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RIVM report 284500 017

Report on the fifth workshop organised by CRL-*Salmonella*

Bilthoven, the Netherlands, 18-19 September 2000

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Samenvatting

Op 18 en 19 september 2000 is door het Communautair Referentie Laboratorium voor *Salmonella* (CRL-*Salmonella*) een workshop georganiseerd in Bilthoven, Nederland. Alle Nationale Referentie Laboratoria voor *Salmonella* (NRLs-*Salmonella*) van de EU lidstaten en Noorwegen waren vertegenwoordigd. In totaal waren er 38 deelnemers.

Het programma van de workshop bestond uit verschillende delen. Het eerste deel bestond uit de bespreking van de nieuwe draft van de zoonose richtlijn. Daarna vond een evaluatie plaats van het bacteriologische ringonderzoek en de bacteriologische detectie in de verschillende lidstaten. Verder werd gesproken over de opzet en resultaten van typeringsringonderzoeken en immunologische methoden. De achtergrond en betekenis van (metingen van) antibioticum resistentie werd als speciaal onderwerp door een gastspreker toegelicht.

Summary

At 18 and 19 September 2000 a workshop was organised by the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*) in Bilthoven, the Netherlands. All National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*) of the EU Member States and Norway participated (in total 38 participants).

The workshop programme allowed discussion on different subjects, starting with the new draft zoonoses directive. Subsequently an evaluation was held on the bacteriological collaborative study and activities on bacteriological detection in the Member States. The set-up and results of collaborative typing studies and immunological methods were also discussed. A guest speaker gave a presentation on the background and significance of (measurements of) antibiotic resistance.

1. Opening and introduction of the participants

Mr. A.M. Henken (Director CRL-*Salmonella*, the Netherlands)

First of all I would like to sincerely welcome you all at this workshop. We are with many people, that is, at least 1 to 3 persons of each of the 15 EU Member States and, for the first time, 2 representatives of Norway. A special word of welcome to Mr. Cavitte and Ms. Mäkelä, who are the representatives of the Commission among us. Ms. Mäkelä is the successor of Mr. Niemi.

Secondly, I would like to emphasise that this is a workshop meaning that we all are working these days, that is actively participating in the presentations and discussions. To bring these two days to a fruitfull end, we all have to spent some energy.

During these days I will be your chairman as the head of the CRL-*Salmonella*. I would appreciate it if we all are willing to use the English language during our sessions.

With these words the workshop is opened!

What can we expect from this workshop? The functions and duties of the CRL-*Salmonella* according to the zoonoses directive are:

1. Providing National Reference Laboratories with details of analytical methods and comparative testing;
2. Coordinating the application by national reference laboratories of the methods, referred to under the first mentioned point, in particular by organising comparative testing;
3. Coordinating research into new analytical methods and informing National Reference Laboratories of advances in this field;
4. Conducting initial and further training courses for the benefit of staff from National Reference Laboratories; and
5. Providing scientific and technical assistance to the Commission of the European Community.

The aims of the workshop were defined as to discuss:

- The proposed new zoonoses directive;
- Results of collaborative studies organised by the CRL-*Salmonella* with NRLs-*Salmonella*;
- Organisational aspects of collaborative studies among and within states;
- Research activities within Member States;
- Whether or not there are specific needs among NRLs-*Salmonella*; and
- Activities CRL-*Salmonella* 2001.

Participating are (See Appendix 2): representatives of the EU Commission, a representative of the CRL-Epidemiology of Zoonoses, representatives of NRLs-*Salmonella*, representatives of CRL-*Salmonella* and invited speakers (part of the programme).

2. Review of the presentations

2.1 Current issues on the New Draft European zoonoses directive

Mr. Jean-Charles Cavitte (European Commission)

The proposals for the review of the Community zoonosis legislation have been prepared in the light of Article 15a of Directive 92/117/EEC[1], which obliges the Commission to submit a report and accompanying proposals concerning the measures to be put in force for the control and prevention of zoonoses. The review of the Community zoonosis policy is also part of the actions in the White Paper on Food Safety adopted by the Commission on 12 January 2000. The principles on food safety laid down in the White Paper have been taken into account as appropriate. The ideas put forward as to the new proposal are the provisional view of the SANCO Directorate concerned and they have not yet been endorsed at upper level.

Proposal for a Directive of the European Parliament and of the Council on the monitoring of zoonoses and zoonotic agents

The proposal on the monitoring of zoonoses would be the part relating to "risk assessment" and to a certain extent "risk communication". It would oblige the Member States to monitor zoonotic organisms in general. The list of organisms covered by the monitoring is mainly based on the opinion on zoonoses of 12 April 2000 of the Scientific Committee on Veterinary Measures relating to Public Health. The monitoring systems should be primarily based on existing systems in Member States. However, there should be procedures available to establish common criteria for data collection. It is suggested also to create a basis for co-ordinated Community monitoring programmes. These co-ordinated monitoring programmes would last a limited time period and the results of surveys could be used for instance as the basis for possible control actions such as establishment of modification of pathogen reduction targets.

The collection of human data on the incidence of zoonotic diseases is of paramount importance to obtain feedback on the effectiveness of the control applied and, when necessary, to redirect these measures. The data collected in the framework of communicable diseases network (Decision 2119/98/EC[2]) should be used for the purposes of zoonoses monitoring and control, as well as data collected gathered from other Community sources like the implementation of animal health legislation. Therefore, the new Directive requires close co-operation between human, veterinary and food safety authorities in Member States.

[1] OJ L 62, 15.3.1993, p. 38. Directive as last amended by Directive 1999/72/EC (OJ L 210, 10.8.1999, p.12).

[2] OJ L 268, 3.10.1998, p. 1.

The monitoring of foodborne outbreaks is included also in the proposal. In the light of the increasing importance of antibiotic resistance in zoonotic organisms, it is proposed to include its monitoring within the new Directive.

Proposal for a Regulation of the European Parliament and of the Council on the control of specified zoonoses and zoonotic agents

This is the part of the proposal dealing with "risk management". The approach follows basically the opinion on zoonoses of the Scientific Committee on Veterinary Measures relating to Public Health of 12 April 2000 and the principles of the White Paper on Food Safety.

The proposal on control of zoonoses creates a framework for an incremental pathogen reduction policy. Community targets for selected zoonotic agents in selected farming animal populations would be established, subject to a preliminary opinion by the scientific committee. The targets would be established by comitology procedure within a fixed timeframe.

The proposal permits future modification of pathogen reduction targets. The targets would progressively be set for certain *Salmonella* in laying hens, broilers, and their breeder flocks, as well as turkey and pig breeders. Other emerging pathogens could be selected as targets based on specified criteria.

The descriptive rules concerning control measures at breeder flocks would be minimised compared to the existing Zoonosis Directive. National control programmes would have to be established. The Commission shall approve the programmes. To take into account that animal production systems are nowadays more and more integrated, it is proposed that the Member States should encourage food businesses to establish their own control programmes.

The Commission would have the capacity to decide that certain control methods should or should not be used as part of control programmes.

Provisions relating to intra-Community trade and imports are proposed, also. The basic element in the proposal is to ensure that the purchaser of live animals (except for immediate slaughter) or hatching eggs knows the status of the holding of origin of the animals, through certification. Through comitology procedure, the Commission may decide that a Member State of destination could decide to apply, for dispatches from other Member States, the same tolerances as are applied domestically as a part of the respective control programme.

Discussion:
The main goal of the Directive is to protect the consumers from pathogens. Most probably it is not possible to really eradicate *Salmonella* from all animals. In several cases serotypes are isolated from food which are not often detected from humans, for example *S*. Dublin.
The percentage of human *S*. Typhimurium cases originating from animals is not known.
It is difficult to decide which serotypes must be put in the new directive. Probably *S*. Enteritidis and *S*. Typhimurium and maybe more, depending on serotype prevalence in the different Member States.

The obliged decrease in *Salmonella* infection can be described as, for example, 50%. This means that a country with 20% positive flocks should reduce this to 10%.
With the new zoonoses directive, the function of the CRL-epidemiology will remain the same, i.e. for collection of data.

2.2 Update on the recent workshop of the CRL-Epidemiology of Zoonoses

Ms. Anne Kaesbohrer (CRL Epidemiology of Zoonoses, Germany)

The yearly workshop on "Analytical methods in the epidemiology of zoonoses" was held in Berlin on 4 - 5 September 2000. Main topics of the agenda were the data needs for risk assessment and risk management, and how to achieve this in a better way within the reporting system on zoonotic diseases. Special consideration was given to the aspect of antibiotic resistance monitoring and reporting. The workshop was divided into three parts, first plenary presentations, then work in five separate groups and a final plenary discussion of the results of each group. Within this summary only those parts with relevance for *Salmonella* are summarised.

In the "Food borne Zoonoses opinion" of the SCVPH the conclusion was made that the methods for detection and reporting are not standardised nor harmonised. Therefore data on human incidence, prevalence of food contamination and prevalence in animals are generally not aligned to be comparable. The recommendation was given, that the reporting system should be revised with the objective to follow epidemiological trends in live animals and food, to estimate the true incidence of disease in each member state, to allow comparisons between EU member states and to provide for the early detection of human outbreaks. For this purpose, common case definitions, terminology, sampling schemes, laboratory protocols and methodology are necessary.

For risk assessment many data are needed on the prevalence of zoonotic agents from primary production, during processing (industrial, consumer), on consumption by amount and frequency, information on the dose-response and on the public health effect. Additionally to the prevalences at different levels, distribution curves instead of point estimates should be taken into account to overcome the uncertainty of the information. Close to the consumer level quantitative data (counts of micro-organisms) are needed additionally to qualitative data.

To provide that kind of information, monitoring programmes are necessary which are applied in all Member States in a harmonised way. When drawing up such a plan, different approaches have to be considered, i.e. a sampling plan to detect a disease in a herd or flock if present, a sampling plan to estimate the prevalence of disease in a herd or flock or a sampling plan to estimate the prevalence of disease in a country. The general principle is that a sample size n is fixed and that these sample units are randomly chosen. There are several aspects which have to be considered in a monitoring programme, i.e. sample size, sampling frame,

transport and handling of samples, analytical method (sensitivity, specificity), targets and objectives of the investigation.

In the *Salmonella* working group, the purpose of the annual trend report was discussed for all the different aspects which are requested in the yearly report. Proposals were developed how to improve the manual and tables for reporting as tools for better reporting. As medium and long term approaches, it was stated that data necessary for risk assessment have to be based on harmonised monitoring systems. The importance of the analytical methods and the necessity of a good quality control for comparable data was emphasised.

In the antibiotic resistance working group the next steps to be taken on antibiotic resistance testing were discussed. The objective was defined as to monitor trends in occurrence of resistance to antimicrobials of public health significance in Europe. At least 60 isolates of each of the 5 most important *Salmonella* serotypes of each of the 3 main species of food animals (cattle, pigs, poultry) should be tested. The isolates should be selected in a randomised way among isolates at NRLs-*Salmonella* and clustering is to be avoided. Information about whether isolates derive from active or passive surveillance should be included. The isolates should be taken as close to the level of primary production as possible. The antimicrobials included in the test panel were discussed and agreed on. Reporting should include details on the test method, the testing standard used, the breakpoints used and whether the laboratory does use quality control strains. The results should be reported as percent resistant among isolates tested for each serotype separately.

Other working groups dealt with reporting of human disease, other zoonotic agents and the reporting in a database format.

Reporting by the Reference Laboratories should cover the results of serotyping stratified by main categories of animal species, feeding stuffs and food together with information on the source of the isolates. Furthermore, results of phage typing and of comparisons of strains using molecular methods would be desirable.

The conclusion was made that there is a need for standardisation and harmonisation of the methods for detection of *Salmonella* where the sensitivity of the method for different specimen (incl. sample size) is regarded. Furthermore, quantitative methods for detection of *Salmonella* have to be developed. Methods for antibiotic resistance testing have to be harmonised too.

As regards the monitoring schemes routinely used emphasis was laid on the need for standardisation and harmonisation of the sampling frame, the type of specimen to be investigated, the sample size (n), the size of specimen (in g) investigated in the laboratory and the interpretation and reporting of the results. Furthermore, specific studies / surveys should be carried out.

Resources have to be made available to do that work. It is hoped that this approach will be assisted by decisions which lay down monitoring schemes to ensure high quality data.

A more complete overview on the contributions and results of the workshop is given in the report available at the Community Reference Laboratory for the Epidemiology of Zoonoses.

Discussion:
The most sensitive method for each laboratory can be another method than the most harmonised method. It is important to have a golden standard. When using another method this must obtain equal or better results than this standard.
Nevertheless, within the European Union, one generally agreed method should be described.

2.3 Early warning for *Salmonella* in humans; the zoonotic connection

Mr. Wilfrid van Pelt (RIVM, the Netherlands)

One of the tasks of the surveillance of infectious diseases is the early detection of explosions of infections in order to timely initiate interventions. For this purpose automated national, region crossing, detection of explosions of laboratory confirmed infections in humans has proved to be of value in several countries. Since April 1998 an algorithm has been implemented for *Salmonella* in an application at the National Reference Laboratory (NRL) at the RIVM. The Dutch NRL-*Salmonella* is reference laboratory for *Salmonella* spp. isolated from both human and non-human sources.

From human patients all first isolates of *Salmonella* are sero- and/or phage typed. These isolates originate from 16 regional public health laboratories, covering about 64% of the Dutch population. In September 1998 *Shigella* spp., *Campylobacter* spp. and *E. coli* O157 were added, the latter two for weekly aggregated data only. From trends in historical data, the algorithm computes prospectively, an expected frequency of occurrence for each *Salmonella* type and a tolerance level for the actual frequency above which a potential outbreak is indicated (Figure 1).

Automated evaluation is especially helpful for the monitoring of a large amount of different micro-organisms: for *Salmonella* alone already 600 different types are discerned since 1984 in humans in the Netherlands and an additional 400 from non-human sources. The algorithm has to be as sensitive as possible for explosions but should minimise the number of false alarms. This is achieved by taking into account seasonal fluctuations and secular trends, and down-weighting past outbreaks in the estimations.

Retrospectively 48 (CI_{95} 25-70) *Salmonella* types were seen on average each week whereas 2 (P_{99}:8) came above the tolerance level for a median explosion period of three weeks (CI_{95} 14-70 days). Explosions that were investigated showed that they were noted about 2 to 3 weeks after the onset of the disease for infections caught within the Netherlands and 3 to 4 weeks when contracted abroad.

The outbreak warning application is an add-on on an extensive information system on *Salmonella* in which historical data on earlier explosions, regional distribution of cases and

trends in the occurrence in sources from both human, (farm) (exotic) animals, foods and the environment can be inspected. The same holds with respect to the development of antibiotic resistance. Together this facilitates the first step in the explosion-signal verification process.

Apart from detection of manifest explosions in humans the system allows a view on emerging infections or developments of resistance to antibiotics in animal husbandry, that may pose a threat to human health (*cf.* Figure 2). The whole information system is updated almost each week and available on internet within the RIVM. The internet-site can be reached from outside the RIVM using a password that is regularly changed. The password is available for the human and veterinary inspections, food inspection service, animal health service, and, in principal, to animal production boards and co-workers in the Netherlands or abroad.

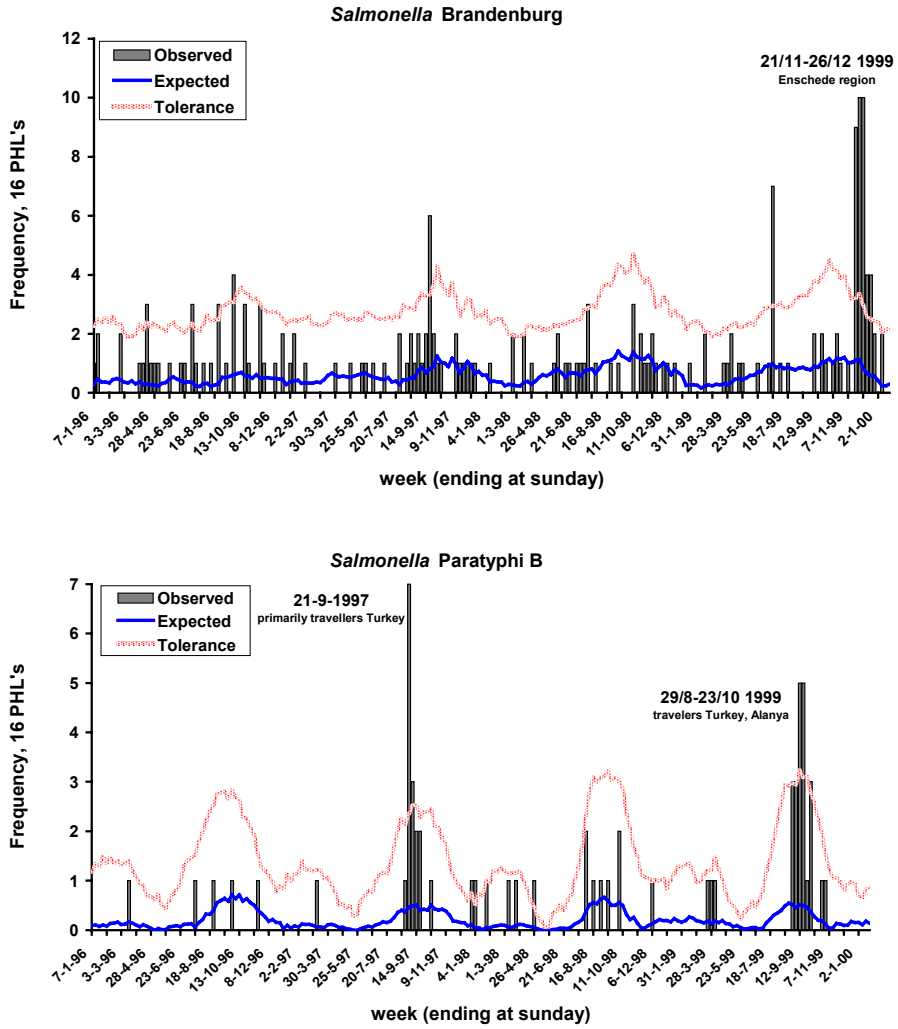

Figure 1. The upper example shows an explosion of infections with S. *Brandenburg at the end of 1999, that after investigation proved to be caused by an imported delicacy named ox-tongue. The lower example concerns the Dutch patients of an international outbreak of* S. *Paratyphi B amongst travellers to Turkey.*

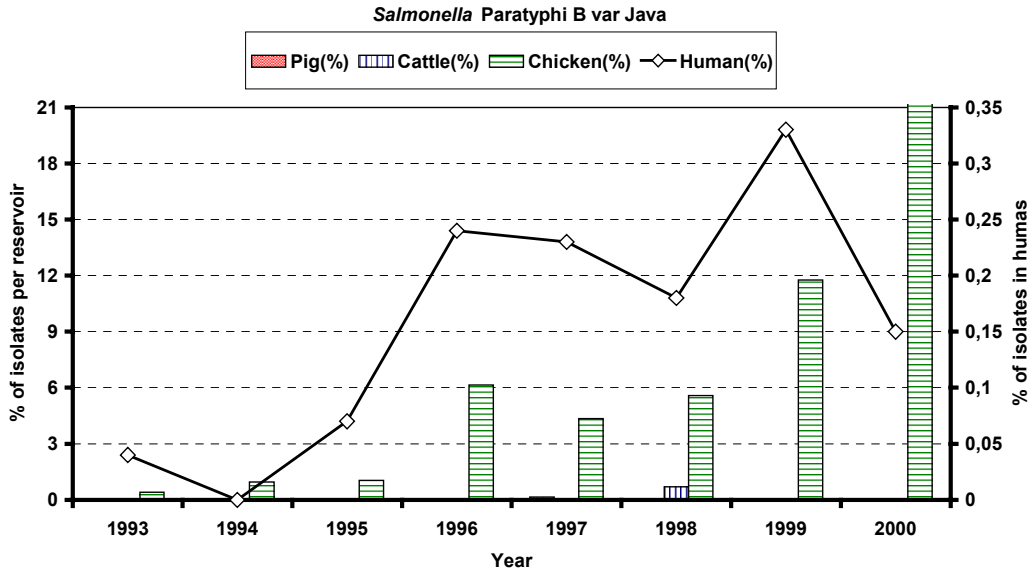

Figure 2. Example that illustrates the emergence of S. *Paratyphi B var. Java in poultry meat which appears to be of negligible impact on humans so far.*

Discussion:

Due to late data reports, prevention is not possible with this warning system. However, if the reaction is not to late, action can be taken to diminish the scale of an outbreak.

