutateladze,

49. Liu, B. L., Clarke, I. N., Caul, E. O., and Lambden, P. R. Human enteric caliciviruses have a unique genome structure and are distinct from the Norwalk-like viruses. Arch Virol. 1995; 140:1345-56.


74. Ohyama, T., Yoshizumi, S., Sawada, H., Uchiyama, Y., Katoh, Y., Hamaoka,


100. Vinjé, J. and Koopmans, M. P. Molecular detection and epidemiology of small


Evidence for quasispecies distributions in the human hepatitis A virus genome

Glòria Sánchez, a Albert Bosch, a, * Gema Gómez-Mariano, b Esteban Domingo, b and Rosa M. Pinto a

a Grup Virus Entèrics, Department of Microbiology, University of Barcelona, 08028 Barcelona, Spain
b Centro de Biología Molecular “Severo Ochoa,” CSIC-UAM, Cantoblanco, 28049 Madrid, Spain

Received 7 April 2003; returned to author for revision 20 May 2003; accepted 12 June 2003

Abstract

Nucleotide sequence analysis of multiple molecular clones of the hepatitis A virus (HAV), generated by reverse transcription-PCR of two capsid-coding regions, revealed a degree of heterogeneity compatible with a quasispecies structure in three clinical samples. Passage of plaque-purified reference strain HAV pHM175 43c in FRhK-4 cells documented the generation of a mutant distribution of HAV genomes. The mutant spectra showed mutation frequencies in the range of $1 \times 10^{-3}$ to $1 \times 10^{-4}$ substitutions per nucleotide, with a dominance of transition over transversion mutations. While in the VP3-coding region, nonsynonymous mutations were predominant; in the VP1-coding region they were uncommon. Around 50% of the amino acid replacements involved residues located at or near antigenic sites. Most of the detected mutations occurred at or in the vicinity of rare codons, suggesting a dynamics of mutation-selection, predominantly at and around rare codons. The results indicate that despite antigenic conservation, HAV replicates as a complex distribution of mutants, a feature of viral quasispecies.

© 2003 Elsevier Inc. All rights reserved.

Keywords: HAV; Quasispecies; Antigenic sites; Codon-usage; Rare codon; Normal codon

Introduction

Hepatitis A virus (HAV), classified as the type species of the genus Hepatovirus within the Picornaviridae family (van Regenmortel et al., 2000), is a hepatotropic virus which represents a significant problem for human health (Battegay and Feinstone, 1997; Hollinger and Emerson, 2001). The virion capsid is composed of the structural proteins VP1, VP2, VP3, and possibly VP4, encoded in the P1 region of the genome (Hollinger and Emerson, 2001; Racaniello, 2001).

Some degree of nucleotide sequence heterogeneity of the P1 genomic region has been observed among independent HAV isolates from different regions of the world (Lemon et al., 1987; Robertson et al., 1992; Taylor, 1997; Arauz-Ruiz et al., 2001; Costa-Mattioli et al., 2001). However, this variability at the nucleotide level is not reflected in an equivalent degree of variation at the amino acid level (Lemon and Robertson, 1993; Hollinger and Emerson, 2001; Sánchez et al., 2003). The high degree of conservation of the amino acid sequences of the capsid proteins of HAV entails a low antigenic diversity, and therefore, only a single serotype of human HAV has been recognized (Hollinger and Emerson, 2001). This suggests the operation of severe structural constraints in the HAV capsid (Sánchez et al., 2003). HAV shows a high codon usage bias, with the repeated occurrence of 22 rare codons for 14 amino acids (Sánchez et al., 2003). Most of the carboxy-terminal regions of β-barrels and α-helices, that are predicted in the capsid proteins (Luo et al., 1988), include residues encoded by conserved rare codons, suggesting a potential function of such codons in a decrease of the rate of translation to facilitate the proper folding of the capsid proteins (Sánchez et al., 2003).

The molecular basis of the genetic variability of RNA
viruses has been associated with the absence of a 3′ → 5′ exonuclease proofreading activity in viral RNA-dependent RNA polymerases and reverse transcriptases, together with lack of postreplicative repair mechanisms that can act on DNA but not on RNA (Holland et al., 1992; Steinhauer et al., 1992; Williams and Loeb, 1992; Domingo and Holland, 1997). Mutation rates for a variety of RNA viruses range between 10⁻⁴ and 10⁻⁵ substitutions per nucleotide copied (Batschelet et al., 1976; Drake, 1993; Drake and Holland, 1999). As a consequence, RNA viruses replicate as complex dynamic mutant distributions, termed viral quasispecies (Eigen and Biebricher, 1988; Holland et al., 1992; Domingo and Holland, 1997; Domingo et al., 2001). The open reading frame of the putative HAV 3D (the RNA-dependent RNA polymerase) does not provide any evidence for the presence of a proofreading or error-correcting activity known to be associated with several DNA-dependent DNA polymerases (Kunkel, 1988; Zimmer, 1988; Friedberg et al., 1995). Therefore, mutation rates and frequencies for HAV are not expected to differ significantly from those of other picorna-viruses and RNA viruses in general (Drake and Holland, 1999). In this view, the 90% or higher amino acid sequence identities among independent strains and isolates of HAV (Hollinger and Emerson, 2001) would be the result of negative selection on many newly arising mutants and convergence of consensus or average sequences (Eigen and Biebricher, 1988; Holland et al., 1992; Domingo et al., 2001). Yet if mutational pressure originated a number of mutants hidden in a mutant spectrum, such mutants would provide evidence of quasispecies dynamics, implying the presence of a variant reservoir for HAV adaptation. Despite the biological significance of this population structure, no such analyses of HAV mutant spectra have been reported.

In the present work, results suggesting the occurrence of distributions of related genomes in HAV are presented, both with clinical isolates of HAV and in populations evolved in cell culture from a biological clone of HAV. The nature of the mutations detected in the mutant spectra supports the operation of structural constraints in the capsid of HAV. Furthermore, the results of HAV evolution in cell culture revealed the presence of antigenic variants that were generated in the absence of immune selection-as-previously observed with other viruses (Domingo et al., 1993).

Results

A mutant spectrum in clinical isolates of HAV

To test whether the limited amino acid variation among consensus sequences of independent HAV isolates was paralleled by a homogeneous mutant spectrum within a HAV population, a clonal analysis of three clinical samples of HAV was performed. The samples were Val9 (stool), Val10 (stool), and Val12 (serum) (Sánchez et al., 2002), whose origin is described under Materials and methods. A mean of 25 molecular clones from a single reverse transcription (RT)-PCR amplification of a fragment of the VP3 (from nucleotides 1470 to 1839, encoding amino acids 1 to 123) and a fragment of the VP1 (from nucleotides 2459 to 2943, encoding amino acids 85 to 245), which span sequences encoding the main antigenic sites of HAV (Nainan et al., 1992; Ping and Lemon, 1992; Bosch et al., 1998), were analyzed for each sample. Minimum mutation frequencies (counting repeated mutations only once) ranged between 1.2 × 10⁻³ and 1.4 × 10⁻⁴ substitutions per nucleotide (Table 1). In some analyses, the maximum mutation frequency (counting all mutations relative to the consensus) increased over the minimal mutation frequency. The values of Shannon entropy (a measure of the proportion of different sequences in the set analyzed) indicated heterogeneity of the mutant spectra. Control experiments (described in detail under Materials and methods) indicated that the mutation frequencies observed cannot be the result of misincorporations during RT-PCR amplification of HAV RNA. Statistical analysis (nonparametric Mann–Whitney U test and ANOVA) revealed significant differences between the mutation frequencies of the patient samples and the error rate of the RT-PCR system, except in the Val10 sample. However, despite this lack of statistical significance, the localization of the mutations (see below) make it extremely unlikely that they could be just due to the occurrence of random misincorporations. The mutation-frequencies and Shannon entropies of the mutant spectra are in the range observed in other viral quasispecies (see Discussion).

A mutant spectrum in serially passaged HAV pHM175 43c

The sequence comparisons indicated the occurrence in the HAV isolates of dominant genomic sequences together with several variants. These results suggest generation of mutants in the course of replication of each individual HAV. However, a definitive proof of mutant generation requires a demonstration that multiple variants are produced de novo upon replication of a single HAV genome (Domingo et al., 1978; Domingo, 1996). To this effect, the reference HAV strain pHM175 43c, adapted to FRhK-4 cells, was subjected to three successive plaque isolations to produce pHM175 43c P0, and then this clonal population was subjected to 26 serial passages in the same cells (about 10⁶ PFU infecting 10⁶ cells per passage), as detailed under Materials and methods. The resulting population is termed pHM175 43c P26. No mutations were detected in the consensus sequences of the VP1- and VP3-coding regions analyzed, at passages 1, 5, 11, 16, and 26. However, a clonal analysis revealed 23 mutations in 100 molecular clones from passage 26 (Table 2). The minimum mutation frequencies were 7.0 × 10⁻⁴ and 3.3 × 10⁻⁴ substitutions per nucleotide for the VP3- and VP1-coding regions, respectively. Only for the VP3-coding region the maximum mutation frequency was slightly higher than the minimum mutation frequency.
synonymous or silent (not inducing an amino acid substitution) and insertion/deletion mutations (inducing an erroneous reading frame)

–

Statistical analysis (nonparametric Mann–Whitney U test and ANOVA) revealed significant differences between these mutation frequencies and the experimental error rate. The values of Shannon entropy indicated heterogeneity of the mutant spectra. The consensus and the dominant sequences were identical. Thus, the results document constancy of the average or consensus sequences, and the generation of a mutant spectrum upon replication of a biological clone of HAV. These are features of quasispecies dynamics (Eigen and Biebricher, 1988). Experiments are in progress to elucidate whether the virus mutant distribution varies with cell passage.

Types and location of mutations: mutation clustering

A dominance of transitions over transversions was observed in all HAV mutant spectra analyzed (Tables 1 and 2). In contrast, the ratio of synonymous-to-nonsynonymous mutations was 33 for the VP1-coding region and 0.5 for the VP3-coding region (average for all mutations detected in the four mutant spectra analyzed, Tables 1 and 2). This suggests higher constraints for variation at the amino acid level for VP1 than for VP3. As expected, no bias was observed in the few mutations recorded in the control amplification of an RNA transcript (basal RT-PCR error, described under Materials and methods).

The positions of the mutations and amino acid substitutions indicate the occurrence of several clusters of mutations in the mutant spectra of both clinical samples and in the clonal population pHM175 43c P26 (Fig. 1). Clusters of substitutions at amino acid positions 37, 40, 41 and at 82, 83, 84 of VP3 in the mutant spectra of pHM175 43c P26, and at 179, 180, and 181 of VP1 in the mutant spectra of the clinical samples were remarkable. Some substitutions were observed in independent mutant spectra (i.e., at positions 93, 104, 116, 131, 140, 181, and 217 of VP1), suggesting

Table 1
Characterization of the mutant spectrum of HAV in clinical samples

<table>
<thead>
<tr>
<th>Genomic region</th>
<th>Mutations&lt;sup&gt;a&lt;/sup&gt; nucleotides sequenced</th>
<th>$T_b$</th>
<th>$T_c$</th>
<th>$N_{syn}$&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Syn&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Indel or stop&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Nucleotide mutation frequency&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Maximum amino acid mutation frequency&lt;sup&gt;c&lt;/sup&gt;</th>
<th>$S_N$&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP3 Val9</td>
<td>6/8773</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>$6.8 \times 10^{-4}$</td>
<td>$6.8 \times 10^{-4}$</td>
<td>0.31</td>
</tr>
<tr>
<td>VP3 Val10</td>
<td>1/6960</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>$1.4 \times 10^{-4}$</td>
<td>$1.4 \times 10^{-4}$</td>
<td>0.05</td>
</tr>
<tr>
<td>VP3</td>
<td>5/9913</td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>$4.0 \times 10^{-4}$</td>
<td>$5.0 \times 10^{-4}$</td>
<td>0.22</td>
</tr>
<tr>
<td>VP3 Val12</td>
<td>40/13,035</td>
<td>38</td>
<td>2</td>
<td>0</td>
<td>40</td>
<td>0</td>
<td>$1.2 \times 10^{-3}$</td>
<td>$3.1 \times 10^{-3}$</td>
<td>0.50</td>
</tr>
<tr>
<td>VP1 Val9</td>
<td>2/9962</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>$2.0 \times 10^{-4}$</td>
<td>$2.0 \times 10^{-4}$</td>
<td>0.12</td>
</tr>
<tr>
<td>VP1 Val10</td>
<td>19/12,248</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>19</td>
<td>0</td>
<td>$6.5 \times 10^{-4}$</td>
<td>$1.5 \times 10^{-3}$</td>
<td>0.26</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mutant residues are those that vary relative to the corresponding consensus sequences

<sup>b</sup> Detected mutations were classified as indicated in footnote b of Table 1.

<sup>c</sup> Mutation frequencies are as defined in footnote c of Table 1.

<sup>d</sup> The normalized Shannon entropy was calculated as indicated in footnote d of Table 1.

Table 2
Characterization of the mutant spectrum of the pHM175 43c P26

<table>
<thead>
<tr>
<th>Genomic region</th>
<th>Mutations&lt;sup&gt;a&lt;/sup&gt; nucleotides sequenced</th>
<th>$T_b$</th>
<th>$T_c$</th>
<th>$N_{syn}$&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Syn&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Indel or stop&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Nucleotide mutation frequency&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Maximum amino acid mutation frequency&lt;sup&gt;c&lt;/sup&gt;</th>
<th>$S_N$&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP3</td>
<td>15/18,500</td>
<td>12</td>
<td>3</td>
<td>10</td>
<td>5</td>
<td>0</td>
<td>$7.0 \times 10^{-4}$</td>
<td>$8.1 \times 10^{-4}$</td>
<td>0.31</td>
</tr>
<tr>
<td>VP1</td>
<td>8/24,150</td>
<td>7</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>1 Stop</td>
<td>$3.3 \times 10^{-4}$</td>
<td>$3.3 \times 10^{-4}$</td>
<td>0.20</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mutant residues are those that vary in comparison with the consensus sequence at P26.

<sup>b</sup> Detected mutations were classified as indicated in footnote b of Table 1.

<sup>c</sup> Mutation frequencies are as defined in footnote c of Table 1.

<sup>d</sup> The normalized Shannon entropy was calculated as indicated in footnote d of Table 1.
Fig. 1. Amino acid sequence of the VP3 and VP1 fragments analyzed in the present study. (A) VP3 sequence of the pHM175 43c strain. (B) VP3 sequence of the clinical samples. (C) VP1 sequence of the pHM175 43c strain. (D) VP1 sequence of the clinical samples. In bold are depicted those amino acids encoded by rare codons. Arrows indicate those positions in which a mutation occurred. Regarding the codon usage (Sánchez et al., 2003), the letter n designates a normal (frequent) new codon; † n, a significantly more normal (frequent) new codon than the old one;  ‡ n, a significantly less normal (frequent) new codon than the old one; and r, a rare new codon. Numbers in parentheses indicate the number of clones with a given substitution. At those positions in which a nonsynonymous substitution occurred, the new amino acid is depicted over the sequence row. At position 72 in (B) either a valine or an isoleucine may be present depending on the clinical sample.
again the operation of constraints that limit the number of positions that tolerate replacements, giving the appearance of mutational hot spots (see Discussion). Seven of 15 amino acid substitutions were either at residues previously identified as belonging to antigenic sites (i.e., 65, 72, and 115 of VP3) or at residues in the neighborhood of antigenic sites (i.e., 78, 83, 84, and 91 of VP3) (Ping and Lemon, 1992; Bosch et al., 1998; Hollinger and Emerson, 2001).

A large proportion of mutations in the mutant spectra analyzed involved either rare codons, defined as those codons used at low frequencies (Gavrilin et al., 2000) or normal codons located in the neighborhood of rare codons (at a maximum distance of three triplets from a rare codon) (Fig. 1). The majority of the mutations involving rare codons (90%) resulted in the change to a normal codon. However, most of the resulting normal codons (78%) were contiguous to a rare codon (Fig. 1). In contrast, most mutations involving normal codons (74%) induced changes which significantly affected the normality of the codon, and a high percentage of them (72%) were again closely located to rare codons (Fig. 1). Therefore, both the types of mutations and the types of codons affected suggest variation constraints in HAV.

Discussion

Despite the invariance of consensus sequences, at the VP3- and VP1-coding regions examined, upon serial passage of a biological clone of HAV, each of the populations analyzed consisted of a mutant spectrum in agreement with a quasispecies population structure for this important human pathogen (Tables 1 and 2). From the minimum mutation frequencies and since the HAV genome is about 7500 nucleotides in size [specifically, the genome of the original HM175 wild-type HAV is 7493 nucleotides long (Cohen et al., 1987) and the genome of the cell culture-adapted strain pHM175 43c is 7503 nucleotides long (Lemon et al., 1991)], the average number of mutations per genome (relative to the consensus sequence) for Val9, Val10, Val12, and the clonal population pHM175 43c P26 is 7.0, 1.2, 3.9, and 3.8, respectively. This calculation assumes that the mutation frequencies for the analyzed genomic regions reflect those of other genomic regions, a point that would require sequencing of other genomic regions from components of mutant spectra. However, comparison of nucleotide sequences from different HAV isolates suggests a considerable similarity in the degree of conservation of different genomic regions (Hollinger and Emerson, 2001; Ching et al., 2002; Sánchez et al., 2003). In these comparisons, the most variable genomic regions are those encoding 2B, 2C, and 3B (representing 25% of the genome), with an average of 1.1-fold the genetic distance calculated for the analyzed VP3- and VP1-coding regions, the most conserved genomic regions are the highly conserved noncoding regions and those encoding 3C and 3D (representing 39% of the genome), with an average of 0.8-fold the genetic distance calculated for the VP3- and VP1-coding regions. Since in other viruses conservation among independent isolates often parallels conservation within mutant spectra (Arias et al., 2001; Domingo et al., 2001), the heterogeneity quantitated on the basis of the VP3- and VP1-coding regions analyzed is unlikely to differ substantially from the heterogeneity for the entire HAV genome. With this assumption, the proportion of genomes with no mutations (the dominant sequence), calculated from the Poisson, distribution, would be 0.09, 30, 2.0, and 2.2% for Val9, Val10, Val12, and pHM 175 43c P26 respectively. These values are comparable to those estimated for clonal populations of other RNA viruses such as bacteriophage Qβ (Domingo et al., 1978) or FMDV (Sobrino et al., 1983; Arias et al., 2001; review in Domingo et al., 2001). Thus, in these HAV populations the nonmutated class of genomes were a minority of the total.

Of the analyzed mutant spectra, only Val10 had a level of heterogeneity that could be influenced by misincorporations during RT-PCR amplification. Several arguments support the fact that the great majority of the mutations found, even in Val10, was present in the RNA populations under study and were not the result of misincorporations during the RT-PCR procedure employed. First, a basal error rate for the RT-PCR procedures was experimentally determined for the same VP3- and VP1-coding regions under study. This control experiment (detailed under Materials and methods) used recombinant clones to prepare VP3 and VP1 transcripts with T7 DNA-dependent RNA polymerase. The transcripts were then subjected to the same RT-PCR amplification with HAV RNA and 50 cDNA clones were sequenced. The final error rate attributable to the system is $7.9 \times 10^{-5}$ by $8.1 \times 10^{-5}$ substitutions per nucleotide for the VP3- and VP1-coding regions, respectively. Moreover, the mutations that occurred during the RNA synthesis and the RT-PCR amplification (three transitions and two transversions, three nonsynonymous and two synonymous mutations) did not show any of the mutational type bias or mutation clustering observed in the HAV populations. For obvious statistical reasons, the probability of mutation clustering as an RT-PCR artifact is negligible. The mutations in the mutant spectrum of Val10 were located in the vicinity of the sites where mutations for the other samples have been mapped. Therefore, the vast majority of mutations scored in the mutant spectra must have been present in the HAV RNA populations examined. Although it cannot be excluded that some mutation could have been generated during the in vitro amplification procedure, its exclusion would not significantly modify the quantifications and conclusions on mutant spectrum complexity of HAV. The narrower mutant spectrum in Val10 could not be the result of a limitation in the number of RNA template molecules in the sample, since in all cases a dilution of at least 1:100 of the preparation of the RNAs used as template produced a positive amplification, which excludes a bias in the sequence repertoire (Airaksinen et al., 2003). The narrower mutant spectrum of
this isolate could result from a shorter time from a clonal origin of the infection, a limited HAV replication, or higher sequence constraints than for the other isolates, among other possibilities (Domingo et al., 2001).

A complex mutant spectrum for HAV isolates poses a problem regarding the concept of strain for this virus. For hepatitis C virus, a genomic distance higher than 5%, in a genomic fragment encompassing the E2-NS2 junction, is required for two HCVs to be considered a different strain (Cabot et al., 2000), and different HAV and poliovirus genotypes are those with higher than 15% nucleotide sequence divergence in the VP1-2A-coding region (Rico-Hesse et al., 1987; Hollinger and Emerson, 2001). As more consensus sequences and data on mutant spectra of HAV become available, it may be necessary to redefine limits for strain and genotype classifications.

The mean ratio of transition to transversion mutations in the RNA of the analyzed populations was 8.6, probably reflecting the misincorporations tendencies of RNA dependent RNA polymerases (Domingo et al., 1978; Kuge et al., 1989; Schnerder and Roossinck, 2000). Also, the proportion of transitions tends to decrease with the divergence of the genes compared (Villanueva et al., 1983; Nei, 1987), supporting a recent occurrence of the mutations observed in these HAV samples. Examination of the sequence context in which mutations are found indicates that 25% of the mutations in clusters are located at the ends of short oligo (A) or oligo (U) stretches, suggesting a possible contribution of polymerase slippage in the generation of some of these mutations (Ripley, 1990; Arias et al., 2001). However not all homopolymeric tracts were associated with mutations only 2 of 14 (2/14) A3 tracts; 3/33 U3; 1/7 A4; 1/10 U4; 2/3 A5; 4/8 U5.

Several amino acid substitutions in components of the mutant spectra are located at or near recognized antigenic sites of HAV (Fig. 1). In particular in population pHM175 43c P26 passaged in cell culture in the absence of immune selection, substitutions at positions 65 (Pro → Ser), 83 (Pro → Ser), and 84 (Tyr → Asn) of VP3 are related to the immunodominant site of HAV (Nainan et al., 1992; Luo et al., 1988), and 115 (Leu → Phe) and 91 (Thr → Lys) affect a continuous epitope of VP3 (Bosch et al., 1998; M. Luo, personal communication). Antigenic variation in the absence of immune selection has been observed in several other viruses (reviewed in Domingo et al., 1993) and has been attributed to higher tolerance to replacements of surface residues which are relatively free of structural constraints (Domingo et al., 1993; Haydon and Woolhouse, 1998). Replacements at or in the neighborhood of antigenic sites were also found in the mutant spectra of Va19 [substitution Val → Ile at position 72 of VP3, which is involved in the epitope defined by the monoclonal antibody K34C8 (Sánchez et al., 2002)] or of Va12 [substitutions Ile → Val at position 72 of VP3, and Val → Leu at position 78 of VP3]. These replacements are in the vicinity of the immunodominant site (Luo et al., 1988).

One of the unusual features of HAV is the abundance of rare codons at some capsid sites, compatible with a modulating effect on HAV translation (Sánchez et al., 2003). The fact that the mutations observed tended to maintain a minimal frequency of rare codons suggests a constraint in the dynamics of mutation-selection to preserve the previously postulated balance between rate of translation and capsid protein folding (Sánchez et al., 2003). In HAV, this may be an important evolutionary constraint, additional to those generally accepted to limit evolutionary rates of RNA viruses despite high mutation rates (Simmonds and Smith, 1999).

A quasispecies dynamics for HAV may be of consequence for the natural history of this pathogen and its control in the human population. Pathogenic manifestations of HAV are quite variable (reviewed in Hollinger and Emerson, 2001). Patients may be asymptomatic despite active viral replication, but relapsing and fulminant forms of hepatitis, as well as extrahepatic manifestations such as encephalopathy, have been described. Although a strong host component undoubtedly must influence disease outcomes, a possible participation of virus variants to respond to host defense mechanisms or to favor replication in the face of physiological alterations cannot be excluded. In this context, it is remarkable that the pattern of mutations found in the analyzed VP3 and VP1 regions differed between the pHM175 and the clinical isolates, likely due to different selective pressures. The existence of variant reservoirs in HAV populations should also be taken into consideration in the design of preventive and therapeutic treatments, despite antigenic conservation of the virus.

Material and methods

Cells, viruses, and infections

The cytopathogenic pHM175 43c strain of HAV (courtesy of T. Cromeans, Centers for Disease Control, Atlanta, GA) was three times plaque-purified in FRhK-4 cells, as previously described (Cromeans et al., 1987), and a biological clone (pHM175 43c P0) was serially passaged 26 times in the same cell line, as previously described (Bosch et al., 1998) to yield population pHM175 43c P26.

Clinical samples

Three HAV strains were isolated from three different patients of an outbreak of acute hepatitis A associated with the consumption of coquina clams in Valencia, Spain, during autumn–winter 1999 (Sánchez et al., 2002). One virus strain (Va19) was isolated from 60 μl of serum, and two additional strains (Va110 and Va112) were isolated from 60 μl of extracted feces as previously described (Sánchez et al., 2002). All strains belonged to genotype IB and presented an overall nucleotide homology of the consensus sequences of
99.7% in a fragment of the 5′ NCR and 99.3% in the region encoding the VP1-2A junction (Sánchez et al., 2002), which indicates an epidemic relationship among them.

Molecular cloning and sequencing

RNA extracted from pHM175 43c P26 or from the clinical samples was retrotranscribed to a cDNA with the M-MLV reverse transcriptase (Promega), and the cDNA was copied and amplified by the thermostable Pwo pol from *Pyrococcus woesei*, which has proofreading activity (error rate of 3.2×10⁻⁶ substitutions/nucleotide) (Mullan et al., 2001). Two genomic regions coding for capsid proteins were amplified. A fragment of the VP3-coding region (nucleotides 1470 to 1839, corresponding to amino acids 1 to 123) and a fragment of the VP1-coding region (nucleotides 2459 to 2943, corresponding to amino acids 85 to 245), which include most of the epitopes so far described in HAV (Nainan et al., 1992; Ping and Lemon, 1992; Bosch et al., 1998). The primers used to copy and amplify the VP3-coding region are NH₂-VP3 (5′ TCTACCTGAAATTGATTTGG 3′) for the cDNA synthesis, and NH₂-VP3 and VP3-1431B (5′ CTTGGATCCCACCCTGATTTGTTTAGCCTAG 3′) for PCR amplification. The primers used to copy and amplify the VP1-coding regions are VP1-2965 (5′ TCTGTCAGACAGACAAAATAAACAC 3′) for the cDNA synthesis, and VP1–2965 and VP1–2428 (5′ GAGGGATCCGACATACATCAGATCATATGTC 3′) for PCR amplification. The synthesis of cDNAs was performed in a final volume of 25 µl containing 8 units of M-MLV RT, 0.2 mM of each nucleotide, 0.5 µM primer, 50 mM Tris–HCl, 75 mM KCl, 3 mM MgCl₂, and 10 mM DTT. Ten microliters of HAV RNA was denatured at 99°C for 5-min and incubated at 45°C for 1 h. DNA amplification was performed following the manufacturer’s specifications in a final volume of 50 µl containing 0.5 U of the Pwo pol, 0.2 mM of each nucleotide 0.5 µM of each primer, 10 mM Tris–HCl, 25 mM KCl, 5 mM (NH₄)₂SO₄, 2 mM MgSO₄, and 10 µl of the RT product, and using an annealing temperature of 50°C in both reactions. Since the DNA fragments produced by Pwo pol are blunt-ended, and primers VP3–1431B and VP1–2428 were designed to include a BamHI restriction site at their 5′ end, the amplification products were cloned into pGEM-3Zf(−). PCR products were digested with BamHI and the plasmid vector with both BamHI and HincII. Digested DNAs were purified with the High Pure PCR Product Purification Kit (Roche) following the directions of the manufacturer. DNA ligations were performed overnight at 16°C using T4 DNA ligase. Ligation products were transformed in *Escherichia coli* DH5α, and transformant clones were screened first by the standard white/blue β-galactosidase colorimetric reaction and then confirmed by colony hybridization with specific digoxigenin-labeled probes. Plasmid DNA from each clone was purified by using the Wizard Plus SV Minipreps Kit (Promega). Nucleotide sequencing was carried out in an ABI PRISM 377 automated DNA sequencer, with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and using vector-derived primers, as described elsewhere (Arias et al., 2001). All mutations were confirmed by sequencing both strands of DNA.

Error rate of the RT-PCR system

To ensure that the observed heterogeneity was due to the HAV polymerase and not to artificial misincorporations introduced by the RT or Pwo polymerases during the amplification procedure, a control experiment to determine the error rate of the system was carried out. One recombinant clone of the VP3 fragment and one recombinant clone of the VP1 fragment, obtained by copying RNA from pHM175 43c, were in vitro transcribed with the T7 polymerase. The RNA transcripts, diluted 1/1000, were subjected to the original RT-PCR procedure and the amplified products were subcloned. The sequence of 50 clones from each VP3- and VP1-coding region yielded an error rate for the amplification system of 1.1×10⁻⁴ and 1.7×10⁻⁴ mutations per nucleotide, respectively. Since the error rate of the T7 RNA polymerase is about 2.9×10⁻⁵ (Remington et al., 1998), the final error rate of the system was calculated to be 8.1×10⁻⁵ and 1.4×10⁻⁴ for the VP3- and VP1-coding regions, respectively. The ANOVA and the nonparametric Mann–Whitney U tests were employed to compare viral mutation frequencies and the system error rates, revealing statistical significance in all but one case (Val10). These data, together with the types and location of the mutations scored (described under Results and Discussion) indicate that the vast majority of mutations detected must have been present in the mutant spectra of the HAV samples analyzed.

Sequence analysis

The quasispecies complexity was analyzed by calculating the mutation frequencies and the Shannon entropy. Minimum and maximum mutation frequencies were determined as previously described (Arias et al., 2001). Normalized Shannon entropies were calculated following the formula $S_\text{e} = -\frac{1}{\ln N} \sum p_i \ln p_i$, where $p_i$ is the frequency of each sequence and $N$ is the total number of sequences in the spectrum of mutants (Airaksinen et al., 2003). $S_\text{e}$ ranges from 0 (no diversity) to 1 (maximum diversity). The codon usage table of HAV defined previously (Sánchez et al., 2003) was used in the analyses of codon abundances.

Acknowledgments

We acknowledge the technical expertise of the Serveis Científic-Tècnics of the University of Barcelona. Work in Barcelona was supported in part by Grants ERB3514PL973098, QLRT-1999-0634, and QLRT-1999-
References


Genome Variability and Capsid Structural Constraints of Hepatitis A Virus

Glòria Sánchez, Albert Bosch,* and Rosa M. Pintó

Grup Virus Entèrics, Department of Microbiology, University of Barcelona, 08028 Barcelona, Spain

Received 17 June 2002/Accepted 24 September 2002

The number of synonymous mutations per synonymous site ($K_s$), the number of nonsynonymous mutations per nonsynonymous site ($K_a$), and the codon usage statistic ($N_c$) were calculated for several hepatitis A virus (HAV) isolates. While $K_s$ was similar to those of poliovirus (PV) and foot-and-mouth disease virus (FMDV), $K_a$ was 1 order of magnitude lower. The $N_c$ parameter provides information on codon usage bias and decreases when bias increases. The $N_c$ value in HAV was about 38, while in PV and FMDV, it was about 53. The emergence of 22 rare codons in front of 8 in PV and 7 in FMDV was detected. Most of the conserved rare codons of the P1 region were strategically located at the carboxy borders of β barrels and α helices, their potential function being the assurance of proper folding of the capsid proteins through a decrease in the translation speed. This strategic location was not observed for amino acids encoded by the conserved rare codons of the 3D region. The percentage of bases with low pairing number values was higher in the latter region, suggesting a role of the conserved rare codons in the maintenance of RNA structure. Many of the rare codons in HAV are among the most frequent in humans, unlike in PV or in FMDV. This fact may be explained by the lack of cellular shutoff in HAV. One hypothesis is that HAV has evolved in order to avoid competition with its host for cellular tRNAs.

The high degree of conservation of the amino acid sequence of the capsid proteins of hepatitis A virus (HAV) correlates with a lack of antigenic diversity; thus, there is only a single serotype of human HAV. However, despite this limited amino acid heterogeneity, a significant degree of nucleic acid variability has been observed in diverse isolates from different regions of the world (3, 8, 23, 25). The molecular bases of this genetic variability may be the high error rate of the viral RNA-dependent RNA polymerase and the absence of proofreading mechanisms. Although no data exist on the error rate of the HAV polymerase, the mutation frequencies for a variety of different RNA viruses range from $10^{-4}$ to $10^{-5}$ substitution per base per round of copying (9). The reason why this nucleic acid heterogeneity does not correspond to amino acid heterogeneity should rely on the lack of nonsynonymous mutations, possibly due to their elimination by negative selection. However, the actual mode of transmission of very small HAV populations, frequently associated with contaminated foods, may lead to the accumulation of debilitating mutations (7). In this context, the strikingly low level of amino acid changes in the capsid region suggests strong structural constraints.

In the present work, we undertook an analysis of the nucleotide and amino acid changes in sequences representing the available strains from GenBank and isolates from a food-borne hepatitis A outbreak. Since HAV structural data exist only for the 3C protein (4), structural models for the VP2, VP3, VP1, and 3D proteins have been deduced from actual data for the structural proteins of poliovirus (13) and foot-and-mouth disease virus (1) and from the actual 3D polymerase of poliovirus (12).

MATERIALS AND METHODS

Viruses. The 15 complete HAV sequences available at GenBank were used throughout this study. These sequences represent a group of geographically and temporally diverse HAV strains (Table 1). Additionally, 18 strains were isolated from patients in an outbreak of acute hepatitis A associated with the consumption of coquina clams (24). Virus RNA was isolated from 60-μl serum samples by guanidine thiocyanate treatment as specified elsewhere (5, 24).

RT-PCR amplification and nucleotide sequencing. The complete P1-2A sequence of the HAV isolates was obtained after their amplification with the Pwo reverse transcription (RT)-PCR system (Roche) by following the manufacturer’s specifications and with primers corresponding to the capsid protein genomic regions (Table 2). Sequencing of RT-PCR products in both directions was performed with a Thermo Sequenase II dye terminator cycle sequencing kit (Amersham Pharmacia Biotech) by following the manufacturer’s instructions and with an ABI Prism 377 automated DNA sequencer (Perkin-Elmer).

Analysis of nucleotide and amino acid sequences. Alignment of multiple sequences was carried out with the ClustalW program (European Bioinformatics Institute). The number of synonymous mutations per synonymous site ($K_s$) and the number of nonsynonymous mutations per nonsynonymous site ($K_a$) were calculated by the Nei-Gogobori method (19) with the DnaSP program (http://www.ub.es/dnaop/) (University of Barcelona).

To create codon usage tables for HAV, poliovirus serotype 1 (PV-1), and foot-and-mouth disease virus serotype C (FMDV-C), the Cusp program (European Molecular Biology Open Software Suite) was used. A rare codon was defined as one whose frequency was less than 30% that of its most abundant synonym in each of the codon usage tables (11). The effective codon usage statistic ($N_c$) measures the codon bias (26). The $N_c$ value is always between 20 (when only one codon is effectively used for each amino acid) and 61 (when codons are used randomly). The $N_c$ value was calculated with the Chips program (European Molecular Biology Open Software Suite).

For the rare codon location study, protein secondary structure wire plot models of VP2, VP3, and VP1 of HAV were calculated from a picornavirus alignment (16) in which actual structural data for the Mahoney strain of PV-1 (http://www.biochem.ucl.ac.uk/bsm/pdbsum/2plv/main.html) were added and aligned. The three-dimensional model for the HAV protease was also deduced by Luo et al. (16; M. Luo, personal communication) from this alignment. To statistically confirm the locations or distributions of rare codons, a χ² analysis of frequencies was undertaken. The different proteins were divided into two regions: (i) the carboxy ends and borders of the highly structured elements (β

* Corresponding author. Mailing address: Department of Microbiology, School of Biology, University of Barcelona, Av. Diagonal 645, 08028 Barcelona, Spain. Phone: (34) 934034620, Fax: (34) 934034629. E-mail: abosch@ub.edu.
TABLE 1. Complete HAV sequences available at GenBank and used in the present study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Geographical location</th>
<th>Date of isolation</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA</td>
<td>IA</td>
<td>United States</td>
<td>1975</td>
<td>K02990</td>
</tr>
<tr>
<td>HAS-15</td>
<td>IA</td>
<td>United States</td>
<td>1979</td>
<td>X15464</td>
</tr>
<tr>
<td>AH2</td>
<td>IA</td>
<td>Japan</td>
<td>1991</td>
<td>AB020565</td>
</tr>
<tr>
<td>AH1</td>
<td>IA</td>
<td>Japan</td>
<td>1992</td>
<td>AB020564</td>
</tr>
<tr>
<td>FH1</td>
<td>IA</td>
<td>Japan</td>
<td>1992</td>
<td>AB020565</td>
</tr>
<tr>
<td>AH3</td>
<td>IA</td>
<td>Japan</td>
<td>1993</td>
<td>AB020566</td>
</tr>
<tr>
<td>FH2</td>
<td>IA</td>
<td>Japan</td>
<td>1993</td>
<td>AB020568</td>
</tr>
<tr>
<td>FH3</td>
<td>IA</td>
<td>Japan</td>
<td>1994</td>
<td>AB020569</td>
</tr>
<tr>
<td>GBM</td>
<td>IA</td>
<td>Germany</td>
<td>1976</td>
<td>X75214</td>
</tr>
<tr>
<td>FG</td>
<td>IA</td>
<td>Italy</td>
<td>1988</td>
<td>X83302</td>
</tr>
<tr>
<td>MBB</td>
<td>IB</td>
<td>Northern Africa</td>
<td>1978</td>
<td>M02073</td>
</tr>
<tr>
<td>HM-175</td>
<td>IB</td>
<td>Australia</td>
<td>1976</td>
<td>M14707</td>
</tr>
<tr>
<td>HAF-203</td>
<td>IB</td>
<td>Brazil</td>
<td>1992</td>
<td>AF268396</td>
</tr>
<tr>
<td>L-A-1</td>
<td>IB</td>
<td>Brazil</td>
<td>1992</td>
<td>AF314208</td>
</tr>
<tr>
<td>SLF88</td>
<td>VII</td>
<td>Sierra Leone</td>
<td>1988</td>
<td>AY032861</td>
</tr>
</tbody>
</table>

TABLE 2. Primers and conditions used for RT-PCR amplification and sequencing of HAV

<table>
<thead>
<tr>
<th>Target region</th>
<th>Sequence</th>
<th>Genomic position</th>
<th>[Mg2+] (mM)</th>
<th>Hybridization temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP0 NH2</td>
<td>CAGCTGGACTTGTCTTTTGGG</td>
<td>668-667</td>
<td>2.0</td>
<td>55</td>
</tr>
<tr>
<td>VP0 NH2</td>
<td>TACACCAGAAACATAGCACAG</td>
<td>1198-1178</td>
<td>2.0</td>
<td>55</td>
</tr>
<tr>
<td>VP0 COOH</td>
<td>TACAAGCAGCTTGTGCAG</td>
<td>1065-1084</td>
<td>2.0</td>
<td>55</td>
</tr>
<tr>
<td>VP0 NH2</td>
<td>GCTTGTCATCCATTTCTTATGG</td>
<td>1543-1552</td>
<td>2.0</td>
<td>55</td>
</tr>
<tr>
<td>VP3 NH2</td>
<td>GGAGAACTACTCCATTTATAC</td>
<td>1380-1402</td>
<td>2.0</td>
<td>50</td>
</tr>
<tr>
<td>VP3 NH2</td>
<td>TCTACCTGAAAGTATATTTG</td>
<td>1859-1840</td>
<td>2.0</td>
<td>50</td>
</tr>
<tr>
<td>Inner VP3</td>
<td>GCTTTGACAGGACCACTATTG</td>
<td>1701-1722</td>
<td>2.0</td>
<td>50</td>
</tr>
<tr>
<td>Inner VP3</td>
<td>TCAGTGATCCATCTGACTTAT</td>
<td>2031-2010</td>
<td>2.0</td>
<td>50</td>
</tr>
<tr>
<td>VP3 COOH</td>
<td>GTGTGATGATGATTGATGATTG</td>
<td>2207-2267</td>
<td>2.0</td>
<td>50</td>
</tr>
<tr>
<td>VP1 NH2</td>
<td>AATGTGTCTTCTTCTTCCAGAT</td>
<td>2136-2155</td>
<td>2.0</td>
<td>53</td>
</tr>
<tr>
<td>VP1 NH2</td>
<td>ACAGCCTCAAGAGCAATG</td>
<td>2751-2770</td>
<td>2.0</td>
<td>53</td>
</tr>
<tr>
<td>VP1 COOH</td>
<td>ATGGGCTGTTGTTACTGCAAG</td>
<td>2673-2691</td>
<td>2.5</td>
<td>45</td>
</tr>
<tr>
<td>VP1 COOH</td>
<td>CCCTTCTTTTACTCAGG</td>
<td>3229-3213</td>
<td>2.5</td>
<td>45</td>
</tr>
</tbody>
</table>

a In wild-type HM-175 (GenBank accession no. M14707).

TABLE 3. Analysis of mutations in HAV sequences

<table>
<thead>
<tr>
<th>Genomic region (nucleotides)</th>
<th>Ka</th>
<th>Ks</th>
<th>Ks/Ka</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP0 (735–1469)</td>
<td>0.30806</td>
<td>0.00037</td>
<td>102.90</td>
</tr>
<tr>
<td>VP1 (1470–2207)</td>
<td>0.03832</td>
<td>0.00089</td>
<td>43.00</td>
</tr>
<tr>
<td>VP1-2A (1208–3119)</td>
<td>0.05608</td>
<td>0.00015</td>
<td>373.90</td>
</tr>
<tr>
<td>P1-2A (735–1391)</td>
<td>0.08776</td>
<td>0.00044</td>
<td>190.30</td>
</tr>
<tr>
<td>VP0 (735–1469)</td>
<td>0.28205</td>
<td>0.00499</td>
<td>56.52</td>
</tr>
<tr>
<td>VP1 (1470–2207)</td>
<td>0.29861</td>
<td>0.00272</td>
<td>109.80</td>
</tr>
<tr>
<td>VP1 (2208–3026)</td>
<td>0.29051</td>
<td>0.00244</td>
<td>119.06</td>
</tr>
<tr>
<td>P1 (139–3026)</td>
<td>0.29304</td>
<td>0.00336</td>
<td>86.40</td>
</tr>
<tr>
<td>2A (3027–3242)</td>
<td>0.30325</td>
<td>0.01296</td>
<td>23.40</td>
</tr>
<tr>
<td>2B (3243–3995)</td>
<td>0.32080</td>
<td>0.01009</td>
<td>31.80</td>
</tr>
<tr>
<td>2C (3996–5000)</td>
<td>0.35559</td>
<td>0.01329</td>
<td>26.75</td>
</tr>
<tr>
<td>3A (5001–5222)</td>
<td>0.24410</td>
<td>0.01291</td>
<td>18.90</td>
</tr>
<tr>
<td>3B (5222–5291)</td>
<td>0.34555</td>
<td>0.00508</td>
<td>68.02</td>
</tr>
<tr>
<td>3C (5922–5948)</td>
<td>0.27352</td>
<td>0.00472</td>
<td>57.95</td>
</tr>
<tr>
<td>3D (5949–7415)</td>
<td>0.27100</td>
<td>0.01097</td>
<td>24.70</td>
</tr>
<tr>
<td>FMDV-C VP1</td>
<td>0.29</td>
<td>0.03</td>
<td>9.70</td>
</tr>
<tr>
<td>FMDV-C P1</td>
<td>0.28</td>
<td>0.02</td>
<td>14.00</td>
</tr>
<tr>
<td>VP-1 VP1 (d)</td>
<td>2.50/18.40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Hepatitis A outbreak sequence.

b HAV sequence from GenBank.

c From Martinez et al. (17) (accession numbers M84360, M90055, and M90367 to M90382 for VP1 and M90367, M90368, M90372, M90376, M90381, and M90382 for P1). (accession numbers AF238098, AF233099, AF233110, and AF233113.)

d From Gasvain et al. (11).
TABLE 4. Percentages of codons with regard to the most abundant synonym in HAV and human cell codon usage tables

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Codon*</th>
<th>Anticodons</th>
<th>% Occurrence in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GenBank</td>
</tr>
<tr>
<td>Arg AGA</td>
<td>UCU, UCI</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>AGG</td>
<td>UCC, UCU</td>
<td>29.2</td>
<td>52.6</td>
</tr>
<tr>
<td>CGC</td>
<td>GGC, GCI</td>
<td>2.7</td>
<td>0.0</td>
</tr>
<tr>
<td>CGU</td>
<td>GCA, GCG, GCI</td>
<td>3.5</td>
<td>4.5</td>
</tr>
<tr>
<td>CGA</td>
<td>GCC, GCI</td>
<td>3.2</td>
<td>0.0</td>
</tr>
<tr>
<td>CGG</td>
<td>GCC, GCU</td>
<td>0.6</td>
<td>0.0</td>
</tr>
<tr>
<td>Leu UUG</td>
<td>AAC, AAU</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>UUA</td>
<td>AAG, AAI</td>
<td>52.0</td>
<td>58.4</td>
</tr>
<tr>
<td>CUU</td>
<td>GAA, GAG, GAI</td>
<td>46.0</td>
<td>32.8</td>
</tr>
<tr>
<td>CUG</td>
<td>GAC, GAU</td>
<td>25.3</td>
<td>35.0</td>
</tr>
<tr>
<td>CUA</td>
<td>GAU, GAI</td>
<td>9.2</td>
<td>4.6</td>
</tr>
<tr>
<td>CUC</td>
<td>GAG, GAI</td>
<td>7.0</td>
<td>10.4</td>
</tr>
<tr>
<td>Ser UCU</td>
<td>AGA, AGG, AGI</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>AGU</td>
<td>UCA, UCG, UCI</td>
<td>31.8</td>
<td>18.2</td>
</tr>
<tr>
<td>UCC</td>
<td>AGG, AGI</td>
<td>27.9</td>
<td>45.3</td>
</tr>
<tr>
<td>UCG</td>
<td>AGC, AGU</td>
<td>4.7</td>
<td>11.3</td>
</tr>
<tr>
<td>AGC</td>
<td>UCG, UCI</td>
<td>4.7</td>
<td>3.6</td>
</tr>
<tr>
<td>Thr ACU</td>
<td>UGA, UGG, UGI</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>ACA</td>
<td>UGU, UGI</td>
<td>90.8</td>
<td>95.7</td>
</tr>
<tr>
<td>ACC</td>
<td>UGG, UGI</td>
<td>18.0</td>
<td>21.2</td>
</tr>
<tr>
<td>ACG</td>
<td>UGC, UGU</td>
<td>4.5</td>
<td>6.7</td>
</tr>
<tr>
<td>Pro CUC</td>
<td>GGA, GGG, GGI</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>CCA</td>
<td>GGU, GGI</td>
<td>89.5</td>
<td>74.4</td>
</tr>
<tr>
<td>CCG</td>
<td>GGG, GGI</td>
<td>20.0</td>
<td>21.0</td>
</tr>
<tr>
<td>CGG</td>
<td>GCC, GGU</td>
<td>1.8</td>
<td>0.0</td>
</tr>
<tr>
<td>Ala GCU</td>
<td>CGA, CGG, CGI</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>GCA</td>
<td>CGU, CGI</td>
<td>60.7</td>
<td>73.2</td>
</tr>
<tr>
<td>GCC</td>
<td>CGG, CCG</td>
<td>30.7</td>
<td>35.0</td>
</tr>
<tr>
<td>GCG</td>
<td>CGC, CGU</td>
<td>1.4</td>
<td>4.8</td>
</tr>
<tr>
<td>Gly GGA</td>
<td>CUC, CCI</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>GGU</td>
<td>CCA, CGG, CCI</td>
<td>58.4</td>
<td>79.1</td>
</tr>
<tr>
<td>GGG</td>
<td>CCC, CCI</td>
<td>34.4</td>
<td>42.7</td>
</tr>
<tr>
<td>GGC</td>
<td>CCC, CCI</td>
<td>28.1</td>
<td>39.1</td>
</tr>
<tr>
<td>Val GUU</td>
<td>CAA, CAG</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>GUG</td>
<td>CAC, CAU</td>
<td>46.3</td>
<td>39.5</td>
</tr>
<tr>
<td>GUA</td>
<td>CAU, CAI</td>
<td>17.7</td>
<td>8.0</td>
</tr>
<tr>
<td>GUC</td>
<td>CAG, CAI</td>
<td>12.8</td>
<td>9.2</td>
</tr>
<tr>
<td>Lys AAA</td>
<td>UUU, UUI</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>AAG</td>
<td>UUC, UUI</td>
<td>58.8</td>
<td>56.5</td>
</tr>
<tr>
<td>Asn AUA</td>
<td>UUA, UUG, UUI</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>AAC</td>
<td>UUG, UUI</td>
<td>19.1</td>
<td>29.0</td>
</tr>
<tr>
<td>Gin CAA</td>
<td>GUU, GUI</td>
<td>88.0</td>
<td>100.0</td>
</tr>
<tr>
<td>CAG</td>
<td>GUC, GUU</td>
<td>100.0</td>
<td>91.6</td>
</tr>
<tr>
<td>His CAU</td>
<td>GUA, GUG, GUI</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>CAC</td>
<td>GUG, GUI</td>
<td>22.1</td>
<td>17.4</td>
</tr>
<tr>
<td>Glu GAA</td>
<td>CUI, CUI</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>GAG</td>
<td>CUC, CUI</td>
<td>76.7</td>
<td>92.2</td>
</tr>
<tr>
<td>Asp GAU</td>
<td>CUA, CUG, CUI</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>GAC</td>
<td>CUG, CUI</td>
<td>19.2</td>
<td>17.0</td>
</tr>
<tr>
<td>Tyr UAU</td>
<td>AUA, AUG, AUI</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>UAC</td>
<td>AUG, AUI</td>
<td>26.5</td>
<td>18.0</td>
</tr>
<tr>
<td>Cys UGU</td>
<td>ACA, ACG, ACI</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>UGC</td>
<td>AGC, ACI</td>
<td>26.5</td>
<td>12.0</td>
</tr>
<tr>
<td>Phc UUU</td>
<td>AAA, AAG, AA1</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>UUC</td>
<td>AAG, AA1</td>
<td>27.0</td>
<td>25.0</td>
</tr>
<tr>
<td>Ile AUU</td>
<td>UUA, UAG, UAI</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>AUA</td>
<td>UAU, UAI</td>
<td>31.7</td>
<td>29.0</td>
</tr>
<tr>
<td>AUC</td>
<td>UAG, UAI</td>
<td>15.9</td>
<td>12.0</td>
</tr>
</tbody>
</table>

* Codons shown in bold type were rare in both sets of HAV sequences and were considered in the location study.

the higher divergence of the GenBank sequences than of the cluster of outbreak sequences. The $K_s/K_a$ ratios for these outbreak sequences were extremely high for the VP0 and VP1-2A regions and considerably high for the VP3 region. The lower ratio observed for the VP3 region reflects the relatively abundant (6 of 18 sequences) occurrence of the antigenic variant described above. For the GenBank sequences, this ratio was also very high for all of the analyzed genomic regions. When the complete capsid regions (P1 regions) of HAV and FMDV-C were compared, it was found that while the $K_s$ values were similar in both viruses, the $K_a$ value was 1 order of magnitude higher in FMDV-C. Consequently, the $K_s/K_a$ ratio was significantly higher in HAV (Table 3). A similar conclusion was drawn after a comparison of the values for the P1 regions of HAV, FMDV-C, and PV-1 (Table 3).

Conod usage. Since synonymous mutations are the most prevalent in HAV and the occurrence of such mutations is subject to the influence of codon usage, an analysis of this usage in HAV was undertaken. The complete coding genome was studied for the GenBank sequences. Fifteen out of the 18 amino acid families containing synonymous codons showed the use of rare codons (Table 4). Overall, 25 rare codons were detected. Similarly, analysis of the codon usage in the P1-2A region of the sequences from the outbreak revealed the existence of 20 rare codons in 14 out of the 18 amino acid families (Table 4). Eighteen out of these 20 rare codons were common with those of the GenBank sequences, and 4 more (Arg: CGC; Arg: CGA; Arg: CGG; Pro: CCG) were not found in the outbreak sequences. However, three (Arg: CGC; Arg: CGG; Pro: CCG) out of these four codons were not detected in the capsid region of the GenBank sequences. Two codons that were rare in the outbreak sequences were not rare in the GenBank group (Ser: AGU; Ile: AUA), while three codons that were rare in the GenBank sequences were not rare in the outbreak group (Arg: AGG; Ser: UCC; Leu: CUG).

No significant differences could be detected in the frequencies of rare codons between the capsid region and the nonstructural region of the genome among the GenBank sequences. These frequencies, defined as the number of rare codons versus the total number of codons, were 9.1% in the former genomic region and of 8.2% in the second. However, as mentioned above, some rare codons were absent from the capsid region. The overall heterogeneity could be expressed as the effective $N_e$ values, which were 39 for the total coding sequence, 38.8 for the capsid region, and 38.7 for the nonstructural region. The $N_e$ value for the capsid region in the outbreak sequences was 38.9. These values indicate that there was no significant difference in the codon usage bias among the closely related outbreak sequences and the GenBank sequences. The $N_e$ values were also calculated for two other picornaviruses, PV-1 and FMDV-C (Table 5). For both of these viruses, markedly higher $N_e$ values were obtained (52.6 and 52.1, respectively, compared to 37.2 for VP1 of HAV; 53.3 and 38.8 for the entire P1 regions of FMDV-C and HAV, respectively), indicating that HAV has a much higher bias in codon usage. This fact was further confirmed by the number of amino acid families containing rare codons or by the clearly higher total number of rare codons in HAV than in the other viruses (Table 5).

Additionally, another important and surprising difference among HAV and the other picornaviruses (PV-1 or FMDV-C)
TABLE 5. Codon usage for three picornaviruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Effective N, for:</th>
<th>No. of amino acid families with rare codons</th>
<th>Total no. of rare codons</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAV</td>
<td>VP1 38.1, P1 38.8</td>
<td>14</td>
<td>22</td>
</tr>
<tr>
<td>PV-1</td>
<td>52.6</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>FMDV-C</td>
<td>52.1 53.3</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

*a The number of amino acid families containing synonymous codons was 18.
*b The number of synonymous codons was 59.
*c Data were calculated with sequences from Gavrilin et al. (11) (accession numbers AF283098, AF233109, and AF233110).
*d Data were calculated with sequences from Martinez et al. (17) (accession numbers M84360, M90055, and M90367 to M90382 for VP1 and M90367, M90368, M90372, M90376, M90381, and L290061 for P1).

could be observed. While the codon usage of PV-1 and FMDV-C was mostly coincident with that of their hosts, the HAV codon usage was quite antagonistic to that of human cells. The codons most abundantly used for Arg, Ser, Thr, Pro, Asn, His, Asp, Tyr, Cys, Phe, and Ile by human cells were rare codons for HAV. Leu, Ala, Gly, Val, and Lys codons were not as rare as the codons just listed but clearly were less abundant. Only 5 out of the 22 HAV rare codons were also rare in human cells (UCG, CUA, ACG, GCG, and GUU). Consequently, the most abundant HAV codons were not the most abundant human codons. Only Gln and Glu were mostly encoded by the same triplets. A closer analysis of this situation reveals that for most twofold degenerate amino acids, namely, Asn, Hys, Asp, Tyr, Cys, and Phe, one codon may bind a different anticodon, and the other codon may bind the same two tRNAs and a third tRNA with a third and different anticodon; human cells more frequently use the first of these codons, while HAV clearly uses the second, which contains the unshared anticodon (Table 4). For the three remaining twofold degenerate amino acids, Lys, Gln, and Glu, each codon may bind two tRNAs and only one anticodon is shared; no clear codon preference is shown by HAV. For the threefold degenerate Ile, the most abundant human codon may bind two tRNAs, whose anticodons are shared with the two alternative codons; the second most abundant codon may bind three anticodons (two shared and one unshared); and the least abundant codon may bind two anticodons (one shared and one unshared). In this situation, HAV chooses as the most abundant codon a codon that has one unshared anticodon and that is not rare for the host cell. The same strategy is observed for most of the four- and sixfold degenerate amino acids, although in some, such as Val and Leu, HAV selects a rare human codon as its most abundant, since the alternative codons with an unshared anticodon correspond to the most abundant human codons, thus representing strong competition.

Locations of rare codons. The locations of the rare codons in the GenBank sequences were studied, although codons considered rare were only those that were present and rare in both the GenBank and the hepatitis outbreak sequences (Table 4). Only rare codons conserved in more than 50% of the analyzed sequences were considered significant in the location study. Overall, 60 rare codons out of 764 total codons were conserved in the P1 region, representing 7.8% conserved rare codons. When the stringency in conservation was increased to either 85 or 100%, 2.7 or 0.9%, respectively, of the total P1 codons were rare codons. It should be noted that 7 out of these 60 rare codons (11.6%) were conserved in the entire group of sequences. In some instances, sequences lacking an individual rare codon had an alternative rare codon in close proximity (distance of one to three codons), increasing the percent conservation. Accordingly, 26 highly conserved positions that should be very critical were recognized in the structural genome.

A tendency to be located within the carboxy limits of the highly structured elements (β barrels and α helices) was observed (Fig. 1). Overall, 52.7% of the rare codons of the capsid region were located in the carboxy limits of the β barrels and α helices, while the residues contained in these limits represented 37.6% of the total polyprotein. This tendency to be located within the carboxy limits was statistically significant (P < 0.05). These same determinations were calculated for the P1 region of FMDV-C and for the VP1 region of PV-1. A significant nonrandom location could not be detected in either FMDV-C or PV-1, although in both viruses a preference for the carboxy limits was also observed.

At certain positions, the conserved rare codons were clustered (Fig. 1). A cluster was defined as a group of at least two contiguous rare codons or a group of at least two rare codons at a distance of one to three codons. In the VP0 region, three clear clusters were observed. The first was located in the VP4 region, for which no structural data exist. The second was located right at the carboxy border of the βD barrel, and the third extended all along the short joining sequence between the βE and the βG1 barrels. In the VP3 region, one cluster was detected starting just before the βG2 barrel and finishing just after this structure. In the VP1 region, one cluster was located at the amino terminus of the protein, and two contiguous clusters were located at the end of the βE barrel and at the joining sequence between the βE barrel and the α3 helix. The outbreak sequences were not included in this analysis to avoid the potential bias due to their close relationships; however, they were used to confirm the existence of critical positions (Fig. 1). Eighty-one percent of the highly conserved rare codon locations detected in the GenBank sequences were also detected as highly conserved in the outbreak sequences. A high degree of correlation was also observed among the rare codon clusters, with the exception of those of VP1. However, since these clusters were not highly conserved in the GenBank sequences, they should not be regarded as actual clusters.

All of the amino acids encoded by these clusters were located in exposed regions of the capsid (Fig. 2), and these clusters included very rare codons. The frequencies of these very rare codons were below 20% that of the most common codon of their families or even below 5% in some instances, such as that of the βG2 cluster of VP3. It should be noted that not only the clusters but also several single rare codons encoded residues located on the surface. Overall, 67% of the total rare codons encoded residues exposed on the capsid surface, more precisely, 81, 72, and 64% of the VP2, VP3, and VP1 rare codons, respectively. On the other hand, 60% of these rare codons were highly conserved in at least 85% of the GenBank sequences.

The occurrences and locations of rare codons in the 3C and 3D regions of the GenBank sequences of HAV were comparatively analyzed. The 3C coding region contained 1.82, 1.37,
FIG. 1. VP2, VP3, and VP1 structural wire plot models deduced from actual data for PV-1 (Mahoney). (A) VP2. (B) VP3. (C) VP1. The first and second rows correspond to the outbreak and GenBank consensus sequences, respectively. Bold type indicates amino acids encoded by conserved rare codons.
and 0.91% rare codons conserved in 50, 85, and 100% of the sequences, respectively. For the 3D region, 6.7% of the total codons were rare codons conserved in 50% of the sequences, 2.86% of the total codons were rare codons conserved in 85% of the sequences, and 1.84% of the total codons were rare codons conserved in 100% of the sequences. While the patterns of conservation of the rare codons were completely different in the 3C and P1 regions, that of the 3D region did not differ significantly from that of the P1 region. However, the strategic location of the HAV P1 region rare codons contrasted with the data obtained for the 3D region, whose rare codons were randomly distributed, instead of being accumulated at the carboxy limits. Overall, the carboxy limits represented 30.5% of the 3D protein, and 33.3% of the rare codons were located at these limits. Thus, it could be concluded that no clear preference for the carboxy limit location exists in the 3D polymerase. For the 3C protein, the statistical analysis was hampered by the low number of rare codons.

**RNA secondary structure.** Although the dynamic nature of the RNA genome avoids an accurate prediction of its secondary structure, P-Num values provide a quantitative estimation of the propensity of a base to pair with alternative partners in a collection of suboptimal folds (20). RNA regions having abundant bases with low P-Num values (P-Num, <100) are predicted to contain secondary structures (20). This parameter was calculated either for the total genome of the HM-175 strain of HAV or for partial RNA regions. Although the percentage of bases with P-Num values of <100 was 24.15%, a distinct pattern was observed among the different genomic regions (Table 6). Remarkably, significantly higher percentages of bases with low P-Num values were observed in the P3 region than in the P1 region and even than in the noncoding regions, suggesting a tighter structure in the P3 region. The ratios between the P-Num values of different regions were calculated for HAV and PV-1. The P3 region/P1 region ratios were 2.3 and 1.7, respectively, the P3 region/5′ noncoding region ratios were 1.6 and 1.7, respectively, and the P1 region/5′ noncoding region ratios were 0.7 and 1, respectively. These ratios suggested that the RNA of the HAV P1 region had a comparatively lower P-Num value and correspondingly a relatively looser structure.

**DISCUSSION**

HAV has low antigenic variability, as reflected by the existence of a single serotype (14). However, antigenic variants have been selected for their resistance to different MAbs (18, 21). Among the group of isolates from the clam-associated outbreak, a natural antigenic variant has been detected which induces a loss of recognition by MAb K34C8 and a second variation in a linear epitope of VP1 (24). However, the frequency of nonsynonymous mutations observed in HAV is sig-
TABLE 6. Effective \( N_e \) values, percentages of rare codons conserved in at least half of the GenBank sequences of HAV, and P-Num values for the HM-175 strain of HAV in association with different genomic regions

| Genomic region | \( N_e \) | % of: | Conserved rare codons | Bases with P-Num values of <100
|
|----------------|---------|--------|----------------------|-----------------------------|
| Whole genome   | 6.55    | 24.1   |                      |                             |
| 5' Noncoding region | 38.8   | 23.4 (7.1) | 7.85               | 16.1 (7.1)                 |
| P1             | 3.7     | 9.2    | 8.16                |                             |
| VP0            | 38.3    | 20.9   | 7.32                |                             |
| VP3            | 38.1    | 18.1   | 8.05                |                             |
| VP1            | 39.0    | 18.4 (8.2) | 6.37               |                             |
| P2             | 35.8    | 6.5    | 5.55                |                             |
| 2A             | 34.3    | 27.0   | 5.55                |                             |
| 2B             | 39.6    | 14.5   | 7.16                |                             |
| 2C             | 37.9    | 12.5   | 5.46                |                             |
| P3             | 33.6    | 68.5   | 9.46                |                             |
| 3A             | 29.1    | 94.2   | 4.35                |                             |
| 3B             | 34.8    | 33.9   | 1.82                |                             |
| 3C             | 37.1    | 30.9   | 6.74                |                             |
| 3D             | 11.1 (94.2) |            | 64% of the P1 region and at the RNA level in the 3D region. However, by using the P-Num value of the HM-175 strain as a reference for HAV, it was observed that for the 3D region, the RNA secondary structure is tighter; consequently, its sequence should be less prone to variability, as has been suggested for other viruses (11). Thirty percent and 64% of the P1 region and 3D region rare codons, respectively, were immersed in RNA regions with low P-Num values (data not shown). Consequently, it can be considered that these rare codons play a dual role in maintaining both the RNA and the protein structures, being more important at the protein level in the P1 region and at the RNA level in the 3D region.

The occurrence of surface residues encoded by rare codons, which account for approximately 15% of the total surface residues (Fig. 2 and data not shown), could contribute to the low variability of the HAV capsid, since it is quite unlikely that the occurrence of a nucleotide substitution in a rare codon will give rise to a new codon of similar rarity (Table 4), in order to maintain the translation kinetics for correct folding without a loss of efficiency. In fact, among the previously mentioned capsid substitutions, only that at position 25 of VP1 (Ile to Met) changed from quasi-rare to non-rare and that at position 271 of VP1 (Ser to Pro) changed from quasi-rare to unmistakably rare. All of the other substitutions affected non-rare codons.

An intriguing issue, however, is the antagonism in the codon usage of HAV and human cells, since the availability of tRNAs is host dependent. The picornavirus models used throughout this study showed codon usage very similar to that of their hosts. However, this situation implies the occurrence of competition for tRNAs, among other factors. For PV-1 and FMDV-C, this competition is avoided by the induction of cellular shutoff of protein synthesis through carboxy cleavage of component eIF4G of the translation initiation complex by 2A and L proteases, respectively (22). The cleaved eIF4G factor is still active for the internal ribosome entry site-dependent initiation of translation of most picornaviruses, although that of HAV requires an intact eIF4G factor (6). The latter is a plausible explanation for why shutoff has not been described for HAV-infected cells. Consequently, HAV competes poorly for cellular factors, among them tRNAs; therefore, the most abun-
dant codons of its host are not its most abundant and, in several instances, even are rare codons.

The lack of a specific shutoff-inducing mechanism and the occurrence of long extracapsid periods are concordant with a special codon usage which prevents direct competition with the host cell system and concomitantly allows a highly compact capsid that ensures a high level of environmental persistence.

ACKNOWLEDGMENTS

We acknowledge the skillful assistance of Àngels Rabassó and the technical expertise of the Serveis Científico-Tècnics of the University of Barcelona.

This study was supported in part by grants ERB3514PL973098, QLRT-1999-0634, and QLRT-1999-0594 from the European Union; BIO99-0455 from the CICYT, Ministry of Science and Technology, Spain; and 1997SGR 00224 from the Generalitat de Catalunya.

REFERENCES

Evolution of Human Calicivirus RNA In Vivo: Accumulation of Mutations in the Protruding P2 Domain of the Capsid Leads to Structural Changes and Possibly a New Phenotype

Mikael Nilsson, Kjell-Olof Hedlund, Margareta Thorhagen, Göran Larson, Kari Johansen, Anders Ekspong, and Lennart Svensson

Department of Virology and Centre for Microbiological Preparedness, Swedish Institute for Infectious Disease Control, Solna, Department of Clinical Chemistry and Transfusion Medicine, Sahlgrenska University Hospital, 413 45 Göteborg, Department of Medicine, Sundsvall County Hospital, 851 86 Sundsvall, and Department of Molecular Virology, University of Linköping, 581 85 Linköping, Sweden

Received 3 June 2003/Accepted 5 September 2003

In the present study we report on evolution of calicivirus RNA from a patient with chronic diarrhea (i.e., lasting >2 years) and viral shedding. Partial sequencing of open reading frame 1 (ORF1) from 12 consecutive isolates revealed shedding of a genogroup II virus with relatively few nucleotide changes during a 1-year period. The entire capsid gene (ORF2) was also sequenced from the same isolates and found to contain 1,647 nucleotides encoding a protein of 548 amino acids with similarities to the Arg320 and Mx strains. Comparative sequence analysis of ORF2 revealed 32 amino acid changes during the year. It was notable that the vast majority of the cumulative amino acid changes (8 of 11) appeared within residues 279 to 405 located within the hypervariable domain (P2) of the capsid protein and hence were subject to immune pressure. An interesting and novel observation was that the accumulated amino acid changes in the P2 domain resulted in predicted structural changes, including disappearance of a helix structure, and thus a possible emergence of a new phenotype. FUT2 gene polymorphism characterization revealed that the patient is heterozygous at nucleotide 428 and thus Secretor+, a finding in accordance with the hypothesis of FUT2 gene polymorphism and calicivirus susceptibility. To our knowledge, this is the first report of RNA evolution of calicivirus in a single individual, and our data suggest an immunity-driven mechanism for viral evolution. We also report on chronic virus excretion, immunoglobulin treatment, and modification of clinical symptoms; our observations from these studies, together with the FUT2 gene characterization, may lead to a better understanding of calicivirus pathogenesis.

Acute diarrheal diseases are common in humans and are associated with significant morbidity worldwide and substantial mortality in developing countries. In recent years, caliciviruses have emerged as an important cause of gastroenteritis in all age groups and are now recognized as the main cause of gastroenteritis in nursing homes and hospitals (3, 8, 12, 14, 15, 27).

Caliciviruses are classified into four genera: norovirus, sapovirus, vesivirus, and logovirus. Human caliciviruses are found within the norovirus and sapovirus genera, which contain a wide range of genetically distinct strains. The sequence heterogeneity of these strains may be a result of the high mutation rate caused by the lack of proofreading of the polymerase. However, the sequence variation has also raised the possibility that calicivirus RNA undergoes constant genetic drift through immune-response-driven mechanisms, leading to the emergence of new strains. However, at present there is no information on human calicivirus mutation rates over time, nor has the importance of immune-response-driven mutations been reported.

The clinical features of a calicivirus infection includes an incubation period of 12 to 48 h characterized by acute onset of nausea, vomiting, abdominal cramps and diarrhea that generally lasts for about 48 h. Recent studies have shown, however, that diarrhea can persist for up to 4 weeks (15, 21, 22). Furthermore, it was previously believed that a person remained contagious for 48 to 72 h after recovery from a Norwalk-like infection (24). This has been challenged, and a recent study reports that virus can be excreted for up to 3 weeks (21). Thus, the more recent studies show clearly that not only can the virus be excreted for much longer periods than previously thought but also that the diarrhea can also last much longer than was previously thought. It should be noted, however, that all current information is obtained from immunocompetent individuals, and no information is yet available about the clinical manifestation of calicivirus infections in immunosuppressed or immunodeficient individuals; it is also not known how calicivirus evolution and excretion is affected in these individuals.

We report here on polymerase and capsid gene evolution of the calicivirus in vivo. The most novel observations from sequencing the capsid gene from 12 consecutive isolates from a single individual were that the vast majority of the cumulative nucleotide changes (8 of 11) appeared within the region from amino acids 304 to 404 located on the surface of the capsid protein and within the hypervariable region, resulting in predicted structural changes in the capsid. To our knowledge, this is the first report of chronic calicivirus shedding and RNA
evolution, and the results support the hypothesis of an immune-response-driven mechanism for calicivirus RNA evolution. We also provide host genetic information that support the hypothesis of specific histo-blood group antigens and calicivirus resistance (6, 13, 23).

MATERIALS AND METHODS

Patient and clinical samples. The patient received a heart transplant in 1994 due to severe heart failure and malignant arrhythmias after a myocardial infarction. After transplantation, he subsequently received immunosuppressive treatment with cyclosporine, azathioprine, and prednisolone. The immunosuppressive treatment resulted in a low total number of lymphocytes (8%; normal range, 50 to 80%), a low number of CD4+ cells (0.15; normal, 0.4), and a low CD4/CD8 ratio of 0.37 (normal, >1.0) but normal immunoglobulin concentrations. During the following years the patient developed renal insufficiency, nephroclerosis, possibly due to cyclosporine toxicity, as shown on renal biopsies. In 1998 he became dialysis dependent. Since 1999 he has been on hemodialysis three times weekly, while waiting a cadaveric renal transplantation.

Fecal samples were collected from July 2000 to July 2001, and 12 urine samples and one serum sample were collected from 1 June 2001 to 31 July 2001. EM. Fecal samples and urine samples were initially screened for viruses by electron microscopy (EM) as previously described (3).

Calicivirus RT-PCR. Twenty-two of the specimens were investigated for the presence of Norwalk-like human calicivirus by a reverse transcription-PCR (RT-PCR) as previously described by Vinje et al. (26, 27). Briefly, RNA was extracted from 50 µl of a 10% stool suspension by using the guanidinium thio- cyanate-silica extraction method (1). RT was performed as follows. First, 5 µl of extracted RNA was annealed with 50 pmol of JV15 (5′-TCA TCA CCA TAG AAA GAG) in a total volume of 9 µl and then added to a reaction mixture containing 10 mM Tris (pH 8.3), 50 mM KCl, 3 mM MgCl2, 1 mM deoxynucleotide triphosphate (dNTP), and 100 U of Moloney murine leukemia virus reverse transcriptase (Superscript; Life Technologies). Reaction mixtures were incubated for 1 h at 42°C. A total of 5 µl of the RT reaction was added to a PCR mix composed of 10 mM Tris (pH 9.2), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTP, 15 pmol of JV12 (5′-ATA CCA TGA TGC AGA TTA), and 2.5 U of Taq polymerase. Forty reaction cycles were carried out for 1 min at 94°C, 1.5 min at 37°C, and 1 min at 74°C, followed by a final incubation at 74°C for 7 min. One-fifth of the reaction volume was analyzed on agarose gels.

RT-PCR and molecular cloning of open reading frame 2 (ORF2). RNA was initially extracted from the stool suspension (sample 2004/00) by using the gua- nidinium thiocyanate-silica extraction method (1). RT was performed as follows. A total of 5 µl of extracted RNA was annealed with 50 pmol of LV13 (5′-AGT ACC TGT TGT CGC TCC A) in a total volume of 9 µl and then added to a reaction mixture containing 10 mM Tris (pH 8.3), 50 mM KCl, 3 mM MgCl2, 1 mM dNTP, and 100 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies). Reaction mixtures were incubated for 1 h at 42°C. Next, 5 µl of the RT reaction mixture was added to a PCR mix composed of 10 mM Tris (pH 9.2), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTP, 15 pmol of LV4092 primer (5′-CAC GGC CCA GCA TTC TAC A), and 2.5 U of Taq polymerase. After an initial incubation at 94°C for 3 min, 25 reaction cycles were carried out with 10 s at 94°C, 30 s at 45°C, and 2 min at 72°C, followed by a final incubation at 72°C for 7 min. Positive PCR amplicons were purified from gels and polished by incubating DNA in polishing buffer, 0.5 mM dNTP, and 0.5 U of Phi29 DNA polymerase (Stratagene) at 72°C for 30 min. The polished DNA fragment was subsequently inserted into the pCR-Script cloning vector (Stratagene). Based on the sequence of sample 2004/00, new primers, DK1 (5′-CTA AAG GAA GGT GCC ATG GA) and DK2 (5′-GTC ACC ACG CAA CCC TG), were designed that were used in the subsequent RT-PCR amplification of the ORF2 sequence of selected isolates.

Sequencing of ORF1 and ORF2. RT-PCR amplicons obtained from ORF1 by using the JV12 and JV13 primers targeting a region of the RNA polymerase and sequences containing the entire ORF2 cloned into pCR-Script (Stratagene) were sequenced. After RT-PCR, ORF1 amplification products were purified by using a Stratagene PCR purification kit (Stratagene). Positive clones containing ORF2 were purified by using a Qiagen plasmid purification kit. The DNA sequences were determined by using the ABI Prism ready reaction BigDye sequencing kit (Applied Biosystems) and an ABI automated sequencer (Applied Biosystems). RT-PCR amplicons from ORF1 were sequenced on both strands, whereas three ORF2 types of each isolate were sequenced on both strands.

Host gene polymorphism characterization. DNA was extracted from saliva samples (100 µl), and the FUT2 (Secretor), FUT3 (Lewis), and ABO genotypes were determined by using sequence-specific primers and PCR (PCR-SSP) as previously described (2, 20).

Sequence analysis. Overlapping nucleic acid sequences were combined for analysis and edited with a laser gene software package (DNASTAR, Inc., Madison, Wis.). Amino acid secondary structure predictions were made by using the PSIPRED secondary structural prediction program, based on position-specific scoring matrices (9).

Nucleotide sequence accession numbers. The nucleotide sequences determined in the present study have been deposited in the GenBank sequence database. In the text below, the designations for the partial ORF1 sequences and the corresponding sequence accession numbers are as follows: July 00, AY247424; Aug 00, AY247425; Sep 00, AY247426; Oct 00, AY247427; Nov 00, AY247428; Dec 00, AY247429; Jan 01, AY247423; Feb 01, AY247430; Apr 01, AY247419; May 01, AY247420; June 01, AY247421; and July 01, AY247422. The ORF2 sequences from July 00 to July 01 have accession numbers in ascending order from AY247431 to AY247442.

RESULTS

Clinical findings. After visiting a local restaurant in June 2000, the patient (born in 1935), along with other visitors to the restaurant, developed extensive vomiting and diarrhea resulting in moderate dehydration. He was admitted to the hospital for intravenous rehydration. Excretion of calicivirus was demonstrated by EM and PCR. The acute symptoms turned into chronic diarrhea with four to eight episodes per day, and severe weight loss developed over the next several weeks.

Immunoglobulin treatment. Due to the severity of the symptoms and prolonged excretion of virus, it was decided to try available treatments. Initially, breast milk treatment (125 ml given three times per day) was evaluated for 3 weeks starting 12 December 2000, followed by oral immunoglobulin treatment (10 g/day) for 15 days beginning 11 January 2001 and subsequent intravenous immunoglobulin treatment (0.4 g/kg) for 5 days beginning 13 February 2001. However, none of these treatments reduced the severity of diarrhea or the secretion of calicivirus, as determined by the number of diarrheal episodes and virus excretion.

To reduce the immunosuppression, the azathioprine dosage was reduced from 125 to 75 mg daily and the cyclosporine dosage was reduced to ca. 120 µg/liter. None of these treatments had any effect on the number of diarrheal episodes or on virus excretion, as determined by EM and PCR.

Persistent viral shedding and modification of clinical symptoms. During the period from July 2000 to July 2001, 51 fecal samples were collected from the patient, with an average of four samples collected per month (Fig. 1). All samples were initially analyzed by EM, and in 49 of 51 fecal samples calicivirus was detected. The two EM-negative samples were shown to be positive for calicivirus RNA when retested by RT-PCR. In addition, 22 other EM-positive samples were confirmed to contain Norwalk-like viruses by PCR. In addition to the stool samples 12 urine and 1 serum sample were collected and examined by EM and RT-PCR, but none of these samples were positive for norovirus.

At present (May 2003), the patient still has more than four diarrheal episodes/day, and symptomatic treatment with parenteral nutrition 5 days per week, loperamide hydrochloride (2 mg given three times per day), and tinidazol are used to maintain body weight and fluid balance. The patient suffered from diarrhea, vomiting, and nausea early in the course of the infection but, although the severity of the diarrhea remains, the vomiting and nausea have disappeared.
Sequence analysis of the polymerase gene (ORF1). Nucleotide sequences of ORF1 were determined from 12 isolates collected in approximately 1-month intervals from July 2000 to July 2001. Sequences with a length of 254 bp were obtained and compared. During the 12-month study period, the individual isolates displayed sequence identities of between 97.6 and 100\% with the two last collected isolates (June/01 and July/01) being most divergent from each other (2.4\%). Over the entire study period nine sporadic nucleotide changes were demonstrated. Two of these nucleotide changes occurred in the last isolate collected in July 2001. The changes were scattered throughout the sequence, but four of the substitutions occurred in isolate June/01. However, repeated RT-PCR and sequencing of this isolate did not reveal any differences from the initially determined sequence. One of the sporadic changes appearing in isolate April/01 reappeared in the same position 2 months later in the last collected isolate (July/01). Only one nucleotide substitution, introduced in the second collected isolate (Aug/00), accumulated throughout the study period.

All of the changes were in the third codon position and were nucleotide transitions in eight of nine cases. Of the eight possible transitions, four were U→C, two were C→U, one was A→G, and one was G→A. Two of the changes resulted in amino acid changes. The sporadic nucleotide change in isolate Nov 00 resulted in a shift from Glu to Asp. Furthermore, a permanent single nucleotide shift introduced in isolate Aug/00 resulted in a change from Thr to Ile.

Nucleotide sequence analyses of the capsid gene (ORF2). The complete nucleotide sequences of ORF2 from the 12 isolates described above were obtained and compared. Sequences were determined from both strands on three clones from each of the 12 isolates. Sequence analysis showed that the RNA was 1,647 nucleotides long and coded for a protein of 548 amino acids with a deduced molecular mass of 59.6 kDa. Sequence comparison showed a 93\% nucleotide identity between the first isolate, July/00, and Norwalk-like human calicivirus/Oberhausen 455/01/DE and 92 and 87\% identity with Arg320 and the Mx strain, respectively. Pairwise comparative nucleotide sequence analysis demonstrated that during the 12-month study period all sequences were different compared to the original sequence. The identity between the individual isolates varied between 97.1 to 99.1\%, and the differences were due to a single nucleotide changes. No deletions or insertions were detected. Not surprisingly, the majority of the changes were transitions rather than transversions. However, of the four possible transitions, one was predominant: 41\% (23 of 56) were C→U, 14\% were U→C, and 14\% were G→A. In addition to these changes, 10 of 12 sequences contained nucleotide microheterogeneities, which are defined as the presence of different nucleotides at the same position in different cDNA clones. There was a range of 4 to 19 microheterogeneities found in each isolate, except in isolates Aug/00 and April/01, where no such changes were found. The vast majority of these nucleotide heterogeneities were transitions, with most changes being from C to U and from U to C. Furthermore, most of the microheterogeneities appeared to be independent from isolate to isolate and were in most cases sporadic, but in 10 cases the microheterogeneity reoccurred at a specific position in one or more sequential isolates.

Accumulation of amino acid changes in the immunodominant protruding P2 domain of the capsid results in structural changes. Nucleotide substitutions and microheterogeneities were found in 94 of 548 codons (17\%). These changes resulted in sporadic or permanent amino acid changes in 32 of the affected codons (Fig. 2). Figure 3 illustrates the sporadic and permanent heterogeneity found in ORF2 and shows that A→R, C→Y, and T→Y were the most frequent alterations. Thirteen of the amino acid changes were sporadic and occurred only once, whereas another four point mutations were detected in the last isolate. At the four amino acid positions 255, 291, 294, and 515, the mutated amino acid varied from among two or three different residues.

Except for sporadic amino acid changes, 11 amino acids were cumulative; i.e., they remained once they had occurred (Fig. 2 and 4). The most notable finding was that 8 of 11 amino acids accumulated within the P2 region of the capsid (Fig. 4), which is located on the external part of the capsid (17, 25) and thus could be subjected to immune-response-mediated mutations. Of the three amino acids located outside the P2 region, one was located in the extreme N terminus of the S domain and the two other changes were located as positions 415 and 452 in the P1 region (Fig. 4). Interestingly, a secondary structure prediction of the mutant sequences revealed the disappearance of a helix structure in the P2 region, where the helix structure was present in the first isolate but absent 1 year later (Fig. 5).

Host gene polymorphism characterization revealed a Secretor\(^*\) status. Certain individuals appear to be resistant to calicivirus infections. Recent studies have shown an association between histo-blood group antigens and virus binding to blood cells, saliva, and gastrointestinal cells (6, 7, 13). To determine whether this individual belongs to the “susceptible” group, we performed polymorphism characterization at three loci on the FUT2 gene located on chromosome 19. As illustrated in Fig. 6, the individual was genotyped as Secretor\(^*\), thus being heterozygous (++−) at nucleotide 428 (10) and homozygous (+++) at positions 385 and 571 of the FUT2 gene (4, 5). This individual was characterized at five loci of the FUT3 gene and typed as Lewis positive with two wild-type alleles. His
ABO genotype was O^1O^1. Phenotypically, he would thus be typed as O Le(a^b^-) by the hemagglutination technique.

**DISCUSSION**

In the present study we analyzed the clinical, virological, and genetic characteristics of an immunosuppressed individual with persistent diarrhea and calicivirus excretion. This study has not only provided new clinical information about the course of the infection but also provided novel genetic information about susceptibility and evolution mechanisms of calicivirus.

Nucleotide sequencing of the polymerase gene revealed nine sporadic and scattered nucleotide changes, with one nucleotide substitution being accumulated. In total these alterations resulted in two amino acid changes. Since the polymerase gene most likely is not subjected to immunological pressure, it is reasonable to believe that most, if not all, of these changes were the result of lack of proofreading by the RNA polymerase. However, since four of the substitutions occurred in the same isolate, questions were raised about PCR amplification errors. Repeated RT-PCR and subsequent sequencing revealed, however, a sequence identical to the original sequence, suggesting that substitutions were not a result of errors introduced by the DNA polymerase used in the PCR but rather a result of lack of proofreading of the viral polymerase.

In contrast to the polymerase gene, the mutations that oc-
curred in the capsid were more interesting. The Norwalk capsid protein contains two distinct domains: the shell (S) domain and a protruding (P) domain, including two subdomains with a distal globular domain called P2 and a more central stem domain called P1 (17–19, 25). The N-terminal 225 residues of the capsid contain the S domain, and residues from 225 to the end of the C-terminal contain the P domain, consisting of two subdomains (P1 and P2). The P1 domain is located between the S and P2 domains and is formed by residues 226 to 278 and the C terminus. Whereas the P1 domain is moderately conserved (17, 25), the P2 domain is highly variable in particular between residues 279 and 405 (17), which is the most exposed region of the structure.

Except for the sporadic amino acid changes, 11 amino acids were accumulated in the capsid (Fig. 4). It is interesting that 10 of 11 amino acids accumulated in the protruding P domain and only 1 amino acid accumulated in the more conserved S domain. A more novel observation was that 8 of 11 amino acids accumulated within the hypervariable P2 domain (Fig. 4), suggesting an immune-response-driven mutation event. Support for this suggestion is that P2 is a domain that previously has been proposed to contain determinants for strain specificity (17) and that a monoclonal antibody that recognizes a region between the residues 300 and 384 in the P2 domain inhibits the binding of Norwalk virus capsid to cells (28).

Secondary structure predictions revealed, as a consequence of the accumulated mutations in P2, the disappearance of a helix structure. To our knowledge, this is the first description of such an event for calicivirus and, by putting all current information together, we speculate that the structural changes of the capsid may have led to the possible emergence of a new phenotype. Although we have no conclusive data that prove that the virus has changed phenotype characteristics during the course of infection, our suggestion is nevertheless supported by a previous prediction that structural variations in the P2 domain could alter host or phenotype characteristics (16, 17).

