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The incidence and genetic variability of Small Round-Structured Viruses (SRSV) in outbreaks of gastroenteritis in 1996 in the Netherlands

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ABSTRACT

Small round-structured viruses (SRSV) are a group of RNA viruses that can cause gastroenteritis in persons of all ages. To determine the incidence of SRSV-associated gastroenteritis in The Netherlands and to study the genetic variability of outbreak strains, all outbreaks that were reported to the epidemiologists of the regional health services in 1996 were investigated using a standardized protocol. In 60 (87%) of the 69 reported outbreaks, SRSV could be detected, showing the etiologic significance of SRSV in outbreaks of gastroenteritis in The Netherlands. Of these outbreaks, 84% occurred in semiclosed communities, such as nursing homes (59%) and hospital wards (25%). Sequence analysis of the outbreak strains revealed that the majority of the strains from January to November 1996 formed a tight cluster within genogroup II SRSV. In November 1996, a shift toward genogroup I SRSV occurred, suggesting a change to a new predominant strain.

SAMENVATTING

Small round-structured viruses (SRSV), ook wel Norwalk-achtige virussen genoemd, zijn belangrijke verwekkers van explosies van gastroenteritis. De detectie van deze groep genetisch uiterst variabele virussen is door de recente ontwikkeling van een generische RT-PCR test sterk vereenvoudigd. Om de incidentie van SRSV bij explosies van gastroenteritis te bepalen, werden in 1996 alle bij de GGD-en gemelde explosies onderzocht volgens een standaardprotocol. Door 28 van de 60 GGD-en werden in totaal 69 explosies gemeld. In 60 van deze explosies (87%), kon met behulp van een RT-PCR SRSV worden aangetoond. Van de 69 gemelde explosies vonden de meeste (78%) in de eerste 3 maanden van 1996 plaats. De meerderheid (59%) van de explosies vond plaats in verpleeg- en verzorgingstehuizen en 25% in ziekenhuizen. Uit sequentie-analyse van de PCR producten bleek, dat stammen van alle explosies in de periode van januari tot november sterk geclusterd waren. Het betrof hier een genogroep II SRSV-type (Grimsby), een type SRSV dat ook al in 1995 in Nederland als predominant type was waargenomen. Vanaf november was een duidelijke shift naar de circulatie van een genogroup I SRSV-type (Venlo) waarneembaar.

Samenvattend zijn SRSV de meest frequent voorkomende pathogenen geassocieerd met explosies van gastroenteritis die in 1996 werden gemeld aan de GGD-en in Nederland. De moleculaire analyse laat zien dat SRSV epidemisch verspreiden.

1. INTRODUCTION

Gastroenteritis contributes significantly to morbidity worldwide and to mortality in developing countries [1]. Advanced age has been described as a risk factor, and over 50% of diarrheal deaths in the USA are in the elderly [2, 3]. Groups at risk are mainly people who resides in semi-closed communities like nursing homes and hospital wards. On the basis of epidemiological criteria, it has been predicted that small round-structured viruses (SRSV) are the single most important viral cause of outbreaks of gastroenteritis [4]. Until recently, in The Netherlands outbreaks of gastroenteritis due to SRSV infection were diagnosed infrequently, mostly because of the lack of simple methods for their detection.

The SRSV are a group of single-stranded RNA viruses that recently have been classified as members of the family *Caliciviridae* [5]. Within the *Caliciviridae*, at least three genogroups have been recognized: SRSV-type or genogroup I and II [6, 7], and classical calicivirus-type or genogroup III [8, 9]. The SRSV are morphologically indistinguishable by electron microscopy (EM), but they are antigenically distinct when examined using solid phase immune-EM (SPIEM). Six antigenic groups have been described so far [10], but several more antigenically distinct strains or clusters of strains exist [6]. Morphologically, epidemiologically, genetically, and antigenetically the classical caliciviruses are quite distinct from the SRSV-type calicivirus. Symptomatic infections with classical caliciviruses or genogroup III human caliciviruses are mostly restricted to childhood, whereas the genogroup I and II or SRSV-type infections are an important cause of gastroenteritis in adults as well as in children [11].