The data originate from laboratories at the RIVM. In the Netherlands, laboratories are obliged to send the *Salmonella* strains they isolate to the Dutch *Salmonella* Centre where they are typed. That causes the delay for the warning system.

The data given with strains from human samples are good. It is more difficult to get good information on strains isolated from animals.

2.4 Outbreak of hedgehog related *Salmonella* Typhimurium infection on the Norwegian West Coast August-September 2000

Mr. Viggo Hasseltvedt (NRL-*Salmonella* Norway)

The first cases were detected in early August. As of 14 September 2000, approx. 30-35 cases have been verified. The Agent is: *Salmonella Typhimurium* variant of PT 42.

There is a great preponderance of cases among individuals with gardens harbouring hedgehogs. This connection has been established using an extended questionnaire including questions on contact with hedgehogs. One patient developed septicaemia after directly injuring his hand while touching a hedgehog. Mostly the clinical picture has been that of diarrhoea lasting a few days. Some patients have been hospitalised. There have been no deaths. Children and young adults are among those affected.

Bergen and other districts have been affected. It is possible that there is an interaction between hedgehogs and seagulls carrying carcasses of other animals that the hedgehogs have consumed. PFGE shows an identical profile with human strains of *S. Typhimurium* from a seagull-associated waterborne outbreak in Western Norway comprising approx. 50 cases in January-February 1999. This strain accounts for some sporadic cases in Western Norway throughout the 1990s.
PFGE from the Bergen strains shows similarity but no identity to strains from a hedgehog-associated outbreak in Central Eastern Norway in 1996.

Recently there has been established by PFGE that in Western Norway identity exists between human- and hedgehog-derived *S. Typhimurium* in two different locations in the greater Bergen area indicating two parallel outbreaks.
Norwegian strains of *S. Typhimurium* show great similarity in PFGE patterns perhaps indicating that some of the clones have diversified in the relatively recent past.
PFGE may not be sufficiently discriminating as a method in this connection. Phage typing and other methods may prove necessary to get a more complete overview.
This outbreak has been published in The National Institute of Public Health's Weekly Report no. 34/00 which has the URL: www.folkehelsa.no.

It is documented that individuals have moved couples of hedgehogs physically from one location in Norway to another many metric miles apart to establish or re-establish a local hedgehog population. The Norwegian hedgehog population has declined during the 1990s but is increasing again. Hedgehogs from the Bergen area may very well be of Central Eastern Norwegian origin.

Discussion:
The differences between the PFGE patterns are very small, only a few bands. Most *S.* Typhimurium strains in Norway are conserved very good. Human isolates have been tested, but more hedgehog isolates should be tested to discriminate within the serotype. The small difference in PFGE pattern can be caused by plasmids in the strain. Therefore it might be good to use RAPD or Plasmid Profiling next to PFGE.

2.5 Overview of all bacteriological collaborative studies

Ms. Nelly Voogt (NRL-*Salmonella,* the Netherlands)

Objective

The CRL *Salmonella* has organised four bacteriological collaborative studies on the detection of *Salmonella* in the period 1995 till 1999. In this overview the results of these four studies are presented to gain insight into the performance of the bacteriological examination for *Salmonella* within and between the NRLs-*Salmonella* over the course of time.

Methods

In the first and second study the ISO 6579 method, which was recommended by the Scientific Veterinary Committee of the European Commission was prescribed. In the second study it seemed that the selectivity of selenite-cystine broth (prescribed in the ISO method) was low in the presence of competitive flora. Therefore, in the third study only Rappaport Vassiliadis broth (RV) had to be used as selective enrichment medium. Modified Semi-solid Rappaport Vassiliadis (MSRV) seemed to be more suitable for the isolation of *Salmonella* from chicken faeces and especially for detection of *Salmonella* Enteritidis (SE). Therefore in addition to RV, MSRV had to be used in the fourth study. Furthermore in all studies the samples could be tested in parallel also with the method routinely used in the laboratory.

Materials

In the first study the ability of the NRLs-*Salmonella* to use the ISO 6579 method successfully was examined by testing capsules containing a low number (5 colony forming units (cfu)) of *Salmonella* Typhimurium (STM). The ability of the laboratories to detect *Salmonella* in the presence of competitive organisms in the form of 1 gram chicken faeces was tested in the second study. A low and high contamination level, 100 cfu and 1000 cfu STM respectively, was used to investigate whether there was a relation in the number of positive isolations and the contamination level. As a consequence of the results the contamination level of the STM capsules was reduced to 10 cfu and 100 cfu in the third study. In this study for the first time capsules containing SE (100 cfu) had to be examined also. In the fourth study instead of 1 gram 10 grams chicken faeces had to be added to the capsules. The contamination levels of the STM capsules were 10 cfu and 100 cfu again, while SE capsules containing 100 cfp and 1000 cfu were included.

Results & conclusions

In study I and IV two and three (different) laboratories respectively isolated *Salmonella* from one to three blank, or negative, capsules. Concerning the positive control capsules, one laboratory found in all studies one or more of these capsules negative for *Salmonella*. For the statistical analysis, the percentage of *Salmonella* positive capsules isolated in the presence of competitive flora with the prescribed method was used.

The results between the four collaborative studies were compared taken the results of the laboratories together for each study. The results of study I and II were significantly better in comparison to those of study III and IV. This may be caused by testing only capsules (study I), the use of high contamination levels STM (study II) and/or using capsules containing STM only (study I and II).

The results between the laboratories were compared taken the results of all studies together for each laboratory. Six laboratories found significantly more *Salmonella* positive samples and 3 laboratories found significantly less *Salmonella* positive samples. Analysing only the three best performing studies, 2 of these 3 laboratories still were less than average.

Within two laboratories no significant difference was found in the results between the studies. However, within 12 laboratories a significant difference between studies was found. The results of the 2 remaining participants were not analysed, because they did not carry out one of the studies.

Coming years the same protocol in the collaborative studies as used in study IV will be used to enable a trend analysis.

Discussion:
No better results for laboratories were revealed if the method routinely used was evaluated next to the prescribed method.
A lot of NRLs-*Salmonella* mostly perform typing of strains but do not isolate the strains in their laboratory. This could be a reason for not performing very well. These data on level of experience are not exactly known at the CRL-*Salmonella*, but should be taken into account for the evaluation of the bacteriological collaborative studies.
No difference has been made between SE and STM for evaluation. The same strains of SE and STM have been used in the different studies.
It should be checked which batch of medium is used per laboratory. This could be a reason for not performing well in a study.
Research revealed that there was no influence of duration of transportation of the parcels.
It is important to have some data on the level of contamination of natural samples to know how to continue with the next bacteriological collaborative studies.

2.6 Investigation of *Salmonella* contamination on commercial egg laying farms

Mr. Rob Davies (NRL-*Salmonella,* United Kingdom)

The workshop presentation gave examples of investigations carried out on layer farms which were infected with *S.* Enteritidis. On a depopulated free range unit, 3 months after depopulation of the site, *Salmonella* was widespread in most of the houses and was found in fresh droppings from mice and cats. Interestingly a group of pullets housed in an uncleaned contaminated pen failed to become infected. At 8 months post depopulation contamination was still present in soil and in most of the pens, particularly in feeders, nest boxes, thinly scattered litter, dust and chunks of dried faeces collected from between slats. After 26 months *Salmonella* was present in some of the houses. In samples taken during the summer after 13 and 26 months garden beetles were present in the feed trough in one house and these and their larvae were found to be carrying *Salmonella.* The large, succulent larvae would be very attractive for birds had the house been populated.

On a large mixed housing type farm, *S.* Enteritidis was found in both of two barn egg houses, and in 2 of 3 free range houses, where birds had been vaccinated with a commercial bacterin. The prevalence of contamination was lower in vaccinated groups however and the best samples were swabs or litter from nest boxes, litter from scratching areas or bulked faeces

comprising aggregated material trapped between slats. Samples were also taken in a large two storey cage layer unit on the site. There was little difference in the prevalence of *Salmonella* in environmental samples in the half of the houses containing vaccinated birds compared with the non-vaccinated half. There was however less *Salmonella* in samples from fresh droppings but more general contamination relating to egg belts and dust in the vaccinated group. *Salmonella* was also found on beetles in litter (*Alphitobius)* and in fresh droppings from starlings. There was also contamination of egg grading equipment, even after cleaning and disinfection the previous day. At post-mortem examination 1.7% of spent hens from the non-vaccinated group were carrying *Salmonella.* After full vaccination of the whole site *S.* Enteritidis was only found in flies.

On a 4 house barn egg production site sampled before and after cleaning and disinfection *Salmonella* was found in 3 of the 4 houses with dust and swabs from nest boxes being the best samples. The egg grading and packing equipment was also substantially contaminated. *Salmonella* Enteritidis was also found in mice. After cleaning to a high standard and disinfection with a combination disinfectant product containing formaldehyde, glutaraldehyde and quaternary ammonium compound applied through a foaming lance there was good elimination of *Salmonella* at floor and slat level, except for within pools of wash water. The undersides of ventilation ducting and interiors of some nest boxes as well as grading equipment were still contaminated as it is difficult to disinfect the undersides of equipment with a foaming disinfectant. These areas were retreated by fogging with formaldehyde and the houses restocked with non-vaccinated birds. Repeat sampling at the end of the laying period showed that there had been no carryover of infection on site.

The prevalence of *S.* Enteritidis in eggs and spent hens from an infected cage layer flock was investigated. Even though there was extensive environmental contamination within the house contaminated eggs were only recovered on one occasion but the prevalence of infected spent hens ranged from 0-42.9%. *Salmonella* was only isolated from egg contents after 72 hours pre-enrichment in Buffered Peptone Water supplemented with 10 g/l beef heart infusion. Moistened sterilised eggs passed through the contaminated packing plant failed to acquire *Salmonella* on this farm and also from another farm where the packing plant was highly contaminated.

In a study with *Salmonella* negative pullets which had been treated with competitive exclusion (CE) culture on entry to the laying house, there was a gradual rise in seroprevalence up to a maximum of 60%, which was identical with the seroprevalence found in non-treated birds in the same house. The prevalence of *Salmonella* in individual droppings collected from the two groups of birds was substantially reduced in the treated groups however. In a further visit carried out to the premises once all replacement birds had received CE, only one *Salmonella* isolate, from dusty material on the floor, was found, suggesting that the flock infection had cleared.

This work, together with studies on other farms, has been valuable in demonstrating the value of vaccination and disinfection with formaldehyde, as well as showing the limited role of the

egg packing plant in dissemination of contamination. Sampling sites for optimising detection of *Salmonella* in laying flocks by environmental monitoring have also been identified.

Discussion:
The number of samples taken per company is 300-500. None of the samples were pooled because this would cause a dilution of the samples, resulting in a decrease of sensitivity.

2.7 Estimation of the percentage of contaminated eggs laid by a naturally contaminated *Salmonella* Enteritidis flock

Ms. Florence Humbert (NRL-*Salmonella,* France)

Eric JOUY, Florence HUMBERT, Karine PROUX and Gilles SALVAT

Evidence obtained from different studies on naturally or artificially *Salmonella* Enteritidis (SE) infected laying flocks suggests that:

1. The number of contaminated eggs is low
   - for natural infection, incidences are typically less than 0.3 ‰ (Schlosser et al., 1995; Kinde et al., 1996)
   - even for experimental infection studies, reported frequencies of egg contamination are between 0.1 and 10 % with an overall prevalence of 0.6 %.
2. The number of *Salmonella* cells in freshly laid egg is very low (Gast and Bear, 1990; Bichler et al., 1996)
   - fewer than 10 *Salmonella* / egg
   - less than 10 *Salmonella* / ml (mean of 600 *Salmonella* / egg)

The purpose of the study was to detect *Salmonella* internal contamination of egg using a simplified bacteriological method based on direct plating from egg yolk, after incubation of these eggs 10-12 days at an elevated temperature of 35°C (in order to multiply an initial low contamination, if this contamination does exist). This method which could be considered as a sterility control test, was applied because it allowed us to analyse large number of eggs.

Materials and method

Eggs

From a naturally infected flock of 35,000 caged laying hens (eggs for consumption) recognised as positive for SE since 2 months (monthly environmental swab and faeces positive by bacteriology) a total of approximately 6,500 eggs were collected from two dates at one week interval. From these 6,500 eggs 6,370 without any apparent external crack or faecal contamination were selected for the study and randomly divided in 12 groups of 540 eggs each. Each egg was individually packaged in a plastic bag in order to prevent cross-contamination in case of egg explosion during incubation.

Negative controls

150 eggs from a laying flock raised at the experimental station of our institute in Ploufragan and controlled as negative for *Salmonella* were used as negative controls.

Incubation of eggs
The 12 groups of 540 eggs and the 150 controls were put into an incubator, on 12 consecutive days, in a protected room usually used to raise contaminated animals. The heating system was settled in order that the room temperature reaches 35°C.

Analysis of incubated eggs
Eggs were daily analysed by group of 540 by :
1- breaking the egg by taping the bag on the bench, at the laboratory
2- opening the bag with scissors, near the flame
3- using a calibrated disposable plastic loop to homogenise egg yolk and spread out an inoculum of approximately 10 µl onto half a plate of brilliant green agar (BGA).

Reading the BGA plates
Each result (2 by plate as each egg used half a plate) was scored as:
1- no growth
2- growth, but only non characteristic colonies present (mainly yellow to green colonies, but also some white coci-like colonies, yeast and mould)
3- Growth, with presence of pink to red colonies, even if not a pure culture or not isolated.

Re-isolation onto more Salmonella specific media
All isolations leading to the presence of doubtful or characteristic colonies on BGA (group 3) were used to pick up these suspected colonies in order to re-isolate them onto Rambach agar. This media, more specific and easier to read than BGA, allowed to more precisely check for the presence of characteristic colonies for *Salmonella*.

Each isolated colony with morphology related to *Salmonella* on Rambach agar (pink) was further identified by inoculation on Kligler agar.

Results

- None of the eggs exploded and very few showed cracks during incubation. None of these cracked eggs were positive for *Salmonella*.
- All eggs used as negative control were classified in group 1 (no growth).
- Approximately 10% of isolations on BGA needed to be re-plated onto Rambach agar, because of the presence of pink to red colonies.
- Only one egg out of 6,370 analysed yielded *Salmonella* typical colonies on Rambach agar (pure heavy culture) which also showed typical Kligler reactions (glucose+; lactose-; H2S+ and gas+) and were confirmed as SE by serotyping.

Conclusion

The percentage of positive eggs laid by this flock is estimated to be 1 out of 6370, *i.e.* 0.1 to 0.2 ‰ which is not very different from the 0.3 ‰ reported in literature.

Our first goal is now to validate our method of recovering low contamination from eggs by performing artificial inoculations of small numbers (<10 cells/egg) of *Salmonella* Enteritidis in a sufficient number of eggs (minimum 300) by 3 routes (in the yolk, in the albumen, just inside the egg membranes). The statistical validation will come from the comparison between

results of recovery of these artificial contaminations with Poisson distribution. In fact, when using high dilution of a pure culture, there is a calculated statistical probability that some inoculum used to artificially inoculate the eggs, does not contain any cell.

Our second objective, if the methodology is validated, is to analyse as much flocks as possible to determine quantitative relations between :
- serological results (yolk and sera)
- environmental contamination (litters and swabs)
- animal contamination (individual or pooled faeces and organs)
- number of positive eggs.

As a conclusion, it is important to have a detection system available that identifies even those flocks where prevalence of eggs with *Salmonella* positive contents is relatively low. One must bear in mind that 1 positive out of 6,000 means a daily delivery of 5 positive eggs from a flock of 30,000 hens.

Discussion:
The incubation of the eggs for 10-12 days enables the *Salmonella* to migrate from the egg white to the egg yolk. Some control experiments will be performed to investigate this.
It is not known how many of the individual chickens are contaminated with *Salmonella* within a flock, and there is no method to examine this.

2.8 Comparison of dung samples and sock samples for surveillance of *Salmonella* in poultry under the Danish *Salmonella* Control Programme

Mr. Mogens Madsen (Danish Veterinary Laboratory)

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In the present EU Zoonoses Directive (Directive 92/117/EEC) surveillance programmes for *Salmonella* in poultry are primarily based on the collection of fresh faeces samples from poultry houses as sample material for bacteriological analysis. Sixty samples have been chosen as the sample unit per flock which conveys a theoretical detection limit of 5% infected birds with 95% confidence.

The collection of 60 individual faeces samples by hand is laborious. Besides, the quality of the combined sample depends heavily on the motivation and ability of the collector to pick fresh droppings, and to collect a sample representative of the area occupied by the flock.

Several studies have investigated the use of alternative sampling methods for *Salmonella* in poultry flocks. One of these methods is the 'sock' method where elastic cotton tubes pulled over the collector's boots absorb faecal droppings while walking through the poultry house. The method has been thoroughly investigated in Danish broiler flocks (Skov *et al*., J. Appl. Microbiol. 1999, 86:695-700), and it was concluded that a sampling protocol of five pairs of socks analysed as five pools had a sensitivity comparable to the hand collection method of 300 faecal samples analysed as 60 pools of five faecal samples. The pre-harvest sampling of broiler flocks for *Salmonella* by five pairs of socks was consequently introduced at the end of 1997, in replacement of the previous collection of 60 faeces samples by hand.

Recently, the Danish *Salmonella* Control Programme (1997-1999) has been revised and extended for a further three-year period (2000-2002). One of the major revisions of the surveillance programme for central rearing and parent layer flocks is the replacement of 4-weekly serological samples with weekly faecal samples for bacteriological analysis.

In order to improve the sensitivity and at the same time optimising the sampling economy, 'sock' samples have replaced the collection of 60 faecal samples in the revised *Salmonella* Control Programme. In this programme, the sampling unit for each poultry house is two pairs of 'socks' analysed as one pooled sample.

The presentation reports the results of a comparison of the two sampling methods when applied to 41 floor-raised poultry flocks infected with *Salmonella*.

2.9 Methodology for detection of *Salmonella* from chicken faeces

Ms. Nelly Voogt (NRL-*Salmonella,* the Netherlands)

Introduction

The semi-solid media Diagnostic Semi-solid *Salmonella* (DIASALM) and Modified Semi-solid Rappaport Vassiliadis (MSRV) seem to be useful for the isolation of *Salmonella* bacteria in poultry faeces. In this study the results of these media were compared with the results of Rappaport Vassiliadis broth (RV) for the detection of *Salmonella* in faecal samples from broiler and layer flocks.

Materials

The samples were derived from poultry layer and broiler flocks. From each flock of poultry 60 fresh faecal samples were randomly collected and pooled into five pool-samples. In this comparative study, each pooled sample was considered as a separate sample.

Method (adapted ISO 6579)

- pre-enrichment
  - 25 gr faeces added to 225 ml BPW
- selective enrichment
  - 0.1 ml pre-enrichment in 10 ml RV-broth
  - 0.1 ml pre-enrichment onto DIASALM
  - 0.1 ml pre-enrichment onto MSRV
- isolation on BGA
- biochemical and serological confirmation

Results

In total, 1,022 faecal samples from poultry layer flocks and 892 faecal samples from broiler flocks were tested. The total cumulative number of samples with *Salmonella* isolated from layer and broiler flocks after 48 h of incubation of the selective enrichment media was 132 and 108, respectively. With DIASALM and MSRV significantly more positive samples were found compared to RV. No significant difference was found between the results of DIASALM and MSRV. A number of samples was only found positive for *Salmonella* using RV or only positive for *Salmonella* using semi-solid media. The serotypes isolated in these cases are shown in Table 1.