Our structural comparisons were based on the structure of Norwalk virus (16), a virus that belongs to genogroup I, in contrast to the virus described in the present study, which belongs to genogroup II. Although structural differences might occur between the genogroups, the overall architecture of the Norwalk virus capsid is similar to that of animal caliciviruses, such as rabbit hemorrhagic disease virus (25).

An interesting observation was that clinical symptoms varied over time, with diarrhea, vomiting, and nausea occurring in the early phase of the illness, followed by the disappearance of
vomiting and nausea later in the course of the illness. Although several studies have shown that the relative frequencies of these symptoms vary among different outbreaks, ages, and settings (15, 21, 22), it has not to our knowledge been previously reported that calicivirus-associated diarrhea can last for years. The disappearance of vomiting and nausea appeared not to be related to reduced excretion of virus or diarrheal episodes. During the chronic excretion, the virus was transmitted to the partner and nursing staff, showing that infectious virus and not merely noninfectious particles were excreted. Furthermore, no pathogenic bacteria or virus other than norovirus was found in the samples, which strongly suggests that the clinical symptoms were the result of an ongoing norovirus infection and not merely that norovirus was asymptomatically excreted concomitant with a pathogenic microorganism.

The patient was in a state of severe immunosuppression, as illustrated by a CD4/CD8 ratio of 0.37. However, his immunoglobulin concentrations were normal for immunoglobulin G (IgG), IgA and IgM, suggesting that his cellular immunity was impaired but that his humoral immunity was intact. With an intact B-cell immunity, antibody-mediated mutations might have been the most reasonable explanation for the cause of the structural changes of the capsid. Further support for this view is the observation that antibodies do recognize this domain and can prevent virus binding (29).

To investigate whether the patient was Secretor+ and thus expressed the native H antigen on mucosa and saliva, polymorphism studies of three different FUT2 loci were undertaken. Particular attention was given to the FUT2 gene 428G→A mutation since this is the most common inactivating Secretor gene mutation among Caucasians (10, 11).

This individual was found to carry one functional and one nonfunctional FUT2 gene mutated at nucleotide 428. He is thus heterozygous (+/−) (Secretor−) and can fucosylate the Galβ1-3GlcNAc core into the H type 1 structure. This is the only fucosyltransferase cloned to date which can synthesize the H type 1 and is thus the necessary prerequisite for secretion of ABH antigens in saliva. However, since this individual also carries functional Lewis alleles, the H type 1 structure is probably fucosylated further to the Lewis b structure. Whether this antigen, also typically found in secretions or on epithelial cells, is a potential receptor for the virus has not yet been carefully tested.

In conclusion, the present study is the first report of RNA evolution of a norovirus and suggests an immune-response-driven mechanism for virus evolution with, in this case, pre-
dicted structural changes of the viral capsid possibly resulting in a new emerging phenotype. Furthermore, our FUT2 polymorphism characterization further supports the hypothesis of host gene determinants in norovirus susceptibility.

ACKNOWLEDGMENTS

This study was partly supported by the European Union (grants QLRT-1999-00634 and QLRT-1999-00594) and grant 8266 from the Swedish Research Council.

REFERENCES


FIG. 6. FUT2 gene polymorphism characterization. The PCR-SSP pattern from saliva shows that the patient is Secretor+, since the pattern indicates heterozygous mutation at nucleotide 428 and no mutations at positions 385 and 571. For allele characterization the following primers were used: allele 385 (primers 385wt [wt] and 385A→T [mt]), allele 428 (primers 428wt [wt] and 428G→A [mt]), and allele 571 (primers 571wt [wt] and 571C→T [mt]), as indicated in the figure. M. Molecular weight markers (6x174). The arrow indicates an internal PCR gene control (human growth hormone) of 428 bp. The right side of the panel (homozygot+428) shows a non-Secretor control being homozygotically mutated at nucleotide 428 of FUT2. PCR products were separated on agarose gels (1%) and stained with ethidium bromide.


Journal of Food Protection, in press.

Research note: Virus detection in food.

Round-robin comparison of methods for the detection of human enteric viruses in lettuce.

Françoise S. Le Guyader1*, Anna-Charlotte Schultz2, Larissa Haugarreau1, Luciana Croci3, Leena Maunula4, Erwin Duizer5, Froukje Lodder-Verschoor5, Carl-Henrik von Bonsdorff6, Elizabetha Suffredini3, Wim M.M. van der Poel5, Rosanna Reymundo7, Marion Koopmans5.

1: IFREMER, Laboratoire de Microbiologie, Nantes, France
2: Danish Veterinary and Food Administration, Division for Food Safety, Soborg, Denmark.
3: Laboratorio Alimenti, Istituto superiore di sanita, Rome, Italy
4: Helsinki University Central Hospital, Division of Virology, Helsinki, Finland
5: National Institute of Public Health and the Environment, Diagnostic Laboratory for Infectious Diseases and Perinatal Screening, Bilthoven, Netherlands
6: Haartman Institute, Department of Virology, Helsinki University, Finland
7: Centro Nacional de Microbiologia, Instituto de Salud Carlos III, Madrid, Spain.

Keywords: human enteric viruses, calicivirus, norovirus, food analysis.

* corresponding author: Laboratoire de Microbiologie, IFREMER, BP 21105, 44311 Nantes cedex 03, France.
Phone: 33 2 40 37 40 52, Fax: 33 2 40 37 40 73, E-mail: sleguyad@ifremer.fr
Abstract

Five methods to detect human enteric virus contamination in lettuce were compared. To mimic multiple contaminations as observed after sewage contamination, the artificial contamination was done using human calicivirus and poliovirus and animal calicivirus strains at different concentrations. Nucleic acid extractions were done at the same time in the same laboratory to reduce assay-to-assay variability. Results showed that the two critical steps are the washing step and removal of inhibitors. The more reliable methods (sensitivity, simplicity, low cost) included an elution/concentration step and a commercial kit. Such development of sensitive methods for viral detection in foods other than shellfish is important to improve food safety.
The importance of food-borne transmission in outbreaks of viral origin is increasingly recognized (6, 8, 16, 18). At present, diagnosis of such outbreaks relies mostly on epidemiological investigations, coupled with identification of the causative pathogen in persons with health complaints following consumption and, occasionally, in food handlers thought to be the source of infection. Final confirmation by detection of the pathogens in food still remains a challenge for various reasons. Firstly, few methods were developed for detection of viruses in foods and limitations exist to isolate and detect viruses in complex food matrixes (7, 9, 27, 28). Secondly, most of the viral food-borne outbreaks are caused by noroviruses. These RNA viruses, which cannot be propagated in cell culture, are antigenically very diverse making the use of immunological methods difficult, and these methods are probably not sensitive enough to detect viruses in foods (2, 10, 25). Moreover, the genetic diversity of these viruses has made the selection of a consensus primer set, and hence the use of RT-PCR more difficult (2, 30, 31).

Various types of food have been implicated in outbreaks: shellfish, vegetables, fruits, delicatessen foods, and bakery products (6, 16-18). Foods served raw may be a risk of transmitting viruses, and lettuce, vulnerable to contamination, has been implicated in gastroenteritis and hepatitis A virus outbreaks (12, 15, 24, 26). Contamination could occur anywhere from the field to the table, and food handler hygiene is an important parameter throughout (8, 12, 18, 28). A simple contact with soiled hands is sufficient for contamination with high levels of virus, and the common enteric viruses can stay infectious for quite a long time, even under modified atmosphere or after disinfection (3, 4, 7, 14). Finally, the dose required for infection may be as low as a few particles for noroviruses (13). Combined, these factors provide a clear explanation for the great transmissibility of noroviruses and other non-enveloped enteric RNA viruses, such as hepatitis A virus, via food.

To improve microbial monitoring of food quality and to assess the real role of food in viral transmission, standardized methods need to be developed for use in reference laboratories. While significant progress has been made in detection of enteric viruses in shellfish, much needs to be done for other foods before this goal can be reached. The aim of our study was to compare methods for virus detection in a high-risk food item for virus transmission under controlled circumstances mimicking multiple contaminations observed after sewage contamination. The main criteria used for
evaluating and comparing these tests were sensitivity, reproducibility, time, cost, and equipment needs. Lettuce was selected as a high-risk food item because it is eaten raw, it is handled extensively during harvest and preparation, its leaves have a large surface area, and it may become contaminated with soil or by irrigation with sewage water.

MATERIALS AND METHODS

Viruses. Three different types of viruses were used: poliovirus Sabin strain type 2 (EV) (kindly provided by A. Bosch, U. Barcelona, Spain), canine calicivirus strain n°48 (CaCV) that can be titrated by cell culture (kindly provided by E. Duizer RIVM, Netherlands) and a human stool RT-PCR positive for norovirus genogroup 2 (NoV) (kindly provided by P. Pothier, CHU Dijon, France). Viruses were extracted using the QIAamp Viral RNA mini kit (Qiagen) and titrated by RT-PCR (end point dilution). The lowest dilution of extract that yielded a detectable product by RT-PCR confirmed by hybridization was considered one RT-PCR unit.

Artificial contamination of lettuce. Sterile water (800 ml) was artificially contaminated using the CaCV, the EV, and the NoV inocula. Approximately 80 g of freshly purchased lettuce (Lactuca sativa) was cut into small pieces (about 5 cm²) and immersed into the contaminated water for 20 min. at room temperature. Then the lettuce was strained and left out to dry for about 1h under a laminar flow. The lettuce was weighed, divided into 10 replicates of 8 g each, and immediately analyzed using 5 different methods in duplicate.

To evaluate sensitivity of one method, the NoV-positive stool suspension was diluted into sterile water, and 100 µl were spread directly onto 10 g of lettuce. After a contact time of 15 min under laminar flow the extraction was done as described. Replicate experiments were done to evaluate the reproducibility of the method, including negative control lettuce samples.
**Virus extraction and nucleic acid purification.** The five methods that were evaluated are summarized in Table 1. Methods were developed independently in the laboratories indicated, and were brought to the IFREMER laboratory for a workshop on comparative evaluation. Each workshop attendee performed his own protocol.

**Method A:** The viruses were eluted by addition of 6 ml of PBS to the lettuce samples in a 50 ml polypropylene (pp) centrifuge tube (Falcon Corp.) and shaking for 5 min by hand. This step was repeated and one volume of Vertrel® (1,1,1,2,3,4,4,5,5,5-decafluoropentane DuPont, France) was added before shaking for 5 min, and centrifugation for 10 min, 7,000 rpm at 4°C. Lettuce was eliminated and PEG 6000 was added to the supernatant at a final concentration of 10% and NaCl (0.3M), incubated for 2 hours at 4°C, and centrifuged for 30 min at 9,500 rpm at 4°C in a 50 ml pp tube. The pellet was suspended in 2 ml of TRIzol® (Invitrogen, Scotland, UK), incubated at room temperature for 5 min, and centrifuged for 20 min at 12,000 rpm at 4°C. The aqueous phase was adsorbed onto RNAMATRIX (Bio101, Carlsbad, CA), mixed for 1 h at room temperature and centrifuged for 2 min at 3,000 rpm. The matrix was washed three times with 400 µl using a wash solution, and dried overnight. The RNA was eluted using 100 µl of RNase free water by heating for 10 min at 65°C.

**Method B:** The viruses were eluted by addition of 21.3 ml (1:3 w/v) of beef extract (3%, pH 9.5), and mixed for 20 min at room temperature in a 50 ml pp tube. The aqueous phase was collected (about 16.5 ml), distributed into four ultracentrifugation tubes (Polyallomer centrifuge tubes, Beckman, CA) and ultracentrifuged for 2 h at 200,000 x g. The pellet was suspended in 1 ml of sterile distilled water. For nucleic acid extraction, 334 µl of sample (equivalent of 1/3 of the sample) was mixed with 666 µl of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% (w/v) sodium lauryl sarcosinate and 0.1 M β-mercaptoethanol) (1), and 100 µl of CsCl (5.7M solution of CsCl in 25 mM sodium acetate, pH5, RI= 1.4000) was added (Baker Analyzed Reagent-Deventer, Holland). After centrifugation for 20 min at 12,000 x g the pellet was suspended in 1 ml of 70% ethanol and centrifuged for 5 min at 12,000 x g. The pellet was then dried and suspended in 100 µl of RNase free water.
**Method C:** The viruses were eluted by addition of 4 ml of glycine-NaCl buffer (0.05M-9g/l, pH 9.5), vortexed for 1 min before adding 3 ml of chloroform –butanol (1:1, vol/vol) and 0.5ml of Cat-Floc (Calgon Corp.) in a 50 ml pp tube. After vortexing, the mixture was centrifuged for 20 min at 12,000 x g at 4°C. PEG 6000 was added to the supernatant to a final concentration of 8% (0.4M NaCl), rocked for 1h at 4°C. After centrifugation for 30 min at 10,000 x g at 4°C, the pellet was suspended directly in the lysis buffer of the RNeasy Plant Mini kit (Qiagen, Courtaboeuf, France) and extracted according to the manufacturer protocol. The nucleic acid was eluted twice in 50µl of RNase free water.

**Method D:** The viruses were eluted by addition of 5 ml of PBS in a 50 ml pp tube, vortexed and incubated rotating for 5 min. After adding 5 ml of Vertrel (DuPont, France), the mixture was rocked for 30 min at room temperature and centrifuged 20 min at 13,000 x g at 4°C. The aqueous phase was then ultracentrifuged for 2 hours at 200,000 x g. The pellet was suspended in 200 µl of PBS, and the nucleic acid was then extracted using the RNeasy Plant Mini kit. The nucleic acid was eluted twice with 50 µl of RNase free water.

**Method E:** The viruses were eluted for 15 min by addition of 5 ml of glycine buffer (pH 8.5) in a 50 ml pp tube. After gravity settling of large particles, the supernatant was collected and clarified by centrifugation for 20 min at 13,500 x g. Three ml of the supernatant was then concentrated using a microconcentrator (Amicon) until volume was reduced to 200 µl. The nucleic acid was extracted using the RNeasy Plant Mini kit. The nucleic acid was eluted twice in 50 µl of RNase free water.

**RT-PCR.** RT-PCR was performed as previously described, according to the instructions of the RT and Taq polymerase supplier (Perkin-Elmer Corp.) (21). Primers used were: P1-PV444 for EV (5), P110 –NI for NoV (20) and CaCV-3-CaCV-4 for the CaCV (11). Extracts were tested undiluted (2 µl) and after one log dilution (0.2 µl). For each primer set, all nucleic acid extracts were assayed in one run including negative controls. RT-PCRs were performed according to the instructions of the murine leukemia virus reverse transcriptase and Taq polymerase suppliers (Applied Biosystems). The RT was done for 30 min at 42°C and the PCR amplification was performed for 40 cycles (94°C for 30 s, 50°C for 30 s, and 72°C for 30 s) with final extension at
72°C for 7 min in a thermocycler (9600 or 2400 Cycler; Perkin-Elmer Corp.). PCR products were visualized by electrophoresis on a 9% polyacrylamide gel and stained with ethidium bromide (21). Positive and several negative controls were included in each RT-PCR run.

Hybridization. PCR products of EV and NoV were hybridized using the dot-blot method and chemiluminescence detection (Roche Applied Science) (21). Specific probes (P357 and NVp117 respectively) were used (5, 20).

Detection of inhibitors. Each nucleic acid extract, undiluted and after one log dilution, was co-amplified with 10 RT-PCR units of a calibrated single strand RNA internal control (IC) (constructed from the EV genome)(21). Briefly, 1µl of a dilution of the IC was mixed with 1µl of each nucleic acid solution and subjected to RT-PCR as described above. The presence of inhibitors means no amplification of the IC amplification, the absence of inhibitor means amplification of the IC, detected after gel electrophoresis.

RESULTS

Artificial contamination. A titer was calculated for each virus stock at 2.5 x 10^7 RT-PCR units/ml for the poliovirus sabin strain type 2, 1 x 10^7 RT-PCR units/ml for the canine calicivirus strain, and 1 x 10^5 RT-PCR units/ml for a NV GGII positive stool. The water used for the lettuce contamination, was seeded at a final concentration of 3 x 10^4 RT-PCR units/ml for the CaCV strain, 1 x 10^5 RT-PCR units/ml for the EV, and 1.2 x 10^2 RT-PCR units/ml for the NoV. A control, done after the lettuce was removed, showed that less than 1% of the viruses can be detected in the water (data not shown).

Inhibitor removal. The IC could not be amplified in undiluted NA extracted using method A and D, indicating the presence of residual inhibitors. However no inhibition persisted after one log
Virus detection. The EV strain was detected in all nucleic acid extracts (Table 2). However method B detected the strain only in the undiluted extract and method A only after dilution (confirming the inhibitor detection). All results observed on the gel were confirmed after hybridization.

For CaCV detection, positive results were observed after gel electrophoresis for all methods except for method B and one replicate after dilution 1/10 for method A. Unfortunately no probe was available for this RT-PCR to increase the sensitivity and confirm the specificity.

For the NoV strain, in all instances except one, product could be detected by both gel electrophoresis and hybridization. In one case (A2), product could not be detected by electrophoresis but was indeed confirmed by subsequent hybridization and in an other case (D2) the virus was not detected after one log dilution. (Table 2).

Method C sensitivity study. Based on results obtained for virus detection and evaluation criteria, method C was selected for further evaluation. To evaluate the reproducibility and sensitivity, several replicate experiments were done using stool sample dilutions directly spread onto lettuce aliquots (Table 3). Of the 10 experiments done using an inoculum of 10 RT-PCR units/g, 9 were found positive, one experiment failed to detect the virus. Among the 9 positive experiments, inhibitors persisted in 4, and the virus could be detected only after dilution. The ability to detect virus after one log dilution suggests that the sensitivity of the amplification method was about 1 RT-PCR unit. However, this does not take into account the effect of inhibitors on the overall detection limit of the assay, which is indeed a function of the combined effect of extraction and amplification efficiency.

DISCUSSION
Method comparison for food analysis is a difficult challenge due to the scarceness of naturally contaminated food and to the variability of the food matrices and the contamination. For in vitro studies, artificially contaminated samples are more convenient to use provided they mimic the natural way of contamination. Lettuce contamination is likely to be related to the use of sewage contaminated water in the fields or when washing before consumption. Similarly, in the case of contamination by a food handler, the virus is likely to be on the surface. Therefore, the immersion of lettuce in water contaminated with different virus strains at various concentrations, as was done here, can be considered to mimic the real situation.

In food contaminated by sewage, multiple viruses can be found and the predominant strain is not always the strain detected in patient’s stool (17, 22, 23, 29). Since an efficient method should be able to detect a variety of pathogenic viruses, we used poliovirus, and human (NoV) and animal caliciviruses (CaCV) for the seeding experiments, with comparatively lower doses for the human Norovirus because of its high infectivity.

To optimize the reliability of the experiment and to avoid any discrepancy in the results possibly due to storage conditions, it was necessary to contaminate all the lettuce samples in the same batch and to do all the extractions at the same time. Therefore, all the extractions were done simultaneously in one laboratory, with all participants using their own reagents. To eliminate any variation that could interfere with the results, one person from the organizing laboratory did the RT-PCR and hybridizations immediately after the extraction.

All methods proposed by the different laboratories consisted of an elution step, followed by a concentration step using PEG precipitation, ultra-centrifugation or ultra-filtration. Whereas PEG precipitation can be done very easily in any laboratory, the ultra-centrifugation step needs a more expensive apparatus and a suitable rotor. As this method was found efficient in a previous study (7), this highlights the importance of comparing different methods under standardized conditions in the same laboratory.

For the nucleic acid extraction, three methods were proposed. One method based on a cesium chloride cushion was quite easy and fast but needs an experienced technician and requires the use of a toxic product (cesium chloride). TRIzol lysis and nucleic acid purification by adsorption
used in method A is quite similar to the RNeasy kit principle, but is more time-consuming and
complicated to perform, and yielded a lower sensitivity. The RNeasy Plant mini kit (Qiagen),
included in three methods, is simple, user-friendly, efficient in eliminating inhibitors and gave the
best results. Residual RT-PCR inhibitors hamper most published methods as reviewed by Sair et al.
(27). These authors reported that this kit was found less efficient than the use of the TRIzol with the
QIAshredder™ homogenizer but more sensitive than TRIzol alone or guanidium isothiocyanate
extraction method (27). Comparison of sensitivity is difficult for norovirus as it depends on the strain
and the primer set used. However, the detection of about 10 RT-PCR units/g of lettuce obtained in
this study, with a good reproducibility, is better or similar to previously described methods for
lettuce analysis. For example, Leggitt and Jaykus (19) and Dubois et al. (9) reported about the same
sensitivity, (i.e. $\geq 1.5 \times 10^3$ RT-PCRU per 50-g food sample and $1.5 \times 10^3$ RT-PCRU per 30-g food
sample) whereas, Sair et al. (27) reported a sensitivity of 50 PCRU per 6 g of food sample for NoV.

Based on the experiments described here, our preferred methods for further evaluation are
methods C and E. Both methods (one elution/concentration step and a standardized commercial
available product) are convenient and, by minimizing error factors are promising for the future
development of a competent viral quantification by real time RT-PCR. More work is needed to
establish more precisely the virus recovery, to extend to other enteric viruses such as hepatitis A
virus and to compare cell culture and RT-PCR analysis. To our knowledge, this is the first study
using the cultivable canine calicivirus, as a surrogate for human norovirus detection in food analysis.

This study was done as part of an European project to identify common source food-borne
virus outbreaks in Europe. It illustrates that it may be possible to develop sensitive methods for virus
detection in food other than shellfish. Analysis of food samples in an outbreak investigation will help
to evaluate the true role of food in the transmission of epidemic viral gastroenteritis. Whether viral
analysis of food samples is of practical value in confirmation of foodborne outbreaks will require
their application. Method validation need to be done on food related to foodborne outbreaks
identified through epidemiological surveillance, which is the future focus of our work. The
adaptation of protocols for different food matrices (e.g. fruits, liquids, meat products, etc) will be
needed prior to their widespread use. When these aspects have been addressed, analysis of food
samples for viral pathogens may become more feasible and provide valuable information.

ACKNOWLEDGMENTS.

This research was supported by European Union Grant: Foodborne viruses in Europe, QLK1- CT-
1999- 00594. The authors thank Christine Moe for helpful advice on the manuscript.
REFERENCES.


TABLE 1. Overview of the different methods used to eluate and concentrate the enteric viruses and to extract and purified the nucleic acids.

<table>
<thead>
<tr>
<th>Method</th>
<th>Elution</th>
<th>Concentration</th>
<th>RNA extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>PBS/Vertrel&lt;sup&gt;R&lt;/sup&gt;</td>
<td>PEG&lt;sup&gt;a&lt;/sup&gt;</td>
<td>TRIzol, RNAMatrix</td>
</tr>
<tr>
<td>B</td>
<td>Beef extract</td>
<td>ultracentrifugation</td>
<td>Guanidium, CsCl&lt;sup&gt;b&lt;/sup&gt; cushion</td>
</tr>
<tr>
<td>C</td>
<td>Glycine/chloroforme/butanol</td>
<td>PEG&lt;sup&gt;a&lt;/sup&gt;</td>
<td>RNeasy Plant mini kit</td>
</tr>
<tr>
<td>D</td>
<td>PBS/Vertrel&lt;sup&gt;R&lt;/sup&gt;</td>
<td>ultracentrifugation</td>
<td>RNeasy Plant mini kit</td>
</tr>
<tr>
<td>E</td>
<td>Glycine buffer</td>
<td>Filtration</td>
<td>RNeasy Plant mini kit</td>
</tr>
</tbody>
</table>

<sup>a</sup>: poly-ethylene glycol precipitation, <sup>b</sup>: cesium chloride
**TABLE 2:** Detection of inhibitors and inoculated viruses on lettuce samples using five virus recovery/nucleic acid extraction methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Replicate</th>
<th>Inhibitor detection&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Enterovirus&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Canine calicivirus&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Norovirus&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>+(-)</td>
<td>+(+)</td>
<td>+(+)</td>
<td>+(+)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+(-)</td>
<td>-(+)</td>
<td>+(+)</td>
<td>+*(+)</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>-(+)</td>
<td>+(+)</td>
<td>-(-)</td>
<td>-(-)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-(+)</td>
<td>+(+)</td>
<td>-(-)</td>
<td>-(-)</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>-(+)</td>
<td>+(+)</td>
<td>+(+)</td>
<td>+(+)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-(+)</td>
<td>+(+)</td>
<td>+(+)</td>
<td>+(+)</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>+(-)</td>
<td>+(+)</td>
<td>+(+)</td>
<td>+(+)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+(-)</td>
<td>+(+)</td>
<td>+(+)</td>
<td>+(+)</td>
</tr>
<tr>
<td>E</td>
<td>1</td>
<td>-(+)</td>
<td>+(+)</td>
<td>+(+)</td>
<td>+(+)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-(+)</td>
<td>+(+)</td>
<td>+(+)</td>
<td>+(+)</td>
</tr>
</tbody>
</table>

<sup>a</sup>: two replicates were analyzed for each method and the results reported are for the undiluted nucleic acid extract or after one log dilution (in brackets). <sup>b</sup>: the inhibitor removal was evaluated by amplification of 10 RT-PCR units of an internal control mixed with 1µl of the nucleic acid extract undiluted or after one log dilution (in brackets). The presence (+) of inhibitors means no amplification of the IC amplification, the absence (-) of inhibitor means amplification of the IC, detected after gel electrophoresis. <sup>c</sup>: the enterovirus and the norovirus were detected by RT-PCR and hybridization as described in the material and methods. All positive results were detected both after gel electrophoresis and confirmed by hybridization except one (+*) that was positive only after hybridization. <sup>d</sup>: the canine calivirus was detected by RT-PCR.
TABLE 3: Detection of Norovirus directly spread onto lettuce samples at various concentrations, by RT-PCR and hybridization after extraction using method C (PEG precipitation and RNeasy plant mini kit extraction).

<table>
<thead>
<tr>
<th>Stool inoculum (RT/PCR units/g)</th>
<th>100</th>
<th>10</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted NA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2/5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6/10</td>
<td>0/3</td>
</tr>
<tr>
<td>One log dilution NA</td>
<td>5/5</td>
<td>9/10</td>
<td>0/3</td>
</tr>
</tbody>
</table>

<sup>a</sup>: NA : nucleic acid,

<sup>b</sup>: Results are indicated as number of positive replicates/number of spiked samples tested.
Foodborne viruses: an emerging problem

Marion Koopmans*, Erwin Duizer

National Institute of Public Health and the Environment (RIVM), Antonie van Leeuwenhoeklaan, 9, NL-3720 BA Bilthoven, The Netherlands

Received 10 October 2002; received in revised form 12 February 2003; accepted 15 March 2003

Abstract

Several groups of viruses may infect persons after ingestion and then are shed via stool. Of these, the norovirus (NoV) and hepatitis A virus (HAV) are currently recognised as the most important human foodborne pathogens with regard to the number of outbreaks and people affected in the Western world.

NoV and HAV are highly infectious and may lead to widespread outbreaks. The clinical manifestation of NoV infection, however, is relatively mild. Asymptomatic infections are common and may contribute to the spread of the infection. Introduction of NoV in a community or population (a seeding event) may be followed by additional spread because of the highly infectious nature of NoV, resulting in a great number of secondary infections (50% of contacts).

Hepatitis A is an increasing problem because of the decrease in immunity of populations in countries with high standards of hygiene.

Molecular-based methods can detect viruses in shellfish but are not yet available for other foods. The applicability of the methods currently available for monitoring foods for viral contamination is unknown.

No consistent correlation has been found between the presence of indicator microorganisms (i.e. bacteriophages, E. coli) and viruses.

NoV and HAV are highly infectious and exhibit variable levels of resistance to heat and disinfection agents. However, they are both inactivated at 100 °C.

No validated model virus or model system is available for studies of inactivation of NoV, although investigations could make use of structurally similar viruses (i.e. canine and feline caliciviruses).

In the absence of a model virus or model system, food safety guidelines need to be based on studies that have been performed with the most resistant enteric RNA viruses (i.e. HAV, for which a model system does exist) and also with bacteriophages (for water).

Most documented foodborne viral outbreaks can be traced to food that has been manually handled by an infected foodhandler, rather than to industrially processed foods. The viral contamination of food can occur anywhere in the process from farm to fork, but most foodborne viral infections can be traced back to infected persons who handle food that is not heated or otherwise treated afterwards. Therefore, emphasis should be on stringent personal hygiene during preparation.

If viruses are present in food preprocessing, residual viral infectivity may be present after some industrial processes. Therefore, it is key that sufficient attention be given to good agriculture practice (GAP) and good manufacturing practice (GMP) to avoid introduction of viruses onto the raw material and into the food-manufacturing environment, and to HACCP to assure adequate management of (control over) viruses present during the manufacturing process.

* Corresponding author. ILSI Europe, 83 Avenue E. Mounier, Box 6, B-1200 Brussels, Belgium. Tel.: +32-2-771-00-14; fax: +32-2-762-00-44.
E-mail address: publications@ilsieurope.be (M. Koopmans).

0168-1605/$ - see front matter © 2003 ILSI. Published by Elsevier B.V. All rights reserved.
doi:10.1016/S0168-1605(03)00169-7
If viruses are present in foods after processing, they remain infectious in most circumstances and in most foods for several days or weeks, especially if kept cooled (at 4 °C). Therefore, emphasis should be on stringent personal hygiene during preparation.

For the control of foodborne viral infections, it is necessary to:
- Heighten awareness about the presence and spread of these viruses by foodhandlers;
- Optimise and standardise methods for the detection of foodborne viruses;
- Develop laboratory-based surveillance to detect large, common-source outbreaks at an early stage; and
- Emphasise consideration of viruses in setting up food safety quality control and management systems (GHP, GMP, HACCP).

© 2003 ILSI. Published by Elsevier B.V. All rights reserved.

**Keywords:** Foodborne viruses; Noroviruses; Hepatitis A; Food processing; Good manufacturing practices

### 1. Introduction

Viruses are very small microorganisms, ranging in size from 15 to 400 nm (examples, see Fig. 1). Viruses cause a wide range of diseases in plants, animals and humans. These infections do not occur at random: each group of viruses has its own typical host range and cell preference (called tropism). Viruses can be transmitted in different ways, for example by droplets generated when an infected person coughs, by contamination with stool samples from a person infected with an intestinal virus, by sexual intercourse, by contact with blood from infected persons with blood-borne viruses, by contact with infected animals with zoonotic viruses, or by vectors, such as mosquitoes or ticks for arthropod-borne (arbo-) viruses. Clearly the most relevant in foodborne infections are those viruses that infect the cells lining the intestinal tract and are dispersed by shedding into the stool or through emesis (Table 1). Some general features of foodborne viral infections and important differences from foodborne bacterial infections are:

- Only a few particles are needed to produce illness;
- High numbers of viral particles are shed in the stools from infected persons (up to $10^{11}$ particles per gram stool reported for rotavirus);

![Fig. 1. Electron micrograph and some structural properties of enteric viruses that are commonly (NoV, HAV) or occasionally (other viruses) associated with foodborne or waterborne transmission (Locarnini et al., 1974). (NoV-Noroviruses, HAV = hepatitis A viruses, PV = poliovirus, EV = enterovirus, HRV = human rotavirus; ss = single-stranded, ds = double-stranded).](image-url)
Viruses need specific living cells in order to replicate and therefore cannot do so in food or water; and foodborne viruses typically are quite stable outside the host and are acid-resistant. Conceivably, current food hygiene guidelines, most of which have been optimised for the prevention of bacterial infections, may be only partially (if at all) effective against viruses. A complicating factor is that most common foodborne viruses grow poorly or not at all in cell culture, so that studies of inactivation of these pathogens are not possible. For this overview, we have reviewed currently available information on foodborne viruses and tried to give an estimate of viral inactivation by looking for parallels in structurally similar viruses that can be grown in cell culture systems in the laboratory.

2. Which viruses are involved?

Numerous viruses can be found in the human gut, but only a few are commonly recognised as important foodborne pathogens. These can be classified into three main groups, according to the type of illness they produce (Table 1):

- Viruses that cause gastroenteritis;
- Enterically transmitted hepatitis viruses; and
- A third group of viruses that replicate in the human intestine but cause illness after they migrate to other organs, such as the central nervous system or the liver.

Foodborne illness has been documented for most of these viruses, but recent studies show that the Noroviruses (NoV) and hepatitis A virus (HAV) are by far the most common cause of illness by this mode of transmission (Cliver, 1997). Some large foodborne outbreaks have occurred with group B and C rotaviruses, and waterborne outbreaks have occurred with hepatitis E virus.

3. Epidemiology

Recent studies have shown that NoV is the single most common cause of gastroenteritis in people of all age groups and is as common as rotavirus in patients who consult their general practitioners for gastroenteritis (Wheeler et al., 1999; Koopmans et al., 2000). The incidence is highest in young children, but illness also occurs regularly in adults. Asymptomatic infections are common. In addition, the majority of outbreaks of gastroenteritis in institutions such as nursing homes and hospitals is caused by NoV (Codex Alimentarius, 1999). Although it is not known what proportion of infections can be attributed to the consumption of contaminated food, several reports have shown that foodborne NoV infections are common. Large, even international foodborne outbreaks of NoV have been described (Berg et al., 2000). Data from seroprevalence studies suggest that NoV infections are found worldwide.

For HAV the picture is different. The incidence of HAV infection varies considerably among and within countries (Mast and Alter, 1993). In much of the developing world, where HAV infection is endemic, the majority of persons are infected in early childhood and virtually all adults are immune. In these areas, HAV transmission is primarily from person to person. Outbreaks are rare because most infections occur among young children who generally remain asymptomatic. In the developed countries, however, HAV infections become less common as a result of increased standards of living. Very few persons are infected in early childhood, and the majority of adults

<table>
<thead>
<tr>
<th>Likelihood of food- or waterborne transmission</th>
<th>Illness</th>
<th>Epidemiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common</td>
<td>Norovirus</td>
<td>Epidemiology</td>
</tr>
<tr>
<td>Occasionally</td>
<td>Enteric adenovirus (types 40/41)</td>
<td>Epidemiology</td>
</tr>
<tr>
<td>Rotavirus (group A–C)</td>
<td>Hepatitis A virus</td>
<td>Epidemiology</td>
</tr>
<tr>
<td>Sapovirus</td>
<td>Enterovirus (waterborne)</td>
<td>Epidemiology</td>
</tr>
<tr>
<td>Astrovirus</td>
<td>Epidemiology</td>
<td></td>
</tr>
<tr>
<td>Coronavirus</td>
<td>Epidemiology</td>
<td></td>
</tr>
<tr>
<td>Aichivirus</td>
<td>Epidemiology</td>
<td></td>
</tr>
</tbody>
</table>

*Enteroviruses (e.g. poliovirus) are associated with a range of symptoms, including neurological symptoms.
remain susceptible to infection by HAV. Because virus shedding starts 10–14 days before the onset of symptoms, there is a clear window for spreading the virus. As a result, the risk of (large) outbreaks of HAV increases in these regions.

In addition, adults are more likely to develop symptoms upon infection, causing enhanced recognition of outbreaks. Indeed, foodborne outbreaks have been reported in most parts of the world and can be large. For example in Shanghai, China, in 1988, 250,000 people had HAV after consumption of contaminated clams (Halliday et al., 1991). Detection of sporadic cases or small clusters of foodborne hepatitis A is problematic because the incubation period can be long. As a result, a possible association with food consumed weeks ago can rarely be investigated at the time of onset of illness. For NoV and HAV, waterborne outbreaks are unusual but have been reported.

4. Are foodborne viruses zoonotic?

For most enteric viruses, host range variants have been found in different animal species. So far, however, the majority appear to be quite host-specific. Recently, NoV was found in a large proportion of calf herds and in some pigs (van der Poel et al., 2000). The strains in animals were genetically distinct from any of the viruses found in people (Sugieda et al., 1998). No calf-to-human or pig-to-human transmission has been documented so far. The animal viruses, however, are quite similar to the human NoV and continue to change as all RNA viruses do. This implies that zoonotic transmission might occur if the right circumstances arise.

Similarly, hepatitis E virus (HEV) variants were found in pigs and, in this case, almost identical viruses were found in some humans (Meng et al., 1997). This was taken as the first evidence of zoonotic transmission of HEV. The pig viruses appear to be quite common, even in countries where HEV is rarely diagnosed in humans. This suggests that the risk of zoonotic transmission is rather low and currently of no practical consequence for food handling procedures. Again, however, given the genetic flexibility of RNA viruses, these viruses should be monitored closely for changes in behaviour.

5. Detection and typing

Infection with gastroenteritis viruses is usually diagnosed by the detection of the pathogen in stool samples from sick people, rather than by measuring the antibody response in serum (Tables 2 and 3). Historically, viruses were diagnosed by scanning a stool suspension under an electron microscope (EM) (Atmar and Estes, 2001). This assay still remains the gold standard for virus diagnosis but is rather insensitive and labour-intensive (Table 2). Routine ELISA assays are available for detection of group A rotaviruses, adenoviruses, and astroviruses, as well as for some of the NoV (Tables 2 and 3). For non-group A rotaviruses, Sapoviruses (SaV), and the remaining NoVs, the diagnosis can be made by detection of viral nucleic acid using reverse transcriptase-polymerase chain reaction (RT-PCR) assays. A problem with NoV is the variability of the viral genome, making it difficult to develop a single generic detection test. For the hepatitis viruses, detection of specific IgM antibodies is diagnostic of recent infection. In addition, viruses can be detected in stool and in serum by RT-PCR, but this is not done routinely. There are great

<table>
<thead>
<tr>
<th>Principle of assay</th>
<th>Example</th>
<th>Infectivity test</th>
<th>Detection limit (particles per gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visualisation of particles</td>
<td>EM</td>
<td>No</td>
<td>(10^{0–1})</td>
</tr>
<tr>
<td>Detection of viral protein</td>
<td>ELISA, latex tests</td>
<td>No</td>
<td>(10^{2})</td>
</tr>
<tr>
<td>Detection of genome</td>
<td>Probe hybridisation</td>
<td>No</td>
<td>(10^{3})</td>
</tr>
<tr>
<td>Detection of genome</td>
<td>RT-PCR</td>
<td>No</td>
<td>(10^{3–4})</td>
</tr>
<tr>
<td>Screen for effect on living cells</td>
<td>Cell culture isolation (where feasible)</td>
<td>Yes</td>
<td>(10^{0–1})</td>
</tr>
<tr>
<td>Measurement of exposure</td>
<td>Antibody assays</td>
<td>Yes</td>
<td>Window of detection varies by type of antibody. IgM indicates recent infection</td>
</tr>
</tbody>
</table>
differences in the detection limit of the different assays, ranging from a few particles (cell culture and RT-PCR) to a million particles per gram as minimum amounts necessary for a positive test (Table 2). This has direct consequences for the interpretation of results. A person with a positive EM test sheds a great number of viral particles, whereas a person with a positive RT-PCR may shed few particles. No clear guidelines are yet available on interpretation of these different test results, and little has been done to standardise tests. Complicating factors are that people who are ill do not necessarily shed more viruses than those who have no symptoms and that the maximum levels of shedding appear to be different for different viruses. Thus, at present, the diagnosis of viral infection is qualitative (yes/no) and does not provide additional information that may help in deciding whether the person presents an important risk factor for the food chain. On the other hand, because the minimum dose required for infection is very low for these viruses, any infected person may spread the disease.

Virus detection in food or water has been problematic, even after the introduction of RT-PCR. Because the most important foodborne viruses do not grow (readily) in cell culture, they must be detected directly in food extracts, with all the problems of standardisation, inhibition of enzymes used in the RT-PCR, false-positive tests, etc. (Lees, 2000; Atmar et al., 2001). Because contamination is often caused by foodhandlers, the level of contamination with virus may vary greatly within a product. The combination of variable virus counts and the lack of a culture system is the main reason why virtually no information is available on the variability of test results from sampling or what would be considered representative samples for monitoring purposes. Furthermore, (molecular) diagnostic methods for food or water are not routinely available in food microbiology laboratories (Table 3). Most successful research has focussed on shellfish, but even with published standard protocols, little is known about the performance of such standards in “the field” (e.g. if a batch of oysters does contain some contaminated

### Table 3
Detection and typing methods for foodborne viral infections

<table>
<thead>
<tr>
<th>Virus</th>
<th>Clinical samples Methods</th>
<th>Category*</th>
<th>Food Methods</th>
<th>Category</th>
<th>Water Methods</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calicivirus</td>
<td>Stool, genome detection, EM&lt;sup&gt;b&lt;/sup&gt;</td>
<td>S-R</td>
<td>Genome detection</td>
<td>S-E</td>
<td>Genome detection</td>
<td>S-R</td>
</tr>
<tr>
<td>NoV</td>
<td>Stool, genome detection, EM&lt;sup&gt;b&lt;/sup&gt;</td>
<td>S-R</td>
<td>Genome detection</td>
<td>S-E</td>
<td>Genome detection</td>
<td>S-E</td>
</tr>
<tr>
<td>SaV</td>
<td>Stool, genome detection, EM serum, antibody detection</td>
<td>R</td>
<td>Genome detection, culture</td>
<td>S-E/R</td>
<td>Genome detection, culture</td>
<td>S-E/R</td>
</tr>
<tr>
<td>Rotaviruses</td>
<td>Stool antigen detection</td>
<td>R</td>
<td>Culture, genome detection</td>
<td>S-E</td>
<td>Culture, genome detection</td>
<td>S-E</td>
</tr>
<tr>
<td>Group A</td>
<td>Stool antigen detection</td>
<td>S-E/R</td>
<td>Culture, genome detection</td>
<td>S-E</td>
<td>Culture, genome detection</td>
<td>S-E</td>
</tr>
<tr>
<td>Non-group A</td>
<td>Stool antigen detection</td>
<td>R</td>
<td>Genome detection</td>
<td>S-E</td>
<td>Genome detection</td>
<td>S-E</td>
</tr>
<tr>
<td>Adenoviruses</td>
<td>Stool, antigen detection</td>
<td>R</td>
<td>Genome detection</td>
<td>S-E</td>
<td>Genome detection</td>
<td>S-E</td>
</tr>
<tr>
<td>Astroviruses</td>
<td>Stool, antigen detection</td>
<td>S-R</td>
<td>Genome detection, culture</td>
<td>S-E</td>
<td>Culture, genome detection</td>
<td>S-E</td>
</tr>
<tr>
<td>Enteroviruses</td>
<td>Stool culture</td>
<td>R</td>
<td>Culture</td>
<td>S-E</td>
<td>Culture, genome detection</td>
<td>S-R</td>
</tr>
<tr>
<td>Hepatitis E virus</td>
<td>Serum, antibody detection</td>
<td>R</td>
<td>Genome detection</td>
<td>NA</td>
<td>Genome detection</td>
<td>S-E</td>
</tr>
</tbody>
</table>

*Category of laboratory: R = routine; S-R is routinely available in specialised laboratories; S-E = experimentally available in specialised laboratories; S-E/R = routinely available in some of the specialised laboratories, experimentally available in more.

<sup>b</sup> EM = particle detection by electron microscope.
Table 4
Food processes, virus inactivation factors, and resulting risk of the product if viruses are present before processing

<table>
<thead>
<tr>
<th>Process</th>
<th>Example of food product</th>
<th>Virus inactivation (log_{10})</th>
<th>Risk of infection of consumer if viruses are present before processing</th>
<th>Likelihood of presence before processing</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thermal treatments</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boiling at 100 °C</td>
<td>Any liquid food (e.g. milk) or solid food boiled in water</td>
<td>HAV and PV&gt;4 (Hollinger and Ticehurst, 1996)</td>
<td>Negligible</td>
<td>Unlikely</td>
<td>Likelihood of presence depending on food; kinetic data lacking</td>
</tr>
<tr>
<td>60 °C, 30 min (liquids or solid foods)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Inactivation in solid foods lower than in liquids; dependent on fat and protein content</td>
</tr>
<tr>
<td>Pasteurisation of solid foods (70 °C or equivalent, 2 min)</td>
<td>Paté and other cooked meats</td>
<td>HAV &lt;2 (Millard et al., 1987) FeCV&gt;3 (Doultree et al., 1999)</td>
<td>Medium</td>
<td>Unlikely</td>
<td>Inactivation dependent on fat and protein content</td>
</tr>
<tr>
<td>Pasteurisation of liquids and immediate packing (e.g. HTST 71.7 °C for 15 sec)</td>
<td>Milk, ice cream</td>
<td>HAV &lt;2 (Bidawid et al., 2000a)</td>
<td>Medium</td>
<td>Unlikely</td>
<td>Inactivation dependent on fat and protein content</td>
</tr>
<tr>
<td>UHT and aseptic filling (&gt;120 °C)</td>
<td>Long-life milk, other dairy products</td>
<td></td>
<td>Negligible</td>
<td>Unlikely</td>
<td></td>
</tr>
<tr>
<td><strong>Other physical/chemical/biological processes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drying (spray and freeze drying)</td>
<td>Dried milk, instant dried soups, dessert mixes, chocolate</td>
<td>HAV, FeCV &lt;1 (Doultry et al., 1999; Mbithi et al., 1991)</td>
<td>High</td>
<td>Unlikely&lt;sup&gt;a&lt;/sup&gt;</td>
<td>No information on commercial drying</td>
</tr>
<tr>
<td>Freezing</td>
<td>Ice-cream, frozen desserts (containing fruit)</td>
<td>HAV, PV, FeCV &lt;1 (Hollinger and Ticehurst, 1996)</td>
<td>High</td>
<td>Possible</td>
<td></td>
</tr>
<tr>
<td>Fermentation</td>
<td>Cheese, yoghurt</td>
<td>No information</td>
<td></td>
<td>Unlikely</td>
<td>Microbial inactivation of viruses is found for sludge (Ward, 1982)</td>
</tr>
<tr>
<td>Acidification</td>
<td>Fruit juices, still fruit drinks</td>
<td>NoV: pH 2.7, 3h incomplete (Dolin et al., 1972) HAV: pH 1, 5h incomplete (Hollinger and Ticehurst, 1996)</td>
<td>Medium</td>
<td>Possible</td>
<td>No quantitative data on inactivation</td>
</tr>
<tr>
<td>Method</td>
<td>Inactivation/Depuration</td>
<td>Probability/Likelihood</td>
<td>Comments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>-------------------------</td>
<td>------------------------</td>
<td>---------------------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenisation</td>
<td>Incomplete</td>
<td>High</td>
<td>Likely depending on type of product</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depuration of oysters and mussels</td>
<td>NoV incomplete</td>
<td>High</td>
<td>Likely depending on type of product</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High hydrostatic pressure</td>
<td>PV &lt; 1</td>
<td>High</td>
<td>Possible depending on type of product</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(drinking water); likely</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(surface water)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virus inactivation in water</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorination</td>
<td>HAV&gt;3, HAV&lt;2, HRV&lt;2, PV&gt;3</td>
<td>Variable</td>
<td>Risk is low for PV but medium for HRV and HAV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.5 mg free chlorine/l, 1 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV radiation</td>
<td>PV 3 or less</td>
<td>Low</td>
<td>Risk is low for PV but medium/high for PV and HRV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(20 mJ/cm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ozone treatment</td>
<td>HAV&gt;3, PV 2 or less, HRV&lt;1</td>
<td>Variable</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.2 mg/l, 10 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cleaning of equipment and surfaces</td>
<td>HAV&lt;2</td>
<td>Medium/low</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rinsing with (lots of) water</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>HAV&lt;2, HRV&lt;3</td>
<td>Medium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(70%, 10 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorhexidine digluconate</td>
<td>HAV&lt;1, HRV&lt;1</td>
<td>High</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.05%, 10 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium hypochlorite</td>
<td>HAV&lt;3, HRV&lt;3</td>
<td>Low</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.125%, 10 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium chlorite</td>
<td>HAV&gt;3, HRV&gt;5</td>
<td>Negligible</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(30%, 10 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catering</td>
<td>Washed salads, Fruits</td>
<td>No substantial removal or inactivation</td>
<td>Any removal of viruses will be by mechanical action only; very difficult to remove any microorganisms from foods by washing alone (Mariam and Cliver, 2000b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Washing, rinsing</td>
<td></td>
<td>High</td>
<td>Possible</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(where water &gt;1% of food) and the food is eaten without additional cooking</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 4 (continued)

<table>
<thead>
<tr>
<th>Process</th>
<th>Example of food product</th>
<th>Virus inactivation (log_{10})</th>
<th>Risk of infection of consumer if viruses are present before processing&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Likelihood of presence before processing&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catering</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Freezing of drinking water to prepare ice</td>
<td>Ice for drinks or for cold foods</td>
<td>No inactivation</td>
<td>High</td>
<td>Possible</td>
<td>Freezing is an excellent way to preserve viruses; therefore best to assume there will be no inactivation after one freeze/thaw cycle</td>
</tr>
<tr>
<td>Chilling of drinking water or use of water from tap without any treatment</td>
<td>No inactivation</td>
<td>High</td>
<td>Possible</td>
<td></td>
<td>Chilling will slow down the inactivation rate of viruses</td>
</tr>
</tbody>
</table>

<sup>a</sup> Viruses for which data were used to assemble this table are the (common) foodborne hepatitis A virus (HAV), Noroviruses (NoV) [and the animal model viruses feline calicivirus (FeCV) and canine calicivirus (CaCV)], human rotavirus (HRV), rhesus rotavirus (RV), and poliovirus (PV). Note: estimates included in this table are based on extrapolation of data from scientific studies and should be regarded as indicative only. Data in this table cannot be used to calculate risks. For precise process calculations or predictions on food manufacturing processes, additional experimental information is needed.

<sup>b</sup> Unlikely = no reports are known in which NoV, HAV, RV, or PV were found on these food items. Possible = sporadic contamination with NoV, HAV, RV, or PV has been reported on these food items. Likely = contamination with NoV, HAV, RV, or PV is reported frequently on these food items. Negligible risk = product highly unlikely to contain infectious viruses; treatment results in at least 4 log_{10} inactivation of common foodborne viruses. Low risk = product unlikely to contain infectious viruses in numbers likely to cause disease in healthy individuals; treatment results in approximately 3 log_{10} inactivation of common foodborne viruses. Medium risk = product may contain infectious viruses in numbers that may cause disease; treatment results in approximately 2 log_{10} inactivation of common foodborne viruses. High risk = products in which the level of viruses is likely to be high enough to cause disease in healthy individuals; treatment results in less than 1 log_{10} inactivation of common foodborne viruses. Variable risk = treatment results in significant differences in inactivation of several common foodborne viruses.

<sup>c</sup> Before spray drying in dried milk processes, a substantial heat step destroys viruses.
ones, how likely is it that a virus test will give the right answer?). Therefore, these methods currently cannot be used reliably for quality control and assurance.

6. Use of molecular epidemiology in virus tracing, pros and cons

For all enteric viruses, strains can be divided into subtypes by analysis of the genome. By doing so, common source outbreaks have been diagnosed, even in cases in which links between different outbreaks had not been suspected on the basis of epidemiological investigation (Berg et al., 2000). Conversely, molecular strain typing has also been used to disprove links between cases and a suspected source (Marshall et al., 2001). At present, a European foodborne virus network, including various public health institutes, uses information on strain typing to trace NoV and HAV outbreaks. The participating groups have agreed to exchange epidemiological and virological information through a central database to identify international common source outbreaks as early as possible (QLK1-1999-00594; for information: marion.koopmans@rivm.nl).

7. Monitoring for the presence/absence of viruses: the problem of infectivity

A problem in drafting recommendations for virus control and prevention is that some enteric viruses grow poorly (HAV) or not at all (NoV) in cultured cells (Atmar and Estes, 2001). In addition, no simple animal models are available for experimental studies of virus inactivation. Thus, detection methods currently rely on genome detection by molecular detection techniques such as RT-PCR. A positive signal indicates an intact segment of viral genomic RNA. This does not provide information on virus infectivity. Completely inactivated particles that pose no threat to public health may still contain intact RNA, thus resulting in a positive virus assay. The RNA will eventually be degraded, but it is unknown how long this will take in different environments. In shellfish, inactivation of the virus was followed by rapid degradation (<1 min) of viral RNA (Slomka and Appleton, 1998).

In seawater, however, RNA persisted for days after inactivation of the virus.

In the absence of a culture system for NoV, a common sense approach is to review information on structurally similar viruses and use these as models for the noncultivatable pathogens. For HAV, a cell-culture-adapted variant has been used, for example in studies addressing heat resistance in different food items (Bidawid et al., 2000a). For NoV, structurally similar viruses are the enteroviruses, HAV, and astroviruses. These are all viruses with a single-stranded RNA genome and are approximately 7-kb long, approximately 30 nm in size, and similar in capsid structure (no envelope) (Fig. 1). Slomka and Appleton (1998) recommended the use of an animal calicivirus (FeCV) for inactivation studies and found that FeCV was clearly less stable than HAV. Because most infectivity and inactivation data are available for the enteroviruses and HAV, we have used these data for our risk estimates (Table 4). It should be clear, however, that these remain estimates and will have to be evaluated carefully.

8. Correlation between indicator organisms and virus presence

In water quality research, the use of indicators for the presence of human pathogenic viruses has been an area of considerable debate (Lees, 2000). It is clear from numerous outbreaks that the presence of “traditional” bacterial indicators of faecal contamination does not consistently correlate with the presence of pathogenic viruses. Many groups have proposed the use of bacteriophages as indicators. Bacteriophages are viruses that infect and replicate in bacteria. They are present in substantial numbers in human stool samples and, in some respects, are similar to viruses pathogenic to humans. Because of similarities in structure, behaviour, and stability, bacteriophages may be of use in assessing cumulative exposure to human faecal waste. However, care must be taken not to provide a false sense of safety by measuring the presence of bacteriophages only. The observed clear differences in stability of different human pathogenic viruses (described later in this document) that reside in the intestine illustrate that extrapolation of data from one virus to another cannot be relied upon (Slomka and Appleton,
Similarly, the possible use of phages as surrogates in evaluating the antiviral effectiveness of processes needs to be carefully validated (Mariam and Cliver, 2000a).

9. How do foods become contaminated?

Foods can be contaminated by (Fig. 2):

- Contact with (human) faeces or faecally contaminated water;
- Contact with faecally soiled materials (including hands);
- Contact with vomit or water contaminated with vomit;
- Contact with environments in which infected people were present, even if the surface was not directly contaminated with stool or vomit; and
- Aerosols generated by infected people.

There is no proof that animal contact, directly or indirectly (pigs, calves, surface-contaminated meat, meat products, or other products derived from those animals), can be a source of foodborne infection.

Central to the issue are infected foodhandlers. These may be:

- Infected foodhandlers with symptoms. Shedding of virus occurs during the period of illness;
- Infected foodhandlers who have recovered from illness. Shedding of NoV may persist for at least 3 weeks after recovery;
- Infected foodhandlers without symptoms. Asymptomatic infections are common for all foodborne viruses. For example, carriers of hepatitis A typically shed high quantities of the virus 10–14 days after infection; in the weeks following this period carriers may or may not develop symptoms; and
- Foodhandlers with contacts with sick people (e.g. people with sick children or relatives).

Note that although most outbreaks can be traced to infected foodhandlers at the end of the food chain, they may be anywhere (e.g. seasonal workers picking berries for use in composite foods, people on recreational boats near shellfish harvesting areas, etc.). A large, multistate outbreak of illness associated with oysters was finally traced back to a sick oyster harvester who had vomited and disposed of the waste overboard (Berg et al., 2000).

Outbreaks have been documented in association with a long list of food items (e.g. deli meat, sandwiches, bread rolls, bakery products, berries, ice cubes). Dishes containing fresh (or fresh frozen) fruits and vegetables have been the source of numerous outbreaks of foodborne illness. Filter-feeding shellfish are a particular risk, as they concentrate viruses...
present in their growing water, and numerous outbreaks linked to the consumption of shellfish have been reported.

10. Spread and persistence of foodborne viruses from farm to fork

It is clear that foods requiring either intensive manual handling, manual handling under poor hygienic conditions (e.g. in orchards), or close-to-fork and end-product manual handling are the products at highest risk (see http://www.who.int/fsf/). Information obtained with HAV suggests that approximately 10% of the virus particles can easily be transferred from faecally contaminated fingers to foods and surfaces (Bidawid et al., 2000b).

Another factor determining risk for contamination of foods is the stability of some of the foodborne viruses in the environment. For example, rotaviruses in aerosols (generated while vomiting and thought to play a role in the transmission of those viruses) were found to survive in the air up to 9 days at 20 °C (Sattar et al., 1984). Viruses also may persist for extended periods (1–60 days for 100-fold reduction in infectivity) on several types of materials commonly found in institutions and domestic environments (e.g. paper, cotton cloth, aluminium, china, glazed tile, latex, and polystyrene; Abad et al., 1997) (Fig. 3). Adenoviruses were found to survive for up to 35 days

Fig. 3. Virus survival in tap water (A), aluminium fomites (B), or vegetables (C). Represented are the number of days after which the virus recovery will be less than 1% (A and B) or 10% (C) of the original contamination. (Data from: Enriquez et al., 1995; Kurdziel et al., 2001; Mbih, 1991; Ward and Irving, 1987).
on a plastic surface in an environment with low relative humidity (Nauheim et al., 1990). This relation with humidity varies among viruses. A high relative humidity favours the survival of enteroviruses, whereas a low relative humidity favours survival of HAV and human rotavirus (HRV) (Mbithi et al., 1991; Sattar et al., 1986, 1988). Furthermore, HAV remained infectious in dried faeces for 30 days when stored at 25 °C and 42% relative humidity (Hollinger and Ticehurst, 1996). This stresses the need for virus-specific studies to address virus inactivation. Finally, in artificially contaminated water, viruses may survive for prolonged periods of time, with over 1-year survival of poliovirus and rotavirus in mineral water at 4 °C (Biziagos et al., 1988). Recent data published by Beuret et al. (2000) on traces of NoV RNA found in bottled water may tend to support this statement. However, as yet, no one has been able to confirm these data.

11. Stability of foodborne viruses during processing

Viruses, unlike bacteria, are strict intracellular parasites and cannot replicate in food or water. Therefore, viral contamination of food will never increase during processing, transport, or storage, and the contaminated products will look, smell, and taste normal. Moreover, because contamination is often caused by foodhandlers, the level of contamination with virus may vary greatly within a product.

Nonetheless, several recent studies were performed to determine the modes of transfer and inactivation profiles of foodborne viruses. Most food- or waterborne viruses are more resistant to heat, disinfection, and pH changes than are most vegetative bacteria. It is no coincidence that most virus groups implicated in outbreaks are small, nonenveloped particles, rather than large, fragile, enveloped viruses (Fig. 1). Numerous studies have addressed the stability of viruses under different circumstances (see Table 4), but little was done to standardise these studies. An overall conclusion is that HAV and HRV are more resistant to inactivation than enteric adenovirus and poliovirus, but it must be noted that significant differences in survival rates were found for different environmental and substrate conditions. Again, these findings stress the need for independent assessment of behaviour for different viruses.

This poses a problem for NoV, which cannot be grown in cell culture and, therefore, cannot readily be tested under the experimental conditions described previously. It remains to be seen whether other viruses that can be grown in tissue culture may serve as models for the NoV, as has been suggested for FeCV. In the interim, we recommend using the inactivation profiles of the most stable enteric RNA virus to assess the safety of a process. Thus, for most processes relevant in the food industry, HAV may be considered a good indicator virus.

Information obtained with HAV shows that more than 1000 virus particles can easily be transferred from faecally contaminated fingers to foods and surfaces (Bidawid et al., 2000b). Based on this information, an inactivation factor of at least 3 log10 during post-manual-treatment processes would be required. Based on these assumptions, we have tried to estimate the likelihood of survival of the most important foodborne viruses for commonly used food processing methods if foods are contaminated before processing. With the exception of ultrahigh temperature treatment, no methods would completely inactivate more than 3 log10 of virus, and we estimate that with foods contaminated after processing, viruses will remain active to a significant extent and thus pose a possible risk factor (Fig. 3, Table 4). Therefore, the emphasis should be strongly on prevention of contamination before or during processing by proper deployment of GHP, GMP, and HACCP. Clearly, the likelihood of virus contamination in primary products will differ for different commodities and is the highest for shellfish and manually handled fruits. For foods contaminated after processing, our estimate is that viruses will remain active in most foods (Kurdziel et al., 2001) (Fig. 3, Table 4).

12. What can be done for prevention?

It is clear that most problems with foodborne viruses occur from contamination of food products during manual handling in combination with minimal processing of foods afterwards. With viral infections (e.g. NoV) being very common, it is wise to assume that the introduction of viruses into the food chain is a
likely event that needs to be prevented by stringent hygienic control. Foodhandlers in contact with people with gastroenteritis (e.g. young children) are at special risk of being contaminated and becoming a source of viruses during food manufacture operations. They must be made aware that specific personal hygiene must be ensured. Increasing the awareness of all foodhandlers about transmission of enteric viruses (including the spread of viruses by vomiting) is needed, with special emphasis on the risk of “silent” transmission by asymptomatically infected persons and via those who continue to shed virus after recovery from illness. At present, insufficient data are available to determine which steps will be critical for all foods in an HACCP system, but it is clear that at least the following points should be addressed:

- Water used in combination with the culturing or preparation of food should be of drinking water quality; and
- Guidelines specifically aimed at the reduction of viral contamination are needed, as it has become clear that current indicators for water and shellfish quality are insufficient as predictors of viral contamination.

Documented outbreaks of foodborne infections could be reported faster using, for example, the European Foodborne Virus Network, the “rapid alert system for food” of the European Union. These networks could operate more effectively if typing information for virus strains were included. A vaccine is available for hepatitis A, and contacts can be treated with the administration of immunoglobulin within 2 weeks after exposure. The Advisory Committee on Immunization Practices (ACIP, 1996) in the United States has suggested that HAV vaccination should be considered for foodhandlers, although risk assessment will be different for each country given the great differences in seroprevalence of HAV. At present, most countries prefer to stress the use of stringent personal hygiene to prevent infections.

13. Recommended areas for research

- More developmental work is required on methods to detect viruses in food. Such methods should be reasonably simple (as few steps as possible), efficient (in terms of recovery of viruses), and reproducible. The crucial (and most difficult to achieve) step is extraction of virus particles from the food matrix. Research should focus on this process.

- A standard method to assess virus survival would allow acquisition of comparative data (e.g. responses of different virus types to the same set of environmental conditions). The features of this method would include similar inoculum size, sample size, sampling time, and statistical analysis. A project to develop such a method and apply it in various environments would provide useful data.

- Efforts to find a cell culture system that will allow propagation of NoV are vital. The above two recommended areas of endeavour will depend upon this to be applicable to NoV.

- Information is needed on virus survival on different food commodities including thermal resistance.

- Information is needed on duration of shedding and levels of virus shedding in persons with and without symptoms.

14. Considerations for governments

- The existing surveillance systems for foodborne viruses are incomplete. Basic virus detection and typing methods are not routinely available in many countries. Rapid detection and reporting networks for foodborne viruses need to be implemented in standard surveillance systems. These networks should combine laboratory and epidemiological information. A reporting strategy for international outbreaks should be established.

- The detection and prevention of foodborne viral infections should be organised. Foodborne viral infections are diagnosed with increasing frequency. This illustrates the existence of regular breaks in the microbial safety of food. Although the most common pathogens cause relatively mild, self-limiting illness, their high incidence illustrates the potential for large, international foodborne viral epidemics. This includes the risk of foodborne spread of more dangerous pathogens, such as HEV or enteroviruses that may cause paralytic illness. Person-to-person spread is very high. As a result,
an initial point-source outbreak may be amplified significantly.

- Incidents (foodborne outbreaks) should be evaluated carefully by governments, WHO, and NGOs to identify whether changes in the guidelines are needed.

15. Considerations for agriculture industries

- The emphasis should be on GAP. Primary products and raw materials, especially those of agricultural origin, must be protected from contamination by human, animal, domestic, or agricultural wastes that are known sources of viruses/microorganisms. Examples of such products are fresh berries and salad.
- Foodhandlers, including seasonal workers, need to be educated specifically about microbial safety guidelines and hygiene rules. This includes education about the risk of exposure to viruses through sick children in the household.
- Managers of agricultural businesses involving produce to be eaten raw need to exclude foodhandlers with symptoms consistent with exposure to infectious foodborne diseases until 48 h after recovery (Cowden et al., 1995). Foodhandlers returning to work need to be instructed that NoV can be shed for weeks following recovery from illness and should be made aware that stringent personal hygiene must be ensured.
- Microbial food safety guidelines should be revised to include viral food safety (e.g. for codes of practice).
- Incidents should be reported to public health authorities through existing networks.

16. Considerations for food manufacturing industries

- Food safety management systems (HACCP, GHP and GMP), safety guidelines, and best-practice documents need to include considerations on the possible risks that infectious foodborne viruses pose during and after processing. This underlines the importance of adherence to good personal hygiene.
- Primary products must not be produced in areas where water used for irrigation might constitute a health hazard to the consumer through the food.
- Foodhandlers, including seasonal workers, need to be educated specifically about the microbial safety guidelines and hygiene rules. This includes education about the risk of exposure to viruses through sick children in the household.
- Managers of food manufacturing industries should consider excluding foodhandlers with symptoms consistent with exposure to infectious foodborne diseases until 48 h after recovery (Cowden et al., 1995). Foodhandlers returning to work need to be instructed that NoV can be shed for weeks following recovery from illness and should be made aware that stringent personal hygiene must be ensured.
- Microbial food safety guidelines should be revised to include viral food safety (e.g. for codes of practice).
- Incidents should be reported to public health authorities through existing networks.

17. Considerations for the catering and food service industries

- Foodhandlers, including seasonal workers, need to be educated specifically about the microbial safety guidelines and hygiene rules. This includes education about the risk of exposure to viruses through sick children in the household.
- Managers of catering and food service industries need to exclude foodhandlers with symptoms consistent with exposure to infectious foodborne diseases until 48 h after recovery (Cowden et al., 1995). Foodhandlers returning to work need to be instructed that substantial numbers of NoV may be shed for weeks after recovery from illness.
- Incidents should be reported to public health authorities through existing networks.

18. Considerations for consumers

- Consumers and physicians need to be specifically educated about microbial safety guidelines and hygiene rules, including those for viruses.
Abbreviations and Definition

ELISA enzyme-linked immunosorbent assay  
EM electron microscopy  
FeCV feline calicivirus  
GAP good agriculture practice  
GHP good hygienic practice  
GMP good manufacturing practice  
HACCP hazard analysis critical control point  
HAV hepatitis A virus  
HEV hepatitis E virus  
HRV human rotavirus  
NGO nongovernmental organisation  
NoV Norovirus  
RT-PCR reverse transcriptase polymerase chain reaction  
SaV Sapovirus  
WHO World Health Organization

Foodhandler A foodhandler is defined as any person who works in an area where food is being prepared, produced, served, or packed, including those who handle immediate wrapping materials, bulk containers, and machines and those responsible for maintaining and cleaning the workplace, implements, machines, and vehicles. On sites handling “high-risk” foods, all personnel who work in food areas should be included. Workers who handle only pre-wrapped, canned, or bottled food are not considered to be foodhandlers.

Acknowledgements

ILSI Europe and the Emerging Pathogen Task Force would like to thank the authors of this report: Marion Koopmans and Erwin Duizer, National Institute for Public Health and the Environment (RIVM), the Netherlands; the scientific reviewer, Prof. Dean O. Cliver, University of California, USA; as well as the members of the Emerging Pathogen Expert Group on Viruses: Dr. Kate Bellamy, Unilever, United Kingdom; Dr. Nigel Cook, MAFF-Central Science Laboratory, United Kingdom; Dr. John Crowther, United Kingdom; Dr. Fritz Kley, Kraft Foods, Germany; Dr. Olivier Mignot, Nestlé, Switzerland; Prof. Louis Schwartzbrod, University of Nancy, France.

This work was supported by a grant from the Emerging Pathogen Task Force of the European branch of the International Life Sciences Institute (ILSI Europe). Industry members of this task force are: Campina, Friesland Coberco Dairy Foods, Groupe Danone, Kraft Foods, Masterfoods, Nestle, and Parmalat. For further information about ILSI Europe, call 32-2-771-0014 or email info@ilsieurope.be. The opinions expressed herein are those of the authors and do not necessarily represent the views of ILSI Europe.

Appendix A. Fact sheet

Foodborne infections by Noroviruses (small round structured viruses, SRSV).

A.1. Introduction

Human enteric caliciviruses cause gastroenteritis in humans. The human caliciviruses are assigned to two groups, the genera Norovirus (NoV) and Sapovirus (SaV). The NoVs are also known as “small-round-structured-viruses” (SRSV) and the SaVs as “typical caliciviruses”. The two virus groups differ epidemiologically. The NoVs cause illness in people of all age groups, whereas the SaVs predominantly cause illness in children.

Foodborne transmission of caliciviruses is well known for viruses in the NoV genus. Within this genus is a great diversity of virus types, with genetic differences and differences in the protein composition of the virus particles. To date, 15 distinct genotypes have been recognised, but their number is likely to increase. Infected persons develop immunity, which is short-lived and predominantly type-specific. As a result, one person can have multiple NoV infections, which in part explains the high incidence of NoV infection.

A.2. Clinical symptoms

After a 1–3-day incubation period, infected persons may develop low-grade fever, vomiting, diarrhoea, and headache as prominent symptoms. Symptoms usually subside within 2–3 days, although the course of illness may be protracted in the elderly. Deaths associated with NoV outbreaks have been reported, but a causative relationship remains to be
proven. The average attack rate is high (typically 45% or more). The virus is shed via stools and vomit, starting during the incubation period and lasting up to 10 days and possibly longer. NoV infections are highly contagious, resulting in a high rate of transmission to contacts.

A.3. Incidence

NoV infections are among the most important causes of gastroenteritis in adults, and often occur as outbreaks that may be foodborne. The spread can be epidemic. In The Netherlands, approximately 80% of outbreaks of gastroenteritis reported to municipal health services are caused by NoVs. More than half of these outbreaks occur in nursing homes, but this may be an overrepresentation resulting from selection bias. In The Netherlands, foodborne outbreaks are also reported through a network of food inspection services. Preliminary results from studies there suggest that NoVs may also cause a significant number of these outbreaks. Based on studies from the UK and the US, it has been estimated that a substantial proportion of foodborne infections may be caused by NoVs (67% estimated for the US by Mead et al., 1999). In addition to outbreaks, NoVs also cause numerous sporadic cases of gastroenteritis. Five percent of patients with gastroenteritis who consult a physician have NoV infection, compared with 4% for Salmonella. In addition, caliciviruses are by far the most common cause of sporadic gastroenteritis (NoV accounts for 11% of all cases). Monitoring of sewage samples confirmed that high levels of NoVs circulate in the general population.

A.4. Epidemiology

It has been established that many different types of NoV cocirculate in the general population, causing sporadic cases and outbreaks. However, occasionally, epidemics occur in which the majority of outbreaks are caused by a single genetic type (e.g. in the Netherlands in 1996).

These epidemics may be widespread and even global. The mechanisms behind the emergence of epidemic types are unknown. Hypotheses range from large-scale foodborne transmission of a single strain to spillover from a reservoir, possibly nonhuman. An indication for the latter was a recent study from Japan in which NoVs were found in stool specimens from pigs, using RT-PCR assays based on caliciviruses of humans (Sugieda et al., 1998). In the 1990s, the reported incidence of NoV increased, probably as a result of improved diagnostic methods and increased awareness.

A.5. Risk groups

Outbreaks of NoV gastroenteritis (not only foodborne) are common in institutions such as nursing homes and hospitals. The high attack rate in both residents and personnel at such institutions leads to major logistic problems (understaffing) during outbreaks. In addition, an unknown but probably large number of sporadic cases occur. The risk factors for these infections are currently under investigation in the United Kingdom and the Netherlands.

A.6. Routes of transmission

NoVs are transmitted by direct contact or indirectly via contaminated water, food, or from the environment. Many foodborne NoV outbreaks have been described, often caused by infected foodhandlers. The NoVs usually are shed in large quantities during the initial stages of the illness, with maximal titres as high as $10^8$ virus particles per gram of stool. Although there are some indications for aerogenic transmission of NoV, the importance of this route is still unclear. Infectious viruses can be transmitted not only at the time of illness but also during the incubation period and after recovery, with 30% of cases shedding virus for up to 3 weeks after infection.

In addition to foodborne transmission, waterborne transmission of NoV is common, both directly (e.g. during recreation) or indirectly. NoVs can survive outside the host, are resistant to common disinfectants and extreme pH fluctuations, and are highly infectious. As a result, transmission of virus via fomites is likely.

A.7. High-risk foods

Filter-feeding shellfish are notorious as a source of foodborne viral infections because they actively concentrate viruses from contaminated water. Infectious
viruses can be detected for up to 6 weeks without any loss in quality of the shellfish. Depuration, a practise that may reduce bacterial contamination, is not as effective in reducing the viral load of shellfish.

In addition to shellfish, many food items have been associated with NoV outbreaks. In the literature, several other manually handled foods have been implicated (desserts, fruits, vegetables, salads), but the message is that any food that has been handled manually and not heated (sufficiently) afterwards may be a source of infection.

A.8. Diagnosis in humans

NoV or SaV infections can be diagnosed by visualisation of virus particles by electron microscopy and with molecular methods (RT-PCR). However, in most countries these methods are not available for routine diagnostics. Stool viruses can be typed by sequence analysis or by reverse-line blotting, and genetic typing may be used to trace common source outbreaks. Using these techniques, outbreaks from geographically distinct regions have been linked.

A.9. Virus detection in food and water

Molecular methods have been adapted for the detection of NoVs in food and water. However, because little is known about their sensitivity under field conditions, these techniques are not yet routinely available. Quality control of food and water on the basis of the detection of indicator organisms for faecal contamination has proven to be an unreliable predictor for viral contamination. When NoVs are detected in food, typing assays can be used to establish transmission routes. However, these techniques are not routinely available in most laboratories.

A.10. Zoonotic transmission

Some groups of animal caliciviruses have a broad host range, and it is currently a matter of debate whether NoVs can be transmitted between humans and animals. Recently, caliciviruses indistinguishable from NoVs have been found in pigs in Japan and cattle in the United Kingdom, Germany, and the Netherlands. Data from the Netherlands suggest a very high prevalence of NoV in calf herds.

A.11. Prevention of foodborne NoV infections

Strict implementation of hygienic rules is currently considered the most important preventive measure. Foodhandlers with gastroenteritis should immediately be removed from the food chain. More problematic are outbreaks linked to asymptomatic, presymptomatic, and postsymptomatic shedders. The kinetics of viral shedding has been studied in only a few infected volunteers and may not reflect real-life situations in which people may have been infected with a low dose of infectious virus. Given the highly infectious nature of NoV and the documented risk of virus transmission to food during the incubation period, it is suggested that guidelines be developed that include the occurrence of gastroenteritis in contacts (e.g., children) of people working at critical points in the food chain. This should be based on data on the kinetics of viral shedding after natural infection.

For prevention of foodborne transmission, it obviously is also essential that food items be not grown or washed in faecally contaminated water. However, the globalisation of the food market has hampered the implementation of control measures to assure safe food, as it is often difficult to exactly trace the food.

Routine monitoring is not yet feasible: first, because there are no good methods, and, second, because end-product testing is not reliable to assure food safety on statistical grounds. Documented outbreaks of foodborne infections could be reported faster using a system such as the “rapid alert system for food” of the European Union. However, this would be much more informative if typing information of virus strains were included.

A.12. Disinfection

Norwalk virus (one of the prototypes NoV) is resistant to low pH and heat treatment (30 min at 60 °C). The virus reportedly is quite resistant to chlorine; the virus remains infectious after 30 min in the presence of 0.5–1 mg free chlorine/l. At higher concentrations (>2 mg free chlorine/l), the virus is inactivated. The effect of other disinfectants on NoV infectivity has hardly been studied because of the lack of a tissue culture system or animal model.
References


Nauheim, R.C., Romanovski, E.G., Araullo-Cruz, T., Kowalski, R.P., Turgeon, P.W., et al., 1990. Prolonged recoverability of
desiccated adenovirus type 19 from various surfaces. Ophthalmology 97, 1450–1453.
Inactivation of Caliciviruses

Erwin Duizer,* Paul Bijkerk, Barry Rockx, Astrid de Groot, Fleur Twisk, and Marion Koopmans

Diagnostic Laboratory for Infectious Diseases and Perinatal Screening, National Institute for Public Health and the Environment, 3720 BA Bilthoven, The Netherlands

Received 8 December 2003/Accepted 5 May 2004

The viruses most commonly associated with food- and waterborne outbreaks of gastroenteritis are the noroviruses. The lack of a culture method for noroviruses warrants the use of cultivable model viruses to gain more insight on their transmission routes and inactivation methods. We studied the inactivation of the reported enteric canine calicivirus no. 48 (CaCV) and the respiratory feline calicivirus F9 (FeCV) and correlated inactivation to reduction in PCR units of FeCV, CaCV, and a norovirus. Inactivation of suspended viruses was temperature and time dependent in the range from 0 to 100°C. UV-B radiation from 0 to 150 mJ/cm² caused dose-dependent inactivation, with a 3 D (D = 1 log₁₀) reduction in infectivity at 34 mJ/cm² for both viruses. Inactivation by 70% ethanol was inefficient, with only 3 D reduction after 30 min. Sodium hypochlorite solutions were only effective at >300 ppm. FeCV showed a higher stability at pH <3 and pH >7 than CaCV. For all treatments, detection of viral RNA underestimated the reduction in viral infectivity. Norovirus was never more sensitive than the animal caliciviruses and profoundly more resistant to low and high pH. Overall, both animal viruses showed similar inactivation profiles when exposed to heat or UV-B radiation or when incubated in ethanol or hypochlorite. The low stability of CaCV at low pH suggests that this is not a typical enteric (calici-) virus. The incomplete inactivation by ethanol and the high hypochlorite concentration needed for sufficient virus inactivation point to a concern for decontamination of fomites and surfaces contaminated with noroviruses and virus-safe water.

The viruses most commonly associated with outbreaks of gastroenteritis are the noroviruses (formerly known as small round structured viruses and Norwalk-like viruses). In the Western world, these highly infectious viruses reportedly cause 68 to 80% of gastroenteritis outbreaks (16, 28), but estimates as high as 90% for outbreaks have been reported (19). The relative contribution of the noroviruses to food- and waterborne outbreaks is estimated to vary between 5 and 15% (16, 28). The resistance of noroviruses to culturing (14) has hampered the development of reliable methods for their detection and viability testing. Therefore, knowledge of efficient inactivation methods and effective intervention in transmission pathways is limited and based on studies with model viruses (13, 22, 39, 40; reviewed in reference 23).

The noroviruses are members of the family Caliciviridae. Caliciviruses are important veterinary and human pathogens and cause a wide range of symptoms by infecting a variety of organs (8, 9). The human caliciviruses, i.e., the noroviruses and the sapoviruses, are all enterotropic and cause gastrointestinal illness (27, 37). Most caliciviruses isolated from domestic cats (feline calicivirus [FeCV]) cause severe respiratory illness and limping disease, but some strains were etiologically associated with diarrhea (20, 30). Calicivirus-related disease in dogs is less common, but some canine calicivirus (CaCV) strains were isolated from dogs with diarrhea and vesicular genital lesions (31, 32). It is plausible to assume that viruses that use the same transmission route and replication site possess comparable stabilities. The (food-borne) enteric viruses survive the harsh conditions in the gastrointestinal tract, such as low pH and high bile concentrations (10, 30), and in general, enteric viruses are considered more resistant to environmental factors and disinfection methods than respiratory viruses. Therefore, in these studies, we determined the inactivation profiles of the widely studied norovirus surrogate FeCV-F9 (respiratory) vaccine strain and the antigenically unrelated reported enteric CaCV no. 48 strain.

In one series of experiments, we applied a variety of physical and chemical conditions to assess the stability of three different caliciviruses (FeCV, CaCV, and norovirus) by three different methods, culture and conventional and quantitative reverse transcription (RT)-PCR. The viruses in suspension were stored at 4°C and room temperature to determine environmental survival. They were exposed to heat and multiple cycles of freeze-thawing to obtain data that may be used to assess the survival of caliciviruses (i.e., noroviruses) during food processing and storage. Resistance to high and low pH was studied to assess the likelihood of stomach passage for the enteric viruses. Viruses were exposed to UV-B radiation as a factor in environmental survival and to 70% ethanol and sodium hypochlorite dilutions (0 to 6,000 ppm) to determine effective inactivation methods for cleaning practices. The methods found to be effective for complete inactivation of FeCV and CaCV were applied to a norovirus (genogroup II.4)-positive stool sample suspension, and the presence of viral RNA was determined by conventional RT-PCR and a newly developed quantitative real time RT-PCR.

MATERIALS AND METHODS

Viruses and cells. Crandell-Reese feline kidney (CRFK) cells and Madin-Darby canine kidney (MDCK) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, gentamicin (50 μg/ml), and 1% (vol/vol) nonessential amino acids.
The maintenance medium for virus propagation was identical but with 0 to 2% (vol/vol) fetal calf serum. FeCV strain F9 (kindly provided by H. Egberink, Utrecht University, The Netherlands) was propagated in monolayers of CRFK cells. CaCV strain no. 48 (kindly provided by F. Roerink, Kyoritsu Shoji Corporation, Japan) was propagated in MDCK cells. Cell and virus cultures were done at 37°C in an atmosphere of 5% CO₂ and 90% relative humidity.

Virus stocks used in the inactivation experiments were obtained by inoculation of young cells (24 to 48 h after seeding) at a multiplicity of infection of 0.1. The suspensions were harvested after 24 h by one cycle of freezing and thawing and centrifuged by clarifying centrifugation (10 min, 1,800 × g, 4°C). The clarified stocks contained 2 to 30 mg/ml of total protein. 

Inactivation by chlorine. Virus stocks were inoculated with 2 to 10% sodium hypochlorite solution to the virus stock solutions. After 2 min, the suspensions were neutralized by 10-fold dilution in DMEM, and samples were analyzed immediately. Samples collected in the thermal inactivation and pH stability series were frozen and stored at −20°C prior to analysis. The titers of infectious virus were determined as TCID₅₀ in cell culture (in the 96-well format).

Thermal inactivation. Virus stocks were dispersed in 250-µl fractions and kept at 4°C for time periods varying from weeks (4°C) to seconds (71.3°C). Samples were collected and stored at −20°C until titration. Effects of freeze-thawing cycles (up to five cycles) were studied by freezing dispensed samples (100 ml) transfer from room temperature to −70°C and subsequent thawing in a water bath at room temperature.

UV irradiation. Viral suspensions (250 µl) were exposed to UV-B radiation in 24-well plates (area: 1 cm²) on melting ice (to prevent heating effects and evaporation) for 0 to 30 min. The UV source (STX-35 ML; UVItec, Cambridge, United Kingdom) emitted UV-B (280 to 320 nm) at 0.43 mJ cm⁻² s⁻¹, measured at the sample level with the Optronic OL-752-PMT spectroradiometer (Orlando). Samples were titrated immediately after exposure.

pH stability. To test stability at pH 3, virus suspensions were incubated in citric acid buffer at pH 3 for 30 min at 37°C. Acidic pH was neutralized through dilution with phosphate buffer, pH 8.1. Additionally, 5 M HCl and 1 M NaOH stock solutions were used to make virus suspensions in DMEM covering the pH range from 1 to 14. The suspensions were incubated at 37°C for 30 min. Low and high pHs were neutralized by 10-fold dilution in DMEM, and samples were titrated immediately.

Inactivation by ethanol. Virus suspensions were diluted in 96% (vol/vol) ethanol to yield a final concentration of 70% (vol/vol) ethanol. Virus was exposed to 70% (vol/vol) ethanol for up to 1 h at room temperature (18 to 22°C). After the exposure time, the suspensions were diluted 10-fold to lower the ethanol concentration, hence reducing cytotoxicity, and titrated immediately.

Inactivation by chloride. Sodium hypochlorite solution (6 to 14% active chlorine; Merck, Darmstadt, Germany) was diluted to 1% in demineralized water to prepare suspensions containing 0 to 300 ppm free chlorine. The free chlorine concentration in the stock was determined with the N,N-diethyl-p-phenylenediamine method and a chlorine test kit according to the manufacturer’s instructions (Hach). The higher hypochlorite concentrations were prepared by adding the undiluted sodium hypochlorite solution to the virus stock solutions. After exposures of 10 and 30 min at room temperature, the free chlorine was neutralized by 1% (wt/vol) Na₂S₂O₃. No decrease in virus titer was detected when a virus suspension was added to a mixture of sodium hypochlorite solution and Na₂S₂O₃.

RT-PCR. Viral RNA was extracted by the Boom method as described before (3, 41). Reverse transcription of the extracted RNA was done for 60 min at 42°C after annealing with an in-house CaCV YGDD primer, FeCV-YGDD primer, or the JV13I primer for CaCV, FeCV, and norovirus (Table 1), respectively. RT primers were used at 50 pmol in 15 µl of 10 mM Tris-HCl (pH 8.3)–50 mM KCl–3 mM MgCl₂–1 mM deoxynucleoside triphosphates–40 U of RNAGuard per ml–5 U of avian myeloblastosis virus reverse transcriptase (Promega, Leiden, The Netherlands); 5 µl of the mix was added to 45 µl of a PCR mix containing 1:0 mM MgCl₂–0.12 mM MgCl₂ (final concentration, 1.5 mM), 0.2 mM deoxynucleoside triphosphates, 2.5 U of AmpliTaq, and 0.3 mM BRI for CaCV and JV12Y for norovirus. Samples were denatured for

<table>
<thead>
<tr>
<th>Table 1. Primers, targets, and amplicon characteristics</th>
<th>Target</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FeCV</strong></td>
<td>5'-CTC AGA ACC ACA TTT GGA TCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3'-GAT GCG GCC TCG GTG GGC</td>
<td></td>
</tr>
<tr>
<td><strong>CaCV</strong></td>
<td>5'-TAC TAC GGT GGC TTT GGT GGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3'-CGC GTA GAT GGC GTG GGC</td>
<td></td>
</tr>
<tr>
<td><strong>Norovirus</strong></td>
<td>5'-CTG GGG WGT YGA YGT TGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3'-TAA TAC GGT GTC TGT GGA TGG</td>
<td></td>
</tr>
<tr>
<td><strong>RT-PCR</strong></td>
<td>5'-CCAT GAG GGT CGG TCA TCT TGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3'-GCT CTC ACT TAC GAG YGA GTA</td>
<td></td>
</tr>
<tr>
<td><strong>ORF1</strong></td>
<td>5'-CTG GGG WGT YGA YGT TGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3'-TAA TAC GGT GTC TGT GGA TGG</td>
<td></td>
</tr>
</tbody>
</table>
3 min at 94°C and subjected to 40 cycles at 94°C for 1 min, 37°C for 1.5 min, and 74°C for 1 min. PCR products were analyzed by agarose gel electrophoresis.