As both an in vitro culture system and an animal model are lacking for these viruses, SRSV detection depended mainly on electron microscopy. The diagnosis of SRSV infections has recently been advanced tremendously by the development of very sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) assays [12, 13, 14]. By sequencing the amplified products (polymerase gene), all known antigenic types of SRSV could be grouped into one of the two genogroups. Norwalk virus, Southampton virus and UK2 viruses were grouped into genogroup I, and Hawaii virus, Mexico virus, Pilgrim virus and Snow Mountain virus were grouped into genogroup II viruses. However, the diversity within each genogroup is probably greater [6, 15, 16, 17], and little is known about the epidemiologic and biologic implications of this genetic variability.

In the present study, the incidence of SRSV-associated outbreaks of gastroenteritis in the Netherlands was investigated by analyzing all outbreaks of gastroenteritis that were reported to the regional public health services (PHSs) in 1996. In addition, the genetic variation of 1996 outbreak strains was analyzed by sequence analysis and was compared with that of 1994 and 1995 outbreak strains [12].

2. MATERIALS AND METHODS

Outbreaks

Outbreaks of gastroenteritis were investigated using a standardized protocol. In the protocol, instructions were given about collecting stool samples according to CDC guidelines [18], which included collection of 10 stool samples from cases and 10 from controls within 72 hr. after onset of symptoms, and storage at +4 °C. Inclusion criteria were acute diarrhoea in at least 2 persons, accompanied by 2 or more of the following symptoms: nausea, vomiting, fever, or blood in the faeces. Of all PHSs, 27 participated in the study. They were located in different parts of the country, covering 52% of the Dutch population. The remaining 33 PHSs were interviewed for nonresponse by telephone questionnaire. In total, 704 stool specimens were obtained. In 22 outbreaks, stool samples from both cases (n=176) and controls (n=128) were collected for laboratory investigation.

Diagnostic evaluation of outbreaks

Routine bacterial culture of the stool samples was performed in the local public health laboratories according to standard procedures. Group A rotavirus and adenovirus were detected by enzyme immunoassays as described [19, 20]. Stool samples from outbreaks in which no pathogen could be detected by any of the assays were also analyzed by electron microscopy [12].

Molecular detection of SRSV by RT-PCR

Viral RNA was extracted from stool specimens after it bound to silica particles [21]. To reduce the risk of contamination, specimens from each outbreak were analyzed on separate occasions, and 1 negative control sample was included for every 2 stool specimens prior to the extraction procedure. A stool sample positive for SRSV by electron microscopy was included as positive control. Extraction, preparation of master mixes, preparation of

reactions, and analysis of PCR products were done in different rooms with designated sets of pipettes.

PCR was performed with the generic primer pair JV12/JV13 as described [12] with some modifications. For reverse transcription (RT), 5 µl of the extracted viral RNA was mixed with 4 µl of 50 pmol JV13 (table 1), and heated for 2 min at 90°C. After quenching on ice for 2 min, 6 µl RT-buffer was added. Reverse transcription was performed in a 15 µl reaction mixture consisting 10 mM Tris-HCl pH 8.3, 50 mM KCl, 3.0 mM $MgCl_2$, 1 mM dNTP, and 8 U AMV-RT (Promega, Leiden). Reaction mixtures were incubated for 60 min at 42 °C. After heat denaturing at 99°C for 5 min., 5 µl of the RT-mix was added to 45 µl PCR-mix containing 10 mM Tris-HCl pH 9.2, 75 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 units ampliTaq and 0.3 µM of each primer (JV12, and JV13; table 1). 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.

Gel electrophoresis and Southern blotting of PCR products

The PCR products were separated on a 2% agarose gel (MP agarose, Boehringer, Almere, Netherlands), stained with ethidium bromide, and visualized with UV light. DNA molecular weight marker V (Boehringer) was used as a DNA size standard. The PCR products in the agarose gel were denatured in 0.5 M NaOH for 30 min. and transferred to a positively charged nylon membrane by a vacuum blotting system (Millipore, Etten-Leur, Netherlands).