Table 1: Serotypes isolated with RV only, or with semi-solid media only

flocks	**RV positive and semi-solid negative**	**RV negative and semi-solid positive**
poultry layer	(n = 3) *S*. Panama (2x) *S*. serologically rough (1x)	(n = 72) *S*. Enteritidis (74 %)
broiler	(n = 6) *S*. Panama (1x) *S*. Enteritidis pt 4 (1x) *S*. spp. enterica I 4, [5],12: non-motile (4x)	(n = 35) *S*. Enteritidis (± 40 %) *S*. Paratyphi B v. Java (13 %) *S*. Infantis (± 20 %)

Conclusion and discussion

A main cause of the large amount of false-negative samples found using RV is the low isolation percentage of *Salmonella* Enteritidis. The results of this study favour the use of semi-solid media for the detection of *Salmonella* in poultry faeces. However, the semi-solid media MSRV and DIASALM are not suitable for all *Salmonella* serotypes. Therefore, it is recommended to use MSRV (instead of DIASALM because MSRV is easier to interpret) in combination with RV to detect *Salmonella* in poultry faeces.

Discussion:
It is better to use MSRV and another enrichment medium instead of RV, since similarity of these two media may be to high. Also because RV is too toxic for selective enrichment.
Some laboratories have problems with the growth of Proteus on BGA plates. However, most laboratories do not have this problem.

2.10 Discussion on future bacteriological collaborative studies and discussion on reference material needs

Mr. André Henken (CRL-*Salmonella*, the Netherlands)

During the previous workshop of the CRL-*Salmonella* in 1999, various NRLs-*Salmonella* asked whether it would be possible for the CRL-*Salmonella* to provide them with reference capsules for their national collaborative trials. Also, the NRLs-*Salmonella* wanted to know whether the price of these capsules was negotiable and whether it was possible to obtain capsules with other serotypes and other concentration levels as up to this moment only capsules with *Salmonella* Panama are available with a concentration level of 5 cfp/capsule.

Following these questions the CRL-*Salmonella* did 2 things: Firstly, a questionnaire was held (via the quarterly newsletter) to get an estimate of the initial needs of the various NRLs-*Salmonella* for numbers of capsules, what serotypes and which concentration levels (see Table 1). As it was clear from the beginning that production and distribution could not be considered a task of the CRL-*Salmonella* an alternative solution had to be found. The CRL-*Salmonella* decided that, although it was not her task, she could play an initiating role as an intermediate negotiator between the NRLs-*Salmonella* and a producer of reference materials. Therefore the second thing that was done was that the CRL-*Salmonella* made contact with the Dutch Foundation for the advancement of Public Health and Environmental Protection (SVM), located at Bilthoven at the RIVM, to discuss the questions raised. SVM sells several reference materials (Table 2).

*Table 1: Needs of the NRLs-*Salmonella *for reference materials*

	S. Panama	STM1000	STM100	STM10	Blank	Other/remarks
1	100	100	100	50	50	Annually
2	-	-	75	-	25	Later, probably more
3	100					Or STM5: 100
4	-	20	20	-	-	SE100: 20
5	75	-	-	75	-	-
6	30	30	30	30	30	Numbers not definite
7	-	-	100	100	-	SE100: 100 SE1000: 100
8	10	-	10	-	-	To start with
9	20	20	20	20	-	-
Total	335	170	355	275	105	STM5: 100 SE100: 120 SE1000: 100

Table 2: Available Microbiological Reference Materials at SVM

SVM- order number	Strain	Contamination level cfp/capsule
701.10	*Salmonella* Panama (ALM41)	5
702.10	*Listeria monocytogenes* (ALM92)	5
703.10	*Enterococcus faecium* (WR63)	500
704.10	*Bacillus cereus* (ATCC 9139)	5000
705.10	*Enterobacter cloacae* (WR3)	500
706.10	*Escherichia coli* (WR1)	500
708.10	*Clostridium perfringens* (D10)	5000

During the discussions 2 things became clear. The first things was that the price is not negotiable as the present level of fl 5.50 per capsule does represent the production cost level already. However, SVM is willing to consider production of capsules with other serotypes and other concentration levels.

In the end it was agreed between SVM and CRL-*Salmonella*:

1. That the process for starting production of RMs containing *Salmonella* Typhimurium 10 and *Salmonella* Typhimurium 100 will start as soon as possible, which will include: discussion with all parties involved in SVM; preparation and official printing of all 'paperwork' for production, control and selling of the new RMs. This process might take about 6 months, so that the new RMs might become available after 1 April 2001.

2. That investigations will be done into the stability of the present available batches of highly contaminated milk powder containing *Salmonella* Enteritidis. If a sufficient stable batch is available, the process for starting production of this type of RM can start at SVM as well following the process as stated under 1.

3. That it will be discussed whether it will be necessary to prepare new batches of highly contaminated milk powder, containing other *Salmonella* strains (depending on the interest). Preparation of new batches highly contaminated milk powder will take at least 1 year before stable RMs can be produced from these powders. At the present workshop it appeared that the NRLs-*Salmonella* would appreciate availability of capsules with *S*. Panama, *S*. Typhimurium and *S*. Enteritidis and had no need for more than these.

From the present workshop the needs for capsules can therefore be specified as:
- *S*. Panama at a level of 5 cfp and a somewhat higher level (≤50 cfp);
- *S*. Typhimurium at a level of 10 and 100 cfp; and
- *S*. Enteritidis at a level of 100 and 1000 cfp.

The CRL-*Salmonella* shall communicate this to SVM.

Discussion:
The samples to be investigated for the bacteriological study should be the same for some time in order to compare the results of the laboratories in time. It might be possible to use Tetrathionate broth for selective enrichment.
It will be evaluated if the number of control capsules that have to be examined without addition of faeces can be decreased. However, there are always some laboratories that fail to detect *Salmonella* from the control capsules.
A portion of RV(S) medium (powder) should be sent to the participants of the study, so that a fair comparison can be made for this medium between laboratories.

2.11 Overview of all typing studies

Ms. Nelly Voogt (NRL-*Salmonella,* the Netherlands)

Introduction

The CRL-*Salmonella* organised four collaborative studies on serotyping of *Salmonella* in the period 1995 till 1999. The main objective of these studies was to compare the test results of the NRLs-*Salmonella* in cooperation with the CRL-*Salmonella* in order to attain harmonisation. In this overview the results of the four studies were analysed to gain insight into the performance of the serotyping for *Salmonella* within and between the NRLs-*Salmonella* over the course of time.

Materials and methods

In all studies the typing method routinely performed in the participating laboratory had to be used. In study II, III and IV the laboratories were allowed to send strains for serotyping to another laboratory in their country.

In the first study the strains included belonged to the species *Salmonella enterica* spp *enterica*, *salamae* or *houtenae*. In study II to IV only strains belonging to the species *Salmonella enterica* spp *enterica* had to be identified. In study II and III frequently found serotypes and in study IV a mix of frequently and infrequently found types were included.

Results and conclusions

Besides the results of the identification of the strains, also the results of the detection of the O and H antigens were compared separately.

The four collaborative studies were compared taking the results of the laboratories together for each study. The identification of frequently occurring strains yields better results than when less frequently occurring strains were included. The O antigens were detected correctly by 50% of the participating laboratories, while detection of the H antigens gave the most incorrect identifications.

The results between the laboratories were compared taking the results of all studies together for each laboratory. Two laboratories identified all strains correctly and compared to the other

participating laboratories 2 laboratories identified significantly less strains correctly. Eight laboratories detected the O antigens correctly in all studies; three laboratories detected significantly less O antigens correctly. Two laboratories detected the H antigens correctly in all studies, while 4 laboratories detected significantly less H antigens correctly.

Within 11 of the 16 laboratories no significant differences were found in the results between the studies. Five laboratories identified/detected significant more strains/antigens correctly over the course of time.

Discussion

The collaborative study must be seen as a challenge and therefore it must be not too easy and not too difficult.

Discussion:
It will be investigated whether accredited laboratories perform better in the typing studies than laboratories which do not have an accreditation.

2.12 Molecular typing of *Salmonella* - Harmonisation and standardisation in Denmark

Ms. Dorte Lau Baggesen (NRL-*Salmonella,* Denmark)

D.L. Baggesen[1], M.N. Skov[1], M. Torpdahl[1], D. Sandvang[2], P. Gerner-Smidt[2]
[1]Danish Veterinary Laboratory and [2] Statens Serum Institute

Epidemiological characterisation has the aim of differentiation of bacterial strains within a species. The differentiation makes it possible to trace the spread of bacteria within animal production, in food and to humans. In Denmark, a surveillance system based on characterisation of *Salmonella enterica* has been established through the last decade, which enables a quantitative risk assessment of the different sources of human salmonellosis (Anon., 2000). In addition, the surveillance system and the strain collection, which is made on the basis of the surveillance, constitute an informative and necessary background for outbreak investigations.

A prerequisite for such a surveillance system is the accessibility of typing methods, which are cheap, stable, and reproducible and where the results can be compared between laboratories and over time (definitive methods). Through decades, the basic method applied for characterisation of *Salmonella enterica* has been serotyping as described in the Kauffmann-White scheme followed by phage typing for further differentiation within the most common serotypes.

Even though serotyping and phage typing have proven to be useful for the purpose, these methods have their limitations. Both methods are based on detection of phenotypical traits, which from time to time are damaged resulting in untypable strains or unspecific types. Also

the discriminatory power of the methods can be insufficient which especially is the case of outbreak investigations.

Alternative methods e.g. molecular methods as pulsed field gel electrophoresis (PFGE) and plasmid profiling have been applied for epidemiological characterisation. These methods are in general more differentiating but also more expensive and laborious, and results can only partly be compared between laboratories as comparison of strains in most cases demands parallel examination. There is, therefore, a need of definitive and highly differentiating methods, which are applicable for typing of a large collection of strains.

In Denmark, Statens Serum Institute and the Danish Veterinary Laboratory collaborate in a project that has the aim of improving the background for tracing of infection sources for human salmonellosis. The project includes a harmonisation and standardisation of methodology applied in the two laboratories where by results, hopefully, can be compared and evaluated in electronic form via the internet.

Two methods are included in the project: pulsed field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP).

PFGE has been used several years in both laboratories and harmonisation of the methodology has been performed during 2000. The method has been useful for investigation of more outbreaks and has shown to differentiate within several serotypes and to a less degree within phage types. Strains of *S.* Senftenberg and *S.* Typhimurium DT104 and U302 that have been cause of more cases of human disease have been typed in both laboratories and compared to strains from animal sources and food. Pictures of gels are transferred via the internet and type assignation compared between the two laboratories.

AFLP has been established in both laboratories and results will be compared between the laboratories in the near future. Results of AFLP are directly registered in electronic form, which make the method a good candidate for a molecular, definitive typing method. The first results show, however, that even though more combinations of enzymes have been evaluated the method has limited discriminatory capacity within important serotypes as *S.* Enteritidis and *S.* Dublin. At serotype level, the results are promising and enable for differentiation between investigated serotypes.

The objective of the Danish project is to obtain optimal typing protocols as well as fast result for detection of outbreaks and trace sources of human *Salmonella* infections. The internet based network for molecular typing is a tool for this objective. The Danish network is in many ways similar to the American PulseNet and it is our hope to extend the network and collaborate with laboratories from other countries e.g. the countries in the European Union. (Sheets of this presentation are printed in Appendix 4)

Discussion:
An extended version of the AFLP method is used in the Danish laboratories. Otherwise it is not possible to make a distinction between outbreak and non-outbreak strains.

2.13 Trends in *Salmonella* isolation from livestock and animal feed Jan-June 2000

Mr. Rob Davies (NRL-*Salmonella,* United Kingdom)

Rob Davies and Sarah Evans

Most (508 of 580) *Salmonella* incidents in chickens originate from non-statutory surveillance of broilers. Although similar surveillance schemes apply to commercial layers only 8 incidents were related to these. There has been a decline in *S.* Typhimurium, particularly DT104, in cattle and a concurrent increase in *S.* Dublin. *S. enterica* subspecies diarizonae, serovar 61:k:1,5,(7), which often appears as incomplete antigenic structures, has increased in sheep. As in cattle, *S.* Typhimurium has fallen, but so has *S.* Dublin.

In pigs there has been a substantial reduction in the number of diagnostic submissions because of the depressed economic state of the industry. The proportion of incidents involving *S.* Typhimurium continues to rise however. In chickens, *S.* Enteritidis has declined so that is no longer represented in the 'top 10'. *S.* Senftenberg and *S.* Give have risen but this largely relates to one poultry company.

S. Typhimurium in turkeys has continued to decline, with *S.* Derby, again company related, increasing. In animal feed there has been a large rise in *S.* Agona in rapeseed oil production and this is reflected in the data for isolation of *Salmonella* for finished feed, where it was the most common serotype isolated from feed from all livestock species. There are also some more recent indications that *S.* Agona may now also be increasing in farm livestock.

The data discussed shows the dynamic state of *Salmonella* infection in livestock and animal feed. Contamination of the cooling systems in vegetable oil extraction and compound feed production remains a significant problem and these factors, combined with persistent contamination of ventilation ducts in hatcheries, are responsible for the majority of *Salmonella* infections in poultry flocks.

Discussion:
The declined number of *Salmonella* Typhimurium DT104 can be caused by different factors e.g. immunity against the micro organisms.

2.14 Increasing number of *Salmonella* Paratyphi B (D-tartrate positive) isolations from broilers

Ms. Christina Dorn (NRL-*Salmonella,* Germany)

Christina Dorn, Andreas Schroeter, Angelika Miko and Reiner Helmuth

In the last years the number of isolations of *Salmonella* Paratyphi B sent to the national *Salmonella* reference laboratory of Germany has increased steadily. Most of the isolates originated from fowl or poultry products. The bacteriological, serological and biochemical properties of the isolates were investigated. Special emphasis was given to the utilisation of d-tartrate which subgroups the serotype. All of them belonged to the d-tartrate positive variant, which is generally considered less virulent for humans and was formerly called *Salmonella* java. The performance of various tests is compared and the lead-acetate-test proved to be the best for testing d-tartrate utilisation. However there is a need for a faster, reliable test (PCR). Currently we work on the development of this test. In addition the possibility of the spread of this *Salmonella* serovar within the production line is discussed. Consequently, veterinary medicine and human medicine should work together in order to solve problems like this.

Discussion:
No numbers are available about the human situation of *Salmonella* Paratyphi B. var. Java. Probably the main cause of the spread of this *Salmonella* serotype are the producers.

The designation of a serovar is not all the same between laboratories, which can cause problems in determination of an outbreak.

2.15 Results of the last typing study and discussion on future typing studies

Mr. Wim Wannet (CRL-*Salmonella*, the Netherlands)

Test results of *Salmonella* serotyping (20 strains) by the participating 17 National Reference laboratories for *Salmonella* and 15 EnterNet laboratories were evaluated and discussed, resulting in the following outlines:

- 50% of the NRLs-*Salmonella* performed serotyping on a daily basis, all using commercial sera;
- 80% of the ENLs performed serotyping on a daily basis, 80% using commercial sera (and 20% using ‘own prepared’ sera).

In general the participating laboratories performed rather well:

- O-antigens: typed correctly by at least 75% of the laboratories (mean 86%);
- H-antigens: typed correctly by at least 70% of the laboratories (mean 84%);
- Name serovar: correctly designated by at least 60% (mean 84%).

There were no significant differences between the performance of NRLs-*Salmonella* versus ENLs. Based on data from 1999 it was confirmed that laboratories typing larger numbers of *Salmonella* performed significantly better than other laboratories.

In the present typing study there was a general problem with the serotyping of *S.* Glostrup. Most laboratories encountered problems with the O-serotyping of this species leading to incorrect designations (mainly as *S.* Chomedey*)* of this strain. The explanation for this phenomenon remains unclear, however, there could be influences from the different culture media used (smooth-to-rough conversion), or from the different batches of antisera used (Glostrup: O6, 8; Chomedey: O8).

During the discussion it was decided that for the next serotyping study, in 2001, the same level of difficulty should be maintained, to keep the collaborative study a challenge for the participating laboratories.

Discussion:
During the workshop it was not clear whether or not the strains send back to the CRL-*Salmonella* were rough. One laboratory received two strains and tested both: the two strains were not rough and identified as *S*. Chomedy. Will be continued.

2.16 Comparison of ELISA and conventional methods for the isolation of *Salmonella* from porcine faeces

Mr. John Ward (NRL-*Salmonella,* Ireland)

J. Ward, A. Murphy, G. Murray and J. Egan

Conventional bacteriological methods are slow and in some cases may take up to 7 days to confirm a sample negative. In the last decade several new methods have been developed to produce fast accurate results for the isolation of *Salmonella* from faeces.

The Vidas *Salmonella* is an automated qualitative test for use on the Vidas analyser for the detection of *Salmonella* in food and environmental samples using ELFA technique (Enzyme linked Fluorescent Assay). In our study, at NRL-*Salmonelle* Ireland, 70 porcine faeces were tested by conventional methods and Vidas.

The following methods were evaluated:

1. Add 0.2 gram of faecal material to 10 ml of RV broth. Incubate for 18 hours at 41°C ±1°C. Transfer 0.1 ml to 10 ml M-broth. Incubate for 6 hours at 41°C ±1°C. Heat 0.5 ml at 100°C for 15 minutes. Carry out Vidas *Salmonella* Elisa. Confirm positive Elisa by culture of the residual M-broth stored at 4°C.
2. Add 1 gram of faecal material to 10 ml Buffered Peptone Water. Incubate at 37°C for 4 hours. Add 0.1 ml to 10 ml RV broth. Proceed as by method 1.

3. Add 1 gram of faeces to 10 ml Buffered Peptone Water. Incubate at 41°C ±1°C. add 0.1 ml to centre of semi solid rappaport plate (MSRV). Incubate at 41°C for 24 hours. Add 10 ml to 100 ml selenite cystine broth. Incubate at 37°C for 24 hours. Selective plating of the enrichment steps was onto brilliant green and mannitol lysine crystal violet brilliant green agars.

Results of the different methods are shown in Table 1.

Table 1: A comparison of the VIDAS with the conventional culture media for the detection of Salmonella in 70 porcine faecal samples.

Method/medium	Number of samples positive (%)	Number of false positive samples (%)	Number of false negative samples (%)
Vidas, method 1	4 (5.7)	1 (1.4)	8 (11.4)
Vidas, method 2	7 (10)	1 (1.4)	5 (7.1)
MSRV	8 (11.4)		4 (5.7)
RV	9 (12.8)		3 (4.3)
Selenite	8 (11.4)		4 (5.7)

Discussion:
The aim of the study was an evaluation of the VIDAS analyser for the detection of *Salmonella* in faecal samples. When the results were acceptable, maybe it could be used as a screening method.

2.17 Validation of an LPS ELISA to detect *S.* Enteritidis antibodies in egg yolk

Mr. Maurice Raes (CRL-*Salmonella,* the Netherlands)

Detection of *Salmonella* Enteritidis (SE) in poultry layer flocks can be performed in several ways, i.e. bacteriological, molecular biological and serological or immunological tests can be performed. Using the bacteriological test, the bacteria concerned are isolated from the samples taken from the flock. However, cultivation of *Salmonella* and serotyping the strain takes a minimum of 4-5 days. A test that obtains a quicker result, to detect *S.* Enteritidis is the detection of antibodies against SE which are excreted in the egg yolk after an infection with SE. The results of the LPS ELISA, which was developed at the RIVM, were compared with the results obtained with bacteriology which was performed besides the ELISA and the results obtained by testing the serum of the flocks which was performed to determine the SE status of the flock.

Samples

From each flock 60 eggs were taken to be examined for anti-bodies and 6 faecal samples for bacteriological detection of *S.* Enteritidis were taken. Each individual yolk was diluted 1:10 in Phosphate Buffered Saline (PBS) and pooled to samples A (pooling 5 egg yolks each

resulting in 12 samples), pool samples B (pooling 10 egg yolks each resulting in 6 samples), and pool sample C (pooling all samples B).