Real-time RT-PCR. Viral RNA was extracted by the Boom method as described before (3, 41). Reverse transcription was performed at 42°C for 60 min with 1 µl of reverse primer (50 pmol/µl), 2.5 µl of genomic RNA, 0.5 µl of avian myeloblastosis virus reverse transcriptase (10 U/µl), 1.5 µl of 10× PCR buffer (Promega), 1.8 µl of MgCl2 (25 mM), 1.5 µl of deoxynucleoside triphosphates (10 mM; Roche), and 6.2 µl of water; 2 µl of the cDNA was used in a Lightcycler (Roche) PCR with forward primer (6 pmol), 2 µl of SyberGreen I (Roche), and water up to 20 µl. The PCR program had an initial denaturation step at 94°C for 3 min, followed by 40 cycles of denaturation for 1 s at 95°C, 10 s of annealing at 42°C (FeCV and CaCV) or 46°C (for norovirus), and 13 s of elongation at 72°C. Immediately following PCR, a melting curve was performed by raising the temperature from 65 to 95°C in 0.1°C increments. See Table 1 for primers and PCR product fragment lengths. The primers used for FeCV were published before (21).

RESULTS

Thermal inactivation. The inactivation of both viruses at 4°C was <1 D (D = 1 log10, calculated by dividing the TCID50 of the treated sample by the TCID50 of the untreated sample) in 2 weeks (data not shown). Data for the times required to achieve 3 D inactivation at higher temperatures are presented in Fig. 1. Virus inactivation was found to be temperature dependent and comparable for both viruses. At 20°C a 3 D reduction in infectivity occurred in 1 week. A steep decrease in infectivity was found between 37 and 56°C over a period of 24 h to 8 min. Heating to 71.3°C (pasteurization temperature) resulted in 3 D inactivation in 1 min for both viruses. The reduction of infectivity by five cycles of freezing and thawing was 0.44 ± 0.12 D and 0.34 ± 0.18 D for CaCV and FeCV, respectively (n = 4).

UV inactivation. The inactivation of FeCV and CaCV by UV-B radiation was dose dependent and comparable for both viruses (Fig. 2). In the setup we used, 2 D inactivation was found at 21 and 22 mJ/cm² for CaCV and FeCV, respectively, and 3 D inactivation was found after exposure to 34 mJ/cm² for both animal caliciviruses.

pH stability. For CaCV, more than 5 D inactivation was found for pH values of 5 and lower and 10 and higher (Fig. 3). At alkaline pHs, the FeCV appeared less resistant than the CaCV, with an almost 4 D reduction at pH 9 for FeCV against a 3 D reduction for CaCV. At acidic pHs, the FeCV is more stable, and at pH 6 the infectivity of FeCV is reduced 2 D, while the CaCV infectivity is down by 4 D. More than 5 D inactivation was found for FeCV only at a pH of 2 and lower or 10 and higher.

Inactivation by ethanol. Viral inactivation by 70% ethanol was comparable for both viruses (Fig. 4). After 8 min, less than a 2 D reduction in TCID50 was found, and a 3 D reduction was seen after 30 min for both viruses.

Inactivation by sodium hypochlorite. Viral inactivation by sodium hypochlorite in the virus-DMEM suspensions was ineffective (<1 D inactivation) up to concentrations of 30 ppm free chlorine (Fig. 5). Inactivation by 300 ppm was effective (>3 D inactivation) for CaCV but clearly less effective for FeCV (<2 D inactivation). An increase in exposure time from 10 to 30 min did not result in a significant increase in the effectiveness of sodium hypochlorite for FeCV inactivation, but CaCV inactivation was increased 1 D at 300 ppm chlorine (data not shown). Complete inactivation (>5 D) of FeCV and CaCV was found for chlorine levels of 3,000 ppm (or higher) in 10 and 30 min at room temperature.

Detection of viral RNA of inactivated viruses. The viral RNA contents of virus stock dilutions and completely inactivated virus suspensions were determined with conventional and real-time RT-PCR methods (Table 2). The virus suspen-
sions contained approximately $6 \times 10^5$, $6 \times 10^6$, and $1.2 \times 10^6$ PCR units/ml for FeCV, CaCV, and norovirus, respectively, as determined by endpoint dilution in conventional RT-PCR. The dilution series of all three virus suspensions showed an increase in cycle threshold ($C_t$ value) in the real-time RT-PCR with increasing dilution and an average difference in $C_t$ value ($dC_t$) of 4.1 cycles per 10-fold dilution for FeCV and CaCV and 3.8 for norovirus (Fig. 6).

A less than 1 $D$ reduction in PCR units by exposure to 37°C for up to 168 h was determined for all three caliciviruses, while for the FeCV and CaCV the infectivity was reduced more than 5 $D$ (Table 2). Boiling for 1 min resulted in a less than 1 $D$ reduction in RNA content for FeCV and norovirus, while the reduction of CaCV RNA was slightly higher. After 3 min at 100°C, the RNA reduction was considerable for all three viruses, and again the effect on CaCV RNA content was higher than the effect on FeCV RNA. Complete destruction of the infectivity of CaCV and FeCV by UV irradiation was concomitant with minor reductions in PCR-detectable RNA units for the two animal viruses and the norovirus. For hypochlorite treatment, the elimination of infectivity was concomitant with substantial reductions in detectable RNA, with higher reductions at the higher hypochlorite concentration. Again, the reductions of CaCV RNA contents were found to be higher than the reductions found for FeCV RNA. Exposure to pH 2 resulted in largely decreased RNA contents of both animal caliciviruses, as determined with both PCR protocols. In contrast, no reduction of RNA content for norovirus could be detected.

**DISCUSSION**

In order to increase insight into transmission routes and methods available to inactivate human enteric noroviruses, we studied the inactivation of the two animal caliciviruses FeCV and CaCV. The respiratory FeCV has been used as a model for norovirus in inactivation studies before (13, 30, 33, 34, 39, 40), but little is known about the stability of CaCV. We found that both animal caliciviruses showed long-term survival at temperatures of up to 20°C when incubated in suspension. The 3 $D$

### Table 2. RT-PCR detection of calicivirus RNA after infectivity-abolishing treatments

<table>
<thead>
<tr>
<th>Treatment leading to complete inactivation</th>
<th>FeCV</th>
<th>CaCV</th>
<th>Norovirus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT-PCR test result</td>
<td>Q-RT-PCR $dC_t$</td>
<td>RT-PCR test result</td>
</tr>
<tr>
<td><strong>Heat</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37°C, 120 h</td>
<td>++</td>
<td>0.8</td>
<td>++</td>
</tr>
<tr>
<td>37°C, 168 h</td>
<td>++</td>
<td>0.9</td>
<td>++</td>
</tr>
<tr>
<td>100°C, 1 min</td>
<td>++</td>
<td>7.8</td>
<td>++</td>
</tr>
<tr>
<td>100°C, 3 min</td>
<td>++</td>
<td>2.2</td>
<td>++</td>
</tr>
<tr>
<td><strong>UV</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>103 mJ/cm², 0°C</td>
<td>++</td>
<td>3.6</td>
<td>++</td>
</tr>
<tr>
<td>206 mJ/cm², 0°C</td>
<td>++</td>
<td>0.2</td>
<td>++</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2, 30 min, 37°C</td>
<td>++</td>
<td>11.3</td>
<td>++</td>
</tr>
<tr>
<td>3, 30 min, 37°C</td>
<td>++</td>
<td>11.3</td>
<td>++</td>
</tr>
<tr>
<td>10, 30 min, 37°C</td>
<td>++</td>
<td>11.3</td>
<td>++</td>
</tr>
<tr>
<td>12, 30 min, 37°C</td>
<td>++</td>
<td>11.3</td>
<td>++</td>
</tr>
<tr>
<td><strong>Hypochlorite</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3,000 ppm, 10 min, RT</td>
<td>++</td>
<td>8.2</td>
<td>++</td>
</tr>
<tr>
<td>6,000 ppm, 2 min, RT</td>
<td>++</td>
<td>13.0</td>
<td>++</td>
</tr>
<tr>
<td>6,000 ppm, 10 min, RT</td>
<td>++</td>
<td>14.0</td>
<td>++</td>
</tr>
</tbody>
</table>

* In infectivity-abolishing treatments gave a reduction of >5 log_{10} in TCID_{50} for FeCV and CaCV.
* One symbol per test. +, viral RNA detected; --, viral RNA not detected.
* $dC_t$: average delay in cycle threshold ($C_t$) (two experiments, $n = 2$). Q, quantitative.
* RT, room temperature.
* Inactivation of FeCV not complete.
inactivation in a week that we found for FeCV F9 was less than the approximately 5 D inactivation reported by Doultree et al. (13) at this temperature, while Allwood et al. (1) reported only a 1 D reduction in a week at 25°C. Another report showed survival (less than a 1 D reduction in infectivity) of other FeCV strains in marine water in 1 month at low temperatures (4°C), and even at 20°C a 1 D reduction took 25 days (22). Even though notable differences in time to 1 D strains in marine water in 1 month at low temperatures ranging from 37 to 100°C, hotels (4, 7), a rehabilitation center (25), and a concert hall (15).

Heat inactivation was highly comparable for both viruses at temperatures ranging from 37 to 100°C. The slope of the temperature inactivation curve becomes less steep at temperatures exceeding 56°C, meaning that small increases in temperature do not lead to significant decreases in time to 3 D reduction of infectivity. This indicates that better inactivation of viruses may be expected from regular batch pasteurization (63°C for 30 min) or classical pasteurization (70°C for 2 min) than from high-temperature, short-time pasteurization (72°C for 15 s). Moreover, our results suggest that high-temperature, short-time pasteurization would accomplish <1 D inactivation of caliciviruses, indicating that this will not be the preferred method to make foods or drinks virus safe.

The data that we show on thermal inactivation of FeCV in aqueous solution are comparable to the data from Lee and Gillespie (26) and Doultree et al. (13). Remarkably, Słomka and Appleton (39) reported a comparable 3 D inactivation in 0.5 to 1 min of FeCV in contaminated cockles at 80°C, indicating that FeCV, unlike hepatitis A virus (2, 29), is not significantly stabilized by factors such as fat and high protein content.

To the best of our knowledge, this is the first study reporting calicivirus inactivation by UV-B (280 to 320 nm) radiation. Inactivation rates of FeCV by 253.7-nm radiation was reported to be highly variable, ranging from 21 to 26 (40) and 12 mJ/cm² (11) for 3 D reduction in infectivity to 48 mJ/cm² for only 1 D inactivation (34). The UV-B dose we found to be effective in reducing FeCV infectivity (34 mJ/cm² for a 3 D reduction) is intermediate to the doses reported to be required to achieve similar reduction by 253.7-nm radiation. However, additional studies applying both radiations (253.7 nm and 280 to 320 nm) in similar doses to identical virus stocks are warranted.

Since the sensitivity of both animal caliciviruses to UV-B radiation was so much alike, we consider the effectiveness of UV radiation for the inactivation of caliciviruses to be intermediate, i.e., comparable to that for the enteroviruses (18), less effective than for vegetative bacteria, but more effective than for phage MS2 (11) and for adenovirus 2 (18), adenovirus 40 (40) and Bacillus subtilis spores (6).

The stability of FeCV in a wide pH range, i.e., less than complete inactivation for 3 < pH < 9, was clearly higher than the pH-dependent stability of CaCV. The low stability of CaCV at low pH was remarkable and suggests that this is not a typical enteric calicivirus, since it seems unlikely that CaCV will survive gastric passage. This might at least partly explain the lack of apparent clinical symptoms after experimental oral inoculation of dogs with CaCV no. 48 (38). With respect to the pH stability of the enteric noroviruses, it was shown that 3 h at pH 2.7 (at room temperature) was not enough to completely inactivate Norwalk virus (12), suggesting that for the modeling of noroviruses in gastrointestinal conditions (i.e., low pH and high bile concentrations), other enteric viruses (e.g., poliovirus, hepatitis A virus, or rotavirus) might be better than the animal caliciviruses.

Overall, the two animal viruses showed similar high stabilities when incubated in 70% ethanol or hypochlorite solutions. The resistance of FeCV to chemical inactivation by ethanol and hypochlorite was shown by Doultree et al. (13) for short contact times. They extrapolated the 1-min contact time to recommend using hypochlorite solutions at 1,000 ppm and a 10 min contact time. We found that at concentrations of up to 300 ppm, the effect of longer exposure times was insignificant for FeCV and only modest for CaCV, indicating that extrapolation in time may be open to discussion. The incomplete inactivation by ethanol and the high chlorine concentrations needed for inactivation of both animal caliciviruses point to a concern for decontamination of fomites and surfaces contaminated with human noroviruses. The high chlorine resistance of the animal caliciviruses might indicate that the chlorine levels used in potable water treatment practices (~20 ppm) do not cause a significant reduction of human norovirus infectivity.

A recognized problem with PCR detection of viral RNA is that the presence of RNA does not necessarily point to the presence of infectious virus (17, 36). We show that for most methods applied at levels where infectivity could no longer be detected, viral RNA remained detectable. Higher concentrations (chlorine), lower or higher pH, or longer exposure times (heat and UV radiation) were needed to decrease the amount of detectable viral RNA than to abolish infectivity.

For most treatments, we found a good correlation between the decline of animal calicivirus RNA and the decline of norovirus RNA. This could indicate that the degree of protection of the RNAs by the viral capsids is comparable for all three viruses in the case of heat treatment, UV irradiation, or exposure to free chlorine. However, a remarkable difference in capsid stability was found after exposure to low pH. The RNA of norovirus was still fully protected after exposure to pH 2 for 30 min at 37°C, while the amount of RNA of FeCV and CaCV was greatly reduced. This indicates that the truly enteric norovirus differs greatly from both animal caliciviruses with respect to acid stability, indicating again that CaCV is most likely not a true enteric pathogen.

Using the PCR protocols described, we found some discrepancies between the detectability of RNA by conventional PCR and by real-time PCR. For example, FeCV exposure to pH 2 resulted in a less than 2 D reduction of real-time PCR-detectable RNA, while RNA detectable by the conventional PCR was reduced over 7 D. Similar but less dramatic effects were seen after boiling FeCV for 3 min and after pH 2 and 3 treatment of CaCV. We suspect that this may at least in part be explained by the much smaller size of the PCR products in the real-time PCR compared to the products of conventional PCR.

Although we showed that abolishment of infectivity was not always correlated to diminished detectability of the genome by PCR, we do believe that quantitative RT-PCR might be a
valuable tool in detecting reduction in infectivity, because a delay in C0 was always correlated to (some degree of) viral inactivation. Another PCR-based method to reduce positive RT-PCR results was based on treatment with proteinase K and RNase of UV-, hypochlorite-, or heat-exposed viruses prior to the reverse transcription step (33). A combination of both methods (i.e., proteinase K and RNase treatment and quantitative RT-PCR) may yield a more sensitive PCR-based method to detect viral inactivation.

On the basis of the data presented here on comparable inactivation rates for two animal caliciviruses (FeCV and CaCV) by a variety of methods, we postulate that the human enteric caliciviruses may display similar inactivation rates. In conclusion, we prefer the use of FeCV as model for the human enteric noroviruses over the use of CaCV due to the greater convenience of FeCV in the laboratory (i.e., higher titers after standard culture and easier recognition of cytopathic effect in titrations) and greater stability at low pH. But even though the value of FeCV as a model for the noroviruses might be significant and preferable to the use of other virus families, extrapolation to a typical enteric virus might lead to over- or under-estimation of the risk of infection, stressing again the need for an in vitro method for the detection of norovirus viability.

ACKNOWLEDGMENTS

This work was supported by the Netherlands Center Alternatives to Animal Use (PAD 97-31), the Netherlands Organization for Scientific Research (NWO 01412028), and the European Commission Quality of Life Program, 5th Framework (QLKI-CT-1999-00594).

We are grateful for the excellent assistance and feedback of Bas van der Veer, Erwin de Bruin, and Harry Vennema.

REFERENCES


The survival of hepatitis A virus in fresh produce

Luciana Croci*, Dario De Medici, Concetta Scalfaro, Alfonsina Fiore, Laura Toti

Laboratorio Alimenti, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy

Received 25 March 2001; received in revised form 27 July 2001; accepted 18 September 2001

Abstract

Fresh produce has been repeatedly implicated as the source of human viral infections, including infection with hepatitis A virus (HAV). The objective of the present study was to evaluate the HAV adsorption capacity of the surface of various fresh vegetables that are generally eaten raw and the persistence of the HAV. To this end, the authors experimentally contaminated samples of lettuce, fennel, and carrot by immersing them in sterile distilled water supplemented with an HAV suspension until reaching a concentration of 5 log tissue culture infectious dose (TCID₅₀)/ml. After contamination, the samples were stored at 4°C and analysed at 0, 2, 4, 7, and 9 days. To detect the HAV, RT-nested-PCR was used; positive samples were subjected to the quantitative determination using cell cultures. The three vegetables differed in terms of their adsorption capacity. The highest quantity of virus was consistently detected for lettuce, for which only a slight decrease was observed over time (HAV titre = 4.44 ± 0.22 log TCID₅₀/ml at day 0 vs. 2.46 ± 0.17 log TCID₅₀/ml at day 9, before washing). The virus remained vital through the last day of storage. For the other two vegetables, a greater decrease was observed, and complete inactivation had occurred at day 4 for carrot and at day 7 for fennel. For all three vegetables, washing does not guarantee a substantial reduction in the viral contamination. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Hepatitis A virus; Vegetables; Survival

1. Introduction

The role of food in the transmission of the hepatitis A virus (HAV) has been extensively documented (Cliver, 1997; Mele et al., 1997; Codex Alimentarius Commission, 1999), with the most commonly implicated foods being molluscs (Richards, 1985; Desenclos et al., 1991; Halliday et al., 1991; Lees, 2000) and fresh produce (Beauchat, 1995; Appleton, 2000). With a particular regard to fresh produce, this type of food has been reported to be the source of a fairly large number of cases of HAV infection in various countries (Ramsay and Upton, 1989; Mauezahl et al., 1996; Hernandez et al., 1997; Pebody et al., 1998; Dentinger et al., 2001), not to mention its involvement in an increasing number of viral infections in general, especially gastroenteritis caused by Caliciviruses (White et al., 1986; Ponka et al., 1999). Particularly dangerous are those products with a relatively brief period of growth and which are eaten raw (e.g., green salad) (Rosenblum et al., 1990; Pebody et al., 1998), for which viral contamination in general has often been reported (Monge and Arias, 1996; Hernandez et al., 1997).

The contamination of fresh produce can occur during growth, as a result of the use of contaminated fertilizers or waste-water (Tierney et al., 1977; Badawy
et al., 1990), and during the preparation and distribution, with the virus being transmitted by infected food-handlers when certain hygienic norms are not followed (Griffin et al., 1982; Rosenblum et al., 1990; Dalton et al., 1996). In fact, experimental studies have shown that approximately 9.2% of the infectious virus can be transferred from the contaminated hands of the handlers to lettuce (Bidawid et al., 2000).

The objective of the present study was to evaluate the adsorption capacity of the surface of various fresh vegetables and the persistence of the HAV. To this end, samples of experimentally contaminated lettuce, fennel, and carrots were stored at 4°C and analysed at selected intervals for the presence of the HAV using RT-nested-PCR before and after washing. The positive samples were subjected to the quantitative determination on cell cultures, both to quantify the presence of infectious virus and to evaluate the eventual decreases in viral concentration.

2. Materials and methods

2.1. Virus

HAV was adapted and titrated, as previously described, in Frp-3 cells (Venuti et al., 1985) derived from FRhK-4. The virus had been isolated from the stool of a person with acute HAV infection and was kindly provided by Professor A. Panân (Tor Vergata University, Rome).

2.2. Samples

Samples of lettuce (Lactuca sativa), fennel (Foeniculum vulgare), and carrot (Daucus carota), were taken directly from produce markets. The samples were cut into small pieces (on the average of not larger than 9 cm²) and experimentally contaminated with HAV, as described below.

2.3. Sample contamination

For each vegetable, 200 g was divided into two aliquots of 100 g each and treated as follows:

1. One aliquot (100 g) was immersed in 800 ml of sterile, distilled water supplemented with a suspension of HAV until obtaining a concentration of 5 log tissue culture infectious dose (TCID₅₀)/ml (Reed and Muench, 1938).

2. The other aliquot (100 g) was also immersed in 800 ml of sterile distilled water yet without the addition of the virus; this aliquot was used as the control.

The samples were immersed for 20 min, strained, and left out to dry.

Both the contaminated and control aliquots were further divided into five aliquots (20 g each) and stored at 4°C. At days 0, 2, 4, 7, and 9, one contaminated aliquot (20 g) and one control aliquot (20 g) were collected and further divided into two aliquots each (10 g). One of the 10-g aliquots was analysed without washing; the other was washed with 100 ml of potable water for approximately 5 min, to simulate domestic practices; it was then left out to dry.

For each vegetable, the experiments were repeated three times.

2.4. Virus extraction

2.4.1. Elution

Ten grams of the sample, supplemented with 25-ml 3% Beef Extract (Lab-Lemco Powder—Oxoid) (pH 9.5), was shaken for 20 min. Then, the Beef Extract, filtered with 0.22-µm millipore filters, was divided into two aliquots of 12.5 ml each (equivalent to 5 g of the vegetable) and stored at −80°C until use. The first aliquot was tested using RT-nested-PCR; if positive for the presence of viral RNA, the second aliquot was used for the quantitative determination on cell cultures.

2.5. RT-nested-PCR

2.5.1. Extraction of viral RNA

The 12.5-ml aliquot of Beef Extract was centrifuged at 3000 × g for 10 min at 4°C, and the supernatant was subjected to ultracentrifugation at 200,000 × g for 2 h at 4°C (Beckman L7-55). The pellet was resuspended in 334 µl of sterile tridistilled water and transferred to an Eppendorf vial (1.5 ml) containing 666 µl of Solution D (Afzal and Minor, 1994), vortexing for 30 s. One hundred microliters of CsCl (Baker Analyzed Reagent-Deventer, Holland)
cushion (5.7 M solution of CsCl in 25 mM sodium acetate, pH 5.0, RI = 1.4000) was gently placed at the bottom of the tube by piercing through the liquid. After centrifugation in an Eppendorf microfuge at 13,000 rpm for 20 min at 4 °C, the supernatant was discarded, and the pellet was washed twice with 1 ml of 70% ethanol and then dried.

2.5.2. RT-PCR

The dried pellet was resuspended in 90 μl of RT reaction mixture containing 1 × PCR Buffer II (Perkin Elmer, NJ, USA—Roche AO1658), 2.5 mM MgCl2 Solution (Perkin Elmer—Roche AO1555), 0.25 mM each of deoxynucleoside triphosphate 100 mM (dNTP) (Takara-Shuzo, Japan), 20 U of Rnase Inhibitor (Perkin Elmer—Roche AO2922), 1.25 U of MuLV reverse transcriptase (Perkin Elmer—Roche A00854), and 100 pmol of primer anti-sense (5′-CAGGGG-CATTAGGTTT-3′ HAV strain FG position 669–685) then, the mixture was incubated at 42 °C for 50 min. The reaction was terminated by heating the mixture at 95 °C for 3 min. One hundred picomoles of primer sense (5′-CATATGTATGGTATCTCAACAA-3′ HAV strain FG position 1063–1084), 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer), and DNase–RNase–free water (Sigma, USA W-4502) were added to a final volume of 100 μl. The mixture was subjected to 30 PCR cycles, each consisting of 25 s at 95 °C, 10 s at 49 °C and 1 min at 70 °C. A final extension was carried out for 5 min at 72 °C.

2.5.3. Nested-PCR

Five microliters of the first amplification reaction was further amplified in 95 μl of the reaction mixture containing 1 × PCR Buffer II (Perkin Elmer), 2.5 mM MgCl2 (Perkin Elmer), 0.25 mM each of dNTP (Takara), 100 pmol of primer anti-sense (5′-TAGGACTGCAGTGACT-3′ HAV strain FG position 807–825), 100 pmol of primer sense (5′-CCAATT-TTGGCAACTTCATG-3′ HAV strain FG position 1000–1018), and 2.5 U of Taq DNA polymerase (Perkin Elmer). The amplification conditions were those described for the first PCR amplification.

2.5.4. Electrophoresis

Ten microliters of PCR and nested-PCR mixture were analysed by agarose gel electrophoresis (2% agarose; Kodak, New Haven, CT, USA—IB70040).

2.6. Quantitative detection of HAV

If the first aliquot was positive for the presence of viral RNA, the second 12.5-ml aliquot of the extract was subjected to ultracentrifugation as described above, and the pellet was resuspended in 1 ml of Eagle minimum essential medium (EMEM) (Imperial, UK), supplemented with 100 μl antibiotics–antimycotic (Imperial) solution (1:100 v/v), and stored at 4 °C overnight.

The assay was conducted as previously described (Franco et al., 1990), using 24-well tissue culture plates and Frp-3 cell culture grown with EMEM

<table>
<thead>
<tr>
<th>Time (in days)</th>
<th>Qualitative determination</th>
<th>Quantitative determination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT-PCR</td>
<td>RT-nested PCR</td>
</tr>
<tr>
<td></td>
<td>Not washed</td>
<td>Washed</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Concentration of HAV in the contaminated water: 5 log TCID<sub>50</sub>/ml.
All samples used as control showed negative results.

<sup>a</sup> Mean of three determinations ± standard deviation.
<sup>b</sup> Two of the three determinations showed positive results.
supplemented with 10% Foetal Bovine Serum (FBS) (Imperial) at 37 °C and in 5% CO2 at 37 °C for 3 days. The cell monolayer was inoculated with sample extract (100 μl/well) and left in contact for 1 h at 37 °C and 5% CO2, in order to eliminate all the virus not infecting the cells. After adding 5 ml of EMEM at 2% FBS, the monolayer was then washed three times with 2 ml of EMEM at 2% FBS, and in 5% CO2 for 15 days. Four replicates were prepared for each dilution. The cytopathic effect was confirmed by RT-PCR, carried out as described above using the PCR primers. The viral titre was calculated using the method of Reed and Muench (1938) and was expressed in TCID₅₀/ml.

### 3. Results

The results of RT-PCR, RT-nested-PCR, and of the quantitative analysis at days 0, 2, 4, 7, and 9 are shown in Tables 1, 2 and 3, for lettuce, fennel, and carrot, respectively. The three vegetables differed in terms of their adsorption capacity. The highest quantity was consistently detected for lettuce, for which a progressive yet a slight decrease was observed over time (HAV titre = 4.44 ± 0.22 log TCID₅₀/ml at day 0 vs. 2.46 ± 0.17 log TCID₅₀/ml at day 9, before washing). The virus remained vital through the last day of storage (Table 1). For fennel, the quantity of virus at day 0 (4.32 ± 0.18 log TCID₅₀/ml before washing) was similar to that of lettuce, though it showed a greater

#### Table 2

<table>
<thead>
<tr>
<th>Time (in days)</th>
<th>Qualitative determination</th>
<th>Quantitative determination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT-PCR</td>
<td>RT-nested PCR</td>
</tr>
<tr>
<td></td>
<td>Not washed</td>
<td>Washed</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>9</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

n.d.: not determined.

Concentration of HAV in the contaminated water: 5 log TCID₅₀/ml.

All samples used as control showed negative results.

*Mean of three determinations ± standard deviation.

#### Table 3

<table>
<thead>
<tr>
<th>Time (in days)</th>
<th>Qualitative determination</th>
<th>Quantitative determination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT-PCR</td>
<td>RT-nested PCR</td>
</tr>
<tr>
<td></td>
<td>Not washed</td>
<td>Washed</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+/− b</td>
<td>−</td>
</tr>
<tr>
<td>4</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>7</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>9</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

n.d.: not determined.

Concentration of HAV in the contaminated water: 5 log TCID₅₀/ml.

All samples used as control showed negative results.

*Mean of three determinations ± standard deviation.

b Two of the three determinations showed positive results.
decrease over time. In fact, at day 7, the virus was not detected by RT-PCR but it could only be detected by RT-nested-PCR and the quantity was lower than the minimum level of detection for the quantitative determination (<1 log TCID₅₀/ml) (Table 2). The quantity of the virus detected on the surface of the carrot at day 0 (3.44 ± 0.24 log TCID₅₀/ml before washing) was approximately one logarithm lower than the quantities detected for lettuce and fennel, and at day 4 the viral load had decreased to nonquantifiable levels (Table 3). For all three vegetables, washing resulted in a decrease of approximately one logarithm in the quantity of virus detected.

4. Conclusions

The results of this study demonstrate that lettuce, fennel, and carrot maintain a high quantity of HAV on their surface after being immersed in contaminated water, though the quantity varies according to the specific vegetable. Lettuce seems to have the highest adsorption capacity and the most favourable conditions for viral persistence, probably because of the size and the wrinkled texture of its leaves (i.e., a larger amount of exposed surface, a greater protection for the virus). For fennel and carrot, the quantity of virus was not only lower than that for lettuce but it also decreased more rapidly, reaching complete inactivation within several days. For carrot, the low quantity of virus detected immediately after contamination and its rapid decline could be due to the presence of specific substances, which have previously been shown to exert antimicrobial activity (Babic et al., 1994). Furthermore, washing apparently does not guarantee the elimination of the virus. In view of the fact that these products are either eaten raw or used as basic ingredients for different recipes, the vegetables represent a potential hazard for consumers. To prevent the spread of the HAV and of other viral infections, effective information campaigns are fundamental and should target both producers and consumers, with the aim of promoting adequate hygienic measures both in the production and preparation (e.g., the use of potable water for irrigation and in the preparation of foods and the use of clean utensils in preparing foods).

The results of this study also demonstrate the ever-increasing importance of sufficiently rapid and sensitive methods for determining the HAV on produce. RT-nested-PCR seems to satisfy this need, especially for screening. However, it must be taken into account that the virus detected by RT-nested-PCR is not necessarily infectious. In fact, although free viral RNA (i.e., lacking the protection provided by the capsid) is known to survive briefly in the environment due to the presence of a high percentage of bacterial endonucleases (Tsay et al., 1995), RT-nested-PCR can reveal RNA originating from viral particles with a capsid that is partially denatured, yet still capable of protecting the RNA from degradation, although the RNA is not infectious (Ma et al., 1994; Hilfenhaus et al., 1997; Arnal et al., 1998). Thus, cell cultures should be used for detecting infectious virus and the consequent risk to consumers (Richards, 1999).

Acknowledgements

The authors gratefully acknowledge Mr. Mark Kanieff for the editorial assistance. This work was carried out as part of the project “Biotecnologie”, was supported by a grant from the National Council of Researches, and of the project “Foodborne viruses in Europe” (EU contract QLK1-CT-1999-00594) supported by EU 5th framework program.

References


The importance of foodborne viral infections is increasingly recognized. Food handlers can transmit infection during preparation or serving; fruit and vegetables may be contaminated by fecally contaminated water used for growing or washing. And the globalization of the food industry mean that a contaminated food item may not be limited to national distribution. International outbreaks do occur, but little data are available about the incidence of such events and the food items associated with the highest risks. We developed a combined research and surveillance program for enteric viruses involving 12 laboratories in 9 European countries. This project aims to gain insight into the epidemiology of enteric viruses in Europe and the role of food in transmission by harmonizing (i.e., assessing the comparability of data through studies of molecular detection techniques) and enhancing epidemiologic surveillance. We describe the setup and preliminary results of our system, which uses a Web-accessible central database to track viruses and provides the foundation for an early warning system of foodborne and other common-source outbreaks.

Food-related illness is common worldwide, and bacterial pathogens have historically been associated with this mode of transmission. In recent years, however, the cause of most outbreaks of foodborne illness remained unknown, although a significant proportion were presumed to be viral (1). Additional research established the importance of viruses, especially the human caliciviruses belonging to the genus *Norovirus* (NV) (2). Transmission of these viruses is primarily from person to person, but numerous examples illustrate that NV are efficiently transmitted in food, water, or contaminated environmental surfaces. NV similar to, but not identical with, human strains have been found in cattle and in pigs (3,4). Studies in which viruses were molecularly characterized have shown that numerous variants co-circulate in the community but that occasional shifts occur in which a single variant dominates over a wide geographic region (5). In 1995 to 1996, a worldwide epidemic was observed (6). The mechanism of emergence of these variants is unclear, but one hypothesis is that they represent widespread common-source events.

While it is clear that enteric viral infections are common, far less established is how common the foodborne mode of transmission is and how important it is in the epidemiology of these viruses. The challenge lies not so much in detecting outbreaks related to foodborne contamination at the end of the chain (the food handler in the nursing home or restaurant), because those are likely to be detected by routine outbreak investigation, with or without molecular typing. Linking NV outbreaks to common-source introductions nationally or internationally may be more difficult because of the high secondary attack rate that results from rapid person-to-person transmission. Thus, an initial seeding event will rapidly be masked by the occurrence of new cases or outbreaks, suggesting that person-to-person transmission is the primary mode of spread. The likelihood of detecting such seeding events relies on effective surveillance, which combines epidemi-

---

**Early Identification of Common-Source Foodborne Virus Outbreaks in Europe**

Marion Koopmans,* Harry Vennema,* Herre Heersma,* Elisabeth van Strien,* Yvonne van Duynhoven,* David Brown,† Marc Reacher,† and Ben Lopman,†

for the European Consortium on Foodborne Viruses

**RESEARCH**

The Netherlands: M. Koopmans, H. Vennena, Y. van Duynhoven, D. E. van Strien, W. van de Poel, National Institute of Public Health and the Environment; Bilthoven; Finland: C.-H. von Bonsdorff, L. Maunula, Helsinki University; Denmark: B. Böttiger, K. Mølbak, F.X. Hanon, Statens Serum Institute, Copenhagen; Sweden: L. Svensson, K.-O. Hedlund, Maria Thorvag, Juan Carrique-Mas, Swedish Institute for Infectious Disease Control, Solna; UK: D. Brown, M. Reacher, J. Green, B. Lopman, Public Health Laboratory Service, London; Germany: E. Schreier, H. Gelderblom, Andrea Ammon, Robert Koch Institute, Berlin; Spain: A. Sanchez-Faquier, G. Hernández-Peazzi, Instituto de Salud Carlos III, Madrid; A. Bosch, Universitat de Barcelona, Barcelona; J. Buesa, Universitat de Valencia; France: F. LeGuyader, IFREMER, Nantes; P. Pothier, E. Kohli, Laboratoire de Virologie, Dijon; Italy: F. Ruggeri, D. DeMedici, Instituto di Superiore di Sanità, Rome. In addition, outside the formal project group, M. Poljsak (Slovenia), and G. Szücs (Hungary) actively participate in the project.

*National Institute of Public Health and the Environment, Bilthoven, the Netherlands; and †Public Health Laboratory Service, London, United Kingdom
ologic assessment of the outbreak and molecular typing to discover and track potential links between outbreaks. Such molecular tracing, however, requires knowledge on diversity of “resident viruses” in the region under study to be able to recognize unusual increases. Therefore, we established a combined research and surveillance network for foodborne viruses that was granted by the European Commission. This project group combines complementary expertise from the fields of diagnostic virology, molecular virology, epidemiology, and food microbiology to study modes of transmission of NV across Europe. Mapping these pathways allows better founded estimates of the proportion of illness that may be attributed to foodborne transmission and identification of high-risk foods, processing methods, or import and transport routes, which subsequently can be a focus of prevention programs. The data are important for assessing the risks associated with consumption of certain food items. Essential to the success of this project is the establishment of a common, central database, which is now used by all partners to compare data across Europe as soon as they are available. We describe this project and results from its first 18 months of operation.

Materials and Methods

The network is a collaboration among 12 laboratories in 9 countries in Europe to allow more rapid and internationally harmonized assessment of the spread of foodborne viral pathogens. The project is coordinated by the National Institute of Public Health and the Environment in Bilthoven, the Netherlands. Participants are virologists and epidemiologists with active research programs in (foodborne) enteric viruses from Spain (Barcelona, Valencia, Madrid), Italy (Rome), France (Nantes, Dijon), Germany (Berlin), the Netherlands (Bilthoven), the United Kingdom (London), Denmark (Copenhagen), Sweden (Solna), and Finland (Helsinki). In addition, groups from Slovenia and Hungary participate.

The overall objectives for the complete study are as follows: 1) to develop novel, standardized, rapid methods for detection and typing of enteric viruses, particularly NV, to be used in all participating laboratories; 2) to establish the framework for a rapid, prepublication exchange of epidemiologic, virologic, and molecular diagnostic data; 3) to study the importance of enteric viruses as causes of illness across Europe, with a special focus on multinational outbreaks of infection with NV and hepatitis A virus; 4) to provide better estimates for the proportion of NV infections that can be attributed to foodborne infection; 5) to determine high-risk foods and transmission routes of foodborne viral infections in the different countries and between countries; 6) to describe the pattern of diversity of NV within and between countries and identify potential pandemic strains at the onset; and 7) to investigate the mechanisms of emergence of these strains, including the possibility of spillover from animal reservoirs.

The central research goal is to better understand the mechanisms of emergence of variant NV strains. We hypothesized that the observed epidemic shifts might be caused by displacement of endemic variants attributable to a large seeding event with a variant that subsequently spread through the population by secondary and tertiary waves of transmission, or possibly by a smaller seeding event of a highly transmissible new variant, generated by genetic mutation or recombination. To address these questions, we built a European surveillance structure for outbreaks of viral gastroenteritis, including food- or waterborne outbreaks. The first phase of the project, described in this report, was designed to review existing surveillance systems for viral gastroenteritis, to design and agree on a minimum dataset for collection during the second phase of the project; to review and evaluate currently used methods for detection and genotyping of NV with the aim of harmonizing methods for virus detection in gastroenteritis outbreaks; and to build a database of combined epidemiologic and virologic data for use by all participants. The overriding aim was to facilitate the early detection of potentially emerging variant strains. Upon completion of this phase, we will begin “enhanced surveillance”, i.e., harmonized surveillance for viral gastroenteritis outbreaks to study objectives 4–7.

Results

Review of Current Methods in Europe

From the outset, it was recognized that the best approach in developing an international surveillance scheme for foodborne viruses would not be the standardization of practice, but rather the harmonization of existing practices. To achieve this, a number of surveys were undertaken to determine diagnostic capabilities, genotyping techniques, and the status of surveillance of viral gastroenteritis outbreaks among project participants. The results of these surveys are highlighted below.

Virus Detection and Genotyping

The scale of diagnostic capability in laboratories varies widely, and a range of diagnostic tests (electron microscopy, reverse transcription–polymerase chain reaction [RT-PCR], and enzyme-linked immunosorbent assay) and characterization methods are used (including heteroduplex mobility assay, reverse line blot, microplate hybridization, and sequencing (7–9). Laboratories in all countries now use molecular techniques (RT-PCR) for NV detection (10).

A comparative evaluation of RT-PCR assays was done by analysis of a coded panel of stool samples that had test-
ed positive (81 samples) or negative (9 samples) for NV. Samples provided by four laboratories were included, as well as a samples representing the currently known diversity of NV genotypes. Full details of this study have been published (11). This evaluation showed that no single assay is best, although sensitivities range from 55% to 100%. Most differences were seen when comparing assay sensitivities by genogroup. Based on pre-set scoring criteria (sensitivity, specificity, assay format, length of sequence), one primer combination was ranked as the assay of first choice for laboratories starting surveillance, and protocols and reagents have been made available to all participants on request.

On the basis of the aggregated data from the sequence database, alignments were made of the regions in the viral RNA that contain the primer-binding sites for the set of primers with the highest ranking for the diagnostic evaluation to generate more optimal designs of primers (12). These primers, protocols, and reference reagents have been made available to several groups in the field.

**Outbreak Investigations**

While all countries in the network now have the diagnostic capability to recognize outbreaks of NV, the structure of their national surveillance differs and therefore, so do the epidemiologic data collected on viral gastroenteritis (10,13). Some countries investigate outbreaks of gastroenteritis irrespective of the size or possible mode of transmission (United Kingdom, the Netherlands); others primarily investigate outbreaks that appear as foodborne from the onset (Denmark, France) (10). Similarly, coverage of the laboratories involved ranges from regional (Italy) to national, although different levels of underreporting are likely to exist (10). These differences, as well as differences in the laboratory test protocols, will be taken into consideration when interpreting aggregated data in the later stages of the project. For the purposes of comparing data across Europe, however, the key finding was that most countries maintain a national database of NV outbreaks (as opposed to individual cases). Although the proportion of the population that these databases effectively survey and the completeness of clinical information collected vary, we recognized that we could network national outbreak surveillance by agreeing on a minimum dataset that would include the causative organism, mode and place of transmission, diagnostic results, case details, food vehicles, and viral typing information.

Also agreed upon were clinical definitions of a case and an outbreak of viral gastroenteritis based on Kaplan’s criteria (14), as follows. A case of gastroenteritis was defined as a person seen with 1) vomiting (two or more episodes of vomiting in a 12-hour period lasting ≥12 hours), or 2) diarrhea (two or more loose stools in a 12-hour period lasting ≥12 hours, or 3) vomiting as defined in 1) and diarrhea as defined in 2). An outbreak was defined as follows: 1) Patients living in more than one private residence or resident or working in an institution at the time of exposure; 2) cases linked by time and place; 3) vomiting in ≥50% of total cases (14); 4) mean or median duration of illness of total cases from 12 to 60 hours; 5) incubation period (if available) of total cases between 15 and 77 hours, usually 24–48 hours (14,15); and 6) testing of stool specimens for bacterial pathogens. (This step is not mandatory, however, if tested, all specimens should be negative for bacterial pathogens.)

**Development of Database**

A major goal of the first year was to build a database into which historic information present in the participating institutes would be collected. The rationale behind this was that by combining this existing information, new observations (on seasonality of outbreaks or patterns of emergence of new variants, for example) might be possible. Without harmonization of data collection, the comparative analysis would clearly be limited. The historic database, however, also served as a pilot phase because the definitive format of the database is used in the enhanced surveillance program. Participants who had historic collections of sequences were asked to submit these, along with additional epidemiologic information, as described in Table 1. Data were entered by using the Bionumerics (BN) package (Applied Maths, Ghent, Belgium), which allows storage, comparative analysis, and clustering of combined epidemiologic and biological experimental data (e.g., sequences, reverse line blot results, enzyme immunoassay data). The entries were either uploaded from the public domain or submitted as unpublished sequences from participating laboratories. Publicly available sequences were included to provide a customized report for database searches, e.g., genotype to which the sequence belongs.

Since September 2001, participants have been able to access the database directly through a password-controlled Internet connection. At present, the database contains >2,500 entries, mostly on NV, but including some hepatitis A virus, astrovirus, and Sapovirus (Tables 2 and 3). Upcoming variants will first be subjected to a search of the historic database to determine if the viruses have been seen before in Europe. An automated search tool is available and has been made accessible through the Internet to participants. Partners interested in analyzing the data can obtain the complete dataset, provided they adhere to the confidentiality agreements signed by every partner. Interested parties outside the project group can access the database under certain conditions through the coordinator or one of the participants. This access is not restricted to groups in the participating countries. The limiting factor is
the target region used for virus characterization, which has not been standardized globally. A database search will be performed upon request (for groups outside the network). Results are then communicated to them and to the person who submitted any matching sequences. After that initial linking, follow-up discussions and investigations of possible common-source events can be done by the groups involved.

Table 1. Requested data fields for entries of viral sequences in the historic database

<table>
<thead>
<tr>
<th>Field name</th>
<th>Field type</th>
<th>Mandatory field</th>
</tr>
</thead>
<tbody>
<tr>
<td>Country</td>
<td>A</td>
<td>Yes</td>
</tr>
<tr>
<td>Institute</td>
<td>A</td>
<td>Yes</td>
</tr>
<tr>
<td>Reference no.</td>
<td>A</td>
<td>Yes</td>
</tr>
<tr>
<td>Type of virus (NV, SV, HAV, ASV, HEV)</td>
<td>N</td>
<td>Yes</td>
</tr>
<tr>
<td>Sequence identifier (e.g., GenBank accession no.)</td>
<td>A</td>
<td>No</td>
</tr>
<tr>
<td>Method no.</td>
<td>N</td>
<td>No</td>
</tr>
<tr>
<td>Genotype/subtype</td>
<td>N</td>
<td>No</td>
</tr>
<tr>
<td>Sequence pol region /VP1/2a</td>
<td>A</td>
<td>Yes</td>
</tr>
<tr>
<td>Sequence capsid region</td>
<td>A</td>
<td>Yes</td>
</tr>
<tr>
<td>Source of isolate (human, calf, swine, environmental, other)</td>
<td>N</td>
<td>Yes</td>
</tr>
<tr>
<td>Specimen</td>
<td>N</td>
<td>No</td>
</tr>
<tr>
<td>Epidemiology</td>
<td>N</td>
<td>No</td>
</tr>
<tr>
<td>Vehicle (food, water, person-to-person, zoonotic, not known)</td>
<td>N</td>
<td>No</td>
</tr>
<tr>
<td>National region</td>
<td>A</td>
<td>No</td>
</tr>
<tr>
<td>Date of receipt in reference laboratory</td>
<td>D</td>
<td>Yes</td>
</tr>
<tr>
<td>Age</td>
<td>N</td>
<td>No</td>
</tr>
<tr>
<td>Sex</td>
<td>N</td>
<td>No</td>
</tr>
<tr>
<td>Travel associated</td>
<td>N</td>
<td>No</td>
</tr>
<tr>
<td>Travel destination</td>
<td>A</td>
<td>No</td>
</tr>
</tbody>
</table>

*NV, Norovirus; SV, Sapovirus; ASV, astrovirus; HAV, hepatitis A virus; HEV, hepatitis E virus; A, letters and numbers; B, numbers; D, data.

Prospective Enhanced Surveillance

Comparative Evaluation of Diagnostic/Genotyping Methods

The different PCR primers used among the European group all target a highly conserved region within the viral polymerase gene. Sequences of the amplicons from the various diagnostic PCRs overlap and therefore, can be compared to gain inferences on the molecular epidemiology and the spread of NV variants (11). Rapid characterization techniques, notably the reverse line blot (9) and heteroduplex mobility assay (7), are also used within the network; the typing data generated by these techniques can also be accommodated by the database.

Comparative Evaluation of Data

After agreement on a minimum epidemiologic and virologic dataset, we made a standard Web-based questionnaire available to all participants behind a password-protected site (available from: URL: www.eufoodbornevirologies.net). Using Web-based Active Server Pages (ASP) technology, investigators have full access to the secure outbreak database (Figure). Investigators are asked to enter information that is available as soon as an investigation begins or an event occurs that meets the outbreak definition. A unique reference number is assigned to each outbreak, which is the key used to access records and to update diagnostic or typing data, as an investigation continues.

The database also collects information on the level of evidence (i.e., microbiologic, epidemiologic, circumstantial) implicating food or water as a mode of transmission. Pop-up windows are used to define these criteria, since a range of public health scientists use the system. Other features of the ASP technology, including drop-down menus,
are used to standardize the data collected. Descriptive information from outbreaks (number of people exposed, number of people ill, number of controls infected, symptoms) is collected when possible, to allow comparisons of the clinical characteristics of different NV genotypes. Preliminary data suggest that such differences exist.

One of our main scientific objectives is to explain the mechanism behind the emergence of new variant strains. Essential for the early detection of such emerging variants is a rapid reporting network. The initial suspicion of “something strange” may be from clinicians who investigate outbreaks (e.g., a sudden increase in the number of reports), or from one of the laboratories (e.g., finding the same variant in several outbreaks). The central database is used to facilitate both types of reports. The real power in this format of data exchange is that immediately after entry or update of information, the data are in the database and can be accessed by other collaborators. The database can be searched for common virologic (sequence) or epidemiologic (e.g., a food vehicle) characteristics that would trigger further investigation of links between outbreaks.

### Recognition of International Outbreaks

This model has proved successful in recognizing a number of internationally linked events. Clusters of cases in Denmark, Finland, and the Netherlands were all linked to oysters imported from France. Another foodborne outbreak traced in part through the network followed the concluding dinner of an international conference in Finland. Symptoms began the day after the conference, when many attendees had returned to their home countries. Approximately 40 persons were affected, and the same NV variant was detected from cases in Finland, Sweden, and the Netherlands. A dessert item was implicated by cohort study. Importations of hepatitis A from Peru into Spain and from Ibiza, Spain, to Germany have also been recognized through the network. Full details of these outbreaks will be published elsewhere.

### Discussion

Microbial food safety is considered an important public health issue but historically has focused on control of bacterial contamination. Several recent publications, however, show that outbreaks of foodborne infection attributable to viruses are common and may in fact be an important public health concern for several reasons: most clinical laboratories involved in outbreak investigations do not have access to routine diagnostic methods for detecting NV, user-friendly methods for use in these laboratories are only now becoming available and need to be validated, foodborne transmission of NV is quite common, and food microbial quality control largely relies on indicators for the presence of fecal bacteria, which may not correlate with the presence of enteric viruses (2,16). Although foodborne viruses are increasingly studied, no validated methods yet exist for reliably detecting them in food items. In all, these facts indicate that through foodborne transmission an

---

**Table 3. Number of entries in the database by country of submission**

<table>
<thead>
<tr>
<th>Country</th>
<th>No. of entries</th>
<th>HAV</th>
<th>ASV</th>
<th>NLV</th>
<th>SLV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argentina</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Australia</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Switzerland</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Czech Republic</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germany</td>
<td>167</td>
<td>1</td>
<td>432</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hungary</td>
<td>93</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denmark</td>
<td>67</td>
<td></td>
<td>67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spain</td>
<td>33</td>
<td>18</td>
<td>102</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Finland</td>
<td>90</td>
<td></td>
<td></td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>France</td>
<td>216</td>
<td>24</td>
<td>203</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Great Britain</td>
<td>509</td>
<td>1</td>
<td>566</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Ghana</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hong Kong</td>
<td>7</td>
<td></td>
<td></td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Japan</td>
<td>363</td>
<td></td>
<td></td>
<td>388</td>
<td>3</td>
</tr>
<tr>
<td>Korea</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mexico</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Netherlands</td>
<td>743</td>
<td>87</td>
<td>24</td>
<td>707</td>
<td></td>
</tr>
<tr>
<td>Norway</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>New Zealand</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Russia</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>South Africa</td>
<td>9</td>
<td>5</td>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Sweden</td>
<td>101</td>
<td></td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Turkey</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>United States</td>
<td>67</td>
<td>6</td>
<td>59</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

*HAV, hepatitis A virus; ASV, astrovirus; NLV, Norwalk-like viruses; SLV, Sapporo-like virus.*
enteric viral pathogen (NV) can escape detection, possibly resulting in large epidemics.

In the United States, molecular detection techniques are being implemented in state public health laboratories under the guidance of the Centers for Disease Control and Prevention (CDC), which is building an infrastructure for reporting of outbreaks of food-related illness attributable to enteric viruses (Calicinet). In Europe, no central institute yet exists with the authority to do this, so the best efforts to date are the voluntary disease-specific surveillance networks, such as Enternet (which monitors trends in foodborne bacterial pathogens), and the European Influenza Surveillance Scheme (designed to monitor influenza virus activity across Europe) (17,18). We have built such a surveillance network for enteric viruses, using NV as a target organism. NV was an obvious choice: an increasing number of publications illustrate that it is one of the most important causes of outbreaks of gastroenteritis, including food- and water-related outbreaks (reviewed in 2). CDC estimates that up to 66% of all food-related illness in the United States may be due to NV (19). From a community-based case-control study in the Netherlands, risk-factor analysis for NV, based on information collected throughout a 1-year cohort study, suggested an association between NV and a complex score that was used as a proxy for food-handling hygiene. On the basis of this approach, an estimated 12% to 15% of community-acquired illness may be due to food- or waterborne modes of transmission (with 85% attributed to contact with a symptomatic person in or outside the household) (de Wit et al., unpib. data). The proportion of foodborne outbreaks reported in the countries participating in our network ranges from 7% to 100%, but that range merely reflects the differences in the selections used in the different surveillance systems and cannot be used to estimate the true impact of foodborne illness caused by NV (10). While definitive data still need to be collected, the consensus is that NV is an important cause of food-related infection and disease.

Foodborne transmission of viral gastroenteritis has not historically been acknowledged as a public health priority, which means that our surveillance system is inevitably restricted to groups that already have an active program in the field. Ideally, we would like to build a network of national institutes represented by both epidemiologists and microbiologists involved in outbreaks of viral gastroenteritis; however, at present this ideal is not possible for all of Europe. By networking the existing information, assessing comparability of data through studies of primers and protocols used, and examining data from current surveillance, we hope to be able to paint a bigger picture from the fragmented information that is available.

The standardized outbreak questionnaire, accessible through the Internet, is designed to collect a minimum dataset about all outbreaks. However, participants who perform more detailed epidemiologic or virologic investigations can also submit additional data. The minimum dataset will suffice to answer the basic questions for the surveillance, i.e., what is the reported incidence of NV outbreaks across Europe, and which proportion is considered to be due to food- or waterborne transmission.

A key feature of any disease surveillance system is its use as an early-warning tool, in this case for international common-source outbreaks. To facilitate this, several features were included in our database setup. Information on outbreaks can be updated with new information as it comes in, to avoid piling up information until the outbreak investigation has been completed (which may be months later). Both the epidemiologic data and laboratory data (mostly sequences) can be searched easily. Thus, participants can be alerted to similarities in disease profiles (e.g., outbreaks with imported fruits) or in sequences. Either signal can lead to contacts between participants to discuss possible indications for a joint investigation. Crucial in this discussion was the issue of confidentiality, both for patient and product information, and for data from investigations. The present modus operandi is that each participant signs a confidentiality agreement, which states that data submitted to the database are owned by the person submitting them (subject to each participant’s national regulations on patient and laboratory data); specific patient and product information is not entered into the database. If necessary for outbreak investigations, the groups involved will decide on a case-by-case basis what information may or may not be used by the consortium. Participants can obtain the complete information from the database for their own analysis, or choose to use it as a search tool and rely on the analysis done by a scientist employed on this aspect of the database, who is stationed in Bilthoven, the Netherlands. So far, five international outbreaks have been detected because of the network.

The food distribution chain in Europe is complex, and therefore the transmission of viruses across borders can occur by means of contaminated food. The surveillance network described here allows early detection of international common-source outbreaks of foodborne viruses. Most of the work to date has involved harmonization of methods for investigating outbreaks and detecting the viruses causing these outbreaks, as well as the development of a database system that facilitates the exchange of information between laboratories and institutes involved in viral gastroenteritis research and surveillance. The system’s strength is that it combines basic epidemiologic and laboratory data into a searchable repository. This network has demonstrated its potential to recognize transnational outbreaks. However, the network is inherently limited by the quality of data available at the national level, which is
a reflection of the priority given to foodborne viruses. At present, we are undertaking a 2-year enhanced surveillance project to study the frequency and modes of transmission of viral gastroenteritis outbreaks across Europe.

Core funding for this project was obtained from the European Union under the 5th framework program, contract no QLK1-1999-C-00594.

Dr. Koopmans is a veterinarian with a Ph.D. in virology. Since 2001, she has chaired the Virology of the Diagnostic Laboratory for Infectious Diseases of the National Institute of Public Health in the Netherlands, which focuses on reference diagnostics, molecular epidemiology, and outbreak management of a range of emerging diseases. She also is coordinator of the European Union–funded Foodborne Viruses in Europe Network.

References


Address for correspondence: Marion Koopmans, Research Laboratory for Infectious Diseases, National Institute of Public Health and the Environment, Antonie van Leeuwenhoeklaan 9, 3720BA Bilthoven, the Netherlands; fax: 31.30.2744449; email: marion.koopmans@rivm.nl

www.cdc.gov/eid

To receive tables of contents of new issues send an email to listserv@cdc.gov with subscribe eid-toi in the body of your message.
Increase in viral gastroenteritis outbreaks in Europe and epidemic spread of new norovirus variant

Ben Lopman, Harry Vennema, Evelyne Kohli, Pierre Pothier, Alicia Sanchez, Anabel Negredo, Javier Buesa, Eckart Schreier, Mark Reacher, David Brown, Jim Gray, Miren Iturriza, Chris Gallimore, Blenda Bottiger, Kjell-Olof Hedlund, Maria Torvén, Carl-Henrik von Bonsdorff, Leena Maunula, Mateja Poljsak-Prijatelj, Janet Zimsek, Gábor Reuter, György Szücs, Béla Melegh, Lennart Svennson, Yvonne van Duijnhoven, Marion Koopmans for the European Food-borne Viruses Network*

Summary

Background Highly publicised outbreaks of norovirus gastroenteritis in hospitals in the UK and Ireland and cruise ships in the USA sparked speculation about whether this reported activity was unusual.

Methods We analysed data collected through a collaborative research and surveillance network of viral gastroenteritis in ten European countries (England and Wales were analysed as one region). We compiled data on total number of outbreaks by month, and compared genetic sequences from the isolated viruses. Data were compared with historic data from a systematic retrospective review of surveillance systems and with a central database of viral sequences.

Findings Three regions (England and Wales, Germany, and the Netherlands) had sustained epidemiological and viral characterisation data from 1995 to 2002. In all three, we noted a striking increase in norovirus outbreaks in 2002 that coincided with the detection and emergence of a new predominant norovirus variant of genogroup II4, which had a consistent mutation in the polymerase gene. Eight of nine regions had an annual peak in 2002 and the new genogroup II4 variant was detected in nine countries. Also, the detection of the new variant preceded an atypical spring and summer peak of outbreaks in three countries.

Interpretation Our data from ten European countries show a striking increase and unusual seasonal pattern of norovirus gastroenteritis in 2002 that occurred concurrently with the emergence of a novel genetic variant. In addition to showing the added value of an international network for viral gastroenteritis outbreaks, these observations raise questions about the biological properties of the variant and the mechanisms for its rapid dissemination.


See Commentary page 671

Introduction

In early 2002, the medical community was alerted to a high number of outbreaks of viral gastroenteritis attributed to noroviruses in hospitals and on cruise ships. These warnings came through several reports on the electronic outbreak reporting system ProMED and national epidemiological bulletins in the USA, and England and Wales.¹–³ Noroviruses are a diverse group of non-enveloped RNA viruses, belonging to a genus within the family Caliciviridae. They are the most common cause of gastroenteritis outbreaks and cause acute, self-limiting gastroenteritis in people from all age-groups.³ They are notorious for a high attack rate, the low dose needed to produce active infection, and their unusual stability outside the host.⁴ Outbreaks of norovirus gastroenteritis occur at any time of the year, but there is a distinct seasonal peak in winter in regions of temperate climate.⁵

Starting in 2001, our group of laboratories across Europe began working towards harmonisation of data collection for surveillance of viral gastroenteritis, with an emphasis on noroviruses.⁶–⁸ Since October, 2001, we had improved surveillance using web-based reporting of epidemiological and laboratory data to a central database. We requested that collaborators within this network bring together the relevant data from around Europe to address the following questions: (1) were there more norovirus reports in 2002 than in previous years; (2) did the seasonal pattern of occurrence in 2002 differ from that in the recent past; (3) what norovirus strains are associated with the recent outbreaks; and (4) were the predominant strains in 2002 different from those in the past?

Methods

To address the above questions, we surveyed the laboratories and surveillance centres of the European Food-borne Viruses network.

Epidemiological trend data

The survey, a spreadsheet distributed by e-mail in December, 2002, allowed systematic collection of data on monthly numbers of outbreaks from January, 1995, to December, 2002. We used a broad definition of an outbreak of norovirus gastroenteritis—ie, two or more linked cases with similar symptoms, at least one of which has microbiological confirmation of norovirus.⁹ Since none of the participating countries has continuous surveillance information that would enable the
combination of epidemiological and laboratory data from the surveyed time period, we requested that the epidemiological trend data be extracted from the most consistent and timely source of information. In Spain, and England and Wales, outbreak reports to the national surveillance centre were used. In all other countries, diagnostic laboratories were the chosen source of data. A more detailed description of European surveillance systems for viral gastroenteritis has been published previously.8,9

---

**Figure 1:** Epidemiological and molecular patterns of norovirus outbreaks in Netherlands, England and Wales, and Germany

Bold lines on line graphs show data from 2002. Other lines represent annual data from 1995–2001, except for Germany where pale lines show annual data for 1999, 2000, and 2001; note that scales differ on y axes. Bar charts show number of genogroup II4 polymerase and genogroup II4 polymerase viruses in 2002. Numbers in bars are number of outbreaks; note that virus genogroup was not established for all outbreaks shown in line graphs and only those of genogroup II4 are shown in the bar chart.
Viral characterisation
The European Food-borne Viruses Network maintains a central sequence database of norovirus strains. We interrogated this database to determine the predominant strains circulating in participant countries before and after the increase of incidents in 2002. Details of the database setup are published elsewhere.\(^\text{10}\)

Briefly, viruses were characterised based on the sequence of the polymerase gene, as determined by RT-PCR and sequencing. Several different primers were used by the collaborating centres;\(^\text{11–12}\) however, all have been shown to be broadly reactive as well as effective in the detection of genogroup II\(^4\) strains.\(^\text{13}\) The target regions of the RT-PCR in the polymerase gene overlap sufficiently to allow phylogenetic analysis. Sequences were loaded into a central database where they were stored and analysed with BioNumerics software (Applied Maths, Ghent, Belgium).

**Role of the funding source**
Funding from the sponsor has supported the setup of the collaborative databases as well as the development of infrastructure and technology exchange for investigations. The sponsor had no role in study design, data analysis, data interpretation, or the writing of the report.

**Results**

**Netherlands**
We used the monthly total of outbreaks investigated by the diagnostic and reference laboratory at the National Institute for Public Health and the Environment in Bilthoven to assess time trends of norovirus infections. 155 norovirus outbreaks were identified by RT-PCR (used for the entire surveillance period) in 2002, compared with the previous high of 68 in 1996, an increase of 128% (figure 1; table). Furthermore, a comparison of data from an ongoing regional surveillance covering 19% of the Dutch population in 2001 and 2002 showed an increase in norovirus outbreaks from 15 in 2001 to 23 in 2002—an increase of 53%. An atypical spring peak was noted in April and May, 2002. A new variant of the norovirus genogroup

![Figure 2: Clustering of genogroup II4 norovirus polymerase gene sequences.](image)

Sequences of 145 nucleotides of the RNA dependent RNA polymerase gene region upstream of the conserved GDD-motif were aligned and clustered with BioNumerics software Applied Mathematics, Kortrijk, Belgium. Clustering was done on a multiple sequence alignment of 782 genogroup II4 sequences, 331 from before 2002 and 451 from 2002. Sequences were collected in the Foodborne Viruses in Europe Project and supplemented with reference strains from GenBank. Most 2002 strains cluster together in a new branch. The occurrence of this new branch correlates strongly with the appearance of a sequence motif in the region under study as shown in the bottom part of the figure. Two main sequence types were noted; AACTTG, mainly in strains before 2002, and AATCTG, mainly in 2002 strains. Some AACTTG strains were also detected in 2002 in the first half of the year. GGII4 strains with the AATCTG motif were not detected before January, 2002.
II4 was first detected in January, 2002. The variant had an unusual set of mutations (AACCTG to AATCTG starting at position 4820 relative to Norwalk-virus M87661) as compared with the previous predominant genogroup II4 viruses also known as Grimsbyvir (figure 2). Henceforth, genogroup II4 viruses containing this motif are referred to as the “new variant”. This motif was not present in any of the genogroup II4 sequences analysed worldwide before 2002 (n=450, from the Foodborne Viruses in Europe database and from GenBank). In total, 153 outbreaks were characterised, 134 (88%) belonging to genogroup II4.

Figure 3: Norovirus activity in six European countries, 1995–2001
Bold lines shows data for 2002. Data are from positive laboratory results of individual cases, except in Hungary where data are from outbreak reports. Note that scales differ on y axes.
The new genogroup II4 variant predominated after February, 2002, preceding the atypical spring peak. 76% (117/153) of outbreaks were caused by the new variant in 2002.

Germany

In Germany, time trends were drawn based on the number of outbreaks investigated by the molecular virology laboratory at the Robert Koch Institute in Berlin. In 2002, there were 161 norovirus outbreaks diagnosed by RT-PCR, a 94% increase from the previous peak of 83 outbreaks in 1999; although, the sensitivity of diagnostics is thought to have increased during these years (figure 1, table). An unusual peak was noted in May, 2002, although in 1999, a summer peak was noted in June. The new variant of the norovirus genogroup II4 was first detected in January, 2002. 161 outbreaks were characterised, 117 (42%) of genogroup II4. The new genogroup II4 variant predominated after March; 63% (102) of all outbreaks were caused by the new variant in 2002.

England and Wales

Since 1995, there have been 2324 reports of laboratory-confirmed outbreaks of norovirus infection captured by the national database for England and Wales (figure 1; table). Although under-reporting to the system has been shown, it provides a timely and consistent source of outbreak data based on reports from public health physicians, environmental health officers, and hospital infection control teams.14 In 2002, 614 norovirus outbreaks were reported, a 77% increase from the previous peak of 347 outbreaks in 1995 (table). Furthermore, an atypical peak was noted in the summer and autumn of 2002, which had not been seen in the previous years of the survey period.1 351 outbreaks were characterised in 2002; 232 (66%) were genogroup II4. The polymerase gene was sequenced from 83 viruses, 38 (46%) of which were the new variant. The new variant of the norovirus genogroup II4 was first detected in March, 2002, and was the predominant type from April onwards. The variant had not been identified in England and Wales before March, 2002.

Other epidemiological evidence

Data on laboratory activity, based on outbreak or individual samples, by month were provided for six other countries (figure 3). In France, norovirus surveillance has been in place since 1999 and, therefore, no historical data were available for comparison. An increase over the previous annual maximum was seen in all countries except for Spain (table; figure 3). The annual increase from all countries was substantial. Like Germany and the Netherlands, a spring peak was noted in Finland in May. The winter seasonality that has been reported in Northern European countries was not seen in Spain, Slovenia, and Hungary before 2002, although this may be artefactual because of small numbers of reports. Spain was the only country where fewer outbreaks of norovirus were reported to the national centre at Instituto de Salud Carlos III in 2002 than in previous years (table).

The setting of norovirus outbreaks in countries with data from at least 50 outbreaks is shown in figure 4. In all such countries, outbreaks in health-care facilities (including hospitals, residential homes, and nursing homes) predominated.

New virus variant in other countries

Information about the type of virus in the outbreak was available from three other countries. In Finland, nine of 12 (75%) characterised outbreaks belonged to genogroup II4, as were 29 of 36 (81%) outbreaks in Spain (Valencia region), and 17 of 31 (55%) outbreaks in Hungary. In addition to the Netherlands, England and Wales, and Germany, the novel variant was detected in France (April), Slovenia (July), Hungary (September), Finland (June), Spain (April), and Denmark (September).

Discussion

Epidemiological trend data and virological typing data from the Netherlands, England and Wales, and Germany show a similar pattern of increased numbers of norovirus outbreaks in 2002 after detection of a new genogroup II4 variant. The variant was first noted in January in Germany and the Netherlands and during the course of the year it became the predominant cause of norovirus outbreaks throughout Europe. Data from five other countries with only epidemiological or molecular data lend support to these observations. Additionally, highly atypical spring or summer peaks were noted in the Netherlands, Germany, Finland, and England and Wales, which were preceded by detection of the new variant.

Noroviruses are a diverse group of viruses segregated into genotypes on the basis of sequence analysis. Predominant strains are occasionally replaced by emerging ones.15–18 However, the genogroup II4 genotype has predominated in outbreaks reported from many countries since 1996.15–18 Here, we have documented the emergence, and subsequent predominance of a new strain within the genogroup II4 genotype over a wide geographical area.

We postulate that this new variant might be more virulent or environmentally stable than previous genogroup II4 viruses. Several enteric viruses (eg, rotavirus, astrovirus) are more likely to have outbreaks in winter. The reason for this seasonality is not well understood, but is probably due to a combination of climatic conditions that favour survival of the virus, social
behaviours that increase the likelihood of person-to-person and foodborne transmission, and, in the case if nosocomial and foodborne transmission, in the case if nosocomial.

Two alternate explanations for our findings are that: (1) the increase is a surveillance artefact—ie, that it is attributable to our increased vigilance; or (2) that there has been a change in the microbiological quality of food or a breakdown of infection control in hospitals and other institutions. First, we cannot exclude the possibility of a surveillance artefact because, in 2002, we established a central reporting scheme of viral gastroenteritis outbreaks in these countries through an EU-funded research activity. However, the observation that the seasonal pattern was clearly different in 2002 in the countries that had increased numbers of outbreaks suggests that there was a true increase. Also, the harmonised surveillance has not directly affected national surveillance systems. An increased number of outbreaks was observed in Denmark, England and Wales, Finland, Germany, the Netherlands, and Sweden, all countries with long standing (>5 years) and consistent sources of surveillance data.

Data from other countries, although based on shorter surveillance periods, lend support to the conclusion that there was an increase of norovirus outbreaks in 2002 compared with previous years. Another potential surveillance weakness is that collaborators used different primers for the detection of noroviruses, and therefore, for the selection of strains to sequence. However, in a comparative assessment of the assays, all allowed detection of genogroup II4 strains. These data have shown that outbreaks in hospitals and nursing homes are common in many European countries and might represent most of the public-health burden attributable to noroviruses. They are consistent with findings in previous reports.

Therefore, a breakdown in infection control could account for an increase in norovirus outbreaks. However, since the increase is so widespread across countries, the breakdown would have had to occur in many European health-care systems simultaneously, which is highly unlikely. Likewise, a widespread failure in the microbiological quality of food or a substantial seeding event could initiate an epidemic. We have previously noted evidence for widespread dissemination of specific norovirus variants after common source introduction via contaminated food. We suspect that the present epidemic could represent another example of this mode of introduction. Is this a problem? We think so: noroviruses are ubiquitous, cause large outbreaks in institutions, and are highly contagious because of the small dose needed for infection, their stability outside the host, and short-term immunity. Foodborne and waterborne transmission of these viruses is common, and food products move all over the world without microbiological control of viral contaminants. Therefore, foods can pass microbiological quality control, but still contain viruses. However, we are currently unable to explain exactly how the new variant spread. Whatever the mechanism of emergence, the dissemination was associated with a wave of person-to-person outbreaks. Most outbreaks in 2002, as previously, were set in health-care institutions, where person-to-person spread predominates. No increase of foodborne transmission was noted in 2002, nor were any large foodborne outbreaks of the genogroup II4 variant detected.

Our findings raise several questions about how the new variant spread so rapidly and what biological differences might have mediated the trend. Further work should focus on whether the disease caused by the new variant causes more severe symptoms in patients or attack rates. Mutations that distinguish the new variant are silent (ie, they do not result in protein changes) and further virological characterisation is ongoing to determine whether there are genomic mutations in other regions (the capsid gene for example) and, if so, whether these mutations confer antigenic or viability changes. In 1995–96, a norovirus strain, genetically similar to the variant described in the present study, was detected and subsequently noted in North and South America, Europe, Asia and Australia. What biologically distinguishes these strains is yet to be determined, but the observation that a specific strain can emerge and have rapid public-health effects across geographical and political boundaries underscores the importance of sustained international norovirus surveillance.

Observations thus far suggest that genogroup II4 noroviruses might have increased the risk of epidemic spread.

Our combined data from ten European countries shows that the striking increase and unusual seasonal pattern of norovirus gastroenteritis in 2002 arose concurrently with the emergence of a new virus variant. Had these observations been made in one country they could be dismissed as aberrations in surveillance, the result of changes in ecological circumstances, or because of the local circulation of a new variant. However, the data collected within our network lend support to anecdotal reports of an increase of an important infection and have allowed us to present a feasible virological explanation for the effect on public health.

**European Food-borne Viruses Network**


**Finland**—K-H von Bonsdorff, I. Maunula, Helsinki University.

**Denmark**—B Böttger, K Molbak, F X Hanon, Statens Serum Institute, Copenhagen.

**Sweden**—I Svensson, University of Linköping; K-O Hedlund, Maria Torvén, Swedish Institute for Infectious Disease Control, Solna.

**England and Wales**—D Brown, M Reacher, J Green, B Lopman, Public Health Laboratory Service, London.

**Germany**—E Schreier, H Gelderblom, M Hoehne, A Ammon, Robert Koch Institute Berlin. Spain—A Sanchez-Faqquier, G Hernandez-Pezzi, Instituto de Salud Carlos III, Madrid; A Bosch, Universitat de Barcelona, Barcelona; J Buesa, Universitat de Valencia.

**France**—F LeGoüeayder, IFREMER, Nantes; P Pothier, E Kohli, Laboratoire de Virologie, Dijon.

**Italy**—F Ruggieri, D DeMedici, Instituto di Superiore di Sanità, Rome.

**Slovenia**—M Poljsak-Prijatelj, Institute of Microbiology and Immunology, University of Ljubljana, Ljubljana; A Hocevar-Grom, Institute of Public Health, Communicable Disease Centre, Ljubljana.

**Hungary**—G Szucs, G Reuter, County Institute of State Public Health Service, Pécs.

**Contributors**

Study design was directed by M Koopmans, M Reacher and D Brown. H Vennema analysed the international virological data and B Lopman analysed the international epidemiological data. National epidemiological studies were performed by K-O Hedlund, A Negredo, G Szucs, M Poljsak-Prijatelj, B Lopman, I Maunula and Y van Duyhnoven. Sequencing and phylogenetic data generation of national virological populations was performed by H Vennema, B Melegh, G Reuter, L Svensson, C-H von Bonsdorff, I Maunula, J Buesa, E Schreier, M Poljsak-Prijatelj, J Zimovcik, E Kohli, P Pothier, B Böttger, M Torvé, J Gray, M Iturriza, C Gallimore, A Sanchez and A Negredo. B Lopman and M Koopmans drafted the report. All researchers took part in the revision of the paper and approved the final version.

**Conflict of interest statement**

None declared.

**Acknowledgments**

This work was supported by the European Commission, Quality of Life Program, 5th Framework (QLK1-CT-1999-00594).
References

Detection of noroviruses in raspberries associated with a gastroenteritis outbreak

Françoise S. Le Guyader a,*, Christian Mittelholzer b,c, Larissa Haugarreau a, Kjell-Olof Hedlund b, Rolf Alsterlund d, Monique Pommepuy a, Lennart Svensson b,e

a Laboratoire de Microbiologie, IFREMER, BP 21105, Nantes cedex 03 44311, France
b Department of Virology, Swedish Institute for Infectious Disease Control, Solna 171 82, Sweden
c Institute of Marine Research Austevoll, Storebø 5392, Norway
d Regional Center of Communicable Disease Control Skåne, Kristianstad 291 85, Sweden
e Department of Molecular Virology, University of Linköping, Linköping 581 85, Sweden

Received 22 December 2003; received in revised form 26 April 2004; accepted 27 April 2004

Abstract

Following an acute foodborne gastroenteritis outbreak in southern Sweden, stool specimens from five of nine ill patients were found positive for norovirus using reverse-transcriptase polymerase chain reaction. Epidemiological data pointed to raspberry cakes as the source of the outbreak. Using a combination of generic and patient-specific primers and novel food analysis methodology (with extraction efficiency control and inhibitor removal), norovirus strains from two different genogroups were directly identified in the contaminated raspberries.

© 2004 Elsevier B.V. All rights reserved.

Keywords: Norovirus; Gastroenteritis; Food analysis; Reversetranscriptase polymerase chain reaction; Sequence analysis

1. Introduction

Noroviruses (NoVs, formerly Norwalk-like viruses) are the most common agents of outbreaks of acute gastroenteritis, and their transmission via contaminated food is increasingly recognized (Bresee et al., 2002; Koopmans et al., 2002; Koopmans and Duizer, 2004). Because these viruses are resistant to environmental degradation, most chemical treatment processes, and are not altered by freezing, they can contaminate all types of food products either by sewage contamination during growth or by food handlers during harvest and packaging (Green et al., 2001; Gulati et al., 2001).

Direct virus strain identification in food implicated in outbreaks has rarely been achieved due to food matrix complexity, low levels of contamination, and genetic diversity (Schwab et al., 2000; Atmar and Estes, 2001; Sair et al., 2002). At present, diagnosis of foodborne outbreaks relies mostly on epidemiological investigations, coupled with the identification of the causative pathogen in persons with health complaints following food consumption. Occasionally, virus detection can also be achieved in food handlers thought to be the source of infection. However, methodologies...
for the direct detection of NoVs in food are improving and recently, sensitive methods have been described for analysis of foods other than shellfish (Schwab et al., 2000; Dubois et al., 2002; Sair et al., 2002). All these methods rely on molecular detection of the agent and several factors such as extraction efficiency controls, avoidance of contamination during the PCR process, optimal RT-PCR conditions, or adequate primer sets, have to be taken into account for reliable and sensitive results to be achieved (Sair et al., 2002; Nishida et al., 2003; Vinje et al., 2003).

In November 2001, a gastroenteritis outbreak occurred in southern Sweden involving bakery products containing either raspberries or pears. Epidemiological investigations soon indicated the raspberry cakes as the suspected source in this outbreak. To directly identify the pathogen, a novel food analysis methodology and outbreak-specific primers were applied. Here we describe the detection of multiple norovirus strains contamination in the raspberries and the difficulties of analyzing complex food items containing low levels of virus contamination.

2. Materials and methods

2.1. Epidemiology data

The outbreak occurred in the beginning of November 2001 in a town in southern Sweden. A local bakery produced cakes with two different kinds of cream topping: green cakes containing pear extract and pink cakes containing frozen whole raspberries. Otherwise the cakes were identical. A preliminary epidemiological study indicated the pink cakes to be the suspected source of the outbreak, as only people (30 persons) who consumed the raspberry cakes got ill with gastrointestinal symptoms, including vomiting, compatible with a norovirus illness.

2.2. Clinical sample analysis

Stool samples were collected from nine symptomatic patients and analyzed as 10% suspensions in PBS. Nucleic acid extraction was done using the guanidinium thiocyanate (GuSCN)—silica method (Boom et al., 1990). Briefly, to each tube 10 µl silica suspension and 500 µl of L6 buffer (5.25 M GuSCN [Merck, Hohenbrunn, Germany], 50 mM Tris-HCl [pH 6.4], 20 mM EDTA, 1.3% [wt/vol] Triton X-100 [Sigma Aldrich, St Louis, MO]) was added. Then 100 µl of either sample suspension, positive control (stool sample tested positive for NoVs by EM and PCR), negative control (stool sample repeatedly tested negative for NoVs by EM and PCR) or extraction control (H2O) were added. The samples were incubated on a rocker platform for 30 min at room temperature. After centrifugation for 1 min at 14,000 × g, the supernatant was removed and the pellet was washed twice with 500 µl of L2 (5.25 M GuSCN, 50 mM Tris/C1HCl [pH 6.4], 20 mM EDTA, 1.3% [wt/vol] Triton X-100 [Sigma Aldrich, St Louis, MO]) buffer, twice with 500 µl 70% ethanol at 4 °C, and once with 500 µl acetone at 4 °C. The pellet was dried in a heating block for 15 min at 56 °C and the nucleic acid was eluted by adding 40 µl H2O, mixing and incubation for 15 min at 56 °C.

RT-PCR was done using primers from the polymerase region of the norovirus genome (JV12/JV13, NVp110/NVp36, NVp110/NVp69, NVp110/NI) (Atmar and Estes, 2001; Johansson et al., 2002; Vinje et al., 2003). Briefly, 5 µl extracted RNA, or H2O as a control, was added to 4 µl of anti-sense mix containing 50 pmol of 5' biotinylated primer JV13 and 50 µM of each dNTP (Invitrogen, Stockholm, Sweden) in DEPC-treated H2O (Biochemical, Cleveland, OH), and denatured for 2 min at 94 °C. After chilling on ice for at least 2 min, 6 µl of an RT-Mix containing 40 units of RNaseOUT recombinant Ribonuclease Inhibitor and 200 units of Superscript RT (Invitrogen, Stockholm, Sweden) was added to give final concentrations of 1 × First-strand buffer and 10 µM DTT before the samples were incubated for 60 min at 42 °C, followed by 5 min at 94 °C. Five microliters of such cDNA was then added to 45 µl PCR-mix (all reagents from Perkin-Elmer). The RT-PCR was done as previously described (Johansson et al., 2002). After amplification, the PCR products were analyzed on a 1.5% agarose gel and detected by ethidium bromide staining.

2.3. Food sample analysis

The suspected frozen berries were collected, sent in a sealed package and arrived frozen in the laboratory for analysis. Extraction of the berries was performed on two occasions: the first extraction was done on duplicates (replicates A and B) and the second ex-
traction on one replicate (C) as a separate experiment. As an extraction control, enterovirus (EV) particles corresponding to 100 RT-PCR units of a poliovirus type 2 (titration made by RT-PCR on the purified particles from cell culture) were added to each replicate (10 g of berries). The berries were washed with 4 ml of glycine-buffer pH 9.5 (0.05 M glycine, 0.3 M NaCl, Sigma, St Quentin, France), shaken vigorously for 1 min before the seeds were eliminated using a sterile strainer, to avoid pectin extraction. The pH was adjusted to 9.5 using 0.1N NaOH (Sigma, St Quentin, France) and the mixture was shaken by vortexing for 2 min to elute viruses. Three milliliters of chloroform–butanol (1:1 vol/vol) were added, and the samples mixed for 30 s. Ninety microliters of CatFloc T (Calgon, Elwood, Pa) was added to the homogenate. After being rocked for 5 min, the samples were centrifuged for 20 min at 12,000 × g at 4 °C. The aqueous phase was collected, adjusted to 6 ml, and added to 3 ml of a polyethylene glycol 8000 (24% wt/vol, Sigma, St Quentin, France)–sodium chloride (1.2 M) solution. Samples were rocked for 1 h at 4 °C, and then centrifuged for 30 min at 10,000 × g at 4 °C. The resulting pellet was used for nucleic acid extraction as previously described (Le Guyader et al., 2000). Briefly, after digestion using proteinase K (Amresco, Solon, OH), extraction using phenol–chloroform and ethanol precipitation, an additional step of purification using cetyltrimethylammonium bromide (Sigma, St Quentin, France) was used before a final ethanol precipitation. The final pellet was suspended in 100 μl of sterile water.

- Primers: EV detection was done using primers located in the 5' non-coding region (Le Guyader et al., 2000). For NoV, different primer sets were used. In the polymerase region, the same four primer sets as for stool analysis were used plus the primer set NVp110/SR48-SR50-SR52 (Atmar and Estes, 2001). In the capsid region, primer sets P155/P156 or SRI-1/SRI-3 were used (Atmar and Estes, 2001; Le Guyader et al., 2003).
- Based on the sequence determined for the virus found in the stool of three patients, outbreak-specific primers were designed: reverse primer Ra1 (5' GAA GTA GGA TTG CGA TTG TA 3’) and forward primer Ra2 (5' GCA TTA ACC ACT GGA TGT TA 3’).

- RT-PCR: RT-PCR was performed according to the instructions of the murine leukemia virus RT and Taq polymerase supplier (Perkin-Elmer). PCR amplification was performed for 40 cycles (94 °C for 30 s, 37 °C, 50 °C, or 55 °C for 30 s, and 72 °C for 30 s) using a 9600 or a 2400 Perkin-Elmer apparatus. The annealing temperature was 37 °C for JV12/JV13 primer set, 55 °C for Ra1/Ra2 primer set and 50 °C for all the other primer sets. All the RT or PCR mix were optimized and validated in a separate experiment to avoid the use of positive controls. Negative controls were included in each RT-PCR run. After amplification, the PCR products were analyzed on a 9% polyacrylamide gel and detected by ethidium bromide staining.
- Nested-PCR: 2 μl of the first amplification reaction using NVp110/RA2 was further amplified with primer set RA1/RA2 under the same conditions of amplification as the first PCR. To avoid cross-contamination, nested-PCR was done in a separate laboratory, using filter tips and several negative controls were included.
- Hybridization: All PCR products were hybridized using the dot blot technique as previously described (Le Guyader et al., 2000). A panel of probes was used both for the polymerase or the capsid region (Le Guyader et al., 2000; Vinjé et al., 2003; Le Guyader et al., 2003). After prehybridization for 30 min at 50 °C, hybridization was performed for 2 h at 50 °C. The hybridized probes were detected by chemiluminescence using CDP-Star (Roche, Meylan, France) according to the manufacturer’s protocol using a Bio-Rad multi-imager.

- Sequencing: (as food samples analysis) For stool samples, sequences of both strands were determined by direct cycle sequencing with primers NVp110 and NVp69. For the raspberries, RT-PCR products were directly sequenced using primers NVp110 and NI or cloned into the pCRII vector in the TA cloning kit (Invitrogen, Carlsbad, USA). Sequencing was performed on at least three different randomly selected clones with an ABI 373A automated sequencer and a Taq Dideoxy Terminator cycle-sequencing kit (Applied Biosystems) (ESGS-Cybergene, Paris, France). Sequence analyses were performed with the DNASTAR program package (DNASTAR, Madison, WI).
3. Results

3.1. Stool results

All stool samples examined gave negative results using the norovirus-specific primer sets JV12/JV13, NVp110/NVp36, and NVp110/NI. Five samples, all from symptomatic patients, gave positive results with primer set NVp110/NVp69. Sequence analysis of three of the samples, for which sufficient quantities of amplicons were obtained for direct sequencing, revealed the presence of 112 unique nucleotides between the two primer sequences (accession number AY316294, Table 1). The sequences were identical in all three patients and were identified as belonging to genogroup I.

3.2. Initial raspberries analysis

- Extraction efficiency: For the first set of extractions, the inoculated EV was detected in replicate A (Fig. 1A), both in the undiluted extract and after one log dilution, but could not be detected in replicate B (data not shown). In the second extraction (replicate C), the EV was detected at the same sensitivity (about 10 RT-PCR units) as in replicate A (data not shown). This highlights the necessity of extraction efficiency controls when dealing with such complex food matrices.

- Norovirus detection: For replicate A, only NVp110/NI gave a positive signal in the undiluted extract (Fig. 1B) and a faint positive signal after one log dilution (not visible in Fig. 2B), whereas all other primer sets did not result in amplicons. Replicate B was found negative with NVp110/NI (Fig. 1B) and all primer sets tested, which is in accordance with the negative results obtained for the extraction control.

The NVp110/NI products hybridized with genogroup II probe (NVp117) (Le Guyader et al., 2000). Direct sequencing of the PCR products or sequence analysis of three plasmid-cloned sequences revealed an identical sequence of 76 unique nucleotides between the primer sites (accession number AY316295, Table 1). Comparisons with published sequences identified this sequence as a genogroup II norovirus with the most closely related sequences belonging to the newly discovered cluster IIb. The second extraction done later, on replicate C, gave the same positive signal using NVp110/NI. After direct sequencing of these PCR products, identical sequences as in replicate A were found.