Hybridization of PCR products

After washing 2 times with 2x SSC (30 mM sodium citrate pH 7.0, 0.3 M NaCl), the DNA was cross-linked by baking in a magnetron oven for 12 min. Hybridization was done with a set of 5'-biotinylated probes (probe UK1-4 from [13]; Applied Biosystems / Perkin Elmer, Nieuwerkerk a/d IJssel, Netherlands) as described previously [22]. The nylon membranes were prehybridized for 5 min. at 40 °C in a solution of 2x SSPE (300 mM NaCl, 20 mM $NaH_2PO_4.H_2O$, 2 mM EDTA pH 7.4) with 0.1% SDS. Twenty picomoles of each of the biotin-labeled oligonucleotides (table 1) were added and hybridization was done for 45 min. at 40 °C,

Table 1 Primers and probes used in this study

primer name		sequence (5'-3')
JV12		ATACCACTATGATGCAGATTA
JV13		TCATCATCACCATAGAAAGAG
UK-1	biotine-	TATGTGCCCTGTCAGAAGT
UK-2	biotine-	TATCACCTGATGTTATACAAATCC
UK-3	biotine-	GTCCCCTGACATCATACAGGCT
UK-4	biotine-	ATCCCCTGACATCGTCCAGGCT
JV-5	biotine-	CTCACCAGAGTGTTGTCAAGC

followed by two 10 min. wash steps at 40°C with 2x SSPE and 0.1% SDS. The membranes were incubated with 1:4000 diluted streptavidine-peroxidase conjugate (Boehringer Mannheim) for 45 min. at 42°C in 2x SSPE and 0.5% SDS and then washed three times (10 min. each) with decreasing concentration SDS (0.5%, 0.1% and 0%, respectively) in 20 ml 2x SSPE. The membranes were incubated with the ECL detection kit (Amersham, 's Hertogenbosch, Netherlands) for 2 min. and was exposed to an ECL hyperfilm (Amersham) for 30 min. to visualize bound probe.

Cycle sequencing and data analysis

To determine the range of genetic diversity among the strains from the 1996 outbreaks, SRSV positive RT-PCR products of strains from 53 outbreaks were sequenced using a dye terminator sequencing kit (Amersham). The nucleotide sequence was determined using an ABI 373A automated sequencer (Applied Biosystems). Each RT-PCR product was sequenced in both orientations using the PCR primers. DNA sequences were edited using Seq Ed (V 1.03, Applied Biosystems), converted to the Geneworks format (V 2.45, Intelligenetics, Mountain View, CA), and aligned using the unweighted pair group method with arithmetic mean [23]. The sequences of prototype strains of SRSV were obtained from Genbank (accession numbers: Norwalk virus (M87661), Southampton virus (L07418), Desert Shield virus (U04538), Hawaii virus (U07611), Mexico virus (U22498), and Bristol virus (X76716).

Statistical Analysis

Statistical analysis of data was performed using the Epi Info Version 6.02 (Centers for Disease Control and Prevention, Atlanta, GA) software package.

3. RESULTS

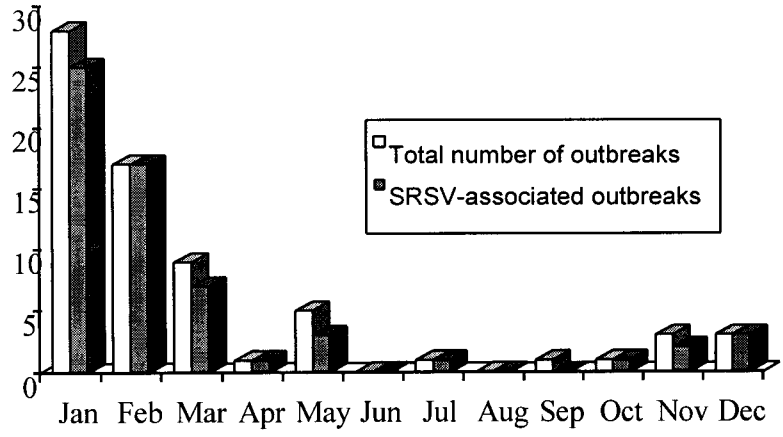

Figure 1 Seasonal occurrence of reported outbreaks of gastroenteritis in the Netherlands in 1996.

Description of outbreaks

In 1996, 69 outbreaks of gastroenteritis were reported to the epidemiologists from 27 of the 60 PHSs, and were investigated using a standardized protocol. In each outbreak, at least 5 persons were affected. The outbreaks occurred in different parts of the country, and there were no obvious epidemiologic links between outbreaks. Most outbreaks (78%) occurred during the first 3 months of 1996. The majority of the outbreaks (84%) occurred in institutions, such as nursing homes for the elderly (59%) and hospitals (25%). All residents of nursing homes (40 of the 69 outbreaks) were ≥ 65 years old.