Method

ELISA

Wells of microtitre plates were coated with lipopolysaccharides (LPS) from *S*. Enteritidis. The samples were added by making a serial threefold dilution, resulting in dilutions of 1:100; 1:300; 1:900; 1:2,700; 1:8,100 and 1:24,300.. The next step consisted of adding 100µl of rabbit-anti-chicken serum conjugate with peroxidase to each well and the trays were incubated for 60 minutes at room temperature. After that the wells were incubated at room temperature with 100µl TMB-substrate. This reaction was stopped after 10 minutes by adding 50 µl 2 M sulphuric acid to each well. The reaction product was measured using a spectrophotometer at 450 nm. A flock was called immunological positive if dilution 1:8,100 of one of the pooled samples tested in the ELISA resulted in an OD450 value >0.100.

Bacteriology

Each portion of faeces (6 per flock) was tested using RV, MSRV and DIASALM. All isolated *Salmonella* strains were serotyped at the Dutch National *Salmonella* Centre.

Results

A total of 104 laying flocks were tested serologically for *S*. Enteritidis. This resulted in 52 serologically positive as well as 52 serologically negative flocks.

Comparing serology with bacteriological results revealed 8 false negative flocks for bacteriology. The specificity of serology compared to bacteriology is 86% (92% sensitivity).

Comparing immunology with bacteriology reveals 3 false negative and 16 false positive flocks testing samples A. Testing of sample C resulted in 34 positive flocks (14 false negative flocks). This is a sensitivity of 71% compared to almost 94% for samples A.

Comparing immunology with serology reveals 1 false negative and 10 false positive flocks testing samples A. Testing sample C obtains only 39 positive flocks. This is a sensitivity of 75% compared to a sensitivity of 98.1% with samples A.

Comparison of immunology with bacteriology and serology can be performed with 92 flocks. Samples A resulted in 1 false negative result and sample C resulted in 10 false negative results. Sample C results in a sensitivity of 77.3% in comparison with 97.7% obtained with samples A.

Conclusion

- The most sensitive results were obtained with samples A.
- The LPS-ELISA is a good screening method; Few false negatives were obtained.

Discussion

Infection with *Salmonella* Enteritidis results in an antibody response of the host. A delay however occurs between the moment of infection and the antibody response. This results in discrepancy between methods of measurement of infection. At the moment that *S*. Enteritidis

can be detected in the faeces, it is possible, that the antibody titre is not yet detectable. A flock can also give a positive titre when *S*. Enteritidis is not excreted in the faeces anymore.

2.18 Use of serology to control the *Salmonella* status in layers and meat breeders

Ms. Karine Proux (NRL-*Salmonella,* France)

Karine PROUX, Eric JOUY and Florence HUMBERT
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As recommended by the Zoonoses Directive 92/117/EEC, serology may be used as it offers guarantees equivalent to the bacteriological analysis. Therefore the aim of this work was to validate the serological method on flocks using bacteriology as the reference method. This study was divided in two parts:

1) A transfer and an inter-laboratory trial with the ELISA developed by the Food safety Agency located in Ploufragan; and
2) A field study in which labs selected during the ring trial tested serological and bacteriological samples of meat breeders and layers.

The ring trial was organised in 1999 and 10 labs were validated within 11 participants. The serological method was an indirect ELISA based on lipopolysaccharides from *S*. Enteritidis and *S*. Typhimurium. Accuracy was tested on 22 samples and the results were correct except for one laboratory which obtained more sensitive results than the others. This lab was excluded from the following field study. Repeatability was considered as correct on 2 sera and 2 yolks which were each tested 10 times (variation coefficient < 20%). Reproducibility observed on two different days was correct but a need for training was necessary for 4 labs as their accuracy was improved on the second day. The dose-effect was tested on serial dilutions of 2 sera from 1:2 to 1:32. The higher the dilution was, the lower the calibrated optical density (COD) was. Hundred percent specificity was obtained on 3 sera and 2 yolks from *Salmonella*-free birds. At last samples which COD was between 0.150 and 0.400 were declared as doubtful for the field study as they were negative or positive depending on the laboratory's results.

For the field study farms were not randomly tested as they were voluntary; 18 layers flocks (2 vaccinated with the *Salmonella* Enteritidis killed vaccine SALENVAC) and 31 meat breeder flocks were investigated. Samples were collected monthly: 2 swabs (1 pooled faeces or faeces collected on a sock and 1 dust) and 20 sera on meat breeders or 20 yolks on layers. When discordance was observed, complementary samples were collected: 16 environmental swabs, 60 sera on meat breeders or 60 yolks plus 20 sera on layers. Among 42 serologically negative flocks, 34 were bacteriologically negative, whereas 8 were bacteriologically positive (Table 1).

Table 1: Bacteriological results on 42 serologically negative flocks

Number of flocks	Bacteriological result	Isolated serovar	Persistance of the serovar
34	-	/	/
2	+	Mbandaka	No
1	+	Willemstad	No
1	+	Anatum	No
1	+	Enteritidis	No
1	+	Enteritidis	? (Slaughtered)
1	+	Braenderup	Continuously
1	+	Hadar	Continuously

One of these flocks was contaminated by *S*. Enteritidis but it was slaughtered, so it was not possible to do further serological screening on it. Two flocks, continuously contaminated respectively with *S*. Braenderup (0: 6,7) and *S*. Hadar (0: 6,8) which do not have common antigens with *S*. Enteritidis or Typhimurium, were consistently serologically negative. The 5 remaining flocks were bacteriologically positive for only 1 month and these results suggest that the *Salmonella* infection was rapidly eliminated by these birds and their immune system was not stimulated. Furthermore 7 flocks were serologically positive with 1 or more sample serologically positive among 20 samples tested (table 2).

Table 2: Bacteriological results on 7 serologically positive flocks

Number of flocks	Bacteriological result	Isolated serovar	Vaccination
2	-	/	Yes (SALENVAC)
3 (meat breeders)	+	Heidelberg	No
2 (layers)	+	Enteritidis	No

Post-vaccinal antibodies were detected on 2 flocks vaccinated with the *S*. Enteritidis killed vaccine SALENVAC and post-infection response were obtained on the yolks issued from 2 layers flocks contaminated with *S*. Enteritidis. At last 3 meat breeders flocks were serologically positive as they were infected by *S*. Heidelberg which have exactly the same somatic antigens as *S*. Typhimurium (O: 1, 4, 5, 12).

To conclude correlated results were observed on 46 flocks: 40 serologically negative and without *S*. Enteritidis or Typhimurium isolations with bacteriological investigation, 5 serologically and bacteriologically positive (3 with *S*. Heidelberg and 2 with *S*. Enteritidis) and 1 flock serologically negative but infected with a non-persistent *S*. Enteritidis strain. This last result may be considered as correct as this strain was not enough invasive or the contamination was not high enough to infect the host, so consequently this strain was eliminated and did not induce an immune response. Discordance occurred on 2 vaccinated

flocks serologically positive and bacteriologically negative. This result was considered as correct as the vaccine induced a good humoral immune response and a good protection preventing contamination. But this protection must be discussed because only 2 bacteriological samples were analysed on these flocks and it is generally known that, as vaccination decrease the contamination pressure, more bacteriological samples are needed to detect an infection on vaccinated flocks. Moreover 1 flock contaminated by *S*. Enteritidis was not confirmed as positive by the serological investigation as it was slaughtered a few days later, but the contamination may be so recent that the antibodies were not yet produced by the birds. These preliminary results must be completed in order to allow serological analyses on layers and meat breeder flocks as the end of our study will occur in July 2001. Whatever the results will be, serologically positive results will be confirmed by a bacteriological investigation.

Discussion:
Sixty faeces samples, according to the zoonoses directive, were taken to carry out the bacteriological detection.

2.19 Report on the first meeting held in Kopenhagen about the PCR EU project

Mr. Reiner Helmuth (NRL-*Salmonella,* Germany)

Reiner Helmuth, Burkhard Malorny, Dr. Jeffrey Hoorfar

The EU 5'th Framework Programme on harmonisation and standardisation of diagnostic polymerase chain reaction for detection of foodborne pathogens has started April 1st 2000 and is financed in the EU 5'th Framework Programme, 1st Thematic Programme on Quality of Life and Management of Living Resources, Key Action 1: Food, Nutrition and Health.

Total cost: 2,326,333 ECU
EC contribution: 1,607,220 ECU
Starting date: 1. Apr. 2000
Duration: 36 months

Objectives - Technological problem

Recognising that sensitive and more cost-effective methods are needed for detecting food-borne pathogens, the consortium has launched a project seeking to validate and standardise use of the polymerase chain reaction (PCR) for this purpose. Although a powerful research tool, the application of PCR for detecting food-borne pathogens is hampered from lack of validation, standard protocols, reagents, and equipment. Additional specific project objectives include validating a simple method for purifying DNA from bacterial cultures, establishing a central collection of certified DNA sample materials, establishing a databank containing key food-pathogen DNA sequence, listing strains for specificity testing, developing standardised reagents, and validating pre-PCR sample treatment methods.

Expected results

To facilitate its routine use for diagnostic purposes, participants in the new project are being asked to undertake a series of specific projects, including to construct a DNA sample library and primer databank, validate widely used thermocyclers and other automated equipment, and to develop uniform guidelines describing how tests should be conducted. Because much of the challenge in applying PCR to food-borne pathogens is technical, there is also a need to develop standardised training manuals and procedures for those who will conduct such tests.

The programme is planned in 2 phases over a 3-year period and will include 6 work packages and 20 tasks. During phase 1, researchers working in 12 separate laboratories will develop data about the basic methods and requisite reagents. During phase 2, they will focus on validation trials, automation issues, and transferring the validated technology to end-users.

As part of this project, 35 participants are being asked to focus on 5 major pathogens (*Salmonella*, Campylobactor, enterohemorrhagic E. coli (EHEC), L. monocytogenes and Y. enterocolitica) and on three sample types (poultry carcass-rinse, pig carcass swab and milk). An expert laboratory is assigned to each pathogen, with the task to prepare defined DNA material, compare the methods and devise and optimise a PCR detection protocol.

During its first general meeting June 26-27th 2000 at the Royal Veterinary and Agricultural University Denmark several conclusions were reached. (Participants: Approx. 60 participants representing all project partners from 21 European countries.)

The meeting was divided in 6 sessions covering all 6 workpackages. The relevant workpackage leader, who also prepared a short summary as follows, chaired each workpackage. The main part of the meeting was devoted to discussion regarding preparation of reference DNA and strains, standardisation work and the milestones for the first year of the project.

The fortunate coincidence of the dates of this meeting and the very recent ISO meeting facilitated the emergence of efforts of FOOD-PCR and the ISO and CEN on standardisation of PCR. Hence, it was decided that fully collaborate with CEN for preparation of general guidelines for European standards under TC275/WP6.

WP1: Construction of DNA banks

The six expert laboratories presented the specific methods used for the isolation and purification of DNA of their specific pathogen bacteria:

1) Universidad Complutense de Madrid - Listeria monocytogenes
2) Danish Veterinary Laboratory – Campylobacters
3) Lund University - Yersinia enterocolitica
4) Central Science Laboratory Listeria monocytogenes
5) Federal Institute for Health Protection of Consumers and Veterinary Medicine – *Salmonella*e
6) Justus-Liebig University – EHEC

In respect to *Salmonella* it was proposed to use the list of *Salmonella* reference strains shown in table 1 for specificity tests. These strains represent the most relevant species, subspecies and serotypes for the whole genus *Salmonella*.

Two partners use commercial available kits for the preparation of DNA, two 'Boiling-methods' and one partner a 'CTAB-method' including the use of phenol.

As all partners achieve high quality results with Quiagen kits, it was agreed to prepare the DNA needed for the production of a DNA reference material in this way. Each of the above mentioned partners should isolate between 0.5 and 1 mg DNA of one important strain using preparative Quiagen kits. The frozen DNA will be send to IRMM on dry ice.

Although the Quiagen kit will be used for isolation, partners were asked to keep their eyes open for isolation and purification methods, which can be scaled up easily. Madrid will try to replace the toxic phenol used in their isolation method by octanol. In July 2000 IRMM will run a preliminary test in order to study different methods for the long-term stabilisation of DNA.

The preliminary test will be carried out with non-pathogenic bacteria strain, which was provided by DVL. After development of a method for the stabilisation of DNA, the final reference material will be processed. The feasibility study on DNA stabilisation using the above mentioned approaches will be finalised approximately until 1 October 2000.

Basic principles of a DNA database were presented by IRMM. The participants of the kick-off meeting are asked to describe the kind of information, which should be included in such DNA database for pathogenic bacteria. A prototype of the database will be set up by IHCP and put on the PCR-homepage. All partners will have the possibility to hand in comment and suggestions during four weeks.

The lists of primers and the corresponding published references, which were already collected by IRMM and will be sent to Madrid in order to merge the information in a single document. The list of epidemiological important strains, also collected by IRMM, will be send to Copenhagen to be put on the homepage of the project (www.pcr.dk).

WP2: Validation of thermal cyclers

Partners 4 and 7 had already a preparatory meeting in Berlin on May 1, 2000, in order to harmonise endeavours to be done in the first year of collaboration. Agreement was established on the way of quantification of DNA yield after amplification (by HPLC high-pressure liquid chromatography).

The mode of preparing target DNA for estimation of thermal cycler amplification efficiency was discussed in detail.

Access to products and product information was revealed studying manufacturer presentations on the Internet, personal consultation and dispatch of a questionnaire. The questionnaire was distributed to all relevant thermocycler producers. It was comprised by three parts asking for detailed technical specifications, for benefits and shortcomings and data and for in - house validations.

WP3: Pre-PCR-Treatment

In order to develop an optimal pre-PCR treatment method, it is important to consider the following three questions:

1) What detection level has to be reached in the original sample?
2) Do living or dead cells or both need to be detected?
3) Will conventional or real-time PCR technology be used?

International standards suggest that the lower detection limit for food-borne pathogens is one living cell per 25 gram of material tested. To obtain such a detection limit with the methods that are currently available, sample pre-treatment will most likely have to involve pre-enrichment of samples. However, other methods to obtain such a detection limit will also be studied in WP3 for carcass-rinse and milk.

Addressing the question of dead versus living cells. Preferably, only living cells should be detected since only living pathogens cause infection risk. The risk of intoxication, dead cells but active toxins, should be taken into account. However, intoxication is not a problem in our project.

The question whether detection of dead cells or free DNA derived from such cells can produce false-positive PCR results will be studied in WP3, since this is frequently stated as one of the disadvantages of diagnostic PCR. Recent studies by partner 24 indicate that the half-life of naked DNA after heat treatment of living cells in food can be very short. A further investigation of this topic would be useful, to rule out the possibility that fragmented DNA from dead cells can still occasionally yield a positive PCR result.

Finally it has been decided that, according to the technical annex, the ring-trail assays in WP4 will be developed for conventional PCR. Nevertheless, pre-PCR treatment strategies for real-time PCR will be included in WP3, wherever appropriate, because of their potential future importance in microbiological routine analysis.

Table 1: Proposed list of Salmonella *reference strains for PCR specificity tests („Food PCR" project)*

Serotype	Serogroup	No. of strains	Comment
Enterica Subspecies I			
Enteritidis	D	60	Most important serotype in Europe
Typhimurium	B	60	Most important serotype in Europe
Hadar	C	5	Frequently isolated serotype in Europe
Virchow	C	5	Frequently isolated serotype in Europe
Infantis	C	5	Frequently isolated serotype in Europe
Heidelberg	B	5	Frequently isolated serotype in Europe
Newport	C	5	Frequently isolated serotype in Europe
Brandenburg	B	5	Frequently isolated serotype in Europe
Saintpaul	B	5	Frequently isolated serotype in Europe
Agona	B	5	Frequently isolated serotype in Europe
Blockley	C	5	Other important serotype
Bovismorbificans	C	5	Other important serotype
Bredeny	B	5	Other important serotype
Derby	B	5	Other important serotype
Dublin	D	5	Other important serotype
Livingstone	C	5	Other important serotype
Montevideo	C	5	Other important serotype
Paratyphi B	B	5	Other important serotype
Salamae Subspecies II			
S. 42:r:-		2	
Others		4	
Arizonae Subspecies IIIa		3	
Arizonae Subspecies IIIb		3	
Houtanae Subspecies IV		3	
Bongori Subspecies V		3	
Indica Subspecies VI		3	
Total No. of strains		221	

Discussion:

The programme also includes the selection and testing of primers. A review article enters a table including all available primers.

More issues can be discussed and are useful, but firstly there must be an agreement on European level about the method used.

2.20 Antibiotic resistance; Use of Antibiotics in Animals and Public Health risks

Mr. Dik Mevius (ID-Lelystad, the Netherlands)

In the past 50 years antibiotics have been used for therapy and prevention of infectious diseases in animals and humans. These use practices have resulted in selection pressure on intrinsically susceptible bacteria. Resistance to antibiotics in bacteria developed either by

mutation of chromosomal DNA or by acquisition of resistance genes from environmental organisms in which these genes were present and combinations of these mechanisms.

Development of resistance can be determined in longitudinal studies. Also during treatment the selective pressure of an antibiotic on bacteria populations in the gut of animals can be studied and the dynamics of this selective effect determined.

Monitoring of resistance in animals should be aimed at potential zoonotic aspects of antimicrobial resistance in animals by transfer of resistant strains or resistance genes from animals to humans. Resistance can be transferred from animals to humans by several routes. The most important route of transfer is considered to be the food chain. Direct contact may be important for specific animal owners but is not considered to be a general public health risk. For this reason the major food animal species (e.g. broilers, slaughter pigs and cattle) should be included and pet animals not included in such a programme. Because of the zoonotic aspect the bacterial species involved are zoonotic food-borne pathogens *Salmonella* spp. and *Campylobacter* spp. Moreover indicator organisms for the normal gut flora are included. *E. coli* as indicator for the Gram-negative flora and *E. faecium* as indicator for the Gram-positive flora.

The purposes of such a monitoring programme are:

- detection of emergence of new resistance phenotypes;
- determination of trends in resistance in time;
- detection of potential public health risks.

Susceptibility of the bacteria should be tested in a quantitative way. Preferably by microbroth dilution. Advantages are that microtitre trays with custom-made panels of antibiotic dilutions can be bought (i.e. Sensititre®) commercially, with optimum quality control and comparability between laboratories and countries. As antibiotics representatives of all relevant classes including antimicrobial growth promoters should be included in the tests.

The panel of antibiotics used in the Netherlands is displayed in Figure 1. In this figure, also the MIC distribution for all antibiotics tested, the interpretive criteria (breakpoint MIC's) and resistance percentages are displayed. This type of presentation facilitates the interpretation of the data.

Resistance in food borne pathogens as a result of the use of antibiotics in animals is a direct public health risk. However, the major risk is the gastro-enteritis itself, if the strains involved are resistant this may contribute to that risk. For certain potentially more virulent phage types of *Salmonella*, the impact of resistance to important antibiotics can be much larger.

Resistance in human pathogens is only indirectly related to use of antibiotics in animals, if related at all.

Discussion:

Although it is hard, it is possible to make a distinction between isolates from a hospital.

The work performed is done as standardised as possible. Each supplement added to media used, can influence the results obtained. The extent of the influence is unknown, no research has been performed on this.

It is hardly seen that a MIC shifts towards the breakpoint. Most of the time a shift is seen after a year of testing, because then a great number of strains is tested.

In the Netherlands flumequine is used instead of nalidixic acid. The same results are obtained with these two antibiotics.