3.3. Confirmation of multiple strain contamination by outbreak-specific primers

The sequences obtained from the stools and the raspberries were clearly identified as norovirus sequences, yet they were not identical. Amplification of stool extracts using NVp110/NI consistently gave negative results, and despite several attempts using primer set NVp110/NVp69 on the raspberry extracts, no amplicons could be obtained. Based on these observations, outbreak-specific primers Ra1 and Ra2 were designed from the stool sequence. When applying these primers directly on the raspberry extracts, no signal was observed after a single round of RT-PCR. However, when a first round of amplification was performed using NVp110/Primer Ra1, followed by a nested PCR using Ra1/Ra2, a clear band of the expected size of 85 bp was observed (Fig. 1C). These positive results were not due to contamination by the stool samples as the analysis of the stools was done in Sweden and the analysis of the raspberries was performed in France. In addition, all PCR steps were performed in separate rooms, with several negative

### Table 1
Norovirus sequences detected in stool and raspberry samples

<table>
<thead>
<tr>
<th>Samples</th>
<th>Sequence (5’ → 3’ )</th>
<th>Size (bp)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>AGCACTGACAGAGGATTAGACATTAACCACTTGAACCTATGTGCACCGCT-CAAGTAGCTGGCCCAGATGATACAAATCGCAATCCTACACATCCTCCTTC</td>
<td>112</td>
<td>AY316294</td>
</tr>
<tr>
<td>Raspberries</td>
<td>CCTCACATATGTGGCAAGGTCTGCTGATACAGGAGCAGGTCTGCTGGACATCACATCCAGGCAAATCATCCTCCTTC</td>
<td>76</td>
<td>AY316295</td>
</tr>
</tbody>
</table>

In stool samples of ill patients, the sequence was obtained using primer set NVp110/NVp69, and in the raspberry samples using primer set NVp110/NI.
controls and filter tips to prevent carryover. Unfortunately, the obtained PCR fragment was too short to give reliable results in direct sequencing, and several attempts to clone the fragment were unsuccessful, presumably due to the low amounts of the PCR products. Nevertheless, the correct size of the PCR product and its absence in control reactions strongly indicate the presence of the same type of norovirus in the raspberries that was detected in the stool samples, in addition to the predominant strain only detected in the raspberries (that cannot be amplified by these primers).

4. Discussion

The importance of food transmission in gastroenteritis outbreaks is increasingly being recognized and a range of different types of food products can be involved as vehicles for noroviruses, such as cakes, deli-meat, salads, vegetables, fruits or shellfish (Bresee et al., 2002; Koopmans et al., 2002; Koopmans and Duizer, 2004). Berries are frequently implicated in gastroenteritis or hepatitis A outbreaks (Koopmans et al., 2003; Calder et al., 2003) and preservation of berries by freezing facilitates such virus persistence (Deng and Cliver, 1995; Croci et al., 2002; Calder et al., 2003). About 15 berry-related epidemics have occurred in Finland between 1998 and 2001, resulting in a ban of the use of unheated berries in all catering and other large-scale kitchens (Pöntä et al., 1999). Contamination of the berries can occur at several steps during the production process, for example, in open orchards, by irrigation of the fruits with sewage-contaminated water, or by handling of the berries by norovirus-infected food workers (Calder et al., 2003). Even contamination of the berries via the roots has been suspected (Katzenelson and Mills, 1984; Kirkham et al., 2002).

Detecting viruses in berries, especially raspberries, is a difficult task due to the presence of various inhibitors and a low pH. Nevertheless, the development of improved methods has recently been published (Bidawid et al., 2000; Schwab et al., 2000; Croci et al., 2002; Dubois et al., 2002; Sair et al., 2002; Calder et al., 2003). However, these reports were based on artificially contaminated berries, and to our knowledge, only two papers describe the detection of viruses in naturally contaminated berries implicated in outbreaks. One paper mentions the detection of a norovirus sequence in a raspberry sauce but fails to describe the methods used (Gaulin et al., 1999), and another paper demonstrates the same hepatitis A virus in blueberries and in stools of infected patients (Calder et al., 2003). We therefore aimed at further developing methods for the direct detection of noroviruses in complex food matrices like raspberries.

Following a gastroenteritis outbreak in Sweden, stool samples from ill patients and the raspberries that were suspected to be the cause of the outbreak were collected. Five out of nine patients turned positive for norovirus, but only after a range of generic primer sets had been applied in the diagnostic RT-PCR. This showed once again that molecular detection of noroviruses is far from easy, mostly caused by the extensive sequence diversity, and it emphasizes the necessity to apply several of the current detection methods to avoid false negative results. Alternative techniques to RT-PCR exist, but electron microscopy is laborious and demonstrates a lower sensitivity, and the ELISAs that are under development show an even lower sensitivity.
compared to RT-PCR, whereas the specificities of these three methodologies are in a comparable range (Rabenau et al., 2003; Richards et al., 2003). This makes RT-PCR the method of choice, especially when low levels of norovirus are expected or when complex food matrices like berries are analyzed.

The sequencing results for the patient samples showed that a single norovirus strain was responsible for the gastroenteritis outbreak, or at least that the RT-PCR only detected one strain that was predominant in the infected patients. However, when we applied the same panel of primers to the raspberry extracts, positive signal was obtained with a different primer set. Possible explanations for this result were either variations in virus infectivity, that the food analysis methodology was not optimal, that the berries were not contaminated with that norovirus strain and consequently were not responsible for the outbreak, or that not enough stools were collected (some other patients may have a different strain). Since noroviruses are not cultivable, it is not possible to test the first hypothesis, but this is unlikely when considering the low infectious dose needed to provoke gastroenteritis symptoms (Green et al., 2001). The second hypothesis was investigated by conducting experiments to evaluate the efficiency of the method adapted from a shellfish procedure (Le Guyader et al., 2000). The most crucial steps appeared to be the elution step, elimination of the seeds and adjustment of the pH (data not shown). To control extraction efficiency and to evaluate test sensitivity (quality control), samples were seeded with enterovirus particles at a low concentration. The use of an enterovirus excluded any mis-priming or competition during RT-PCR. Introducing quality control in environmental or food analysis is important when contamination levels are low and inhibitors are numerous, which was obvious when the EV control was negative for one of the three replicates analyzed. This sample was also found negative for norovirus, showing that results obtained using this extract had to be considered a false negative. This emphasizes the requirement for an extraction control when analyzing complex food items.

The two other replicates were found positive for norovirus, but sequence analysis revealed that this sequence was not identical to the patient strain. This would support the third hypothesis, namely that the raspberries were not responsible for the outbreak. However, multiple contaminations of shellfish with different strains of norovirus have been reported, with several strains detected in ill people (Sugieda et al., 1996), or multiple contaminations in patients with a single contamination in shellfish (Le Guyader et al., 1996, 2003). The fact that we successfully obtained a PCR fragment when applying the outbreak-specific primers on the raspberry extract indicates that the “outbreak” strain was also present on the raspberries, although presumably in lower quantities. Unfortunately, we failed to sequence the short fragment and were thereby not able to unambiguously prove that the berries were indeed contaminated with the outbreak strain as well, but the specificity of the primers makes it reasonably safe to conclude that the signal obtained by the nested PCR corresponds to the outbreak strain.

Why then did the people not get ill with the predominant strain detected by RT-PCR in the raspberries? Susceptibility to norovirus infection possibly has a genetic component (Harrington et al., 2002; Hutson et al., 2003), and recent work has demonstrated that the genetic background of patients could influence the clinical outcome of the infection (Hutson et al., 2003; Lindesmith et al., 2003). However, more genetic and immunological data from people exposed to currently circulating strains are needed for the better interpretation of these data. In addition, one could speculate that a variability of infectivity could be determined at least partially by the virus, one strain being more infectious for humans than the other. Also, when considering the most likely sources of the raspberry contamination, it can be envisaged that the noroviruses were not evenly distributed in the batch of frozen berries imported into Sweden. The berry sample collected belonged to the same lot as the one that was consumed, but variation in the contamination has previously been described for oyster contamination (Sugieda et al., 1996; Le Guyader et al., 2000) and blueberries (Calder et al., 2003). We therefore cannot exclude that some of the berries consumed were contaminated predominantly by the outbreak strain and the berries extracted for analysis were contaminated with the other strain detected in the raspberry analyses. Unfortunately, no further information could be obtained on the batch of berries, concerning either the production area or the production process, and therefore, we were not able to better elucidate the possible ways the berries could have been contaminated.
Recently, recombinant noroviruses have been identified (Koopmans et al., 2003) and it is possible that multiple strain contamination of food or water that result in co-infections with more than one strain of norovirus may facilitate the emergence of such recombinant viruses. Sensitive methods for the detection of noroviruses in various sources of infection, combined with powerful tools for genetic analysis, are crucial to improve our understanding of the evolution of these viruses. It is also important to better analyze the modes of transmission, how viruses are circulating between countries and to link variations between different outbreaks to genetic information about both hosts and viruses. We think that our study is contributing to this process and at the same time stimulates further improvements in the research on noroviruses.

Acknowledgements

This research was supported by European Union Grant: Foodborne viruses in Europe, QLK1-CT-1999-00594.

References


susceptibility and resistance to Norwalk virus infection. Nat. Med. 9, 548–553.
Norovirus Outbreak among Primary Schoolchildren Who Had Played in a Recreational Water Fountain

Christian J. P. A. Hoebe,1 Harry Vennema,2 Ana Maria de Roda Husman,3 and Yvonne T. H. P. van Duynhoven4

1Department of Infectious Diseases, Western and Eastern South Limburg Municipal Health Service, Heerlen, and 2Diagnostic Laboratory for Infectious Diseases and Perinatal Screening, 3Microbiological Laboratory for Health Protection, and 4Centro for Infectious Diseases Epidemiology, National Institute of Public Health and the Environment, Bilthoven, The Netherlands

Background. A gastroenteritis outbreak was associated with playing in a norovirus-contaminated recreational fountain.

Objective and study design. A retrospective cohort study was performed to estimate the magnitude of the outbreak and identify its source. Epidemiological investigation included standardized questionnaires about sex, age, school, class, risk exposures, and illness characteristics. Stool samples and environmental water samples were analyzed for the presence of bacteria, viruses, and parasites.

Results. Questionnaires were returned for 191 schoolchildren (response rate, 83%) with a mean age of 9.2 years, of whom 47% were ill (diarrhea and/or vomiting). Children were more likely to have been ill if they had played in the recreational fountain (relative risk, 10.4). Norovirus (Birmingham) was detected in 22 (88%) stool specimens from ill children and in 6 (38%) specimens from healthy children. The water sample from the fountain contained a norovirus strain that was identical to the RNA sequence found in stools.

Conclusions. Recreational water may be the source of gastroenteritis outbreaks. Adequate water treatment can prevent these types of outbreak.

In recent years, noroviruses (previously designated as “Norwalk-like viruses” or “small round-structured viruses”) have emerged as an important cause of food- and waterborne gastroenteritis outbreaks [1]. Reported waterborne outbreaks caused by norovirus have been associated with private wells, small water systems, and community water systems [2–7]. Recreational surface water, including lakes and swimming pools, has also been associated with norovirus outbreaks [8–10]. However, recreational fountains have so far been associated with gastroenteritis outbreaks caused by Shigella and Cryptosporidium species but not by norovirus [11, 12].

In the present article, we describe the investigation of the first outbreak caused by norovirus among schoolchildren who played in a recreational fountain. On a hot summer day in June 2002, ~200 children in 3 primary schools had their annual preholiday school outing at a playground. Two days later, the principals of the schools informed the Dutch Municipal Health Service that ~100 children had symptoms of vomiting, diarrhea, abdominal pain, and headache. Food was not a probable cause of the illness, because most children had eaten their own homemade lunches. The only common food exposure was commercially packaged ice cream, purchased at the playground by most children. One common source of water exposure was a recreational fountain (figure 1). In addition, some children might have drunk from a water tap close to the fountains and inside the sanitary facility. The inside and outside water taps both contained regular drinking water.

All parents of ill children were given information about hygiene, risk of dehydration, and the application of oral rehydration solution. In addition, a retrospective cohort study was performed to estimate the magnitude of the outbreak among these schoolchildren and to identify its source.

METHODS

Epidemiological investigation. All 3 schools reporting ill schoolchildren had taken part in a visit to a
Figure 1. Photograph of children playing in a recreational water fountain

playground, featuring a recreational fountain, on 18 June. A retrospective cohort study was conducted among the 231 Dutch children attending the 3 primary schools who had visited the playground that day (referred to below as playground children). Standardized questionnaires were sent to their homes to obtain information about sex, age, school, class, possible risk exposures (playing in or drinking from the fountain, drinking from water taps, or eating ice cream), the onset and nature of symptoms, the duration of illness, contact with a general practitioner or hospital, and prior illness. A primary case was defined as illness in any child or adult who had visited the playground and who had developed diarrhea (≥3 loose stools in any 24-h period) or vomiting (at least 1 episode) or both within 72 h after the visit. An exposed child was defined as a child who had played in the recreational fountain at the playground.

Six weeks after the first questionnaire was sent out, we sent a second questionnaire to all children’s homes to obtain details about family size and possible secondary cases caused by person-to-person transmission. A secondary case was defined as illness in any child or adult—within a family with a playground child—who had not visited the playground and who had experienced diarrhea (≥3 loose stools in any 24-h period), vomiting (at least 1 episode), or both during the 6-week period after the visit. This 6-week period was chosen because the duration of virus shedding can be long and there might be even tertiary waves of cases [13]. We calculated relative risks (RRs) with 95% confidence intervals (CIs) to assess any association between illness and individual exposure factors. The secondary attack rate was calculated as the number of secondary cases divided by the total number of household members, excluding playground children.

Environmental investigation. Two days after the visit, on the day of the notification, the playground was visited so that an environmental assessment could be conducted. Hygiene policies and procedures were reviewed. The recreational fountain covers an area of ~40 m², in which ~40 jet spray nozzles squirt water jets whose height varies over time. Children playing in the fountain—frequently in underwear—are completely soaked by the water. The fountain system uses recirculated water that drains from the wet play floor into an underground reservoir. A filter is used for coarse materials like sand, grass, and hair, and part of the water is subjected to sand filtration. The reservoir water is manually chlorinated using hypochlorite tablets, and no continuous water analysis is performed to check chlorination levels. The reservoir is replenished with tap water each day, to compensate for water losses. The nearby water taps provide drinking water.

Environmental samples (100 mL) of the recreational fountain water and the drinking water were obtained for bacterial analysis according to the European recreational and drinking water guidelines, respectively. The water samples were cultured for coliform bacteria, *Escherichia coli*, and *Enterococcus, Salmonella,*
and Campylobacter species, by standard culture methods. In addition, large volumes (300 L) of drinking and recreational water were concentrated for rotavirus and norovirus detection by polymerase chain reaction (PCR) [14]. Commercially packaged ice cream sold at the playground was also obtained for microbiological investigation but was not examined any further after the cause of the outbreak became clearer. In consultation with the manager of the playground, the recreational fountain and water taps were closed on the day of sampling until further research had clarified the cause of the illness. The water reservoir was emptied and disinfected.

Laboratory investigation. Stool specimens were collected 3–6 days after the playground visit and were cultured for Salmonella, Shigella, Yersinia enterocolitica, enterohemorrhagic E. coli (O157), and Staphylococcus aureus, by standard culture methods. A random sample of isolates of E. coli was serotyped. Fecal smears were examined by direct microscopy for ova and parasites, including Cryptosporidium and Giardia species. Fecal examination of a random selection of 11 second samples was performed for Giardia species, because it was expected that more positive samples would be detected after 3 weeks if Giardia species were the cause of the outbreak. Stool samples were examined for rotavirus and adenovirus by commercially available ELISA kits, and RNA amplification by reverse-transcription (RT) PCR was used to test for astrovirus and caliciviruses (i.e., noroviruses and sapoviruses), as described elsewhere [15], using primers JV12Y/JV13I [16]. The initial results indicated the presence of norovirus genotype Birmingham in some fecal samples. Because it has been found that this genotype is difficult to detect with primers used in routine diagnostic procedures, we used an alternative RT primer and designed a specific primer for the PCR, designated NVp110 [17] and JV12BH (5′-GTT TCA TTA TGA TGC TGA-3′), respectively.

RESULTS

Epidemiological investigation. Questionnaires were returned for 191 schoolchildren (response rate, 83%) and for 1 parent who had accompanied the children on the school trip (table 1). These data represent 160 different households. The mean age of the children was 9.2 years (SD, 1.5 years; range, 4–12 years), and 53% (102/191) were girls. Symptoms of diarrhea and/or vomiting were present in 47% of the children (90/191). No children had
symptoms of diarrhea and/or vomiting before or during the playground visit. The main symptoms among cases were abdominal pain (89%), nausea (78%), vomiting (75%), diarrhea (70%), headache (70%), and abdominal cramps (49%). Only 8% of the children (7/90) had visited a general practitioner, and none had been admitted to a hospital. The onset of symptoms in most children was 1–2 days after the trip (84%). The mean incubation period was 30 h, with a range of 7 h (18 June) to 72 h (21 June; the see epidemic curve in figure 2). The duration of symptoms was known for 67% of the cases. The remainder still had symptoms at the time of completing the questionnaire. The mean duration for those who had already recovered was 1.7 days (SD, 0.8 days; range, 0.5–5 days). This implies that the estimate for all ill children, including those with illness of longer duration (33%), would be higher (at least 2 days).

There was no relationship between developing illness and drinking from the water taps or consumption of ice cream (table 2). However, schoolchildren were more likely to have become ill if they had played in the recreational fountain (RR, 10.4; 95% CI, 1.5–70.8) or had ingested water from it (RR, 2.0; 95% CI, 1.4–3.0). The question as to whether they had ingested the water could not be answered (“unsure”) by 43 (23%) of the children. The attack rate in children exposed to the recreational fountain was 54% (90/167). Attack rates were similar for both sexes (45% male vs. 50% female) and for all age groups.

Table 2. Univariate analyses of risk factors studied in outbreak of gastroenteritis.

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Exposed to risk factor</th>
<th>Not exposed to risk factor</th>
<th>RR (95% CI)</th>
<th>Cases exposed to factor, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial ice cream</td>
<td>Ill 72 NOT 76</td>
<td>Ill 17 NOT 22</td>
<td>1.1 (0.8–1.7)</td>
<td>80</td>
</tr>
<tr>
<td>Consumption of water from taps near recreational fountain</td>
<td>Ill 3 NOT 4</td>
<td>Ill 78 NOT 89</td>
<td>0.9 (0.4–2.2)</td>
<td>4</td>
</tr>
<tr>
<td>Consumption of water from taps near sanitary facility</td>
<td>Ill 18 NOT 32</td>
<td>Ill 68 NOT 64</td>
<td>0.7 (0.5–1.0)</td>
<td>21</td>
</tr>
</tbody>
</table>

NOTE. Data are no. of children, except where noted. CI, confidence interval; RR, relative risk.

Environmental investigation. No enterococci or E. coli bacteria were detected in the drinking water samples taken at the sanitary facility, in compliance with the European drinking water regulations. The sample taken from the recreational fountain, however, had very high bacterial counts that exceeded the standards of the European recreational water guidelines. Numbers of coliform bacteria exceeded the detection limit of 1000 organisms/mL, and enterococci were found at a rate of 3500 organisms/100 mL, whereas the concentration of E. coli bacteria was 7700 organisms/100 mL. No Salmonella, Campylobacter, or rotaviruses were detected in any of the water samples. The presence of norovirus RNA in the fountain water was ascertained using the primer pair JV12BH/NVp110. The sequence of the PCR product derived from the fountain water was identified as norovirus type Birmingham.

Microbiological results. Stool specimens were available from 25 children who had fallen ill and 16 children without symptoms of diarrhea and/or vomiting. Two stool samples were available from adults: a teacher and a parent who had accompanied the children on the playground trip. All stool samples were negative for bacterial pathogens. Of the 9 fecal specimens cultured for E. coli, all showed possible strains of E. coli; a random sample of 7 were serotyped. There was no similarity in serotypes: samples with serotypes O7, O45, O86, O88, O141, and O166, and 1 turned out not to be E. coli. This outcome
Table 3. Positive findings from stool sample analysis (43 stool samples tested) from an outbreak of gastroenteritis.

<table>
<thead>
<tr>
<th>Diagnostic assay</th>
<th>Children exposed to fountain</th>
<th>Adults exposed to fountain</th>
<th>Total exposed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ill</td>
<td>Healthy</td>
<td></td>
</tr>
<tr>
<td>Norwalk PCR</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>JV12Y/JV13I</td>
<td>6/25 (24)</td>
<td>4/36 (11)</td>
<td>10/27 (37)</td>
</tr>
<tr>
<td>JV12BH/NVp110</td>
<td>17/71 (24)</td>
<td>2/18 (11)</td>
<td>19/51 (37)</td>
</tr>
<tr>
<td>All tests combined</td>
<td>22/92 (24)</td>
<td>6/45 (11)</td>
<td>27/76 (37)</td>
</tr>
<tr>
<td>Giardia species</td>
<td>3/13 (24)</td>
<td>1/9 (11)</td>
<td>4/11 (37)</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. positive/no. tested (% positive). PCR, polymerase chain reaction.

Norovirus in a Water Fountain

---

**DISCUSSION**

**Norovirus outbreak.** This outbreak in children with a clinical profile of abdominal pain, nausea, vomiting, and diarrhea, with an attack rate of 54%, a mean incubation period of 30 h, a duration of illness of ~2 days, and 39 clear secondary cases, fits in well with norovirus. An identical norovirus strain (Birmingham) was detected in stool specimens from ill persons and in a water sample derived from the fountain. It is very unusual that we were able to detect the causative agent in the water, because the source is usually only identified epidemiologically [18]. The duration of playing in the fountain per school suggests a dose-response relation, because the primary attack rate was significantly higher for the school with the longest visit, compared with the school with the shortest visit. The evidence of the source of this outbreak was provided by the complimentary nature of the epidemiological analytical and microbiological findings and was reinforced by the specific norovirus molecular-sequencing analysis in stool specimens and water samples, which shows the great value of these techniques in discovering new relationships.

**Exposure to recreational fountain.** Recreational fountains are becoming more frequent in traditional playgrounds (and in public parks or town centers), because they are very popular among children. Children like to stand directly over the nozzles (jets), so their entire bodies become soaked (figure 1). Recreational water may be at high risk for contamination by enteric pathogens through overt fecal accidents or the rinsing of contaminated bodies in the water. Operators of recreational fountains should monitor levels of effective chlorine and microbiological water-quality indicators routinely. Public health officials should regularly inspect public recreational fountains about these water-quality procedures. European Union guidelines are based on bacterial indicators (general contamination), but these are not always able to detect contamination by viruses—although, in our outbreak, there was bacterial contamination as well. It is not reasonable to routinely monitor for norovirus, because PCR assays are difficult to perform and interpret. Thus, preventive measures should be taken to reduce the risk of contamination, including adequate chlorination of the water and supervision of the chlorination system, the frequent replacement of the water (especially after hot days with heavy use), and the presence of adequate, clean sanitary facilities. An automatic water-handling system was later installed at the recreational fountain described here that maintains continuous chlorination levels of 1.2 mg/L. Chlorine levels are registered in a journal 3 times/day. Procedures to fill the reservoir with fresh water every 2 days are documented and carried out, and microbiological analysis of water samples is performed by an authorized laboratory every 14 days. Additional measures—which would, however, be more difficult to maintain—include forcing visitors to shower at home or at the fountain area before entering the fountain, excluding patients with di-
Surveillance and mandatory notification. Studies on gastroenteritis outbreaks are regularly conducted in The Netherlands, as they are in many countries. Interim results from a study of gastroenteritis outbreaks in The Netherlands in 2002 showed that ~53% of 119 microbiologically investigated outbreaks were caused by norovirus, and only 1 outbreak—described in the present report—was waterborne. In the United States, 3%–6% of the norovirus outbreaks have been reported to have originated from water consumption. During the 4-year period from 1997 to 2000, 54 waterborne disease outbreaks of gastroenteritis associated with recreational water were reported in the United States [19, 20]. Five (9%) were caused by norovirus and were associated 3 times with lake-water, once with motel pool water, and once with hot springs in a resort. In the United Kingdom, none of the norovirus outbreaks originated from a water source [21]. Prior outbreak surveillance in England and Wales showed that only 1 of 26 waterborne gastroenteritis outbreaks was caused by norovirus; it originated from recreational water sports on a river [22]. In Ireland, only 1 of 67 gastroenteritis outbreaks studied was waterborne (through water consumption) [23]. This shows that recreational waterborne norovirus outbreaks are reported rarely. These outbreaks go easily unnoticed and are most likely underreported because norovirus outbreaks are so common, with >90% caused through person-to-person transmission, that it is very difficult to motivate local authorities to stay alert and investigate them in order to distinguish the food and waterborne outbreaks in an early stage from the bulk of person-to-person outbreaks. Also, in waterborne outbreaks, affected people often do not know each other and do not visit the same general practitioner or school; therefore, connections are easily missed between the different cases. Furthermore, in The Netherlands, as in most European Union countries, the testing capacity for norovirus is not routinely available in the primary diagnosing laboratories that received stool samples from the diarrheal cases. Norovirus outbreaks often show up as the outbreaks that are unexplained by well-known and routinely tested enteric bacteria, some viruses, and protozoa. Confirmation of the outbreak being caused by norovirus fully depends on these laboratories passing on the stools to the 1 or 2 national laboratories that are able to perform norovirus tests [24]. The large outbreak described in the present report would have probably remained undetected if the primary schools had not reported the cases to the Municipal Health Service. This shows the value of the mandatory notification of gastroenteritis clusters by institutions like primary schools. Outbreak investigations are important in public health to identify the source, implement control measures, and prevent future illness; in ad-
dition, they frequently yield new knowledge that may lead to amended control policies. In the outbreak described, the novel source of norovirus was a recreational fountain.

Acknowledgments

We thank J. Gronenschild (Public Health Laboratory at the Atrium Hospital Heerlen); B. M. de Jongh (Medical Microbiology Department of the Lorentz Hospital Zeist); W. Wannet, T. Kortbeek, and D. Hoek (Research Laboratory for Infectious Diseases and Perinatal Screening of the National Institute of Public Health and the Environment), for their participation in the microbiological analysis of the stool samples; W. J. Lodder, H. A. M. de Bruin, and M. During (Microbiological Laboratory for Health Protection of the National Institute of Public Health and the Environment), for sampling and analysis of water from the recreational fountain and nearby taps; and public health nurses M. Henssen and C. de Jager, who provided valuable help in the management of the outbreak.

References

Epidemiology of Norwalk-like virus infections in cattle in The Netherlands

Wim H.M. van der Poel\textsuperscript{a,*}, Reina van der Heide\textsuperscript{a}, Froukje Verschoor\textsuperscript{a}, Hans Gelderblom\textsuperscript{d}, Jan Vinje\textsuperscript{b}, Marion P.G. Koopmans\textsuperscript{c}

\textsuperscript{a}Microbiological Laboratory for Health Protection (MGB), National Institute for Public Health and the Environment (RIVM), P.O. Box 1, Antonie van Leeuwenhoeklaan 9, 3720 BA Bilthoven, The Netherlands

\textsuperscript{b}Laboratory for Infectious Disease Research (LIO), National Institute for Public Health and the Environment (RIVM), P.O. Box 1, Antonie van Leeuwenhoeklaan 9, 3720 BA Bilthoven, The Netherlands

\textsuperscript{c}Laboratory for Infectious Disease Diagnostics and Screening (LIS), National Institute for Public Health and the Environment (RIVM), P.O. Box 1, Antonie van Leeuwenhoeklaan 9, 3720 BA Bilthoven, The Netherlands

\textsuperscript{d}Robert Koch-Institut, Nordufer 20, D-13353 Berlin, Germany

Received 18 March 2002; received in revised form 9 October 2002; accepted 17 November 2002

Abstract

“Norwalk-like viruses” (NLVs) are the most common cause of acute non-bacterial gastroenteritis in humans. Cattle may be a reservoir of NLVs although never bovine NLVs have been found in humans. To gain more insight into the epidemiology of NLV, infections in cattle in The Netherlands were studied. Individual faecal samples from a large dairy herd and 243 pooled samples from veal calf farms were analysed for NLV by RT-PCR. Calves under 3 months of age in the dairy herd were sampled three to five times with 3-week intervals, whereas dairy cattle were sampled twice with a 2-month interval. In 31.6\% (77/243) of the veal calf farm samples and in 4.2\% (13/312) of the individual dairy cattle samples NLV was detected. The mean age of virus positive dairy cattle was 2.5 months. The highest numbers of NLV positive veal calf farms in The Netherlands were found in the regions with the highest number of veal calf farms. NLV infected veal calf farms were detected in every month throughout the study period. Cattle appeared to be hosts of NLVs, and virus shedding was weakly associated with diarrhoea. Complete ORF2 sequences were obtained from two calf NLVs and phylogenetic analyses suggested that these strains belong to a distinct cluster (GGIII/2) in between GGI and GGI\textsubscript{I} NLVs of humans. Overall, genetic variation between strains as determined by

\textsuperscript{*}Corresponding author. Tel.: +31-30-2743666; fax: +31-30-2744434.

E-mail address: wim.van.der.poel@rivm.nl (W.H.M. van der Poel).
sequence analysis of the P1/P2 capsid region was limited to 14.6%. Our data shows that NLV is endemic in the cattle population in The Netherlands and genetically distinct from NLVs in humans. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** Cattle virus; Norwalk-like virus; Calicivirus; Epidemiology

### 1. Introduction

Within the family *Caliciviridae*, four genera have been distinguished, named vesivirus, lagovirus, “Norwalk-like viruses” (NLV) and “Sapporo-like viruses” (SLV) (Green et al., 2000). Norwalk-like viruses, have been emerging as the single most common cause of infectious gastroenteritis in people of all age groups, and the main cause of outbreaks of gastroenteritis in institutions such as nursing homes, hospitals, and restaurants (De Wit et al., 2001; Fankhauser et al., 1998; Green, 1997; Vinjé et al., 1997). Every winter season, distinct genetic types of NLVs are detected, causing gastroenteritis cases and outbreaks, and sometimes epidemics. In some epidemics, the greater number of outbreaks are caused by a single genotype (Vinjé et al., 1997), and such a single NLV strain can have a global distribution (Noel et al., 1999). Person-to-person, foodborne, or zoonotic transmission have been suggested, which could explain the sudden emergence and global spread of an epidemic strain of NLV.

Based on RNA polymerase sequences (ORF1), NLVs have been divided into two major genetic groups: GenogroupI (GGI) and GenogroupII (GGII) (Vinjé et al., 2000b). Based on ORF2 sequences, Ando et al. (1994) have proposed a genetic classification of NLVs into three major genetic groups: GGI and GGII comprising almost all human strains. The two bovine strains, Jena (Liu et al., 1999) and Newbury (Dastjerdi et al., 1999) would constitute a newly defined third major genetic group (GGIII). This was supported by Schuffenecker et al. (2001) who proposed a uniform classification of SLVs.

Molecular characterizations of calicivirus strains from cattle have been reported. The strain described by Neill et al. (1995) Tillamook virus; BCV-Bos1, caused respiratory symptoms, and was phylogenetically related to San Miguel sea lion virus (SMSV) and vesicular exanthema of swine virus (VESV), both viruses within the genus vesivirus. Jena virus (Liu et al., 1999) and Newbury agent type 2, were detected in feces from two newborn calves with diarrhoea (Dastjerdi et al., 1999). Recently, the presence of NLVs was detected in 44% of calf farms in The Netherlands. Phylogenetic analysis of the detected polymerase sequences showed a distinct cluster, although at considerable difference, most closely related to GGI. It, therefore, has been suggested that the bovine species may be a normal host for NLVs (van der Poel et al., 2000).

To study the epidemiology of NLV in bovines, faecal samples were collected from veal calf farms and from individual cattle in a large dairy herd in The Netherlands. All samples were tested for NLV by RT-PCR and the dynamics of NLV infections in cattle were evaluated. Furthermore, the genetic classification of these viruses was investigated by sequencing ORF1 and ORF2 PCR amplification products as well as the entire ORF2 of two strains.
2. Materials and methods

2.1. Animals and faecal specimens

Between 1 January 1997 and 31 December 1999, 243 faecal samples were collected from veal calf farms as part of an ongoing surveillance study of potential zoonotic microorganisms associated with gastroenteritis in humans (Giessen et al., 1992). Results of NLV screening of faecal calf herd samples over the first 3 months of 1998 have been described previously (van der Poel et al., 2000). Data of other micro-organisms will be described elsewhere. The age of the calves ranged from 1 to 52 weeks (average age 12 weeks), and farm sizes ranged from 38 to 930 animals (average 296). Calf farms were geographically distributed all over The Netherlands. Between October 1999 and June 2000 faecal specimens were also collected from individual cattle of a large dairy herd.

The sampling strategy of the calf herds was designed to allow monitoring for the presence of pathogens in a large number of animals and allows detection of microorganisms at farm level with a prevalence of 5% with a 95% confidentiality (Giessen et al., 1992). Faecal samples from veal calves were collected from animals housed in one randomly chosen farm building. Pooled samples of maximum 12 individual veal calf samples were assigned as the farm sample. In the dairy herd (240 animals), the animals were sampled individually. Twice, with a 2-month interval, the following samples were collected: (1) 0–6 months age: 25 samples (all animals), (2) 6–12 months: 24 samples (all animals), (3) 1–2 years: 30 samples, (4) >2 years: 47 samples. In addition individual samples of dairy calves under 3 months of age, of the same herd were collected longitudinally, three to five times with a 2–3-week interval. A total number of 88 faecal samples from 19 young dairy calves were tested. In this dairy herd all cases of diarrhoea at the time of sampling were recorded.

Until tested, faecal samples were stored at −70 °C in 15 g/l of Trypton Soya broth (TSB) (Oxoid CM 129) and 10% glycerol. All samples were analysed in the study unless indicated differently.

2.2. Virus detection

Molecular detection of calf NLVs was performed by RT-PCR and Southern blotting as described previously (van der Poel et al., 2000). For comparison, all samples were also screened for the presence of rotavirus group A by RT-PCR as described by Husain et al. (1995). To reduce the risk of contamination, a negative control sample was included for every two faecal specimens. Bovine faecal samples positive for NLV and rotavirus by EM were included as positive controls. Extraction, preparation of master mixes, preparation of reactions, and analysis of PCR products was done in different rooms with designated sets of pipettes. To avoid false-positive PCR results the precautions described by Kwok and Higuchi (1989) were strictly followed.

NLV positive samples containing high concentrations of viral RNA, resulting in RT-PCR end-point dilution titres of at least 10^5, were screened for NLV by electron microscopy (EM). EM procedures were performed as recommended by Flewett (1978) and Doane and Anderson (1987). Briefly, a 10% faecal suspension in phosphate-buffered saline was
clarified by centrifugation for 10 min at 3000 × g at 4 °C. The supernatant fluid was centrifuged for 1 h at 90,000 × g at 4 °C. The resulting sediment was resuspended in a drop of distilled water, adsorbed to carbon reinforced pioloform coated grids and negative contrasted using uranyl acetate (Biel and Gelderblom, 1999). Samples were evaluated using a ZEISS EM 10 Å transmission electron microscope at 40,000 primary magnification at 80 kV. Based on size and characteristic surface morphology (Caul and Appleton, 1982), particles were assigned to specific virus families. Measurements of virus diameters were taken directly on negatives with the help of a 10× measuring magnifier.

2.3. Sequencing

The NLV polymerase gene RT-PCR products of expected size (327 bp) were excised from a 2% agarose gel and purified using a Qiaquick gel extraction kit (Qiagen, Hilden, Germany). Purified RT-PCR products were sequenced with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Applied Biosystems, Foster City, CA) by the use of the PCR primers.

First strand c-DNA was generated from polyadenylated viral RNA of two samples using T20VN-linker primer (Liu et al., 1995) and Superscript II RNase H-reverse transcriptase (Life Technologies). The complete calfcalciivirus ORF2 gene was amplified using a nested RT-PCR based on primers calfpol1 and calfpol2 (Table 1) derived from the previously obtained calf-NLV RNA polymerase nucleotide sequences (van der Poel et al., 2000) and primer JenaORF3 from the ORF3 sequence of Jena virus (Liu et al., 1999) using the Expand Long Template PCR system (Roche, Mannheim, Germany) for 35 cycles of 94 °C for 10 s, 40 °C for 30 s, and 68 °C for 2 min 10 s in a Perkin-Elmer 9700 thermocycler.

The 2.7 kb products were cloned using a TA cloning kit (Invitrogen, Leek, The Netherlands) and sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Reaction Kit (Perkin-Elmer, Nieuwerkerk a/d IJssel, The Netherlands) on an automated sequencer (Applied Biosystems model 377; Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Orientation</th>
<th>Nucleotide sequence (5′–3′)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TVN-linker</td>
<td>–</td>
<td>TAG TAC ATA GTG GAT CCA GC(T)20VN</td>
<td>Liu et al. (1995)</td>
</tr>
<tr>
<td>BAMHI linker</td>
<td>–</td>
<td>TAG TAC ATA GTG GAT CCA GC</td>
<td>Liu et al. (1995)</td>
</tr>
<tr>
<td>Calfpol1</td>
<td>+</td>
<td>TTC CTG CTG GCA GTG CAC G</td>
<td>This study</td>
</tr>
<tr>
<td>Calfpol2</td>
<td>+</td>
<td>CAG AGG TCA CCA ACT TGG AC</td>
<td>This study</td>
</tr>
<tr>
<td>JenaORF3</td>
<td>–</td>
<td>CAG TGG TAA ACG ATC TGT ATT</td>
<td>This study</td>
</tr>
<tr>
<td>CCV1</td>
<td>–</td>
<td>CAA AGG TCA AAG AGC AAT CGG A</td>
<td>This study</td>
</tr>
<tr>
<td>CCV2</td>
<td>+</td>
<td>CTC AGT TCA ATT TCA AAA CGG C</td>
<td>This study</td>
</tr>
<tr>
<td>CCV3</td>
<td>+</td>
<td>GGC TTT CCA GAT TTT TCC GAT TG</td>
<td>This study</td>
</tr>
<tr>
<td>CCV4</td>
<td>–</td>
<td>GGC AGC TCT GGA AAC AAA ATG</td>
<td>This study</td>
</tr>
<tr>
<td>Probe CV12a</td>
<td>+</td>
<td>CCC TCA TCT CCT TCC GAC CTG</td>
<td>This study</td>
</tr>
<tr>
<td>Probe CV34a</td>
<td>+</td>
<td>TGT TCC TGC CCC AGG TGT CGG TG</td>
<td>This study</td>
</tr>
</tbody>
</table>

CV1 CV2 amplicon: expected size 217 bp; CV3 CV4 amplicon: expected size 387 bp.

* Probes are 5′-biotin labeled.
2.4. Capsid RT-PCR

Based on the complete ORF2 consensus capsid nucleotide sequences of the two calf NLV strains, we selected several primer pairs that correspond to the protruding (P1) and structural domains (P2) of Norwalk virus capsid protein (Prasad et al., 1999), and thus potentially would be the most sensitive for detecting the genetic variability among the calf-NLVs. RT-PCR assays were developed using the same format that has been used previously (Vinjé et al., 1997, 2000a). The assays were optimized for concentrations of deoxynucleotide triphosphates (dNTPs) and MgCl₂. Of the RT-PCR positive faecal samples, complete sequences were obtained of 69 PCR products of the hypervariable region of the capsid gene.

2.5. Phylogenetic analysis

Multiple alignment of partial NLV polymerase nucleotide sequences and complete capsid sequences were created using Clustal W (v1.4) alignment program in the Bioedit (v5.0.9) sequence alignment editor and analysis package (Hall, 1999), using its default settings. Distance calculations were done using Jukes and Cantor correction for evolutionary rate. The confidence values of the internal nodes were calculated by performing 100 bootstrap analyses. Evolutionary trees for nucleotide sequences were drawn using the UPGMA method, for ORF1 and for ORF2 (nt 550–2085) capsid gene. To address the proper phylogenetic classification of calf NLVs detected in this study, we compared the entire ORF2 sequences of two Dutch calf-NLVs (CH126, CH131) with Jena virus (complete ORF2) and Newbury agent (partial ORF2).

2.6. Data analysis

NLV positive as well as rotavirus positive calf herd samples were counted for each quarter of the years 1997–1999. Percentages of positives were calculated and graphed together with total numbers of positives and the numbers of collected samples. NLV positive and rotavirus positive calf herd samples were also counted per postal area and depicted together with the numbers of collected samples and the distribution of veal calf farm in The Netherlands. Birth dates of all dairy calves were recorded and ages at the time of NLV shedding were calculated. Statistical analysis was performed with the SAS software system, using the GENMOD procedure and the Cochran–Mantel–Haenszel test.

3. Results

3.1. Virus detection

In total, in 77 (32%) of 243 veal calf farm samples and in 13 (4%) of 312 individual dairy cattle samples (including calves) NLV was detected by RT-PCR including hybridisation with a specific probe (according to van der Poel et al., 2000).
Of all 13 NLV positive individual dairy cattle (including dairy calves) samples, 12 contained high concentrations of NLV RNA, resulting in RT-PCR end-point dilution titres of $10^5$ or more. Particles with NLV morphology were found in 42% (5/12) of these specimens (Fig. 1). Norwalk-like virus RNA as well as Rotavirus group A RNA was detected in veal calf farms in every month from April 1997 to December 1999. The positivity rate ranged from 6 to 54% for NLV and from 4 to 40% for rotavirus (Fig. 2).

3.2. Association with gastrointestinal disease

Of all surveyed calves under two months of age ($n = 83$), 27 calves showed diarrhoea at the time of sampling. In 5 of these 27 calves, NLV was detected, whereas in 9 of the 56 non-diarrhoeic calves NLV was also detected. Three of these nine NLV positive non-diarrhoeic calves had manifested diarrhoea before. NLV shedding was slightly associated with diarrhoea but this association was not statistically significant ($P > 0.05$).
3.3. Age distribution and duration of virus shedding

In 0.15% (2/13) of NLV positive dairy calves viral RNA could still be detected after a 2-month interval. Duration of NLV shedding in calves was estimated between 1 and 4 weeks. The mean age of calves shedding virus was 2.5 months, with the youngest being 1 month of age and the oldest 9 months of age. Reinfections were not observed.

3.4. Seasonal distribution

Norwalk-like virus infections in veal calves in The Netherlands did not show a clear seasonal distribution like the seasonality of rotavirus, observed in countries with a temperate climate. However, a seasonality was also not observed for rotavirus in this study. While a decrease in the the proportion of NLV positive samples was observed in the second quarter of 1997, the third quarter of 1998 and the first quarter of 1999, virus positive farms were found throughout the year (Fig. 2).

3.5. Geographical distribution

In The Netherlands, the highest concentrations of veal calf farms are located in the centre and the mid-east part of the country. The highest numbers of NLV positive veal calf farms in The Netherlands were found in the same regions as where the highest concentration of veal calf farms are located. A significant increase in relative incidence in specific regions of the country were not observed (Fig. 3).
3.6. Genetic classification and diversity of calf-NLVs

Based on a 145 nt fragment of the ORF1 polymerase gene, all bovine calicivirus sequences clustered with Jena virus and Newbury agent on a separate branch in between GGI and GGII NLVs (Fig. 4). The maximum divergence between the bovine NLV ORF1 sequences in The Netherlands and the Newbury strain was 37% (25% on amino acids) as previously described (van der Poel et al., 2000). Based on a 568 nt fragment of the ORF2 nucleocapsid gene (CCV2/CCV4 amplicon) the genetic variability among 69 calf-NLVs in The Netherlands was 0–14.6% (Fig. 5). All NLV positive samples from the dairy herd showed exactly the same sequence (DH1 in Fig. 4) but closely related calf herd sequences did not show any association regarding region or year of origin.

For sequencing of the complete ORF2 gene, two calf herd strains were picked: Bo/NLV/ Calf126/NL/1998 and Bo/NLV/Calf131/NL/1998. The complete capsid sequences of these two calf-NLV strains had uncorrected amino acid distances of only 2% with each other, and 2% with a partial capsid sequence of the Newbury strain (Dastjerdi et al., 1999). For NLVs from humans, 20% amino acid difference across the capsid is thought to constitute a
Fig. 4. Topology of NLV The Netherlands calf herd 1998 strains (including CH126 and CH131) in different regions of the genome. GenBank accession numbers of the complete capsid sequences used for the trees: Norwalk (M87661), AJ011099, L23831, L070418, U04469, X86557, X81879, Toronto (U02030), Hawaii (U07611), AF320625, and AF320113.
distinct genotype. Based on the amino acid distances with Jena virus (31%), the Dutch strains form a new cluster or genotype within the third major genetic group (GGIII) proposed by Ando et al. (1994). The Genbank sequences of the entire ORF2 of two strains (CH126 and CH131) are AF320625 and AF320113. A phylogenetic tree of complete capsid sequences is shown in Fig. 4.

4. Discussion

To study the epidemiology of NLV in cattle, fecal samples were collected from calf farms and from individual cattle in a large dairy herd in The Netherlands. All samples were tested for NLV by RT-PCR including hybridisation. In 32% (77/243) of veal calf farm samples and in 4% (13/312) individual dairy cattle samples NLV was detected. A similar prevalence in veal calf farms has been observed previously (van der Poel et al., 2000). The observed NLV veal calf farm prevalence (32%) cannot be compared to the individual dairy cattle prevalence (4%). But from the results in veal calves and dairy cattle in this study it could be concluded that NLV infections were mainly found in young cattle. Reinfections were not observed and older cattle (>9 months) did not shed detectable amounts of NLV.
This observation differs from the reported findings in humans where NLV infections are found in all age groups (Koopmans et al., 2000). The absence of NLV sequences in adult cattle may be explained by an acquired strain-specific immunity in older animals that prevents repeated infections, or by mismatches of the used primer pair which was optimised for detection of NLVs of humans (Vinjé and Koopmans, 1996). Not all samples have been tested by EM, and there could be cattle NLVs that are not detected by the used JV12/13 primer pair and the probe we selected. A third possibility is that levels of shedding in adult animals are very low, precluding virus detection in faecal specimens. A difference in epidemiology and/or immunogenicity of NLVs between species could explain different findings between cattle and humans, but extensive immunological studies will be needed to confirm this.

In this study no clear seasonal distribution in NLV infections in veal calves was observed. This may be due to the fact that the veal calves, were kept indoors all year round and climatic conditions inside barns were quite stable. This assumption is supported by the observed pattern of rotavirus infections, which did not show a clear seasonal distribution either. As expected, geographical influences on NLV presence in cattle within The Netherlands could not be demonstrated. Like human NLVs, cattle NLVs are most likely detectable wherever there is properly looked for.

Pathogenicity of specific NLVs in calves have repeatedly been reported (Dastjerdi et al., 1999; Günther et al., 1984; Liu et al., 1999; Reynolds et al., 1986; Bridger et al., 1984). In our study, we found a slight association of NLV shedding with diarrhoea, but this was not statistically significant. However the sampling interval, in our study, of 2–3 weeks was too long to study the virus’ pathogenicity adequately. Intensive clinical and virological monitoring will be needed to study the pathogenicity of NLVs in cattle in the field appropriately. In some calves in the study calicivirus excretion was observed without clinical diarrhoea. This may be due to subclinical disease but it might be also be due to a carrier state after infection as reported for feline caliciviruses (Ellis, 1981).

Phylogenetic analysis of partial ORF1 and ORF2 sequences of all calf NLV strains in this study and complete ORF2 sequences of two strains, showed that these viruses form a new cluster or genotype within the proposed GenogroupIII NLV: GIII.2. Jena virus and Newbury virus, of which only a partial ORF2 sequence is available (Dastjerdi et al., 1999) are the two most closely related strains from abroad. The genetic variation between the different strains was very limited even in the hypervariable region of the capsid. Nevertheless, the possibility of a cattle reservoir for human infection cannot be excluded, because of the close relationship with human ORF1 sequences. NLV recombination can play a role (Jiang et al., 1999; Vinjé et al., 2000b) and NLVs from an animal reservoir may pose a public health risk.

From this study, it can be concluded that a particular NLV strain belonging to the proposed GGIII NLVs is most likely continuously circulating in bovines in The Netherlands. Based on ORF1 and ORF2 sequences, we did not find indications for NLV sequences in calves, more closely related to human GGI or GGII strains than to previously detected bovine sequences. Nevertheless, NLV may be a potential zoonotic infection and bovines may be an animal reservoir for human infection. Therefore, it is indicated to continue the study of NLVs in animals and to develop methods for early detection of interspecies transmissions.
Acknowledgements

This research was financially supported and approved by the Dutch Inspectorate for Health Protection, Commodities and Veterinary Public Health. We thank Dr. A.W. van de Giessen, W.D.C. Deisz and Dr. C. Huetink for collecting and volunteering fecal specimens of cattle from ongoing studies. Dr. W. van Pelt and Dr. N. Nagelkerke contributed to data and statistical analysis. Cattle farmers are thanked for their cooperation. Dr. Ir. A.M. Henken is thanked for critically reading the manuscript.

References


**Campylobacter** spp., *Giardia* spp., *Cryptosporidium* spp., Noroviruses, and Indicator Organisms in Surface Water in Southwestern Finland, 2000-2001

Ari Hörmann,1,2* Ruska Rimhanen-Finne,1 Leena Maunula,3 Carl-Henrik von Bonsdorff,3 Niina Torvola,3 Annamari Heikinheimo,1 and Marja-Liisa Hänninen1

Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine,1 and Department of Virology and HUCH Laboratory Diagnostics, Division of Virology, Haartman Institute,3 University of Helsinki, Helsinki, and The Finnish Defense Forces Medical School, Lahdi,2 Finland

Received 9 July 2003/Accepted 18 September 2003

A total of 139 surface water samples from seven lakes and 15 rivers in southwestern Finland were analyzed during five consecutive seasons from autumn 2000 to autumn 2001 for the presence of various enteropathogens (*Campylobacter* spp., *Giardia* spp., *Cryptosporidium* spp., and noroviruses) and fecal indicators (thermotolerant coliforms, *Escherichia coli*, *Clostridium perfringens*, and F-RNA bacteriophages) and for physicochemical parameters (turbidity and temperature); this was the first such systematic study. Altogether, 41.0% (57 of 139) of the samples were positive for at least one of the pathogens; 17.3% were positive for *Campylobacter* spp. (45.8% of the positive samples contained *Campylobacter jejuni*, 25.0% contained *Campylobacter lari*, 4.2% contained *Campylobacter coli*, and 25.0% contained *Campylobacter* isolates that were not identified). 13.7% were positive for *Giardia* spp., 10.1% were positive for *Cryptosporidium* spp., and 9.4% were positive for noroviruses (23.0% of the positive samples contained genogroup I and 77.0% contained genogroup II). The samples were positive for enteropathogens significantly (*P* < 0.05) less frequently during the winter season than during the other sampling seasons. No significant differences in the prevalence of enteropathogens were found when rivers and lakes were compared. The presence of thermotolerant coliforms, *E. coli*, and *C. perfringens* had significant bivariate nonparametric Spearman’s rank order correlation coefficients (*P* < 0.001) with samples that were positive for one or more of the pathogens analyzed. The absence of these indicators in a logistic regression model was found to have significant predictive value (odds ratios, 1.15, 108, 7.57, and 2.74, respectively; *P* < 0.05) for a sample that was negative for the pathogens analyzed. There were no significant correlations between counts or count levels for thermotolerant coliforms or *E. coli* or the presence of F-RNA phages and pathogens in the samples analyzed.

Surface freshwater is widely used as a source for drinking water production; the majority of the world’s human population uses surface water as drinking water. In 2001 42% of the drinking water consumed in Finland originated directly from various surface water sources (41). Treated or untreated surface water is also one of the main sources of drinking water under field conditions when there is no sanitary or municipal infrastructure, both for military use and civil organizations and for individual hikers. Historical and present data on raw water quality combined with data on purification capacity play an essential role when water safety plans recommended by the World Health Organization are applied to drinking water production (10, 19, 22). Surface waters are also used a great deal for leisure and recreational activities, and unintended ingestion of microbiologically contaminated water poses a potential health risk (40).

Extensively collected and documented monitoring data are available in Finland for the hygienic quality of surface water sources based on counts of fecal indicator microbes, mainly thermotolerant coliforms and *Escherichia coli* (32, 36). According to these monitoring studies coastal rivers tend to have higher counts of thermotolerant coliforms than lakes have, which probably indicates that there is a higher fecal contamination load in rivers. However, monitoring programs have not included data on the prevalence of various enteric pathogens in surface water. Few systematic studies have been undertaken in other countries to determine the simultaneous prevalence of various enteric pathogens in surface water (4, 16, 27, 28, 34). Epidemiological studies of waterborne outbreaks have indicated that the most important waterborne pathogens in Finland are the noroviruses (NVs) (formerly referred to as the Norwalk-like viruses) and campylobacters; 11 of 14 reported waterborne outbreaks that occurred during 1998 and 1999 were caused by these microbes (31). One documented outbreak also occurred when consumption of untreated surface water caused severe campylobacter gastroenteritis in military conscripts during a field exercise (1). Enteric parasites, such as *Giardia* spp. and *Cryptosporidium* spp., have not been reported to cause waterborne epidemics in Finland according to the National Infection Register (42), but these parasites are well recognized as organisms that are able to cause severe waterborne enteric infections even at small doses, especially in immunocompromised persons (13, 33). Some data on the occurrence in Finland of *Giardia* spp. and *Cryptosporidium* spp. in surface waters are available (37).
In addition to data on the occurrence and prevalence of various enteropathogens, it is crucial to have data on the correlation between fecal indicators and enteric pathogens in surface water. Thermotolerant coliforms, *E. coli*, fecal enterococci, and *Clostridium perfringens* are used as indicator organisms worldwide for microbial water hygiene (12, 39). Bacteriophages, such as somatic coliphages, F-RNA bacteriophages, or phages of *Bacteroides fragilis*, have also been proposed as indicator organisms (9, 35), but data on their prevalence in Finnish surface waters are limited (9). The ecological and environmental survival characteristics of bacterial, viral, and parasitic enteropathogens vary, indicating that probably no single indicator organism can predict the presence of all enteric pathogens. Furthermore, whether there is a true correlation between the indicator organisms generally used and pathogens and to what extent and under which circumstances these organisms can be used as reliable determinants in water hygiene have been discussed previously (11, 12, 26, 43).

In this study one of our aims was to investigate simultaneously the occurrence of various enteropathogens belonging to different microbial groups, including *Campylobacter* spp., *Giardia* spp., *Cryptosporidium* spp., and NVs, in diverse types of surface water in southwestern Finland during different consecutive seasons. Our second aim was to analyze the correlation between the pathogens and selected indicator parameters, including counts of thermotolerant coliforms and *E. coli*, the presence of *C. perfringens* and F-RNA bacteriophages, and turbidity.

**MATERIALS AND METHODS**

**Sampling sites and sampling.** A total of 139 water samples were collected at 30 different sites (Fig. 1) on five separate sampling occasions within a 2- to 3-week period in consecutive seasons. The sampling sites were selected to include the most important rivers and lakes representing various contamination sources and catchment areas in southwestern and coastal Finland. Primary selection was made on a macro level (lake or river) and on a micro level (sampling site) by using map reconnaissance. The sites included seven lakes and 13 rivers and an additional 10 separate sites in two rivers (5 sites in each river), the Aura River and the Kokemäki River. These two rivers were included in the study due to their importance as local drinking water sources and due to the differences in their total flow rates, water volumes, and catchments areas. Exact sampling points were recorded by using a Garmin global positioning system 12XL (Garmin, Olathe, Kans.) satellite device.

The first samples were collected in September and October (autumn) 2000; this was followed by collection of samples in February and March (winter), May (spring), August (summer), and finally October (autumn) 2001. During the first sampling period 19 samples were collected from 19 sites (Aura River and Kokemäki River were not included), and during the four subsequent sampling periods the number of samples and sampling sites was increased to 30.

The sampling protocol consisted of collecting four separate subsamples at each sampling site at every sampling time; three separate 1-liter samples were used for bacteriological, virological, and physicochemical analyses, and one 10-liter grab sample was used for parasitological analysis. The samples were taken from the nearshore areas of lakes or rivers at depths of 0.5 to 1 m (20 to 30 cm beneath the surface), and sediment that would contaminate the samples was avoided. During the winter sampling was done by collecting the samples with a pump through holes made in the ice cover. The water samples were transported within 24 h after collection to laboratories and were stored cooled (at 5 to 8°C) prior to analysis, which was begun within 24 h.

**Analyses of *E. coli* and thermotolerant coliforms.** *E. coli* counts were obtained from 100 ml of a sample by using the Colilert-18/Quanti-Tray 2000 most-probable-number (MPN) test (IDEXX Laboratories, Inc., Westbrook, Maine); the test procedure described by the manufacturer was used. Thermotolerant coliform counts were determined by filtering a 100-ml sample through a 0.45-μm-pore-size cellulose filter (Millipore Corporation, Bedford, Mass.). The filter was placed on an incubation patch impregnated with liquid m-FC broth containing 0.4 mg of D-cycloserine per g. The plate was incubated microaerobically at 37°C for 48 h. The sample was positive for *E. coli* if 10 or more colonies were formed CCDA (end of the 10th day) or 20 or more colonies were formed CCDA (end of the 11th day).

**Analysis of *C. perfringens*.** For the *C. perfringens* analysis 100 ml of a sample was first heat treated in a water bath at 70 ± 2°C for 10 min to kill vegetative bacteria and cooled (2). The heated and cooled sample was then filtered through a 0.45-μm-pore-size cellulose filter (Millipore), and the filter was placed on a Shahidi Ferguson perfringens agar plate (Becton Dickinson Microbiology Systems, Franklin Lakes, N.J.) containing 0.4 mg of ti-cycloserine per g. The plate was incubated anaerobically at 44 ± 0.5°C for 24 h. A sample was determined to be *C. perfringens* positive if typical black colonies were present on the plate, and the colonies were confirmed to be *C. perfringens* colonies by using the method described in the ISO 6461-2 standard (2).

**Analysis of Campylobacter spp.** A 100-ml sample was filtered through a 0.45-μm-pore-size cellulose filter (Millipore), and the filter was placed in a petri dish filled with 20 ml of liquid Bolton campylobacter enrichment broth without antibiotics (LabM, Bury, Lancashire, United Kingdom). After incubation in a microaerobic atmosphere at 37 ± 1.0°C for 6 h, 0.2 ml of a selective supplement containing cephapirzone, vancomycin, trimethoprim, and cycloheximide (LAB M XI31; LabM) was added to the enrichment broth. Incubation was continued microaerobically for a further 24 h at 42 ± 0.5°C. After the selective enrichment phase, a 10-μl portion of broth was spread onto the surface of a modified CCDA agar plate (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) and incubated microaerobically at 37 ± 1.0°C for 48 h. The sample was positive for campylobacters if small grayish colonies were detected, if it was oxidase and...
catalase positive, and if a typical curved cell morphology was observed after Gram staining. A typical colony was inoculated onto blood agar and incubated microaerobically at 42 ± 0.5°C for 24 h. Bacterial isolates were stored at −70°C for further analysis. The cultures were identified to the species level by using catalase, oxidase, and indole hydrolysis tests (17).

Analysis of NVs and F-RNA bacteriophages. Virological analysis for detection of NVs was performed by reverse transcriptase PCR (29) after concentration of the water samples (15, 25). Briefly, 1,000 ml of water was filtered through a positively charged membrane (AMF-Cuno; Zetapore, Meriden, Conn.) by using a fiberglass prefIlter. Possible viruses were eluted from the membrane with 50 mM Tris–HCl–NaOH (pH 9.5) containing 1% beef extract. Further concentration (to 100 µl) was performed with a Centricon-100 microconcentrator (Amicon, Beverly, Mass.). RNA was extracted from concentrated water samples with a phenol-containing reagent (TriPure; Roche Diagnostics, Basel, Switzerland) and was precipitated with 75% ethanol. After a common reverse transcription step, separate PCRs for NV genogroups I and II were performed; PCR amplifiCations that were 152 and 117 bp long were obtained with primers NVp110 and NVp69 and with primers NVp110 and NVII, respectively. The amplifiCed products were confiMed with using a probe panel in a microplate hybridization assay (29).

Analysis for the presence of F-RNA bacteriophages was performed as described in the ISO 10705-1 standard (3). The samples were mixed with host strain Salmonella enterica serovar Typhimurium WG49, and they were grown on a semisolid extract glucose-based agar. MS2 was used as a positive control. For exclusion of DNA bacteriophages, samples were also grown in the presence of RNase.

Analysis of Giardia spp. and Cryptosporidium spp. For concentration of cysts and oocysts, 10-liter grab samples were filtered through a polycarbonate filter, and the concentrates were further purifiMed by immunomagnetic separation (37). After immunomagnetic separation, each 50-µl product was divided and used for immunofluorescence assay (IFA) microscopy and PCR. For PCR, the immunohead-oocyst complexes were rinsed twice with phosphate-buffered saline–Tween 20, and the DNA of the parasites was released by repeated freeze-thaw cycles. The amplifiCation reaction mixture (total volume, 50 µl) contained 2.5 U of HotStarTaq DNA polymerase, 1.5 mM of dNTPs, 1.5 mM of MgCl2 per liter, and 200 nmol of each dNTPs (HotStarTaq Master Mix; Qiagen, Hilden, Germany) per liter, 12.6 pmol of primers cry15 and cry9 (Cryptosporidium spp.), 50 pmol of primers GDH1 and GDH4 (Giardia spp.), and 5 µl of the template. The PCR was performed with a thermal cycler (DNA Engine PTC-200; MJ Research, Waltham, Mass.) by using the following temperature cycles: for Cryptosporidium spp., 94°C for 15 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min and then a final extension at 72°C for 10 min; and for Giardia spp., 94°C for 15 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min and then a final extension at 72°C for 7 min.

During microscopy, cysts and oocysts were detected by direct IFA by using an Aqua Flores (G/C kit; Waterborne, New Orleans, La.). The slides were fiCered with an epifioreCence microscope (Nikon type 115) at a magnifiCation of ×200, and the particles with green fioreCence were confiMed by using a magnifiCation of ×400. Objects that were ovoid or spherical and had a diameter of 4 to 6 µm were recorded as Cryptosporidium spp., and particles that were round to oval and had a diameter of 5 to 16 µm were recorded as Giardia spp. A sample was determined to be positive if a positive result was obtained either by PCR or by IFA or if both tests were positive.

Physicochemical analysis. Temperature, pH, and conductivity were measured with portable devices; temperature was measured with a Delta Ohm HD8601P (Delta Ohm, Padua, Italy), pH was measured with a Eutech Cyberscience pHScanWP2 (Eutech Instruments, Singapore), and conductivity was measured with a HACH model CI050 conductivity meter (HACH Company, Loveland, Colo.) immediately after sampling at the sampling site. Turbidity was measured in the laboratory for each thoroughly stirred sample on the day after sampling by using a Eutech CyberScan WL TB1000 turbimeter (Eutech), and the results were expressed in nephelometric turbidity units.

Statistical analyses. All individual results were recorded by using Microsoft Excel 2002 software (Microsoft Corporation, Redmond, Wash.), and a statistical analysis was performed with the Statistical Package for Social Sciences 11.5 for Windows (SPSS Inc., Chicago, Ill.) software. Prevalences were calculated for each microbe analyzed, and arithmetic means and standard deviations were calculated for MPNs of E. coli, turbidity, and temperature separately for each sampling time and type of sampling site (all lakes plus the Kokemäki River, the Aura River, and other rivers). Analysis of variance (ANOVA) was used to determine possible signifiCant differences at a P level of <0.05 for prevalences and means for different sampling times and sites. If signifiCant differences were observed, Duncan’s post hoc test was performed to determine which values differed from all other values. All samples were grouped according to the thermotolerant coliform and E. coli counts at four different levels, and the prevalence of enteropathogens and indicator microbes at each level was analyzed by using ANOVA and Duncan’s post hoc test.

A nonparametric Spearman rank order correlation coefCicient with a two-tailed P value was calculated for cross-correlations between different indicator parameters (coliform and thermotolerant coliform counts, MPNs of E. coli, turbidity values, and presence or absence of C. perfringens and F-RNA bacteriophages). Spearman’s correlation coefCicient was also computed for bivariate correlations between indicator parameters and pathogen fiCings. The odds ratio (OR) with a 95% confiCence interval was calculated for each sample, and the value was positive for pathogens analyzed according to the outcome for various indicator parameters.

All the samples were divided into two groups: pathogen absent or pathogen present (i.e., the sample was either negative for Campylobacter spp., Giardia spp., Cryptosporidium spp., and NVs or was positive for at least one of the pathogens). Pathogen absence was considered a dependent variable in the logistic multivariable regression model. In this model various indicator parameters (i.e., levels of E. coli and thermotolerant coliforms and the presence of C. perfringens and F-RNA bacteriophages) were considered independent variables, and their predictive values for the dependent variable were analyzed by computing the coefCient estimates (B values), P values for the B values, and ORs with 95% confiCence intervals from the B values.

RESULTS

Enteropathogens. A total of 57 of 139 samples (41.0%) were found to be positive for at least one of the enteropathogens analyzed (Campylobacter spp., Giardia spp., Cryptosporidium spp., or NVs). Of these 57 positive samples, 46 contained one of the pathogens analyzed; two pathogens were isolated from 10 samples, and three pathogens were isolated from one sample. Pathogens were isolated less frequently (3 of 30 samples; 10.0%) during the winter (P < 0.05) than during the other sampling periods (Table 1). There was not a signifiCant difference (P > 0.05) in the prevalences of positive samples for pathogens at different types of sampling sites (lakes or the Aura River, the Kokemäki River, or other rivers) (Table 2). Only 1 of the 30 individual sampling sites was found to be negative for all enteropathogens studied at all fiVe sampling times. Ten sites were found to be positive at only one sampling time, 10 sites were positive at two sampling times, and nine sites were positive at three sampling times. None of the sampling sites was found to be positive for one or more of the pathogen analyzed at all fiVe sampling times.

Campylobacter spp. were isolated from 24 samples (17.3%) and were the most frequently isolated enteropathogens in this study (Table 1). Of 24 campylobacter-positive samples, 11 (45.8%) were found to contain Campylobacter jejuni, 6 (25.0%) were found to contain Campylobacter lari, and 1 (4.2%) was found to contain Campylobacter coli, while 6 (25.0%) of the strains were undetermined Campylobacter spp. More samples were positive for campylobacters in May 2001 (43.3% of the samples were positive) than at other sampling times (P < 0.05). Lakes and the Aura River were positive for campylobacters significantly (P < 0.05) more often than the Kokemäki River or other rivers (Table 2). NVs were detected in 13 samples (9.4%); 3 of the positive samples contained genogroup I NVs, and 10 of the positive samples contained genogroup II NVs. There were no differences in the prevalence of NVs when sampling times were compared (Table 1), but the Kokemäki River was found to be positive for NVs more frequently (P < 0.05) than the other
### Table 1: Indicator parameters and proportions of samples positive for various pathogens for 139 surface water samples collected between September 2000 and October 2001 in southwestern Finland according to the sampling time

<table>
<thead>
<tr>
<th>Indicator parameters</th>
<th>% of positive samples</th>
<th>Sampling time</th>
<th>No. of samples</th>
<th>No. of sampling sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli (MPN/100 ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Campylobacter spp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Giardia spp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptosporidium spp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F-RNA phages</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NVs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temp (°C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Campylobacter spp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Giardia spp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptosporidium spp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F-RNA phages</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NVs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>September and October 2000</td>
<td>19</td>
<td>19</td>
<td>21.1</td>
<td>19.9</td>
</tr>
<tr>
<td>February and March 2001</td>
<td>30</td>
<td>30</td>
<td>19.1</td>
<td>19.9</td>
</tr>
<tr>
<td>May 2001</td>
<td>30</td>
<td>30</td>
<td>16.9 (1.2)</td>
<td>25.3</td>
</tr>
<tr>
<td>October 2001</td>
<td>30</td>
<td>30</td>
<td>15.7 (1.3)</td>
<td>25.3</td>
</tr>
<tr>
<td>Total</td>
<td>139</td>
<td>139</td>
<td>19.7 (2.4)</td>
<td>17.7 (17.8)</td>
</tr>
</tbody>
</table>

The values for the differences between means are 0.020, as determined by ANOVA. The P-value for the differences between values is 0.351, as determined by ANOVA. The P-value for the differences between values is 0.006, as determined by ANOVA. The P-value for the differences between values is 0.001, as determined by ANOVA.

Campylobacter spp., Giardia spp., or NVs. The presence of NVs was more frequently (P < 0.05) than the remaining 28 sites.

Giardia spp. were isolated from 19 (13.7%) of the 139 samples, and Cryptosporidium spp. were isolated from 14 (10.1%) of the 139 samples (Tables 1 and 2). Both Giardia spp. and Cryptosporidium spp. were found more frequently during the summer of 2001 and less frequently during the winter of 2001 than at the other sampling times (P < 0.05). No significant differences between the proportions of positive samples at different types of sampling sites were detected.

### Results for indicator microbes

The MPNs of *E. coli* were below the detection limit of the test used (one microbe per 100 ml) for 13 samples (9.3%), while 110 samples (79.1%) had MPNs of *E. coli* of 100 CFU per 100 ml or less (Table 3). Only five samples (3.6%) had MPNs of >1,000 CFU per 100 ml. The MPNs of *E. coli* varied widely for individual samples; thus, there were no significant differences at a P level of 0.05 in mean MPNs when sampling times (Table 1) or sites (Table 2) were compared. However, the MPNs tended to be higher in the Aura River and most other rivers than in the lakes and the Kokemäki River (Table 2). The samples are arranged according to thermotolerant coliform counts in Table 4.

A total of 44 samples (31.7%) were positive for *C. perfringens*, and 47 samples (33.8%) were positive for F-RNA bacteriophages (Tables 1 and 2). *C. perfringens* was isolated most frequently during the autumn of 2000 and spring of 2001 (P < 0.05). No sample from the Kokemäki River was found to be positive for *C. perfringens*. There were no differences (P > 0.05) in the occurrence of F-RNA bacteriophages when sampling sites were compared, but phages were isolated more often during the winter of 2001 and less frequently during the autumn of 2001 (P < 0.05).

### Physicochemical results

The turbidity varied between 1.2 and 222.0 nephelometric turbidity units, and the variations both between sampling times (Table 1) and between types of sampling site (Table 2) were significant (P < 0.05). The temperature varied during the study period from 0.1 to 22.0°C, but the variation within each sampling season was very limited (Table 1). The conductivity (mean, 143.4 μS/cm²; standard deviation, 65.9 μS/cm²; minimum, 64.0 μS/cm²; maximum, 398.0 μS/cm²) and pH (mean, 7.51; standard deviation, 0.44; minimum, 6.4; maximum, 9.5) did not vary significantly (P > 0.05) for different sampling times or for different types of sampling site.

### Correlation between indicator parameters and enteropathogens

The indicator parameters used in this study (turbidity, thermotolerant coliform counts, MPNs of *E. coli*, and presence of *C. perfringens*) showed significant cross-correlations with each other (correlation coefficients, 0.30 to 0.86; P < 0.05). However, the presence of F-RNA phages showed no significant correlation (P > 0.05) with any other parameter except turbidity and MPNs of *E. coli*, for which there were low but significant levels of correlation (correlation coefficients, 0.30 and 0.21; P < 0.05). The presence of any of the pathogens analyzed did not correlate significantly with the presence of other pathogens (P > 0.05 for Spearman correlation coefficients [data not shown]).
TABLE 2. Indicator parameters and proportions of samples positive for various pathogens for 139 surface water samples collected between September 2000 and October 2001 in southwestern Finland according to the sampling site

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>No. of samples</th>
<th>No. of sampling sites</th>
<th>Turbidity (NTU)</th>
<th>E. coli count (MPN/100 ml)</th>
<th>% of positive samples</th>
<th>% of samples positive for pathogens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Campylobacter spp.</td>
</tr>
<tr>
<td>Lakes</td>
<td>35</td>
<td>7</td>
<td>15.8 (14.6)</td>
<td>75.2 (407.9)</td>
<td>25.7</td>
<td>20.0</td>
</tr>
<tr>
<td>Rivers</td>
<td>58</td>
<td>13</td>
<td>43.7 (43.4)†</td>
<td>232.6 (548.8)</td>
<td>41.4</td>
<td>41.4</td>
</tr>
<tr>
<td>Aura River</td>
<td>26</td>
<td>5</td>
<td>76.2 (43.5)*</td>
<td>148.0 (275.9)</td>
<td>42.3</td>
<td>42.3</td>
</tr>
<tr>
<td>Kokemäki River</td>
<td>20</td>
<td>5</td>
<td>8.4 (4.0)</td>
<td>45.9 (151.9)</td>
<td>0</td>
<td>25.0</td>
</tr>
<tr>
<td>Total</td>
<td>139</td>
<td>30</td>
<td>37.7 (41.4)</td>
<td>150.3 (433.9)</td>
<td>31.7</td>
<td>33.8</td>
</tr>
</tbody>
</table>

* Mean (standard deviation). NTU, nephelometric turbidity units. The P value for the differences between values is <0.001, as determined by ANOVA.
† Mean (standard deviation). The P value for the differences between values is 0.278, as determined by ANOVA.
‡ The P value for the differences between values is 0.003, as determined by ANOVA.
† The P value for the differences between values is 0.112, as determined by ANOVA.
§ The P value for the differences between values is 0.010, as determined by ANOVA.
¶ The P value for the differences between values is 0.381, as determined by ANOVA.
† The P value for the differences between values is 0.431, as determined by ANOVA.
‡ The P value for the differences between values is 0.044, as determined by ANOVA.
§ The P value for the differences between values is 0.590, as determined by ANOVA.
¶ Sample was positive for at least one of the following pathogens: Campylobacter spp., Giardia spp., Cryptosporidium spp., or NVs. The P value for the observed differences between values is 0.590, as determined by ANOVA.
§ Value is significantly different at the 0.05 (95%) level from other values for individual types of sampling sites in the same column.
¶ Aura River and Kokemäki River data are not included.

Significant (P < 0.05) bivariate nonparametric Spearman rank order correlation coefficients and ORs for various indicator parameters and pathogens in samples are shown in Table 5. Based on these bivariate correlations, four variables (levels of E. coli and thermotolerant coliform counts, absence of C. perfringens, and absence of F-RNA bacteriophages) were selected for use in a multivariate logistic regression model (Table 6) in which the absence of E. coli, thermotolerant coliforms, and C. perfringens had a significant (P < 0.05) predictive value (ORs, 1.15 × 10^8, 5.75, and 2.74, respectively) for a sample being negative for any of the pathogens analyzed.

DISCUSSION

Our results showed that all the enteropathogens analyzed can be detected in surface water from lakes and rivers located in southwestern Finland. This was the first systematic study done in Finland in which enteropathogens representing different microbial groups (NVs as a model for enteric viruses, campylobacters as a model for enteric bacterial pathogens, and Giardia spp. and Cryptosporidium spp. as models for resistant protozoan parasites) were analyzed for diverse surface water sites during several consecutive seasons. The point prevalences of the various enteropathogens in our study, as well as in all biological and environmental studies, are dependent on sampling time and site, but findings can serve as rough general estimates for the prevalences in surface water as a whole and can provide qualitative data for risk assessment. The results showed that there was some tendency for seasonal variation: during the winter the pathogens were detected less frequently than during the spring, summer, or autumn. This pattern was observed especially for the occurrence of Campylobacter spp.,

TABLE 3. Proportions of surface water samples positive for indicators and pathogens at various levels of E. coli in samples

<table>
<thead>
<tr>
<th>E. coli count (MPN/100 ml)</th>
<th>No. of samples (%)</th>
<th>Indicators</th>
<th>Pathogens</th>
<th>% of positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C. perfringens*</td>
<td>F-RNA phages*</td>
<td>Campylobacter spp.</td>
</tr>
<tr>
<td>&lt;1</td>
<td>13 (9.3)</td>
<td>0*</td>
<td>23.1</td>
<td>0</td>
</tr>
<tr>
<td>1–10</td>
<td>55 (39.0)</td>
<td>18.2</td>
<td>23.6</td>
<td>21.8</td>
</tr>
<tr>
<td>101–100</td>
<td>42 (30.2)</td>
<td>35.7*</td>
<td>38.1</td>
<td>21.4</td>
</tr>
<tr>
<td>100.1–1,000</td>
<td>24 (17.3)</td>
<td>58.3*</td>
<td>54.2</td>
<td>12.5</td>
</tr>
<tr>
<td>&gt;1,000</td>
<td>5 (3.6)</td>
<td>100.0*</td>
<td>40.0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>139 (100.0)</td>
<td>31.7</td>
<td>33.8</td>
<td>17.3</td>
</tr>
</tbody>
</table>

* The P value for the differences between values is <0.001, as determined by ANOVA.
† The P value for the differences between values is 0.088, as determined by ANOVA.
‡ The P value for the differences between values is 0.249, as determined by ANOVA.
§ The P value for the differences between values is 0.207, as determined by ANOVA.
¶ The P value for the differences between values is 0.129, as determined by ANOVA.
# Sample was positive for at least one of the following pathogens: Campylobacter spp., Giardia spp., Cryptosporidium spp., or NVs. The P value for the observed differences between values is 0.590, as determined by ANOVA.
* Value is significantly different at the 0.05 (95%) level from other values for E. coli count ranges in the same column.
Giardia spp., and Cryptosporidium spp., and the pattern was not affected when we excluded the samples obtained during the first sampling period, during which fewer samples were collected than at other sampling times, from the statistical analysis. The major discriminating factors between seasons in water bodies in Finland and regions with similar climatic conditions are temperature, ice cover, and solar radiation. Low temperatures in water during the winter and high solar radiation during the summer are known to have effects on the survival and recovery of Campylobacter spp. In studies done in Norway (6, 21) and Finland (23), campylobacters in natural water exhibited seasonal patterns, and the number of positive samples was highest in the winter and lowest in the summer. C. jejuni and C. coli survive in cold water (at temperatures below 10°C) much longer than they survive in water at temperatures higher than 18°C (23, 24). A confounding factor in the assessment of campylobacter seasonality in water is birds, which are carriers of C. jejuni, C. lari, and C. coli (17, 44) and contaminate watershed areas where they live. Birds were probably the cause of the rather high numbers of campylobacter-positive samples in August 2001, even when the temperature of the water was >18°C in areas where positive samples were collected.

There have been few studies on the possible seasonality of the intestinal parasites Giardia spp. and Cryptosporidium spp. in surface waters. Lower numbers of samples positive for these parasites during the cold winter months than during other seasons have been found in some studies (45). In one study the highest frequencies of samples positive for Giardia spp. and Cryptosporidium spp. were found during the autumn and winter in surface waters affected by agricultural discharges due to heavy rains (5), but no clear seasonality has been found in some other studies (38).

Possible seasonal or time-related variation in the occurrence of various groups of enteric pathogens in surface water appears to be dependent on the source of contamination and the conditions facilitating the discharge of contaminants into surface water. If the major sources are sewage plants that treat human wastes, seasonal patterns similar to those observed for human infections for a particular pathogen are detected in effluents and downstream water samples (25). If the watershed is contaminated by discharges resulting from agricultural runoff, the highest numbers of zoonotic enteric pathogens are found during the pasture season after snowmelt, floods, and heavy rainfalls (5). Even though most of the NV infections in Finland occur (according to the Finnish Infection Register) in the winter and early spring (42), no clear winter peak in the number of samples positive for NVs was detected in our study, and positive samples were detected in all seasons.

We obtained no evidence that enteric pathogens are more frequent in certain types of surface water in Finland. However, we did observe a trend for higher E. coli counts in the Aura River and other rivers than in lakes and the Kokemäki River, confirming the results of previous monitoring studies (32, 36). There was also a tendency for higher turbidity values in rivers (except the Kokemäki River) than in lakes; this result reflects the general appearance of coastal rivers with high loads of inorganic material and clay in the water combined with relatively low flow rates. The sampling sites were selected to represent diverse types of lakes and rivers with variable contamination sources and catchment areas. However, the statistical analysis did not reveal any significant correlation between the enteropathogens analyzed as a whole and any particular type of surface water sampling site; enteropathogens were found to the same extent at all sampling sites. This may have resulted from the fact that all sites were in relatively densely populated areas of Finland subject to discharges from human activities, as well from agriculture.

The occurrence of the various pathogens did not correlate significantly with traditionally used fecal indicator parameters (counts or count levels for E. coli or thermotolerant coliforms per 100 ml) or turbidity. In some studies a significant correlation has been found between the level of thermotolerant coliforms and the number of samples positive for certain pathogens (4, 34). However, a poor correlation similar to that shown in the present study has also been demonstrated elsewhere (8,

<table>
<thead>
<tr>
<th>Thermotolerant coliform count (CFU/100 ml)</th>
<th>No. of samples (%)</th>
<th>Indicators</th>
<th>Pathogens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C. perfringens</td>
<td>F-RNA phages</td>
</tr>
<tr>
<td>&lt;1</td>
<td>11 (7.9)</td>
<td>0b</td>
<td>27.3</td>
</tr>
<tr>
<td>1–20</td>
<td>45 (32.4)</td>
<td>15.6</td>
<td>28.9</td>
</tr>
<tr>
<td>20.1–200</td>
<td>48 (34.5)</td>
<td>41.7</td>
<td>33.3</td>
</tr>
<tr>
<td>200.1–2,000</td>
<td>30 (21.6)</td>
<td>43.3</td>
<td>46.7</td>
</tr>
<tr>
<td>&gt;2,000</td>
<td>5 (3.6)</td>
<td>80.0b</td>
<td>20.0</td>
</tr>
<tr>
<td>Total</td>
<td>139 (100.0)</td>
<td>31.7</td>
<td>33.8</td>
</tr>
</tbody>
</table>

- The P value for the differences between values is <0.001, as determined by ANOVA.
- The P value for the differences between values is 0.511, as determined by ANOVA.
- The P value for the differences between values is 0.296, as determined by ANOVA.
- The P value for the differences between values is 0.273, as determined by ANOVA.
- The P value for the differences between values is 0.231, as determined by ANOVA.
- The P value for the differences between values is 0.019, as determined by ANOVA.
- Sample was positive for at least one of the following pathogens: Campylobacter spp., Giardia spp., Cryptosporidium spp., or NVs. The P value for the differences between values is 0.047, as determined by ANOVA.
- Value is significantly different at the 0.05 (95%) level from other values for thermotolerant coliform count ranges in the same column.
longer than that of most enteropathogenic bacteria (7). Cysts persist for a long time in water; this persistence is considered to be much higher than that of most enteropathogenic bacteria (7). Cysts of *G. duodenalis* and *C. parvum* are known for their capacity to survive for several months in water (25), and it has been suggested that *C. perfringens* is similarly not a suitable indicator for enteric viruses (11, 18). There was a positive correlation between the presence of *C. perfringens* and *E. coli* and thermotolerant coliforms in a 100-ml sample (2, 14, 26, 43). The presence or absence of a correlation between different indicator parameters and pathogens could reflect the occasional occurrence of enteropathogens in surface waters and the different rates of survival and recovery of the pathogens compared to the low correlation values presented for the correlation analysis by the present study. The presence or absence of a correlation between various indicator parameters and pathogens was not significant for a sample being positive for one or more of the pathogens analyzed. However, a considerable number of samples that did not contain any of the pathogens analyzed had a positive predictive value for a sample being positive for one or more of the pathogens analyzed. Moreover, a considerable number of samples that did not contain any of the pathogens analyzed had a positive predictive value for a sample being positive for one or more of the pathogens analyzed. Therefore, the presence or absence of *E. coli* and thermotolerant coliforms appears to be a better predictor for the occurrence of enteropathogens than a certain level of these microbes is not sufficient for a sample being positive for one or more of the pathogens analyzed. The present study provided valuable qualitative data for assessing microbial risks in surface waters in Finland. The occurrence of enteropathogenic bacteria in surface waters was linked directly to possible contamination sources, while environmental conditions affect only the survival of these microbes in water. The presence of F-RNA bacteriophages could not be linked to the presence of *C. perfringens* (25), and it has been suggested that F-RNA bacteriophages are suitable indicators for enteric viruses (11, 18). The presence of F-RNA bacteriophages could not be linked to the presence of *C. perfringens* (25), and it has been suggested that F-RNA bacteriophages are suitable indicators for enteric viruses (11, 18). There was a positive correlation between the presence of *C. perfringens* and *E. coli* and thermotolerant coliforms in a 100-ml sample (2, 14, 26, 43). The presence or absence of a correlation between various indicator parameters and pathogens could reflect the occasional occurrence of enteropathogens in surface waters and the different rates of survival and recovery of the pathogens compared to the low correlation values presented for the correlation analysis by the present study. The presence or absence of a correlation between various indicator parameters and pathogens was not significant for a sample being positive for one or more of the pathogens analyzed. However, a considerable number of samples that did not contain any of the pathogens analyzed had a positive predictive value for a sample being positive for one or more of the pathogens analyzed. Therefore, the presence or absence of *E. coli* and thermotolerant coliforms appears to be a better predictor for the occurrence of enteropathogens than a certain level of these microbes is not sufficient for a sample being positive for one or more of the pathogens analyzed. The present study provided valuable qualitative data for assessing microbial risks in surface waters in Finland. The occurrence of enteropathogenic bacteria in surface waters was linked directly to possible contamination sources, while environmental conditions affect only the survival of these microbes in water. The presence of F-RNA bacteriophages could not be linked to the presence of *C. perfringens* (25), and it has been suggested that F-RNA bacteriophages are suitable indicators for enteric viruses (11, 18). There was a positive correlation between the presence of *C. perfringens* and *E. coli* and thermotolerant coliforms in a 100-ml sample (2, 14, 26, 43). The presence or absence of a correlation between various indicator parameters and pathogens could reflect the occasional occurrence of enteropathogens in surface waters and the different rates of survival and recovery of the pathogens compared to the low correlation values presented for the correlation analysis by the present study. The presence or absence of a correlation between various indicator parameters and pathogens was not significant for a sample being positive for one or more of the pathogens analyzed. However, a considerable number of samples that did not contain any of the pathogens analyzed had a positive predictive value for a sample being positive for one or more of the pathogens analyzed. Therefore, the presence or absence of *E. coli* and thermotolerant coliforms appears to be a better predictor for the occurrence of enteropathogens than a certain level of these microbes is not sufficient for a sample being positive for one or more of the pathogens analyzed. The present study provided valuable qualitative data for assessing microbial risks in surface waters in Finland. The occurrence of enteropathogenic bacteria in surface waters was linked directly to possible contamination sources, while environmental conditions affect only the survival of these microbes in water. The presence of F-RNA bacteriophages could not be linked to the presence of *C. perfringens* (25), and it has been suggested that F-RNA bacteriophages are suitable indicators for enteric viruses (11, 18). There was a positive correlation between the presence of *C. perfringens* and *E. coli* and thermotolerant coliforms in a 100-ml sample (2, 14, 26, 43). The presence or absence of a correlation between various indicator parameters and pathogens could reflect the occasional occurrence of enteropathogens in surface waters and the different rates of survival and recovery of the pathogens compared to the low correlation values presented for the correlation analysis by the present study. The presence or absence of a correlation between various indicator parameters and pathogens was not significant for a sample being positive for one or more of the pathogens analyzed. However, a considerable number of samples that did not contain any of the pathogens analyzed had a positive predictive value for a sample being positive for one or more of the pathogens analyzed. Therefore, the presence or absence of *E. coli* and thermotolerant coliforms appears to be a better predictor for the occurrence of enteropathogens than a certain level of these microbes is not sufficient for a sample being positive for one or more of the pathogens analyzed. The present study provided valuable qualitative data for assessing microbial risks in surface waters in Finland. The occurrence of enteropathogenic bacteria in surface waters was linked directly to possible contamination sources, while environmental conditions affect only the survival of these microbes in water. The presence of F-RNA bacteriophages could not be linked to the presence of *C. perfringens* (25), and it has been suggested that F-RNA bacteriophages are suitable indicators for enteric viruses (11, 18). There was a positive correlation between the presence of *C. perfringens* and *E. coli* and thermotolerant coliforms in a 100-ml sample (2, 14, 26, 43). The presence or absence of a correlation between various indicator parameters and pathogens could reflect the occasional occurrence of enteropathogens in surface waters and the different rates of survival and recovery of the pathogens compared to the low correlation values presented for the correlation analysis by the present study. The presence or absence of a correlation between various indicator parameters and pathogens was not significant for a sample being positive for one or more of the pathogens analyzed. However, a considerable number of samples that did not contain any of the pathogens analyzed had a positive predictive value for a sample being positive for one or more of the pathogens analyzed. Therefore, the presence or absence of *E. coli* and thermotolerant coliforms appears to be a better predictor for the occurrence of enteropathogens than a certain level of these microbes is not sufficient for a sample being positive for one or more of the pathogens analyzed. The present study provided valuable qualitative data for assessing microbial risks in surface waters in Finland. The occurrence of enteropathogenic bacteria in surface waters was linked directly to possible contamination sources, while environmental conditions affect only the survival of these microbes in water. The presence of F-RNA bacteriophages could not be linked to the presence of *C. perfringens* (25), and it has been suggested that F-RNA bacteriophages are suitable indicators for enteric viruses (11, 18). There was a positive correlation between the presence of *C. perfringens* and *E. coli* and thermotolerant coliforms in a 100-ml sample (2, 14, 26, 43). The presence or absence of a correlation between various indicator parameters and pathogens could reflect the occasional occurrence of enteropathogens in surface waters and the different rates of survival and recovery of the pathogens compared to the low correlation values presented for the correlation analysis by the present study. The presence or absence of a correlation between various indicator parameters and pathogens was not significant for a sample being positive for one or more of the pathogens analyzed. However, a considerable number of samples that did not contain any of the pathogens analyzed had a positive predictive value for a sample being positive for one or more of the pathogens analyzed. Therefore, the presence or absence of *E. coli* and thermotolerant coliforms appears to be a better predictor for the occurrence of enteropathogens than a certain level of these microbes is not sufficient for a sample being positive for one or more of the pathogens analyzed.
TABLE 6. Multivariate analysis by logistic multiple regression model with no pathogen detected as the dependent variable

<table>
<thead>
<tr>
<th>Model or variable</th>
<th>Model-fitting information</th>
<th>Parameter estimates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>–2 Log likelihood</td>
<td>Chi square</td>
</tr>
<tr>
<td>Models</td>
<td>92.546</td>
<td>64.423</td>
</tr>
<tr>
<td>Intercept only</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level of E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level of thermotolerant coliforms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence of C. perfringens (ND)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence of RNA bacteriophages (ND)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a When no pathogen was detected, the sample was determined to be negative for each of the following pathogens: Campylobacter spp., Giardia spp., Cryptosporidium spp., and NVs.