Table 2. Attack rates calculated from residents and staff in 12 SRSV-associated outbreaks of gastroenteritis

	Residents	Staff
symptoms	603 (45%)	280 (29%)
no symptoms	736 (55%)	699 (71%)
total	1339 (100%)	979 (100%)

NOTE: Yates 's corrected Chi-Square = 64.1.; $p < .001$; odds ratio= 2.1 (95% confidence interval 1.7-2.5)

Nonresponse investigation

Epidemiologists from 33 PHSs were interviewed for nonresponse by telephone questionnaire. Twenty-two had not received any reports on outbreaks of gastroenteritis in 1996. The remaining 11 had, in total, 17 outbreaks reported. On the basis of epidemiologic criteria [12], in 12 of the 17 outbreaks, a viral etiology was suspected, but no virologic examination was done. *Salmonella. enteritidis* was isolated from stool samples of 2 outbreaks. The cause of the remaining 3 outbreaks could not be identified, although food contamination by a bacterial toxin was suspected as the most likely cause.

Table 3 Detection rate of SRSV RT-PCR in stool samples from cases and controls from 22 outbreaks of gastroenteritis in 1996 in the Netherlands

RT-PCR	Cases		Controls	
positive	132	(75%)	24	(19%)
negative	44	(25%)	104	(81%)
Total	176	(100%)	128	(100%)

Yates 's corrected Chi-Square = 91.6.; p < 0.001; odds ratio= 13.0 (95% confidence interval 7.2-23.7)

Diagnostic results of outbreaks

A total of 704 stool samples was sent to the National Institute of Public Health and the Environment (Bilthoven, The Netherlands) for virologic investigation. A possible causative microorganism was detected in 94% of all outbreaks. By RT-PCR, SRSV were detected as the sole pathogen in stool samples from 60 (87%) of the 69 investigated outbreaks. In these outbreaks, 70% (range 20% - 100%) of the stool samples were positive by RT-PCR. In addition, individual stool samples from 3 outbreaks (2 day care centers, 1 boys camp) contained SRSV or rotavirus group A, indicating that both viruses did circulate independently in these settings. In 22 outbreaks, stool samples from both cases and controls were obtained. Control specimens were selected on the basis of absence of symptoms. SRSV were detected significantly more frequently in stool samples from cases (75%) than from controls (19%) (χ^2 = 91.6; $P < .001$, table 1).

Routine bacterial cultures were all negative, with the exception of 1 outbreak, in which *Clostridium perfringens* toxin was detected. In samples from this outbreak, no SRSV were found. All stool samples from the remaining 5 outbreaks were negative for SRSV by RT-PCR. Group A rotavirus was found in 9 of the 11 stool samples tested from 1 of these outbreaks, and no bacteria and viruses could be demonstrated in stool samples from 4 outbreaks (6%).

Genetic variability

To investigate the genetic variability of SRSV strains from outbreaks in 1996, we determined the nucleotide sequence of a 145-nucleotide stretch of the RNA polymerase region (pol). Strains from all outbreaks were sequenced (2 or 3 samples / outbreak). Strains from 53 of 55 consecutive outbreaks formed a tight cluster with the viruses circulating in The Netherlands in 1995 [10], and were closely related to the Bristol / Pilgrim cluster within genogroup II of SRSV [10]. The exceptions were a strain isolated from a day care center, clustering with Hawaii virus, and strains isolated from outbreak 96-61, which belong to SRSV genogroup I (figure 1A). The nucleotide sequences of the remaining 5 outbreaks also formed a tight cluster (Venlo-type, figure 1A) but clearly belonging to a totally different genotype within genogroup I viruses. These outbreaks all occurred in November and December 1996. Within outbreaks, identical sequences were found except in outbreak 96-61 (boys camp) in which 2 different sequences (genogroups I and II) were obtained from different stool samples. In addition, rotavirus group A was also detected in some stool samples. Between outbreaks of the NET96 (Netherlands 1996) cluster, 24 had identical nucleotide sequences, and small differences (maximum, 2,5 %, figure 2) were seen for 29 outbreaks.