(Sheets of this presentation are printed in Appendix 5)

Figure 1. MIC distribution of Salmonella's isolated in 1998 and 1999 in The Netherlands

MIC	0,008	0,015	0,03	0,06	0,12	0,25	0,5	1	2	4	8	16	32	64	128	256	512	N	R (%)
Human																			
Amoxicilline						1	239	345	7			1	3	1	99			696	15
Amox/clav						2	302	285			12	67	21	3	4			696	4
Piperacilline							1	66	491	33	3					7	95	696	15
Cefotaxime			3	305	343	37	3					1	4					696	0,7
Ceftazidime				3	33	564	87	4						5				696	0,7
Imipenem				27	213	359	93	3	1									696	0,1
Gentamicine					29	339	259	58	9		1		1					696	0,1
Doxycycline								18	368	151	69	49	35	6				696	23
Trmethoprim						3	61	172	75	83	133	122	11	36				696	24
TmpS					74	243	233	68	21	10	6	7	34					696	6
Ciprofloxacin	51	463	150	10	9	11	2											696	0
Nitrofurantoine										1	14	164	234	247	33	3		696	-
Chlooramfenicol									1	116	491	23		1	64			696	9
Florfenicol								1	27	536	67	9	43	8	5			696	8
Carbadox									7	71	275	281	61	1				696	0
Flumequine						3	372	265	31	3	8	9	5					696	3

MIC	0,008	0,015	0,03	0,06	0,12	0,25	0,5	1	2	4	8	16	32	64	128	256	512	N	R (%)
Food-animals																			
Amoxicilline							46	144	4			1			14			209	7
Amox/clav							56	137	1		2	12	1					209	0,4?
Piperacilline								16	167	12						5	9	209	7
Cefotaxime				68	129	12												209	0
Ceftazidime					3	138	63	5										209	0
Imipenem				12	46	115	36											209	0
Gentamicine					23	115	47	11	2		1	3	7					209	5
Doxycycline								4	144	43	6	3	9					209	9
Trmethoprim							25	74	30	18	9	17	4	28				205	23
TmpS				1	24	87	55	9	4	3	6	3	17					209	10
Ciprofloxacin	14	147	37	5		6												209	0
Nitrofurantoine											3	58	71	55	2	20		209	-
Chlooramfenicol										80	118	8	1		2			209	1
Florfenicol									7	183	16	3						209	0
Carbadox										11	89	89	19	1				209	0
Flumequine							132	66	5	1		5						209	2

= Breakpoint MIC

2.21 Use of sensititre to determine MIC trends in STM DT104

Mr. Rob Davies (NRL-*Salmonella,* United Kingdom)

Yvette Jones and Rob Davies

Four hundred and forty one poultry strains of *Salmonella* Typhimurium DT 104 chosen from a seven year period were tested by Sensititre. Each sample was tested against a panel of sixteen antimicrobials prepared as a customised 'Sensititre' plate.

There appears to have been a small increase in the MIC values over the seven year period for the majority of antimicrobials tested. The range of dilutions chosen for ampicillin did not identify an end point, however only one strain from 1993 showed an MIC of less than 256. Plate MICs on strains from 1998 identified all strains to have MICs greater than 512, thus showing all strains to be highly resistant to this antimicrobial.

The MIC values of samples tested with nalidixic acid show the most marked increase in MIC values over the seven year period. In 1994 the majority of strains had an MIC of 4, by 1996 56 strains out of 87 were resistant (64%). By 1999 all but three strains had a MIC of 128 or greater. NCCLS interpretations used by Sensititre identify a MIC of 32 or greater as resistant.

A very gradual shift in MIC is seen in amoxicillin/clavulanic acid. A value of 32/16 is considered resistant. Only a few samples showed MICs at or above this level, and were found in all years after 1993.

Salmonella is considered resistant to Tetracycline when a MIC of 16 is identified. All strains tested with the exception of one in 1994 had a MIC value in excess of this, but again a gradual increase in MIC was clearly identified over the seven year period.

No strains tested against gentamicin from 1993 to 1996 showed an MIC greater than 1. In 1997 one strain had an MIC of 2 and three strains in 1998. Two strains in 1999 had a MIC in excess of 2. Although the numbers are small a trend is evident. Intermediate resistance was identified at a MIC of 8 and full resistance at 16, thus gentamicin resistance was not evident from these results. The number of strains identified with resistance to neomycin (those with MIC of 32 and above) has shown a slight increase in 1998, however the vast majority of strains for all years tested remain sensitive with MICs of 2 and below. Resistance to streptomycin, MIC of 128 and above, has remained stable over the seven year period, however the 1999 data shows the proportions of strains in MIC values 64, 128 and 256 are fairly even. This demonstrates an overall increase in resistance.

For furazolidone MIC values show there has been a gradual increase in resistance, although the level at which the strain is considered resistant (at MIC 32) was only found in three strains within the seven year period.

Cefoperazone resistance remained consistent throughout the seven year period. NCCLS breakpoint is set at MIC 32 and above.

Cefuroxime again showed a gradual shift toward higher MIC values from 1993 to 1999. The resistant value of 32 was only seen after 1996, although intermediate resistance (MIC 16) was evident from 1994 onward.

Low MIC values for Apramycin were apparent for all tests and MICs remained fairly consistent throughout the seven year period. Resistance at MIC 64 has not been seen except for two strains in 1998 which also showed resistance to gentamycin.

A definite increase in MIC values of Trimethoprim/sulphamethoxazole from 1993 to 1999 can be identified. No breakpoint is quoted by NCCLS for this combination drug.

A shift toward higher MIC values is clearly identifiable from the Enrofloxacin data. The resistance MIC breakpoint of 2 is evident from 1996 onward. In 1999 almost half the strains showed resistance.

MIC values for chloramphenicol increased, from 1993 to 1999. Most of the strains would be identified as resistant as they had an MIC greater than 32.

The MIC resistance value of 64 was not reached in the case of amikacin and MIC values have remained stable over the seven year period

2.22 Application within the 5th framework for resistance monitoring in *Salmonella* according to the ARBAO guidelines

Mr. Reiner Helmuth (NRL-*Salmonella,* Germany)

Antimicrobial resistant bacteria of animal origin (ARBAO, www.fougeres.afssa.fr:80/arbao/) is a concerted action gathering 25 contractants from 14 countries of the European Community. This project entitled FAIR PL 97 3654 is funded by the European Commission (DGVI) for a period of 3 years beginning on 1st January 1998 and ends in 2000.

The objectives are :

- Set up of a European scientific network on the resistance of bacteria from animal origin;
- Surveillance of the resistance to antibiotics in these bacteria. Writing of recommendations;
- Harmonisation of antibiotic resistance studying methods; and
- Promote the development of European research programmes on emergence and diffusion of the resistance as well as on risk for public health.

After the group adopted in several meetings minimal requirements for the monitoring of bacteria of animal origin, it was decided to propose an application for a project entitled:

Bacterial resistance to antibiotics in food animals in Europe - *Salmonella,*

Acronym: ARBAO-SAL, within the 5th EU framework programme of the EU.

Its basic principles involve the collection of data on 900 *Salmonella* isolates originating from three major food producing animal species as outlined in the ARBAO conclusions and recommendations.

The project involves the collection of raw data from all 15 member countries. In addition some NRLs-*Salmonella* will receive financial support to perform MIC determinations with an automated test system and supplied consumables. Such systems are already used in three member countries who will receive consumables only.

Selected isolates will be sent to specialised molecular biology laboratories. The NRL-*Salmonella* Germany will perform an overall clonal analysis and molecular characterisation. Special investigations on fluorochinolone and beta lactamase resistance will be performed in the laboratories of INRA Station de Pathologie Aviaire Parasitologie Chaslus Dancla and FAL Schwartz.

The total cost estimates will be in the order of about 0.75 to 1.5 M€.

The objectives of the project are:

- to obtain data on the prevalence of resistance in *Salmonella* from food producing animals;
- to introduce a standardised and harmonised micro-broth dilution method in NRL-*Salmonella*; and
- to characterise molecular mechanisms for antibiotic resistance and their clonal spread.

Expected achievements:

- Feasibility study on the recommendations elaborated by ARBAO.
- The prevalence of resistance in *Salmonella* serotypes causing human infection and originating from the main food producing animal species will be obtained on an European level.
- These data can be used as a basis for licensing and control of antimicrobial agents in animal husbandry.
- By introduction of a standard method for quantitative susceptibility testing the results of different European countries and laboratories within those countries can be pooled and compared in a database.
- By harmonising the results obtained with traditionally used methods the quality of these results will be improved and facilitate the interpretation of the data for resistance surveillance purposes.
- Description at a molecular level of the occurrence of resistance among *Salmonella* from farm animals.
- Identification of reservoirs of resistance genes.
- Identification of predominating resistant *Salmonella* clones in EU member states.

Project leader:
Needs to be nominated.

Project partners: National Reference Laboratories for *Salmonella* of EU member countries.

2.23 Risk analysis and data

Mr. Eric Evers (RIVM, the Netherlands)

Eric G. Evers, André M. Henken

Quantification of public health aspects is important in order to develop a good strategy to improve public health. Using quantification, it can be decided which zoonoses are of importance and which measures are suitable to control and reduce important zoonoses. A good tool for quantification is risk analysis. The outcome of a risk analysis, the risk, consists of two parts: the probability and the severity of a certain occurrence. The process of risk analysis consists of risk assessment, risk management and risk communication.

Risk assessment consists of hazard identification (choice of the relevant micro-organism), exposure assessment (estimated intake of the hazard per person per time unit), hazard characterisation (dose-response relationship and infection-illness relationship) and risk characterisation (synthesis of the preceding three). It is important to include uncertainty and variability in risk assessment, as it is not a worst case approach. This includes e.g. the uncertainty of the fraction of broiler flocks infected with *Salmonella* Enteritidis in the Netherlands given the result of 100 sampled flocks. Another example is the variability in susceptibility to *S.* Enteritidis infection between people.

Prior to risk assessment a statement of purpose must be made. This statement defines which combination of pathogen, product, process and population is to be considered, and the exact form of the output. In this way, the risk assessor investigates exactly the subject intended by the risk manager. The value of risk assessment is that we can estimate the risk of a certain predefined combination, compare risks and calculate the effect of a certain intervention on the risk.

Reality is too complex to be incorporated into a mathematical risk assessment model. Therefore, reality has to be simplified into conceptual frameworks for risk assessment (using e.g. chain models) and also in more general frameworks for e.g. cost-utility analysis. Ideally, a risk assessment is based on many data, but in practice data are only partly available. Missing data are replaced by assumptions. The most important gap in available data are counts of micro-organisms (e.g. no. of *S.* Enteritidis per gram of faeces or per egg). They are necessary to perform a risk assessment, because they are input for the dose-response relationship, but also because they allow for modelling prevalence.

The following recommendations with regard to data can be given:
1) A (rough) risk assessment model must be made prior to data collection;
2) Data must be collected for every link in the chain that you want to consider;
3) Prevalence data must be collected at animal level, not only at group level;
4) Counts of micro-organisms are needed, not only presence/absence – measurements;
5) Detection limit values of measurements must be reported;
6) Measurements must be done throughout the year and at several locations;
7) Preferably, measurements in food just prior to consumption must be done;
8) When considering the whole chain, data should be spread evenly over the links;
9) An exception to the previous recommendation are links that will be subject to intervention measures. For these links, relatively more data are needed.

(Sheets of this presentation are printed in Appendix 6)

Discussion:
There are different manners in which measurements can be carried out and data will be obtained. A number of approaches are possible, but this depends e.g. on the case. Therefore, a close collaboration between risk assessor and data manager is a good starting point.

2.24 Discussion about activities CRL-*Salmonella* 2001

Mr. André Henken (CRL-*Salmonella*, the Netherlands)

The regular yearly working programme of CRL-*Salmonella* consists of the following activities:
- The organisation of two collaborative studies, one on bacteriological detection and one on typing;
- The publication of 4 newsletters, one at the end of each 3-month period;
- The organisation of a workshop with the NRLs-*Salmonella*;
- Performance of ad hoc activities upon request of the Commission or NRLs-*Salmonella*; and
- Performance, to some extend, of research on detection methods and reference materials in relation to the collaborative studies organised.

These activities were discussed at the workshop. The activities are much appreciated. There is no need for big changes. Suggestions were made with respect to some issues relating especially to the first 3 above-mentioned points. The participants agreed as stated below:

With respect to the collaborative studies
The participants agreed that the CRL-*Salmonella* should continue with its work on making two overviews (one on bacteriology and one on typing) of all previous collaborative studies done in the CRL-NRL network. It would be nice to have next year the concept overviews ready for publication.

Remarks made more specifically with respect to the bacteriological studies:
- Continue with the present set-up;
- Consider adding also tetrathionate instead of using RV(S);
- Consider sending RVS powder in order to exclude variation in its composition as source of variation between laboratories;
- In the year 2000 bacteriological study NRLs-*Salmonella* were invited to perform PCR analysis. The results of this will be collected and analysed by the CRL-*Salmonella*. Depending on the results it might be decided to organise a 'hands-on' course on PCR in cooperation with the German NRL-*Salmonella* as this lab is actively involved in the EU Food PCR project; and
- As presented at this workshop a Foundation related to the RIVM will start producing capsules with other serotypes than *S*. Panama and with more than one concentration level making it possible for NRLs-*Salmonella* to buy specific capsules for their national studies on *Salmonella* detection.

Remarks made more specifically with respect to the typing studies:
- Continue with the present set-up, i.e. using mostly more frequently occurring serotypes and a few less frequently occurring ones;
- Laboratories that are interested can perform antibiotic resistance typing on these strains using their own methods. As presented on this workshop there are initiatives, i.e. proposals for the EU 5th framework programme, to standardise on the quantitative methods used in which several NRLs-*Salmonella* are involved;
- For phage typing again 10 S. *Typhimurium* and 10 S. *Enteritidis* strains will be included.

A few general remarks were made:
- to choose preferably less busy periods of the year for the collaborative studies to be held, i.e. not in the summer and not around Christmas and New Year;
- to investigate the possibility for using fewer capsules in the studies to decrease the work load;
- whether or not the laboratories participating in the studies were accredited. It was suggested to include in one of the next newsletters an inventory on this issue.

Newsletter and pr in general

The newsletter is much appreciated. There is a need to be able to refer to the newsletter. The CRL-*Salmonella* shall investigate the possibility to get an ISBN or ISSN number for the newsletter.

It is suggested to make a website for CRL-*Salmonella* with e.g. links to the newsletter.

With respect to the workshop in 2001 and 2002

It is suggested to have the 2002 workshop in combination with the international *Salmonella* symposium in Ploufragan. So, this 2002 workshop will then be held in France, in May. The workshop for 2001 should therefore be held somewhat earlier than autumn. It is decided to have our 2001 workshop in June.

A presentation and discussion on quantitative methods is suggested as special item for the next workshop in 2001. In preparation of this the NRLs-*Salmonella* are invited to submit their knowledge and experience for publication in the December 2000 issue of the newsletter.

Appendix 1 Mailing list

01	European Commission, Director of Directorate D	P. Testori-Coggi
02	European Commission, head of Unit D.2	E. Poudelet
03	European Commission	J.C. Cavitte
04	European Commission	P. Makela
05	President of the Council of Health, the Netherlands	prof. dr. J. J. Sixma
06	Veterinary Public Health Inspector	drs. H. Verburg
07-43	Participants of the workshop	
44	Dutch National Library for Publications and Bibliography	
45	Board of Directors RIVM	dr. G. Elzinga
46	Head of Microbiological Laboratory for Health Protection and Director CRL-*Salmonella*	dr. ir. A.M. Henken
47	Authors	
48	SBD/Information and Public Relations	
49	Registration agency for Scientific Reports	
50	Library RIVM	
51-65	Sales department of RIVM Reports	
66-75	Spare copies	

Appendix 2 Participants

National Reference Laboratories for *Salmonella*

Austria
Christian Berghold
Belgium
Hein Imberechts
Denmark
Jens Chr. Jorgensen
Dorte Lau Baggesen
Mogens Madsen
Finland
Tuula Johansson
Sirkka Kivela
France
Florence Humbert
Karine Proux
Germany
Reiner Helmuth
Andreas Schroeter
Greece
Maria Passiotou-Gavala
Ireland
John Ward
Bernard Bradshaw
Italy
Antonia Ricci
Luxembourg
Joseph Schon
The Netherlands
Nelly Voogt
Norway
Viggo Hasseltvedt
Trine Lise Stavnes
Portugal
Maria Do Rosario Vieira
Alice Amado
Spain
Consuelo Rubio Montejano
Francisco Javier Garcia Pena
Sweden
Ingrid Hansson
Anna Aspan
United Kingdom
Stanley McDowell
Robert Davies

CRL-*Salmonella*
André Henken
Maurice Raes
Wim Wannet
Hennie Maas

CRL-Epidemiology of Zoonoses
Annemarie Kaesbohrer

Commission
Jean-Charles Cavitte
Pia Mäkelä

Invited speakers/guests
Dik Mevius
Eric Evers
Wilfrid van Pelt

Appendix 3 Programme of the Workshop

Sunday 17 September 2000

21.00 – 22.00 Social get together in the bar of Hotel Mitland

Monday 18 September 2000 (Hotel Mitland, hall: Tureluur and Zwaan)

8.30 - 8.50 Opening and introduction of participants (André Henken)

8.50 - 9.50 Current issues on the New Draft European zoonoses directive (Jean-Charles Cavitte)

9.50 - 10.10 Update on the recent workshop of the CRL-Epidemiology of Zoonoses (Anne Kaesbohrer)

10.10 - 10.30 Early warning for *Salmonella* in humans; the zoonotic connection (Wilfrid van Pelt)

10.30 - 10.50 Outbreak of hedgehog related *Salmonella* Typhimurium infection on the Norwegian West Coast August-September 2000 (Viggo Hasseltvedt)

10.50 - 11.05 Coffee / tea

11.05 - 11.25 Overview of all bacteriological collaborative studies (Nelly Voogt)

11.25 - 12.40 National activities with respect to bacteriological detection

- Investigation of *Salmonella* contamination on commercial egg laying farms (Rob Davies, 20 min)
- Estimation of the percentage of contaminated eggs laid by a naturally contaminated *Salmonella* Enteritidis flock (Florence Humbert, 20 min)
- Comparison of dung samples and sock samples for surveillance of *Salmonella* in poultry under the Danish *Salmonella* Control Programme (Mogens Madsen and Jens Joergensen, 20 min)
- Methodology for detection of *Salmonella* from chicken faeces (Nelly Voogt, 15 min)

12.40 - 13.00 Discussion on future bacteriological collaborative studies and discussion on reference material needs (André Henken)

13.00 - 14.00 Lunch

14.00 - 14.20 Overview of all typing studies (Nelly Voogt)

14.20 - 15.10 National activities with respect to typing of *Salmonella*

- Molecular typing of *Salmonella* - Harmonisation and standardisation in Denmark (Dorte Lau Baggesen, 20 min)
- Trends in *Salmonella* isolation from livestock and animal feed Jan-June 2000 (Rob Davies, 10 min)
- Increasing number of *Salmonella* Paratyphi B D tartrate positive isolations from broilers, received at the national *Salmonella* reference laboratory in Germany (Christina Dorn, 15 min)

15.10 - 15.40 Results of the last typing study and discussion on future typing studies (Wim Wannet)

15.40 - 16.10 Coffee / tea
16.10 - 16.55 National activities with respect to immunology / serology
- Comparison of ELISA and conventional methods for the isolation of *Salmonella* from porcine faeces (John Ward, 20 min)
- Validation of an LPS ELISA to detect S. Enteritidis antibodies in egg yolk (Maurice Raes, 15 min)
- Use of serology to control the *Salmonella* status in layers and meat breeders (Karine Proux, 20 min)

16.55 - 17.10 report on the first meeting held in Kopenhagen about PCR EU project. (Reiner Helmuth)

Evening programme

18.30 Departure from hotel Mitland to canal ride Utrecht. Dinner and optionally walk through Utrecht back to the hotel

Tuesday 19 September 2000 (RIVM, hall A10.014)

9.00 Departure from hotel Mitland to RIVM by RIVM cars
9.30 - 10.30 Antibiotic resistance (Dik Mevius)
10.30 - 11.15 National activities with respect to antibiotic resistance typing
- Use of sensititre to determine MIC trends in STM DT104 (Rob Davies, 10 min)
- Application within the 5th framework for resistance monitoring in *Salmonella* according to the ARBAO guidelines (Reiner Helmuth, 30 min)

11.15 - 11.45 coffee / tea
11.45 - 12.20 Risk analysis and data (Eric Evers)
12.20 - 12.40 Discussion about activities CRL-*Salmonella* 2001 (André Henken)
12.40 - 13.00 Closing remarks (André Henken)
13.00 – 14.00 Visit to phagetyping laboratory (optionally)
13.30 – 14.30 lunch
15.00 Departure to airport

Appendix 4 Sheets presentation 2.12

Molecular typing of Salmonella - harmonisation and standadization in Denmark

Danish Veterinary Laboratory
Statens Serum Institute
D.L. Baggesen, M.N. Skov, M. Torpdahl, D. Sandvang, P. Gerner-Smidt