A Norwalk-like virus waterborne community outbreak in a Swedish village during peak holiday season

J. Carrique-Mas, Y. Andersson, B. Petersén, K.-O. Hedlund, N. Sjögren and J. Giecke

1 Swedish Institute for Infectious Disease Control, 17182 Solna, Sweden
2 European Programme for Intervention Epidemiology Training (EPIET), 17182 Solna, Sweden
3 Dalarna County Council of Communicable Disease Control, Falu lasarett, 79182 Falun, Dalarna, Sweden

(Accepted 14 February 2003)

SUMMARY

An outbreak of gastroenteritis due to Norwalk-like virus (NLV) affecting approximately 500 people occurred in a Swedish ski resort during February–March 2002. Epidemiological investigations were performed on cohorts of schoolchildren, permanent residents and skiers visiting the area. Attack rates were respectively 39.7, 29.9 and 38.5%. Drinking un-boiled water originating from one of the three communal water systems was a significant risk factor for all groups. For schoolchildren, the risk of illness increased with increasing amount of water consumed. Nine of 12 stool samples of patients analysed tested positive for NLV. The water tested negative for indicator bacteria and results of NLV tests were inconclusive. In the absence of microbiological findings, the environmental authorities were reluctant to act based on the epidemiological analysis alone, and intervention was delayed until mid-April, following the discovery of a crack in a sewage pipe 10 m from the well.

INTRODUCTION

Norwalk-like viruses (NLV) have been recognized as a leading cause of acute, non bacterial gastroenteritis worldwide [1]. The viruses are transmitted by the faecal–oral route from person to person, and also by contaminated food and water [2]. Several waterborne NLV outbreaks have been described previously [3–5].

A problem in confirming the source of viral waterborne outbreaks is the technical difficulty in detecting viruses in patients [6], and especially in the water vehicle [7]. Because of this latter difficulty, investigators have usually relied on the detection of viruses from patient samples and ‘indicator bacteria’ in the water and the establishment of statistical association with the consumption of the water vehicle.

In Sweden, waterborne outbreaks involving different pathogens have been reported with relative frequency [8–10]. The present paper describes a large NLV water-borne outbreak which was investigated using epidemiological methods in the absence of microbiological confirmation in the water vehicle and makes the case for a more vigorous intervention as soon as epidemiological findings are available in order to prevent further cases.

The outbreak

During the last week of February 2002 (designated ‘winter sports break’ in Sweden) the Dalarna Office for Infectious Disease Control was informed by a district health centre clinician of several cases of gastroenteritis among guests of a hotel situated in a village in the County of Dalarna (west of Sweden). The area was in peak tourist season during this period, with...
many visitors from Sweden and abroad. Precautionary recommendations were given advising residents and visitors to boil the water.

In spite of these recommendations, additional cases were found among residents as well as visitors to the area. A school (60 pupils), located in the village, had remained closed for holidays during the winter sports break, and several schoolchildren were also affected soon after returning to school. All schoolchildren live in a nearby village and travel to the school by bus. Visitor cases identified included several members of a group of skiers from Southern Sweden who had arrived to participate in the 1-day long Vasa cross-country ski competition ('Vasalopp') during 1–3 March.

Initially, NLV was confirmed in stool samples from four hotel guests. Given the high number of cases scattered across the area, the drinking water was the suspected vehicle of infection. An epidemiological investigation was conducted with the aim of determining the magnitude of the event, describing the disease in the different age groups and determining the source in order to control the outbreak.

MATERIALS AND METHODS

The study area

Transtrand, with a population of 605 residents, is located in the Western mountain range of Sweden. Within the village there are two hotels and several guesthouses, which provide accommodation to visiting winter sports fans.

Once a year, thousands of skiers gather in the area to participate in the Vasalopp. Most of them stay overnight in Transtrand and other nearby villages. The village has a food shop, a school and a petrol station, and is served by three different community water supplies (A, B, C). In addition there are a number of households with a private well. The school and the two hotels are served by water supply A.

Epidemiological studies

The Swedish Institute for Infectious Disease Control and the Dalarna County Office for Communicable Disease Control carried out a cohort study among the 60 schoolchildren (age range 10–13 years). This was followed by a second cohort study with the 605 permanent residents in the village. A third cohort study was performed with the 107 skiers belonging to the above-mentioned ski group.

Information for the school survey was collected by telephone. Schoolchildren were asked directly about exposure to food and water during the first 2 days after returning to school (5–6 March). Given that the school gym had been used as temporary accommodation for some visitor cases, the questionnaire also gathered information about participation in the class of gymnastics. For the population survey, questionnaires were mailed to all 214 households in the village. Heads of family were asked to provide information about water consumption, illness in any family members and number of guests lodged during the period 16 February to 19 March. The environmental office provided data on the type of water supply for each address. Information for the ski group study was collected by a questionnaire mailed to all 107 skiers.

A case was defined as a person who developed either diarrhoea (equal to or greater than three loose stools per day) or vomiting or abdominal pain plus one of: muscle pain, fever, and headache between 5 and 9 March (schoolchildren group), between 16 February and 19 March (resident group) and between 2 and 6 March (ski group). In view of the shape of the epidemic curve of resident cases, these were further divided into ‘early cases’ (with disease onset in February) and ‘late cases’ (with onset in March).

Dose–response model

After having established that consumption of one type of community water (A) was associated with illness and that there was a dose response among schoolchildren, the ED$_{50}$ (effective dose 50%) was calculated from a logistic regression model for the water consumption data for schoolchildren. The exposure was the average amount of water drunk per day (5–6 March) and the outcome was ‘being a case’.

Temporal spread of cases in the household

The transmission within the households was described by studying the temporal spread of the dates of onset of symptoms in both types of households (those with community water A and those with water source other than A). For households containing two or more cases, these were classified into primary (with the earliest date of onset) and non-primary (all subsequent cases). The number of days between disease onset in the primary case and non-primary cases (time lag) was calculated and compared between both types of households. In households with co-primary cases, only one of them was chosen as primary case.
Statistical methods

Exposure-specific attack rates and relative risks (RR) with Taylor 95% confidence intervals (CI) were calculated using Epi-Info 6.04 (Centre for Disease Control, Atlanta/World Health Organisation, Geneva).

A Mann–Whitney test was used to compare time lags from index cases. All reported $P$ values are two-sided and calculated by Fisher’s exact method. The software used was SAS version 8 (SAS Institute, Cary, USA).

Microbiological studies

Stool samples obtained from schoolchildren and visitors were tested for NLV by electron microscopy (EM) and RT–PCR. Water samples obtained from the school were tested for *Escherichia coli* and other indicator bacteria (cultures) as well as NLV (RT–PCR).

RESULTS

Epidemiological studies

Survey responses

Information was gathered for 58 schoolchildren (response = 96.7%). In the resident study, a total of 165/214 (77%) heads of family returned completed questionnaires. The questionnaire from one household was very incomplete and therefore rejected. In addition two families had been away for longer than 1 week during the study period and were therefore excluded from the study. This left us with 162 families and 387 individuals (64% of population) in our study. Ninety-six of 107 (89.7%) ski participants returned completed questionnaires.

Descriptive study

There were 23, 115 and 37 cases among schoolchildren, residents and skiers, respectively. The ARs were 39.7% for schoolchildren (42.8% and 35.3% for girls and boys respectively); 29.7% for residents (30.5% and 28.9% for females and males, respectively) and 38.5% for skiers (55.5% and 36.8% for females and males, respectively). The epidemic curve showed a clear peak for the residents, with most cases occurring during the last week of February, but the curve drops abruptly in March. All pupils became sick within 2 days after returning to school (Fig. 1). The majority (93.7%) of cases from the ski group became sick within 3 days after arriving at the village.

Analytical study

Among schoolchildren, ARs were higher among those who drank water from the school (RR = 2.6; 95% CI = 1.0–6.5). Other exposures like eating food items
served in the school canteen, having a shower at the school or having attended the class of gymnastics were not associated with the development of illness (Table 2).

Persons living in a household connected to community water A had higher ARs than those with other water supplies. The ARs were higher among people living in households with 3 and 4 persons (Table 3). Stratification of household size by type of water supply revealed that all households of 5–7 persons had their own well (data not shown). A logistic regression model fitted with the variables ‘having community water supply A’, ‘living in a household of 3’ and ‘living in a household of 4’ showed that only community water supply A and living in a household of 4 were significant independent risk factors (adjusted odds ratios, OR = 16.7; 95% CI = 9.6–30.1 and OR = 2.4; 95% CI = 1.3–4.6, respectively).

For the ski group, the AR for those staying in a guesthouse served by water supply A was 57.5% (23/40), compared to 25.5% (14/55) for those staying in a guesthouse served by other water systems (RR = 2.3; 95% CI = 1.3–4.6).

Dose–response

For schoolchildren, the probability of illness increased with the amount of water consumed at the school ($\chi^2$ for linear trend = 8.6; $P=0.003$), but not for residents

---

**Table 1.** Distribution of symptoms among resident cases by age agroup (n = 113). Transtrand NLV waterborne outbreak, Sweden, February–March 2002

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>No. people</th>
<th>Diarrhoea (%)</th>
<th>Vomiting (%)</th>
<th>Abdominal pain (%)</th>
<th>Headache (%)</th>
<th>Muscle/body pain (%)</th>
<th>Fever (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–9</td>
<td>18</td>
<td>67</td>
<td>94</td>
<td>44</td>
<td>17</td>
<td>6</td>
<td>39</td>
</tr>
<tr>
<td>10–19</td>
<td>13</td>
<td>54</td>
<td>92</td>
<td>54</td>
<td>54</td>
<td>31</td>
<td>23</td>
</tr>
<tr>
<td>20–29</td>
<td>16</td>
<td>75</td>
<td>88</td>
<td>69</td>
<td>56</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td>30–39</td>
<td>15</td>
<td>100</td>
<td>67</td>
<td>80</td>
<td>60</td>
<td>40</td>
<td>13</td>
</tr>
<tr>
<td>40–49</td>
<td>16</td>
<td>63</td>
<td>56</td>
<td>38</td>
<td>19</td>
<td>38</td>
<td>25</td>
</tr>
<tr>
<td>50–59</td>
<td>10</td>
<td>70</td>
<td>50</td>
<td>50</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>60–69</td>
<td>16</td>
<td>81</td>
<td>56</td>
<td>56</td>
<td>31</td>
<td>44</td>
<td>31</td>
</tr>
<tr>
<td>Over 69</td>
<td>8</td>
<td>88</td>
<td>37</td>
<td>38</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Total</td>
<td>112</td>
<td>74</td>
<td>70</td>
<td>54</td>
<td>35</td>
<td>29</td>
<td>26</td>
</tr>
</tbody>
</table>

**Table 2.** Attack rates according to food, drink and other school exposures for schoolchildren (n = 53). Transtrand NLV waterborne outbreak, Sweden, February–March 2002

<table>
<thead>
<tr>
<th>Exposures</th>
<th>Exposed</th>
<th></th>
<th></th>
<th>Not exposed</th>
<th></th>
<th>RR</th>
<th>95% CI</th>
<th>% cases exposed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases</td>
<td>Total</td>
<td>AR</td>
<td>Cases</td>
<td>Total</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>18</td>
<td>33</td>
<td>52.3</td>
<td>4</td>
<td>19</td>
<td>17.6</td>
<td>2.6*</td>
<td>1.0–6.5</td>
</tr>
<tr>
<td>Milk</td>
<td>19</td>
<td>46</td>
<td>41.3</td>
<td>3</td>
<td>5</td>
<td>60.0</td>
<td>0.0</td>
<td>0.0–4.0</td>
</tr>
<tr>
<td>Gymnastics 5 March</td>
<td>23</td>
<td>48</td>
<td></td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>NC†</td>
<td>—</td>
</tr>
<tr>
<td>Shower 5 March</td>
<td>20</td>
<td>41</td>
<td>48.8</td>
<td>3</td>
<td>10</td>
<td>30.0</td>
<td>1.6</td>
<td>0.6–4.8</td>
</tr>
<tr>
<td>Lunch 5 March</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fish</td>
<td>21</td>
<td>43</td>
<td>48.8</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>NC</td>
<td>—</td>
</tr>
<tr>
<td>Potatoes</td>
<td>20</td>
<td>44</td>
<td>45.5</td>
<td>1</td>
<td>3</td>
<td>33.3</td>
<td>1.4</td>
<td>0.3–7.0</td>
</tr>
<tr>
<td>Raw vegetables</td>
<td>6</td>
<td>16</td>
<td>37.5</td>
<td>14</td>
<td>30</td>
<td>46.7</td>
<td>0.8</td>
<td>0.4–1.7</td>
</tr>
<tr>
<td>Dessert</td>
<td>5</td>
<td>11</td>
<td>45.5</td>
<td>15</td>
<td>36</td>
<td>41.7</td>
<td>1.1</td>
<td>0.5–2.3</td>
</tr>
<tr>
<td>Lunch 6 March</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rice</td>
<td>20</td>
<td>43</td>
<td>46.5</td>
<td>2</td>
<td>5</td>
<td>40.0</td>
<td>1.2</td>
<td>0.4–3.6</td>
</tr>
<tr>
<td>Bread</td>
<td>21</td>
<td>45</td>
<td>46.7</td>
<td>1</td>
<td>4</td>
<td>25.0</td>
<td>1.9</td>
<td>0.3–10.5</td>
</tr>
<tr>
<td>Ham</td>
<td>11</td>
<td>23</td>
<td>47.8</td>
<td>11</td>
<td>26</td>
<td>42.3</td>
<td>1.1</td>
<td>0.6–2.1</td>
</tr>
<tr>
<td>Sausage</td>
<td>10</td>
<td>19</td>
<td>52.6</td>
<td>12</td>
<td>29</td>
<td>41.4</td>
<td>1.3</td>
<td>0.7–2.3</td>
</tr>
<tr>
<td>Whey cheese</td>
<td>5</td>
<td>9</td>
<td>55.5</td>
<td>16</td>
<td>38</td>
<td>42.1</td>
<td>1.3</td>
<td>0.7–2.6</td>
</tr>
</tbody>
</table>

* Statistically significant 95% confidence level.
† NC, not calculated.
Table 3. Attack rates according to sex, age, household size and type of water among residents (n=387). Transtrand NLV waterborne outbreak, Sweden, February–March 2002

<table>
<thead>
<tr>
<th></th>
<th>Case</th>
<th>Total</th>
<th>AR (%)</th>
<th>RR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>58</td>
<td>188</td>
<td>30.8</td>
<td>1.1</td>
<td>0.8–1.5</td>
</tr>
<tr>
<td>Male</td>
<td>54</td>
<td>187</td>
<td>28.9</td>
<td>Ref</td>
<td>—</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–19</td>
<td>32</td>
<td>100</td>
<td>32</td>
<td>1.5</td>
<td>0.7–3.1</td>
</tr>
<tr>
<td>20–49</td>
<td>47</td>
<td>134</td>
<td>35.1</td>
<td>1.6</td>
<td>0.8–3.3</td>
</tr>
<tr>
<td>50–69</td>
<td>26</td>
<td>92</td>
<td>28.3</td>
<td>1.3</td>
<td>0.6–2.8</td>
</tr>
<tr>
<td>70–89</td>
<td>7</td>
<td>33</td>
<td>21.1</td>
<td>Ref</td>
<td>—</td>
</tr>
<tr>
<td>Household size</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>11</td>
<td>43</td>
<td>25.6</td>
<td>Ref</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>100</td>
<td>25.0</td>
<td>1.0</td>
<td>0.5–1.8</td>
</tr>
<tr>
<td>3</td>
<td>31</td>
<td>75</td>
<td>41.3</td>
<td>1.6</td>
<td>0.9–2.9</td>
</tr>
<tr>
<td>4</td>
<td>37</td>
<td>99</td>
<td>37.4</td>
<td>1.5</td>
<td>0.8–2.6</td>
</tr>
<tr>
<td>5–7</td>
<td>11</td>
<td>68</td>
<td>16.2</td>
<td>0.6</td>
<td>0.3–1.3</td>
</tr>
<tr>
<td>Source of domestic water</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Comm water A</td>
<td>74</td>
<td>101</td>
<td>73.3</td>
<td>6.4*</td>
<td>3.9–10.7</td>
</tr>
<tr>
<td>Comm water B</td>
<td>18</td>
<td>88</td>
<td>20.4</td>
<td>1.8</td>
<td>0.9–3.4</td>
</tr>
<tr>
<td>Comm water C</td>
<td>4</td>
<td>31</td>
<td>12.9</td>
<td>1.1</td>
<td>0.4–3.2</td>
</tr>
<tr>
<td>Other type</td>
<td>5</td>
<td>41</td>
<td>12.2</td>
<td>1.1</td>
<td>0.4–2.8</td>
</tr>
<tr>
<td>Own well</td>
<td>14</td>
<td>123</td>
<td>11.4</td>
<td>Ref</td>
<td>—</td>
</tr>
</tbody>
</table>

* Statistically significant 99% confidence level.

living in households served by water supply A (water drank at home) ($\chi^2$ for linear trend = 1.7; $P=0.2$) nor for the skiers ($\chi^2$ for linear trend = 0.29; $P=0.6$). The ED$_{50}$ (effective dose 50%) for schoolchildren was 1.25 glasses; 95% CI = 0.6–1.9, equivalent to 250 ml; 95% CI = 116–380 ml (Fig. 2).

Risk factors for early and late cases

Table 4 shows the ARs by type of water supply (water system A compared to water systems other than A) for early and late cases. The RR for illness for those having water supply A decreased from 17.4 (95% CI = 9.0–33.9) for early cases down to 3.2 (95% CI = 1.8–5.6) for late cases. Having community water supply A explained 86% of early cases but only 34.1% of late cases. No dose response was found for early or late cases analysed separately (data not shown).

Temporal spread of cases in the household

For households connected to water supply A, the median time lag between non-primary and primary cases was 1 day (interquartile range = 0–4). For households with water supply other than A the median value of the time lag was 6 days (interquartile range = 2–7). In these households, only 2/14 (14%) non-primary cases had occurred within 1 day after onset in their respective primary cases. We concluded that non-primary cases from households served by community water supply A occurred earlier after primary cases compared to those served by other water supplies ($P=0.006$).

Estimation of the magnitude of the outbreak

The questionnaire for the residents also inquired about lodgers during the outbreak period. For visitors
to the area an AR similar to that of the ski group (38%) was assumed. From these responses, together with the assumed incidence in non-responders, it could be estimated that 500 people were ill in the outbreak. This is a conservative estimate, since it assumes that the non-respondents did not lodge any guests and does not take into account visitors staying in the two local hotels, for which there are no available data.

Microbiological studies

Nine of 12 faecal samples analysed tested positive for NLV by EM and RT–PCR. In addition, water samples tested negative for coliforms, E. coli, sulphite-reducing clostridia, faecal streptococci, heterotrophic bacteria and E. coli phages. RT–PCR tests for NLV in the water were inconclusive.

DISCUSSION

The present study describes a large NLV waterborne outbreak associated with the consumption of communal drinking water that may have affected over 500 people. The epidemiological findings, the laboratory analysis of the faecal samples and the later discovery of a technical failure in the sewage pipe, are all factors which are consistent with a NLV waterborne outbreak. The epidemiological study of the outbreak was facilitated by the fact that the village was served by independent water distribution systems and ARs for different water sources could be compared. The similar (high) attack rates in all age groups is also typical of a community waterborne aetiology. In spite of the lack of microbiological findings in the water vehicle, the outbreak can be categorized as ‘strongly associated with water’ according to the classification devised by Tillett et al. [11].

No other exposures were suspected to confound this association (i.e. closeness to the village centre), since households served by the different water supplies were part of the same urban area (with the possible exception of those with a well, which were few and tended to live further away from the centre).

Recall biases are common in the investigation of waterborne outbreaks [12]. In the resident group we cannot rule out some biased responses leading to a better dose–response relationship as a result of the boil notice in the village, which was posted as early as the first week of March. And yet, no dose–response relationship was found in this group. Conversely we did not suspect a recall bias in the schoolchildren group. They did not reside in the area and we could confirm that the school managers were unaware of the boil water notice. Indeed, un-boiled tap water was available for the children at the school. Moreover, questions about water consumption were buried among other food exposures.

Four school cases replied that they had not drunk tap water. We investigated this further and found out that these students had attended a lesson of ‘home economics’ where they had been learning to dilute syrup to prepare refreshments using tap water. The high ARs among those exposed to water from system A (73·3% in the population cohort) is
consistent with ARs found in experimental studies, where 68% of exposed volunteers were found to develop illness [13]. In our study, 26.7% of the population did not develop illness, in spite of having consumed presumably contaminated tap water. It has been shown that for NLV infections some people seem to show a natural resistance to the disease, even after direct challenge [14].

A dose–response was detected among the school group, but not among any of the other two groups served by communal water A, in spite of their larger sizes. This discrepancy may be partly explained by the fact that only 63% of the children drank water from the school, whereas in the resident cohort all but five people answered positively that they had drunk un-boiled water from home. Given that the contaminated water was available at home for a longer period, it is conceivable that they also drank a much larger total amount compared to the schoolchildren. Likewise, it is also conceivable that the ski group drank water from different sources and in larger quantities as a result of their physical exercise. Skiers who drank from the area reported drinking a median of eight glasses of un-boiled water (=1.6 litres) per day.

In most Swedish households, unlike in other European countries, drinking tap water is considered to be safe. It is estimated that about 96% of Swedes drink regularly tap water from their household [15]. Information about households that switched to drinking bottled water or that paid heed to the water boil notice was regrettably not gathered, and questions were only asked about consumption of un-boiled water.

An interesting but explainable finding was that cases of households served by water system A tended to fall sick within a shorter period compared to those living in households with other water supplies, reflecting differences in the availability of the infectious source. It is likely that people living in households served by non-contaminated water had the opportunity to become infected through drinking water through visits to friends, relatives or by working in places which may have been connected to water supply A, as well as through person-to-person transmission.

Although most members of individual households became sick almost simultaneously, not all households served by water supply A were affected necessarily on the same days. A possible explanation may be that the contaminated water reached different households at different times, probably reflecting low contamination levels in the water. One may hypothesize that only marginal increases in the contamination may have been sufficient to trigger sickness in a given household. These low levels of contamination may also explain the negative bacteriological and virological results in the water samples. At the time of writing this article, techniques for detecting viruses in environmental samples are still in an early stage of development. Only few papers have reported the successful detection of NLV in water [4, 16].

The analysis performed dividing cases into early and late shows that water system A remained a risk factor throughout, suggesting that the water was contaminated over a prolonged period of time, and is consistent with anecdotal evidence of visitors to the area falling sick well until the end of March. Our study shows that among village residents, the water-borne route of transmission may have been more important during the first period (February) whereas person-to-person transmission may have become more important in the second period (March) of the outbreak.

The relatively large sample size of the populations surveyed allowed the study of the clinical differences across age groups. A high frequency of vomiting among cases has been used as a rudimentary indication to suggest NLV as the aetiology of an outbreak of gastrointestinal disease. In our study, vomiting was more prevalent in younger age groups, a finding consistent with previous studies [17]. However this proportion drops to levels of below 40% among the oldest age group. One of the explanations for this low frequency may be a low sample size in this age group (eight individuals). A frequency of vomiting over 50%, one of the four Kaplan criteria [18], is commonly used for recognizing NLV outbreaks in the field. Hedberg and Osterholm [2] suggested that the increased frequency of vomiting in relation to fever may be a better criterion for distinguishing outbreaks caused by NLV from those caused by bacteria. In our study this ratio ranged from 5:1 in the 30–39 year-old age group to just about 1.5 in the over 70 year-old age group.

The described outbreak highlights a problem that is all too common in viral food- or waterborne outbreaks, namely a strong statistical association that implicates the vehicle of infection (in this case the water), but a lack of success by the laboratory in detecting the pathogen in the suspected vehicle. Most routine laboratory testing of water relies on the detection of ‘indicator bacteria’ as a proxy for ‘sewage contamination’. It has been shown, however, that these indicators do not have an unequivocal correlation with the presence of enteric viruses in ground water [7].
The apparent potability of the water, when ‘indicator bacteria’ are absent, creates an additional difficulty in persuading environmental authorities of the convenience of carrying out a more vigorous public health intervention (i.e. chlorination of the communal water supply). We believe that at least one-third of the cases, including disease in schoolchildren, could have been prevented if preventive recommendations at the early stages of the investigation had been more thoroughly followed. The boil water notice, issued during the first week of March was not sufficiently adopted, as demonstrated by the subsequent occurrence of cases among schoolchildren, skiers and visitors. The effective conveyance of this preventive message to the visitors was indeed hampered by their high turnover, but sporadic cases in the community also occurred since then. The water was not chlorinated until mid-April, when videofilming of a sewage pipe by the environmental authorities revealed a sizeable crack located 10 m from one of the wells supplying water to water system A. Until then, the authorities had opposed to chlorination of the water due to lack of conviction of the waterborne nature of the outbreak and the general reluctance of the population to regular chemical treatment of the water supplies.

ACKNOWLEDGEMENTS

The authors wish to thank Alain Moren for his useful comments on the manuscript. We are also grateful to Maria Torvén and Görel Allestam for their work with laboratory analysis of samples. The work of Juan J. Carrique-Mas was supported by the European Programme of Intervention Epidemiology Training (EPIET), a programme funded by the DG Health and Consumer Protection of the European Commission.

REFERENCES


From May through June 2001, an outbreak of acute gastroenteritis that affected at least 200 persons occurred in a combined activity camp and conference center in Stockholm County. The source of illness was contaminated drinking water obtained from private wells. The outbreak appears to have started with sewage pipeline problems near the kitchen, which caused overflow of the sewage system and contaminated the environment. While no pathogenic bacteria were found in water or stool specimens, norovirus was detected in 8 of 11 stool specimens and 2 of 3 water samples by polymerase chain reaction. Nucleotide sequencing of amplicons from two patients and two water samples identified an emerging genotype designated GGIIb, which was circulating throughout several European countries during 2000 and 2001. This investigation documents the first waterborne outbreak of viral gastroenteritis in Sweden, where nucleotide sequencing showed a direct link between contaminated water and illness.

Viruses have emerged as important causes of foodborne and waterborne diseases in recent years, with numerous outbreaks associated with Norwalk viruses. This virus is the prototype in the genus Norovirus, family Caliciviridae, which includes a large number of genetically related strains associated with acute gastroenteritis. Longitudinal surveys have shown that caliciviruses and especially noroviruses are common causes of nosocomial and community-associated outbreaks of acute gastroenteritis worldwide (1–5). Norovirus-associated gastroenteritis is transmitted by the fecal-oral route. It occurs both as sporadic community cases and as large outbreaks in, for example, nursing homes, hospitals, schools, and ships. The outbreaks often are associated with ingestion of food or contaminated water. Norovirus-associated waterborne outbreaks (6) have been associated with contamination of septic tanks, industrial water system (7–9), and swimming water (10–12) as well as drinking contaminated drinking water (13–18).

We describe a waterborne outbreak caused by contaminated drinking water. While no pathogenic bacteria were found in collected samples, identical noroviruses belonging to genogroup II (GGIIb) were identified in both stool and water samples.

Methods

Outbreak Description

An outbreak of acute gastroenteritis occurred in a combined activity camp and conference center in Stockholm County from May to the end of June 2001. During the summer, the center caters to both overnight guests and daytime visiting groups. A separate cafe for outside visitors to the nearby beach is also on the premises. Environmental and microbiologic investigations were conducted to determine the source of the outbreak and implement control measures to stop the outbreak and prevent similar situations in the future.

Environmental Investigation

The municipal environmental health unit was first contacted on June 12. The facilities were inspected, and water and food samples were collected. On June 15, the Stockholm County Council Department of Communicable Disease Control and Prevention was contacted, and the premises were reinspected on June 25 and July 3. Additional water samples were taken on several occasions during June and July.

Microbiologic Investigation

Bacteriologic Investigation

A total of 11 stool specimens were collected (2 from staff and 9 from visiting guests) and cultured for bacterial enteropathogens, including Salmonella, Shigella, Campylobacter, and Yersinia. Ten water samples were...
examined for fecal coliforms, total coliforms, fecal streptococci, and sulphite-reducing clostridia. Seven food products were examined for aerobe microorganisms, enterobacteriaceae, enterococci, fecal coliforms, Salmonella, Bacillus cereus, Clostridium perfringens, coagulase-positive staphylococci, yeast, and mold. Approved standard laboratory methods were used for all bacteriologic investigations.

**Virologic Investigation**

Stool samples were examined for norovirus by electron microscopy and reverse transcription–polymerase chain reaction (RT-PCR), as previously described (4,19,20). Briefly, viral RNA was extracted from 100 µL of a 10% stool suspension with the guanidine thiocyanate–silica extraction method (21) followed by RT-PCR with primer pair JV12/JV13, which yields a 326-bp product, located in the gene for RNA-dependent RNA polymerase.

Three water samples collected from the kitchen, the water works, and the public beach were tested for norovirus. These water samples were concentrated by a method slightly modified from Gilgen et al. (22). Briefly, 0.5 L of water was filtered through a positively charged 0.45-µm membrane (Zetapor, Millipore Corp., Bedford, MA) followed by virus elution from the membrane with 50 mM glycine–NaOH, pH 9.5, containing 1% beef extract as described (16). A Centricon-100 microconcentrator (Amicon, Millipore) was used for further concentration to 100 µL.

For the water samples, a nested PCR was used. RNA-extraction and first-round PCR were performed as described in this section. For the nested PCR, new inner primers were designed from alignment of sequences circulating in Sweden and sequences from the GenBank database. The inner primers were designated n12 (5’-TGG GAY TCM ACD CA-3’) and n13 (5’-CTT CAG ANA GNG CAC ANA GAG T-3’). These primers yield a 234-bp product.

**Nucleotide Sequencing**

The PCR products from two human and two water samples were sequenced. The samples were sequenced from both directions by using primer pair n12/n13 (water samples) and primer pair JV12/JV13 (patient samples) by ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) on an ABI 310 automated sequencer. Sequences from prototype strains of caliciviruses from the GenBank database were aligned with the sequences from patient and water samples. Programs from the PHYLIP program package (National Institutes of Health, Bethesda, MD) were used to construct the phylogenetic trees. SEQBOOT (NIH) was used for bootstrap resampling to produce 100 different datasets from the aligned sequences. From these datasets, phylogenies were estimated by DNAMLK (NIH). CONSENCE (NIH) was used to construct a consensus tree from the obtained data and to obtain bootstrap values. The tree was drawn with Treeview (Page RD. TREEVIEW, University of Glasgow, Glasgow, Scotland). The nucleotide sequence accession number assigned by GenBank is AY240939.

**Results**

**Environmental Investigation**

The activity camp, conference center, and nearby cafe were supplied with ground water from their own private wells, located at the premises. Six months before the outbreak, they had started to use water from two newly drilled wells located within 20 m of each other. Only chemical parameters had been analyzed before the new wells were put in use. The water from both wells was held in a common reservoir and was not disinfected before distribution. According to personnel at the camp, the wells were approximately 80 m deep, and the soil layer was 18 m at the location of the wells. A third well was drilled at the same time and located close to the other two but was not put in use. Previously, water had been obtained from an old well located further away from the facilities. Since this old well had limited capacity, and sometimes its water was not potable, new wells with enough capacity to fulfill increased demands had been drilled. For practical and economical reasons, the new wells had been placed closer to the center facilities.

Sewage from the camp was connected to the community system and was transported to the nearest sewage treatment facility. The sewage pipes were old, and personnel reported that on several occasions problems with the capacity of the system had occurred. In April 2001, a blockage of the overflow in the low-pressure-system well, located near the kitchen facilities, occurred, and sewage had spilled out on the ground. On this site, located approximately 100 m from the ground water wells, the rock was covered by only 1–2 m of soil. Sewage had also overflowed on the ground near the kitchen in the autumn 2000 because of a stoppage in the sewage pipeline connection to the community system.

**Epidemiologic Investigation**

Approximately 200 people contracted gastroenteritis after consuming tap water. They had clinical symptoms of vomiting, diarrhea, abdominal pain, and fever (mostly a combination of these symptoms). Duration of symptoms varied from several hours to 2 to 3 days. The first known cases of illness occurred in a group of adults participating in a 1-day conference on May 31. Of 16 persons (all adults), 8 became ill (attack rate 50%) with gastrointestinal
symptoms. Nearly 2 weeks later (June 9–10), a school class with 28 pupils (8–13 years of age) arrived for an overnight stay; approximately half became ill (attack rate 50%) with similar symptoms. The following day (June 10), the first participants of a sport-training camp arrived. The camp lasted for 10 days, during which a total of 150 children (9–12 years of age) and 20 adults stayed at the facilities in three overlapping periods. The first cases of illness in this group occurred the day after arrival; approximately 100 persons became ill (attack rate 58%). During the next 2 weeks, several more guests and visiting groups reported illness after visiting the center; some of these persons had not eaten but had just drunk the center’s tap water. Two of these groups were children (8–13 years of age); the attack rate in both groups was 40%. The outbreak was not controlled until the facilities closed for >1 week in the end of June. Some of the personnel working at the center also reported gastrointestinal symptoms, including one of the kitchen personnel, who became ill on June 13 and was taken off duty.

**Control Measures**

On the first visit, general recommendations regarding kitchen hygiene and cleaning of the environment were given. When the results of the first water samples were ready, additional recommendations on boiling all water used for drinking and food preparation were given. At the same time, the environment was thoroughly sanitized. In spite of these measures, new cases continued to occur, so the facilities were closed for >1 week at the end of June to interrupt possible continuous transmission among guests. After this measure, no new cases occurred. Different alternatives to prevent similar situations in the future were discussed, and the decision was made to close the wells and connect to the municipal water supply.

**Microbiologic Investigation**

None of the stool samples collected from the two staff or nine visitors were positive for *Salmonella, Shigella, Campylobacter*, or *Yersinia*, nor were any viruses other than calicivirus found by electron microscopy. Of the 11 samples examined by norovirus-specific PCR, 8 had an amplified PCR product of the expected size. No foodborne pathogens were found in any of the food items investigated. The first samples were collected from tap water in the kitchen on June 12 and water collected from the water works on June 18 showed strong indication of fecal contamination (Table). Samples collected from the wells 1 and 3 on June 20 and 27 showed evidence of fecal contamination, as did sampling of well 2 in July (Table). Water samples from the tap in the kitchen and the water works, collected on June 18, were positive for norovirus with a nested PCR and showed evidence of fecal contamination (Table). The water samples collected from the beach were negative for norovirus. PCR amplicons from two visitors (samples collected at different time points) and the two positive water samples were sequenced and compared. The strains were identical to each other and identical to strain “Gothenburg” (Figure) and had 97%-98% nucleotide identity to Spanish GGIIb strains (AJ487474, AJ487794, AJ487795, AJ487789, AJ487787) (23).

**Discussion**

We describe an epidemiologic and microbiologic investigation of a waterborne outbreak in which at least 200 persons became ill after staying at a combined activity camp and conference center in the Stockholm area. A large number of daytime visitors to the beach and nearby cafe may also have become ill, so the actual number of cases has likely been underestimated. The visitors in different groups did not eat the same food items, and some visitors did not eat any food. Several of the short-stay visitors consumed only camp tap water, which was fecally contaminated. The source of illness was drinking water obtained from ground water wells that had been contaminated by sewage. Person-to-person transmission and transmission through contaminated surfaces probably contributed to the rapid spread among the overnight visitors. While no pathogenic

---

**Table. Results from bacteriologic analysis of water samples, Sweden, 2001**

<table>
<thead>
<tr>
<th>Place</th>
<th>Date (2001)</th>
<th>Heterotrophs/100 mL</th>
<th>Coliforms/100 mL</th>
<th>E. coli*/100 mL</th>
<th>Sulphite-reducing clostridia/100 mL</th>
<th>Fecal streptococci/100 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tap water, kitchen</td>
<td>6/12</td>
<td>80</td>
<td>140</td>
<td>47</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Water works</td>
<td>6/18</td>
<td>690</td>
<td>100</td>
<td>32</td>
<td>&lt;1</td>
<td>2</td>
</tr>
<tr>
<td>Tap water, kitchen</td>
<td>6/18</td>
<td>530</td>
<td>130</td>
<td>40</td>
<td>&lt;1</td>
<td>1</td>
</tr>
<tr>
<td>Well 1</td>
<td>6/20</td>
<td>&gt;300</td>
<td>430</td>
<td>&gt;100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Well 2</td>
<td>6/20</td>
<td>&gt;300</td>
<td>1</td>
<td>&lt;1</td>
<td>-</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Well 3</td>
<td>6/27</td>
<td>2,100</td>
<td>19</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Old well</td>
<td>6/27</td>
<td>1,100</td>
<td>630</td>
<td>22</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Storm water</td>
<td>6/27</td>
<td>2,000</td>
<td>190</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Well 2</td>
<td>7/03</td>
<td>1,300</td>
<td>160</td>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Beach</td>
<td>7/17</td>
<td>16,000</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*/Escherichia coli.*
bacteria were found in water or stool samples, norovirus belonging to genogroup II with identical nucleotide sequence in the polymerase region was obtained from both stool and water samples. The strain was identical to strain Gothenburg, previously identified in Sweden and belonging to the emerging genotype cluster GGIIb. These strains have circulated in several European countries during 2000 and 2001 (23). While the GGIIb outbreak in this study was associated with contaminated water, previously reported GGIIb strains have been associated with school, nursing home, and rural village outbreaks (23). That all were identified during 2000 and 2001 further supports the hypothesis of an emerging strain or cluster of strains.

The drinking water was obtained from deep ground wells close to the cafe. Before the outbreak, this cafe had had problems with low pressure in its well, which caused blockage of the sewage system. As a consequence sewage spilled out and led to contamination of the environment. At the contamination site, the soil was only 1–2 m deep, and cracks in the rock may have facilitated migration of microorganisms from the sewage to the ground water. Norovirus can migrate through soil and contaminate well water and cause gastroenteritis outbreaks (7,24).

One possible explanation for the protracted duration of the outbreak could be a continuous leak from the sewage system, which would have caused persistent contamination of the environment. The ill persons staying at the facilities might have contributed to increased viral load in the sewage, and problems with the sewage collection system would then have further aggravated contamination of the water supply. Another possibility was that the water initially caused the outbreak, but person-to-person spread contributed to the continuous transmission.

The low infectious dose of norovirus readily allows transmission through environmental contamination and aerosols. Boiling the water used for drinking and food preparation was recommended. Since the risk for transmission through aerosols generated when showering with possibly contaminated water is not well established, no recommendations were made in this regard. Another problem was how to decontaminate bed linen and other fabrics. Washing at high temperatures is the recommended procedure to eliminate viral contamination. However, if the water used for washing is contaminated, the rinsing process may lead to recontamination of the fabrics. We recommended boiling or heating water for washing to >90°C in the presence of detergents.

This outbreak illustrates some problems related to private water supply. In Sweden, approximately 15% of the population has a private water supply, and the extent of gastrointestinal illness related to water is not clearly identified. Problems with person-to-person transmission of noroviruses are well known; however, risks related to exposure through contact with contaminated water and environment through vomit and aerosols are not well established.

In summary, detecting identical virus in both drinking water and stool specimens from ill persons strongly indicated that norovirus was the principal pathogen of this outbreak. Nucleotide sequence analysis identified a norovirus designated GGIIb (23).

This study was supported by the European Union (QLRT-1999-00634 and QLRT-1999-00594

Dr. Nygård is an epidemiologist. Her areas of interest are epidemiology, water control, and foodborne and vector-borne infectious diseases.

References


Address for correspondence: Lennart Svensson, Department of Molecular Virology, University of Linköping, 581 85 Linköping; fax: +46 13 22 47 89; email: lensv@imk.liu.se

Many believe that art and science, if coordinated in some way, might expose wider vistas of natural truth. After all, the aim of artist and scientist alike is to communicate a new and valuable way of regarding the natural world around us.

–Graeme L. Stephens

In "The Useful Pursuit of Shadows"
Food-Borne Outbreak of Gastroenteritis Associated with Genogroup I Calicivirus

P. J. Hugo Johansson,1* Maria Torvén,2 Ann-Christin Hammarlund,1 Ulla Björne,1 Kjell-Olof Hedlund,2 and Lennart Svensson2

Hospital Infection Control Unit, Clinical Microbiology Laboratory, University Hospital of Lund, Lund,1 and Department of Virology, Swedish Institute for Infectious Disease Control, Stockholm,2 Sweden

Received 16 April 2001/Returned for modification 9 September 2001/Accepted 15 December 2001

An outbreak of gastroenteritis affecting 158 of 219 (72%) guests and employees at a hotel is described. Food served at the hotel restaurant is believed to have been the source of the outbreak and to have been contaminated by sick employees working in the restaurant. A secondary attack rate of 22% was seen involving 43 persons in all. In stool specimens from seven of eight patients, Norwalk-like viruses (NLVs) were detected by electron microscopy. While NLV-specific PCR using primers JV12 and JV13 were negative, all specimens examined with primers NVp69 and NVp110 were positive. The failure of primers JV12 and JV13 was attributed to several mismatches in the JV12 primer. Genotyping and sequence analysis revealed that all samples had identical sequences and clustered with genogroup I, and the most closely related well-characterized genotype is Desert Shield. This is the first described food-borne outbreak associated with genogroup I virus in Sweden.

Since the discovery of the Norwalk virus in 1972 (26), Norwalk-like viruses (NLVs) have been recognized as the leading cause of acute nonbacterial gastroenteritis (6, 10, 15, 27, 36). The Norwalk virus was cloned and sequenced in the early 1990s and was found to belong to the human Caliciviridae (20, 23). On the basis of their sequences within the putative RNA-dependent RNA polymerase region and the capsid region, members of the genetically and antigenetically diverse NLVs can be divided into the two genogroups I and II (2, 13, 39). The genogroups can be further divided into various genotypes, but whether the various genogroups and genotypes differ in epidemiological, clinical, or other characteristics is presently unknown.

NLV-associated gastroenteritis is transmitted by the fecal-oral route and occurs both as sporadic community cases and as large outbreaks in, e.g., nursing homes, hospitals, schools, universities, vacation camps, cruise ships, hotels, and restaurants (10, 27). Many of the sporadic cases in the community are spread by person-to-person contacts, while outbreaks often are associated with the ingestion of food or water contaminated by the virus (4, 7, 12, 14, 25, 28).

Here we describe a food-borne outbreak of gastroenteritis associated with a genogroup I, Desert Shield-like calicivirus. Desert Shield virus was first isolated from U.S. military troops who had gastroenteritis while stationed in Saudi Arabia during operation Desert Shield in 1990 (17), and it was characterized a few years later (30). Transmission of the Desert Shield-like calicivirus has been suggested to occur by contaminated water as well as by person-to-person spread (11, 18, 33). We believed that our outbreak originated from an infected food handler.

* Corresponding author. Mailing address: Hospital Infection Control Unit, University Hospital of Lund, Lund, Sölvegatan 23, S-223 62 Lund, Sweden. Phone: 046-17 32 72. Fax: 046-18 91 17. E-mail: hugo.johansson@skane.se.

MATERIALS AND METHODS

Description of outbreak and infection control measures. On 2 and 3 May 2000, a major outbreak of gastroenteritis occurred at a hotel located near a university hospital in southern Sweden. The majority of the hotel guests were present or recently discharged patients from the hospital and their relatives. Not only guests living at the hotel but also medical staff working at the nearby university hospital ate at the hotel restaurant.

The outbreak began early on the morning of 2 May when the chef of the hotel restaurant started vomiting and had diarrhea while preparing breakfast and other meals for the guests. He left the hotel at 8 a.m. after working for only 1.5 h. The chef had met a friend with gastrointestinal symptoms the day before. On 3 May, five additional employees working in the hotel kitchen fell ill with gastroenteritis while preparing lunch for the hotel guests. Later that evening, infection control personnel at the university hospital were alerted when hotel guests who had eaten food prepared by the sick employees and served in the restaurant, but with no direct contact with the kitchen, started to fall ill with gastroenteritis. The food served at the restaurant on 2 and 3 May (breakfast buffet, ground beef, chicken, vegetarian paella, goulash, salad buffet, and desserts) was suspected to be contaminated and the source of the infection. The restaurant was closed, and guests at the hotel were not allowed to visit the hospital in order to limit the spread of the infection. On the morning of 4 May, when the magnitude of the outbreak was apparent, the hotel was closed and the hotel guests and employees were sent home. The hotel and the restaurant were not opened until 14 days later after a thorough disinfestation of all contaminated settings. Medical staff working at the university hospital who had eaten at the restaurant were sent home immediately at the slightest signs of gastrointestinal symptoms and were not allowed to go back to work until 3 days after the symptoms had ceased to exist.

Epidemiological investigation and guests at the hotel. On 2 and 3 May, 144 guests and employees and 13 newborn babies were either living or working at the hotel. During the same days, 102 outside guests not living at the hotel but eating at the restaurant could be identified. These 246 persons were contacted and either asked to fill out a questionnaire or interviewed by telephone. They were asked whether they had eaten at the restaurant, about clinical symptoms, the start and duration of the symptoms, and whether secondary cases had occurred among their social contacts. Information was obtained from 219 of the 246 contacted guests and employees. In addition, information about the 13 newborn babies was given by their parents. The mean age of the 219 guests and employees (the 13 newborns not included) was 46, with a range of 7 months to 83 years. In addition to the 13 newborn babies, two guests were below 10 years of age (8 and 14 months old) and three were 80 years old or older (80, 82, and 83 years old). The 13 newborn babies were born 1 to 5 days old.

Case definitions. A patient with clinical symptoms was defined as a patient with one or more of the following symptoms: nausea, vomiting (one or more times in 24 h), diarrhea (more than two watery stools in 24 h), abdominal cramping, fever (≥38.0°C), headache, and/or muscle pains. A patient with gas-
trointestinal symptoms was defined as a patient with one or more of the following symptoms: nausea, vomiting, abdominal cramping, and/or diarrhea. A patient with gastroenteritis was defined as a patient with vomiting and/or diarrhea.

Environmental investigation. Public health inspectors conducted an environmental assessment of the hotel restaurant and collected samples of foods served on 2 and 3 May (ground beef, chicken, vegetarian paella, goulash, salads, and desserts). The food samples were analyzed for pathogenic food-borne bacteria like *Salmonella*, *Escherichia coli*, *Enterobacteriaceae*, *Staphylococcus*, *Bacillus cereus*, and *Clostridium perfringens* and for yeast and mold species.

Laboratory investigation. Bacterial cultures of stool samples from eight patients (seven hotel guests and one employee) were performed at the clinical microbiological laboratory at the University Hospital in Lund, Sweden. The stool samples were cultured for *Salmonella*, *Shigella*, *Campylobacter*, and *Yersinia* species.

EM. Electron microscopy (EM) was performed essentially as previously described (15). Briefly, a 10% fecal suspension was prepared in phosphate-buffered saline, and a drop of the suspension was incubated for 1 min on a Formvar-coated grid and then stained with 2% tungstophosphoric acid. To increase the sensitivity, a second step was performed in which the fecal suspension was clarified at 20,000 × g for 30 min and the supernatant was pelleted directly on the grid at 150,000 × g for 10 min in a Beckman (Palo Alto, Calif.) Airfuge. RNA extraction. RNA was extracted from 100 μl of a 10% fecal suspension by use of the guanidinium thiocyanate-silica extraction method (5).

Detection of NLVs by RT-PCR. Reverse transcriptase (RT)-PCR was performed with the primer pair JV12-JV13, which generates a 326-bp product (36, 37), and with primers designated NVp69 (upstream; 5'-GGG CTG CCA TCT GGA TGG CC-3') and NVp110 (downstream; 5'-AC[A/T/G] AT[CG] TCA TCA TCA CCC AA-3'), which yielded a 151-bp fragment of the RNA polymerase region (nucleotides [nt] 4733 to 4884, corresponding to equivalent locations within the Norwalk sequence, M87661) (29).

Genotyping. All PCR-positive samples were genotyped by reverse line blot hybridization essentially as described previously (38) with the modification that primer pair NVp110-NVp69, with primer NVp110 biotinylated, was used instead of primer pair JV12-JV13.

Sequencing. The RT-PCR-positive samples were further characterized by sequence analysis using the NVp69 primer and the ABI Prism BigDye Terminator Cycle-Sequencing Ready Reaction kit on an ABI 310 automated sequencer. Sequences were edited using Seq Ed version 1.0.3 software and aligned using Se-al software. A 108-nt stretch of the RNA polymerase region was obtained and aligned against sequences obtained from the GenBank database. The dendrogram was produced by PAUP version 3.1.1 software.

In order to investigate why primer pair JV12-JV13 failed to amplify the samples, a new primer was designed to cover the JV12 region. The BLAST search program was used to find the sequence in GenBank most closely related to the sequence obtained by NVp69. A new primer (5'-TGC TAT AGA GGA TGG TGG CCC TT-3') was designed from this sequence and used in RT-PCR together with primer JV13, resulting in a 375-bp fragment. This fragment was sequenced with the JV13 primer, as described above, and a 320-nt stretch of the polymerase region was obtained.

Statistical analysis. For statistical analysis, the nonparametric chi-square test and Fisher’s exact test were utilized.

Nucleotide sequence accession number. Sequence data have been submitted to GenBank and assigned accession number AF356599.

RESULTS

Incidence and clinical symptoms. Information was obtained from 219 of the 246 (89%) contacted guests and employees; 214 responded to the questionnaire, and for an additional 5, information was obtained by interview. In all, 158 of the 219 (72%) guests and employees fell ill with clinical symptoms like nausea (89%), vomiting (77%), diarrhea (74%), abdominal cramping (61%), headache (39%), fever (30%), and myalgias (27%) (Table 1). There were no significant differences in attack rate, symptoms, or onset and duration of disease for the 78 guests living at the hotel and the 80 guests, of which 46 were hospital employees, only eating at the restaurant. A total of 156 patients had gastrointestinal symptoms (nausea, vomiting, gastrointestinal cramping, and/or diarrhea), and 147 had gastroenteritis (vomiting and/or diarrhea).

Environmental assessment of the hotel restaurant and collected samples of foods served on 2 and 3 May (ground beef, chicken, vegetarian paella, goulash, salads, and desserts). The food samples were analyzed for pathogenic food-borne bacteria like *Salmonella*, *Escherichia coli*, *Enterobacteriaceae*, *Staphylococcus*, *Bacillus cereus*, and *Clostridium perfringens* and for yeast and mold species.

EM. Electron microscopy (EM) was performed essentially as previously described (15). Briefly, a 10% fecal suspension was prepared in phosphate-buffered saline, and a drop of the suspension was incubated for 1 min on a Formvar-coated grid and then stained with 2% tungstophosphoric acid. To increase the sensitivity, a second step was performed in which the fecal suspension was clarified at 20,000 × g for 30 min and the supernatant was pelleted directly on the grid at 150,000 × g for 10 min in a Beckman (Palo Alto, Calif.) Airfuge.

RNA extraction. RNA was extracted from 100 μl of a 10% fecal suspension by use of the guanidinium thiocyanate-silica extraction method (5).

Detection of NLVs by RT-PCR. Reverse transcriptase (RT)-PCR was performed with the primer pair JV12-JV13, which generates a 326-bp product (36, 37), and with primers designated NVp69 (upstream; 5'-GGG CTG CCA TCT GGA TGG CC-3') and NVp110 (downstream; 5'-AC[A/T/G] AT[CG] TCA TCA TCA CCC AA-3'), which yielded a 151-bp fragment of the RNA polymerase region (nucleotides [nt] 4733 to 4884, corresponding to equivalent locations within the Norwalk sequence, M87661) (29).

Genotyping. All PCR-positive samples were genotyped by reverse line blot hybridization essentially as described previously (38) with the modification that primer pair NVp110-NVp69, with primer NVp110 biotinylated, was used instead of primer pair JV12-JV13.

Sequencing. The RT-PCR-positive samples were further characterized by sequence analysis using the NVp69 primer and the ABI Prism BigDye Terminator Cycle-Sequencing Ready Reaction kit on an ABI 310 automated sequencer. Sequences were edited using Seq Ed version 1.0.3 software and aligned using Se-al software. A 108-nt stretch of the RNA polymerase region was obtained and aligned against sequences obtained from the GenBank database. The dendrogram was produced by PAUP version 3.1.1 software.

In order to investigate why primer pair JV12-JV13 failed to amplify the samples, a new primer was designed to cover the JV12 region. The BLAST search program was used to find the sequence in GenBank most closely related to the sequence obtained by NVp69. A new primer (5'-TGC TAT AGA GGA TGG TGG CCC TT-3') was designed from this sequence and used in RT-PCR together with primer JV13, resulting in a 375-bp fragment. This fragment was sequenced with the JV13 primer, as described above, and a 320-nt stretch of the polymerase region was obtained.

Statistical analysis. For statistical analysis, the nonparametric chi-square test and Fisher’s exact test were utilized.

Nucleotide sequence accession number. Sequence data have been submitted to GenBank and assigned accession number AF356599.

Patients with gastroenteritis. The first to fall ill with gastroenteritis was the chef of the restaurant on 2 May, followed the next day by five employees, all working in the restaurant kitchen. In all, 9 of 11 employees working in the kitchen had gastroenteritis on that and the following days. On 3 May, guests who had eaten at the restaurant, but with no direct contact with the kitchen, started to fall ill. The dates of onset of gastroenteritis for guests and employees were from 2 to 10 May, with a peak on 4 May (Fig. 1). Of the 147 patients with gastroenteritis, 98 (67%) were females and 49 (33%) were males. The mean and median ages were 46 and 45 years, respectively, with a range of 1 to 83 years. The age distribution can be seen in Fig. 2: one patient was below 10 years of age (14 months old), and two patients were >80 years old (82 and 83 years old).

The duration of symptoms was known for 139 of the 147 patients. It was less than 24 h for 37 of the patients, 24 to 48 h for 61 patients, 48 to 72 h for 27 patients, and more than 72 h for 14 patients. One patient had to receive intravenous fluids to treat dehydration, but all patients recovered.

The food served at the hotel restaurant was suspected to be contaminated and the source of the outbreak. For 211 of the 219 guests and employees, information about whether they had been eating at the restaurant on 2 and/or 3 May was available. In all, 200 of the 211 had been eating at the restaurant, and of these, 142 (71%) had fallen ill with gastroenteritis while 58 (29%) remained well. A significant correlation was seen be-

| TABLE 1. Clinical symptoms of patients falling ill from a food-borne calicivirus infection |
|---------------------------------------------------------------|----------------|
| Symptom                                           | No. of patients (percentage) |
|Clinical symptoms .......................................................... | 158 |
|Nausea ............................................................................... | 140 (89) |
|Vomiting ........................................................................... | 121 (77) |
|Diarrhea ............................................................................ | 117 (74) |
|Abdominal cramping ........................................................ | 96 (61) |
|Headache ............................................................................ | 61 (39) |
|Fever .................................................................................. | 48 (30) |
|Myalgia .............................................................................. | 43 (27) |
|Gastrointestinal symptoms* ................................................ | 156 |
|Gastroenteritis* ................................................................ | 147 |

* Defined as nausea, vomiting, diarrhea and/or gastrointestinal pains.
* Defined as vomiting and/or diarrhea.

FIG. 1. Onset of symptoms for 147 patients with gastroenteritis from 2 to 10 May 2000.
between eating at the restaurant and falling ill with gastroenteritis ($P < 0.001$; Fisher's exact test), but no specific food could be associated with disease. Of the 58 guests who remained well despite eating at the restaurant, some had avoided the salads while others had eaten the same food as those who had fallen ill. Only one patient with gastroenteritis had not been eating at the restaurant. This patient worked as a cleaning person at the hotel.

For 61 of the patients with gastroenteritis who had been eating at the restaurant on either 2 or 3 May, an incubation period could be calculated. All of these patients fell ill within 2 days after eating at the restaurant; 2 patients fell ill on the day they ate at the restaurant, 44 patients fell ill the day after, and 15 patients fell ill 2 days after eating at the restaurant. This suggests that the infection was food borne until 6 May (Fig. 1) and thereafter was transmitted by person-to-person contact or from a contaminated environment.

Secondary gastrointestinal infections. Of the 147 patients with gastroenteritis, 117 had social contacts with one or more persons, mostly family members, with no direct connection to the restaurant or hotel; 26 of these contacts resulted in secondary gastrointestinal infection (a secondary attack rate of 22%) affecting 43 persons in all. All secondarily infected persons were identified on clinical grounds alone, and no genotype identification was performed. The mean age of the secondarily infected persons was 36 years, with a range of 2 to 80 years. No case of gastroenteritis was observed among the 13 newborns at the hotel, although five parents had symptoms.

One probable secondary case was seen among patients at the university hospital. Two of the hotel guests with gastroenteritis had to be transferred to the cardiology department at the hospital, and one of them shared a wardroom with another patient who 2 days later had vomiting and diarrhea. Although no diagnostic tests were performed, this was judged to be a probable secondary case of calicivirus infection.

Environmental investigation. No pathogenic bacteria, such as Salmonella, E. coli, Enterobacteriaceae, Staphylococcus, B. cereus, and C. perfringens, and no yeast or mold species could be detected from the collected food samples. The food was not examined for viruses.

**Laboratory investigation.** Cultures of stool specimens from the eight tested patients did not yield any pathogenic bacteria, such as Salmonella, Shigella, Campylobacter, or Yersinia species. In stool specimens from seven of the eight patients, NLVs were identified by EM. Six of the seven positive specimens contained sufficient material to also be examined by an NLV-specific PCR utilizing primers JV12 and JV13 (37), and surprisingly, these primers failed to give a positive reaction. This led us to retest with a second set of NLV-specific primers designated NVp69 and NVp110, which gave a specific reaction in six of six specimens. Sequence analysis revealed that primer JV12 had six mismatches with the target sequence and primer JV13 had two mismatches, which may explain the failure with these primers.

A modified reverse line blot hybridization assay (38) including primer pair NVp110-NVp69, with primer NVp110 biotinylated, was then used to genotype the six specimens as Desert Shield-like viruses. Sequence analysis of the six RT-PCR-positive samples showed that all of the samples had identical sequences (321 nt) and clustered with genogroup I and the most closely related Desert Shield virus with 82% identity on the nucleotide level (Fig. 3). The BLAST search showed that the strain in GenBank most closely related to the sequence obtained by NVp69 and JV13 was from a food-borne outbreak in Hokkaido, Japan, where oysters were the probable source of infection (accession number ABO19262). It has 98% identity on the nucleotide level.

**DISCUSSION**

NLVs represent the major cause of acute nonbacterial gastroenteritis outbreaks among adults and the elderly (6, 10, 15,
we made this observation we have identi
ever, belong to genogroup II. It should be mentioned that since
break was unexpected, as these primers work very well for
sequencing of samples from six patients revealed the same
that caliciviruses were detected by EM and PCR and that
hospital in southern Sweden and involving 158 of 219 (72%)
with calicivirus genogroup I, at a hotel located at a university
I and II can be further subdivided into subgroups and geno-
types as more extensive information becomes available (1, 3,
In Sweden, genogroup II, Lordsdale-like viruses (8),
has up to now been the dominant genotype (unpublished).
We describe a major outbreak of gastroenteritis associated
with calicivirus genogroup I, at a hotel located at a university
hospital in southern Sweden and involving 158 of 219 (72%)
guests and employees. The diagnosis was based on the facts
that caliciviruses were detected by EM and PCR and that
of calicivirus. Antibodies to NLVs are acquired in early child-
hood and can be detected in up to 90% of older children and
adults (16, 35). Studies of the role of serum antibodies in
mediating protection against NLVs, however, have yielded
conflicting results. In adults, preexisting serum antibodies do
not seem to be associated with protective immunity, but anti-
body levels become associated with protection after repetitive
exposure (24).
Very few viruses are needed for infection, and therefore, trans-
mission is possible by droplets and person-to-person con-
tacts and from a contaminated environment, as well as from
contaminated food. The only patient with gastroenteritis who
did not eat at the restaurant worked as a cleaning person at the
hotel and could have been infected from the environment when
cleaning up after sick guests. It is known that caliciviruses can be
persistent in the environment for a rather extended time and
that they are relatively resistant to a variety of disinfectants (9).
Secondary transmission and a high secondary attack rate for
family members and friends is a prominent feature of calici-
viruses (27). We noted a secondary attack rate of 22% affecting
most family members. Only one secondary case was seen at the
university hospital, which may be attributed to the fact that
guests at the hotel, as soon as an outbreak was suspected, were
prohibited from visiting the hospital. Additionally, medical
staff who had eaten at the restaurant and who worked at the
hospital were sent home immediately at the slightest sign of
gastrointestinal symptoms.
This is the first recorded outbreak of a Desert Shield-like
virus in Sweden. Our data illustrate the limitations of using
only one pair of PCR primers for diagnostic purposes and
point to the importance of good food hygiene practices and the
immediate exclusion of infected food handlers from work.

ACKNOWLEDGMENTS

We thank Jan Vinje and Marion Koopmans for the reverse line blot
hybridization kit and Margareta Thorhagen for excellent technical
assistance.
This project (L. S.) received financial support from the EU: QLK1-CT-1999-00624 (Virus Safe Seafood) and QLK1-CT-1999-00594 (Foodborne Viruses in Europe).

REFERENCES


Two Epidemiologic Patterns of Norovirus Outbreaks: Surveillance in England and Wales, 1992–2000

Benjamin A. Lopman,* Goutam K. Adak,* Mark H. Reacher,* and David W.G. Brown*

In the period 1992–2000, the Public Health Laboratory Service Communicable Disease Surveillance Centre collected standardized epidemiologic data on 1,877 general outbreaks of Norovirus (formerly “Norwalk-like virus”) infection in England and Wales. Seventy-nine percent of general outbreaks occurred in health-care institutions, i.e., hospitals (40%) and residential-care facilities (39%). When compared with outbreaks in other settings, those in health-care institutions were unique in exhibiting a winter peak (p < 0.0001); these outbreaks were also associated with significantly higher death rates and prolonged duration but were smaller in size and less likely to be foodborne. These data suggest that Norovirus infection has considerable impact on the health service and the vulnerable populations residing in institutions such as hospitals and residential homes. A distinct outbreak pattern in health-care institutions suggests a combination of host, virologic, and environmental factors that mediate these divergent epidemiologic patterns.

Recent population-based studies have shown that Noroviruses ([NVs] formal name: Norovirus; formerly “Norwalk-like viruses”) are the most commonly identified cause of infectious intestinal diseases in Western European communities (1,2). These viruses account for an estimated 6% and 11% of all infectious intestinal diseases in England and the Netherlands, respectively (1,2) and for an estimated 23 million cases of NV in the United States each year (3). NVs are also the most common cause of outbreaks of infectious intestinal diseases in Western Europe and North America (3–7).

Three factors contribute to the considerable impact of disease caused by NV: a large human reservoir of infection (2,8), a very low infectious dose (9), and the ability to be transmitted by a variety of routes. Person-to-person spread by means of the fecal-oral route or aerosol formation after projectile vomiting is the most commonly recognized mode of transmission (4,10), although foodborne (3,11) and waterborne (12–14) transmission are also well documented.

Gastroenteritis caused by NV is mild and self-limiting in the absence of other factors. Kaplan et al. and others have proposed that NV outbreaks can be recognized on clinical symptoms (short duration and incubation) and epidemiology (high attack rates and high frequency of vomiting) alone (4,15–17).

Unlike rotavirus, NVs affect all age groups (2,8) The highest incidence is in children <5 years of age (2,18), but the greatest impact of NV is probably an economic one among the elderly in health-care institutions (4,6,19,20).

We describe the epidemiology of NVs in different outbreak settings. The data we present were collected by routine surveillance of general outbreaks of infectious intestinal diseases in England and Wales from 1992 to 2000 (4,21). Laboratory report surveillance of NV has been shown to be subject to a high degree of underascertainment (8) and age bias (4). Therefore, routine laboratory reporting of cases does not serve as a reliable sample for illness due to NV. For this reason, we describe only outbreak data.

**Methods**

Since January 1992, the Public Health Laboratory Service Communicable Disease Surveillance Centre has operated a standardized comprehensive surveillance system for general outbreaks of infectious intestinal diseases (see Appendix). The details of how this system operates are described elsewhere (4,21). In 1995 and 1996, the Public Health Laboratory Service instituted an active reporting program for outbreaks of NV through the Electron Microscopy Network. Ten electron microscopy units, representing the principal regional diagnostic centers for viral gastroenteritis in England, reported to the Centre all general outbreaks for which clinical specimens had been submitted. These reports were then integrated into the existing outbreak surveillance system, and standardized epidemiologic data were sought from investigating public health physicians. The public health physicians contacted were asked to return completed questionnaires when investigations were concluded. Data from these questionnaires were entered and stored on an Epi Info 6.0 database (23).

**Statistical Analysis**

We used the statistical software package STATA 6.0 for these analyses (24). Chi-square tests were used to compare proportions, and the Student t test was used to compare means. Data on persons affected and duration of outbreaks were observed to follow a non-normal distribution. Therefore, a natural log transformation was performed on the persons affected and duration of outbreak data to normalize the distribution of variables and satisfy the normality assumption for the t test (25). A reverse natural log transformation was then performed; results are presented as geometric means.
Results

Completed outbreak questionnaires were returned for 5,241 general outbreaks occurring from January 1, 1992, to December 31, 2000 (response rate 73%). Laboratory confirmation of NV was recorded for 1,877 (36%) outbreaks (Figure 1). The median number of laboratory-confirmed cases in NV outbreaks was 2 (range 1–36). Another 731 outbreaks (14% of all outbreaks) were suspected of being caused by viral agents; 8 outbreaks were attributed to NV plus other pathogens; these outbreaks were excluded from these analyses.

Settings

Information on setting was available for every NV outbreak (n=1,877). The most common settings were health-care institutions: 754 (40%) outbreaks occurred in hospitals and 724 (39%) in residential-care facilities. Information on the type of unit affected was available for 648 (86%) of 754 hospital outbreaks and 190 (26%) of the 724 in residential-care facilities. NV infection was centered on elderly care and geriatric units in 251 (39%) of 648 hospital outbreaks and 169 (89%) of 190 residential home outbreaks. A total of 147 (7.8%) outbreaks occurred in hotels, 73 (4%) occurred in schools, and 105 (6%) were linked to food outlets (Appendix). Seventy-four outbreaks (3.9%) occurred in other settings such as private homes, holiday camps, and military bases.

Illness and Death

A total of 57,060 people were affected in the 1,877 NV outbreaks. After excluding hospital outbreaks (n=711), we recorded 128 hospitalizations (case-hospitalization rate = 33/10,000 cases) from 52 outbreaks (mean hospitalizations per outbreak 0.19; range 0–38). Forty-three deaths (case-fatality rate 7.5/10,000 cases) occurred in 38 outbreaks (mean deaths per outbreak 0.07; range 0–2); all were associated with outbreaks in hospitals (24 deaths) and residential-care facilities (19 deaths).

Time Trends and Seasonality

Reports of NV outbreaks peaked in 1995 (367 outbreaks) (Figure 1), falling to 139 outbreaks in 1997. Since then, outbreaks have steadily increased; 281 outbreaks were reported in 2000. Since 1995, outbreaks have shown a strong seasonal peak (Figure 1). Outbreaks begin increasing in September and peak in the months of January, February, and March. Outbreaks in hospitals and residential facilities occur more commonly in the 6 months from November to April than the rest of the year (994/421; ratio 2.36) (Figure 2). Outbreaks in other settings display no winter peak (189/205; ratio 0.92). This difference in the seasonality between outbreaks in health-care institutions and those in other settings is significant ($\chi^2$ 51.1, p<0.0001)

Mode of Transmission

The reported modes of transmission were as follows (Table 1): person to person in 1,599 (85%) outbreaks; foodborne in 93 (5%) outbreaks; foodborne followed by person-to-person spread in 91 (5%) outbreaks; waterborne in 1 outbreak; unknown in 92 (5%) outbreaks.

Person-to-person spread was reported in 716 (95%) of the 754 hospital outbreaks. This figure was a significantly higher proportion than observed in food outlets (22%; 23/105 [$\chi^2$ 551.3; p<0.0001], hotels (64%; 94/147 [$\chi^2$ 175.9; p<0.0001], schools (89%; 65/73 [$\chi^2$ 27.6; p<0.0001]), or residential facilities (91.0%; 658/723 [$\chi^2$ 13.9; p=0.0002]). Food outlets were the only setting where foodborne transmission predominated (67%; 70/105).

Person-to-person outbreaks occurred more commonly from November to April than in the rest of the year (1,020/ 514; ratio 1.98). Foodborne outbreaks showed a significantly weaker seasonality (105/73; ratio 1.43) than person-to-person outbreaks ($\chi^2$ 3.99; p=0.05).

Food Vehicles

Specific vehicles were implicated in 72 (39.1%) of the 184 NV outbreaks reported to be transmitted by food. In 12 of these outbreaks, multiple food vehicles were reported, for a total of 86 implicated items. A wide range of food types were reported as vehicles of infection, including oysters, salad vegetables, poultry, red meat, fruit, soups, desserts, and savory snacks. The evidence implicating these food vehicles included...
cohort studies (55%; 47/86), case-control studies (8%; 7/86), and microbiologic studies (6%; 5/86) (Table 2). Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to confirm viral contamination in oysters in all five outbreaks where microbiologic evidence was reported.

**Contributory Factors**

Contributory factors were reported in 113 (61%) of the 184 foodborne outbreaks. Infected food handlers were more commonly identified in food-related NV outbreaks (32%; 58/184) than in those caused by other pathogens (9%; 164/1750) ($\chi^2$ 80.39; $p<0.0001$). Contamination by an infected food handler was reported less frequently in outbreaks involving oysters than other foods (oysters 0%, other foods 47%; $\chi^2$ 14.69; $p<0.0001$). Cross-contamination was also reported less frequently in outbreaks involving oysters than other foods (oysters 5%, other foods 17%; $\chi^2$ 3.35; $p=0.07$).

**Duration**

The median duration of outbreaks was 8 days (range 1–139 days). By setting, data on the duration of outbreaks were right-skewed since some outbreaks persisted for exceptionally long periods. The following results are therefore presented as geometric means. The duration of hospital outbreaks (8.8 days; 95% confidence intervals [CI] 8.4 to 9.3) was greater than those in food outlets (3.3 days; 95% CI 2.8 to 3.8; $t = -12.699$; $p<0.0001$) and hotels (4.3 days; 95% CI 3.6 to 5.1; $t = -7.025$; $p<0.0001$). However, the duration of hospital outbreaks and those in residential facilities did not differ significantly (8.7 days; 95% CI 8.1 to 9.4; $t = -0.321$; $p=0.7$) or schools (8.1 days; 95% CI 6.8 to 9.7; $t = -0.879$; $p=0.4$) (Table 3).

**Numbers of Persons Affected**

The median number of persons affected per outbreak was 21 (range 2–1,200).

Data on the number of people affected in outbreaks were right-skewed since a number of outbreaks were exceptionally large. The following results are therefore presented as geometric means. The number affected in hospital outbreaks (17.5; 95% CI 16.4 to 18.5) was significantly lower than for other settings (geometric means 21.5 to 26.5; Table 3).

**Discussion**

Examination of the features of NV outbreaks by setting reveals that outbreaks in health-care facilities have a distinctive epidemiologic profile. When compared with outbreaks in other settings, those in health-care institutions were unique in exhibiting a winter peak; they were also associated with higher death rates and prolonged duration but were smaller in size and were less likely to be foodborne. School outbreaks shared some but not all of the features that characterize outbreaks in health-care institutions.

Several epidemiologic and biologic reasons may contribute to the divergent seasonality. The respiratory infections season, which increases activity in health-care institutions, occurs concurrently with the peak in NV outbreaks in these facilities. Greater admission of patients in hospitals increases both the population at risk and the opportunities for NV to be introduced. An increase in transfers of people between residential-care facilities and hospitals also facilitates the movement of viruses between institutions. Populations in health-care facilities differ from the rest of the population in that they require nursing care. Health-care settings are semi-closed environments where patients and residents are subject to person-to-person spread and potentially contaminated environments.

Biologic differences between strains may also result in different clinical patterns. NVs from outbreaks in health-care institutions have less genetic diversity compared with those

### Table 1. Primary modes of transmission of Norovirus outbreaks, England and Wales, 1992–2000

<table>
<thead>
<tr>
<th>Setting of outbreak</th>
<th>Foodborne*</th>
<th>Person to person*</th>
<th>Other/unknown*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hospital</td>
<td>10 (1.3)</td>
<td>716 (95.0)</td>
<td>28 (3.7)</td>
<td>754</td>
</tr>
<tr>
<td>Residential facilities</td>
<td>33 (4.5)</td>
<td>658 (91.0)</td>
<td>32 (4.4)</td>
<td>723</td>
</tr>
<tr>
<td>School</td>
<td>4 (5.5)</td>
<td>65 (89.0)</td>
<td>4 (5.5)</td>
<td>73</td>
</tr>
<tr>
<td>Food outlet</td>
<td>70 (66.7)</td>
<td>23 (21.9)</td>
<td>12 (11.4)</td>
<td>105</td>
</tr>
<tr>
<td>Hotel</td>
<td>42 (28.6)</td>
<td>94 (63.9)</td>
<td>11 (7.5)</td>
<td>147</td>
</tr>
<tr>
<td>Other</td>
<td>25 (33.8)</td>
<td>43 (58.1)</td>
<td>6 (8.1)</td>
<td>75</td>
</tr>
<tr>
<td>Total</td>
<td>184 (9.9)</td>
<td>1,599 (85.2)</td>
<td>93 (5.0)</td>
<td>1,877</td>
</tr>
</tbody>
</table>

*No. of outbreaks (% of all outbreaks in setting).
from other settings (26) or sporadic cases (7), and certain variants are more commonly found in health-care facilities than in other settings (26). Thus, the strong seasonality in health-care institutions may be the result of complex interaction between host, pathogen, and environment. If and how these factors contribute to the divergent patterns of health-care-associated and community outbreaks are unknown, but we believe that our findings warrant focused investigation in the UK and elsewhere.

The observation that a hospitalization was associated with 1 in every 40 outbreaks and a death with 1 in every 50 outbreaks calls into question the belief that NV gastroenteritis is a trivial disease. Although we have no information about the other health conditions of patients who were hospitalized or died, these figures are generated from laboratory-confirmed outbreaks. Previous estimates generated by Mead et al. (which were derived from Mounts et al.) were based on the assumption that NV causes a certain proportion of gastroenteritis hospitalizations and deaths (11%), an assumption that was not based on diagnostic results (3,27).

Deaths were only reported from outbreaks in health-care institutions. The populations in these institutions differ from those found in other settings by virtue of their greater age or presence of other underlying diseases. While NV infection is not likely the principal cause of death in most cases, this infection might constitute an additional burden on patients already weakened by other conditions and thus become an important contributory factor. In hospital outbreaks, attack rates among staff are similar to those among patients (4,28), suggesting that health status is not related to acquisition of disease but to severity of outcome. Therefore, efforts to control NV infection should be directed towards vulnerable persons who already require nursing care because of illness or injury.

The only settings in which foodborne transmission predominated were food outlets. That setting was the only category in which the purchase or consumption of food was the

<table>
<thead>
<tr>
<th>Implicated food</th>
<th>Microbiologic evidence</th>
<th>Cohort study</th>
<th>Case-control study</th>
<th>Any evidence</th>
<th>Total no. of outbreaks in which food vehicle implicated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oysters</td>
<td>5 (25%)</td>
<td>9 (45%)</td>
<td>0</td>
<td>14 (70%)</td>
<td>20</td>
</tr>
<tr>
<td>Poultry</td>
<td>0</td>
<td>6 (67%)</td>
<td>0</td>
<td>6 (67%)</td>
<td>9</td>
</tr>
<tr>
<td>Meat</td>
<td>0</td>
<td>3 (60%)</td>
<td>0</td>
<td>3 (60%)</td>
<td>5</td>
</tr>
<tr>
<td>Fish</td>
<td>0</td>
<td>3 (50%)</td>
<td>1 (16%)</td>
<td>4 (67%)</td>
<td>6</td>
</tr>
<tr>
<td>Salads and vegetables</td>
<td>0</td>
<td>10 (59%)</td>
<td>3 (18%)</td>
<td>13 (76%)</td>
<td>17</td>
</tr>
<tr>
<td>Other items</td>
<td>0</td>
<td>16 (55%)</td>
<td>3 (10%)</td>
<td>19 (65%)</td>
<td>29</td>
</tr>
<tr>
<td>Total</td>
<td>5 (6%)</td>
<td>47 (55%)</td>
<td>7 (8%)</td>
<td>59 (68%)</td>
<td>86</td>
</tr>
</tbody>
</table>

*Percentages represent outbreaks with evidence per total outbreaks where food vehicle was implicated.
*In certain outbreaks more than one form of evidence was reported.

Table 3. Outbreak characteristics compared by setting of outbreak, England and Wales, 1992–2000

<table>
<thead>
<tr>
<th>Setting</th>
<th>Median (days)</th>
<th>N</th>
<th>Geometric mean of duration (days)(95% CI)*</th>
<th>t test</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Duration of outbreaks</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hospital</td>
<td>8</td>
<td>679</td>
<td>8.8 (8.4 to 9.3)</td>
<td>−0.321</td>
<td>0.73</td>
</tr>
<tr>
<td>Residential facilities</td>
<td>9</td>
<td>664</td>
<td>8.7 (8.1 to 9.4)</td>
<td>−0.879</td>
<td>0.40</td>
</tr>
<tr>
<td>School</td>
<td>8</td>
<td>63</td>
<td>8.1 (6.8 to 9.7)</td>
<td>−12.699</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Food outlet</td>
<td>3</td>
<td>94</td>
<td>3.3 (2.8 to 3.8)</td>
<td>−7.025</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hotel</td>
<td>5</td>
<td>133</td>
<td>4.3 (3.6 to 5.1)</td>
<td>−8.043</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Other</td>
<td>4</td>
<td>69</td>
<td>4.3 (3.6 to 5.1)</td>
<td>−8.043</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>All settings</td>
<td>8</td>
<td>1,702</td>
<td>7.7 (7.5 to 8.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Numbers affected per outbreak</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hospital</td>
<td>17</td>
<td>751</td>
<td>17.5 (16.4 to 18.5)</td>
<td>4.895</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residential facilities</td>
<td>23</td>
<td>723</td>
<td>21.5 (19.8 to 23.3)</td>
<td>3.594</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>School</td>
<td>24</td>
<td>73</td>
<td>24.9 (20.5 to 30.3)</td>
<td>3.444</td>
<td>0.001</td>
</tr>
<tr>
<td>Food outlet</td>
<td>23</td>
<td>104</td>
<td>23.4 (19.8 to 27.6)</td>
<td>5.729</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hotel</td>
<td>29</td>
<td>147</td>
<td>24.5 (20.2 to 29.7)</td>
<td>3.432</td>
<td>0.001</td>
</tr>
<tr>
<td>Other</td>
<td>29</td>
<td>74</td>
<td>24.5 (20.2 to 29.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All settings</td>
<td>21</td>
<td>1,872</td>
<td>20.3 (19.7 to 21.1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*CI, confidence interval.
main factor linking at-risk populations. In other settings, living, working, or recreational areas were shared by at-risk populations for varying lengths of time, thus increasing the opportunities for person-to-person spread. Even in those instances where foodborne transmission initiated an outbreak within a health-care institution, high levels of person-to-person spread usually followed. Therefore, prolonged levels of contact between persons in semi-closed institutions such as hospitals, residential-care facilities, and schools facilitate person-to-person spread to an extent not seen in other settings, which in turn leads to more prolonged outbreaks. However, schools differ from health-care institutions in terms of the seasonality and duration of NV outbreaks. In this respect, schools are more like hotels, food outlets, and other settings.

The number of affected persons was smaller in hospital outbreaks than in all other settings. This finding may reflect the lack of a universally employed definition of the spatial boundaries of an outbreak. In some hospitals, each unit affected was reported as a separate outbreak, resulting in smaller but more numerous outbreaks. In addition, cases that occur in institutions are more easily recognized as part of an outbreak than cases in open settings or the community. Thus, smaller outbreaks occurring in open settings might not be recognized or reported to investigating agencies.

The peak in recorded outbreaks seen in the winter of 1995–1996 can largely be seen as a consequence of enhanced surveillance through the development of the Public Health Laboratory Service electron microscopy network. However, there are anecdotal reports of an increase in workload in these laboratories, and other countries also recorded an increase in NV activity during the same period (11). The steady increase of reports from 1998 to 2000 may be due to increased awareness, increasing use of the molecular diagnostics RT-PCR and enzyme immunoassays, or a real increase in the occurrence of outbreaks.

Biases in different surveillance systems partly explain the wide variation in estimates of the levels of foodborne transmission in NV outbreaks. The data presented in this report suggest foodborne transmission in 10% of outbreaks in England and Wales. Estimates in Sweden (16%) (6), the Netherlands (17%) (11), and the United States (40%) (3) were all higher; however, figures from these countries are derived from much smaller datasets. In the United States, foodborne outbreaks were more likely to be reported because surveillance may be focused on detecting foodborne outbreaks (3).

The data sources that contribute to a surveillance system are a key factor affecting the estimate of the importance of foodborne transmission. In England and Wales, surveillance is broad-based and collects reports on outbreaks spread by all modes of transmission from a range of public health professionals such as physicians, environmental health officers, and diagnostic laboratories. By contrast, FoodNet, a U.S. network, is designed to detect foodborne infections (29). Since hospitals in England and Wales are in the public sector, they might be expected to readily report outbreaks to the national surveillance scheme. However, by this logic, residential homes (which are privately operated) would not be expected to report outbreaks since they might be under commercial pressures to keep information on infection confidential. The fact that nearly as many outbreak reports came from residential homes as from hospitals in the survey period suggests that reporting predominantly from the public, not the private sector, is not the case. The biases on a passive surveillance system are multiple and cannot be expected to act in only one direction.

The importance of NV as a cause of gastroenteritis outbreaks in U.S. nursing homes has been demonstrated by Green et al. (30), although the role of this virus in hospital settings has not. Aside from bias, other reasons such as variability in infection control practices in different health-care systems could result in a real difference in the importance of foodborne transmission or transmission in health-care facilities. Although NV has been estimated to cause 67% of all such illness caused by identified microbial agents (3), only 5% of public health professionals considered this pathogen to be “one of the three most common pathogens causing foodborne illness in the United States” (31); this lack of awareness probably affects outbreak investigation.