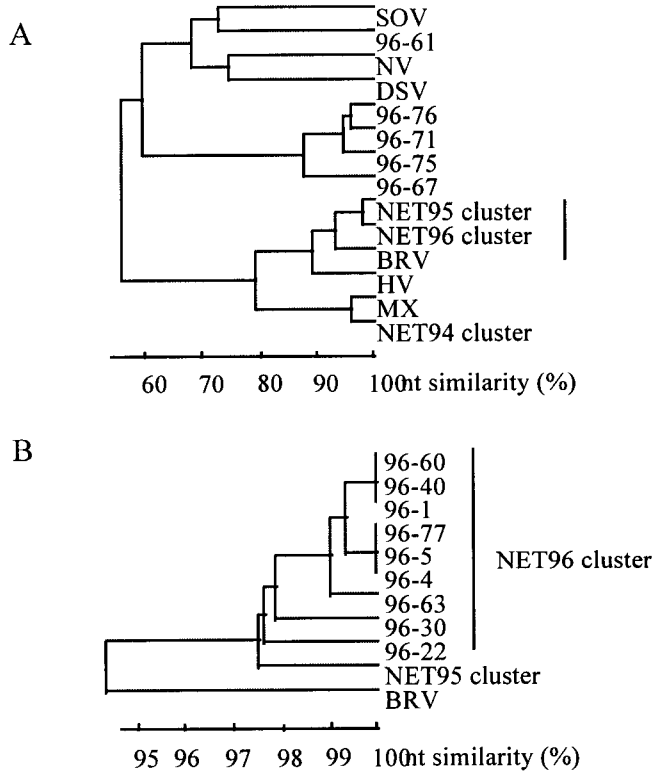

Figure 2 Phylogenetic relationships among SRSV from genogroup I and II (**A**), and within the predominant 1996 cluster (**B**), based on a 145 bp nucleotide stretch within the RNA polymerase gene [10]. **A)** Dendrogram includes sequences from Norwalk virus (NV), Desert Shield virus (DSV), Southampton virus (SOV) , "Venlo"-type cluster (96-67, 96-71, 96-72, 96-75 and 96-76), and outbreak strain 96-61 (all GG I strains) and Mexico virus (MX), Hawaii virus (HV), Bristol virus (BRV), and the predominant strains circulating in the Netherlands in 1994 (NET94 cluster), 1995 (NET95 cluster) and 1996 (NET96 cluster) (all GG II strains). **B)** Dendrogram includes a selection of strains from the NET96 cluster (96-1, 96-4, 96-5, 96-22, 96-30, 96-40. 96-60, 96-63, and 96-77) and Norwalk virus (NV), Southampton virus (SOV), Mexico virus (MX) and Bristol virus (BRV).
The sequences of prototype strains of SRSV were obtained from Genbank (accession numbers: NV (M87661), SOV (L07418), DSV (U04538), HV (U07611), MX (U22498), and BRV (X76716).

4. DISCUSSION

The incidence of SRSV-associated outbreaks of gastroenteritis in The Netherlands has not been investigated previously. Here we describe the results of a study in which we analyzed all outbreaks of gastroenteritis that were reported to the PHSs during a 1-year period. Twenty-seven of the 60 PHSs participated in this study, covering 52% of the total Dutch population. There was a clear selection bias in this study, since the 27 participating PHSs reported 69 outbreaks and the remaining 33 only 17; therefore, extrapolation of the data found in our study to all PHSs is not possible. Nonetheless, in 1996, SRSV were the single most common pathogen identified in outbreaks of gastroenteritis (87%).

The local epidemiologists at the nonresponding PHSs, were interviewed by telephone questionnaire and reported 17 outbreaks, of which 2 could be attributed to *S. enteritidis*. On the basis of epidemiologic criteria, a viral etiology in 12 (71%) of the remaining outbreaks was very likely. Assuming that the figures in the telephone questionnaire are correct, there were 86 total outbreaks reported to all PHSs, and 70% of them were confirmed as SRSV outbreaks. This is a higher proportion than that described by Kaplan et al. [3], who estimated, on the basis of epidemiologic criteria, that 32% - 42% of the total number of outbreaks may be viral, and that SRSV cause 67% of outbreaks in nursing homes.

Our study clearly indicates that SRSV outbreaks are quite common in The Netherlands. The total number of outbreaks of gastroenteritis is probably higher due to underreporting to the PHSs. This may also cause selection bias toward nursing homes and hospitals, where such outbreaks may be more alarming due to the presence of known risk groups. An important setting where underreporting is thought to occur day care centers. Although, we analyzed 3 outbreaks from day care centers, many PHS officers confirmed that only a fraction of the total number of outbreaks of gastroenteritis occurring in this setting is reported. Also, suspected foodborne outbreaks in The Netherlands are usually not reported to the PHS but to food inspection services.