Epidemiological typing of *Salmonella enterica* i Denmark

- Surveillance
- Representative strain collection
- ”Annual *Salmonella* account”
  - quantitative risk assessment
- Outbreak investigations

Definitive typing methods

- Reproducible
- Cheap
- Stable
- Comparison of results between laboratories and over time
- Limited discriminatory power
- Typability < 1

Comparative methods

- High discriminatory power
- Typability = 1
- Expensive
- Laborious
- Difficulties in comparison of results between laboratories and over time

Aim of Danish collaborative project

- To evaluate and implement genotypical methods for characterisation of *Salmonella enterica*
  - Standardisation and harmonisation between two laboratories
  - Internet based network for exchanges of results
- Improvement of methods for surveillance of salmonella infection and for quantitative risk assessments

Pulsed Field Gel Electrophoresis

- Standard protocol
- Harmonisation
  - Restriction enzymes
  - Reference strains
  - Electrophoretic conditions
- Evaluation on common strain collection
- Electronic database of profiles

Pulsed field gel electrophoresis

STATUS

- Standard protocol established
- Selection of enzymes and reference strains
  - Based on PulseNet (USA)
  - European standard ???
- Harmonisation of method between the two laboratories
- Results from both laboratories included in common data base (Bio Numerics)
- *S.* Senftenberg and *S.* Typhimurium DT104 and DT U302 – outbreak investigations

Amplified Fragment Length Polymorphism

- Method established in both laboratories
- Evaluation of enzymes
- Development of standard protocol
- Evaluation on common strain collection
  - *S.* Enteritidis and *S.* Dublin (DVL)
  - *S.* Typhimurium DT104 (SSI)
  - Common serotypes (TOP 20) (DVL & SSI)
- Electronic database of profiles

Amplified Fragment Length Polymorphism

STATUS

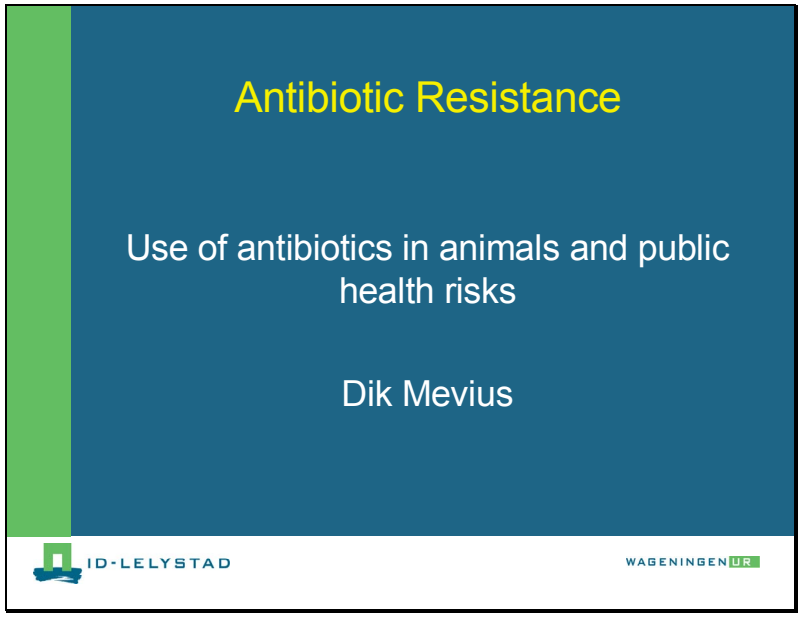
- Method established in both laboratories
- Selection of enzymes (*Bgl*II + *Mfe*I/ *BstBI*/ *Bsp*DI) Selection of reference strains
- Results from each laboratory included in common data base (Bio Numerics)
- Good differentiation at serotype level
- No or limited differentiation within serotypes (SD/SE)

Future perspectives

- Danish project: 1999 – 2002
  - Internet based network for outbreak investigations (~ PulseNet)
  - Internet based network for surveillance
- European collaboration ? – projects ?
  - EnterNet
  - CRL / NRLs
  - Informal network

Appendix 5 Sheets of presentation 2.20

Slide 1



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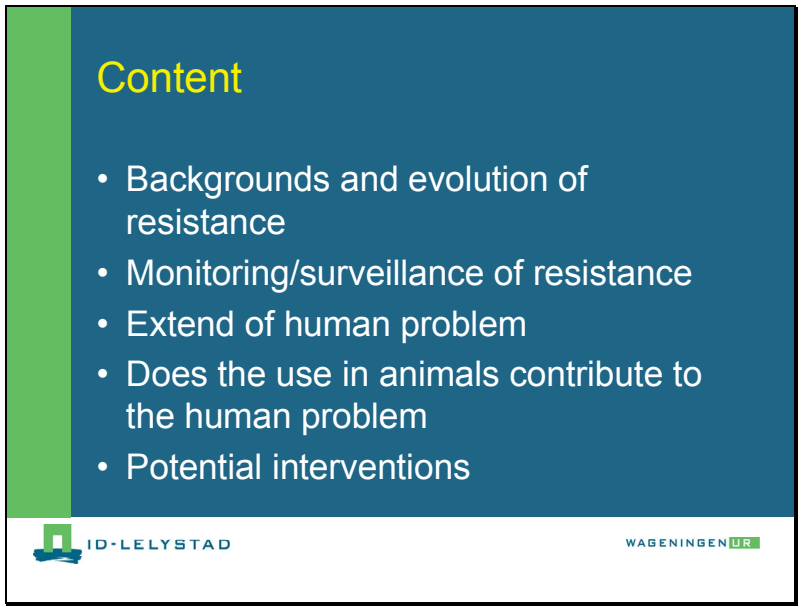

Slide 3

Backgrounds and evolution of resistance

- Use of antibiotics results in development of resistance
  - mutation
  - acquisition
    - conjugation, transduction, transformation
  - combination
- Selection!!!!!!!

ID-LELYSTAD WAGENINGEN UR

Slide 4

Table 4: Resistance mechanisms found in bacteria with biochemical homologues in antibiotic producing organisms (in soil)

Antibiotic	Resistance mechanisms
Penicillins, Cephalosporins	ß-lactamases, Penicillin-binding proteins
Aminoglycosides	Acetyltransferases Phosphotransferases Nucleotidyltransferases
Chloramphenicol	Acetyltransferases
Tetracyclines	Efflux system Ribosomal protection
Macrolides Streptogramins Lincosamines	Ribosomal RNA methylation Esterases Phosphotransferases Acetyltransferases
Glycopeptides	vanA-Ligase

ID-LELYSTAD WAGENINGEN UR

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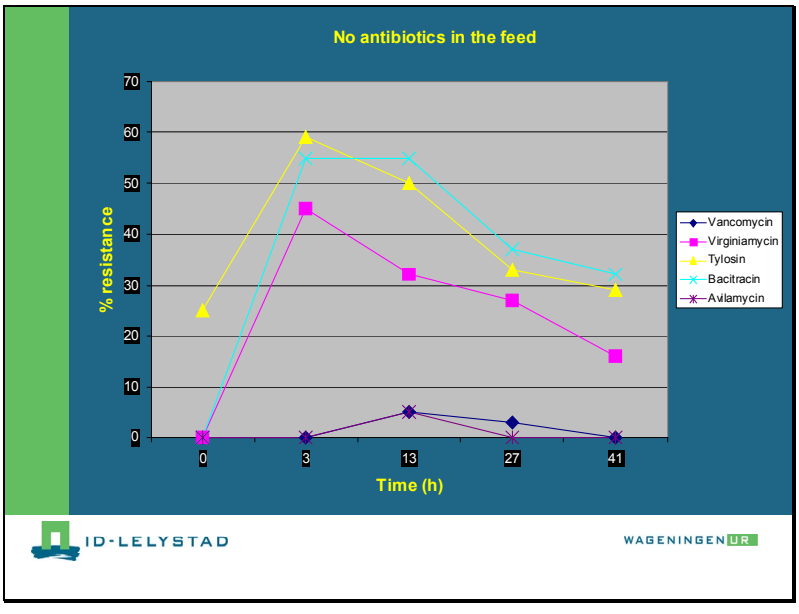

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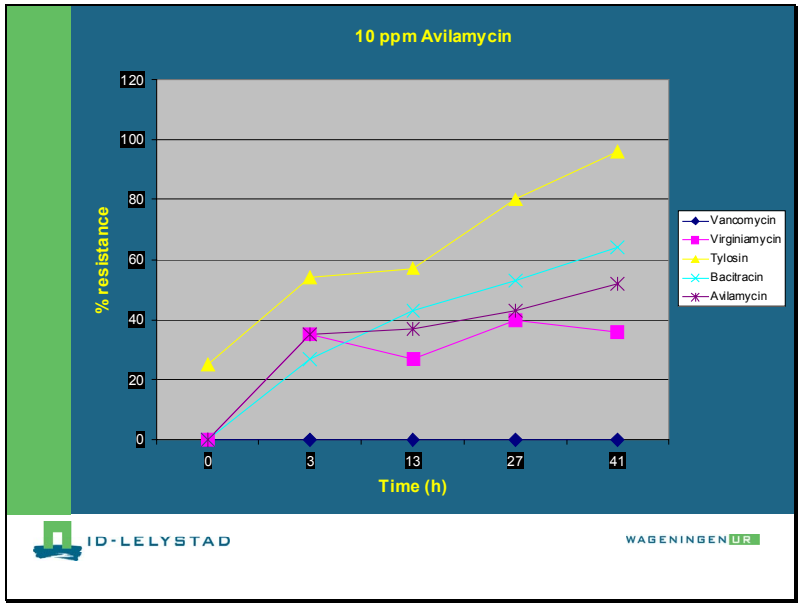

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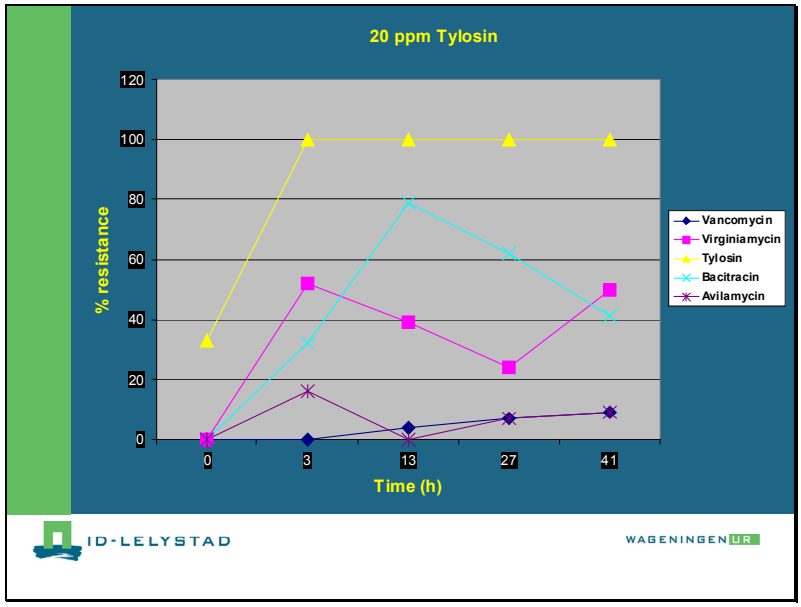

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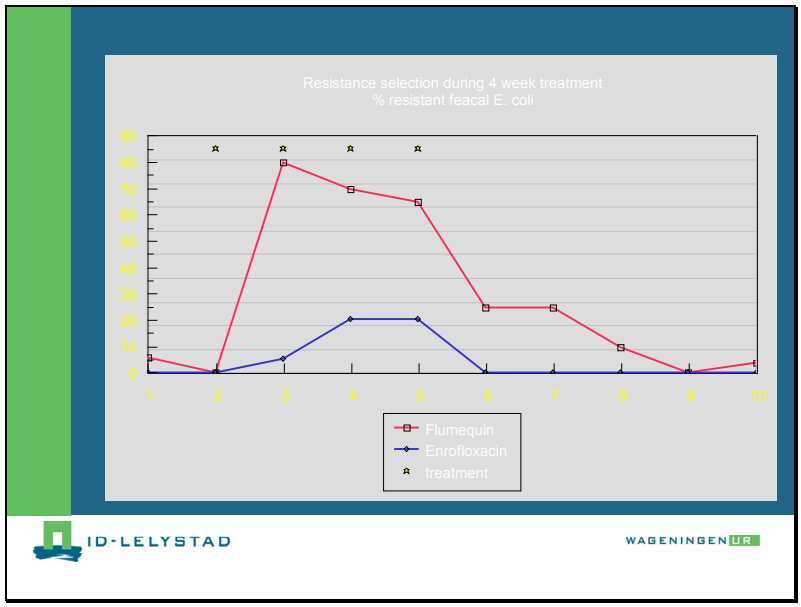

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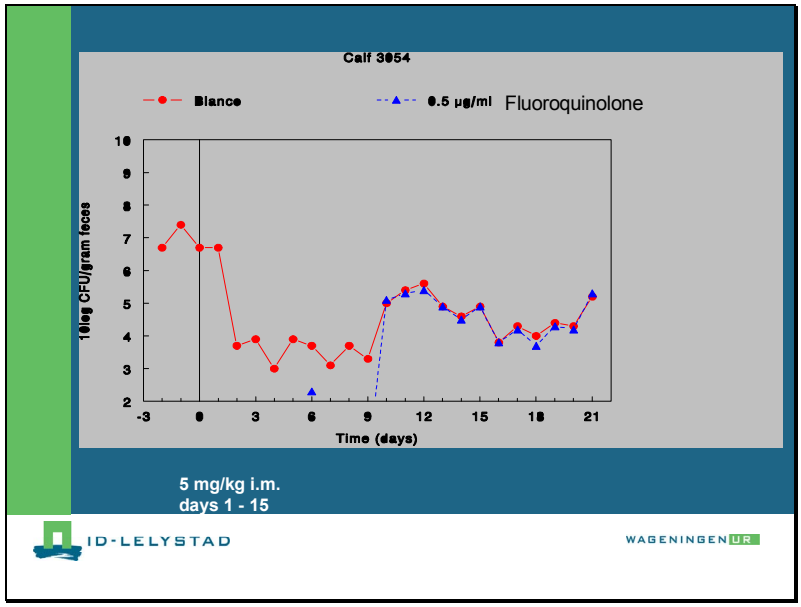

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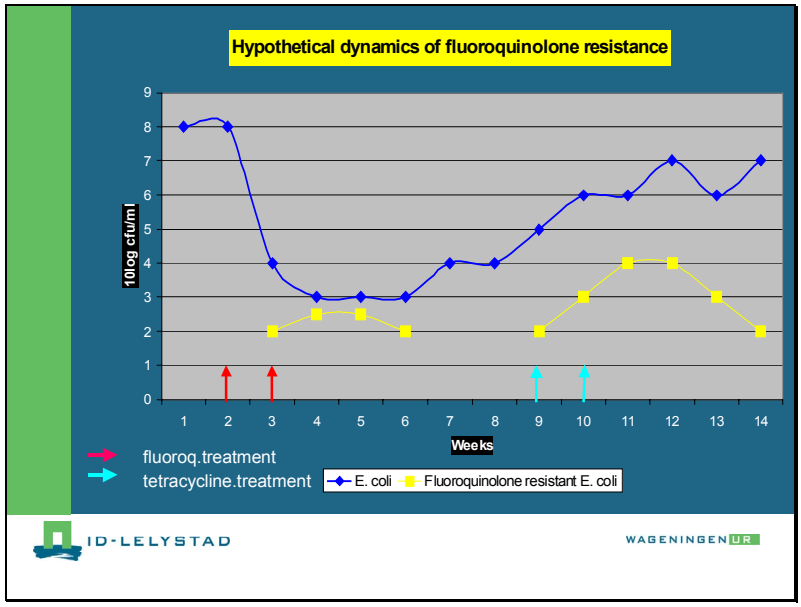

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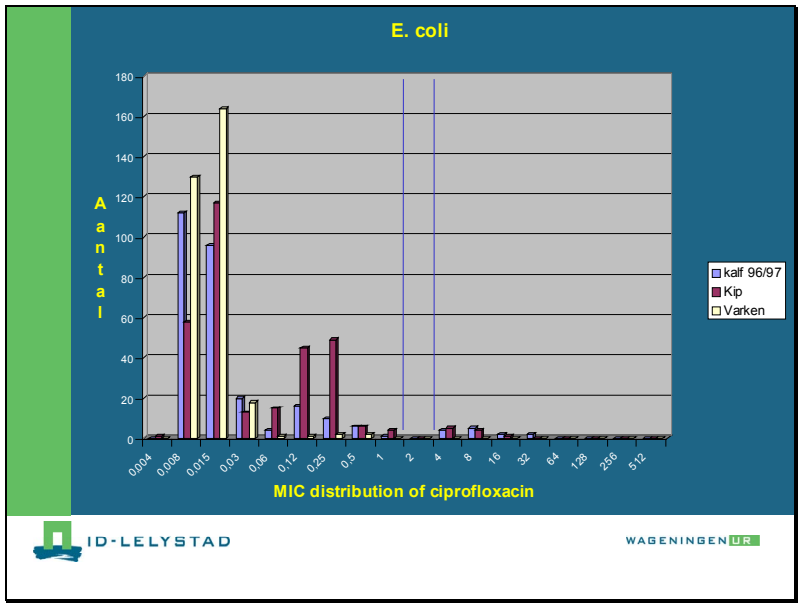

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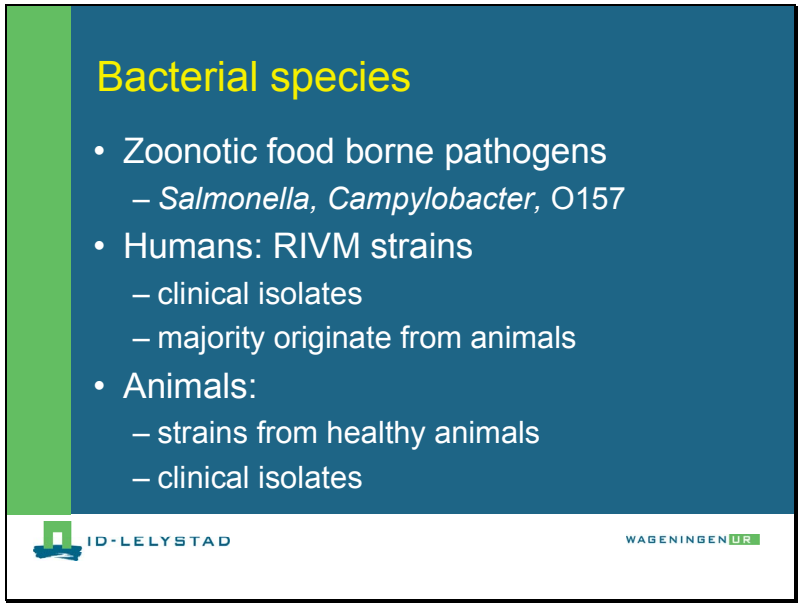

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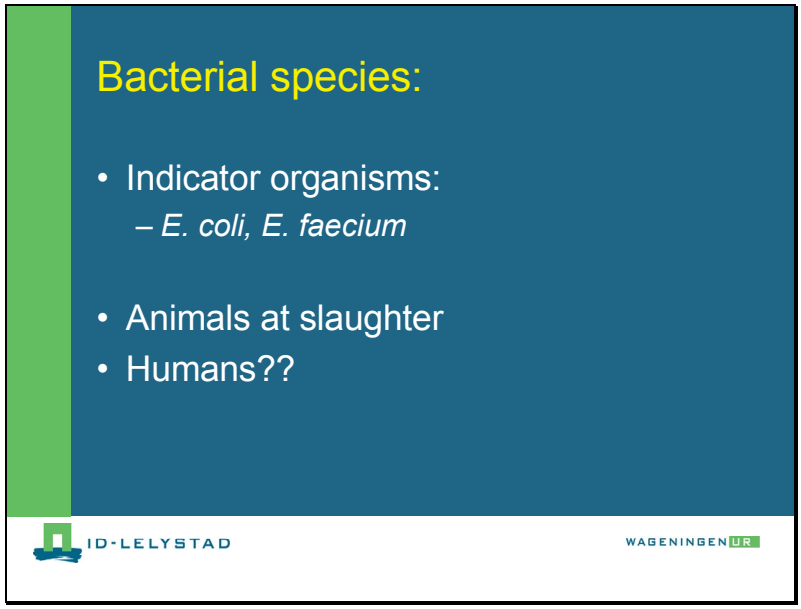