The link between oysters and NV infection is well described (32–35). These filter feeders become contaminated during growth or transport in sewage-contaminated water (33), unlike other food products that become contaminated by an infected food handler or cross-contamination. However, oysters were implicated as the vehicle of infection in <25% of the foodborne outbreaks, and a wide range of other vehicles were also reported. The greatest proportion of these outbreaks was attributed to ready-to-eat foods contaminated by infected food handlers. In the absence of a known zoonotic reservoir for NVs, the main reservoir of infection appears to be humans. Thus, reducing the incidence of foodborne NV infection requires interventions designed to prevent infected persons from contaminating prepared food and sewage from contaminating oyster beds.

These data, which show NV as the causative agent in 36% of outbreaks, support previous reports that NVs are the most common cause of infectious intestinal diseases in industrialized nations (6,11,20,36). NV accounts for a substantial extent of disease and potential economic loss, particularly to the health service where a large proportion of outbreaks occur. Wider consequences include ward closure, delayed discharge, and postponement of operations. Although NVs cause mild symptoms in healthy adults, the consequences of infection in vulnerable populations may be more serious. Considering that the populations of developed countries are aging, ensuring high levels of infection control in institutions caring for vulnerable groups is important.

Conclusions

These analyses demonstrate the value of maintaining standardized outbreak surveillance over an extended period. By examining the epidemiologic characteristics of general out-
breaks of NV by setting, we demonstrated that this pathogen is not merely an extremely common cause of infectious intestinal disease but that its effects vary widely according to the population at risk. Within health-care institutions, NV contributes to substantial illness and is associated with substantial numbers of deaths. The elucidation of a distinct outbreak pattern that is characteristic of health-care institutions suggests that a combination of host, virologic, and environmental factors mediate these divergent epidemiologic patterns. Focused research studies need to be developed to investigate the population as well as the microbiologic and behavioral processes that might explain these observations. In addition, population-based studies incorporating virus typing are required to gain a deeper understanding of the epidemiology of sporadic NV infection in the wider population. Such studies are a prerequisite to the development of firm evidence-based and targeted control strategies.

Acknowledgments

We thank André Charlett for reviewing the statistical methods of this report and Sue LeBaigue and Sally Long for their assistance with the gastrointestinal diseases outbreak database. We thank the environmental health officers, consultants in communicable disease control, infection control officers, and virologists who conducted the outbreak investigations presented here.

Mr. Lopman is an epidemiologist at the Gastrointestinal Diseases Division of the Public Health Laboratory Service Communicable Disease Surveillance Centre. His work focuses on the epidemiology of viral gastroenteritis in health-care settings and coordination of epidemiologic surveillance for the Foodborne Viruses in Europe Consortium.

Appendix. Surveillance and analysis definitions

Outbreak: an incident in which two or more people, thought to have a common exposure, experience a similar illness or proven infection, at least one of them being ill (22).

General outbreak: an outbreak that affects members of more than one household, or residents of an institution (36).

General outbreak of Norovirus: a general outbreak in which Norovirus is determined to be the causative agent by electron microscopy, RT-PCR, or enzyme immunoassay in one or more affected persons.

Residential facilities: includes residential homes, which provide some assistance in day-to-day living, and nursing homes, which provide care for persons whose infirmity or illness requires nursing care on a regular basis.

Food outlets: commercial food retailers including restaurants, pubs, bars, cafeterias, mobile food vendors, and caterers.

References


Address for correspondence: Benjamin Lopman, Communicable Disease Surveillance Centre, 61 Colindale Avenue, London NW9 5EQ, United Kingdom; fax: 44-0-200-7868; e-mail:blopman@phls.org.uk

Search past issues of EID at www.cdc.gov/eid
Viral Agents of Acute Gastroenteritis in German Children: Prevalence and Molecular Diversity

Djin-Ye Oh, Gerhard Gaedicke, and Eckart Schreier*

1Robert Koch-Institute, Berlin, Germany
2Charité Children’s Hospital, Humboldt University, Berlin, Germany

Acute gastroenteritis is a major source of morbidity and mortality among young children in developed and developing countries. Enteropathogenic viruses are regarded as particularly relevant causative agents. Between February 2001 and January 2002, fecal specimens were obtained from German children admitted to hospital with acute gastroenteritis and examined for rotaviruses, Noroviruses, enteric adenoviruses, and astroviruses using (RT-)PCR methods. Of the 59% (129/217) samples positive for ≥1 viral agent, 79% (102/129) carried rotavirus, whereas Norovirus was detected in 35% (45/129), enteric adenovirus in 14% (18/129), and astrovirus in 4% (5/129). Thirty-eight specimens contained at least two enteropathogenic viruses, with the majority of coinfections attributable to rotavirus/Norovirus dual infections. Sequence analysis revealed a cocirculation of G1, G3, G4, and G9 type rotavirus with G1 being the most common and G9 the second most common rotavirus G-type. Emergence of G9 rotaviruses in Germany may have implications for future vaccine development. A variety of Norovirus genotypes, most belonging to GGII, were found. Apart from subgenus F, adenovirus related genetically to subgenera A–C were detected. All astroviruses belonged to genotype 1. This is the first study concerning German children admitted to hospital that assesses the relative importance of these viruses by nested (RT-)PCR methods. J. Med. Virol. 71:82–93, 2003.

KEY WORDS: rotavirus; Norovirus; adenovirus; astrovirus; pediatrics; coinfection

INTRODUCTION

Acute gastroenteritis is a common disorder in young children. The associated dehydration is a leading cause of admission to hospital in industrialized countries and a major source of mortality in developing countries. Enteric viruses have been recognized as the most important etiologic agents of the disease [Kapikian, 1996; de Wit et al., 2001], and four categories of viruses are being considered as relevant: Group A rotavirus (RoV, family Reoviridae), Norovirus (NV, family Caliciviridae), adenovirus 40/41 (AdV, subgenus F) and astrovirus (AstV). Rotavirus is acknowledged unanimously as the major cause of severe diarrheal illnesses of infants and children worldwide. Data on the relative importance of the other agents as pediatric pathogens depend on variants such as the diagnostic assay or the geographical setting chosen [Uhnno et al., 1984; Maldonado et al., 1998; Pang et al., 2000; Waters et al., 2000].

Reverse transcription polymerase chain reaction [(RT-)PCR] methods are characterized by their high sensitivity; for instance, when applied for the diagnosis of astroviruses, the detected prevalence of AstV-associated diarrhea would significantly exceed the prevalence detected by enzyme immunoassay (EIA) [Mitchell et al., 1995; Glass et al., 1996; Cubitt et al., 1999]. Sequence analysis of PCR products obtained from clinical specimens renders valuable data on the genotype distribution and sequence variance within the studied population. Therefore, the introduction of molecular techniques in the etiologic study of acute gastroenteritis has advanced markedly the knowledge on the molecular epidemiology of these viruses. The information thus provided is indispensable for future vaccine development [Estes et al., 2000; Peter and Myers, 2002].

Our interest was to determine the prevalence of the above-named viruses in clinically relevant (i.e., hospitalization-requiring) infantile gastroenteritis, using highly sensitive molecular techniques as the diagnostic tool. A second aim was to determine the distribution of mono- and coinfections between these viral agents. Therefore, stool specimens from children admitted...
to hospital throughout a one-year period for acute gastroenteritis underwent consecutive (RT-) PCRs specific for each virus. A third aim of our study was to gain insight into the genetic variability of the enteric viruses circulating among young German children.

**MATERIALS AND METHODS**

**Specimen Collection**

A total of 217 stool samples from infants and children admitted to the Berlin Charité Children’s hospital with diagnosis of acute gastroenteritis were submitted to the laboratory between February 2001 and January 2002. One hundred forty-one (65%) of the specimens were obtained within the first/fourth annual quarters. During the last two months of the study, another 22 specimens were collected from pediatric inpatients admitted for non-enteric illnesses; a third group of 28 fecal samples was obtained from children attending a Berlin kindergarten who did not suffer from gastroenteritis. RNA preparation would take place within two days after collection or, in the case of individual samples, after storage at −20°C.

**Extraction of Nucleic Acids From Fecal Specimens**

Viral nucleic acids were extracted from 140 µl of a 10% fecal suspension by the use of a spin column technique according to the manufacturer’s instructions (QIAGEN, Hilden, Germany) and RNA/DNA was eluted in 50 µl 0.01 Tris-HCl, pH 7.0. All samples were tested for the presence of RoV, NV, AdV, and AstV nucleic acids by (RT-)PCR.

**Rotavirus Diagnosis**

**VP6.** RoV specific diagnostic nested RT-PCR was targeted for a 189-nt region corresponding to nt 135–323 (inner PCR; outer PCR, nt 1–421) of the human Wa rotavirus VP6 gene (accession no. K02086). Primers were deduced from an alignment of the VP6 genes of human and animal group A rotaviruses [Gorziglia et al., 1988] and are listed in Table I. In a 10-µl reaction volume, 5 µl of the extracted RNA were reverse transcribed using 50 U M-MLV-RT (Moloney Murine Leukemia Virus reverse transcriptase; Life Technologies, Gaithersburg, MD) and primers RoV1 and RoV2;

**TABLE I. (RT-)PCR Primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Original sequence (5’→3’) designation</th>
<th>Map position</th>
</tr>
</thead>
<tbody>
<tr>
<td>RoV1</td>
<td>GGCTTTWAAACGAAGTCTTC</td>
<td>1–20</td>
</tr>
<tr>
<td>RoV2</td>
<td>GATGAATTATCAAATTTATT</td>
<td>421–400</td>
</tr>
<tr>
<td>RoV3</td>
<td>ATAATTACTATGAATGGAAGAATGA</td>
<td>135–157</td>
</tr>
<tr>
<td>RoV4</td>
<td>CATTTCATTACATACTACATT</td>
<td>323–303</td>
</tr>
<tr>
<td>RoV5a</td>
<td>GGCCTTTAAAGAGAGAATTTCGTTCTGG</td>
<td>1–28</td>
</tr>
<tr>
<td>RoV5b</td>
<td>GGCCTTTAAAACGAGAATTTCGTTCTGG</td>
<td>1–28</td>
</tr>
<tr>
<td>RoV6a</td>
<td>GGTCCCCATACATAAAATCTAAYCTA</td>
<td>1,062–1,038</td>
</tr>
<tr>
<td>RoV7</td>
<td>GTATGGTTATGAAATATACACC</td>
<td>51–71</td>
</tr>
<tr>
<td>RoV8a</td>
<td>TCTTTAAATGATGATGACCC</td>
<td>407–388</td>
</tr>
<tr>
<td>RoV8b</td>
<td>TCTTTAAATGATGATGACCC</td>
<td>407–388</td>
</tr>
<tr>
<td>RoV8c</td>
<td>TCTTTGAAGTACACAGGCTCC</td>
<td>407–388</td>
</tr>
<tr>
<td>RoV9a</td>
<td>CTGGAAATCTCATTTTACCTTCTTG</td>
<td>331–308</td>
</tr>
<tr>
<td>RoV9b</td>
<td>CATTTACTTTCTCTTTGTGTCTTCAG</td>
<td>331–308</td>
</tr>
<tr>
<td>NV32</td>
<td>ATGAAATATGAAATGAGATG</td>
<td>4,226–4,245</td>
</tr>
<tr>
<td>NV32a</td>
<td>ATGAAACAAATAGARGATG</td>
<td>4,226–4,245</td>
</tr>
<tr>
<td>NV33</td>
<td>TACCACTATGATGACGATTA</td>
<td>4,280–4,299</td>
</tr>
<tr>
<td>NV33a</td>
<td>TATCCTATGATGCTGACTA</td>
<td>4,280–4,299</td>
</tr>
<tr>
<td>NV35</td>
<td>GTTGACAAATCATCTATCATC</td>
<td>4,617–5,498</td>
</tr>
<tr>
<td>NV35a</td>
<td>ACAATCTCATCATCICCAT</td>
<td>4,611–5,493</td>
</tr>
<tr>
<td>NV36</td>
<td>ATTTGTCTCTTTGTTGTC</td>
<td>4,707–6,688</td>
</tr>
<tr>
<td>AstV1a</td>
<td>TCAGATGCATTTRTCTTTG</td>
<td>5,004–4,985</td>
</tr>
<tr>
<td>AstV1b</td>
<td>TCTGAAAGCTATTTRTTCTTG</td>
<td>5,004–4,985</td>
</tr>
<tr>
<td>AstV2</td>
<td>CAACCTCAGGAAACAGGCTG</td>
<td>4,556–4,575</td>
</tr>
<tr>
<td>AstV3</td>
<td>TTTGTIARCCACACICCRTC</td>
<td>4,953–4,934</td>
</tr>
<tr>
<td>AstV4 Mon269</td>
<td>GGTGTCACAAGGCCAAAACC</td>
<td>4,541–4,560</td>
</tr>
<tr>
<td>AdV1</td>
<td>CAAGATGCCACCCCTTCTG</td>
<td>17,639–17,657</td>
</tr>
<tr>
<td>AdV2</td>
<td>CGATCCAGCCCGGCGGGATGTC</td>
<td>17,968–17,946</td>
</tr>
<tr>
<td>AdV3</td>
<td>AATGGTCTTACATGCACAT</td>
<td>17,668–17,686</td>
</tr>
<tr>
<td>AdV4</td>
<td>ACCGGGTGTCTGGCCACGGCCAG</td>
<td>17,920–17,898</td>
</tr>
</tbody>
</table>

*aFor primers identical to previously described primers, the original designation is given. bRoV1–4: referring to K02086, Human Wa RoV segment 6 RNA, complete VP6 gene; RoV5–10: referring to K02033, Human Wa RoV gene 9 for serotype-specific antigen VP7; NV32–36: referring to X86557, Lordsdale virus complete genome; AstV1–4: referring to AF141381, Human astrovirus type 3 total sequence; AdV1–4: referring to NC_001454, Human adenovirus F, complete genome. cIdentical to/derived from primers Beg, End [Gouvea et al., 1990]. dIdentical to/derived from primers given by Schreier et al. [2000]. eIdentical to/derived from primers given by Noel et al. [1995].
the reaction was performed at 42°C for 5 min and 37°C for 20–55 min, with subsequent denaturation for 5 min at 94°C. Five microliters of the RT reaction product were amplified by PCR with Taq-polymerase (InVitek, Berlin, Germany) and the same pair of primers. Two microliters of the first-round product were subjected to nested PCR, using primers RoV3 and RoV4. PCR was carried out for 35 cycles (94°C, 45 sec; 42°C, 15 sec; 72°C, 1 min), with a final extension step at 72°C for 5 min.

**VP7.** In order to amplify part of the RoV VP7 gene and by this means G-type RoV-positive isolates, RT and first-round PCR were carried out by a single-tube method. The 25-μl reaction mixture contained 5 μl of the RNA preparation, both the antisense primer RoV6 and mixed equimolar sense primers RoV5a/RoV5b and OneStep RT-PCR Enzyme Mix (QIAGEN®, Hilden, Germany) containing Omniscript and Sensiscript reverse transcriptases as well as HotStar DNA polymerase. Reverse transcription (30 min at 42°C) was followed by a 15-min incubation period at 95°C to inactivate transcriptases and activate HotStar DNA polymerase. Thermal cycling conditions were as follows: 35 cycles of denaturation (94°C, 30 sec), annealing (42°C, 30 sec) and extension (72°C, 1 min), final extension for 5 min at 72°C. Subsequent nested PCR mix contained primers RoV7 (sense) and an equimolar mixture of primers RoV8a–c (antisense), to which Taq-polymerase and 1 μl of template were added. Amplifications were undertaken for 35 cycles (94°C, 30 sec; 42°C, 30 sec; 72°C, 45 sec; for the last 5 min, extension at 72°C).

Those RoV samples yielding negative signals in the described single-tube PCR underwent nested RT-PCR using equimolar mixes of sense primers RoV5a/RoV5b and antisense primers RoV9a–c for RT and first-round PCR. Inner PCR was done with sense primer RoV7 and a mixture of antisense primers RoV10a–c. RT conditions equalled those of RoV (VP6) reverse transcription described above, while PCR was carried out under the same conditions as RoV (VP7) nested PCR. Primers RoV 5-6 are identical to/derived from primers Beg and End [Gouvea et al., 1990] as noted in Table I.

**NV Diagnosis**

The RT-PCR method used to detect NVs was performed over part of the polymerase region and has been described elsewhere [Schreier et al., 2000]. In order to detect a broad range of sequence variants of NV genomes, primer systems were complemented as follows: For the first round of amplification, an equimolar mixture of sense primers NV32 and NV32a was used in combination with antisense primer NV 36. The second round reaction was carried out using the antisense primer mixture NV35 and NV35a and the sense primer mixture NV33 and NV33a. A complete overview of the primers used is given in Table I.

**AstV Diagnosis**

For AstV diagnosis, reverse transcription was carried out under the same conditions as RoV (VP6) RT, using an equimolar mixture of antisense primers AstV1a and AstV1b (Table I). Part of the c-DNAs underwent PCR with sense primer AstV2 and antisense mixed primers AstV1a and AstV1b. One microliter of the first-round PCR product was added to a second-round nested PCR mix containing sense primer AstV4 and antisense primer AstV3a. Amplifications were undertaken for 35 cycles (94°C, 30 sec; 42°C, 30 sec; 72°C, 45 sec; for the last 5 min, extension at 72°C). The primer system used here, amplifying a 412 nt fragment (inner PCR; first round PCR, 449 nt) of open reading frame (ORF) 2, corresponds to oligonucleotides Mon244, Mon245, Mon269, and Mon270 [Noel et al., 1995]. Two of these (Mon 245, Mon 270) were optimized in order to include current sequence data.

**AdV Diagnosis**

For molecular diagnosis of AdV, first-round PCR was performed using 5 μl of the extracted DNA and outer primers AdV1 (sense) and AdV2 (antisense). Two microliters of the first-round PCR products underwent nested PCR with primers AdV3 (sense) and AdV4 (antisense). The 253 nt genome region thus amplified is located close to the 5’ end of the hexon gene of the virus and corresponds to nucleotides 17,668–17,920 of the AdV subgenus F (AdV-F) reference sequence (NC 001454). Primers are shown in Table I and PCR conditions were the same as those described for AstV.

**Quality Control Measures and Carryover Prevention**

In order to avoid false-positive results, which is particularly relevant for nested PCR assays, quality control measures were taken as recommended by Kwok and Higuchi [1989]. Negative sample controls were included with each set of amplifications, accordingly.

**Molecular Sequencing and Phylogenetic Analysis**

Nested products of NV-, AdV-, AstV-, and RoV (VP7)-PCRs were sequenced using an ABI Prism 377 DNA sequencer and Big Dye Terminator Cycle Sequencing Mix (Perkin Elmer, Wellesley, MA). For each virus, inner PCR primers were used to sequence nested amplions. Sequence analysis provided the opportunity to ensure the specificity of the nested PCR assays used here. Data were analysed with the Sequencher sequence analysis program version 3.1.1 (Gene Codes Corp., Ann Arbor, MI). Sequence alignments and phylogenetic analysis were carried out using the CLUSTAL W program and the Phylogeny Interference Package (PHYLIP) as included in the BioEdit program, version 5.0.6 [Hall, 1999]. Genetic distances were estimated using the DNADIST program, and unrooted phylogenetic trees were constructed by the neighbor-joining method. Bootstrap analysis of 100 replicate data sets was carried out using the SEQBOOT and CONSENSE programs [Felsenstein, 1985].
Nucleotide sequences of the German isolates have been deposited in GenBank.

RESULTS

Epidemiology of Viral Infections in Children Hospitalized With Acute Gastroenteritis and Controls

During the 12 months of this study (February 2001 through January 2002), fecal specimens from 217 children admitted to the Charité Children's Hospital for acute gastroenteritis were examined for enteric viruses. The age of the children examined ranged between 29 days and 15.5 years (median age: 13 months, mean age: 21.5 months) and 98% were aged 5 years and younger. Stool samples from two control groups were collected during December 2001 and January 2002: The first group (28 children) attended kindergarten and did not suffer from any gastrointestinal symptoms in the two weeks preceding specimen collection; the second group consisted of Charité Children's Hospital patients admitted for a cause other than gastrointestinal illness.

For each child, a single stool specimen was obtained and tested for rotavirus (RoV), Norovirus (NV), astrovirus (AstV) and adenovirus (AdV) by specific nested PCR systems.

A nested PCR assay amplifying a short, conserved 188-nt part of the VP6 gene was used to determine the overall RoV prevalence among pediatric patients admitted for acute gastroenteritis. For NV diagnosis, RT-nested PCR screening was performed using primer pairs reactive to both genogroup I and genogroup II NV RNA, which amplify a 338-nt segment of ORF 1. AstV prevalence was determined from RT-nested PCR of a 413-nt part of the ORF 2 region of the viral genome. The AdV-nested PCR system utilized in this study yields a 253-nt DNA amplicon located close to the 5’ end of the hexon gene. Primers were designed to detect enteric as well as non-enteric AdV serotypes; to identify enteropathogenic subgenus F adenovirus (AdV-F) among all AdV isolates, nested PCR products were sequenced directly and sequence comparison to reference sequences was carried out.

Enteropathogenic viral agents were detected in 129 of the children hospitalized for acute gastroenteritis (59%); of these, 62 (48%) were boys. Eighty-one percent of the infections were diagnosed during 1st/4th annual quarters. Rotavirus could be identified in 102 (79% of the 129 specimens containing at least one enteropathogenic virus) samples. Norovirus was found in 45 (35%) samples. Astrovirus was responsible for 5 (4%) infections and enteric adenovirus F (AdV-F) was detected in 18 (14%) samples. To note, the overall number of adenovirus-positive specimens (non-enteric subgenera included) was 31 (Figs. 1 and 2).

The percentages of monoinfections were 51% (66 samples), 13% (17 samples), 4% (5 samples), and 2% (3 samples) for RoV, NV, AdV-F, and AstV, respectively. Eighteen percent of all specimens examined contained two or three enteropathogenic viruses. Dual infections between RoV and NV accounted for the largest portion of coinfections (24 of the 129 samples with positive diagnoses, representing 19%), followed by dual infections between RoV and AdV-F (nine samples, i.e., 7%). Double infections between enteric AdV and either NV or AstV occurred in one sample each (2%). Another three (2%) of the infected samples were triple-infected with RoV, NV, and either AdV-F or AstV. Figure 2 demonstrates the relative proportions of mono- and coinfections based on the total number of enteropathogenic virus infections detected.

Of the 28 fecal specimens obtained from kindergarten control subjects, 27 (96%) were negative for all four viruses whereas one sample was diagnosed NV-positive. Among the 22 stool samples from children admitted to hospital for non-gastrointestinal disorders, five (23%) contained RoV and one (5%) sample contained NV. AdV was detected in three (14%) samples. In sequence comparison, two (9%) of these were related to subgenus C sequences; the third one, together with rotavirus, was involved in the only double infection observed in this control group and assigned to subgenus A (see Fig. 5). None of the samples yielded astrovirus (Fig. 1).

Molecular Typing of Rotavirus, Norovirus, Adenovirus and Astrovirus

Rotavirus. For assessment of G-type distribution among rotavirus circulating in Germany, the 102 samples yielding positive signals in the described RoV (VP6) PCR were subjected to nested PCR amplification of a 357-nt region located close to the 5’ end of the VP7 gene. Of the 39 specimens not detected by this PCR method, 36 were further assayed with antisense primers RoV9a–c and RoV10a–c as described above. Seventy-one percent of the isolates positive in the (VP6) PCR could be amplified by one of the consecutively-applied (VP7) PCRs. Phylogenetic analysis was performed over an internal region located at base positions 138–248 of one of the RoV VP7 reference sequences (Human Wa RoV gene 9 for serotype-specific antigen VP7, GenBank acc. No. K02033), and included known sequences of all G-types except G7 and G10, for which suitable sequence information is unavailable in Genbank. As demonstrated in Figure 3, the German RoV isolates clustered with reference sequences of G-types G1 (63 isolates, 62% of the rota-positive samples), G3 (1 isolate, 1%), G4 (3 isolates, 3%), G9 (5 isolates, 5%). Coinfections had occurred mainly between G1-rotaviruses and the other viruses; within two specimens, however, NV and G4-/G9-RoV were found. According to this typing method, all of the 5 RoV-positive samples obtained from the control group admitted to hospital contained G1-rotaviruses.

Noroviruses. For investigation of the genetic variability of the detected Noroviruses, the nucleotide sequence of 45 of the 47 PCR amplicons obtained from the study specimens (including controls) was determined. A 145-nt segment of the RNA polymerase gene (ORF 1) corresponding to nts 4,705–4,849 of the Norwalk prototype sequence (GenBank accession no. NC 001959) was
compared to a large selection of published NV sequences. Only one isolate belonged to genogroup I (type Birmingham), while the remaining 43 isolates were assigned to genogroup II of the Norwalk group of viruses. The genogroup II isolates were found to cluster with four prototype strains. The majority (24 isolates, 53% of the NVs detected among acute gastroenteritis patients) were classified as type Tarragona, which was designated according to the reference sequence. Fifteen (33%) isolates were assigned to type Grimsby, which, in addition, comprised the two isolates obtained from control subjects. Two (4%) viruses corresponded to the Melksham and one (2%) to the Lordsdale prototype virus (Fig. 4).

**Adenovirus.** To identify enteropathogenic adenovirus isolates among all samples yielding positive signals in the AdV PCR, PCR products were subjected to genetic analysis. Sequences of a hexon gene region (106 nt, corresponding to nts 17,731–17,836 of the subgenus F reference sequence; GenBank accession no. NC 001454) were compared to published sequence data of all adenovirus subgenera.

Of the 31 German adenoviruses isolated from gastroenteritis patients, 30 could be clearly assigned to the established subgenus clusters (Fig. 5). Eighteen sequences were grouped together with the reference sequences of serotypes 40/41 (subgenus F). Nine isolates clustered with the subgenus C reference strains and 3 isolates fell into the same clade as known viruses classified as subgenus B adenoviruses. One isolate displayed a high degree of nucleotide distance towards reference sequences of all subgenera and could not be unambiguously assigned.

**Astroviruses.** Five samples contained astrovirus. All AstV isolates belonged to serotype 1, as was demonstrated by sequence analysis over a 348-nt ORF2 region (nt 4,601–4,948 of a total AstV sequence deposited in GenBank, accession no. AF141381), which shows a high correlation of geno-/serotype (data not shown).

**DISCUSSION**

This study was designed to assess the prevalences of viral agents and their molecular diversity among young German children admitted to hospital with acute gastroenteritis. Highly sensitive (RT-)PCR methods were established and applied to detect four enteropathogenic viruses esteemed to be of major importance in this age group: Group A rotavirus (RoV), Norovirus (NV), adenovirus 40/41 (AdV 40/41), and astrovirus (AstV).

Recently, several surveys have been published that look for several of the presently known gastroenteritis viruses in ambulant and/or clinical pediatric settings [Bon et al., 1999; Pang et al., 2000; Bereciartu et al., 2002; Marie-Cardine et al., 2002]. Mostly, diverse diagnostic techniques (EIA and PCR) were used to
detect the viruses. The exception is a large-scale study conducted in Finland, focusing on viral gastroenteritis in the community, by the use of PCRs specific for each viral agent [Pang et al., 2000]. This seemed the most valuable diagnostic approach to us, because the sensitivities of EIA and PCR have been reported to differ significantly [Wilde et al., 1991; Glass et al., 1996; Bon et al., 1999]. We present the results of what is, to our knowledge, the first survey concerning children admitted to hospital in Germany that uses (RT-)PCR for the detection of each of the above-named four viral agents.

Concerning the epidemiology of gastroenteritis viruses among German children, the existing data include three viruses at most [Putzker et al., 2000; Schulz et al., 2000; Fruhwirth et al., 2001; Ehlenk et al., 2002]. To date, no information on the importance of NV in a pediatric German population has been available.

Of the specimens examined, the majority (59%) were positive for viral pathogens. As in many other countries, RoV was found to be the single most important enteric viral pathogen associated with clinically severe, hospitalization-requiring gastroenteritis in German children. This highlights the socio-economic importance of effective prevention, including the currently underway development of RoV vaccines.

The second-most frequent pathogen was NV, which was detected at a higher rate (21% of all examined specimens) than it was among children admitted to hospital in France and Finland (~10%) [Pang et al., 2000; Marie-Cardine et al., 2002] and at a rate approximately equal to that found in Argentinian pediatric outpatients (24%) [Bereciartu et al., 2002]. These results confirm recent observations indicating that NV is the second most common enteropathogenic virus among young children [Bon et al., 1999; Pang et al., 2000; Bereciartu et al., 2002].

The frequency determined for enteric AdV-F (8%) corresponded to that found in another recent study relying on PCR diagnosis (7% for hospitalized children) [Pang et al., 2000]. Comparable surveys using EIA have reported lower AdV detection rates (<3%) [Bereciartu et al., 2002; Marie-Cardine et al., 2002], which is not a surprise given the higher sensitivity of PCR as compared to EIA [Raty et al., 1999].

Surveys of the prevalence of AstV among children hospitalized for acute gastroenteritis have reported rates of up to 7% [Dennehy et al., 2001]; the comparatively low detection rate of AstV in this study may be due to the fact that AstV prevalence undergoes significant annual shifts [Mustafa et al., 2000].
The high incidence of coinfections, especially between RoV and NV, is consistent with the results of other recent investigations on the viral etiology of pediatric gastroenteritis [Bon et al., 1999; Pang et al., 2000; Bereciartu et al., 2002]. It is possible that the synergetic action of more than one enteropathogenic virus increases the clinical significance of diarrheal disease. However, the results of a study applying a clinical severity score indicate that the severity of diarrheal illness is not reflected in the proportion of mixed infections [Pang et al., 2000]. Another likely explanation might be that virus from an earlier episode is still being excreted while another virus is causing the acute disease. In an individual case, the decision which of two or even three viruses detected by highly sensitive (RT-)PCR is the particular etiologic agent of a gastroenteritis episode may be difficult.

In 41% of the diarrheal samples, no etiologic agent could be established, despite the use of PCR. In addition to the viruses tested, other pathogens may have been present, such as Sapporo-like viruses, group C Rotaviruses, Toroviruses, and, of course, bacteria and

Fig. 3. Phylogenetic relationships of Rotaviruses collected in Berlin, 2001, and selected RoV reference strains (given in bold letters), based on a 111-nt VP7 segment. Branch lengths of the unrooted phylogenetic tree represent the genetic distance between sequences. The scale indicates nucleotide substitutions per position. Bootstrap values >70% are shown. Viruses of identical sequences to those shown are omitted. Reference types G11, G13 and G14 are animal rotaviruses. The corresponding GenBank accession numbers for the reference sequences are as follows: M21843 (G1), U36241 (G2), D86284 (G3), AB039032 (G4), L79916 (G5), AF207062 (G6), J04334 (G8), AB045372 (G9), M23194 (G11), M58290 (G12), D13549 (G13), M61876 (G14). German RoV sequences have been deposited in GenBank (accession numbers AY274303 to AY274318).

88 Oh et al.
bacterial toxine [Duckmanton et al., 1997; Pang et al., 2000; Dennehy et al., 2001].

Concerning the two control groups, specimen collection was limited to the first/fourth annual quarters; during this time span, 81% of the viral infections were diagnosed among the study group. The high rate of RoV detection within the hospitalized control group as compared to the kindergarten group is not surprising. Nosocomial RoV infections have been described before [Cone et al., 1988]; asymptomatic excretors, infected with RoV previously to being hospitalized, may also contribute.

Fig. 4. Phylogenetic analysis of Noroviruses (NV) isolated in Berlin, 2001, and selected NV reference strains over a 145-nt section of the RNA-polymerase gene (ORF 1). Only non-redundant sequence data were included in construction of the unrooted phylogenetic tree. Bold letters indicate reference strains. The Tarragona reference sequence has recently been determined by a Spanish group [Buesa et al., 2002] whereas all the other reference strains are those published by Vinje and colleagues [Vinje et al., 2000, Vinje and Koopmans, 2000]. German NV sequences have been deposited in GenBank (accession numbers AY274319 to AY274328).
For the 102 RoV-positive specimens, PCR amplification, sequencing and sequence comparison was done over a short portion of the VP 7 gene. This approach led to G-type determination of the majority (70%) of the German RoV isolates.

G1-RoV were the most prevalent by far (62%), a finding that corresponds to the results of numerous studies focusing on RoV G-type distribution. Unexpectedly, G9-RoV was the second most common G-type found, at a prevalence as high as 5%. This genotype has not been previously detected in Germany [Fruhwirth et al., 2001; Ehlken et al., 2002], but recent reports from developing [Ramachandran et al., 1996; Unicomb et al., 1999] and developed countries [Ramachandran et al., 1998; Cubitt et al., 2000; Griffin et al., 2000; Oka et al., 2000] indicate a global emergence of this G-type viewed over years as unusual [Gentsch et al., 1996]. Of particular importance, G9 RoV has been associated with serious neonatal diarrhea outbreaks; it has been discussed that neonates may lack maternal protective antibodies to a genotype their mothers have not been exposed to and are, therefore, especially vulnerable [Cicirello et al., 1994; Widdowson et al., 2000]. The tetravalent RoV vaccine (“Rotashield”), which was withdrawn recently because of a link with intussusception, was based on G1-G4 strains. The development of new candidate RoV vaccines should take the apparent emergence of G9 RoV into account.

Even though specimens yielding negative signals in the original (VP7) PCR underwent subsequent...
PCR with a mixed primer system specific for G2–G4 rotaviruses, these types were rarely, if at all, detected. On the other hand, during 1997–1998, a prevalence of G4-RoV as high as 17% was observed in Germany [Ehikien et al., 2002]. This contrast supports the results of Bishop et al. [2001] who, in a 4-year-spanning multicenter survey, found that G-type frequency may show marked fluctuations, depending on the area and the year studied.

G-typing of RoV is usually done over the whole length of the VP 7 gene and often up to six sequencing primers are required [Ramachandran et al., 1998]. In this study, sequence analysis of an extremely short portion of the gene would suffice to G-type 70% of the RoV isolates. The remaining 29% were viruses that yielded negative results in both consecutively-applied PCRs; this may be due to the pronounced variability of the RoV genome described by others, rendering primer hybridization difficult [Iturriza-Gomara et al., 2000]. Another likely explanation is that the sensitivity of the VP6 assay used for screening supersedes that of the VP7 assays, which amplify longer genome fragments.

NV genotyping was based on a 145-nt section of the polymerase gene. Based on this (and other) genome regions, two genogroups (GGI and GGI) comprising at least 15 genotypes have so far been distinguished [Ando et al., 2000; Vinje and Koopmans, 2000; Vinje et al., 2000]. Our investigations revealed a clear preponderance of GGI strains. This corresponds with the majority of previous reports identifying GGI strains as more prevalent than GGI strains in sporadic and epidemic gastroenteritis [Levett et al., 1996; Schreier et al., 2000; Buesa et al., 2002; Chikhi-Brachet et al., 2002]. The reason for this is unclear; studies on possible differences in biological properties, such as virulence, transmission routes, or stability of the virus in the environment might provide explanations.

The majority of NV isolates in phylogenetic analysis would cluster with NV type Tarragona, which has been isolated from gastroenteritis episodes and outbreaks among Spanish children [Buesa et al., 2002]. One third of the isolates were genetically similar to the Grimsby prototype, the strain isolated predominantly in the United States and the United Kingdom during one or more winter seasons in the 1990s [Hale et al., 2000] and dominating adult outbreaks in Germany in 2000–2002 (data not shown). This indicates that NV type distribution in the Berlin pediatric population reflects the molecular epidemiological pattern on a wider geographical scale and in different age groups. Hale et al. have suggested that the predominance of one NV type indicates its recent introduction into an epidemiologically naive population; they predicted that as immunity to Grimsby-like virus increases, the strain would be superseded by antigenetically distinct NVs [Noel et al., 1999; Hale et al., 2000]. On the assumption that this hypothesis is true, it is possible, based on our results, that in the near future, the Tarragona-like virus will replace the Grimsby-like virus as the predominating NV type in Europe.

PCR systems have been designed for the general detection of AdVs as well as for the selective detection of the enteric types [Allard et al., 1992; Avellan et al., 2001]. In the context of this study, a relatively universal AdV PCR was established, allowing the detection particularly of enteropathogenic but also of other AdV serotypes. The differentiation of AdV-F from all other AdV that may be present in feces was done by phylogenetic analysis over a section of the hexon gene, the high inference quality of which produces a phylogeny compatible with subgenus classification [Bailey and Mountner, 1994]. While AdV diagnosis is routinely done by specific EIAs, our approach was comparable in sensitivity to the diagnostic methods used to detect the other virus families regarded in this study; additionally, it would provide information on non-enteric AdVs circulating in Germany. Concerning the uncategorized isolate, further sequence information is needed for classification.

The five AstV isolates found in the context of this study underwent comparison with database reference sequences over a short region located near the 5' end of ORF 2, for which a firm correlation of geno-/serotype has been established [Noel et al., 1995; Monroe et al., 2001]. Their classification as geno-/serotype 1 corresponds with numerous studies observing a preponderance of this genotype in Europe and worldwide [Koopmans et al., 1998; Mustafa et al., 2000; Schulz et al., 2000; Oh and Schreier, 2001]. However, a continuous observation of AstV type distribution in Germany is desirable, as sample size was small and AstV type predominance may vary depending on the region studied [Walter et al., 2001].

In conclusion, our study has demonstrated a high prevalence of enteropathogenic viruses in the stools of German children suffering from acute gastroenteritis, with a considerable proportion of coinfections. Even though the PCR methods used in this study may be unsuitable in clinical routine diagnosis, they offer the unique opportunity to perform molecular characterization of the viruses isolated. The finding of diverse NV types and particularly the detection of G9 RoV, which have been increasingly reported from different countries, stress the need for enhanced surveillance of gastroenteritis agents with more active characterization of the virus strains isolated.

ACKNOWLEDGMENTS

We thank the Charité Children’s Hospital staff and Gabriele Sinn (Gesundheitsamt Berlin-Charlottenburg/ Wilmersdorf) for providing clinical and control specimens used to conduct this study. We are grateful to Jan Vinje (University of North Carolina) and Harry Vennema and Marion Koopmans (RIVM, Bilthoven, NL) for providing NV reference sequences. We thank J.C. Heidrun Roeske, Kathrin Stanossek, Ute Pätzold, Siegfried Poculi, and Horst Emmel for excellent technical assistance and Stefan Mertens for support on graphics design.


Molecular Epidemiology of Caliciviruses Causing Outbreaks and Sporadic Cases of Acute Gastroenteritis in Spain

J. Buesa,1* B. Collado,1 P. López-Andújar,1 R. Abu-Mallouh,1 J. Rodríguez Díaz,1 A. García Díaz,1 J. Prat,2 S. Guix,3 T. Llovet,4 G. Prats,4 and A. Bosch3

Department of Microbiology, School of Medicine, Hospital Clínico Universitario, University of Valencia, 46010 Valencia,1 Laboratory of Microbiology, Hospital de Sagunto, 46500 Valencia,2 Department of Microbiology, School of Biology, University of Barcelona, 08028 Barcelona,3 and Department of Microbiology, Hospital Santa Creu i Sant Pau, Universitat Autònoma de Barcelona, 08025 Barcelona,4 Spain

Received 25 March 2002/Returned for modification 3 May 2002/Accepted 24 May 2002

The molecular epidemiology of human caliciviruses (HuCVs) causing sporadic cases and outbreaks of acute gastroenteritis around eastern Spain (Catalonia and the Valencian Community) was studied by reverse transcription-PCR (RT-PCR) and by sequencing part of the RNA polymerase gene in open reading frame 1. HuCVs were detected in 44 of 310 stool specimens (14.19%) negative for other enteric pathogens obtained from children with acute gastroenteritis. Norwalk-like viruses (NLVs) were the most common cause of the gastroenteritis outbreaks investigated here. They were detected in 14 out of 25 (56%) outbreaks with an identified pathogen. Genotypes producing both sporadic cases and outbreaks were diverse, with a predominance of GGII strains related to genotypes Melksham and Lordsdale. Five strains clustered with a “new variant” designated GGIIb, which was detected circulating throughout quite a few European countries in the years 2000 and 2001. The emergence mechanism of these strains might be the occurrence of intertypic recombinations between different viruses. The nucleotide sequence part of the capsid gene (ORF2) from three of these strains demonstrated their relationship with Mexico virus.

Human caliciviruses (HuCVs) are considered the most common cause of nonbacterial gastroenteritis outbreaks in persons of all ages worldwide (14, 17). These viruses can phylogenetically be divided into two genera, Norwalk-like viruses (NLVs), with genogroups I and II, and Sapporo-like viruses (SLVs), a group of typical human caliciviruses with distinctive morphology by electron microscopy. NLVs are the viruses most commonly associated with food- and waterborne outbreaks of gastroenteritis (9, 22). The course of the disease is usually mild and self-limiting, but the viruses are highly infectious and gastroenteritis outbreaks can involve small family groups or hundreds of individuals (19). Several studies have found human caliciviruses second only to rotaviruses as a cause of viral gastroenteritis in young children, and seroprevalence studies suggest that childhood infections in both developing and developed countries are common (4, 21, 30). However, their relative importance in mild or severe infantile gastroenteritis compared to other viruses has seldom been evaluated.

Molecular characterization of NLV strains are now an essential tool in our attempts to understand the epidemiology of this group of viruses. Genomic analysis of HuCVs causing outbreaks and sporadic cases of acute gastroenteritis reveals diversity, even in the RNA polymerase gene that is considered to be quite well conserved (1, 13, 37). Less sequence diversity in the polymerase gene has led to the widespread use of this genomic region as a target to detect and conduct molecular epidemiological studies of calicivirus infections (2, 3, 39, 40). According to this sequence divergence, NLVs from the two genogroups have been grouped so far into 15 clusters or genotypes and SLV into four genotypes (2, 12, 20, 37). NLV genogroup I (GGI) includes Norwalk, Southampton, Desert Shield, Queens Arms, and Winchester viruses, whereas NLV GGII includes Hawaii, Mexico, Lordsdale, Melksham, Hillingdon, and Grimsby viruses, among others (7, 25). Criteria for defining those strains belonging to the same genotype have been (i) more than 80% amino acid sequence identity in the complete capsid gene sequence and (ii) nucleotide sequence similarities of more than 85% (GGI) or 90% (GGII) when considering the polymerase gene in ORF1 (37).

The aim of this study was to investigate the role of HuCVs, both NLVs and SLVs, which cause sporadic cases of infantile diarrhea and outbreaks of acute gastroenteritis throughout eastern Spain, in particular Catalonia and the Valencian Community, and to characterize the genotypes of the calicivirus strains involved.

MATERIALS AND METHODS

Stool samples. A total of 310 fecal samples obtained from children under 5 years of age with sporadic acute gastroenteritis were submitted to the laboratory between January 2000 and December 2001. Another 91 specimens were collected from patients involved in 30 outbreaks which occurred in different places during 2001. All the samples had already been proved negative for intestinal pathogenic parasites.

Bacteriological studies. Salmonella, Shigella, Yersinia, and Campylobacter species were investigated by conventional bacterial culture procedures (10). The presence of enteropathogenic, enterotoxigenic, and enterohemorrhagic categories of diarrheagenic Escherichia coli was studied during outbreak investigations by PCR-based methods (8, 16, 31). When it was suggested by the clinical data, staphylococcal enterotoxin and Bacillus cereus enterotoxin were also investigated.

* Corresponding author. Mailing address: Departament de Microbiologia, Facultat de Medicina, Universitat de València, Avda. Blasco Ibáñez, 17, 46010 Valencia, Spain. Phone: 34 96 386 46 58. Fax: 34 96 386 41 73. E-mail: javier.buesa@uv.es.
VOL. 40, 2002 MOLECULAR EPIDEMIOLOGY OF CALICIVIRUSES IN SPAIN 2855

by enzyme immunoassays (EIs) (TECRA Diagnostics, New South Wales, Australia).

**Viral assays.** EIs for group A rotaviruses and adenoviruses 40 and 41 (Rotatrace and Adenoclonel; Meridian Diagnostics, Cincinnati, Ohio) and astroviruses (IDEA Astrovirus; Dako Diagnostics, Cambridgeshire, United Kingdom) were performed.

**RNA extraction.** Viral RNA was extracted by binding to size-fractionated silica particles (RNaid; Q-Biogene, Carlsbad, Calif.) in the presence of guanidinium (2000 to 2001).

**Hybridization of PCR products.** The DNA transferred to the membrane was cross-linked in a UV Cross-linker (Hoefer). Hybridization was performed with a set of three different NLV-specific oligonucleotide probes labeled at the 5' end with digoxigenin (Roche Molecular Biochemicals). The probes used were NVP110 (24) and SR47D and SR61D (1). Hybridization and chromogenic detection of the hybrids with nitroblue tetrazolium (NBT) and BCIP (5-bromo-4-chloro-3-indolylphosphate) were carried out according to the protocols recommended by the manufacturer.

**Nucleotide sequencing.** The 326-bp products from the RT-PCR were excised from the gel, extracted, and purified by using the Concert rapid gel extraction system (Life Technologies). Sequencing was carried out in both directions with the JV12 and JV13 primers using the ABI PRISM Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) and an automated sequencer (Applied Biosystems ABI PRISM 377).

**Phylogenetic analyses.** Prior to any phylogenetic analyses, translated amino acid sequences were aligned using CLUSTAL-X program (34) with the default parameters. Using this alignment as a template, nucleotide sequences were aligned with the DAMBE program (41). Only positions determined without uncertainty for at least 90% of the isolates were included in the following analyses. The phylogenetic content of this data set was assessed using the four-cluster likelihood-mapping method (32) as implemented in the program TREE-PUZZLE. The method is based on an analysis of the maximum likelihood for the three fully resolved tree topologies that can be computed from four taxa (individual sequences or groups of sequences). Finally, phylogenetic reconstructions used the minimum evolution and the maximum parsimony methods implemented in the MEGA2 program (23). For the minimum evolution method, the nucleotide substitution model employed was the p-distance. We used the first and second positions of the protein-coding regions because the substitutions in the third codon positions are likely to be saturated among highly diverged taxa. Gaps were removed in pairwise comparisons when we used the minimum evolution method but employed as a fifth character when the tree reconstruction method was maximum parsimony. Statistical confidence for the evolutionary trees was assessed by bootstrap (1,000 replicates). The phylogenetic tree was drawn using the MEGA2 program.

**Nucleotide sequence accession numbers.** The nucleotide sequences determined in this study have been deposited in GenBank under accession numbers AJ487474 and AJ487789 to AJ487811.

**RESULTS**

HuCVs were detected in 44 cases out of 310 cases of sporadic acute gastroenteritis in children (14.19%) (Table 1). A clear predominance of NLV GGII (33 out of 40 NLV-positive samples) was found. SLVs were detected in only four cases. Other enteric viruses detected were group A rotaviruses (25.3%), astroviruses (3.15%), and adenovirus type 40 or 41 (3.15%) (Fig. 1). The etiologic agent was identified in 25 of 30 gastroenteritis outbreaks investigated in this study (Table 2). Bacterial pathogens were detected in specimens obtained from patients involved in 11 outbreaks. *Salmonella enterica* was the bacteria most frequently found (seven outbreaks), followed by *S. aureus* (two outbreaks), *Campylobacter jejuni*, and *B. cereus* (one outbreak caused by each bacterial species). No diarrheagenic *E. coli* strains were detected.

NLVs were identified as the etiologic agents in 14 gastroen-

---

### TABLE 1. Detection of human caliciviruses by RT-PCR*

<table>
<thead>
<tr>
<th>Test group</th>
<th>No. of samples</th>
<th>No. (%) RT-PCR positive</th>
<th>No. of NLV</th>
<th>No. of GGI</th>
<th>No. of GGII</th>
<th>No. of SLV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporadic cases</td>
<td>310</td>
<td>44 (14.19)</td>
<td>40</td>
<td>7</td>
<td>33</td>
<td>4</td>
</tr>
<tr>
<td>Patients in outbreaks</td>
<td>91</td>
<td>71 (78)</td>
<td>71</td>
<td>23</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>401</td>
<td>115 (28.67)</td>
<td>111</td>
<td>30</td>
<td>81</td>
<td>4</td>
</tr>
</tbody>
</table>

* Fecal specimens from sporadic cases of infantile acute gastroenteritis and from 14 gastroenteritis outbreaks were collected from January 2000 to December 2001.
teritis outbreaks (56%). Eleven outbreaks were caused by NLV GGII strains and three were caused by NLV GGI strains (Table 3).

Some epidemiological characteristics of the calicivirus-associated outbreaks and the viral genogroups and genotypes identified as the causative agents are also shown in Table 3. The majority of these outbreaks occurred in either schools (42.8%) or nursing homes (21.4%). Genotypes detected producing both sporadic cases and outbreaks were diverse, with GGII strains belonging to genotypes Melksham (eight strains), Lordsdale (five strains), Hillingdon (one strain), Mexico (one strain), and five viral strains that clustered with a “new variant” designated GGIIb, which has been detected circulating throughout several European countries during 2000 and 2001 (M. Koopmans, personal communication).

Three of the five GGIIb strains were detected in patients involved in outbreaks (Tarragona/238/01, Suria/312/01, and Castell/217/01), whereas the remaining two strains caused sporadic cases. In order to identify these strains more accurately, the capsid gene (ORF2) from three of them was sequenced and appeared to be related to Mexico genotype (results not shown).

Viral sequences determined in samples from patients in the same outbreak were highly homogeneous. However, the sequence of the virus involved in the Barcelona outbreak could not be established because the PCR product was not pure enough, presumably due to the presence of more than one single strain.

Six NLV GGI strains could not be clearly grouped with any of the previously described genotypes based on the sequence of the polymerase gene, although two of them (Lleida/235/01 and Sagunt/338/01) were related to Norwalk and Southampton viruses.

Prior to the phylogenetic inference, we applied the four-cluster likelihood-mapping method, which strongly confirmed the tree-likeness of the data set (81.8%) (i.e., the likelihood of the data set generating a true phylogenetic tree). Because a tree-likeness value of well above 50% can apparently be trusted (33), we may conclude that despite the high heterogeneity observed among GGI and GGII, the quality of the data still allows us to estimate the relationship between all calicivirus isolates.

Figure 2 shows a phylogenetic tree of sequences of a 326-nucleotide region of the RNA polymerase (ORF1) of 27 NLV strains isolated in Spain during 2000 to 2001 and 14 reference strains available either in GenBank or in the database of the European Union-funded project “Foodborne viruses in Europe.” Information about this database is available upon request. The GenBank accession numbers for the calicivirus reference strains are as follows: Lordsdale, x86557; Hawaii, HCU07611; Melksham, x81879; Hillingdon, AB 020558; Norwalk, M87661; Southampton, L07418; and Desert Shield, DSU04469.

Our results confirm a significant incidence of calicivirus infections, mainly NLV, among young children, causing gastroenteritis which needs medical attention. Only rotaviruses were found more frequently (25.3%) than calicviruses. Mixed viral or viral-bacterial infections were not investigated in our study, and this may reduce to some extent the final figures of prevalent pathogens.

**DISCUSSION**

Molecular detection of HuCVs in stool specimens has become a more common diagnostic procedure of acute gastroenteritis in clinical laboratories (3). The application of PCR-

<table>
<thead>
<tr>
<th>Location</th>
<th>No. of cases</th>
<th>Setting</th>
<th>No. of samples</th>
<th>No. of positives</th>
<th>Genogroup</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castell (Alicante)</td>
<td>20</td>
<td>Rural village</td>
<td>4</td>
<td>4</td>
<td>NLV GGII</td>
<td>GGIIb Mexico</td>
</tr>
<tr>
<td>Meliana (Valencia)</td>
<td>32</td>
<td>School</td>
<td>3</td>
<td>2</td>
<td>NLV GGII</td>
<td></td>
</tr>
<tr>
<td>La Bisbal (Tarragona)</td>
<td>14</td>
<td>Nursing home</td>
<td>4</td>
<td>3</td>
<td>NLV GGII</td>
<td>GGIIb Mexico</td>
</tr>
<tr>
<td>Figueres (Girona)</td>
<td>25</td>
<td>Nursing home</td>
<td>4</td>
<td>3</td>
<td>NLV GGI</td>
<td>ND*</td>
</tr>
<tr>
<td>Lleida (Lleida)</td>
<td>6</td>
<td>Catering service</td>
<td>6</td>
<td>3</td>
<td>NLV GGI</td>
<td>ND</td>
</tr>
<tr>
<td>Sant Feliu (Girona)</td>
<td>21</td>
<td>Vacation camp</td>
<td>3</td>
<td>3</td>
<td>NLV GGII</td>
<td>Melksham</td>
</tr>
<tr>
<td>Vilafranca (Barcelona)</td>
<td>17</td>
<td>School</td>
<td>2</td>
<td>1</td>
<td>NLV GGII</td>
<td>Melksham</td>
</tr>
<tr>
<td>Ullastrell (Barcelona)</td>
<td>37</td>
<td>School</td>
<td>21</td>
<td>17</td>
<td>NLV GGI</td>
<td>ND</td>
</tr>
<tr>
<td>Montblanc (Tarragona)</td>
<td>34</td>
<td>Nursing home</td>
<td>4</td>
<td>3</td>
<td>NLV GGI</td>
<td>Lordsdale</td>
</tr>
<tr>
<td>Suria (Barcelona)</td>
<td>75</td>
<td>School</td>
<td>15</td>
<td>15</td>
<td>NLV GGII</td>
<td>GGIIb</td>
</tr>
<tr>
<td>Les Borges (Lleida)</td>
<td>96</td>
<td>School</td>
<td>6</td>
<td>5</td>
<td>NLV GGII</td>
<td>Melksham</td>
</tr>
<tr>
<td>Esteni d’Aneu (Lleida)</td>
<td>7</td>
<td>Rural village</td>
<td>9</td>
<td>6</td>
<td>NLV GGII</td>
<td>Melksham</td>
</tr>
<tr>
<td>Util (Valencia)</td>
<td>7</td>
<td>Private home</td>
<td>2</td>
<td>1</td>
<td>NLV GGII</td>
<td>Lordsdale</td>
</tr>
<tr>
<td>Barcelona (Barcelona)</td>
<td>80</td>
<td>School</td>
<td>8</td>
<td>5</td>
<td>NLV GGI</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, not determined.
based methods to screen stool specimens has not only shown that the overwhelming majority of outbreaks of acute gastroenteritis are attributable to NLVs but also that HuCVs also cause numerous cases of sporadic gastroenteritis (35, 39).

Our results show that human caliciviruses, mainly GGII of the NLVs, are indeed a major cause of both outbreaks and sporadic cases of acute gastroenteritis in infants and young children in eastern Spain. The prevalence rate detected...
(14.19%) is similar to that reported by other authors, 14% in Dijon, France (4), 9% in Melbourne, Australia (21), and 6.5% in the United Kingdom (35).

To our knowledge, this is the first Spanish study in which genogroups and genotypes of Norwalk-like viruses have been investigated as etiologic agents of sporadic gastroenteritis cases in children and in gastroenteritis outbreaks. We found that among viral pathogens rotaviruses are the most frequent agents causing sporadic gastroenteritis cases (25.3%), followed by HuCVs (14.19%). In previous studies, we characterized the genotypes of group A rotavirus and the serotypes of astrovirus detected in our geographical area (6, 15) as well as the presence of astroviruses in wastewater (28). Our study confirms that NLVs are the main cause of gastroenteritis outbreaks in Spain, as in other EU countries (4, 26, 29, 36). Interestingly, although it is well known that group A rotaviruses are the primary cause of sporadic cases of gastroenteritis in children, they are not frequently found as the causative agents of gastroenteritis outbreaks.

The predominance of NLV GGI strains during this period of study agrees with previous reports of a higher prevalence of GGI strains than of GGI (4, 21). A notable difference has also been found in antibody acquisition to Mexico virus (GGII), to which over 70% of children older than 2 years are seropositive, whereas only 12% of these children had been infected with Norwalk virus (GGI) (27). Molecular epidemiological studies of outbreaks have revealed that GGI strains dominate GGI strains (11, 39). The reason for this is unknown, although differences in biological properties, such as virulence, routes of transmission, or stability of the virus in the environment, are possible explanations.

The alignments of the RNA polymerase nucleotide sequences of the seven GGI strains isolated in this study with reference strains of the accepted GGI genotypes showed similarities of less than 85%. This, together with the results of the reference strains of the accepted GGI genotypes showed similarities of less than 85%. This, together with the results of the phylogenetic analysis, suggests that some of these isolates might represent new variants of GGI or even new genotypes. This possibility awaits confirmation by capsid gene sequence analysis.

In our study, SLV strains were rarely detected (only four cases [1.29%]) as a cause of severe gastroenteritis in young children, perhaps reflecting the idea that SLVs cause less severe symptoms than NLVs. Similar results were obtained by Kirkwood and Bishop in Melbourne, Australia (21), who also consider that SLV-associated gastroenteritis may be clinically milder, not requiring hospitalization.

The number of gastroenteritis outbreaks included in this study does not reflect the real figure of outbreaks which occurred during the period of study, because many of them are not reported to the Regional Health Services. Even so, not all outbreaks are tested for a viral etiology and, consequently, calicivirus infections are underdiagnosed.

Strains isolated from particular outbreaks where highly homogeneous and no sequence differences among specimens investigated within one outbreak were found. However, in some specimens, a mixture of sequences was detected, both from patients involved in an outbreak and from sporadic cases of infantile diarrhea. This finding was presumably due to mixed infections with more than one single viral strain. We have not detected any remarkable difference in the viral genotypes producing outbreaks or sporadic cases in the community. It has been suggested that there are two distinct epidemiological patterns of NLV strains, one producing endemic infections (i.e., Grimsby virus strains considered endemic in the United Kingdom) and another causing epidemic infections (Norwalk and Mexico viruses) (17). Our data do not support such hypotheses, although the number of strains sequenced is limited. The same genotypes were found causing both sporadic cases and gastroenteritis outbreaks in 2000 to 2001, and it seems that multiple viral genotypes are circulating at any given time. Continued NLV surveillance across Europe and investigations of the biological differences between strains are needed in order to clarify their molecular epidemiology.

The genetic relatedness of caliciviruses does not always correspond when regions in different open reading frames are sequenced, this may originate by recombinations in the evolution of caliciviruses (29, 36). The detection of differences in phylogenetic tree topology between different parts of the genome, like the RNA polymerase and the capsid genes, suggests the existence of the recombination process among HuCVs (18). To identify recombinants more clearly, one must compare the sequence of different regions of the genome against a panel of well-characterized representative sequences in a database, such as the one created by the EU-funded project on “Food-borne viruses in Europe” to search for matching strains.

Capsid gene sequence analysis can be used to verify the viral genotyping based on the sequence of the polymerase gene fragment produced by the diagnostic PCR. There is a correlation between antigenic grouping based on the use of virus-like particles generated using baculovirus expression systems and genomic grouping based on capsid amino acid sequences (12, 18). Further characterization of the genetic and antigenic relationships will help the classification of human caliciviruses.

Understanding the real prevalence of these viruses, the mechanisms that lead to the emergence of new viral variants and how they spread within populations will improve the prevention of the diarrheal diseases that they cause.

ACKNOWLEDGMENTS

This study was supported by the EU grant QLK1-CT-00594 (EU 5th Framework Program “Quality of life and management of living resources”).

We thank Marion Koopmans, Jan Vinje, and Harry Vennema (RIVM, National Institute of Public Health and the Environment, Bilthoven, The Netherlands) for providing reference strains and viral sequences and Santiago F. Elena for assistance with phylogenetic analyses.

REFERENCES


Group A Rotavirus in Sewage Samples from Barcelona and Cairo: Emergence of Unusual Genotypes

Cristina Villena, Waled Morys El-Senousy, F. Xavier Abad, Rosa M. Pintó, and Albert Bosch*

Enteric Virus Group, Department of Microbiology, University of Barcelona, Barcelona, Spain

Received 29 October 2002/Accepted 31 March 2003

The presence of rotavirus strains in sewage samples from Cairo, Egypt (November 1998 to October 1999), and Barcelona, Spain (November 1998 to December 2002), was investigated by using a generic molecular detection method based on amplification of a VP6 gene fragment. Overall, 85.7 and 66.9% of the sewage samples from Cairo and Barcelona, respectively, were positive. Positive samples were characterized further, and VP7 and VP4 genotypes were determined. Although 30% of the positive samples from Cairo were G untypeable, the distribution of G types in the positive samples was 69.6% G1, 13% G3, 8.7% G4, and 8.7% G9. The percentage of untypeable samples was much higher for the Barcelona samples (56.5%), and the distribution in the positive samples was 56.4% G1, 31.5% G3, 6% G9, 4% G2, and 2% G5. When the P types were examined, 26.7% of the positive samples from Cairo were untypeable, and the distribution of types in the positive samples was 53.3% P[8], 30% P[6], and 16.6% P[4]. In Barcelona, 27.2% of the samples were P untypeable, and the frequencies of the types detected were 49.7% P[8], 37.2% P[4], 8.8% P[6], and 4.2% P[9]. The distribution for strains from Cairo was 38.5% P[8]G1, 27% P[6]G1, 11.5% P[4]G1, 11.5% P[8]G3, 7.7% P[6]G4, and 3.8% P[8]G9. Strikingly, equivalent frequencies of common and uncommon strains were observed for Barcelona samples, and the distribution was 38.8% P[8]G1, 30.6% P[4]G1, 11.6% P[8]G3, 6.6% P[4]G3, 5.8% P[6]G1, 1.6% P[6]G3, 1.6% P[9]G1, 0.8% P[4]G2, 0.8% P[6]G9, 0.8% P[8]G9, and 0.8% P[8]G5. Additionally, two P[−]G5 strains were isolated in Barcelona, and the porcine or human origin of these strains was unclear. Rotavirus variability exhibited not only a geographic pattern but also a temporal pattern.

MATERIALS AND METHODS

Sewage samples. Raw sewage from three sewage treatment plants (Balaks, Zenin, and El Berka) in Cairo, Egypt, was sampled monthly from November 1998 to October 1999 (n = 35). Raw sewage from the Sant Adrià del Besòs sewage treatment plant in Barcelona, Spain, was sampled during a 4-year period. From November 1998 to October 1999 a mean of 10 samples per month were obtained (n = 125), from November 1999 to May 2000 a mean of 25 samples per month were analyzed (n = 174), and from November 2000 to October 2001 and from November 2001 to December 2002 a mean of two samples per month were obtained (n = 30 and n = 28, respectively).

Different concentration procedures were used in Cairo and Barcelona. In the first case, viruses from 3 liters of sewage were concentrated in 75 ml of 50 mM glycine buffer with 3% beef extract (Oxoid) by adsorption-elution to negatively charged nitrocellulose membranes (Schleicher and Schuell) (31) and were re-concentrated by organic flocculation in 1 ml of 0.14 N NaH₂PO₄, pH 7 (25). Lipoxyphilization was employed to concentrate raw sewage in Barcelona. Samples were concentrated by freeze-drying 50 ml and resuspending the dried material in 500 μl of distilled water (13, 28).

Rotavirus detection. RNA was purified from 50-μl portions of concentrated sewage samples by guanidine thiocyanate extraction, as previously described (5).

For genetic detection of rotaviruses, a reverse transcription (RT)-PCR-hybridization method based on amplification of a VP6 gene fragment and confirmation by Southern blot hybridization with a digoxigenin-labeled internal probe was used. Primers VP6-3 (5′-GCTTTAAAACGAAGTCTTCAACG-3′; positions 2 to 23 of human strain Wa [accession number K02086]) and VP6-4 (5′-GCTTTAAAACGAAGTCTTCAACG-3′; positions 187 to 166 of human strain Wa [accession number K02086]), each at a concentration of 1 μM, were used in an RT reaction in a 10-μl (final volume) mixture containing 4 U of Moloney murine leukemia virus enzyme (Promega), each deoxynucleoside triphosphate at a concentration of 0.2 mM, and 5 μl of a denatured (5 min at 90°C) double-stranded RNA sample. The reaction mixture was incubated for 60 min at 50°C. Five microliters of the RT product was processed by PCR by using 3.5 U of the Expand High Fidelity PCR system (Roche) in a 50-μl (final volume) mixture...
supplemented with each primer at a concentration of 1 μM and each deoxynucleoside triphosphate at a concentration of 2 mM. The PCR program included a 9-min denaturation step at 95°C and 40 cycles of amplification for 1 min at 94°C, for 1 min at 50°C, and for 1 min at 72°C, followed by a final elongation step of 7 min at 72°C. In order to confirm the rotaviral nature of the 186-bp amplimer, Southern blot hybridization with a digoxigenin-labeled probe (5'-CAAAATGAGTGTTACTAGAATGTTG-3'; positions 129 to 151 of human strain Wa [accession number K02866]) was performed.

**Rotavirus typing.** VP6-positive samples were analyzed further, and the G type and P type were determined by using the methods described by Gouvea et al. (16) and Gentsch et al. (14), respectively. The cocktail of primers used in the multiplex reaction allowed determination of the G1, G2, G3, G4, G5, G8, and G9 VP7 types (16, 19) and the P[4], P[6], P[8], and P[9] VP4 types (14). Additionally, a monoplex reaction for G2 typing was performed with the samples that were P[4] positive and G2 negative as determined by the multiplex nested PCR. Two separate monoplex nested PCR G2 typing reactions, either with the RVG9 and aCT2 primers (16) or with the 9 Con 1 and 9T1-2 primers (10), were used. On the other hand, a monoplex nested PCR with the P[8]-specific primer 1T-1D was performed with all the P[8]-negative samples (22).

**TABLE 1.** Distribution of rotavirus G types in sewage samples from Cairo and Barcelona

<table>
<thead>
<tr>
<th>City</th>
<th>Sampling dates</th>
<th>G1</th>
<th>G2a</th>
<th>G2b</th>
<th>G2 total</th>
<th>G3</th>
<th>G4</th>
<th>G5</th>
<th>G8</th>
<th>G9</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cairo</td>
<td>Nov. 1998–Oct. 1999</td>
<td>16 (69.6)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>3 (13.0)</td>
<td>2 (8.7)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>2 (8.7)</td>
<td>23 (100.0)</td>
</tr>
<tr>
<td>Barcelona</td>
<td>Nov. 1998–Oct. 1999</td>
<td>7 (38.9)</td>
<td>0 (0.0)</td>
<td>2 (11.1)</td>
<td>2 (11.1)</td>
<td>8 (44.4)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>1 (5.5)</td>
<td>18 (99.9)</td>
</tr>
<tr>
<td>Nov. 1999–May 2000</td>
<td>59 (62.1)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>30 (31.6)</td>
<td>0 (0.0)</td>
<td>2 (2.1)</td>
<td>0 (0.0)</td>
<td>4 (4.2)</td>
<td>95 (100.0)</td>
<td></td>
</tr>
<tr>
<td>Nov. 2000–Oct. 2001</td>
<td>15 (55.5)</td>
<td>0 (0.0)</td>
<td>4 (14.8)</td>
<td>4 (14.8)</td>
<td>7 (25.9)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>1 (3.7)</td>
<td>27 (99.9)</td>
</tr>
<tr>
<td>Nov. 2001–Dec. 2002</td>
<td>3 (33.3)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>2 (22.2)</td>
<td>0 (0.0)</td>
<td>1 (11.1)</td>
<td>0 (0.0)</td>
<td>3 (33.3)</td>
<td>9 (99.9)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>84 (56.4)</td>
<td>0 (0.0)</td>
<td>6 (4.0)</td>
<td>6 (4.0)</td>
<td>47 (31.5)</td>
<td>0 (0.0)</td>
<td>3 (2.0)</td>
<td>0 (0.0)</td>
<td>9 (6.0)</td>
<td>149 (99.9)</td>
<td></td>
</tr>
</tbody>
</table>

| a | All samples were tested with primers RVG9 and aCT2. | b | All samples that were P[4] positive and G2 negative with primers RVG9 and aCT2 were also tested with primers 9 Con 1 and 9T1-2. | c | All samples were tested with primers RVG9 and aCT2. | d | All samples that were P[8] positive and G2 negative as determined by the multiplex nested PCR. Two separate monoplex nested PCR G2 typing reactions, either with the RVG9 and aCT2 primers (16) or with the 9 Con 1 and 9T1-2 primers (10), were used. On the other hand, a monoplex nested PCR with the P[8]-specific primer 1T-1D was performed with all the P[8]-negative samples (22). Several samples were specifically analyzed for the presence of the porcine P[6] (P2B), P[7], and P[13] genotypes. The P[6] (P2B) and P[7] genotypes were detected by using the procedure described by Gouvea et al. (16), while the P[13] genotype was detected by using the specific primer 501-P[13] (5'-CCTCCRTATTTCAACGCCTCAGT-3'; positions 524 to 501 of the Po/A46 strain [accession number AY050274]) and the RVG9 common primer described by Gouvea et al. (16). Positive controls for the following human reference strains were used: Wa (G1P[11]), VA70 (G4P[8]), and DS-1 (G2P[4]). The OSU strain (G5P[7]) was used as a control for porcine viruses.

**Sequencing.** The sequences of the amplimers generated in the nested G typing reaction were determined for 100 and 60% of the G5 and G2 samples, respectively. The sequences of the nested products of the P typing reaction for 15% of the P[4] samples and 7% of the P[8] samples were also determined. Portions (40 μl) of the nested PCR products were electrophoresed on a 1% agarose gel, and the DNA was purified with a High Pure PCR product purification kit (Roche) used according to the manufacturer’s instructions. The nucleotide sequences were determined by using 2 to 7 μl of the purified DNA with an ABI PRISM Big Dye terminator cycle sequencing Ready Reaction kit (version 2.0; Perkin-Elmer) and an ABI Prism 7700 automated DNA sequencer (Perkin-Elmer). Sequences of both chains were used for genetic analysis of the fragments mentioned above.

**Phylogenetic analysis of the G5 sequences.** Each nucleotide sequence was compared to the sequences of reference strains by using the BLAST program (National Center for Biotechnology Information). Multiple alignments of nucleotide sequences were constructed by using the CLUSTALW program (European Bioinformatics Institute). Nucleotide distance matrices were calculated by the pairwise distance method using the Molecular Evolutionary Genetics Analysis 2.1 software (MEGA, version 2.1) (26). Phylogenetic relationships were analyzed with and without bootstrap analysis of 100 replicates.

**RESULTS**

**Rotavirus detection.** Overall, 30 (85.7%) of 35 sewage samples from Greater Cairo were positive for rotavirus, as determined by a generic VP6 detection method. In Barcelona, 239 (66.9%) of 357 samples were positive. Negative samples were randomly distributed throughout the year in both studies.

**Rotavirus G typing.** A total of 21 of 30 Egyptian rotavirus-positive samples could be G typed, so 30% of the samples were untypeable. Overall, the most frequent type was G1 (69.6%), followed by G3 (13.0%), G4 (8.7%), and G9 (8.7%) (Table 1). The percentage of samples containing more than one type was 9.5%.

In the case of the Spanish samples, only 104 of 239 samples could be typed, and thus the percentage of untypeable samples was much higher (56.4%). The most frequent type again was G1 (56.4%), followed by G3 (31.5%), G9 (6.0%), G2 (4.0%), and G5 (2.0%) (Table 1). Since the G2 type was detected only by using primers 9 Con 1 and 9T2-1, the sequence of 60% of the amplimers, randomly chosen, was used to confirm the actual G2 nature. In this analysis 33.9% of the positive samples contained mixed types.

**Rotavirus P typing.** Overall, 22 of 30 rotavirus-containing samples from Cairo could be P typed, and thus the percentage of untypeable samples was 26.7%. The frequencies of P types were as follows: P[8], 53.2%; P[6], 30.0%; and P[4], 16.6% (Table 2). Mixed types were detected in 31.8% of the sewage samples.

A total of 174 of 239 Spanish samples could be P typed, and...
More than one P type was detected in 64.9% of the samples. A family of primers (14, 16) was used to screen the amplified DNA sequences of rotavirus strains. Furthermore, since most of the P[4]-positive samples unexpectedly did not contain G2 sequences, we randomly chose the sequence of 15% of the P[4] amplimers, which could contain several strains from different individuals, only the samples containing either a single G or a single P type were used for evaluation of potential strains and P-typed samples (71.5%). The most frequent combination found in Cairo was P[8]G1 (38.5%), followed by P[6]G1 (27.0%), P[4]G1 (11.5%), P[8]G3 (11.5%), P[6]G4 (7.7%), and P[8]G9 (3.8%) (Table 2).

The distribution of strains in the Barcelona area was 38.8% P[8]G1, 30.6% P[4]G1, 11.6% P[8]G3, 6.6% P[4]G3, 5.8% P[6]G1, 1.6% P[9]G1, 1.6% P[6]G3, 0.8% P[4]G2, 0.8% P[8]G9, 0.8% P[6]G9, and 0.8% P[8]G5 (Table 3). Interestingly, three G5-containing samples were detected. One of these samples was associated with a P[8] type, while the other two could not be associated with any P type. To rule out the possibility that the latter two strains could be of porcine origin, specific typing reactions with primers for the P[6] (P2B), P[7], and P[13] genotypes were performed, and all of the assayed samples were again negative. To further analyze these strains, a phylogenetic analysis was performed to compare the sequences of these G5 Barcelona isolates with the G5 sequences available in the GenBank database. The closest strains were OSU and Po/A34, both of porcine origin.

Thus, the percentage of untypeable samples was 27.2%. The distribution of the P types found was as follows: P[8], 49.7%; P[4], 37.2%; P[6], 8.8%; and P[9], 4.2% (Table 2). In a first screening performed with primers Con 3 and 1T-1, the P[8] type was detected at a low frequency (14.4%). However, the frequency of this type significantly increased when primers Con 3 and 1T-1D were used. Several (7%) of the additional P[8] strains were confirmed by sequencing. Furthermore, since most of the P[4]-positive samples unexpectedly did not contain G2 types (see below), the sequence of 15% of the P[4] amplimers, randomly chosen, was used to confirm the actual P[4] nature. More than one P type was detected in 64.9% of the samples.

### Table 3. Distribution of common and uncommon rotavirus strains in different countries and in Cairo and Barcelona

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Common strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P[8]G1</td>
<td>76</td>
<td>15</td>
<td>29</td>
<td>12/23</td>
<td>50</td>
<td>43</td>
<td>38.5</td>
<td>38.8</td>
</tr>
<tr>
<td>P[8]G3</td>
<td>2</td>
<td>0</td>
<td>13</td>
<td>0/0.2</td>
<td>0</td>
<td>2</td>
<td>11.5</td>
<td>11.6</td>
</tr>
<tr>
<td>P[8]G4</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>4/11</td>
<td>0</td>
<td>32</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P[4]G2</td>
<td>11</td>
<td>22</td>
<td>31</td>
<td>43/30</td>
<td>8</td>
<td>6</td>
<td>0</td>
<td>0.8</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>43</td>
<td>75</td>
<td>59/64</td>
<td>58</td>
<td>83</td>
<td>50</td>
<td>51.2</td>
</tr>
<tr>
<td>Uncommon strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P[6]G1</td>
<td>0.5</td>
<td>0</td>
<td>2.4</td>
<td>—/—1.4†</td>
<td>0</td>
<td>0</td>
<td>27</td>
<td>5.8</td>
</tr>
<tr>
<td>P[4]G1</td>
<td>0.1</td>
<td>0</td>
<td>4.1</td>
<td>14/2.7</td>
<td>21</td>
<td>0</td>
<td>11.5</td>
<td>30.6</td>
</tr>
<tr>
<td>P[6]G4</td>
<td>—</td>
<td>0.7</td>
<td>2.8</td>
<td>—/—0.2</td>
<td>21</td>
<td>0</td>
<td>7.7</td>
<td>0</td>
</tr>
<tr>
<td>P[8]G9</td>
<td>1</td>
<td>5</td>
<td>0.3</td>
<td>—/—0</td>
<td>0</td>
<td>—</td>
<td>3.8</td>
<td>0.8</td>
</tr>
<tr>
<td>P[4]G3</td>
<td>—</td>
<td>0</td>
<td>4.1</td>
<td>0/—</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6.6</td>
</tr>
<tr>
<td>P[9]G1</td>
<td>—</td>
<td>—</td>
<td>2.8</td>
<td>—/—</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.6</td>
</tr>
<tr>
<td>P[6]G3</td>
<td>—</td>
<td>0.7</td>
<td>1.7</td>
<td>—/—</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.6</td>
</tr>
<tr>
<td>P[9]G3</td>
<td>0.2</td>
<td>—</td>
<td>0</td>
<td>—/—</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.8</td>
</tr>
<tr>
<td>P[6]G9</td>
<td>3.2</td>
<td>9</td>
<td>3.8</td>
<td>——/—0.4</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>0.8</td>
</tr>
<tr>
<td>P[8]G5</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—/—0.4</td>
<td>—</td>
<td>—</td>
<td>0</td>
<td>0.8</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
<td>15.4</td>
<td>24</td>
<td>14/5.1</td>
<td>42</td>
<td>0</td>
<td>50</td>
<td>48.6</td>
</tr>
</tbody>
</table>

* Data from reference 20.
† Data from reference 23.
‡ Data from reference 32.
§ Data from reference 3/data from reference 4.
¶ Data from reference 32.
† Data from reference 7.
⁎ Data from reference 7.
→ no data available.

**DISCUSSION**

In this work, the presence of rotavirus strains in sewage samples from Cairo and Barcelona was investigated. Although a higher percentage of positive samples was obtained for the samples from Cairo than for the samples from Barcelona, negative samples were randomly distributed throughout the year in both areas, which corroborated previous data for Barcelona raw sewage, in which seasonal distribution of rotavirus could not be detected (6), and clinical data for Cairo, in which reduced levels of detection were obtained both in winter months (January and February) and in a summer month (July) (29).

Although serotyping is a classification method based on neutralization of virus infectivity, the available information for gene sequences of rotavirus strains allows prediction of the serotype of a given strain by PCR with type-specific primers (14, 16).

When G typing was used, a significant proportion of the rotavirus-positive samples could not be characterized. In the case of samples from Cairo, the percentage of untypeable samples is consistent with previously published data (29), which showed that 38.7% of stool samples collected from August
1992 to October 1993 were untypeable with an enzyme immunoassay covering serotypes G1 to G4. The overall distribution of rotavirus G types in Cairo is similar to the distributions reported in other parts of the world. However, the results presented here reveal the occurrence of G9 strains in Egypt for the first time, although this does not necessarily imply emergence of new strains, since this serotype was not investigated in previous studies (21, 29). The absence of G2 strains in sewage from Cairo could be the result of either a lack of G2 strains circulating in the population or, more likely, low-level circulation of such strains, as has been reported previously (29).

Another interesting observation was the shift from G4 strains to G1 strains; in a study performed in 1992 and 1993 (29) these two serotypes were found at equal frequencies, but in the present survey, performed in 1998 and 1999, G1 was detected at a significantly higher frequency.

The number of untypeable strains in sewage from Barcelona was significantly higher than the number of untypeable strains in samples from Cairo. Whether this was the result of technical problems associated with the concentration of RT-PCR inhibitors in the freeze-dried samples or reflected the actual occurrence of untypeable strains is not known. However, in a previous study (13) conducted to evaluate different methods for removing inhibitors, lysis followed by PCR appeared to be a satisfactory procedure. Additionally, it should be kept in mind that all the samples assayed were previously selected as VP6-positive samples by RT-PCR and Southern blotting. However, the procedure involved a different RT-PCR, performed with different primers and amplifying a much shorter fragment. A more plausible explanation may be the level of rotavirus in sewage. The proportions of samples that were determined to be positive only after Southern blot hybridization (no gel band corresponding to the amplimer was visualized) were 3% in Cairo and 39% in Barcelona. Rotavirus typing was difficult in these samples having very low virus contents.

The lack of G8 and G4 strains in sewage from Barcelona was surprising, since the G4 type appeared to be one of the most common types in previous studies conducted in Barcelona (October 1996 to October 1997) (33) and Valencia (September 1996 to May 1999) (7). However, in the latter study, the G4 frequency decreased during the period studied, and the prevalence of this type at the conclusion date, just when our sampling started, was significantly lower. The occurrence of this type as the second most common type is also remarkable, since similar findings have been described previously only for the People’s Republic of China (12).

From a technical point of view, it should be noted that the G2 type was not detected, either in a multiplex or a monoplex nested reaction, by using the RVG9-aCT2 pair of primers. In contrast, when primers 9 Con 1 and 9T1-2 were used in a monoplex nested reaction, several samples were G2 positive.

The present study provided the first available information on the distribution of P types in Egypt, and the data are similar to those found in other countries, such as Tunisia (32) and India (23). There was a strikingly high frequency of P[6], which was not detected either in Spain (7) or in Ireland (27), and only reduced frequencies of P[6] were detected in the United States (20) and the People’s Republic of China (12).

The P-type data for Barcelona sewage isolates suggest that there was a shift from P[8] to mainly P[4] and also to P[6] and P[9] in Spain, since Buesa and coworkers (7) described the occurrence of 82% P[8] strains, 5.5% P[4] strains, and 12.4% untypeable strains in Valencia, which is located 300 km south of Barcelona, and Wilhemi and colleagues (33) described the occurrence of 100% P[8] strains in a rural area located 100 km north of Barcelona. High levels of the P[4] genotype have also been described for the People’s Republic of China (12) and India (23); in the latter country there was also a high level of the P[6] genotype.

Interestingly, completely different epidemiological patterns for the P[8] type were obtained depending on the primers used. When the degenerate 1T-1D primer (22) was used instead of the 1T-1 primer in the nested PCR, a significant increase in the level of the P[8] type was observed, mainly in Barcelona, indicating that there was a higher incidence of strains whose sequences could not be amplified with 1T-1 in Barcelona than in Cairo.

The distribution of P-G combinations in Cairo isolates is more or less in agreement with the distribution in Tunisia (32), the only preexisting data for North Africa, where P[8]G1 was the most prevalent strain and where P[6]G4 and P[4]G1 strains emerged. However, while P[6]G4 strains were detected at a low frequency in Cairo, in Tunisia such strains appeared to be quite common. The P[8]G9 combination, detected at a rate of 4.8% in Cairo sewage, is being increasingly detected worldwide (1, 20) since its first description in the United States (8), and very recently it has been implicated in a large waterborne outbreak (C. Villena, R. Gabrieli, R. M. Pinto, S. Guix, D. Donia, E. Buonomo, L. Palombi, F. Cenko, S. Bino, A. Bosch, and M. Divizia, submitted for publication).

The most surprising conclusion of the Barcelona study was the similar frequencies observed for the combinations considered common (51.2% in Barcelona) and uncommon (48.6%) worldwide, mainly because P[4]G1 and P[4]G3 strains are still considered novel or reassortant strains. The reason for the replacement of P[4]G2 strains by these two novel [P4] combinations is not known. However, these novel strains have been detected worldwide since the early 1990s (3, 15), and in some countries they are the most common combinations, among G1 and G3 genotypes, after P[8]G1 and P[8]G3, respectively (42). In particular, P[4]G1 strains are being detected increasingly; they accounted for 4 and 14% of the strains in the People’s Republic of China (12) and Argentina (3), respectively, and they also are considered emerging in Tunisia (32). With this background, the fact that the immigrant population in Barcelona has dramatically increased in the last few years must be taken into account, and the immigrants have originated mainly from North Africa, South America, and East Asia. Studies performed in other parts of Spain (i.e., Valencia) did not result in the same conclusions (7), probably because most of the immigrants in Barcelona started to arrive in 1999 or 2000.

The other uncommon strains detected in sewage from Barcelona have also been found in other countries; these include P[6]G1 strains in the People’s Republic of China (12) and the United States (20), P[9]G1 strains in the People’s Republic of China (12), P[6]G3 strains in the United Kingdom (9) and the People’s Republic of China (12), P[6]G9 strains in India (22), the People’s Republic of China (12), and the United States (20), P[8]G5 strains in Brazil (2, 15) and Argentina (4), and P[8]G9 strains worldwide, as previously mentioned.
Interestingly, three G5 strains were isolated from Barcelona sewage. The G5 strains were originally derived from pigs and are not very common in humans, since they have been found only in Brazilian (17) and Argentinean (4) children. One of the Barcelona G5 isolates was associated with a P[8] type, as previously described in Argentinean (4) and Brazilian children (15). The other two G5 strains were P[8]G5. The VP7 genes of these two Barcelona G5 strains were closely related to the VP7 genes of porcine rotavirus OSU and Po/A34. The actual origin of the these two Barcelona G5 strains is still unknown, since although they are closely related to porcine strains, the sewage sample was from an urban area and consequently most likely was of human origin.

Antigens for the prevalent rotavirus genogroups all over the world should be included in vaccine preparations to ensure protection against all circulating strains. The emergence of new rotavirus strains, which occur in developing countries and also occur in developed countries, and the strain variability, which may exhibit not only a geographic pattern but also a temporal pattern, pose additional difficulties in the design of efficient new rotavirus vaccines.

Important environmental virology issues, such as the differential stability of a given rotavirus genotype, could result in a temporal pattern, posing additional difficulties in the design of efficient new rotavirus vaccines.

ACKNOWLEDGMENTS
C. Villena is a recipient of a BRD fellowship from the University of Barcelona. This study was supported in part by grants QLRT-1999-0634 and QLRT-1999-0594 from the European Union, by grant 1999SGR0022 from the Generalitat de Catalunya, and by the Centre de Referència de Biotecnologia de Catalunya (CeRBa), Generalitat de Catalunya.

We acknowledge the technical expertise of the Serveis Científics Tècnics of the University of Barcelona.

REFERENCES
9. Das, B. K., J. R. Gentsch, H. G. Ciencillo, P. A. Woods, A. Gupta, M. Rama-
23. Pintó, R. M., C. Villena, F. Le Guyader, S. Guix, S. Caballero, M. Pom-
Molecular Detection of Human Calicivirus among Spanish Children with Acute Gastroenteritis

E. Roman, A. Negredo, R. M. Dalton, I. Wilhelmi, and A. Sánchez-Fauquier*

Servicio de Pediatría and Servicio de Microbiología, Hospital Severo Ochoa, Leganés, and Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, Spain

Received 24 May 2002/Returned for modification 26 June 2002/Accepted 17 July 2002

A survey was conducted among Spanish children with gastroenteritis treated in an emergency room. Reverse transcription-PCR with specimens negative for other enteric pathogens was used. The minimum incidence of human calicivirus infection was 7.7%, with Lordsdale as the predominant genotype. The clinical features and severity of calicivirus and rotavirus were similar.

Norwalk-like virus (NLV) and Sapporo-like virus (SLV) are members of the family Caliciviridae (7), which has been considered one of the most common causes of nonbacterial gastroenteritis outbreaks and sporadic cases in adults and children (3, 6, 11, 25).