The high number of PCR-positive samples, and the findings that roughly half of the outbreak strains within the NET96 cluster had identical sequences raises concern about contamination of samples with PCR products. We feel, however, that this is not the case, since

control samples (every third sample) were all negative, samples from outbreaks attributed to other pathogens were negative, there was a clear difference in positivity rates between cases and controls, and in stool samples from a sentinel physician-based survey that are analyzed in the same laboratory by the same person in parallel with outbreak specimens, only few SRSV-positive samples have been found.

Our data support earlier findings in that the SRSV-associated outbreaks were clearly seasonal, as has been described previously for SRSV [15] and other enteric viruses. The majority of reported outbreaks occurred in homes for the elderly (59%) and hospitals (25%). Reportedly, wards with children were not affected during the hospital outbreaks, and in the day care center outbreaks that were studied, SRSV could not be considered the main cause of gastroenteritis. This finding was somewhat surprising. An explanation may be that the average age of the children in the affected groups was too low. SRSV infections do occur in older children, but infants are less commonly affected [9]. In a comparison of the attack rates for staff and residents (table 2), the number of affected residents (45%) was significantly higher than the number of affected staff (29%) ($\chi^2 = 64.1$; $P < .001$, table 2). This could reflect exposure differences or indicate that old age or underlying illness are risk factors for clinical illness following SRSV infection.

Apart from the high incidence of SRSV in outbreaks, a second striking finding was the limited genetic variability of outbreak strains, with a predominance of a single type of genogroup II strains in the first 10 months of the year, with a shift to a genogroup I strain in November. The 1996 predominant strains are highly similar to the Grimsby / Pilgrim-like SRSV-type strains that circulated in The Netherlands in 1995. This confirms and extends our previous studies, showing that the vast majority of outbreaks in institutions in 1994 - 1996 seem to be caused by a single predominant strain [10]. On the basis of this pattern, which we now have observed for 3 years, we predict that the genogroup I strain appearing in outbreaks in early winter of 1996-01997 will be the new predominant strain circulating in The Netherlands in 1997. This Venlo-type SRSV is clearly distinct from all described genogroup I prototype strains. Although our generic primer pair (JV12 / JV13) is able to detect Venlo-like SRSV, the confirmation of these viruses can be missed by hybridization with the UK1-4 probes. Therefore, we included a Venlo-type-specific probe (JV5), which proved very useful in detecting this genogroup I type of SRSV (data not shown).

In conclusion, SRSV were the pathogen most frequently associated with outbreaks of gastroenteritis in The Netherlands in 1996. The epidemic spread of individual strains circulating in the population for a certain period, which we described previously [10], is confirmed in this study.

5. CONCLUSIONS AND RECOMMENDATIONS

1. To determine the incidence of SRSV-associated outbreaks of gastroenteritis, we examined all outbreaks reported to the public health services in the Netherlands during a one-year period. Twenty-seven of the 60 PHSs participated in this study, covering 52% of the total Dutch population. In total, 69 outbreaks were reported and investigated using a standardized protocol.

2. Epidemiologists from the 33 remaining PHSs were interviewed for non-response by telephone questionnaire. Twenty-two had not received any reports on outbreaks of gastroenteritis in 1996 and 11 PHSs, had in total 17 outbreaks reported.

3. In 60 (87%) of the 69 reported outbreaks SRSV could be detected, showing the etiologic significance of SRSV in outbreaks of gastroenteritis in the Netherlands.

4. Most outbreaks (78%) occurred during the first 3 months of 1996 and the majority of the outbreaks (84%) occurred in institutions, such as nursing homes for the elderly (59%) and hospitals (25%).

5. The causal relationship between SRSV infection and symptoms was investigated by analyzing the stool samples from cases and controls from 22 outbreaks. SRSV could be detected significantly more in the stool samples from cases (75%) than in the controls (19%).

6. Apart from the high incidence of SRSV in outbreaks, a second striking finding was the limited genetic variability of outbreak strains, with a predominance of a single type of genogroup II strains in the first 10 months of the year, and a shift to a genogroup I strain in November.

7. The mechanism of selection of a predominant strain is intriguing and will be subject for further study. Therefore, molecular typing of SRSV strains from outbreaks remains important.

8. To investigate if epidemic spread of a single SRSV-type occurs only in outbreaks, we recently started a sentinel physician-based case-control study to obtain information on the genetic variability of SRSV strains found in other, presumably less high-risk populations.

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