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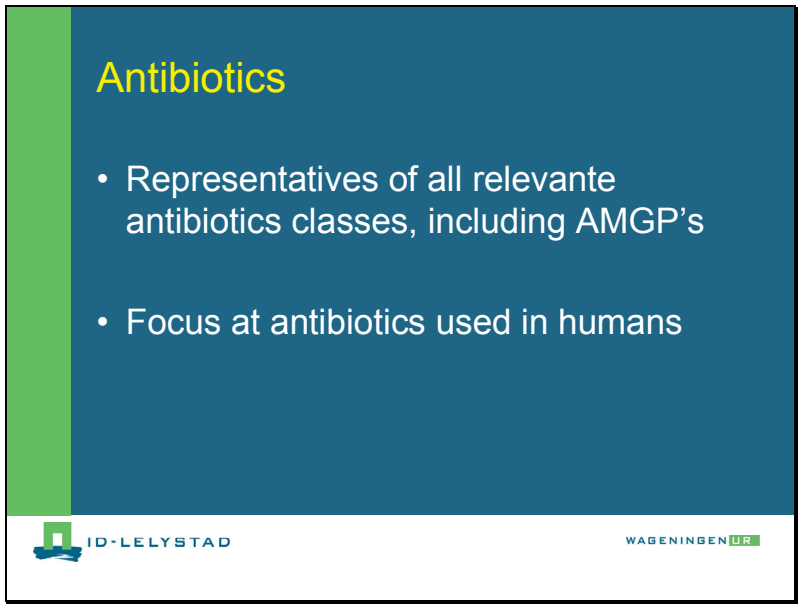

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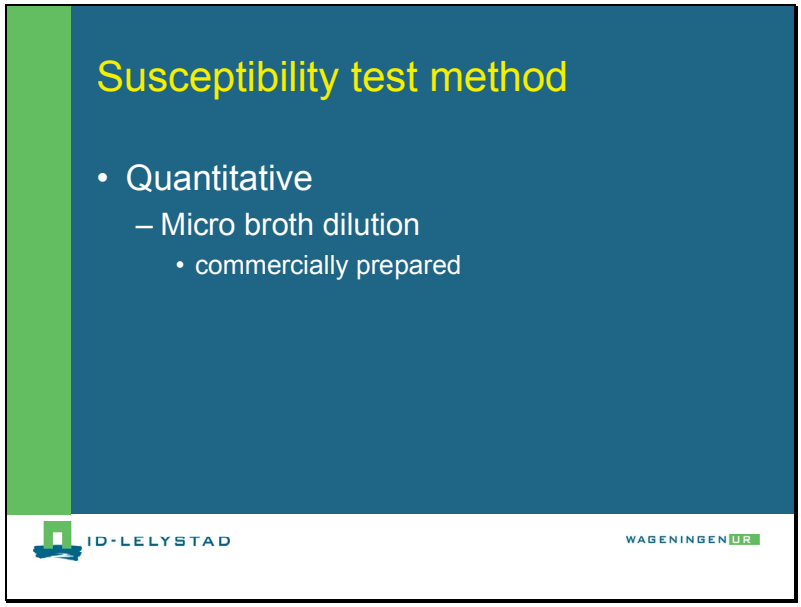

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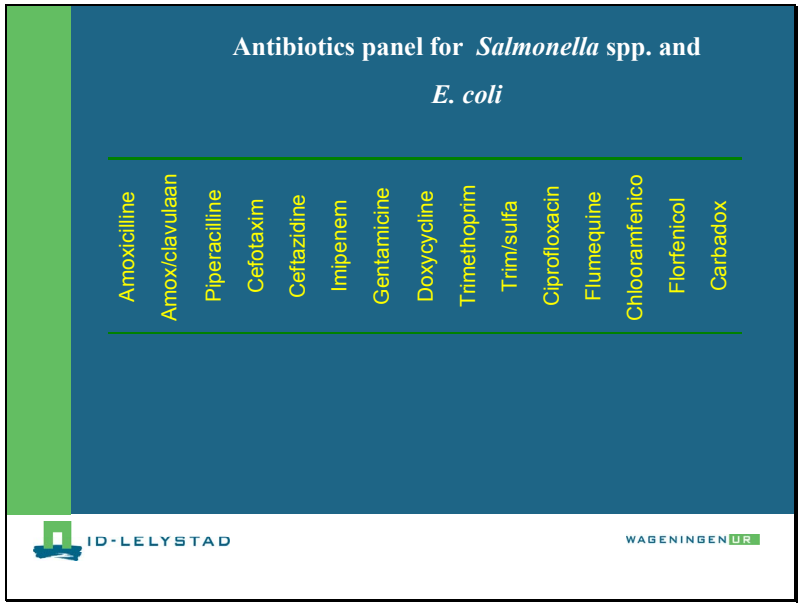

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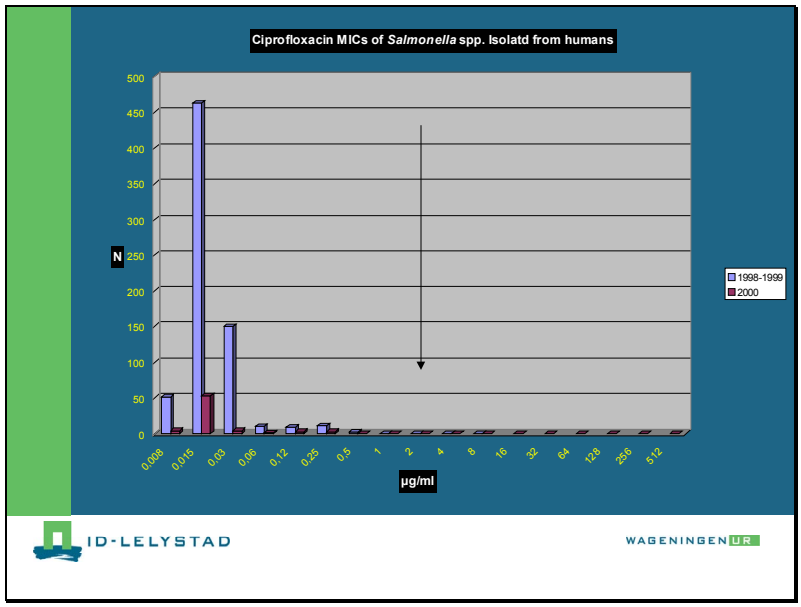

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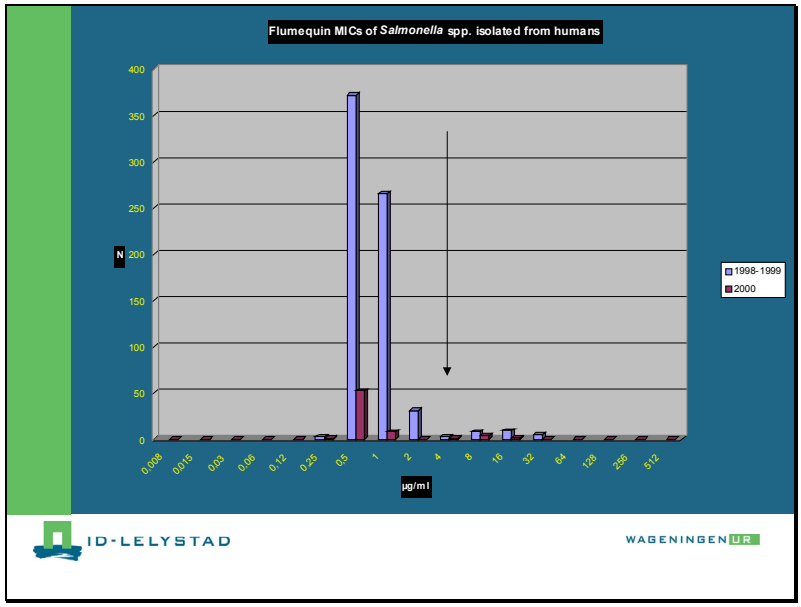

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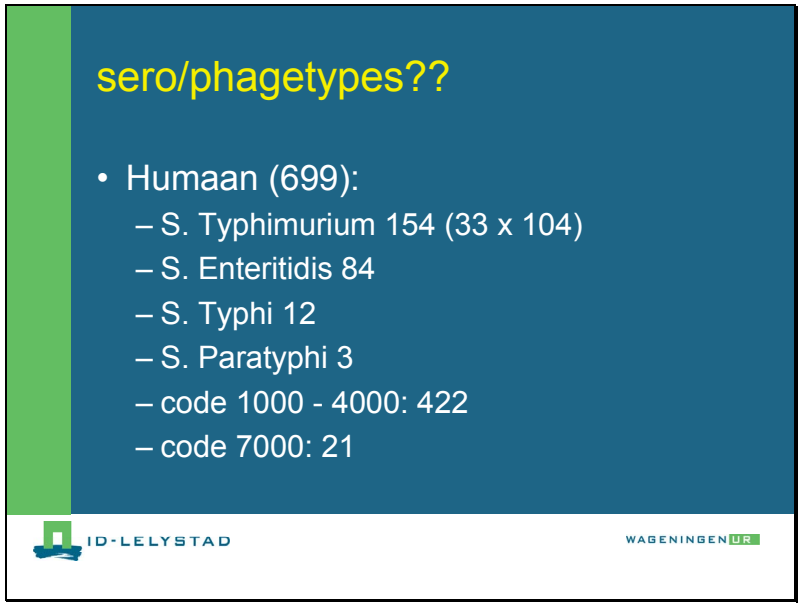

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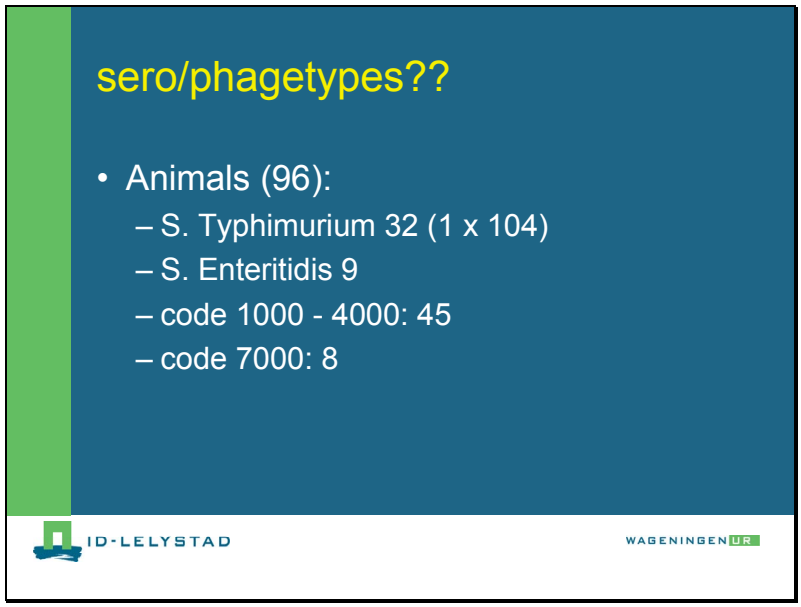

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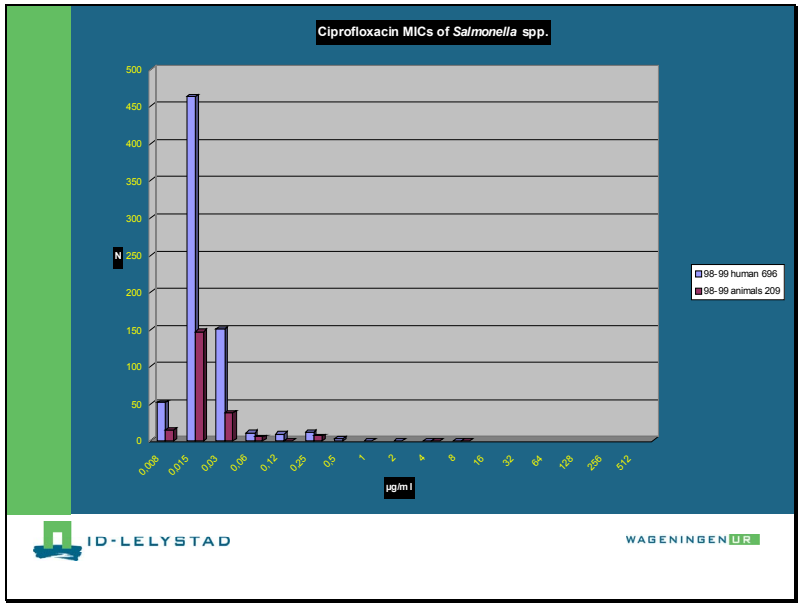

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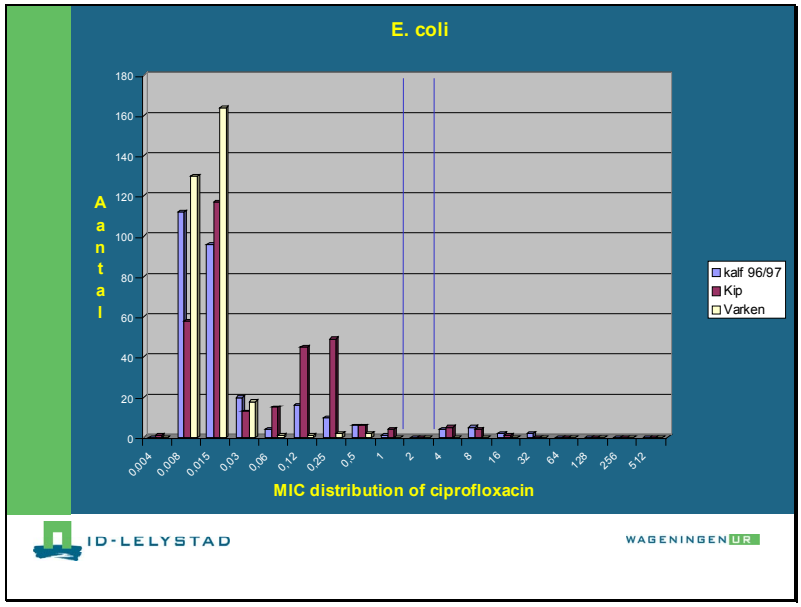

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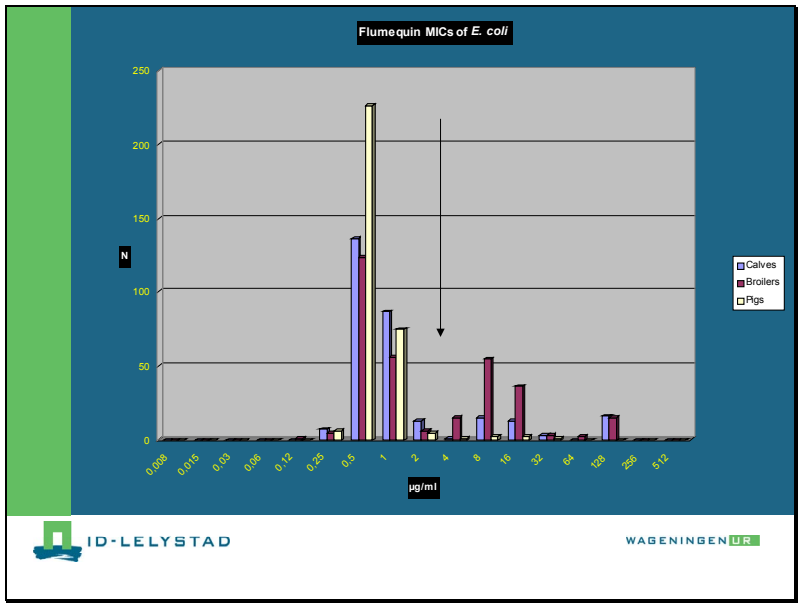

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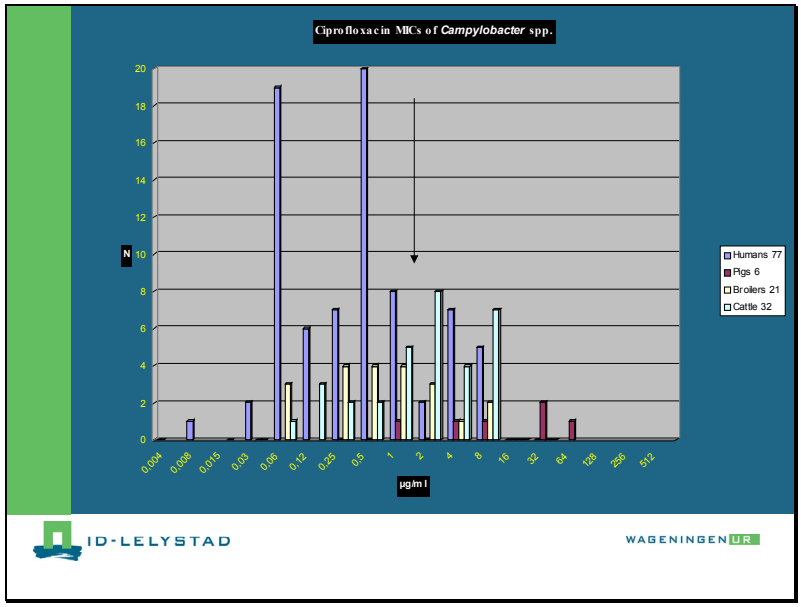

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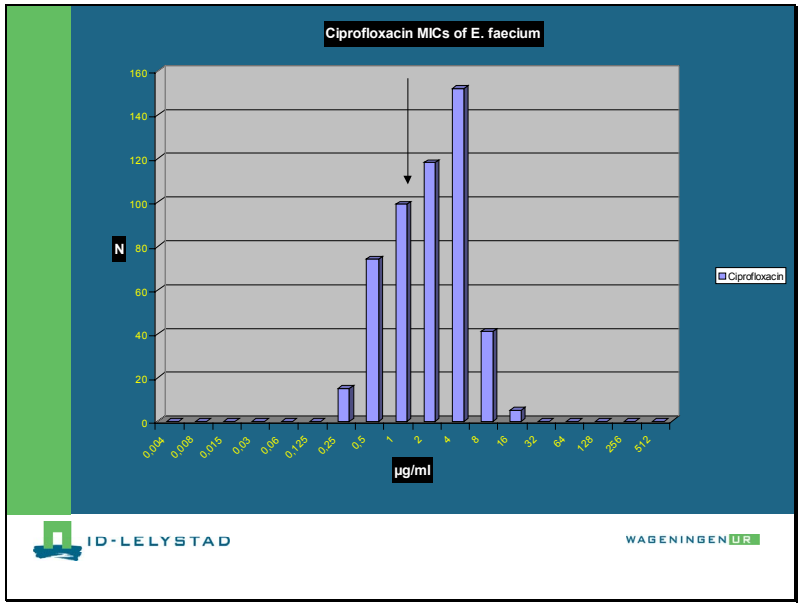

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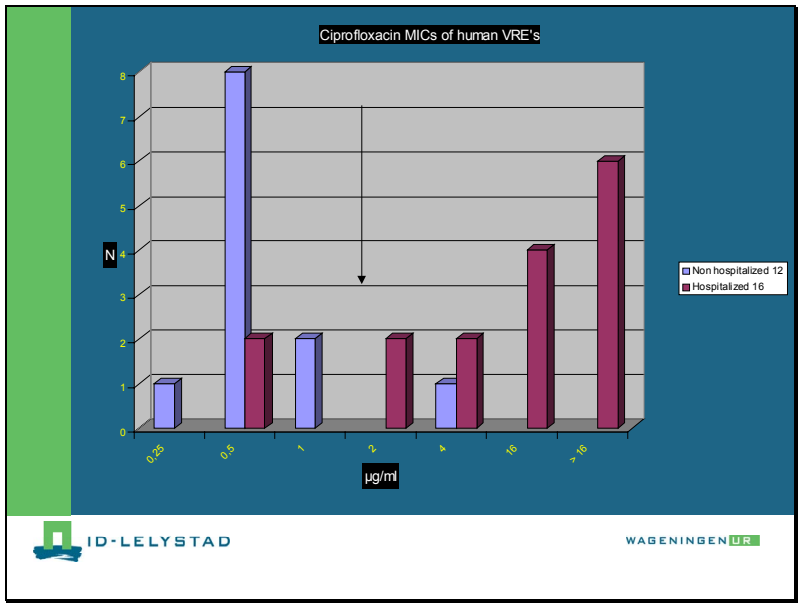