Routine detection is not yet possible due to the lack of a simple diagnostic test. Thus, the most-used human calicivirus (HuCV) detection assay is generic reverse transcription-PCR (RT-PCR) with the RNA polymerase gene (pol) as the target. Thus far, the RT-PCR assay has not been routinely used for the detection of childhood gastroenteritis etiologic agents. Although HuCV is emerging as a cause of sporadic gastroenteritis as a sole pathogen in young children, there are not many data about the incidence of this viral infection. During previous studies in other countries, this infection was detected in 3.5 to 20% of sporadic cases among young children (1, 2, 5, 9, 10, 12, 14, 15, 16, 22, 24). To date, there is to our knowledge a lack of data from Spain. In order to clarify the epidemiologic role and clinical significance of calicivirus-associated gastroenteritis in our country, we have conducted a surveillance study among children with gastroenteritis treated in an emergency room in Madrid, Spain. Our objectives were to determine the incidence of HuCV infection and the clinical characteristics of the disease and to establish the genetic diversity of HuCV strains. A total of 822 fecal specimens were collected from children of less than 4 years of age with gastroenteritis who visited the emergency room of Severo Ochoa Hospital in Madrid between October 1996 and September 1997. A gastroenteritis episode was defined as at least three looser-than-normal stools within a 24-h period or an episode of forceful vomiting with any loose stools. Clinical information was collected for all patients, including age, sex, presenting symptoms, and duration of illness prior to admission. Each episode was graded by using a 20-point severity score scale as previously described (17). Fecal specimens were screened for etiologic agents of diarrhea. Bacteria (Salmonella, Shigella, Yersinia, and Campylobacter spp. and Vibrionaceae) were detected by routine cultivation methods, and viruses (group A rotavirus, adenovirus, and astrovirus) were detected by commercial enzyme immunoassays.

No pathogens were detected in fecal specimens from 292 (35.5%) children. A subset of 201 of these samples with an adequate volume of fecal specimen was tested for the presence of HuCVs (NLV and SLV) by using RT-PCR with the RNA polymerase gene (pol) as the target. For this, RNA was extracted from 20% of the stool suspensions in phosphate-buffered saline by using the guanidine thiocyanate method (23) and the RNaip Spin kit (Q-BioGene; Bio 101). Samples were first tested with the JV12-JV13 primer pair, which detects only NLV agents (21), and the samples that tested negative with this test were then analyzed with the p289-290 primer pair, which detects NLV and SLV agents (8). PCR products were analyzed by electrophoresis in 2% (wt/vol) agarose–Tris-borate-EDTA gels and detected by UV illumination after staining with ethidium bromide.

A subset of HuCV-positive samples was genetically characterized by either reverse line blot hybridization (RLB)
Diarrhea (days) 2 (1–13) 1 (1–8) <0.05
Diarrhea (maximum no. of times/day) 4.5 (1–15) 5 (1–20) NS
Vomiting (days) 1 (0–7) 1 (0–5) NS
Vomiting (maximum no. of times/day) 3 (0–15) 3 (0–20) NS
Maximum fever (°C) 37 (37–40) 37 (36–40) NS
Severity score (points) 10 (2–16) 10 (1–16) NS
Dehydration (%) 26 41 <0.05
Hospitalization (%) 12.6 19.5 NS

TABLE 1. Clinical characteristics of acute gastroenteritis associated with HuCV or rotavirus

The median age of children with HuCV was 15.12 months (range, 1 to 47 months). The seasonal distribution of gastroenteritis episodes caused by HuCV is shown in Fig. 2. HuCV was detected year-round, with two peak seasons in October and in March and April.

From the 63 cases positive for HuCV, 52 (82.5%) were associated with vomiting, 20 (31.7%) were associated with fever, 15 (24%) were associated with mild dehydration, and 1 (1.6%) was associated with severe dehydration. Hospitalization was required in 8 (12.6%) cases.

To further assess the medical importance of HuCV infection, the clinical characteristics of children infected with HuCV were compared with those found in patients infected with rotavirus (205 of 822) (Table 1). The clinical features and the severity of HuCV and rotavirus gastroenteritis episodes were similar, although children with HuCV infection were less likely to be dehydrated ($P < 0.05$), despite the longer duration of diarrhea observed among these patients.

This is the first report, to our knowledge, of HuCV detection among Spanish children with sporadic gastroenteritis. Our results demonstrate that HuCV might be an important and under-appreciated cause of diarrhea in Spanish children. This study provides the lowest possible estimate of the magnitude of the problem (63 of 822 cases, 7.7%) because we screened only those specimens which had no other pathogens. In addition, some positive samples could escape detection with the primer pairs used.

Our results confirm those of studies done in The Netherlands (10), South Africa (24), France (Rouen) (12), and China (16). However, this incidence is lower than that found in studies from Australia (9), France (Dijon) (1), Finland (14, 15), Mexico (4), and Chile (13). The reasons for this discrepancy could be attributable to the different methods used to diagnose HuCV as well as the diversity of the studies.

As described in previous studies, most of the HuCVs identified worldwide belonged to the NLV genus (89.6%) and the majority of the strains were related to the Lordsdale virus (4, 5, 9, 19). SLV appears to be an uncommon cause of severe gastroenteritis in young Spanish children, although further studies are needed to confirm this data.

The clinical severity of HuCV infection has been reported only in Finnish (14) and Japanese (26) studies up until now. Our findings showed that the mean HuCV clinical severity was equal to that in rotavirus gastroenteritis, as in the Japanese
study (26). However, our data are at variance with those of the Finnish study (15).

Undoubtedly, future studies should be applied to determine the true epidemiologic role of HuCV diarrhea among Spanish children.

Nucleotide sequence accession numbers. GenBank accession numbers for the SLV sequences analyzed in this study are as follows: AY102703 (Madrid01/97), AY102704 (Madrid02/97), and AY102705 (Madrid03/97).

This study was supported in part by FIS grant no. 200/2000 and EU grant no.QLK1-199-00594 for the study of food-borne viruses in Europe.

We thank E. Cubero, R. Ramiro, and V. Montero for technical assistance. We are also grateful to H. Vennema and M. Koopmans (RIVM, Bilthoven, The Netherlands) for providing an RLB membrane and to J. Colomina and M. Koopmans for critical readings of the manuscript. We are grateful to Roger Glass for fruitful discussion.

REFERENCES


Persistent Gastroenteritis in Children Infected With Astrovirus: Association With Serotype-3 Strains

Santiago Caballero,1 Susana Guix,1 Waled Morsy El-Senousy,1 Ignasi Calico,2 Rosa M. Pintó,1 and Albert Bosch1*

1Enteric Virus Group, Department of Microbiology, University of Barcelona, Barcelona, Spain
2Enteric Virus Group, Laboratory of Microbiology, Hospital de la Vall d’Hebron, Barcelona, Spain

The relationship between cases of persistent diarrhoea and the levels and type of human astrovirus was investigated. The potential correlation between human astrovirus excretion levels and the occurrence of protracted gastroenteritis was elucidated after quantifying astroviruses in faecal samples by a competitive RT-PCR. This assay was developed employing an internal RNA standard constructed for this purpose and showed a threshold of positivity of $3.4 \times 10^6$ genomes per gram of faeces. By this procedure, the levels of astrovirus, belonging to serotypes 1, 2, 3, 4, and 8, in faecal samples could be ascertained to range from $3.4 \times 10^8$ to $1 \times 10^{13}$ per gram of faeces. The mean viral titre in the serotype 3-containing faeces was higher than in any of the other serotype-containing samples. In children with no background disease, persistent gastroenteritis cases were detected in 8.5% of the astrovirus infections, and 37.5% of those were associated with astrovirus type 3 infection. In addition, 42.9% of astrovirus 3 isolates were implicated with persistent cases, some of them lasting for 3 months. Other type 3 isolates, detected in the faeces in very large numbers, caused severe gastroenteritis. J. Med. Virol. 71:245–250, 2003. © 2003 Wiley-Liss, Inc.

KEY WORDS: epidemiology; quantification; competitive RT-PCR; stool

INTRODUCTION

Human astroviruses were first described in 1975 in stool specimens of newborns with gastroenteritis using electron microscopy [Appleton and Higgins, 1975]. Astrovirus infections occur worldwide and are most frequent in young children and the elderly [Matsui and Greenberg, 2001]. These viruses constitute a family of 27–40-nm non-enveloped viruses, Astroviridae, with a single-stranded positive RNA genome of about 6.8 kb [van Regenmortel et al., 2000]. The astrovirus genome includes three open reading frames (ORFs), ORF1a with a protease motif, ORF1b with a polymerase motif, and ORF2 coding for the capsid proteins [Matsui and Greenberg, 2001].

Human astroviruses are classified into seven serotypes based on the reactivity of capsid proteins with type-specific antibodies [Kurtz and Lee, 1984]. These 7 antigenic groups (serotypes) perfectly correlate with 7 genotypes that may be determined according to the nucleotide sequence of a 348-bp region of the ORF2 [Noel et al., 1995]. The existence of an eighth type has been suggested based on three complete capsid protein gene sequences deposited in GenBank.

Astrovirus infections induce a mild watery diarrhoea that typically lasts for 2–3 days, associated with vomiting, fever, anorexia, abdominal pain, and various constitutional symptoms that last for less than 4 days [Kurtz and Lee, 1987; Kurtz and Cubitt, 1989; Guix et al., 2002]. Protracted diarrhoea and viral shedding in immunocompromised patients have been described in several studies [Kurtz and Lee, 1987; Wood et al., 1988; Björkholm et al., 1995; Coppo et al., 2000]. Additionally, astroviruses have been associated with persistent gastroenteritis in non-immunocompromised individuals in a study conducted in Bangladesh, although no data were given on any specific serotype or strain link [Unicomb et al., 1998].

In a previous study [Guix et al., 2002], we determined the astrovirus prevalence in the Barcelona area. The overall incidence was 4.9%, with type 1 as the most prevalent followed by types 4, 3, 8, and 2. This study allowed us to gather a collection of strains belonging to the above-mentioned serotypes.
In the present study, the incidence of persistent diarrhoea among the astrovirus-associated gastroenteritis cases and the potential relationship with a specific serotype were evaluated. The potential correlation between the virus numbers in faeces and some pathogenic aspects were also analysed. For this purpose, an internal RNA standard has been constructed and included in the amplification method used [Guix et al., 2002], in order to develop a competitive RT-PCR system to quantify astroviruses in faecal samples. This internal standard control may be used as a tracer of the presence of inhibitors of the RT-PCR procedure.

MATERIALS AND METHODS
Viruses and Cells
A cell-adapted strain (p23795) of human astrovirus serotype 4 (HAsV-4) was kindly provided by W.D. Cubitt (Great Ormond Street Hospital for Children, London), and propagated in CaCo-2 cells, as described previously [Pinto´ et al., 1994]. Viral stocks were obtained from the cell fraction at 72 hours post-infection by lysing the cells in TNE buffer (Tris-HCl 50 mM pH 7.4, NaCl 100 mM, EDTA 10 mM) supplemented with 1% of NP40.

Purification of HAsV-4 by Sucrose Gradient Centrifugation
Viral stocks were subjected to sonication (3 × 50 W for 30 s) and purified further in a sucrose gradient (0–45% wt/wt) in TNE buffer. The gradient was spun at 205,000g for 2 hr and 45 min at 4°C, and separated in fractions of 0.3 ml.

To detect the antigenicity of each fraction, a sandwich ELISA consisting of an antigen-capture through the MAb 8E7 and detection with a rabbit polyclonal anti-astrovirus antibody (kindly provided by Dorsey Bass from the Department of Pediatrics, Stanford University, Palo Alto, CA) was used. All the incubations were carried out in blocking buffer (PBS, 0.3% BSA, and 0.05% Tween-20) at 37°C, and the final detection performed with a peroxidase-labelled goat anti-rabbit antibody.

Nucleic acids from the antigenic fractions were extracted by Boom’s method [Boom et al., 1990], blotted onto a nylon membrane and fixed under UV light. The presence of astrovirus genomes was assayed by hybridisation with a digoxigenin-labelled probe from a region of the ORF2 (nt 4,544–4,956 of the HAsV-1 Oxford reference strain Accession number L23513) at a final concentration of 25 ng/ml at 42°C. After washing the membrane under stringent conditions, detection was performed following the manufacturer’s specifications with a chemiluminescent substrate (CSPD: Disodium 3-(4-methoxyspiro [1,2-dioxetane-3,2’-(5-chloro)tricyclo[3.3.1.1^{3,7}]decan]-4-yl) phenyl phosphate, Roche, Barcelona, Spain).

The number of infectious viruses in the antigenic fractions was determined by a method described previously [Abad et al., 1997] based on an integrated cell culture RT-PCR (CC-RT-PCR). Briefly, tenfold dilutions of the antigenic fractions were inoculated on CaCo2 cell monolayers. At 72 hr post-infection, the presence of astrovirus RNA in the infected cell suspension was assayed by RT-PCR (see below), and expressed as CC-RT-PCRU/ml [Abad et al., 1997].

To confirm the presence of viral particles, the antigenic fractions were examined by electron microscopy (EM) of negatively stained suspensions. Fractions from each of the antigenic peaks were pooled separately and dialysed against TNE buffer. Ten microliters of the viral suspension were placed on carbon copper grids (400 mesh) for 15 min and stained with 2% phosphotungstic acid pH 6.5 (PTA) for 1 min. The grid was dried and observed in a Hitachi HT600 AB microscope. To quantify the number of physical particles, 10 μl of the viral suspension were mixed with 1 μl of a colloidal gold particle (42 nm) suspension (7.35 × 10¹⁰ colloids/ml). The number of viruses and colloidal gold particles present in several fields were counted and the number of viruses was estimated in relation to the known number of colloidal gold particles.

Construction of an Internal RNA Standard
An internal standard for the RT-PCR reaction described by Guix and co-workers [Guix et al., 2002] was generated. The starting material was the amplicer obtained after the RT-PCR of the cell-adapted HAsV-4 strain mentioned above. This RT-PCR procedure is based on the use of the previously described primers A1 (5’-CCTGCCCCGAGAACCAACG-3’) and A2 (5’-GTAAGATTCCAGATGGTG-3’) [Willcocks et al., 1994], which amplify a specific region, of variable size (192–237 bp) depending on the strain, of the ORF1a. Five microliters of a HAsV-4 suspension were heated at 99°C for 5 min and the cDNA was synthesised at 42°C for 60 min by adding 2 μM of primer A2, 0.2 mM of each dNTP, and 4 U of M-MLV reverse transcriptase (Promega, Inogenetics, Barcelona, Spain) in a 10-μl final volume containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, and 10 mM DTT. Five microliters of the RT product were amplified by using 0.5 U of Pwo polymerase (Roche) in a final volume of 50 μl containing 1 μM of each primer A1 and A2, 2 mM MgSO₄, and 0.2 mM of each dNTP. After a denaturation step of 3 min at 95°C, 40 cycles of amplification (94°C, 30 s; 55°C 30 s; 72°C, 30 s) followed by a final extension of 7 min at 72°C were performed. The use of the Taq polymerase was avoided in order to generate blunt-ended fragments.

The resulting 192-bp amplicer contained two DdeI restriction sites separated by 57 bp. After digestion of the amplicer with this restriction enzyme, a ligation of the resulting DNA fragments, of 71, 57, and 64 bp, was performed with the T4 ligase (Roche). Among all ligated products, a 135-bp DNA band, corresponding to the ligation of the 71- and 64-bp fragments, was purified from an agarose gel by using the High-Pure PCR Product (Roche). This DNA was cloned in the pCAP™ cloning vector using the Blunt-Ended PCR Cloning Kit (Roche) and transformed into competent Escherichia coli cells.
Clones containing the proper insert were identified by sequencing and used to produce RNA in an in vitro transcription reaction with the T7 RNA polymerase. A PCR using 0.5 U of the *Pwo* polymerase and 50 nM of the primers P1 and P2, supplied with the Blunt-Ended PCR Cloning Kit, was carried out in a final volume of 50 µl, containing 0.2 mM of each dNTPs and 2 mM MgSO₄. Cycling conditions were performed following the manufacturer’s instructions. The *Taq* polymerase was not used in order to avoid the generation of protruding ends, which could interfere in the in vitro transcription reaction [Triana-Alonso et al., 1995]. The resulting 511-bp blunt-ended amplicon was used in the in vitro transcription reaction with the T7 RNA polymerase. One microgram of the amplicon was in vitro transcribed in a 50-µl volume containing 50 U RNasin (Promega), 10 mM DTT, 0.5 mM rNTPs, and 20 U of the T7 RNA polymerase, for 2 hr at 37°C. The DNA was removed by digestion with RNase-free DNase I, and the RNA purified with phenol:chloroform:isoamylc alcohol (25:24:1), and precipitated with 7.5 M ammonium acetate: absolute ethanol (1:5). The RNA was suspended in 50 µl of RNase-free water and quantitated by spectrophotometry at a wave length of 260 nm. The amount of internal standard usually obtained after the in vitro transcription reaction was around 100 ng RNA/µl which corresponded to 3.4 × 10¹¹ RNA molecules/µl.

**Competitive RT-PCR**

Quantitation of human astroviruses was carried out by RT-PCR with primers A1/A2 in the presence of the internal RNA standard, thus establishing competitive conditions. The 135-bp internal standard amplicon is easily distinguishable from the viral amplimers whose length ranges from 192 to 237 bp, depending on the strain (Guix et al., 2002). The sensitivity of detection of the RNA internal standard in this RT-PCR, in the absence of viral RNA, was 17 molecules per reaction. A fixed number of RNA molecules of the internal standard was added to each of the threefold serial dilutions of the viral RNA. Five microliters of the viral RNA dilution were heated to 99°C for 5 min and placed on ice, and 5 µl of a RNA suspension of the internal standard containing 1,700 molecules each added to virus RNA dilution. An RT-PCR assay was carried out with each of these virus/internal standard dilutions under the conditions described above, but replacing the *Pwo* pol by the Expand High Fidelity PCR System enzyme mix (Roche) in the PCR amplification. Twenty microliters of the PCR product were analysed on a 9% polyacrylamide gel and detected by ethidium bromide staining. The number of astrovirus contained in the sample was estimated from the dilution where the viral PCR product (192–237 bp length) showed similar fluorescence intensity to that of the internal RNA standard PCR product (135 bp length). In this way, it could be assumed that in this dilution there would be an approximately equal number of molecules of the RNA internal standard (1,700 molecules) and of the viral RNA. Having in mind the sample dilution and the processed volume, the number of viral genomes in a given unit of volume could be estimated.

**Preparation of the Stool Samples**

Faecal samples from children with astroviral gastroenteritis (HAsV-1, HAsV-2, HAsV-3, HAsV-4, and HAsV-8) were quantified by the competitive RT-PCR. RNA from 50 µl of 10% faecal suspensions was extracted by Boom’s method [Boom et al., 1990] and resuspended in 50 µl of TE buffer (Tris-HCl pH 8 and EDTA 1 mM). Five microliters of the extracted RNA were used in the competitive RT-PCR described above.

The time elapsed between the onset of symptoms and sample collection was 1–3 days in the acute cases, and around two weeks in the protracted diarrheas, with the sole exception of one sample taken three months after the initial discharge.

**RESULTS AND DISCUSSION**

**Quantitation of a Sucrose Purified HAsV-4 Suspension**

Sucrose gradient purified viral suspensions were used to evaluate the potential usefulness of the competitive RT-PCR procedure hereby described for the quantitation of astroviruses. Sucrose gradient centrifugation of HAsV-4 suspensions revealed the presence of three different antigenic peaks (Fig. 1), with densities of 1.05, 1.12, and 1.14 g/ml. Only the 1.05 and 1.14 g/ml antigenic peaks were positive by the molecular hybridisation assay (Fig. 1). These results suggested that the 1.14 g/ml peak corresponded to the nucleic acid-containing virus particle fraction (infectious viruses), the 1.12 g/ml peak corresponded to the nucleic acid-free virus particle fraction (non-infectious empty viruses), and the 1.05 g/ml corresponded to non-assembled viral compounds (proteins and nucleic acids). To confirm the nature of these fractions, the associated infectivity was assayed by CC-RT-PCR. The 1.14 g/ml peak contained 1 × 10⁸ CC-RT-PCR units/ml, which represents 98.5% of the total infectious viruses, the 1.12 g/ml peak contained 1 × 10⁶ CC-RT-PCR units/ml, and the 1.05 g/ml peak 5 × 10⁵ CC-RT-PCR units/ml. Finally, the EM observation of the three antigenic peaks showed no structured material in the 1.05 g/ml fraction and the presence of viral particles in both the 1.14 g/ml and 1.12 g/ml peaks, with no clear morphological differences between them (Fig. 1). Additionally, the average concentration of physical particles was 3 × 10¹⁰ particles/ml in the 1.14 g/ml and 2 × 10¹⁰ particles/ml in the 1.12 g/ml peak. These results confirmed the existence of two viral capsid structures corresponding to low sedimentation empty particles void of RNA, and high sedimentation RNA-containing particles, as demonstrated previously by separation of astroviruses structures in CsCl gradients [Willcocks et al., 1990; Matsui et al., 1993].

The number of HAsV-4 genomes, determined by competitive RT-PCR, in the 1.14 g/ml peak was estimated as...
9 × 10^{10} \text{ genomes/ml}. Although there is not an exact agreement between both physical measures, i.e., E.M. and competitive RT-PCR counting, it should be borne in mind that the microscopic technique is highly dependent on the colloidal gold suspension status (monodispersed or aggregated colloids) and thus less accurate.

**Quantitation of Human Astroviruses (HAsVs) in Faecal Samples by Competitive RT-PCR**

Thirty-two faecal samples, positive for astrovirus by RT-PCR, were quantified by competitive RT-PCR. These astrovirus strains belonged to serotype 1 (n = 6; 18.75%), 2 (n = 4; 12.50%), 3 (n = 7; 21.87%), 4 (n = 9; 28.12%) and
8 (n = 6; 18.75%). Figure 2 shows the image of an stained polyacrylamid gel in which the quantitation of a faecal specimen containing HAsV-8 was performed. The intensities of fluorescent of the viral and the internal standard amplimers were equivalent at dilution 10⁻⁵. Since 1,700 molecules of the internal standard were competing in the RT-PCR assay, it could be assumed that the number of viral RNA molecules contained in this sample was 1.7 × 10^8 molecules/reaction. Having in mind that the volume of viral RNA processed in each competitive RT-PCR was 5 µl, a concentration of 3.4 × 10¹¹ genomes/ml of faecal suspension, or 3.4 × 10¹¹ genomes/g of faeces could be estimated to be present in the sample.

A minimum number or a cut-off value of 3.4 × 10⁴ genomes/g of faeces is required to be able to be quantified by the present competitive RT-PCR method.

The titres of viral RNA genomes in the samples included in the present study ranged from 3.4 × 10⁸ to 1 × 10¹³ per gram of faeces, as shown in Table I. An analysis of variance (ANOVA) of the number of genomes per gram of faeces in samples belonging to different serotypes, revealed significant (P < 0.05) differences between serotypes 1 (10.28 ± 0.88) and 3 (11.95 ± 0.93), and between serotypes 3 and 4 (10.74 ± 1.64). The mean viral titre in the serotype 3-containing faeces was higher than in any of the other serotype-containing samples. However, the lack of significance in astrovirus levels between serotype 3, and serotypes 2 and 8 could probably arise from the low number of samples. The time of collection, which could be relevant in terms of the viral load, should not influence the differences regarding virus titres observed between the serotypes in acute gastroenteritis, since all samples, independently of serotype, were collected between days 1–3. Regarding the protracted diarrhoea cases, sample collection was usually around two weeks of the onset of symptoms. However, in a sample taken after 3 months of gastroenteritis, the titre was still very high (3.4 × 10¹¹ molecules per gram of faeces).

To confirm that differences in the viral titre between serotypes were not due to different primer annealing efficiencies in viral and internal standard RNA molecules, depending on the viral serotype, the sequences corresponding to the annealing regions were determined by means of a third external primer (data not shown). These sequences were determined for those viral strains showing maximum differences in titre. The number of mismatches detected, for all serotypes, between the viral and primer sequences was 1 or 2, either in the A1 or A2 region.

The clinical significance of the higher viral titres in the faeces of individuals infected with HAsV-3 is not clear. However, these results suggest higher virus production levels of these strains. Interestingly, although persistent gastroenteritis was only detected in 8.5% of the astrovirus infections diagnosed during the period 1997–2000 (unpublished results), 42.9% of the HAsV-3 isolates (3 out of 7 total samples) were associated with persistent gastroenteritis. On the contrary, only 16.7% (1 out of 6 total samples) and 5.5% (1 out of 18 total samples) of the HAsV-8 and HAsV-1 isolates, respectively, were associated with persistent diarrhoeas, and 3.4 × 10¹⁰ genomes/ml of faecal suspension, or 3.4 × 10¹¹ genomes/g could be made (see text). M: molecular weight marker; I.S.: sample containing only the RNA internal standard; Neg.: RT-PCR negative control.

![Image](image.jpg)

**Fig. 2.** Quantification of a faecal human astrovirus type 8 by competitive RT-PCR. The intensities of fluorescence of the viral (210 bp) and the internal standard amplimers (135 bp) were equivalent at the 10⁻⁵ dilution. Since 1,700 molecules of the internal standard were competing, an estimation of 1.7 × 10⁸ RNA molecules/reaction, or

### Table I. Analysis of Faeces From Individuals Infected With Different HAsV Serotypes

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Genomes/g</th>
<th>Mean ± SD of log genomes/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAsV-1</td>
<td>3.4 × 10⁹ (3)*</td>
<td>10.28 ± 0.88</td>
</tr>
<tr>
<td></td>
<td>3.4 × 10¹⁰ (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 × 10¹¹ (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.4 × 10¹² (1)</td>
<td></td>
</tr>
<tr>
<td>HAsV-2</td>
<td>1.0 × 10¹⁰ (1)</td>
<td>10.77 ± 0.65</td>
</tr>
<tr>
<td></td>
<td>3.4 × 10¹⁰ (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 × 10¹¹ (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.4 × 10¹² (1)</td>
<td></td>
</tr>
<tr>
<td>HAsV-3</td>
<td>3.4 × 10¹⁰ (1)</td>
<td>11.95 ± 0.93</td>
</tr>
<tr>
<td></td>
<td>1.0 × 10¹¹ (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.4 × 10¹¹ (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.4 × 10¹² (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 × 10¹³ (1)</td>
<td></td>
</tr>
<tr>
<td>HAsV-4</td>
<td>3.4 × 10⁸ (1)</td>
<td>10.74 ± 1.64</td>
</tr>
<tr>
<td></td>
<td>1.0 × 10⁹ (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.4 × 10¹⁰ (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.4 × 10¹¹ (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 × 10¹² (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.4 × 10¹² (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 × 10¹³ (1)</td>
<td></td>
</tr>
<tr>
<td>HAsV-8</td>
<td>3.4 × 10⁹ (1)</td>
<td>10.86 ± 0.75</td>
</tr>
<tr>
<td></td>
<td>3.4 × 10¹⁰ (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 × 10¹¹ (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.4 × 10¹¹ (2)</td>
<td></td>
</tr>
</tbody>
</table>

*The number of samples is shown in parenthesis.
no serotype 2 or serotype 4 associated cases were reported. Overall, 3 out of 8 persistent gastroenteritis samples (37.5%) contained HAsV-3, one contained HAsV-1 (12.5%), one contained HAsV-8 (12.5%), and the other three could not be typed (37.5%), probably because the peak of viral excretion was over at the sampling time. The mean ± SD of the logarithm of the genomes/gram of faeces of these HAsV-3-associated persistent gastroenteritis samples was 12.20 ± 0.58 and the gastroenteritis could last as long as over 3 months. No other pathogens or basal diseases were reported in these children. Further studies are required, with an increased number of samples, to verify whether the association of these HAsV-3 isolates with persistent diarrhoeas is a frequent phenomenon of this serotype or whether it is just a specific characteristic of these particular strains. However, since serotype 3 represents less than 20% of the total astrovirus isolates in our area [Guix et al., 2002], it will be difficult to conduct these studies. Two out of the other four HAsV-3 isolates were associated with severe gastroenteritis, which required admission to hospital. The mean ± SD of the log of genomes/gram of faeces in these samples was 12.76 ± 0.33.

It is not clear whether this higher viral productivity is linked to a capsid-related phenomenon such as the interaction with the cellular receptor/s or to other viral protein functions, such as a protein coded in the ORF1a which includes a nuclear localization signal and that has been related to the adaptability of astrovirus to cell culture [Willcocks et al., 1994]. The higher stool titres of the HAsV-3 isolates could be the consequence not only of a higher replicative potential but also of some enhanced immune resistance of these strains. In this sense, a relationship between long-lasting astrovirus infection and a chemotherapeutic CD4⁺ T-cell depletion has been suggested [Coppo et al., 2000]. It may then be speculated that a central role of anti-astrovirus specific immunoglobulins is required to discontinue the viral infection. Consequently, any mechanism decreasing the immunoglobulin concentration, including a high viral load, may result in protracted or severe diarrhoea. However, the function of other T-cell populations such as CD16⁻ and CD8⁺ in the control of the infection should not be neglected [Cubitt et al., 1999].

Although human astrovirus is associated usually with cases of mild and self-limiting gastroenteritis, its relation with severe and/or protracted diarrhoea, in patients with no other basal disease, deserves further attention.

ACKNOWLEDGMENTS

Santiago Caballero is recipient of a BRD fellowship of the University of Barcelona.

REFERENCES


Viral gastroenteritis is a common illness in humans of all age groups, with a high morbidity reported worldwide (20). Four types of viruses often have been considered the cause of this disease all over the world: group A rotaviruses, adenovirus types 40 and 41, astroviruses, and human caliciviruses, including Norwalk-like and Sapporo-like viruses (2, 12, 13, 20). However, it was not known to what extent community-acquired gastroenteritis was caused by these viruses, as most studies were conducted in hospitals and nursing homes or in the context of outbreaks.

The recent development of sensitive molecular tools, such as reverse transcription (RT)-PCR, led to improved diagnostic assays, particularly for human caliciviruses, allowing etiologic surveys in the community. Consequently, in a previous study (3), Bon et al. looked for the four most important gastroenteritis viruses in children consulting a pediatrician for gastroenteritis and highlighted the importance of viruses as etiologic agents as well as the high rate of Norwalk-like viruses (second after rotaviruses). Similar results were also reported by Pang et al. for young children in the community (33). To our knowledge, two etiologic surveys in the community have included patients of all age groups, in The Netherlands (7) and in England (35). These authors conducted large-scale physician-based studies and detected viruses in 15 and 22% of the cases, respectively.

In France, where acute diarrhea (AD) has been monitored by the Sentinel network of general practitioners (GPs) since 1991 (5), an epidemic peak is observed each year in winter; it was estimated that about 2% of the French population consulted their GPs for AD during January 1995 (8). The origin of these forms of gastroenteritis remained unknown, although the results of a case-control study by Letrilliart et al. (24) suggested a viral etiology.

Here, we conducted a physician-based case-control survey of AD nested in the French Sentinel surveillance network during the winter of 1998 to 1999. It included subjects from all age groups from 23 December 1998 to 5 May 1999. Both clinical data and stool samples were collected. Virologic analyses were performed, including screening for group A rotaviruses, human caliciviruses, astroviruses, and adenovirus types 40 and 41 and typing of group A rotaviruses, caliciviruses, and astroviruses. We also investigated the geographical distribution of the identified strains. In addition, we examined the prevalence of group C rotaviruses, which have been reported sporadically as human pathogens in children and adults in several parts of the world (18, 22, 28).

**MATERIALS AND METHODS**

**Design, case definition, and selection of controls.** The 103 GPs with the highest rate of participation in the Sentinel network during the previous 12 months were asked to take part in this study. Eighty GPs responded between 23 December 1998 and 5 May 1999. The cases included during this period were patients experiencing AD, defined as losing at least three soft or aqueous stools per 24 h for less than 2 weeks; 90% of these patients were enrolled before 1 March 1999. Controls were defined as people consulting for any other conditions and not reporting any gastrointestinal symptoms during the month preceding the consultation; these patients were enrolled between 27 February and 5 May 1999. To maintain the same level of precision for estimations for each age group, GPs were asked to include one patient from each of the following age groups: 0 to 3, 4 to 15, 16 to 65, and more than 65 years.

During the consultation, a standardized questionnaire was completed by GPs for each patient after the latter had given informed consent. Each patient was also asked to send by express mail a stool sample to a virology laboratory (Dijon, France). The questionnaire concerned the date and location of the consultation.
FIG. 1. Distributions of the four viruses among the patients according to age groups. Bars indicate the following viruses: ■, calicivirus; □, rotavirus; △, astrovirus; □, adenovirus types 40 and 41. The line shows the total percentage of infected patients.

Statistical methods. To estimate the relative risk of AD attributable to viruses, we used binary logistic regression to calculate odds ratios (ORs) and their 95% confidence intervals with LogXact software (CYTEL version 4). We compared categorical data by the chi-square test. Two-tailed tests with a significance level (\(P\) value) of \(<0.050\) were used. The proportion of AD in the general population which could actually be attributable to one of the four screened viruses corresponds to the attributable risk and was calculated as follows (36): \[AR = \frac{\text{OR} - 1}{\text{OR}}\], where \(\text{OR}\) is the attributable risk and \(P\) is the proportion of infections in the sample.

RESULTS

Detection of viruses among patients and controls. GPs interviewed a total of 200 patients and 45 individuals serving as controls, but stool samples were received only from 161 patients and 45 controls. Patients and controls were almost equally distributed among the four age groups. Viral detection was positive for at least one virus in 63 patients (39.1%), including seven dual infections. As shown in Fig. 1, infections tended to decrease significantly with age, as the proportion of infected patients in each age group decreased by about 10% (the \(P\) value for the trend was \(<0.001\)): 54.5% in children 0 to 3 years old, 45.5% for patients who were 4 to 15 years old, 32.6% for patients who were 16 to 65 years old, and 22.8% for patients who were more than 65 years old. Caliciviruses were detected in 31 (19.2%) of AD patients, and group A rotaviruses were detected in 28 (17.4%). Astroviruses and adenovirus types 40 and 41 were detected in seven and four patient samples, respectively (4.3 and 2.5%, respectively), but no group C rotavirus was detected. Among these viruses, two rotaviruses and one astrovirus were detected only by RT-PCR.

Among the seven dual infections, six involved a calicivirus: three dual infections with a rotavirus, two with adenovirus type 40, and one with an astrovirus. The last one was a dual
infection with both a rotavirus and an astrovirus. Among the youngest patients (0 to 3 years old), caliciviruses were as frequent as rotaviruses (29.5 and 27.3%, respectively) (Fig. 1), and two patients were dually infected by both a rotavirus and a calicivirus. There was no significant difference in the distributions of rotaviruses in the other three age groups (P = 0.9). Viral detection was positive for 3 of the 45 controls (6.7%). Two of them tested positive for group A rotavirus (0 to 3 and more than 65 years old), and the third one (more than 65 years old) tested positive for an astrovirus, which was detected by RT-PCR.

As shown in Table 1, there was a significant association between any of the four viral identifications and AD (OR, 9.0; 95% confidence interval, 2.7 to 47.4). Subgroup analysis showed a significant association between AD and either caliciviruses (OR, 15.2; 95% confidence interval, 2.6 to infinity) or rotaviruses (OR, 4.5; 95% confidence interval, 1.1 to 40.9). Taken together, the four viruses were responsible for an attributable risk of AD of 34.7% of the general population during the period considered.

Clinical characteristics of viral diarrhea. For the 161 patients who sent in stool samples, complete clinical data were available for only 137, including the description of the diarrhea and associated symptoms, such as nausea or vomiting, fever, abdominal pains, and respiratory difficulties. More patients found positive for at least one virus than patients found negative suffered from nausea or vomiting (P = 0.02) and had aqueous stools (P = 0.045) (Table 2). Among the infected patients, more rotavirus-positive than calicivirus-positive patients had fever (57 versus 28%, respectively; P = 0.045). This was the only significant difference between these two groups. These analyses excluded the seven dual infections and the seven single infections with either an astrovirus (five infections) or an adenovirus (two infections). In general, fewer children less than 4 years old than patients over that age had nausea or vomiting (47 versus 50 to 77%; P = 0.003) and abdominal pains (52 versus 83 to 95%; P < 0.0001).

**Genotyping of group A rotaviruses, astroviruses, and caliciviruses.** Genotyping of group A rotaviruses by multiplex RT-PCR showed that 17 (61%) of the 28 rotavirus strains detected were P[8],G1; 4 (14.5%) were P[8],G4 and 4 (14.5%) were P[4],G2. The three remaining strains could not be typed. No geographic pattern was observed for the distribution of the different genotypes, and there was no association between a given genotype and age or severity of the illness.

The genotypes of each astrovirus strain was determined by sequence analysis of a 348-bp region of open reading frame 2 (nucleotides 4571 to 4918). The strains exhibited a diversity of genotypes. The seven strains detected in the patients were distributed as follows: type 1, one strain; type 2, one strain; type 3, one strain; type 4, two strains; and type 8, two strains. The strain detected in the controls was also classified as type 8 and had 97.4% nucleotide identity with the two type 8 strains detected in the patients. The three type 8 strains were detected in the same geographic area. No association was observed between a given type and age.

The genotypes of 27 of the 31 calicivirus strains were determined by sequencing a 76-bp fragment of the polymerase gene used for detection. As for astroviruses, the results showed a great diversity of strains clustered in eight different groups (Fig. 2): 23 of the 27 strains (85%) were Norwalk-like viruses, and the other 4 (15%) were Sapporo-like viruses. Among the 23 Norwalk-like virus strains, five related to Southampton virus (>92% nucleotide identity) were genogroup I strains. Three other genogroup I strains could not be classified into one of the clusters described: two strains were related to strain JPNV23 (97% identity), and the other strain was related to strain H104-94J (93% identity) (GenBank accession numbers D82331 and AB020553, respectively). The other Norwalk-like virus strains belonged to genogroup II and could be further divided into four genetic groups represented by Lordsdale virus (eight strains), Melksham virus (three strains), Mexico virus (three strains), and Hawaii virus (one strain). The Sapporo-like virus strains could be divided into two groups, the Sapporo/82 virus group (96% identity with Plymouth virus; GenBank accession number X86559) and the London/92 virus group (95% identity with London/92 virus). As most of the strains were located in geographic regions far from one another, it was difficult to propose a geographic pattern of distribution. However, four out of the five Southampton-like viruses were found in the same northwestern region (Côtes d’Armor), the three Melksham-like viruses were found in the southwestern region, and the three Mexico-like viruses were found in the northern region (Fig. 3). Finally, all Sapporo-like viruses were detected in children less than 4 years old, whereas for Norwalk-like viruses, no relationship between a genetic cluster and age was observed.

**DISCUSSION**

This study reports a physician-based study of AD in France, including 161 patients and 45 controls 0 to more than 65 years old and virologic analyses including group A and C rotaviruses, caliciviruses, astroviruses, and adenovirus types 40 and 41. Our
investigation was conducted from December 1998 to May 1999. Here we show that 39% of the patients were infected by at least one virus and that this epidemic was associated not with a single strain but with a large number of strains exhibiting great molecular diversity, as shown by molecular characterization of rotaviruses, caliciviruses, and astroviruses.

Calicivirus genotyping revealed a great diversity of strains, including Sapporo-like and Norwalk-like virus strains. Norwalk-like virus strains were the most prevalent and were found in all age groups, whereas Sapporo-like virus strains were found in children less than 4 years old, as previously reported (6). Among the Norwalk-like viruses, genogroup II strains predominated, and 53% of them (8 of 15) were related to Lordsdale virus. The predominance of genogroup II strains and especially of Lordsdale virus-related strains has been reported in various countries in the last several years (19, 21). However, whereas the cocirculation of different outbreak strains was observed during certain years (21), the diversity of strains evidenced here was not previously reported in the general population of industrialized countries over such a short period. Most of the strains described here were detected in various regions, although others, like the Melksham virus-related strains, seemed to be clustered in space and time, suggesting that they might have been recently introduced into the population. However, such clustered strains might also represent rare strains seldom encountered in our study.

The group A rotaviruses that we detected displayed cocirculation of P[8],G1, P[8],G4, and P[4],G2 strains, with the P[8],G1 strains predominating. It was previously reported in a 3-year study (4) that the predominant strain circulating in France was P[8],G1, followed by P[8],G4. The predominance of P[8],G1 has been reported in many studies (20). Genotyping of astroviruses in our study also disclosed a great diversity of cocirculating types, because the eight strains detected in seven patients included five different types (1, 2, 3, 4, and 8). As in many worldwide studies, a previous pediatrician-based study conducted from 1995 to 1998 (3) showed that type 1 was predominant (9 out of the 10 strains; unpublished data). The diversity described in the present investigation may have been due to the fact that all age groups were tested, whereas most studies reporting type 1 predominance were conducted with young children (9, 26, 29, 32). Here, six of the eight strains detected were found in patients more than 3 years old, and both of the astroviruses detected in

![Phylogenetic tree based on a 76-nucleotide region of the polymerase gene of calicivirus strains detected in France from December 1998 to May 1999. Designations consisting of the letter S followed by a number indicate the patient sample number positive for a calicivirus. GenBank accession numbers for Norwalk-like virus (NLV) strains representative of genogroup I strains were M87661 (Norwalk), L07418 (Southampton), and U04469 (Desert Shield [DS]); those for Norwalk-like virus strains representative of genogroup II strains were U07611 (Hawaii), L23831 (Snow Mountain), U22498 (Mexico), U02030 (Toronto), X86557 (Lordsdale), U46500 (Camberwell), X76716 (Bristol), and X81879 (Melksham). GenBank accession numbers for Sapporo-like virus (SLV) strains were S77903 (Sapporo/82), U95643 (Houston/86), U67858 (London/92), U95644 (Houston/90), and U73124 (Parkville).]
young children were not type 1 but were types 4 and 8. The uncommon type 8 was detected in three samples from patients living in the same region. This type has been described in the United Kingdom (GenBank accession number Z66541, showing 93.7 to 94.4% identity with the three type 8 strains described here) and was recently reported in Australia, Egypt, and Mexico (25, 26, 27). An important diversity of astrovirus strains, including type 8, was also reported in Barcelona during the same period as our study (17). The prevalence of type 8 astrovirus may have been previously underestimated, or type 8 may constitute an emerging type in France.

In this report, we showed that group A rotaviruses and caliciviruses were the most frequent, as they were detected in 17.4 and 19.2% of patients, respectively. Until now, few data were available concerning the prevalence of these viruses in nonhospitalized patients in all age groups, even when sensitive methods were used. In their physician-based study, de Wit et al. (7) found both Norwalk-like viruses and rotaviruses in 5% of patients, and these were the most prevalent viruses. Tompkins et al. (35) also found rotaviruses and small round-structured viruses in 7.7 and 6.5% of patients, respectively, consulting a GP. An evident reason for the higher percentages observed in our study is that we investigated a period shorter than 6 months, including the
winter epidemic peak (23 December 1998 to 5 May 1999), and not an entire year.

Controls were included in this study even though they were not matched to patients, because it was far more difficult for GPs to induce control individuals than patients to send in stool samples. Consequently, in this study, only one control was included for four patients, and enrollment began later for controls than for patients. However, when we restricted the patient sample to the 11 patients recruited during the same period as the controls, 4 (36%) were positive, leading to a relative risk of a similar magnitude (OR; 95% confidence interval, 1.5 to 43.4). Therefore, our results do not seem to be due to a bias in the selection of controls.

The present results for the four age groups showed that viral infection was more frequent in children less than 15 years old than in adults. Similar results were obtained in physician-based studies in The Netherlands (21) and the United Kingdom (35). These results may reflect a nonviral origin for an important part of the gastroenteritis cases among adults. The maximum duration of the diarrhea, as given in the case definition for our study (less than 14 days), makes unlikely a chronic illness as a cause of the gastroenteritis.

The importance of caliciviruses as causative agents of acute gastroenteritis in young children was recently emphasized in a pediatrician-based study conducted in Dijon (3) and in a community-based study conducted with children less than 2 years old in Finland (33). Another finding concerns the prevalence of rotavirus infection in patients more than 3 years old, which was found to be stable in all age groups. This result indicates that symptomatic infections caused by rotavirus in children more than 3 years old and in adults are not rare, although subclinical infections may be the most common outcome (20). In our study, only 2 out of 45 controls were infected by a rotavirus, including 1 more than 65 years old. Probably because of the limited number of controls and the recruitment period, which began after the epidemic peak, we did not observe more asymptomatic rotavirus infections.

A viral etiology could not be established for 61% of the cases, despite the use of RT-PCR for caliciviruses, rotaviruses, and astroviruses. The time between sampling and the beginning of clinical symptoms was in most cases less than 7 days and thus may not have accounted for the lack of sensitivity of the virologic methods. In addition to the four viruses tested, other viruses may have been present, such as Aichi virus, suggested to be a novel viral gastroenteritis agent and recently reported in the United Kingdom (10), or other microorganisms may have been present, such as pathogenic bacteria, as previously shown (7, 35).

Our results indicate that, under the assumption of a causal role of group A rotaviruses, caliciviruses, astroviruses, and adenovirus types 40 and 41, these viruses accounted for an attributable risk of AD of 34.7% for the general French population between 23 December 1998 and 5 May 1999; the most important viruses were caliciviruses and group A rotaviruses. The high incidence of caliciviruses in AD provides important clues to the etiology of large-scale epidemics, especially for undiagnosed gastroenteritis.

In conclusion, the finding that so many virus strains circulated during a single epidemic at a national level raises about their origins, such as contaminated food or environmental reservoirs, as these viruses are known to be very resistant to environmental factors. Their interactions with the environment are currently under investigation in a limited area in France and over a longer period.

ACKNOWLEDGMENTS

We thank Laurent Lettrilliart and Cécile Viboud for help in writing the article and the Sentinel physicians and their patients for participation in this study.

This work was supported by the Centre Hospitalier Universitaire, Dijon, France, and by grant QLK1-CT-1999-00594 from the European Community.

REFERENCES


Molecular Cloning, Expression, Self-Assembly, Antigenicity, and Seroepidemiology of a Genogroup II Norovirus Isolated in France

Béatrice Nicoller-Jamot, Valérie Pico, Pierre Pothier, and Evelyne Kohli*

*Corresponding author. Mailing address: Laboratoire de Microbiologie Médicale et Moléculaire, Facultés de Médecine et Pharmacie, Université de Bourgogne, 21033 Dijon cedex, France. Phone: 33 3 80 29 34 37. Fax: 33 3 80 29 36 04. E-mail: Evelyne.Kohli@u-bourgogne.fr.

Norwalk-like viruses recently designated noroviruses (Caliciviridae family) represent the most important cause of acute gastroenteritis outbreaks in industrialized countries (8, 17) and are also now recognized as a frequent agent of gastroenteritis in the community in all age groups (4, 5, 19). Noroviruses are divided into two genogroups, genogroup I and genogroup II, each including different genotypes or genetic clusters (1, 20).

Expression of Norwalk virus (NV) ORF2, encoding the major capsid protein, in insect cells infected with recombinant baculovirus has been reported previously (15). The capsid protein obtained self-assembled spontaneously into virus-like particles (VLPs) which are morphologically and antigenically similar to the native particles. VLPs have been subsequently prepared for different strains, notably Southampton genotype I (18), Hawaii genotype II (10), Mexico genotype II (14), Toronto GII (16), and Lordsdale genotype II (6). Because noroviruses are difficult to propagate, these VLPs represent an important source of antigen that can be used in place of the native virus to study seroprevalence and to better understand immunity to caliciviruses. The purpose of this study was to clone and express the recombinant capsid protein of a genogroup II Grimsby-like strain (Dijon171/96) (Lordsdale genotype) detected in France in a child during the winter of 1995–1996. The VLPs obtained were used in a seroepidemiological study in the population conducted between February 2000 and June 2001. In addition, we showed that mouse antibodies generated against recombinant Dijon171/96 virus, and human antibodies recognized discontinuous epitopes on the particles.

Virus-like particles of Dijon171/96 virus, a genogroup II norovirus, were expressed in a baculovirus system and were used for a seroepidemiological study of 1,078 age-stratified human sera collected in Dijon, France. The results showed a seroprevalence of 74.1%. Furthermore, we showed that murine antibodies generated against recombinant Dijon171/96 virus, and human antibodies recognized discontinuous epitopes on the particles.

The amplified product was sequenced, and a second PCR allowing the generation of the amplified ORF2 was carried out with the following primers, including EcoRI and BglII restriction sites (underlined): forward primer, 5′-GAATCTGAATG-3′ (positions 4768 to 4788 [9]), primer 2721, and Taq Pwo polymerase (Roche Molecular Biochemicals). The amplified product was sequenced, and a second PCR allowing the generation of the amplified ORF2 was carried out with the following primers, including EcoRI and BglII restriction sites (underlined): forward primer, 5′-GAATCTGAATG-3′ and reverse primer 5′-AAAAGAGATCTCCAGCCATTA-3′ (positions 4768 to 4788). A 1,620-bp fragment was cloned into the baculovirus transfer vector pVL1393 (Invitrogen). The ORF2 sequence predicted a 539-amino-acid capsid, which exhibited 98.7% nucleotide and 98.7% amino acid identity with Grimsby virus and exhibited 91.9% nucleotide and 96.1% amino acid identity with Lordsdale virus. Sf9 (Spodoptera frugiperda) insect cells were cotransfected with baculovirus linear DNA (Autographa californica nuclear polyhedrosis virus) and a recombinant plasmid (baculoGold kit; Pharmingen). At 5 days posttransfection the cells were harvested and a single recombinant virus clone was selected from the supernatant by plaque purification.

Production of the capsid protein was performed by infecting Sf9 cells at a multiplicity of infection of 1 and harvesting of cell cultures at 5 days postinfection. Purification was performed separately from both supernatant and infected cells after two freeze-thaw cycles. The recombinant capsid was first concentrated by ultracentrifugation. The resulting pellets were centrifuged through a 40% (wt/wt) sucrose cushion for 2 h at 28,000 rpm (Beckman SW28 rotor) and then through a preformed CsCl gradient (28 to 36% [wt/wt] in water; 1.2644 to 1.3661 g/cm³) for 22 h at 35,000 rpm (LKB RPS56T rotor). The capsid protein was identified in CsCl fractions by sodium deoxycholate-polyacrylamide gel electrophoresis (SDS-PAGE) and electron microscopy examination after negative staining with 2% phosphotungstic acid, pH 7.0. VLPs were quantified by the Lowry method by using bovine serum albumin as a standard.

Peptides obtained from tryptic digestion of the 35-kDa protein purified from cell lysates were analyzed by Proteomic Solutions (France) with a matrix-assisted laser desorption ionization—
A time of flight (MALDI-TOF) analysis after two-dimensional electrophoresis (13). Briefly, the purified CsCl fraction containing the 35-kDa protein (~450 μg of protein) was mixed with sample buffer containing 9 M urea, 4% 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate, 0.6% ampholytes, and 20 mM dithiothreitol. A pH linear gradient (3 to 10) was used for the first-dimension electrophoresis, and an SDS–12% polyacrylamide gel was used for the second dimension. Upon completion of electrophoresis the polypeptides were stained with Coomassie blue. Several spots were visualized, and the cores of two of them were manually excised before being desiccated for 30 min in a Speed Vac and washed with a solution of 50% acetonitrile, 50 mM ammonium hydrogencarbonate (pH 8). Trypsinolysis was performed by using porcine trypsin (0.5 μg; Promega) for 16 to 18 h at 37°C. The peptides obtained were analyzed by using MALDI-TOF (Voyager, DE super STR; Applied Biosystems).

Five adult female BALB/c mice (5 weeks old; Iffa-Credo, L’Arbresle, France) were immunized intranasally with 10 μg of VLPs on days 0 and 14 and with 10 μg of the mucosal adjuvant Escherichia coli heat-labile toxin (LT) as previously reported (7). Control mice received phosphate-buffered saline (PBS). Blood and fecal samples were collected from each mouse on days 0 and 35.

Serum samples (n = 1,078; 55.9% female, 44.1% male; age range, 2 months to 96 years) were collected at the Laboratory of Virology (Dijon’s Hospital) between February 2000 and June 2001. There was no association between collection of the samples and the presence or absence of known recent gastrointestinal disease.

Enzyme-linked immunosorbent assay for detecting Dijon171/96 virus-specific serum immunoglobulin G (IgG) and fecal IgA antibodies in mice sera was carried out with VLPs as antigen (100 ng in each well) as previously described for rotavirus VLPs (7). For human sera, 1:100 dilutions were used and wells were also coated without VLP (100 μl of PBS). Specific antibodies were revealed by using 100 μl of a 1:2,000 dilution of peroxidase-labeled goat anti-human IgG (Bio-Rad, Marnes-la-Coquette, France). The positive threshold was at least two-fold the optical density of the serum tested without VLP (cutoff value of >0.2).

The purified capsid protein was resolved by a SDS–10% PAGE in denaturing (samples were boiled for 1 min before SDS-PAGE) or nondenaturing conditions, transferred to a nitrocellulose membrane by using a Bio-Rad Transblot apparatus, and analyzed by protein immunoblotting. Human and mouse sera diluted 1:100 in PBS were added and revealed by using goat anti-human (Bio-Rad) or anti-mouse (Biosys) IgG peroxidase.

Sf9 cells infected with the rDijon171/96 virus released into the supernatant a protein which migrated on a Coomassie blue-stained 10% polyacrylamide gel as a 55- to 59-kDa doublet after CsCl purification (Fig. 1A) as already reported (2, 18, 21). The 55-kDa protein might represent a possible cleavage product of the expressed protein as previously suggested (2, 21). The doublet was observed at a density of ~1.30 g/cm³, and when this fraction was examined by electron micrograph, virus-like particles ~38 nm in diameter were observed (Fig. 2). The protein was also present in the cell lysates, and a minor band with an apparent molecular mass of 35 kDa was also observed (Fig. 1B). Such a protein has been shown to be a C-terminal cleavage product of the capsid protein (12, 15) and has been reported to be mainly cell associated (15, 18). A MALDI-TOF analysis of this minor band yielded 13 peptides which all matched, except one, with peptides of the C-terminal end of Dijon171/96 capsid protein. The latter matched with a peptide...
located at the N-terminal end of the protein (amino acids 54 to 69). Of course, the MALDI-TOF method does not allow for affirming that the peptide obtained from trypsinolysis is really the N-terminal peptide. Nevertheless, this result remains difficult to explain. Although this event may be unlikely after a two-dimensional electrophoresis, a contamination by another cleavage fragment of the capsid protein cannot be excluded. Of interest, monoclonal antibodies specific for epitopes localized on an N-terminal fragment of the protein (amino acids 31 to 70) have been shown unexpectedly by Yoda et al. to react with the small-molecular-weight proteins derived from fecal Norwalk strains (22). Such proteins have been shown also to be C-terminal fragments of the capsid protein.

Mice immunized twice intranasally with purified Dijon171/96 VLPs developed specific high-titered serum IgG as well as fecal IgA, whereas control mice immunized intranasally with PBS did not develop any antibody response by day 35. For serum IgG, the geometric mean titers for immunization by VLP plus LT and by PBS plus LT were 5.49 and 1.70, respectively. For fecal IgA, the geometric mean titer for immunization by VLPs plus LT was 2.44, with a standard deviation of 0.33, and for immunization by PBS plus LT was 1.30. Analysis of Dijon171/96 VLPs by immunoblotting with sera from two children as well as with a murine serum showed an immunoreactivity only when VLPs were resolved on SDS-PAGE under nondenaturing conditions. Three bands with an apparent molecular mass between 80 and 97 kDa, probably representing oligomers as suggested by Hardy et al. (11), were recognized by the human and murine sera, whereas the 55× to 59-kDa doublet was not reactive (Fig. 3), suggesting that epitopes recognized by such antibodies are discontinuous. Finally, the minor 35-kDa protein observed on SDS–10% PAGE gels when VLPs were prepared from infected cells was not immunoreactive by immunoblotting (data not shown). Testing of a higher number of sera might have shown different patterns of reactivity; however, these results are consistent with previous results reported for murine antibodies by Hardy et al. (11), which showed that

![FIG. 2. Electron micrograph of CsCl-purified recombinant VLPs (from fraction 3 of Fig. 1A; buoyant density, 1.30g/cm³) stained with phosphotungstic acid, pH 7.0. Bar, 100 nm.](image)

![FIG. 3. Immunoreactivity of rDijon171/96 was analyzed by immunoblotting with a murine serum and serum from a 2-year-old child after migration on a SDS–10% PAGE gel in denaturing (lanes a) and nondenaturing (lanes b) conditions. For SDS-PAGE, arrows indicate the 55- to 59-kDa doublet and possible oligomers of the capsid protein located between 80 and 97 kDa. For immunoblotting, arrows indicate the immunoreactive bands. mw, molecular weight markers.](image)
7 out of 10 monoclonal antibodies induced against recombinant NV VLPs recognized discontinuous epitopes, whereas only 3 out of 10 recognized continuous epitopes. However, here we did not report any reactivity of a 63-kDa oligomer of the minor protein as reported by Hardy et al. for recombinant CF99S01.

The prevalence of Dijon171/96 virus-specific antibodies by age group is shown in Table 1. The majority of sera from children younger than 6 months were reactive due to the presence of maternal antibodies. For children between 6 and 11 months of age, the percentage of reactive sera fell to 35.5% and increased during the next years of life to reach 75% in children aged 5 to 9 years. By these ages the percentage of positively reacting sera increased and reached a peak value of 89% in the third decade. Finally, it is of note that the seroprevalence had a decreasing trend for the groups of more than 50 years of age. Overall, 799 of 1,078 (74.1%) serum samples were positive for Dijon171/96 virus-specific antibodies. The use of antigens produced by recombinant baculoviruses in insect cells allowed for the examination of the seroprevalence to different prototype noroviruses in previous studies in industrialized countries (17). The strains used were mainly NV strain for genogroup I and Mexico strain for genogroup II, and the seroprevalence to these strains generally varied from 75 to 100%. One recent study conducted in Italy used Lordsdale virus (18) and reported a seroprevalence of 91%, higher than the seroprevalence to Dijon171/96 strain (Lordsdale genotype) we report here. Nevertheless, strains close to Dijon171/96 virus in polymerase region comparisons have been found to circulate in France since 1996.

Nucleotide sequence accession number. The GenBank accession number for ORF2 of Dijon171/96 virus is AF472623.

This work was supported by grants from the Conseil Regional de Bourgogne, the Ministere de la Recherche, and ECOS-Nord (no. CF9801).

REFERENCES


<table>
<thead>
<tr>
<th>Age range</th>
<th>No. of samples</th>
<th>Positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>&lt;6 mo</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>6–11 mo</td>
<td>31</td>
<td>11</td>
</tr>
<tr>
<td>1–2 yr</td>
<td>52</td>
<td>30</td>
</tr>
<tr>
<td>3–4 yr</td>
<td>41</td>
<td>28</td>
</tr>
<tr>
<td>5–9 yr</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>10–19 yr</td>
<td>88</td>
<td>71</td>
</tr>
<tr>
<td>20–29 yr</td>
<td>194</td>
<td>159</td>
</tr>
<tr>
<td>30–39 yr</td>
<td>146</td>
<td>130</td>
</tr>
<tr>
<td>40–49 yr</td>
<td>131</td>
<td>107</td>
</tr>
<tr>
<td>50–59 yr</td>
<td>112</td>
<td>82</td>
</tr>
<tr>
<td>60–69 yr</td>
<td>119</td>
<td>69</td>
</tr>
<tr>
<td>&gt;70 yr</td>
<td>116</td>
<td>77</td>
</tr>
</tbody>
</table>

Total 1,078 799 74.1
A semiquantitative approach to estimate Norwalk-like virus contamination of oysters implicated in an outbreak

Françoise S. Le Guyader,*, Frederick H. Neill, Eric Dubois, Fabienne Bon, Fabienne Loisy, Evelyne Kohli, Monique Pommepuy, Robert L. Atmar

Laboratoire de Microbiologie, IFREMER, BP 21105, 44311 Nantes cedex 03, France
Department of Molecular Virology and Microbiology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA
Unité de Virologie, Agence Française de Sécurité Sanitaire des Aliments, BP 67, 94703 Maison Alfort cedex, France
Laboratoire de Virologie et Microbiologie Médicale, UPRES EA562, UFR Médecine et Pharmacie, 7 Bd Jeanne d’Arc, 21034 Dijon cedex, France

Received 13 July 2002; received in revised form 23 November 2002; accepted 16 December 2002

Abstract

Gastroenteritis outbreaks linked to shellfish consumption are numerous and Norwalk-like viruses (NLVs) are frequently the responsible causative agents. However, molecular data linking shellfish and clinical samples are still rare despite the availability of diagnostic methods. In a recent outbreak we found the same NLV sequence in stool and shellfish samples (100% identity over 313 bp in the capsid region), supporting the epidemiological data implicating the shellfish as the source of infection. A semiquantitative approach using most-probable-number-RT-PCR (MPN-RT-PCR) demonstrated the presence of a hundred of RT-PCR units per oyster. Follow-up of the oysters in the harvest area, for approximately 2 months, showed persistence of NLV contamination of the shellfish at levels up to a thousand RT-PCR units per oyster prior to depuration of the shellfish. This finding is useful in beginning to understand shellfish contamination and depuration for use in future hazard analyses.

Keywords: Shellfish; NLV; Outbreak; MPN-RT-PCR

1. Introduction

Norwalk-like viruses (NLVs) are among the most important causes of gastroenteritis in all age groups, and foodstuffs are a major source of infection (Koopmans et al., 2002). Shellfish are notorious as a source of NLVs infection and the first outbreaks were reported almost 20 years ago. For a long time, analysis could be done using only electron microscopy or epidemiological data (Lees, 2000). Now, the development of sensitive and specific methods allows the precise characterization of the viral strain not only in patient stool specimens but also in shellfish (Atmar et al., 1995; Le Guyader et al., 2000; Goswami et al., 2002). However, due to NLV genetic diversity, the optimal strategy to detect the greatest number of NLVs in clinical and environmental samples remains to be determined (Atmar and Estes, 2001). This has two important consequences: several primer sets need...
to be used to detect the greatest number of strains (especially when detecting low quantities of virus, as is needed for shellfish samples), and quantitative assays using real-time RT-PCR are not yet available.

A recent outbreak linked to shellfish consumption in France highlighted the complexity of NLV detection. The first challenge was to find a primer set able to amplify the strain both in stool and in shellfish samples, and the second one was to estimate the NLV contamination in shellfish. For this approach, most-probable-number-RT-PCR (MPN-RT-PCR) was applied to shellfish samples implicated in the outbreak and collected from the producer over the next 1.5 months. This is the first semiquantitative analysis of NLV contamination of shellfish samples implicated in an outbreak.

2. Materials and methods

2.1. Epidemiological data

In March 2000, the French Ministry of Agriculture and Fishery reported several small clusters of gastroenteritis outbreaks. Epidemiological investigations rapidly implicated oysters bought in two stores as the possible source of the outbreaks. Oyster consumption was responsible for at least 14 cases, but only one outbreak involving four consumers could be clearly identified and investigated.

A woman (subject 1, 46 years old) bought oysters on March 23 for herself and her parents (subjects 2 and 3, 75 years old each). Her sister (subject 4, 48 years old) bought oysters from the same batch at the same store on the same day. Subject 1 ate seven oysters at dinner on March 23 and seven at dinner on March 24 and became ill approximately 12 h later. Subject 4 ate 18 oysters at dinner and became ill 24 h later. The two parents (subjects 2 and 3) each ate nine oysters at dinner on March 25 and became ill about 12 h later. Clinical signs were diarrhea, vomiting, abdominal pain for 2 to 3 days.

2.2. Stool sample analysis

Stool samples were collected about 10 days after the onset of illness and were sent to the clinical laboratory in Dijon. Analysis of the stool samples, including sequencing of virus-specific amplicons, was done as described (Vinje and Koopmans, 1996; Hafliger et al., 1997; Bon et al., 1999).

2.3. Shellfish sample analysis

Oysters (Crassostrea gigas) suspected to be responsible for the outbreak were collected from two stores on March 30 (21 oysters from store 1 and 32 oysters from store 2). Samples were collected from the harvesting area on March 31, April 3, 5, 18 and May 17. Each sample consisted of 15 oysters (except for the sample collected on April 18 that contained only 13 oysters).

Samples, kept at 4 °C during shipment, arrived at the laboratory 1 day after collection. On arrival, the shellfish were washed, counted, shucked, and the total weight recorded. The stomach and digestive diverticula were removed by dissection, cut into small portions, mixed, divided into 1.5-g portions (corresponding to one to four oysters), and frozen until analysis. The method used was as previously described (Atmar et al., 1995). Briefly, digestive diverticula were homogenized, extracted with chloroform-butanol, and precipitated with Cat-floc (Calgon, Ellwood City, PA), followed by polyethylene glycol 6000 (Sigma, St. Quentin, France, or BDH Laboratory Supplies, Poole, England) precipitation. Viral nucleic acid was extracted and purified from the suspended polyethylene glycol pellet by digestion with 0.2 mg of proteinase K (Amresco, Solon, Ohio) per milliliter, phenol-chloroform (Applied Biosystems, Foster city, CA) extraction, ethanol precipitation, 1.4% (wt./vol.) cetyltrimethylamonium bromide (Sigma) precipitation, and a final ethanol precipitation. Viral nucleic acid was suspended in 100 μl of RNase-free H₂O.

For NLV detection, primers were selected either in the polymerase coding region (P110/P36, Le Guyader et al., 1996a; P110/N1, Green et al., 1995; P110/SR, Ando et al., 1995; JV12/JV13, Vinje and Koopmans, 1996) or in the capsid coding region (SRI-1/SRI-2, Hafliger et al., 1997; P155: 5’-ccacccacaccctatacttg-3’, nt 5649–5671/ P156: 5’-atgatgatggcgtctaaggacg-3’, nt 5358–5380). Ten probes were used to hybridize the RT-PCR NLV products (Vinje and Koopmans, 1996; Hafliger et al., 1997; Le Guyader et al., 2000). RT-PCRs were performed according to the instructions of the avian myeloblastosis virus reverse tran-
scriptase (Life Sciences, St. Petersburg, FL), or the murine leukemia virus reverse transcriptase and Taq polymerase suppliers (Applied Biosystems). For hybridization, the DNA was denaturated and blotted onto a positively charged nylon membrane, hybridized for 2 h at 50 or 55°C. Probes were labeled with digoxigenin using the 3V tailing kit (Roche, Meylan, France), or the Genius kit 5 (Roche Applied Science, Indianapolis, IN, USA). The hybridized probes were detected by chemiluminescence (Roche) according to the manufacturer's protocol using a Bio-Rad multi-imager, or by the genius 3 nucleic acid detection kit (Roche Applied Science) (Le Guyader et al., 2000; Atmar et al., 1995).

Samples were considered to contain NLVs only if the amplicons were detected by hybridization using NLV-specific probes. NLV-positive RT-PCR amplicons were directly sequenced. Sequence homologies were evaluated using the BLAST-search program.

MPN-RT-PCR conditions: NLV capsid primers were used to perform a semiquantitative detection by a MPN-RT-PCR assay. The MPN of RT-PCR units, where 1 RT-PCR unit is the amount of virus genome needed to yield a positive result, was calculated using the methods of Barkworth and Irwin (1938) and Cochran (1950). Tenfold dilutions of nucleic acid extracts (1/10, 1/100, 1/1000) were amplified in replicates of 5 at each dilution. Positive or negative results were determined by Southern hybridization. The MPN, expressed as RT-PCR U/1.5 g of oyster sample, was estimated. Taking into account the volume of nucleic acid analyzed, and the number of oysters used to obtain 1.5 g of digestive organs, results were then expressed in units per oyster.

3. Results

3.1. Stool results

Stool samples from subjects 1 and 4 yielded virus-specific amplicons with polymerase primers (P110/SR and JV12/JV13) and capsid-specific primers (Table 1). After sequencing, these two samples gave different sequences both in the polymerase and capsid region. Stool samples from subjects 2 and 3 were found positive only using capsid primers and amplicon sequences were identical (Table 1).

3.2. Shellfish results

For NLV detection, all primer sets that amplify a part of the NLV polymerase region gave negative results for all the shellfish sample extracts. Using the two sets of primers targeting the capsid region, virus-specific amplicons were obtained with samples from the two stores and from the additional four samples collected from the harvesting area between March 31 and April 18. The last sample, collected on May 17, was RT-PCR negative. The same sequence was detected in all six positive samples detected from March 31 to April 18, and the sequence was identical to the one found in stools from subjects 2 and 3.

Table 1

<table>
<thead>
<tr>
<th>NLV detection in stool samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Polymerase region</strong></td>
</tr>
<tr>
<td>Primers</td>
</tr>
<tr>
<td>P110/SR</td>
</tr>
<tr>
<td>Patient 1</td>
</tr>
<tr>
<td>Patient 2</td>
</tr>
<tr>
<td>Patient 3</td>
</tr>
<tr>
<td>Patient 4</td>
</tr>
</tbody>
</table>

--- Negative result obtained after RT-PCR and hybridization.

a The sequence identity is given in % after a Blast-search program compared to reference strains. The genogroup and genetic cluster are indicated after the reference strain. Genbank accession numbers are as follows: Desert shield virus (U04469), Saratoga strain (U07614), Southampton virus (L07418), and Norwalk virus (M87661).

Table 2

<table>
<thead>
<tr>
<th>Semi-quantification of NLVs in shellfish samples by MPN-RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
</tr>
<tr>
<td>Store 1</td>
</tr>
<tr>
<td>Store 2</td>
</tr>
<tr>
<td>Field area</td>
</tr>
<tr>
<td>Field area</td>
</tr>
<tr>
<td>Field area</td>
</tr>
<tr>
<td>Field area</td>
</tr>
<tr>
<td>Field area</td>
</tr>
</tbody>
</table>

nc: sampling date not communicated, - : negative result obtained after RT-PCR and hybridization.

Contaminated shellfish were further analyzed using MPN-RT-PCR (Table 2). The amount of NLV per oyster collected in the stores ranged from 85 to 237 RT-PCRU. The amount of NLV from oysters collected in the harvesting area over the next month ranged from 40 to 1740 RT-PCRU per oyster (Table 2).

4. Discussion

Shellfish are commonly implicated as a vehicle for the transmission of NLVs, but precise data demonstrating the same sequence in shellfish and stool samples are still rare. The main difficulty in such a demonstration is to have the epidemiologic information in time to collect the appropriate shellfish implicated in the outbreak (from the table, the retailer, or the producer) and to collect stool samples from ill people. Even when these samples are collected, the amount of NLV contamination of the shellfish may be too low to allow for sequence analysis to be performed (Shieh et al., 2000). There are reports of virus being detected in shellfish without stool sample data being available (Green et al., 1998) and other reports of virus being detected in stools without shellfish data being available (Lees, 2000). In other instances, NLVs were detected in both stool samples and shellfish but no sequence analysis is reported (Sugieda et al., 1996; Christensen et al., 1998). Analysis of NLV sequences detected in stool samples and shellfish from a large outbreak in the United States showed consistent and unexplained differences in the sequence derived from implicated shellfish compared to the stool-derived sequence (Le Guyader et al., 1996b). It was only in 1998 that the first clearly established link between clinical and environmental samples was identified in a California outbreak, demonstrating the same sequence in two shellfish samples and one clinical sample (among two analyzed) (Shieh et al., 2000). These data demonstrate that despite improvements in the ability to identify NLVs in shellfish and extensive epidemiologic data linking shellfish consumption to NLV-associated gastroenteritis, there is still little direct evidence linking NLVs in shellfish to the affected shellfish consumers using molecular methods.

In this study, samples were collected from two different retailers and from the same field source. The first challenge was to identify an NLV-specific primer pair capable of amplifying the causative strain. The shellfish samples were analyzed in three different laboratories and all initially obtained negative results using several different NLV polymerase-specific primer pairs. However, the AFSSA laboratory routinely used capsid-specific primers (Hafliger et al., 1997) in their assays and were able to obtain a positive signal after nested-PCR. This finding was subsequently confirmed by the IFREMER and the US laboratories obtaining an NLV-specific signal after single round PCR using a previously designed capsid-specific primer pair, P155/P156. Finding a strain amplified only by capsid-specific primers is unusual, but is consistent with the genetic diversity of NLVs. Although a large number of primer pairs have been described, many of them targeting a portion of the polymerase gene, no single primer pair is able to detect all strains of NLV (Atmar and Estes, 2001). The use of multiple primer sets enhances the chance to detect a greater number of strains, and the homology of the primers with the NLV strain is important in the analysis of environmental samples with low levels of virus (Le Guyader et al., 1996a; Atmar and Estes, 2001).

The causal link between oyster consumption and gastroenteritis in patients was demonstrated by finding exactly the same NLV sequence in the shellfish samples from the two retailers, in two clinical samples, and from the oyster collected directly from the harvest area. With the epidemiological data (InVS, personal communication), there is no doubt that the oyster consumption was responsible for the outbreak. An unresolved question is why this strain was not identified in stool samples of the two other subjects (1 and 4) with a similar illness and exposure to shellfish from the same source, instead detecting two different NLV strains? A contributing factor may have been the late collection of the stools, allowing NLV infection from another source to occur (to explain why two different strains were detected). A second possibility is that the shellfish may have been contaminated with multiple NLV strains, as reported previously by several investigators (Sugieda et al., 1996; Henshilwood et al., 1998; Le Guyader et al., 2000). However, despite several attempts by all three laboratories, no other NLV strains could be detected in the shellfish. Interestingly, the shellfish and shellfish-growing water did not show evidence of contamination with fecal...
coliforms, with the shellfish meeting the EEC standard of less than 230 E. coli/100 g of shellfish meat, and the harvest area showed no increase in bacteria concentrations since November 1999 (REMI, IFREMER surveillance network). The failure of bacterial indicators to identify virus contamination of shellfish implicated outbreaks has been noted numerous times previously (Lees, 2000; Shieh et al., 2000; Bosch et al., 2001).

From previous studies, the only available data on the risk linking illness occurrence to oyster consumption is the number of shellfish eaten by consumers, with one oyster in some cases being sufficient to induce illness (Lees, 2000). In this outbreak, patients consumed from 9 to 18 oysters. We extended these observations further by applying semiquantitative method to estimate the level of NLV contamination in shellfish samples. Shellfish collected from retail stores and harvested area at approximately the same time as those consumed by subjects 2 and 3 contained about 85–237 RT-PCR units of NLV per oyster. The number of genomic copies per RT-PCR unit has been estimated to be as low as 10–50 (Atmar et al., 1995; Schwab et al., 2001), so that the shellfish were likely contaminated with more than 1000 virions each. This is more than enough to induce illness given the low estimated infectious dose (Atmar and Estes, 2001). Other methods of virus quantitation were not used. Methods for detecting NLVs from shellfish using real-time PCR assays have not been reported and likely must be designed to be strain-specific, given the genetic diversity of these viruses. However, after this study was completed, two Taq-Man-PCR procedures for the detection of NLVs have been described (Shinohara and Kageyama, 2002; Katayama et al., 2002). Another quantitative approach could have been the use of competitive PCR (cPCR) (Schwab et al., 2001). However no internal standard was available in this genomic region, and a recent report noted that MPN-PCR and cPCR assays obtain similar results (Michotey et al., 2000). So, we selected the MPN-PCR for its ease of use. This semi-quantification approach showed that the contamination in the harvesting area persisted for several weeks (same strain) before disappearing. It is unclear whether the persistent contamination was due to continued exposure of the shellfish to virus in the environment or failure of the shellfish to depurate the NLV strain following an exposure in March. Future studies will need to collect environmental and epidemiological data (rain, malfunctioning sewage disposal system, previous outbreak or endemic gastroenteritis in the population, etc.) at the date of the possible shellfish contamination to allow for the evaluation of these possibilities. Such information in combination with quantitative analyses of NLV will lead to a better understanding of shellfish in situ virus contamination and depuration.

In summary, several points are highlighted by the investigation of this shellfish-borne viral outbreak:

1. shellfish were linked to the illness of two patients by epidemiologic and molecular methods, but the use of multiple primer sets was necessary to identify the outbreak strain;
2. 85–237 NLV RT-PCR units were present in shellfish linked to the outbreak. This is the first demonstration of a semiquantitative approach for a NLV-contaminated food to induce illness; and,
3. shellfish viral contamination in the harvest area persisted for several weeks before disappearing.

The collection of similar data in the evaluation of future NLV outbreaks will be useful in the development of models for hazard analyses and for the risks associated with viral contaminated foods.

Acknowledgements

This research was supported in part by European grants: Foodborne Viruses in Europe (QLRT-1999-00594) and Virus Safe Seafood (QLK1-1999-00634).

We thank G. Mouillard and collaborators (Ifremer St Malo laboratory) for sampling; L. Miossec (Lab. Microbiologie, Ifremer Nantes) for environmental investigation, V. Vaillant (INVS) for epidemiological data, and P. Aubert (DGAL) for outbreak information. We are indebted to M.K. Estes (Baylor College of Medicine, Houston) for helpful advice and discussion.

References


Waterborne Outbreak of Norwalk-Like Virus Gastroenteritis at a Tourist Resort, Italy

Delia Boccia,* Alberto Eugenio Tozzi,* Benvon Cotter,* § Caterina Rizzo,† Teresa Russo,‡ Gabriele Buttinelli,* Alfredo Caprioli,* Maria Luisa Marziano,* and Franco Maria Ruggeri*

In July 2000, an outbreak of gastroenteritis occurred at a tourist resort in the Gulf of Taranto in southern Italy. Illness in 344 people, 69 of whom were staff members, met the case definition. Norwalk-like virus (NLV) was found in 22 of 28 stool specimens tested. The source of illness was likely contaminated drinking water, as environmental inspection identified a breakdown in the resort water system and tap water samples were contaminated with fecal bacteria. Attack rates were increased (51.4%) in staff members involved in water sports. Relative risks were significant only for exposure to beach showers and consuming drinks with ice. Although Italy has no surveillance system for nonbacterial gastroenteritis, no outbreak caused by NLV has been described previously in the country.

Norwalk virus is the prototype of the genus Norwalk-like virus (NLV) in the Caliciviridae family, which includes a large number of genetically related strains that together represent the most important cause of gastroenteritis outbreaks worldwide (1–2). NLV accounts for up to 96% of outbreaks of nonbacterial gastroenteritis in the United States (3) and has been implicated in 43% of all foodborne outbreaks in England, 67% in Sweden, and 80% in the Netherlands (4–6).

Outbreaks of NLV gastroenteritis more frequently affect adults and children >5 years of age. Because of the low infectious dose of the agent (10–100 viral particles can induce symptoms), outbreaks are characterized by a high secondary attack rate (7). In most documented outbreaks, the incubation period has been reported as 24–48 hours; the average duration of symptoms is 12–60 hours. During an outbreak, >50% of infected persons have symptoms of vomiting, most often in combination with diarrhea (8). The main source of infection is usually contaminated food or water (9–13), while the usual mode of transmission is direct person-to-person contact with saliva, vomit, or aerosols. Transmission may also occur through contact with contaminated objects and surfaces such as showers, sinks, mats, and floors (3).

In Italy, which has no surveillance system for nonbacterial gastroenteritis, the impact of NLV infection is unknown, and no previous outbreaks of confirmed NLV infection have been reported. We describe a large outbreak of gastroenteritis caused by NLV at a resort in Italy.

Methods

The outbreak occurred at a tourist resort in the Gulf of Taranto, southern Italy, during July 7–31, 2000 (Figure 1). The resort has an area of 122 hectares with 456 guest rooms in 19 buildings, in addition to staff quarters. The buildings are situated around a central area where a restaurant, a swimming pool, and the resort management office are located. The resort can accommodate 1,000 guests, who usually arrive on a Saturday and depart 1 or 2 weeks later, resulting in approximately 50% turnover of guests each weekend.

The resort’s water tank is supplied via a 1-km pipe connected to the main public water supply (Figure 2). On July 13, a break in this water pipe was observed (Figure 2, point 2). Inspection also showed a bypass connecting the tank to an unused irrigation system (Figure 2, points 4 and 5).

On July 18, 2000, the local health unit and the Institute of Hygiene of the Faculty of Medicine in Bari were notified about an outbreak of gastroenteritis at the resort. An epidemiologic investigation was initiated the same day to identify the agent and the mode and vehicle of transmission and to
implement control measures. By July 20, when the local health unit notified the Istituto Superiore di Sanità in Rome, the outbreak had already been in progress for approximately 2 weeks and >150 persons were ill.

Outbreak Investigation

A case was defined as illness in any guest or employee who stayed at the tourist resort during the period July 1–31 and who had diarrhea (three or more loose stools in any 24-hour period) or vomiting (at least one episode) or both, in the same period. Case finding was done by checking records of the resort medical center; after July 20, a door-to-door search was initiated. Demographic data and information on symptoms were collected in face-to-face interviews by the medical staff of the local health unit and the University of Bari.

Because of the high number of cases in resort staff members, a retrospective cohort study was performed to assess risk factors associated with illness in this group. Persons eligible for the study were staff members employed at the resort from July 1 to 31. Standard questionnaires were sent to all 224 staff members in the first week of August. Information requested included name, date of birth, sex, room number, job type, date of onset and type of symptoms, and water and food preferences. A month had elapsed between onset of symptoms and distribution of the questionnaires. We did not inquire about actual food history and activities of staff members during the outbreak but rather about their food preferences and usual activities.

Statistical Analysis

The questionnaires from guests and staff members were returned to the Istituto Superiore di Sanità, where the data were analyzed by using SPSS Base 10.0 (SPSS Inc., Chicago, IL) and Epi-Info 6.04 (Centers for Disease Control and Prevention, Atlanta, GA). Information collected on cases was used to construct the epidemic curve and describe the clinical presentation of the disease. Attack rates, denominator data, personal characteristics, and clinical symptoms of cases were compared between guests and staff members by chi square or Fisher exact test when appropriate; the Mann-Whitney U-test was used for comparisons of age. The room location of ill persons was plotted on a map of the resort that included water pipelines in an attempt to identify any clustering of cases along the pipeline. Statistical test for clustering was performed by the cluster k-means method with SPSS Base 10.0 (SPSS Inc.).

In the cohort study, the attack rate was calculated for the total staff and also by specific job type. Relative risks and 95% confidence intervals were also calculated for job type, behaviors and activities, and food preferences.

Laboratory Investigations

From July 18 to 28, samples (28 fecal and 2 vomit specimens) were collected from 30 participants whose illness met the case definition. Part of each specimen was stored at -20°C until examination for viral particles and free fecal cytotoxins, and the rest was refrigerated and processed within 12 hours of collection.

Ova and parasites were detected by direct microscopy, and Salmonella, Shigella, Campylobacter, Yersinia enterocolitica, Staphylococcus aureus, and enteropathogenic E. coli were sought by standard methods (14). The presence of Clostridium perfringens enterotoxin (CPE) was determined either by assaying the cytopathic effect on Vero cells or by reverse passive latex agglutination (RPLA) test (Oxoid Italia Spa, Garbagnate Milanese, Milan) according to the manufacturer’s instructions (14).

Stool and vomit suspensions were examined by NLV-specific reverse transcription/polymerase chain reaction (RT/PCR) with generic primers JV12–JV13 to a consensus sequence on the RNA polymerase segment of the genome shared by most NLV strains (15). For confirmation of the diagnosis, gels were further analyzed by Southern blot with a mixture of NLV-specific probes (15). The 327-bp amplification product was subjected to sequence analysis with PCR primers, and the sequences obtained were aligned with those in the European Molecular Biology Laboratory Nucleotide Data Bank.

After July 13, water samples were repeatedly collected from the main public water supply and from various points outside and inside the resort. Samples from food in the kitchen and the refrigerators were collected and sent to the University of Bari on July 18. Water and food samples were subjected to culture tests for enteric bacterial pathogens, according to standard methods.

Results

Descriptive Epidemiology

Of 344 cases identified from July 1 to 31, 69 (20%) were in staff members. Information on personal characteristics and clinical presentation was available for 248 ill persons (Table 1). Diarrhea, vomiting, and abdominal pain were observed in
>70% of all cases. Five patients were hospitalized; all recovered rapidly and were discharged within a few hours. None of the patients had any further sequelae. Attack rates did not differ by age, sex, or symptoms for cases in guests or staff members. For cases in guests, the median interval from the arrival date at the resort and onset of symptoms was 4 days, and symptoms developed in 77% within 5 days.

The epidemic curve shows three distinct peaks in each of the 3 weeks, beginning on July 12 (70 cases), July 18 (26 cases), and July 27 (55 cases). Over the total outbreak period, 275 cases occurred in guests and 58 in staff (Figure 3). Fifty-seven percent of cases in staff members occurred before July 15. The outbreak lasted 24 days, and no cases were observed after July 31.

Because of the rapid turnover at the resort, attack rates for guests were calculated separately for each week: an attack rate of 102 (10.5%) of 970 was observed in week 1; 66 (8.7%) of 760 in week 2; and 105 (10.1%) of 1,034 in week 3. Ill guests occupied 157 of the resort’s 456 rooms. No significant evidence of either clustering by the cluster k-means methods (p=0.392) or increased frequency of cases in rooms near the water pipeline was observed. Attack rates by sex, age group, and week of stay were similar.

### Analytical Epidemiology

For the analysis of risk factors in the cohort study, 181 questionnaires from 224 staff members were completed and analyzed. The attack rate in this group was 69 (38.1%) of 181. The lowest attack rates were observed in staff members who worked in the kitchen or the office, and the highest were in waiters, sports trainers, entertainers, and cleaning staff (i.e., staff members who have close contact with guests) (Table 2). Staff members who took showers on the beach or consumed drinks with ice were more likely to become ill than those who did not. No association was found between disease and eating any particular type of food or with being at work on July 8–11 (the first days of the outbreak) (Table 3).

### Microbiologic Results

Stool samples from 28 patients were negative for ova and parasites and bacterial enteropathogens. Of the 28 stool samples examined by NLV-specific RT-PCR, 22 had an amplified DNA of the size expected for NLV. The 327-bp amplification product was also confirmed for all samples by Southern blot hybridization with NLV-specific probes. Vomit specimens from two other subjects were negative.

A readable common sequence of 290 bp was obtained with sequence analysis and found to be the same for eight samples, indicating a single outbreak virus strain. The sequence was analyzed against the European Molecular Biology Laboratory Nucleotide Data Bank, yielding a best fit with the RNA polymerase sequence of the Lordsdale strain of NLV (16). Nucleotide identity between the two strains was 93.1% (270/290 residues), indicating that the outbreak NLV strain belongs to GGII.