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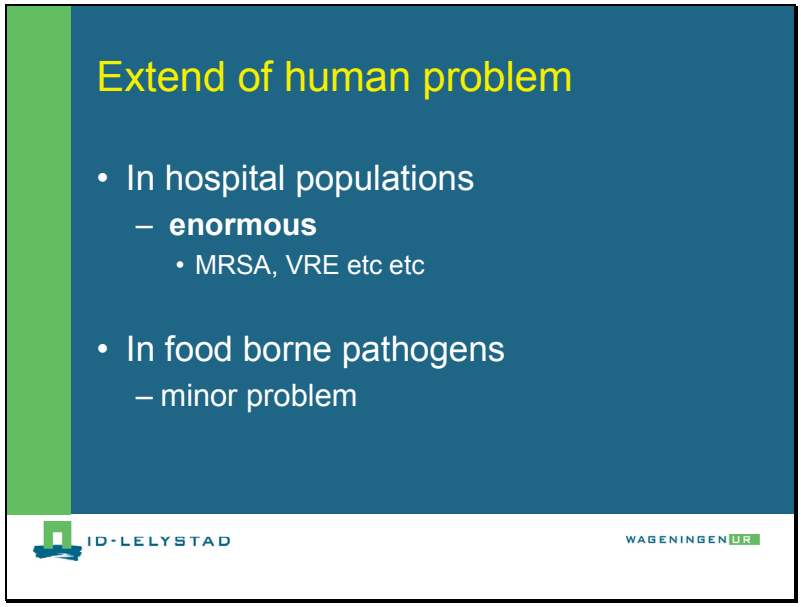

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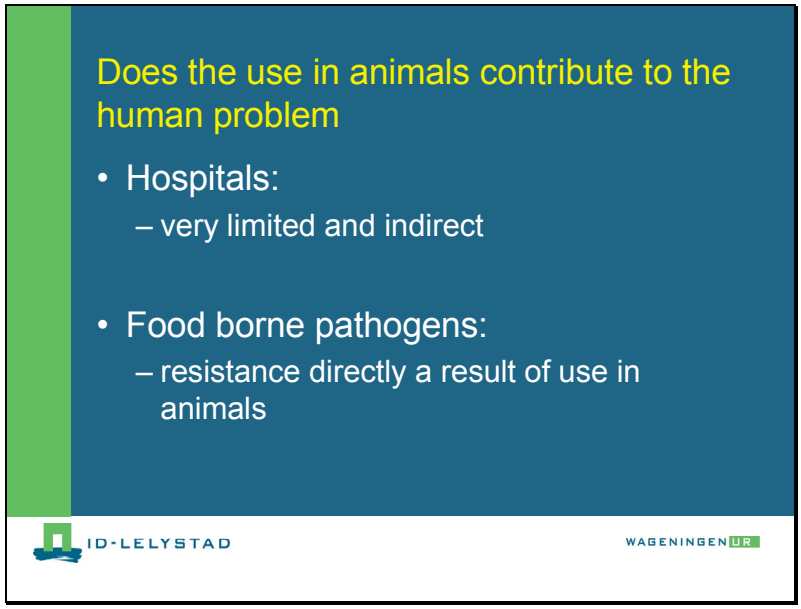

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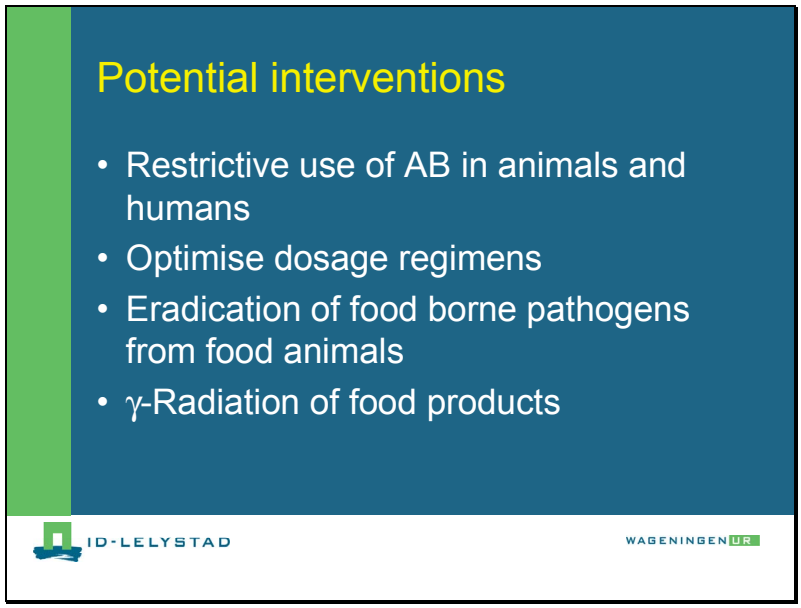


Appendix 6 Sheets of presentation 2.23

Slide 1

Risk assessment and data

Eric G. Evers, André M. Henken
Microbiological Laboratory for Health Protection
RIVM
The Netherlands

Slide 2

Presentation structure

- Risk analysis general
- Risk assessment general
- Risk assessment models
- Data
- Data recommendations

28 total 2

Slide 3

Risk analysis (1)

- Improve public health
- Quantify public health
- Which zoonoses are of importance
- Control and reduce important zoonoses
- Tool: risk analysis

28 total 3

Slide 4

Risk analysis (2)

- Risk: probability and severity
- Risk analysis:
  - Risk assessment
  - Risk management
  - Risk communication

28 total 4

Slide 5

Risk analysis (3)

- Risk assessment: science
- Risk management: weighing policy alternatives in the light of risk assessment
- Risk communication: communication between risk assessors, risk managers, consumers and others

28 total 5

Slide 6

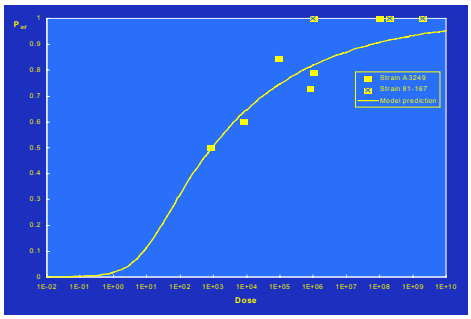
Risk assessment

- Hazard identification: (toxin from) micro-organism
- Hazard characterisation: dose response and health effects
- Exposure assessment: estimated intake
- Risk characterisation: based upon above, probability of occurrence for various health effects

28 total 6

Slide 7

Dose-response relation of infection with *Campylobacter jejuni*



28 total 7

Slide 8

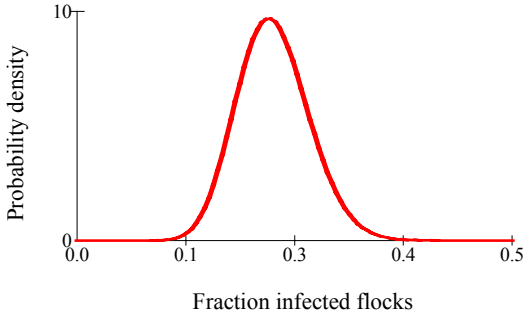
Uncertainty(1)

- Risk assessment should include uncertainty
- Distributions instead of point estimates
- Example: 22 of 100 flocks infected with Salmonella

28 total 8

Slide 9

Uncertainty (2)



28 total 9

Slide 10

Risk assessment

- Statement of purpose: define the combination of pathogen, product, process and population for which risk is assessed
- Statement of purpose is the first thing to do in a risk assessment

28 total 10

Slide 11

Value of risk assessment

- Estimation of the risk of the predefined combination
- Opportunity to compare risks
- Opportunity to evaluate the effect of interventions

28 total 11

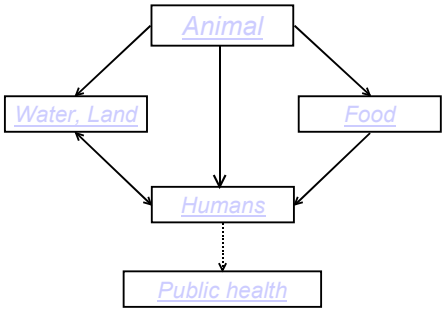
Slide 12

Risk Assessment models: reality is complex

28 total 12

Slide 13

Simplification



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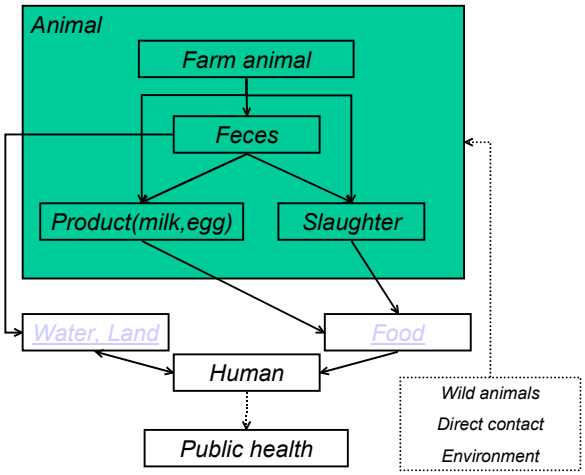
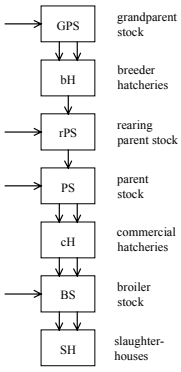


Slide 15

Chain model broilers (1)

Nauta MJ, Van de Giessen AW, Henken AM (2000). Epidemiology and infection 124, 365-373.



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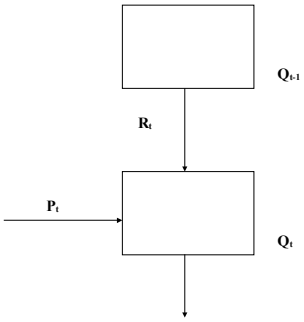
Slide 16

Chain model broilers (2)



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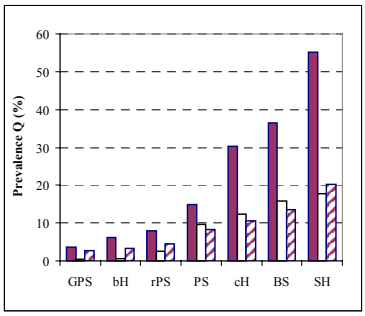
Slide 17

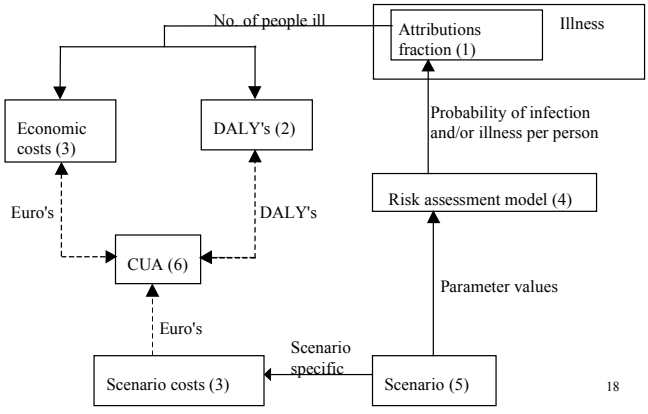
Chain model broilers (3)



28 total 17

Slide 18

A generalised conceptual approach



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Slide 19

Risk assessment model

- Production
- Industrial processing
- Consumer processing and frequency
- Dose-response

28 total 19

Slide 20

Data

- An ideal risk assessment requires many data
- Data are partly available
- Assumptions replace missing data
- Minimum data set cannot be set

28 total 20

Slide 21

Example: data needs for risk assessment Salmonella in eggs (1)

- **Productie module**
- - de prevalentie van SE-positieve koppels
- - de fractie hoog-prevalente (vs laag-prevalente) koppels van de SE-positieve koppels
- - de fractie ruiende SE-positieve koppels
- - de koppelgrootte
- - het aantal dagen na de rui met een hogere fractie SE-positieve eieren
- - het aantal eieren per koppel per dag
- - de fractie SE-positieve eieren in hoog-prevalente, niet ruiende koppels
- - de fractie SE-positieve eieren in hoog-prevalente, ruiende koppels
- - de fractie SE-positieve eieren in laag-prevalente, niet ruiende koppels
- - de fractie SE-positieve eieren in laag-prevalente, ruiende koppels
- - de fractie eieren die als ei wordt verwerkt (vs. de fractie die als eiproduct wordt verwerkt)

- **Eiverwerking en distributie**
- - het aantal SE per positief ei bij de leg
- - de fractie eieren die ter plaatse (zgn. in-line) wordt verwerkt (vs elders = off-line)
- - bewaartemperatuur voor transport voor off-line eieren
- - bewaartijd voor transport voor off-line eieren
- - omgevingstemperatuur gedurende transport voor off-line eieren
- - transporttijd voor off-line eieren
- - bewaartemperatuur voor off-line verwerking
- - bewaartijd voor off-line verwerking
- - omgevingstemperatuur gedurende off-line verwerking
- - off-line verwerkingstijd
- - bewaartemperatuur na off-line verwerking
- - bewaartijd na off-line verwerking
- - bewaartemperatuur voor in-line verwerking
- - bewaartijd voor in-line verwerking
- - omgevingstemperatuur gedurende in-line verwerking
- - in-line verwerkingstijd
- - bewaartemperatuur na in-line verwerking
- - bewaartijd na in-line verwerking

- - transporttemperatuur naar de eigebruiker
- - transporttijd naar de eigebruiker

- - parameter(s) voor een model dat de interne temperatuur van het ei beschrijft
- - parameter(s) voor een model dat de tijd tot stukgaan van het dooiermembraan beschrijft
- - parameter(s) voor een SE-groeimodel

- - de fractie eieren die alsnog naar eiproducten gaat

- **Eiproductverwerking en distributie**
- - het aantal vogels in een koppel (zie productie module)
- - de fractie SE-positieve koppels (zie productie module)
- - positieve eieren per SE-positief koppel (output productie module)
- - aantal SE per positief ei bij de leg (zie eimodule)
- - eiproductie per vogel per dag
- - fractie dooier/eiwit
- - eigewicht
- - verdeling SE over dooier/eiwit
- - pasteurisatietijd
- - pasteurisatietemperatuur

28 total 21

Slide 22

Example: data needs for risk assessment Salmonella in eggs (2)

- - aantal SE in 10.000 lbs ongepasteuriseerde vloeibare eieren
- - hoeveelheid vloeibare eieren in een bulk eitank
- - parameter(s) voor een model m.b.t. de log reductie van SE in vloeibare eieren door pasteurisatie
- - parameter(s) voor een model m.b.t. de log reductie van SE in vloeibaar eiwit door pasteurisatie
- - pH van vloeibaar eiwit bij 'eibreekbedrijf'
- - parameter(s) voor een model m.b.t. de log reductie van SE in vloeibare dooier door pasteurisatie
- **Bereiding en consumptie**
- - aantal SE per positief ei (vanuit eimodule)
- - fractie kapotte dooiermembranen (vanuit eimodule)
- - fractie eieren dat naar een instelling gaat

- - bewaartijd in de kleinhandel
- - bewaartemperatuur in de kleinhandel
- - bewaartijd thuis
- - bewaartemperatuur thuis
- - bewaartijd in een instelling
- - bewaartemperatuur in een instelling

- - fractie eieren dat gepooled wordt in de thuissituatie
- - aantal eieren in een pool in de thuissituatie
- - bewaartijd na poolen in de thuissituatie
- - bewaartemperatuur na poolen in de thuissituatie
- - fractie gepoolde eieren dat gebruikt wordt als ei in de thuissituatie
- - fractie van het voorgaande die onvoldoende wordt gekookt
- - fractie van gepoolde eieren die gebruikt worden als ingredient in de thuissituatie, die niet gekookt wordt

- - fractie eieren dat gepooled wordt in een instelling
- - aantal eieren in een pool in een instelling
- - bewaartijd na poolen in een instelling
- - bewaartemperatuur na poolen in een instelling

- - fractie gepoolde eieren dat gebruikt wordt als ei in een instelling
- - fractie van het voorgaande die onvoldoende wordt gekookt
- - fractie van gepoolde eieren die gebruikt worden als ingredient in een instelling, die niet gekookt wordt

- - fractie niet gepoolde eieren dat gebruikt wordt als ei in de thuissituatie
- - fractie van het voorgaande die onvoldoende wordt gekookt
- - fractie van niet gepoolde eieren die gebruikt worden als ingredient in de thuissituatie, die niet gekookt wordt
- - aantal maaltijden per ei in de thuissituatie met ei als ingredient
- - fractie niet gepoolde eieren die gebruikt worden als ei in een instelling
- - fractie van niet gepoolde eieren die gebruikt worden als ei in een instelling, die onvoldoende gekookt wordt
- - fractie van niet gepoolde eieren die gebruikt worden als ingredient in een instelling, die niet gekookt wordt
- - aantal maaltijden per ei in een instelling met ei als ingredient

- - aantal logs afname in aantal SE door koken in eieren gebruikt als ei
- - aantal logs afname in aantal SE door onvoldoende koken in eieren gebruikt als ei
- - aantal logs afname in aantal SE door koken in eieren gebruikt als ingredient

- - bewaartemperatuur na koken voor gekookte eieren en eiproducten in de thuissituatie en in instellingen

28 total 22

Slide 23

Example: data needs for risk assessment Salmonella in eggs (3)

- - bewaartijd na koken voor gekookte eieren in de thuissituatie
- - bewaartijd na koken voor thuis bereide eibevattende producten
- - bewaartijd na koken voor gekookte eieren in een instelling
- - bewaartijd na koken voor in een instelling bereide eibevattende producten

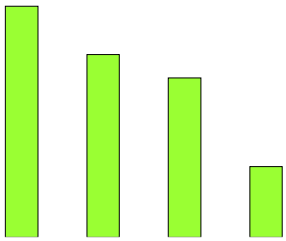
- **Volksgezondheidsmodule**
- - dosis = aantal levensvatbare en infectieuze SE na voedselbereiding (vanuit bereiding/consumptie module)
- - aantal blootgestelde personen (vanuit bereiding/consumptie module)
- - frequentieverdeling van dosis als functie van aantal blootgestelde personen

- - de fractie personen die extra gevoelig is voor ziekte door blootstelling aan SE
- - twee parameters voor de dosis-respons relatie, per subpopulatie
- - de kans op doktersbezoek gegeven ziekte, normale subpopulatie
- - de kans op doktersbezoek gegeven ziekte, gevoelige subpopulatie
- - de kans op ziekenhuisopname gegeven doktersbezoek, normale subpopulatie
- - de kans op ziekenhuisopname gegeven doktersbezoek, gevoelige subpopulatie
- - de kans op overlijden gegeven ziekenhuisopname, normale subpopulatie
- - de kans op overlijden gegeven ziekenhuisopname, gevoelige subpopulatie
- - de kans op reactieve arthritis gegeven genezing van de oorspronkelijke ziekte veroorzaakt door SE

28 total 23

Slide 24

Incidence of *Salmonella* in various data sources



28 total 24

Slide 25

Data

- Data requirement of risk assessment includes data that are presently not obtained

- **Counts of micro-organisms!**

28 total 25

Slide 26

Data recommendations (1)

- First a risk assessment model, then data collection, not the other way round
- Measurements for every link to be considered
- Measurements at animal level not only herd/flock/etc. level
- Not only presence/absence but also CFU's

28 total 26

Slide 27

Data recommendations (2)

- detection limits
- Measurements spread in time and place
- Farm to fork: measurements just prior to consumption
- Data in balance between links
- Extra attention to links with possible intervention

28 total 27

Slide 28

And:

- Import and export of animals and products
- Processing data of food industry
- Food handling data of the consumer
- Consumption data
- Dose-response relationship
- Probability of illnesses given infection, DALY's