When the stool supernatants stored at -20°C were examined by the Vero cell assay for free bacterial toxins, a CPE consistent with that of C. perfringens enterotoxin was induced by seven samples. The RPLA test confirmed the presence of C. perfringens enterotoxin in all seven samples. The positive specimens had been collected July 18–21, and all were also positive for NLV (Table 4).
All food samples tested were negative for enteropathogenic bacteria. Water samples collected on July 13 from faucets in the bar, the kitchen, and a guest room (Figure 2, point 6) had high levels of coliforms (up to 130 CFU/mL) and fecal streptococci (up to 22 CFU/mL). The same level of contamination was observed in water samples from the pipe connecting the resort to the public water supply (Figure 2, point 2); samples collected from the public water supply outside the resort (Figure 2, point 1) were always negative. After July 15, when chlorine was added to the tank, the level of contamination of tap water inside the resort steadily decreased; no contamination was detectable after superchlorination on July 22.

**Discussion**

Although NLV gastroenteritis epidemics likely occur as frequently in Italy as in the rest of Europe, to our knowledge this is the first outbreak of NLV infection to be confirmed in the country. It affected many guests and employees at a summer vacation resort and involved high attack rates in all age groups. The actual number of cases has likely been underestimated since persons with a mild illness may not have sought medical attention. In fact, the retrospective investigation of staff members showed an attack rate three times higher than in guests.

This outbreak had an unusual pattern, with three regular peaks occurring at constant intervals for 3 weeks. This pattern, which is compatible with a point-source infection (3), may be explained by the rapid turnover of guests and their periodic replacement with susceptible persons in the presence of a constant exposure to infection. Most guests arrived at the resort on a Saturday and stayed 1–2 weeks. Guests who became ill did so a few days after their arrival, suggesting that exposure to a source of infection was relatively constant during the whole period. Moreover, a large proportion of staff members had onset of illness in the first week of the outbreak. The hypothesis of a common source of infection is further supported by the identical nucleotide sequence detected in viruses from eight patients during the outbreak.

Water was the likely source of this outbreak. Environmental inspection identified a breakdown in the water system of the resort, and tap water samples from different places in the resort showed contamination with fecal bacteria. Although microbiologic testing for NLV could not be performed on drinking or recreational water, the presence of fecal bacteria suggests that the water system may have been the actual source of NLV. Despite the possible passage of the virus through several hosts during the outbreak, the genome segment used for diagnosis showed complete stability, suggesting that a very high number of human passages may be required to produce the known nucleotide variability for NLV, at least in the RNA polymerase region.

### Table 2. Attack rates and relative risks for staff members (n=69) according to type of work, tourist resort, Italy, July 2000

<table>
<thead>
<tr>
<th>Type of work</th>
<th>Attack rate (%)</th>
<th>Relative risk</th>
<th>95% CI (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kitchen staff</td>
<td>4/34 (11.8)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Office staff</td>
<td>3/14 (21.4)</td>
<td>1.8</td>
<td>0.5–7.1</td>
</tr>
<tr>
<td>Bar staff</td>
<td>3/11 (27.3)</td>
<td>2.3</td>
<td>0.6–8.8</td>
</tr>
<tr>
<td>Shop assistant</td>
<td>7/21 (33.3)</td>
<td>2.8</td>
<td>0.9–8.5</td>
</tr>
<tr>
<td>Cleaning staff</td>
<td>16/36 (44.4)</td>
<td>3.8</td>
<td>1.4–10.2</td>
</tr>
<tr>
<td>Sports trainers and entertainers</td>
<td>19/37 (51.4)</td>
<td>4.4</td>
<td>1.6–11.5</td>
</tr>
<tr>
<td>Waiters</td>
<td>17/28 (60.7)</td>
<td>5.2</td>
<td>2.0–13.6</td>
</tr>
</tbody>
</table>

\(^a\) CI, confidence interval.

### Table 3. Attack rates and relative risks according to usual behaviors and activities of staff members, tourist resort, Italy, July 2000

<table>
<thead>
<tr>
<th>Exposure</th>
<th>No. (n=69)</th>
<th>No. exposed</th>
<th>Attack rate (%)</th>
<th>Relative risk</th>
<th>95% CI (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shower on the beach</td>
<td>22</td>
<td>14</td>
<td>63.6</td>
<td>1.8</td>
<td>1.2–2.6</td>
</tr>
<tr>
<td>Swimming in the pool</td>
<td>45</td>
<td>22</td>
<td>48.9</td>
<td>1.4</td>
<td>0.9–2.0</td>
</tr>
<tr>
<td>Drinking tap water</td>
<td>104</td>
<td>47</td>
<td>45.2</td>
<td>1.4</td>
<td>0.9–2.2</td>
</tr>
<tr>
<td>Drinks with ice</td>
<td>128</td>
<td>55</td>
<td>43.0</td>
<td>1.8</td>
<td>1.0–3.2</td>
</tr>
<tr>
<td>Swimming in the sea</td>
<td>72</td>
<td>31</td>
<td>43.0</td>
<td>1.2</td>
<td>0.8–1.7</td>
</tr>
<tr>
<td>Eating at resort restaurant</td>
<td>159</td>
<td>64</td>
<td>40.2</td>
<td>1.5</td>
<td>0.5–3.9</td>
</tr>
<tr>
<td>Eating ice cream</td>
<td>140</td>
<td>56</td>
<td>40.0</td>
<td>1.1</td>
<td>0.6–1.9</td>
</tr>
<tr>
<td>Eating meat</td>
<td>151</td>
<td>60</td>
<td>39.7</td>
<td>1.2</td>
<td>0.6–2.4</td>
</tr>
<tr>
<td>Eating salad</td>
<td>123</td>
<td>48</td>
<td>39.0</td>
<td>1.0</td>
<td>0.6–1.6</td>
</tr>
<tr>
<td>Eating fruit</td>
<td>139</td>
<td>54</td>
<td>38.8</td>
<td>1.0</td>
<td>0.6–1.8</td>
</tr>
<tr>
<td>Eating pasta</td>
<td>142</td>
<td>55</td>
<td>38.7</td>
<td>1.2</td>
<td>0.6–2.1</td>
</tr>
<tr>
<td>Consuming drinks on draught</td>
<td>91</td>
<td>35</td>
<td>38.5</td>
<td>1.0</td>
<td>0.7–1.4</td>
</tr>
<tr>
<td>Eating fish</td>
<td>112</td>
<td>40</td>
<td>35.7</td>
<td>0.7</td>
<td>0.5–1.1</td>
</tr>
<tr>
<td>Eating seafood</td>
<td>85</td>
<td>28</td>
<td>32.9</td>
<td>0.7</td>
<td>0.5–1.1</td>
</tr>
</tbody>
</table>

\(^a\) CI, confidence interval.
Some specimens showed evidence of simultaneous infection with NLV and enterotoxigenic \emph{C. perfringens}. The food item(s) that could have been the source of infection by \emph{C. perfringens} remained unknown. However, the presence of \emph{C. perfringens} enterotoxin in a small, defined cluster of patients (7 of 28 stool samples) and the concomitant presence of NLV in the 7 positive stools suggests that \emph{C. perfringens} played only a minor role, if any, in the outbreak.

Control measures to limit the spread of the infection had no effect, probably because they did not address the point source and failed to prevent person-to-person transmission. After July 15, 2000, the consumption of tap water was banned, and only bottled mineral water was served in the resort restaurant and used to wash vegetables. Water from the main tank, however, continued to be used for showers, to make ice for consumption (through July 28), and for irrigation. Furthermore, on July 22, the bypass pipe was removed, the water inside the resort tank underwent superchlorination, and the pipe connecting the resort to the public water supply was shut down. However, NLV do survive high levels of chlorination (3, 8), and the treatment was performed only once, at a late stage of the outbreak, and at only one point upstream of the resort water system. Finally, although the resort was serviced by a mobile tank truck that provided water from the main public water supply, the resort main tank was never emptied and cleaned before treatment. Therefore, after July 22, contaminated residual water could have been gradually diluted by consumption (through July 28), and for irrigation. Furthermore, on July 22, the bypass pipe was removed, the water inside the resort tank underwent superchlorination, and the pipe connecting the resort to the public water supply was shut down. However, NLV do survive high levels of chlorination (3, 8), and the treatment was performed only once, at a late stage of the outbreak, and at only one point upstream of the resort water system. Finally, although the resort was serviced by a mobile tank truck that provided water from the main public water supply, the resort main tank was never emptied and cleaned before treatment. Therefore, after July 22, contaminated residual water could have been gradually diluted by refilling the resort tank with clean water from mobile tanks.

In the cohort study of staff members, having showers on the beach was identified as a risk factor, while consuming drinks with ice was only weakly associated with illness. No exposure to other water sources, including drinking tap water (the use of which was forbidden after July 15) was significant. Our analysis found no evidence that contaminated food was the source of infection: no food preference was associated with an increased risk of being ill, and personnel working in the kitchen had the lowest attack rate.

In addition to water contamination, person-to-person transmission may have played a role in this outbreak. Typically staff members of tourist resorts share the same living quarters and have frequent contact with guests during meals, sport training, entertainment, and other activities. Person-to-person transmission may also explain the fact that the time between arrival and onset of symptoms in guests was longer than the incubation period expected for NLV. Person-to-person transmission of NLV infection is well documented (8), and secondary cases may occur. Airborne and fomite transmission also may facilitate the spread of the virus during outbreaks. Such hypothesis is confirmed by the higher attack rate in the cleaning staff and in staff members working in close contact with guests.

This investigation had several limitations. Since the cohort study was carried out after the outbreak had ended, we could inquire only about food preferences and usual activities rather than actual food histories and activities before the outbreak. Recall bias may have occurred, which may have led to nondifferential misclassification of exposure and underestimation of the observed relative risks. If NLV had spread through water and person-to-person transmission had occurred, virtually everyone in the resort would have been exposed to the agent and any epidemiologic association would be difficult to find. Finally, no test specific for NLV was performed on water samples, and the hypothesis of water as the actual source of infection cannot be confirmed.

In conclusion, this event confirms that large outbreaks due to NLV may be occurring in Italy, but without the use of appropriate diagnostic methods this pathogen may go unrecognized. This occurrence highlights the need for a surveillance system of such outbreaks in cooperation with laboratories capable of diagnosing viral gastrointestinal infections.

This investigation was partly supported by the European Union, under the 5th Framework, Quality of Life Programme (grant QLK1-CT-1999-00594, Food-borne Viruses in Europe).

Dr. Boccia is a microbiologist with the National Public Health Institute in Rome. Her main research interest is the epidemiology of antimicrobial resistance, and she has been frequently involved in outbreak investigations caused by viral and bacterial agents.

### References


Address for correspondence: Delia Boccia, Department of Communicable Disease—Unit of Epidemiology and Biostatistics, Istituto Superiore di Sanità, Viale Regina Elena 299 00161, Roma, Italy; fax: 06 4938 7292; e-mail: boccia@iss.it
Contamination of mussels by hepatitis A virus: 
a public-health problem in southern Italy

L. Croci a,*, D. De Medici a, M. Ciccozzi b, S. Di Pasquale a, E. Suffredini a, L. Toti a

a Laboratorio Alimenti, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy
b Laboratorio Epidemiologia e Biostatistica, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy

Received 12 May 2002; received in revised form 5 October 2002; accepted 7 October 2002

Abstract

The frequency of hepatitis A virus (HAV) contamination in mussels sold in south of Italy, where a high incidence of HAV infection both in resident people and in travelers is reported every year, was investigated during a three-year period. Mussels, collected from the markets of five big cities, were analysed by RT-nested-PCR to detect RNA-HAV and by an integrated method, cell culture-RT-PCR, to confirm the presence of infectious virus. On a total of 180 samples, 15.6% resulted contaminated by infectious HAV.

The high percentage of mussels, potentially dangerous for consumers, and the diffuse habit to consume them raw or slightly cooked, can contribute to maintain the endemic status of HAV infection in some areas of the south of Italy.

© 2003 Elsevier Ltd. All rights reserved.

Keywords: Hepatitis A virus; Contamination; Mussels

1. Introduction

In southern Italy, infection with hepatitis A virus (HAV) continues to be endemic, constituting not only an important public-health problem for the area’s resident population but also a health risk for people travelling to this area. Specifically, according to the National Surveillance System for Acute Viral Hepatitis (referred to as “SEIEVA”), the 464 new cases of HAV infection reported in southern Italy in the year 2000 represented 71.2% of the 652 total hepatitis cases in the same area. Although this percentage represents a decrease with respect to the data for 1998 (i.e., 862/1066 cases; 80.9%), it is obviously still quite high, especially when compared to the national-level data (i.e., 1461/2828 cases (51.7%) in 1998 and 928/1959 cases (47.4%) in 2000).

Besides a high percentage of the persons with HAV infection living in the non-endemic northern Italy have reported travel to an endemic zone as the main risk factor (ranging from 30% in 1998 to 31% in 2000) (http://www.iss.it/english/sanita/index.htm). At the same time shellfish, especially mussels, turn out to be the major risk factor for HAV infection (reported by 68% of infected persons in 2000) (http://www.iss.it/english/sanita/index.htm).

During their natural feeding process, mussels are able to filter large quantities of water, retaining and concentrating in their bodies not only bacteria that are present in the environment but also viruses (Burkhardt III & Calci, 2000; Jofre, 1994; Murphree & Tamplin, 1995; Richards, 1988). However, the European legislation on the safety of shellfish for consumption (Directive of Council 91/492/CEE 1991) only mentions testing for salmonella and coliform bacteria, despite the fact that bacteriological tests do not reveal the presence of viruses (Douglas, Hackeney, Carrick, Lovelace, & Sobsey, 1983; Goyan, Gerba, & Melnick, 1979; Wait, Hackney, Carrick, Lovelace, & Sobsey, 1983) and that HAV has been detected in mussels that otherwise met bacteriological quality standards (Croci et al., 2000). Moreover, the depuration processes used to remove bacteria from mussels before their distribution and sale are not effective for eliminating viruses (Croci, De Medici, Gabrieli, Franco, & Di Pasquale, 1992; De Medici, Ciccozzi, et al., 2001; Franco et al., 1990; Sobsey, Davis, & Rullman, 1987).
The objectives of the present study were to determine the frequency of HAV contamination in mussels sold in southern Italy and to discuss these findings in the light of the incidence of infection and the percentage of hepatitis cases represented by HAV infection among both the resident population of southern Italy and travelers to this area.

2. Materials and methods

2.1. Samples

In the period 1999–2001, mussels (Mytilus galloprovincialis) were collected once a month in five cities in southern Italy (Bari, Foggia, Brindisi, Reggio Calabria and Napoli). In each city, the samples were collected in one authorised food market located in a neighbourhood with a high population density. The samples, which were accompanied by the official certificate of their microbiological suitability for consumption, were stored at −20 °C until use.

The samples were analysed by means of RT-nested-PCR to detect viral RNA. Positive samples were then subjected to an integrated cell culture-RT-PCR procedure to confirm the presence of infectious virus.

2.2. Virus extraction

Mussels were rinsed with sterile distilled water: the body and the liquor were removed and homogenised in a blender (Osterizer Pulse Magic 16, Milwaukee, WI, USA; 30 s at maximum speed). Seventy-five grams of a homogenate were diluted 1:2 in glycine buffer (0.05 M, 2.2 V, USA; 30 s at maximum speed). Seventy-five grams of a blender (Osterizer Pulse Magic 16, Milwaukee, WI, USA) were subjected to an integrated cell culture-RT-PCR procedure.

2.3. RT-nested-PCR

Oligonucleotides: The primers were selected as previously reported (De Medici, Croci, Di Pasquale, Fiore, & Toti, 2001). The primer sequences for PCR (primer pair A, primers 1 and 2) and for nested-PCR (primer pair B, primers 3 and 4) are reported in Table 1. Primer pair A amplified a 415 base pair (bp) region, and primer pair B amplified a 211 bp region.

Negative extraction and purification: RNA extraction and purification were performed as previously described (Croci, De Medici, et al., 1999). In accordance with Afzal and Minor (1994), 334 μl of supernatant were added to a 1.5 ml Eppendorf tube containing 666 μl of 1.5 solution D, and the tubes were vortexed for 20 s to 1 min. One hundred microliters of CsCl cushion (5.7 M solution of CsCl in 25 mM sodium acetate pH 5.0) were gently placed at the bottom of the tube by piercing through the liquid. After centrifugation in an Eppendorf microfuge at 13,000 rpm for 20 min at 4 °C, the supernatant was discarded, and the pellet was washed twice with 1 ml of 70% ethanol and dried.

RT-PCR: The dried pellet was resuspended in 90 μl of RT reaction mixture containing 1X PCR Buffer II (Perkin Elmer, New Jersey, USA), 2.5 mM MgCl2 (Perkin Elmer), 0.25 mM of each deoxynucleoside triphosphate (dNTP) (Takeara-Shuzo, Japan), 20 U of Rnasin (Promega-UK), 1.25 U of AMV reverse transcriptase (Promega) and 100 pmoles of primer antisense; the mixture was incubated at 42 °C for 50 min. The reaction was terminated by heating the mixture at 95 °C for 3 min. One hundred pmoles of primer sense,

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer 1</td>
<td>5'-CAT ATG TAT GGT ATC TCA ACA A-3'</td>
<td>1092–1113</td>
</tr>
<tr>
<td>Primer 2</td>
<td>5'-CAG GGG CAT TTA GGT TT-3'</td>
<td>698–714</td>
</tr>
<tr>
<td>Primer 3</td>
<td>5'-CCA ATT TTG CAA CTT CAT G-3</td>
<td>1029–1047</td>
</tr>
<tr>
<td>Primer 4</td>
<td>5'-TGA TAG GAC TGC AGT GAC T-3'</td>
<td>836–854</td>
</tr>
</tbody>
</table>

*Primers 1 and 2 (primer pair A) were used for PCR, primers 3 and 4 (primer pair B) were used for nested-PCR.

*Position refers to the HM 175 strain (Cohen, Ticehurst, Purcell, Buckler-White, & Baroudy, 1987).
2.5 U of Taq DNA polymerase (Perkin Elmer) and DNase-RNase-free water (Sigma, USA) to a final volume of 100 µl were added. The mixture was subjected to 30 PCR cycles, each consisting of 25 s at 95 °C, 10 s at 49 °C and 1 min at 70 °C. A final extension was carried out for 5 min at 72 °C.

**Nested PCR.** Five microliters of the first amplification reaction were further amplified in 95 µl of reaction mixture containing 1X PCR Buffer II (Perkin Elmer), 2.5 mM MgCl₂, 0.25 mM of each dNTP, 100 pmol of primer anti-sense, 100 pmol of primer sense and 2.5 U of Taq DNA polymerase (Perkin Elmer). The amplification conditions used were those described for the first PCR amplification.

For the purpose to avoid cross-contamination, which can invalidate the results, the different phases of the RT-Nested-PCR were carried out in different rooms, using safe materials (aerosol resistant tips, master solutions etc.) and including negative controls in the protocols.

**Electrophoresis:** Ten microliters of PCR and nested-PCR mixture were analysed by agarose gel electrophoresis (2% agarose; Kodak, New Haven, CT, USA).

### 2.4. Integrated cell culture-RT-PCR procedure for detection of infectious HAV

The positive samples were subjected to determination by means of integrated cell culture-RT-PCR procedure, in order to determine whether the detected viral RNA belonged to infectious viruses, that is, viruses capable of reproducing in cell cultures (Richards, 1999).

Frp3 cells culture (Venuti et al., 1985) were grown with EMEM (Euroclone) supplemented with 10% Fetal Bovine Serum (FBS) (Imperial) at 37 °C and in 5% CO₂ in 25-cm² flasks for three days. To 1 ml of mussel extract, 100x antibiotics—antimycotic (Imperial) solution (1:100 v/v) was added and stored at 4 °C overnight. The same amount of antibiotics—antimycotic (Euroclone) solution was then added, and the sample was maintained at 37 °C for 2 h. This solution was then used to inoculate the cell monolayer, leaving it in contact for 1 h at 37 °C and 5% CO₂. The monolayer was then washed three times with 2 ml of EMEM at 2% FBS, in order to eliminate all of the virus not infecting the cells. After adding 5 ml of EMEM at 2% of FBS, the monolayer was incubated at 37 °C and in 5% CO₂. The cells were observed at regular intervals until the appearance of the cytopathic effect (yet in no case for more than 15 days). Whether the cytopathic effect was present or not, RT-PCR was performed to show the propagation of the virus within the cells, using the PCR primers reported in Table 1 and the conditions described above.

### 2.5. Sequencing

Samples positive for viral RNA, but in which infectious virus was not observed (negative at the integrated method), were subjected to sequencing in order to exclude false positives. The samples were cloned into vector PCR II (TA-Cloning System Invitrogen Co., Carlsbad, California, USA) and sequenced (Sanger, Nicklen, & Coulson, 1992) using the Sequenase PCR Product kit (Amersham Pharmacia Biotech AB, Upsala, Sweden) to confirm their identity.

### 3. Results

The results of the laboratory analysis for detecting HAV in mussels are reported in Table 2.

Of the 180 samples analysed, 113 (62.8%) were negative and 67 (37.2%) contained HAV-RNA; of these 28 (15.6% of the total samples) also resulted positive in the integrated procedure, demonstrating the presence of whole virus capable of infecting the cells, hence the dangerousness of the sample (Lees, 2000; Richards, 1999).

In the remaining 39 samples resulting positive by the screening method, PRT-nested-PCR, the sequencing confirmed that the positivity was due to the presence of HAV-RNA.

During the three year study period, the percentage of positive samples for the presence of infectious virus basically remained stable, varying from 18.3% in 1999 to 13.3% in 2001.

### 4. Discussion

The presence of HAV-RNA in a great number of samples (37.2%), although most of them probably does

---

Table 2

<table>
<thead>
<tr>
<th>Year</th>
<th>Samples size</th>
<th>Samples negative</th>
<th>Samples positive for HAV-RNA</th>
<th>Samples positive for infectious HAV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999</td>
<td>60</td>
<td>39 (65%)</td>
<td>21 (35%)</td>
<td>11 (18.3%)</td>
</tr>
<tr>
<td>2000</td>
<td>60</td>
<td>36 (60%)</td>
<td>24 (40%)</td>
<td>9 (15%)</td>
</tr>
<tr>
<td>2001</td>
<td>60</td>
<td>38 (63.4%)</td>
<td>22 (36.7%)</td>
<td>8 (13.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>180</td>
<td>113 (62.8%)</td>
<td>67 (37.2%)</td>
<td>28 (15.6%)</td>
</tr>
</tbody>
</table>
not represent a health risk for the consumer (Lees, 2000; Richards, 1999), does indicate on origin of the mussels from a contaminated environment and, at the same time, a high circulation rate of the virus. The percentage of samples containing the infectious virus (15.6%) is still quite high and, although it was not possible to quantify the virus, its presence in mussels represents a health hazard, since even a few viral particles can cause disease, depending on the susceptibility of the host (Cuthbert, 2001).

Given that the mussels were already commercially available, and thus packaged with a certificate of suitability for consumption, which, for microbiological requirements, is based on the determination of bacteriological parameters (Es. coli and Salmonella), the results of our study confirm that the presence of E. coli is inadequate as an indicator of viral contamination (Croci et al., 2000) and that the depuration processes used for mussels prior to their sale are not completely effective against viruses (Croci et al., 1992; De Medici et al., 2001; Richards, 1988). Despite this, European current legislation does not include the detection of enteric viruses as part of routine controls.

On these grounds, it can be assumed that the widespread habit of consuming raw or slightly cooked shellfish contributes to maintaining the incidence of hepatitis A cases in the southern Italy at high level. At the same time there has been an increase on the percentage of cases reported among people living in non-endemic zones who had travelled to endemic zones, that for example was 31% in 2000, of which 57.7% reported shellfish consumption (http://www.iss.it/english/sanita/index.htm).

Thus, to reduce the incidence of HAV infection, targeted consumer-information campaigns must be strengthened (Cuthbert, 2001), including the promotion of suitable procedures of food preparation and consumption. In particular, mussels should be eaten only after they have been adequately cooked, paying attention to the duration and temperature of cooking, given that the flesh of molluscs can protect the virus from the inactivating effects of heat (Croci, Ciccozzi, et al., 1999; Millard, Appleton, & Parry, 1987). Moreover, immunisation against HAV for people travelling to endemic areas is strongly recommended, as well as is the use of vaccine in the prevention of household secondary cases (Stroffolini, Mele, & Sagliocco, 2001).

Acknowledgements

The authors gratefully acknowledge Mr. Mark Kanieff for the editorial assistance. This work was carried out as part of project “Foodborne viruses in Europe” (EU contract QLKI-CT-1999-00594) supported by EU 5th framework program.

References


Molecular Epidemiology of Human Calicivirus Gastroenteritis Outbreaks in Hungary, 1998 to 2000

Gábor Reuter, Tibor Farkas, Tamás Berke, Xi Jiang, David O. Matson, and György Szűcs

1Regional Laboratory of Virology, Baranya County Institute of State Public Health Service, Pécs, Hungary
2Department of Medical Microbiology and Immunology, Faculty of General Medicine, University of Pécs, Pécs, Hungary
3Center for Pediatric Research, Children’s Hospital of The King’s Daughters and Eastern Virginia Medical School, Norfolk, Virginia

Between November 1998 and November 2000, 196 stool specimens from 21 outbreaks of acute nonbacterial gastroenteritis occurring in 11 of the 19 counties of Hungary were collected and tested for human caliciviruses. Human caliciviruses were detected and characterized by a type-common enzyme-linked immunosorbent assay (EIA) and reverse transcription-polymerase chain reaction (RT-PCR) followed by cloning and sequencing. Twenty (95%) and 14 (67%) outbreaks were positive by EIA and RT-PCR, respectively, and 12 RT-PCR-positive outbreaks were also confirmed by sequencing. Comparative sequence analysis revealed 13 Norwalk-like virus sequences in the 12 outbreaks, including 11 Norwalk-like virus genogroup II (seven in Hawaii-like, two Lordsdale-like, one Melksham-like, and one Hillingdon-like) and two Norwalk-like virus genogroup I (related to Southampton-like and Desert Shield-like clusters) viruses. Multiple Norwalk-like virus clusters, with a predominance of Hawaii-like viruses, played an important role in nonbacterial gastroenteritis outbreaks during the study period. This is the first country-wide molecular epidemiological investigation of human calicivirus-associated, gastroenteritis outbreaks in Hungary and Central-Eastern Europe. J. Med. Virol. 68:390–398, 2002. © 2002 Wiley-Liss, Inc.

KEY WORDS: EIA; RT-PCR; sequencing; Norwalk-like viruses

INTRODUCTION

Human caliciviruses are major causes of acute, nonbacterial gastroenteritis in all age groups worldwide [Glass et al., 2000]. These viruses include two genera within Caliciviridae, “Norwalk-like viruses” (NLVs) and “Sapporo-like viruses” (SLVs) [Berke et al., 1997; Green et al., 2000]. “Norwalk-like viruses” have been divided tentatively into genogroups I and II and both genogroups can be divided further into several genetic clusters (examples in genogroup I include: Norwalk/68 [Jiang et al., 1990], Desert Shield/90 [Lew et al., 1994], Southampton/91 [Lambden et al., 1993] viruses; in genogroup II, Snow Mountain/76 [Hardy et al., 1997], Hawaii/71 [Green et al., 1997], and Lordsdale/93 [Dingle et al., 1995] viruses) suggesting that human caliciviruses are diverse genetically and probably antigenically [Ando et al., 2000; Jiang et al., 2000]. Gastroenteritis due to human caliciviruses is usually mild to moderately severe and self-limited; many infections are subclinical [Greenberg et al., 1990; Sakai et al., 2001]. The worldwide incidence of human calicivirus infections is unknown. Human calicivirus-associated outbreaks can involve thousands of persons and result from common exposures to contaminated food and water [Mead et al., 1999; Deneen et al., 2000]. Person-to-person spread is also important [Caul, 1996]. In 1999, human caliciviruses caused an estimated 23 million food-related illnesses just in the United States [Mead et al., 1999]. Most human calicivirus infections are detected in winter months in temperate regions [Mounts et al., 2000].

RT-PCR and EIA methods developed recently for diagnosing human calicivirus infections have changed markedly our understanding of human calicivirus epidemiology. Norwalk-like viruses have been found in 70–90% of selected nonbacterial gastroenteritis outbreaks in surveillance studies from several countries [Vinje and Koopmans, 1996; Vinje et al., 1997; Fankhauser et al., 1998; Maguire et al., 1999; Gonin et al., 2000; Hedlund et al., 2000; Iritani et al., 2000; Nakata et al., 2000].

Grant sponsor: Ministry of Health, Hungary; Grant number: ETT (T-08) 118/2000; Grant sponsor: U.S. Public Health Service; Grant numbers: AI37093, HD13021.

*Correspondence to: György Szűcs, MD, PhD, Regional Laboratory of Virology, Baranya County Institute of State Public Health Service, Szabadság u. 7., Pécs, Hungary, H-7623. E-mail: gszuocs@main.antszbar.hu

Accepted 21 May 2002
DOI 10.1002/jmv.10216
Published online in Wiley InterScience (www.interscience.wiley.com)
These methods were applied in Hungary, where surveys for human caliciviruses as etiologic agents of gastroenteritis cases and outbreaks had not been done. According to the Hungarian infectious disease surveillance database, in 1999 and 2000, 48 and 37%, respectively, of reported enteric infectious diseases were caused by bacterial pathogens, 0.2 and 0.1%, respectively, by parasitic agents, and more than half (51 and 62%, respectively) by undetermined pathogens [Csohán et al., 2000, 2001]. The objectives of this study were to investigate the incidence and epidemiological characteristics of human calicivirus-associated gastroenteritis outbreaks in Hungary and characterize the detected strains by sequence and phylogenetic analysis.

MATERIALS AND METHODS

Outbreak Stool Specimens and Epidemiological Data

Specimens from outbreak cases designated as non-bacterial “enteritis infectiosa” of unknown etiology were sent to our laboratory by county or city epidemiologists (Institutes of State Public Health Service). Stool samples collected from patients with gastroenteritis were tested for rotavirus (latex agglutination) and cultured for Salmonella spp., Shigella spp., Campylobacter spp., E. coli, Y. enterocolitica, and S. aureus at the referring public health laboratory, then stored at −20°C until human calicivirus testing. After verification of human calicivirus as an etiologic agent in the samples, a standard questionnaire form was sent soliciting epidemiological and clinical data about the outbreaks according to Centers for Disease Control and Prevention (CDC) protocols [CDC, 1990]. Specimens collected before 2000 also were tested for adenovirus (latex agglutination) and enteroviruses (culture) in our laboratory.

RT-PCR

RNA was extracted from stool specimens using TRIzol Reagent (Life Technologies Gibco BRL, Grand Island, NY) according to the manufacturer’s protocol. Primer pairs p289/290 and p289/290A based upon the RNA polymerase sequences (in open reading frame 1) of Norwalk-like viruses and Sapporo-like viruses, designed by Jiang et al. were used [Jiang et al., 1999]. The RT reaction was carried out in 50 μl of reaction mixture containing 5 μl of 10× PCR buffer (100 mM Tris-HCl, pH 8.3, 20 mM MgCl₂, 500 mM KCl; 0.01% gelatine; Sigma, Saint Louis, MO), 2 μl of dNTP (10 mM), 2 μl of negative-sense primer (p289, 0.1 μg/μl), 0.25 μl rRNAse (40 U/μl), 0.7 μl M-MLV-RT (200 U/μl), 36 μl of nuclease-free water, and 3 μl of the extracted RNA for 1 hr at 42°C. For PCR, 50 μl of 1× PCR buffer containing 2 μl positive-sense primer (p290 or p290A, 0.1 μg/μl) and Taq polymerase (5 U/μl; Dupl-A-Taq DNA Polymerase, Zenon Biotechnologie Kft., Szeged, Hungary) were added. Where not separately indicated, all reagents were purchased from Promega, Madison, WI. The thermocycle program included 3 min at 94°C, 40 cycles for 30 sec at 94°C, 90 sec at 49°C, and 60 sec at 72°C, and a final 10-min extension at 72°C, in a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Watertown, MA). RT-PCR products were analysed after gel electrophoresis using a 1.5% agarose gel (NuSieve 3:1 agarose, FMC Bioproducts, Rockland, ME), TBE buffer with ethidium bromide, and ultraviolet light visualization by BioCapt Gel Documentation program (Version 97.05 for Windows, 1997). Nuclease-free water and RNA from a human calicivirus-positive stool were used as negative and positive controls, respectively, in each RT-PCR reaction. Both primer pairs produce RT-PCR products of 319 bp for Norwalk-like viruses and 331 bp for Sapporo-like viruses [Jiang et al., 1999].

Cloning and Sequencing of RT-PCR Products

One to three RT-PCR products from each outbreak were cloned into pGEM-T II (Promega) according to the manufacturer’s protocol. Positive clones were identified by PCR screening. Two purified [Sambrook et al., 1989] clones per sample were sequenced using M13 forward and reverse primers by the chain termination method (SequiTherm EXCEL™ II Long-Read™ DNA Sequencing Kit-ALF™, Epicentre Technologies, Madison, WI) on an automated sequencer (Pharmacia Biotech ALFexpress™ DNA Sequencer, Amersham Pharmacia Biotech, Uppsala, Sweden).

Sequence and Phylogenetic Analysis

The genetic identity of the strains was determined by comparison of their sequences with sequences in the GenBank. Pair-wise alignments and comparative sequence analysis were run in the OMIGA 2.0 program (Oxford Molecular Ltd, Oxford, UK). A dendrogram was constructed using the UPGMA clustering method with distance calculation using the Jukes-Cantor correction for evolutionary rate by Molecular Evolutionary Genetics Analysis (MEGA version 2.1) [Kumar et al., 2001]. The 274-nr of polymerase region sequence of the HUNo1, HUNo2, HUNo4, HUNo10, HUNo11a, and HUNo11b strains were obtained from the GenBank with accession numbers AF472566 to AF472571, respectively. Reference strains and accession numbers from GenBank used in this analysis are: Norwalk/68/US (NV, M87661), Southampton/91/UK (South, L07418), Desert Shield/90/Saudi Arabia (DSV, U04469), Lordsdale/93/UK (LV, X86557), Mexico/89/MX (MX, U22498), Hawaii/71/US (HV, U07611), Snow Mountain/76/US (SMV, U70059), Melksham/94/UK (Melk, X81879), 12C/92/UK (L25111), TI-96-J (Hillingdon-like, AB020558), and MOH/99/HUN (AF397156). The outgroup strain was Sapporo/82/JP (SAPP, S77903).

EIA Specimens

One to four stool specimens from each outbreak, including only RT-PCR-positive samples from RT-PCR-positive outbreaks, were tested for Norwalk-like virus.
antigens utilizing a type-common antigen-detection EIA [Jiang et al., 2001]. The assay uses pooled hyper-immune antisera from rabbits as capture antibody and as detector antibody antisera from guinea pigs cross-immunized with recombinant baculovirus-expressed human calicivirus capsid antigens (virus-like particles) from nine representative Norwalk-like virus strains [three genogroup I: Norwalk virus (NC_001959), VA98115 (AY038598), and C59 (AF435807) and six genogroup II: MX virus (U22498), HV virus (U07611), VA97207 (AY038599), VA98387 (AY038600), Grimsby virus (AJ004864), and MOH strain (AF397156)] [Jiang et al., 2001]. Positive reactions were determined by an optical density (OD 450) value >0.2 in the well coated with hyper-immune antisera and a post-immune/pre-immune ratio (P/N) >5.0 between OD450 values in the wells coated with hyperimmune and pre-immune sera, respectively.

**Statistical Methods**

Epidemiological and clinical data were analyzed for statistical significance using the chi-square test with Yates’ correction or the chi-square for the trend (Epi Info, version 6.0, CDC). P values of ≤0.05 were considered statistically significant.

**RESULTS**

**Investigations for Bacteria and Viruses Other Than Caliciviruses in Outbreaks**

Samples from 21 acute nonbacterial gastroenteritis outbreaks between November 1998 and November 2000 were submitted and came from 11 of 19 counties of Hungary (15 outbreaks in six counties in Transdanubia, West-Hungary; five outbreaks in four counties in East-Hungary; and one in Budapest, the capital). These outbreaks had epidemiological (short duration of illness) and clinical signs (predominance of vomiting and diarrhoea) characteristic of human caliciviruses. Of the 21 outbreaks, three were investigated in 1998, seven in 1999, and 11 in 2000 (Table I). Bacterial cultures and rotavirus testing of all outbreaks were negative except for one outbreak in which *Campylobacter jejuni* (N = 1) and *C. lari* (N = 2) were detected in 3 of 51 stool samples (Table I). Before January 2000, no outbreaks contained adenovirus, but in the outbreak at Székesfehérvár in 1999, *Reuter et al.*

**Table I. Description, Epidemiological Findings, and RT-PCR and EIA Laboratory Diagnosis of Acute Gastroenteritis Outbreaks in Hungary Between 1998 and 2000**

<table>
<thead>
<tr>
<th>Outbreaks (locations)</th>
<th>Mo/yr</th>
<th>Settings</th>
<th>Attack rate (ill/risk)</th>
<th>Mode of transmission**</th>
<th>RT-PCR (pos./tested)a</th>
<th>EIA (pos./tested)b</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RT-PCR-positive</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Szeged and Algőy</td>
<td>11/1998</td>
<td>2 nurseries/1 school</td>
<td>80/NA</td>
<td>F</td>
<td>5/5c</td>
<td>2/2</td>
</tr>
<tr>
<td>2. Kiskunhalas</td>
<td>1/1999</td>
<td>Hospital (U)*</td>
<td>&gt;5/NA</td>
<td>NA</td>
<td>4/5d</td>
<td>4/4</td>
</tr>
<tr>
<td>3. Székeszéd</td>
<td>5/1999</td>
<td>Elderly home</td>
<td>&gt;5/NA</td>
<td>W</td>
<td>1/5e</td>
<td>1/1</td>
</tr>
<tr>
<td>4. Pak</td>
<td>6/1999</td>
<td>Elderly home</td>
<td>13/NA</td>
<td>NA</td>
<td>4/5</td>
<td>1/1</td>
</tr>
<tr>
<td>5. Oroslány</td>
<td>8/1999</td>
<td>Family</td>
<td>8/NA</td>
<td>PP</td>
<td>1/1</td>
<td>1/1</td>
</tr>
<tr>
<td>14. Tab</td>
<td>11/2000</td>
<td>Primary school</td>
<td>36/204</td>
<td>F, PP</td>
<td>2/7</td>
<td>2/2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>73/133 (55%)</td>
<td>33/34 (97%)</td>
</tr>
</tbody>
</table>

| **RT-PCR-negative**  |       |          |                       |                        |                        |                   |
| 15. Eger             | 11/1998 | Hospital (U) | 0/6 | NA | 0/6 | 3/3 |
| 16. Boly             | 12/1998 | Nursery | 0/5 | NA | 0/3 | 3/3 |
| 17. Székesfehérvár   | 3/1999  | Hospital (U) | 0/14 | NA | 0/3 | 3/3 |
| 18. Balatonnelle     | 7/1999  | Children’s camp | 0/9 | NA | 0/9 | 2/2 |
| 19. Kozármisleny     | 10/1999 | Nursery | 0/9 | NA | 0/9 | 1/1 |
| 20. Pécs             | 3/2000  | Secondary school | 0/15 | NA | 0/3 | 1/3 |
| 21. Szeged           | 5/2000  | Mentally challenged/Elderly home | 0/5 | NA | 0/5 | 2/3 |
| **Total**            |        |           |                       |                        | 0/63 (0%) | 12/18 (67%) |

*a*: chronic psychiatric; *M*: 8 wards and 4 services; *R*: motion rehabilitation; *H*: heart surgery; *U*: unknown.

**A**: aerosol; *F*: food-borne; *PP*: person-to-person; *W*: waterborne; *NA*: not available data.

**RT-PCR-positive (with p289/p290 and/or p289/290A)/all tested stool specimens.

**RT-PCR-negative (with p289/p290 and/or p289/290A)/al tested stool specimens.

*Positive by EIA if the OD_{450} value >0.2 and post-immune/pre-immune ratio >5.0.

*Other 3/51 samples contained *Campylobacter jejuni*.

*Primer-set p289/p290A designed for detection of human caliciviruses detected also astrovirus type 1 only from the fifth sample by sequencing.

*3/5 samples also contained untyped enteroviruses.
60% of the stool samples contained enteroviruses (not typed).

Detection of Human Caliciviruses by RT-PCR

A total of 196 specimens were tested by RT-PCR. Amplicons with an expected size for human caliciviruses were found in 73 (37%) of the 196 stool samples and another sample contained RT-PCR products not of the predicted size (Table I). All of the human calicivirus-associated RT-PCR products were 319-bp in size. From 1 of 5 stool samples collected in Kiskunhalas, three different amplicons of unexpected sizes (268-bp, ~360-bp, and ~1.6-kb), were amplified by p289/p290A. The two smaller amplicons were cloned and sequenced and both products were derived from an astrovirus, representing nts 3,716 to 3,939 and 667 to 864 of open reading frame 1b (polymerase) and open reading frame 1a regions of human astrovirus type 1 (NC_001943), respectively. Seventeen (67%) of the 21 outbreaks were RT-PCR-positive for human caliciviruses: 1 (33%) of 3 in 1998, 4 (57%) of 7 in 1999, and 9 (82%) of 11 in 2000 ($\chi^2 = 2.8; P = .095$, for trend of increasing human calicivirus detection by year). Nine human calicivirus outbreaks were diagnosed successfully concurrently with the outbreak and five outbreaks were identified retrospectively. In the RT-PCR-positive outbreaks, 73 (55%) of 133 stool samples tested positive (range 20 to 100%).

Sequence Analysis and Genetic Variability of Human Caliciviruses

The internal 274-nt between the two primers of the RNA polymerase region were sequenced. There were 13 Norwalk-like virus sequences in 12 outbreaks, including 11 from genogroup I and two from genogroup II clusters (Table II). Except for the outbreak in Veszprém, in which we characterized two strains (one each genogroup I and genogroup II) from two individuals, all other outbreaks were caused by a single Norwalk-like virus type. In pair-wise comparisons, the majority of outbreak strains (7/12; 58%) circulating in Transdanubia (West-Hungary; Fig. 1) between 1999 and 2000 were most closely related to prototype Hawaii virus (90–91% nucleotide [nt] and 96–97% amino acid [aa] sequence identity) and closely related to each other (96–99% nt and 98–100% aa sequence identity). Two strains from East-Hungary (Fig. 1) had 91% nt and 95–96% aa sequence identity with the reference Lordsdale prototype. HUNo11b was most closely related to Melksham (92% nt and 98% aa sequence identity) and 12C/92/UK strains (L25111; 93% nt and 96% aa sequence identity) in GenBank. MOH was most closely related (98% nt and 100% aa identity) to TI-96-J, a Hillingdon-like strain. The genogroup I strains, which have less than 80% nucleotide identity to prototype strains (HUNo2 79/97% nt/aa identity to Desert Shield and HUNo11a 77/94% to Southampton; Table II), matched well in nucleotide and amino acid identity to the following strains published in the GenBank: HUNo2 strain from January 1999 with 95% nt and 100% aa identity to Cts1A-1995-JP (AB019262) from Japan and HUNo11a strain from October 2000 with 98% nt and 100% aa identity to HakiE-2000-JP (AB046350) Japanese strain, respectively (data not shown). The phylogenetic relationships among the study strains and the prototypes in the 274-bp fragment of the RNA polymerase region are shown in Figure 2. HUNo11a and two genetically identical HUNo11b strains were detected in three stool samples from the largest outbreak (253 ill persons) in Veszprém. “Sapporo-like viruses” were not detected in the outbreaks.

Detection of Norwalk-Like Viruses by EIA

No primer pair amplifies all circulating human caliciviruses. Accordingly, an EIA broadly reactive for Norwalk-like viruses was employed to detect human

<table>
<thead>
<tr>
<th>Outbreak</th>
<th>Strain</th>
<th>NV</th>
<th>South</th>
<th>DSV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Szeged-Algyo</td>
<td>HUNo1</td>
<td>58/58</td>
<td>58/61</td>
<td>60/61</td>
</tr>
<tr>
<td>2. Kiskunhalas</td>
<td>HUNo2</td>
<td>76/83</td>
<td>71/85</td>
<td>[79/97]</td>
</tr>
<tr>
<td>3. Szekszard</td>
<td>HUNo3</td>
<td>57/59</td>
<td>58/62</td>
<td>59/62</td>
</tr>
<tr>
<td>4. Pak</td>
<td>HUNo4</td>
<td>57/59</td>
<td>58/62</td>
<td>59/62</td>
</tr>
<tr>
<td>5. Oroszlany</td>
<td>MOH</td>
<td>58/59</td>
<td>60/61</td>
<td>57/57</td>
</tr>
<tr>
<td>6. Szkesfehervar</td>
<td>HUNo6</td>
<td>57/59</td>
<td>58/62</td>
<td>59/62</td>
</tr>
<tr>
<td>7. Budapest</td>
<td>HUNo7</td>
<td>57/59</td>
<td>58/62</td>
<td>59/62</td>
</tr>
<tr>
<td>8. Debrecen</td>
<td>HUNo8</td>
<td>59/58</td>
<td>58/62</td>
<td>59/61</td>
</tr>
<tr>
<td>9. Siofok</td>
<td>HUNo9</td>
<td>56/59</td>
<td>57/62</td>
<td>58/62</td>
</tr>
<tr>
<td>10. Patalom</td>
<td>HUNo10</td>
<td>57/59</td>
<td>57/62</td>
<td>59/62</td>
</tr>
<tr>
<td>11. Veszprém</td>
<td>HUNo11a</td>
<td>72/89</td>
<td>[77/94]</td>
<td>71/81</td>
</tr>
<tr>
<td>12. Veszprém</td>
<td>HUNo11b</td>
<td>55/58</td>
<td>56/59</td>
<td>58/60</td>
</tr>
<tr>
<td>13. Szigtavár</td>
<td>HUNo12</td>
<td>58/59</td>
<td>58/62</td>
<td>59/62</td>
</tr>
</tbody>
</table>

*The boxes indicated the highest nucleotide identity in a row. MOH strain detected by T. Farkas and T. Berke. NV, Norwalk; South, Southampton; DSV, Desert Shield; SMV, Snow Mountain; LV, Lordsdale; HV, Hawaii; Melk, Melksham; Hill, Hillingdon-like (TI-96-J, AB020558); MX, Mexico.

TABLE II. Percent Nucleotide/Amino Acid Identity in the RNA Polymerase Region Between the Outbreaks Strains and the Representative Reference Strains of Norwalk-Like Viruses

<table>
<thead>
<tr>
<th>Outbreak</th>
<th>Strain</th>
<th>Genogroup I</th>
<th>Genogroup II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SMV</td>
<td>LV</td>
</tr>
<tr>
<td>1. Szeged-Algyo</td>
<td>HUNo1</td>
<td>81/93</td>
<td>[91/96]</td>
</tr>
<tr>
<td>2. Kiskunhalas</td>
<td>HUNo2</td>
<td>83/60</td>
<td>60/63</td>
</tr>
<tr>
<td>3. Szekszard</td>
<td>HUNo3</td>
<td>82/95</td>
<td>87/98</td>
</tr>
<tr>
<td>4. Pak</td>
<td>HUNo4</td>
<td>82/95</td>
<td>86/98</td>
</tr>
<tr>
<td>5. Oroszlany</td>
<td>MOH</td>
<td>77/86</td>
<td>75/87</td>
</tr>
<tr>
<td>6. Szkesfehervar</td>
<td>HUNo6</td>
<td>81/95</td>
<td>86/98</td>
</tr>
<tr>
<td>7. Budapest</td>
<td>HUNo7</td>
<td>82/95</td>
<td>86/98</td>
</tr>
<tr>
<td>8. Debrecen</td>
<td>HUNo8</td>
<td>80/92</td>
<td>[91/96]</td>
</tr>
<tr>
<td>9. Siofok</td>
<td>HUNo9</td>
<td>82/95</td>
<td>87/98</td>
</tr>
<tr>
<td>10. Patalom</td>
<td>HUNo10</td>
<td>82/95</td>
<td>87/98</td>
</tr>
<tr>
<td>11. Veszprém</td>
<td>HUNo11a</td>
<td>75/80</td>
<td>58/60</td>
</tr>
<tr>
<td>12. Veszprém</td>
<td>HUNo11b</td>
<td>75/80</td>
<td>57/59</td>
</tr>
<tr>
<td>13. Szigtavár</td>
<td>HUNo12</td>
<td>81/94</td>
<td>86/97</td>
</tr>
</tbody>
</table>
caliciviruses. Forty-five (86%) of 52 (34 RT-PCR-positive and 18 RT-PCR-negative) tested stool samples in 20 (95%) of 21 outbreaks were positive in the type-common EIA. In other words, all 14 of the RT-PCR-positive outbreaks and all of the RT-PCR-negative outbreaks, except one outbreak in a hospital in Székesfehérvár, were positive by EIA (Table I). Thirty-three (97%) of 34 RT-PCR-positive stool samples were positive by EIA, the exception being one of two stool samples received from Veszprém, which contained HUNo11b strain ($P = 0.171$ and $P/N = 6.8$ in the EIA) genetically identical to that in an EIA-positive stool specimen from the same outbreak. OD$_{450}$ values of the RT-PCR/EIA-positive samples were between 0.22 and 3.55 and the P/N ratios were between 6.1 and 161. The OD$_{450}$ values of RT-PCR-negative/EIA-positive stool samples were between 0.5 and 3.29 and the P/N ratios were between 10.4 and 48.8 (data not shown).

Fig. 1. Geographic location of the investigated human calicivirus outbreaks (N = 14) with RT-PCR-positive samples in 10 of 19 Hungarian counties. Each marker represents one outbreak and the genetic cluster (squares: Hawaii, triangles: Lordsdale, circles: other Norwalk-like viruses) of the outbreak strain. White markers represent outbreaks in 1998, gray in 1999, and black in 2000.

Fig. 2. Phylogenetic relationships based on a 274-nt region of the RNA-dependent polymerase gene (Norwalk virus 4590 to 4865 nt) showing the relationships among the Hungarian outbreak strains and prototype human caliciviruses. Bootstrapped datasets (N = 1,000) were analyzed and evolutionary trees were drawn using the UPGMA clustering method. Bootstraps values of the internal nodes are indicated.
The single stool sample from the outbreak in Kiskunhalas that contained human astrovirus by RT-PCR for human caliciviruses was positive in the type-common Norwalk-like virus EIA ($P = 1.456$ and $P/N = 20$) and positive by an EIA for human astroviruses (F. Jakab, personal communication).

**Epidemiological Data**

The 14 outbreaks with RT-PCR-positive samples were located in 10 of 19 counties and in 14 different towns and villages in Hungary (Fig. 1) in closed and semi-closed communities. Ten of 15 outbreaks were in six counties in Transdanubia, West-Hungary; three of five outbreaks in three counties in East-Hungary; and one outbreak in Budapest, the capital. Table I shows the epidemiological characteristics of the outbreaks. Most of the human calicivirus outbreaks occurred in hospital-social welfare institutions (5/14, 36%) and homes for the elderly (4/14, 29%). Two outbreaks were in child care facilities (nursery and elementary school, 2/14, 14%) and one outbreak each was in a camp for children and in a family (1/14, 7%). In the 20 outbreaks with EIA-positive samples, hospitals (6, 30%), nurseries, schools (6, 30%), homes for the elderly (5, 25%), camps (2, 10%), and family (1, 5%) were involved. Between 28 and 64 persons were affected in most of the RT-PCR-positive human calicivirus outbreaks; altogether more than 730 persons were ill. The smallest outbreak was in 1999 in a family with eight ill persons [Farkas et al., 2002] and the largest outbreak was in 2000, at an elementary school in Veszprém with 253 ill persons. Most of the illnesses were in children of preschool age (4 to 6 years; N = 84, 14%), young adults and adolescents (10 and 23 years; N = 338, 56%), and the elderly (70 years; N = 87, 14%) among 604 ill persons with known age. Except for one outbreak observed in January, 13 outbreaks occurred between May and November, mainly in May (Table I). The probable mode of transmission was known in 10 of 14 RT-PCR-positive outbreaks (Table I). In seven outbreaks, the virus spread mainly by indirect and person-to-person contact. Three outbreaks were food-borne and one was probably waterborne. Secondary spread by aerosolized vomitus apparently occurred in two outbreaks. Secondary infections were reported in nursery and school outbreaks, from child to family members. Large nosocomial hospital outbreaks lasted more than two weeks and started partly from and were spread by hospital staff, according to epidemiological data.

Attack rates in the human calicivirus outbreaks were between 7 and 40%. The main symptoms generally were more pronounced in late evening to midnight and were, by order of occurrence: diarrhoea (median: 75%; range: 16–100%), vomiting (median: 68%; range: 47–94%), nausea (median: 60%; range: 8.6–95%), abdominal pain (median: 52%; range: 20–85%), fever ($\geq 37.5^\circ\text{C}$; median: 24%; range: 3–55%) and mild fever ($< 37.5^\circ\text{C}$; median: 20%; range: 2–50%). Generally, patients had some, but not all symptoms. In the two age groups most affected, the clinical picture was different: vomiting occurred more frequently in children (85%) than adults (51%; $\chi^2 = 27$; $P < .001$), diarrhoea was more common in adults over 18 years of age (91%) than in children (35%; $\chi^2 = 67$; $P < .001$), and abdominal pain was more common in children (61%) than adults (45%; $\chi^2 = 5$; $P < .05$). Fever (26 and 21%) and mild fever (20 and 19%) were comparably prevalent among children and adults, respectively. The mean incubation period was 1–3 days and the mean duration of illness was 1–4 days. Hospitalization occurred in 1–3% of cases in children and in 1–2% of persons $>70$ years old. No association was found between the clinical symptoms, signs, or severity and the Norwalk-like virus genotype. About a quarter (23%) of the contact persons had a clinical symptom.

**DISCUSSION**

This is the first report of the molecular epidemiology of human calicivirus outbreaks in Hungary and covers a two-year period. Norwalk-like viruses were detected by EIA in 95% and by RT-PCR in 67% of the 21 nonbacterial outbreaks investigated. The RT-PCR detection rate of Norwalk-like viruses in the outbreaks was 55% of stool samples collected from RT-PCR-positive outbreaks, which is slightly higher than the 49% rate obtained by Fankhauser et al. [1998], but less than the 65 and 66% positivity rates reported by Gonin et al. [2000] and Iritani et al. [2000], respectively. Norwalk-like viruses have been found in one- to three-year surveillance studies to cause 70–90% of selected gastroenteritis outbreaks in the United States, Canada, the Netherlands, UK, Sweden, and Japan [Vinjé and Koopmans, 1996; Vinjé et al., 1997; Fankhauser et al., 1998; Maguire et al., 1999; Gonin et al., 2000; Hedlund et al., 2000; Iritani et al., 2000; Nakata et al., 2000]. Our results indicate that human caliciviruses also cause a high percentage of nonbacterial gastroenteritis outbreaks in a moderately developed Central-European country. The percentage of human calicivirus gastroenteritis outbreaks increased year-by-year in our study period as municipal health services became aware of our investigations and as specimens from bacteria- and rotavirus-negative outbreaks were selected.

We investigated human caliciviruses as a cause of “enteritis infectiosa” outbreaks of unknown etiology. In Hungary, between 1998 and 2000, 13,879; 25,629; and 35,080 cases of this syndrome were reported each year, which accounted for more than a half (51–62%) of reported gastroenteritis cases in a population of 10.2 million persons. These number would yield morbidity rates of 254 and 349/100,000 persons (range 90 to 991 in different counties) in 1999 and 2000, respectively [Csohán et al., 2000, 2001]. Because human calicivirus infections usually are of mild-to-moderate severity, the incidence of the disease in the community likely is higher. Annual reported gastroenteritis outbreaks in Hungary numbered 74, 94, and 82 from 1998 to 2000, with more than 10 affected persons per outbreak.
strains [Vinje´ and Koopmans, 1996; Vinje´ et al., 1997; group II strains over Norwalk-like virus genogroup I that Norwalk-like virus clusters were predominant Hawaii-like virus. Molecular epidemiological studies of genogroup I and 4 genogroup II clusters) were present that a minimum of six different genetic clusters (2 respectively. Sequence and phylogenetic analysis revealed of the 13 detected strains in genogroup II and I, respec-
''Norwalk-like virus'' genus, with 11 (85%) and 2 (15%) our study showed that all strains belonged to the of the polymerase region of the human caliciviruses in
Although this region is variable, it is the most con-
derived from both Norwalk-like virus and Sapporo-like viruses. target region of most diagnostic primers for detection of
represent two genera in a genetically and antigenically diverse family of positive-sense RNA viruses [Berke et al., 1997; Green et al., 2000]. Open reading frame 1 of the viral genome encodes a RNA polymerase that is the target region of most diagnostic primers for detection of both Norwalk-like viruses and Sapporo-like viruses. Although this region is variable, it is the most conserved region of the genome suitable for RT-PCR assays [Ando et al., 1995; Vinjé et al., 2000]. A partial sequence of the polymerase region of the human caliciviruses in our study showed that all strains belonged to the “Norwalk-like virus” genus, with 11 (85%) and 2 (15%) of the 13 detected strains in genogroup II and I, respectively. Sequence and phylogenetic analysis revealed that a minimum of six different genetic clusters (2 genogroup I and 4 genogroup II clusters) were present in the outbreaks, with a predominance of genogroup II Hawaii-like virus. Molecular epidemiological studies of outbreaks in most other countries have also revealed that Norwalk-like virus clusters were predominant over Sapporo-like viruses and Norwalk-like virus genogroup II strains over Norwalk-like virus genogroup I strains [Vinjé and Koopmans, 1996; Vinjé et al., 1997; Fankhauser et al., 1998; Wright et al., 1998; Maguire et al., 1999; Noel et al., 1999; Gonin et al., 2000; Iritani et al., 2000; Nakata et al., 2000; Schreirer et al., 2000; Greening et al., 2001]. Analysis of this short polymerase region can be useful for epidemiologic purposes, but its application for genotype determination is questionable without the sequence of capsid region in the case of newly or poorly characterized clusters. The capsid region of MOH/99 was sequenced and showed 97/98% nucleotide/ amino acid identity with the previously described NLV/Hillingdon/90/UK strain [Farkas et al., 2002].

In 1999–2000, closely related strains of Hawaii-like viruses caused the majority (58%) of human calicivirus outbreaks in West-Hungary, and occurred in different settings and distinct age groups, with no obvious common source of exposure. Because Hungary is land-locked and seafood is not commonly consumed, oysters and shellfish can be excluded as shared sources of our regional human calicivirus exposures, which is not the case in the United States [Fankhauser et al., 1998] and Japan [Iritani et al., 2000]. Similar observations of a single strain emerging as the predominant circulating virus in a community have been made, but not of a Hawaii-like virus. Between 1995 and 1997, closely related Lordsdale and Lordsdale-like viruses were detected concurrently at a high frequency among outbreaks in five continents and in seven countries, probably representing a global spread [Noel et al., 1999]. In Europe, particularly in the Netherlands, Mexico virus in 1994, and in eastern England, Lordsdale/Bristol/Pilgrim/Grimsby strains predominated in outbreaks in 1995 and 1996 [Vinjé and Koopmans, 1996; Vinjé et al., 1997; Maguire et al., 1999].

Six (30%) of the outbreaks evaluated were positive by EIA but negative by RT-PCR. Similar detection rates were also observed by Huang et al. [2001] utilizing this EIA. Discrepancies between the assays could be explained by a degree of genetic variation among human caliciviruses that precludes detection by primers p289, p290, or p290A. The negative RT-PCR results also could be explained by the presence of unidentified inhibitors in stools or loss of viral RNA under long-time storage. The last possibility is supported by the increasing RT-PCR detection rate as the time from collection to testing diminished.

Two outbreaks of co-infection by Norwalk-like viruses were observed with, in one instance, an untyped enterovirus and in another a human astrovirus. Primers p289/p290A generated at least two amplicons from human astrovirus type 1 in one specimen (Table II). The size of these amplicons differed from that expected for human caliciviruses. That sample also was EIA-positive for astrovirus. The other samples in that outbreak were EIA-negative for human astrovirus, but EIA-positive for Norwalk-like viruses. By sequence analysis, the 22 bp-long p289 primer aligned with 12 to 15 nt of the open reading frame 1b polymerase region of human astrovirus types 1 to 8 at nt 3,894 to 3,916 of human astrovirus type 1 (J. Walter, personal communication). The low annealing temperature during our PCR reaction probably gave an opportunity for the primers to bind to conserved astrovirus sequences but produced products uncharacteristic for human caliciviruses.

The results for Hungary are similar to observations published previously on human calicivirus outbreaks. In Hungary, about 64 and 55% of the RT-PCR and EIA-positive outbreaks occurred in hospitals or social welfare institutions, homes for the elderly, a rate similar to that observed in the United States (43%) and UK (87%) [Fankhauser et al., 1998; Maguire et al., 1999]. Most of the affected persons were in the first two decades of life or older than 70 years of age; ages when antibody prevalence increases from new exposures (younger ages) or decreases from waning immunity [Greenberg et al., 1990; Szu¨cs et al., 1995; Matsui et al., 2000]. The four diagnostic criteria described previously for human calicivirus outbreaks of vomiting in more than 50% of the cases, a short (24–48 hr) incubation
period, a short (12–60 hr) duration of illness and absence of bacterial and parasitic pathogens [Greenberg et al., 1990], were also characteristic of the Hungarian outbreaks. A difference in clinical presentation between children and adults also was observed. In temperate climates, human calicivirus outbreaks accumulate in winter-early spring (“winter vomiting disease”) from November to May [Mounts et al., 2000]. However, in Hungary, the majority of the outbreaks occurred between May and November but infections occurred every season during a year. In 2001, 29 (69%) of 42 detected human calicivirus outbreaks were between May and November (data not shown). This “summer” seasonality might reflect vehicles of transmission that differ from those predominant in other regions, as suggested elsewhere [O’Ryan et al., 1998]. A longer examination period is needed to clarify the seasonal pattern, if any, of human calicivirus infections in Hungary.

In summary, multiple Norwalk-like viruses, with a predominance of genogroup II strains, played an important role in nonbacterial gastroenteritis outbreaks during the study period in Hungary. Our results, and the reported number of gastroenteritis outbreaks and cases of unknown etiology, indicate the necessity for assaying for human caliciviruses in this country. The continued and expanded application of molecular techniques to the study of human calicivirus outbreaks will be indispensable in the acquisition of needed epidemiological information to manage outbreaks and to plan and implement control measures for human calicivirus-associated gastroenteritis.

ACKNOWLEDGMENTS

We thank the epidemiologists and public health officers in Institutes of Baranya, Bács-Kiskun, Csongrád, Fejér, Hajdú-Bihar, Komárom-Esztergom, Somogy, Tolna, Veszprém County, and Budapest State Public Health Service for their contribution to this work. G. Reuter acknowledges a visiting scientist fellowship from the Center for Pediatric Research. Presented at the 5th Annual Meeting of the European Society for Clinical Virology, September 2001, Lahti, Finland.

REFERENCES


Molecular Detection and Sequence Analysis of Human Caliciviruses From Acute Gastroenteritis Outbreaks in Hungary

T. Farkas, T. Berke, G. Reuter, G. Szűcs, D.O. Matson, and X. Jiang

1Center for Pediatric Research, Eastern Virginia Medical School, Children’s Hospital of The King’s Daughters, Norfolk, Virginia
2Regional Laboratory of Virology, Baranya County Institute of State Public Health Service, Pécs, Hungary

Three viral gastroenteritis (VGE) outbreaks that occurred in 1998–1999, in Hungary were investigated for the presence of human caliciviruses (HuCVs). HuCVs in stool specimens were detected by reverse transcription-polymerase chain reaction (RT-PCR) using primer pair 289/290, which was designed based on the RNA-dependent RNA polymerase (RdRp) sequence. RT-PCR results were confirmed by sequencing showing that all three outbreak strains belonged to genogroup II of “Norwalk-like viruses” (NLVs). Two strains had high sequence identity with strains in known genetic clusters (Hawaii and Lordsdale clusters). The third strain (MOH) had distinct RdRp sequence, sharing 77/86% (nt/aa) identity with Snow Mountain virus (SMV), the closest genogroup II virus. To characterize MOH further, we cloned, sequenced, and expressed in baculovirus its capsid gene. It had 75/79% (nt/aa) identity with SMV, but 97/98% (nt/aa) identity with NLV/Hillingdon/90/UK, a recently identified genetic cluster of HuCVs. The recombinant MOH (rMOH) capsid protein self-assembled into virus-like particles (VLPs), which is antigenically distinct from other recombinant HuCV capsid antigens available in our laboratory. Further study of this VLP will have important applications in antigenic characterization and diagnosis of HuCVs. J. Med. Virol. 67:567–573, 2002. © 2002 Wiley-Liss, Inc.

KEY WORDS: Norwalk virus; gastroenteritis; diarrhea; RT-PCR; baculovirus-expression; VLP

INTRODUCTION

Human caliciviruses (HuCVs) are a major cause of non-bacterial gastroenteritis outbreaks in humans. These viruses represent two genera within Caliciviridae, “Norwalk-like viruses” (NLVs) and “Sapporo-like viruses” (SLVs). NLVs are also known as “small round structured viruses” (SRSVs), based on their distinct morphology. The NLVs are divided into two genogroups. Each genogroup is divided further into genetic clusters with uncertain biological significance [Berke et al., 1997; Green KY et al., 2000]. In surveillance studies from different countries, HuCV strains from different genetic clusters were found to co-circulate in the same geographic location [Vinje and Koopmans, 1996; Fankhauser et al., 1998, 2000; Noel et al., 1999; Farkas et al., 2000a]. New genetic clusters continue to be identified in many studies, suggesting that HuCVs are genetically more diverse than previously thought. For example, in a recent study of the complete capsid sequences of 20 NLVs from outbreaks in the United Kingdom, eight novel, previously unknown genetic clusters were identified (four in genogroup I and four in genogroup II) [Green J et al., 2000]. New strains of HuCVs also were reported in other studies [Vinje and Koopmans, 2000; Vinje et al., 2000]. Currently, based on capsid sequences, at least 15 genetic clusters of NLVs and 4 genetic clusters of SLVs have been reported [Green J et al., 2000; Vinje and Koopmans, 2000; Vinje et al., 2000].

Recent molecular epidemiology studies of HuCVs further highlight the importance of HuCVs as a cause of gastroenteritis in children and adults. Surveillance of HuCVs by RT-PCR showed that HuCVs were associated with 83 to 96% of selected non-bacterial gastroenteritis outbreaks in the United States in 1995 to 1999 [Fankhauser et al., 1998, 2000] and 15 and...
29% of sporadic diarrhea episodes in children in Mexico and Finland, respectively [Pang et al., 2000; Farkas et al., 2000a]. HuCV infection has been studied in many regions of the world, but poorly in Central and Eastern Europe. This report describes three HuCV-associated gastroenteritis (GE) outbreaks that occurred in 1998–1999 in Hungary. The study also describes the genetic and antigenic characterization of one outbreak strain (NLV/MOH/99/HU), by cloning and baculovirus-expression of its viral capsid protein.

**MATERIALS AND METHODS**

**Outbreaks of Acute Gastroenteritis**

**Outbreak 1.** Between November 25 and 28, 1998, 80 persons were affected in the outbreak, 72 of whom were children. Twenty-six children and one adult were affected in a child-care center in the city of Algyó, 22 children and five adults in an elementary school in Algyó, and 24 children and two adults in a child-care center in the city of Szeged. None were hospitalized. Stool samples from 51 patients and 49 workers associated with food-handling were collected and submitted for routine bacteriology. Stool samples from five children, three from Szeged and two from Algyó, were tested for HuCVs. These stool samples were also tested for Salmonella, Shigella, *E. coli*, *S. aureus*, *Y. enterocolitica*, Campylobacter, rotavirus (gel electrophoresis “PAGE”), adenovirus (latex assay “LA”) and enteroviruses (expression of its viral capsid protein).

**Outbreak 2.** This outbreak took place in a home for the elderly in Szekszárd and lasted two days (May 20 to 21, 1999). None were hospitalized. Five stool samples were submitted for routine bacteriology and were tested for the presence of HuCVs. The stools were also tested for Salmonella, Shigella, *Escherichia coli*, *S. aureus*, *Y. enterocolitica*, Campylobacter, rotavirus, and adenovirus.

**Outbreak 3.** Two children and six adults in four households in Oroszlány were affected in this outbreak. The outbreak lasted for six days (August 28 through September 2, 1999). One young child (2 years) visited the emergency department and one adult was seen by a primary care physician because of their severe illness. Reports from the primary care facilities of this town to the national health authorities indicated a significant increase of gastroenteritis complaints during the same period.

**Laboratory Methods**

**Detection of HuCVs by RT-PCR.** RNA was extracted from stool specimens using the Trizol reagent (Gibco BRL, Gaithersburg, MD) as described previously [Farkas et al., 2000a]. Primer pair p289/p290 was used to amplify the HuCV RNA polymerase region [Jiang et al., 1999]. This primer pair produces a 319-bp RT-PCR product for NLVs and a 331-bp product for SLVs. To amplify the capsid gene of the MOH strain we used oligo-dT primer paired with primer p301 or p290. Primers used in amplifications are summarized in Table I. RT-PCR was performed as described previously [Farkas et al., 2000a]. For amplification of the capsid cDNA, a longer extension time (2 min and 30 sec) was used.

**Cloning and sequencing of RT-PCR products.** RT-PCR products were cloned into pGEM-T (Promega, Madison, WI) according to the manufacturer’s instructions. Positive clones were identified by PCR screening. The 3.2-kb amplicon of MOH obtained by p290/oligo-dT containing the partial RNA-dependent RNA polymerase (RdRp) region, ORF2 and ORF3 were sequenced by generating deletion clones for both orientations (Kilo-Sequencing Deletion Kit, TaKaRa Biomedicals, Japan). The insert DNA of each clone was sequenced using M13 forward and reverse primers by the chain termination method on an automated sequencer (ALFexpress™, Pharmacia, Uppsala, Sweden).

**Phylogenetic Analysis**

The genetic identity of the strains was determined by comparison of their sequences to those in GenBank and our local CV sequence database. Pairwise alignments were run using the OMIGA 2.0 package (Oxford

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sense</th>
<th>Location(^a)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>p290</td>
<td>Positive</td>
<td>4568–4590</td>
<td>GATTACTCCAAGTGAGACTCCAC</td>
</tr>
<tr>
<td>p289</td>
<td>Negative</td>
<td>4865–4886</td>
<td>TGAACATGTAAATCATCCACATA</td>
</tr>
<tr>
<td>p301(^b)</td>
<td>Positive</td>
<td>5354–5371</td>
<td>ATGCAGGATCCGGTGAATGAGATCGGTC</td>
</tr>
<tr>
<td>p255(^c)</td>
<td>Negative</td>
<td>poly-A-tail</td>
<td>AGTAGCCTCGAGGGCCCTTT-(T)(_{23})</td>
</tr>
</tbody>
</table>

\(^a\)Nucleotides in the NV genome (M87661).

\(^b\)p301 has BamHI, SmaI, and XhoI overhangs. The ORF2 initiation codon is underlined.

\(^c\)p255 is an oligo-dT with NotI and XhoI overhangs.
Molecular Ltd, Oxford, UK). The 3.2-kb nt sequence of the MOH isolate was submitted to GenBank under accession number: AF397156.

**Baculovirus expression.** In order to construct recombinant baculoviruses, the 2.4-kb MOH RT-PCR product was cloned into the pFastBac vector utilizing BamHI restriction sites on the vector and p301 and NotI sites on the vector and oligo/dT. Recombinant baculoviruses were generated using the Bac-To-Bac baculovirus expression system (GIBCO BRL, Life Technologies, Gaithersburg, MD). Bacmid DNA was transfected into insect cells (*Spodoptera frugiperda* Sf9 cells). Clones with high levels of expression were selected and viral stocks were prepared. Large-scale

<table>
<thead>
<tr>
<th>nt/aa(%)</th>
<th>HUN6</th>
<th>HUN10</th>
<th>SMV</th>
<th>MxV</th>
<th>Lord</th>
<th>HV</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOH</td>
<td>75/87</td>
<td>76/88</td>
<td>77/86</td>
<td>74/86</td>
<td>76/87</td>
<td>73/86</td>
</tr>
<tr>
<td>HUN6</td>
<td>—</td>
<td>85/97</td>
<td>82/95</td>
<td>74/84</td>
<td>87/98</td>
<td>91/96</td>
</tr>
<tr>
<td>HUN10</td>
<td>—</td>
<td>—</td>
<td>81/93</td>
<td>74/85</td>
<td>91/96</td>
<td>86/94</td>
</tr>
</tbody>
</table>

*MOH, strain from outbreak in Oroshlany; HUN6, strain from outbreak in Szekszard; HUN10, strain from outbreak in Algyo and Szeged. The five amplicons (two from Algyo and three from Szeged) yielded identical sequences. SMV, Snow Mountain virus; MxV, Mexico virus; Lord, Lordsdale virus; HV, Hawaii virus.

### TABLE II. Pairwise Sequence Identity in the RNA-Polymerase Region of the New Strain and the Closest Reference Strains*

<table>
<thead>
<tr>
<th>nt/aa(%)</th>
<th>HUN6</th>
<th>HUN10</th>
<th>SMV</th>
<th>MxV</th>
<th>Lord</th>
<th>HV</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOH</td>
<td>75/87</td>
<td>76/88</td>
<td>77/86</td>
<td>74/86</td>
<td>76/87</td>
<td>73/86</td>
</tr>
<tr>
<td>HUN6</td>
<td>—</td>
<td>85/97</td>
<td>82/95</td>
<td>74/84</td>
<td>87/98</td>
<td>91/96</td>
</tr>
<tr>
<td>HUN10</td>
<td>—</td>
<td>—</td>
<td>81/93</td>
<td>74/85</td>
<td>91/96</td>
<td>86/94</td>
</tr>
</tbody>
</table>

*MOH, strain from outbreak in Oroshlany; HUN6, strain from outbreak in Szekszard; HUN10, strain from outbreak in Algyo and Szeged. The five amplicons (two from Algyo and three from Szeged) yielded identical sequences. SMV, Snow Mountain virus; MxV, Mexico virus; Lord, Lordsdale virus; HV, Hawaii virus.

---

**Fig. 1.** Maximum likelihood phylogram of capsid gene nucleic acid sequences of selected NLVs. The tree is rooted by the other genera of Caliciviridae. Scale bar represents the phylogenetic distances expressed as units of expected nucleotide substitutions per site. The tree pattern has a confidence level >0.95 at each branch point. GenBank accession numbers for strains used: Camberwell (AF145896), Chiba (AB022679), C59 (AF435807), Desert Shield (U04469), Hawaii (U07611), Hillingdon (AJ277607), Jena (AJ011099), Lordsdale (X86557), Mexico (U22498), MOH (AF397156), NorwalkUS (M87661), Norwalk DE (NC-001959), Snow Mountain (U75682), Southampton (L07418), Thistlehall (AJ277621), VA115 (AY038598), VA207 (AY038599), VA387 (AY038600), Valetta (AJ277621).
expression of the MOH capsid protein and purification of recombinant VLPs were done as described elsewhere [Jiang et al., 2002].

Production of antisera against recombinant MOH capsid antigen. Sucrose gradient-purified MOH VLPs, with complete or incomplete Freund's adjuvant, were used to immunize one rabbit and two guinea pigs. Blood was collected before immunization and after four immunizations at 2–3-week intervals. The sera were titered in EIAs to monitor antibody response. The final dose of antigen was administered without adjuvant and two weeks later the animals were sacrificed and antisera collected.

Enzyme immune assay (EIA). EIAs were used to characterize the antigenic relationship between MOH and other previously characterized capsid proteins [Jiang et al. 2002]. Recombinant proteins coated as antigens in the EIAs were: NV, VA115, and C59 from genogroup I NLVs and MxV, HV, GrV, VA207, and VA387 from genogroup II NLVs. EIAs were performed as described elsewhere [Jiang et al., 1995, 2000].

**RESULTS**

**Outbreak Characteristics**

**Outbreak 1.** The predominant symptoms were abdominal cramps (85%) and vomiting (84%) followed by nausea (38%), diarrhea (22%), and fever (7%). All five stools tested for HuCVs were positive and the amplicon sequences were identical (HUN10). These stools were negative for Salmonella, Shigella, E. coli, S. aureus, Y. enterocolitica, Campylobacter, rotavirus, adenovirus, and enteroviruses. Epidemiological investigation revealed that all three institutions affected in the outbreak were catered by the same company. They had “A,” “B,” and “C” menus for lunch and gastroenteritis was associated only with menu A. The incubation period, calculated from the time lunch was consumed to symptom onset, was less than 16 hr in 74% of the patients. Attack rates at the affected institutions ranged from 13 to 25%.

**Outbreak 2.** One of the five stools collected was positive for HuCV (HUN6). These stools were nega-
tive for Salmonella, Shigella, *E. coli*, *S. aureus*, *Y. enterocolitica*, Campylobacter, rotavirus, and adenovirus. The HuCV-positive stool, along with two others, was positive for enteroviruses.

**Outbreak 3.** The only stool sample tested from the outbreak was HuCV positive (MOH).

**Sequence analysis.** Pairwise sequence analysis results for the RdRp region of the new isolates and the closest reference strains showed that all three isolates belonged to genogroup II of NLVs (Table II). Isolate HUN6 had 91/96% (nt/aa) identities with Hawaii virus and 87/98% (nt/aa) identities with Lordsdale virus. Isolate HUN10 shared 91/96% (nt/aa) identities with Lordsdale virus and isolate MOH had 77/86% (nt/aa) identities with Snow Mountain virus (Table II).

The 3.2-kb product of MOH obtained by p290/oligo-dT revealed typical NLV genomic structure: a partial RdRp gene (ORF1; 784 nt) followed by a complete capsid gene (ORF2; 1614 nt) and a small open reading frame (ORF3; 657 nt) that encodes for a minor structural protein [Glass et al., 2000], and an untranslated region (154 nt) between ORF3 and the poly-A tail. Blast search of the GenBank database showed that MOH has 97/98% (nt/aa) identities with the Hillingdon isolate (NLV/Hillingdon/90/UK) in the capsid region [Green et al., 2000]. Phylogenetic analysis of the MOH capsid gene showed the close proximity of MOH and Hillingdon isolates (Fig. 1).

**Baculovirus expression.** High yield of the MOH capsid protein was obtained when expressed in Sf9 and H5 cultures. The full-length capsid proteins appeared on the second day and their concentration peaked 4 to 6 days after infection (data not shown). A significant amount of the capsid protein was released into the culture media. Peak fractions containing the 60-kDa capsid protein were found in the middle of the sucrose gradients, suggesting that the baculovirus-expressed MOH capsid proteins assembled into VLPs (Fig. 2).

**Immunogenicity and antigenic relationship of the MOH capsid protein to previously characterized HuCV capsid proteins.** Hyperimmune antisera with high titers against the MOH capsid protein were generated in rabbits and guinea pigs. Antigenic relationships between MOH and previously characterized HuCV capsid proteins were studied in EIAs. VA115 and C59 were capsid proteins that did not form VLPs during baculovirus-expression [Farkas et al., 2000b; Jiang et al., 2002]. Pairwise amino acid identities of the capsid genes, the EIA antigens and reference strains are shown in Table III. Highest antibody titers were

**Table III. Pairwise Sequence (%) Identities of Deduced Amino Acid Sequences of the Capsid Genes of HuCVs Used as Coating Antigen in the EIA and Closely Related Reference Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>GI NV</th>
<th>DSV</th>
<th>South</th>
<th>115*</th>
<th>C59</th>
<th>Lord</th>
<th>GrV</th>
<th>387*</th>
<th>MxV</th>
<th>HV</th>
<th>SMV</th>
<th>MOH</th>
<th>207*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NV</td>
<td>100</td>
<td>66</td>
<td>69</td>
<td>64</td>
<td>64</td>
<td>42</td>
<td>42</td>
<td>42</td>
<td>44</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>43</td>
</tr>
<tr>
<td>DSV</td>
<td>100</td>
<td>66</td>
<td>87</td>
<td>64</td>
<td>64</td>
<td>42</td>
<td>42</td>
<td>42</td>
<td>44</td>
<td>44</td>
<td>44</td>
<td>44</td>
<td>43</td>
</tr>
<tr>
<td>South</td>
<td>100</td>
<td>64</td>
<td>99</td>
<td>42</td>
<td>42</td>
<td>42</td>
<td>42</td>
<td>42</td>
<td>41</td>
<td>41</td>
<td>41</td>
<td>41</td>
<td>41</td>
</tr>
<tr>
<td>115</td>
<td>100</td>
<td>63</td>
<td>33</td>
<td>41</td>
<td>41</td>
<td>41</td>
<td>41</td>
<td>41</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>C59</td>
<td>100</td>
<td>64</td>
<td>99</td>
<td>42</td>
<td>42</td>
<td>42</td>
<td>42</td>
<td>42</td>
<td>41</td>
<td>41</td>
<td>41</td>
<td>41</td>
<td>41</td>
</tr>
<tr>
<td>Lord</td>
<td>100</td>
<td>64</td>
<td>99</td>
<td>66</td>
<td>66</td>
<td>66</td>
<td>66</td>
<td>66</td>
<td>66</td>
<td>66</td>
<td>66</td>
<td>66</td>
<td>66</td>
</tr>
<tr>
<td>GrV</td>
<td>100</td>
<td>98</td>
<td>65</td>
<td>65</td>
<td>65</td>
<td>65</td>
<td>65</td>
<td>65</td>
<td>65</td>
<td>65</td>
<td>65</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>387</td>
<td>100</td>
<td>65</td>
<td>63</td>
<td>63</td>
<td>63</td>
<td>63</td>
<td>63</td>
<td>63</td>
<td>63</td>
<td>63</td>
<td>63</td>
<td>63</td>
<td>63</td>
</tr>
<tr>
<td>MxV</td>
<td>100</td>
<td>71</td>
<td>67</td>
<td>67</td>
<td>67</td>
<td>67</td>
<td>67</td>
<td>67</td>
<td>67</td>
<td>67</td>
<td>67</td>
<td>67</td>
<td>67</td>
</tr>
<tr>
<td>HV</td>
<td>100</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>SMV</td>
<td>100</td>
<td>79</td>
<td>63</td>
<td>63</td>
<td>63</td>
<td>63</td>
<td>63</td>
<td>63</td>
<td>63</td>
<td>63</td>
<td>63</td>
<td>63</td>
<td>63</td>
</tr>
<tr>
<td>MOH</td>
<td>100</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>207</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*Lord, Lordsdale; MxV, Mexico; DSV, Desert Shield; NV, Norwalk; GrV, Grimsby; HV, Hawaii; SMV, Snow Mountain; South, Southampton. C59, Isolate from a shipboard outbreak [Farkas et al., 2000b]. *115, 207, 387, Isolates at the CPR from viral gastroenteritis outbreaks in Virginia [Jiang et al., 2002].

**Table IV. Reciprocal of Titers of Anti-MOH Rabbit and Guinea Pig Hyperimmune Antisera Reactivities Against Recombinant Capsid Proteins of Other HuCVs**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Genogroup I</th>
<th>Genogroup II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C59</td>
<td>NV</td>
</tr>
</tbody>
</table>
| Rabbit 
*α*-MOH  | 32  | 8  | <1  | 1,024 | 8  | 64  | 32  | 32 | 32 |
| Guinea pig 
*α*-MOH | 32  | 4  | 1   | 2,048 | 16 | 128 | 32  | 64 | 32 |

*ELISA titers, ×10⁻³.
detected in hyperimmune anti-MOH rabbit or guinea pig sera against the homologous capsid antigen. Low levels of cross-reactivity were detected with other capsid antigens from genogroup I and genogroup II HuCVs (Table IV) [Jiang et al., 2002].

**DISCUSSION**

In this study, we investigated three viral gastroenteritis outbreaks that occurred between 1998 and 1999 in Hungary. The nature of the outbreaks and the bacteriology and virology test results showed that HuCVs were the most possible cause of the outbreaks. One outbreak, that occurred at three different institutions in two towns, could be related to a lunch menu that was distributed from the same kitchen. The menu items that might have played a role as the source of contamination were not investigated.

Sequence analysis of the amplicons revealed that all three outbreak strains belong to genogroup II NLVs. According to the pairwise nucleotide sequence analysis of the RT-PCR products from the RNA polymerase region, HUN6, HUN10, and MOH belong to clusters represented by the Hawaii, Lordsdale, and Snow Mountain viruses, respectively (Table I). However, MOH showed only 77/86% (nt/aa) identities with Snow Mountain virus, the most closely related cluster representative strain.

We successfully amplified and sequenced the capsid gene of the MOH isolate. Analysis of the MOH capsid gene revealed 97/98% (nt/aa) identities with the Hillingdon isolate (NLV/Hillingdon/90/UK), for which the RNA polymerase region sequence is not published [Green J et al., 2000]. Because HuCVs cannot be propagated in tissue culture or an animal model for studies of virus neutralization, others have suggested that HuCVs with less than 80% aa identity in the complete capsid gene represent antigenically distinct capsid types [Hardy et al., 1997; Vinje et al., 2000]. For example, Queens Arms virus, which has an 80% capsid sequence identity with Norwalk virus, failed to react in the nRV EIA [Green J et al., 2000]. According to this assumption, Hillingdon and MOH represent a new genetic and antigenic cluster within the genogroup II NLVs. MOH and Hillingdon isolates are virtually identical, despite the temporal and geographic distance (Fig. 1).

We successfully expressed the MOH capsid protein using a baculovirus-expression system. The capsid proteins expressed in high yield and self-assembled into VLPs. According to our knowledge, there is no other VLP available representing this cluster.

Hyperimmune rabbit and guinea pig antisera produced against purified MOH VLPs have been obtained and the reactivity of these sera was of higher titer against homologous MOH VLPs, but of significantly lower titer against other capsid antigens. These low cross-reactivities indicate shared antigenic epitopes among different HuCV preparations because these animals were immunized with only MOH VLPs and there were no HuCV-specific antibodies in their pre-immune sera. However, the low level cross-reactivity could be due to insect cell cellular protein contamination in the antigen preparations. Incubation of the anti-MOH sera with excess insect cell lysate before titration in the EIAs did not change the titer readings.

Finally, the MOH VLPs and antisera extend the available new EIAs for the detection of HuCVs in a cluster previously without antigen and antibody detection reagents.

**ACKNOWLEDGMENTS**

We thank Drs. A. Kátaí and M. Kálmán from Csongrád County Institute of State Public Health Service, Szeged, for collecting stool specimens and epidemiological data and public health officer Dr. R. Brunner in Oroshzány for providing epidemiological data from outbreak 3.

**REFERENCES**


