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RIJKSINSTITUUT VOOR VOLKSGEZONDHEID EN MILIEU
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RIVM report 284500 012

Report on the third workshop organized by CRL-*Salmonella*

Utrecht, the Netherlands, 28 - 29 September 1998

M. Raes and A.M. Henken (editors)

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RIVM, P.O. Box 1, 3720 BA Bilthoven, telephone: 31 - 30 - 274 91 11; telefax: 31 - 30 - 274 29 71
European Commission, Legislation Veterinaire et Zootechnique, Rue de la Loi 86, B-1049 Bruxelles, Belgique, telephone: 32-2-2959 928; telefax: 32-2-2953 144

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Samenvatting

Op 28 en 29 september 1998 is door het Communautair Referentie Laboratorium (CRL) voor *Salmonella* een workshop georganiseerd in Utrecht, Nederland. Alle Nationale Referentie Laboratoria (NRLs) voor *Salmonella* waren vertegenwoordigd. In totaal waren er 35 deelnemers.
Het programma van de workshop bestond uit vier delen. Het eerste deel was bacteriologisch gericht en bestond uit een evaluatie van bacteriologische ringonderzoeken. Het tweede deel van de workshop was een bespreking van detectiemethoden voor *Salmonella* en de validatie van deze methoden. Het derde deel was immunologisch gericht waarin de immunologische methoden besproken werden. In het laatste deel van de workshop werden diverse typeringsmethoden besproken.

Summary

On 28 and 29 September 1998 a workshop was organised by the Community Reference Laboratory (CRL) for *Salmonella* in Utrecht, the Netherlands. All National Reference Laboratories (NRLs) for *Salmonella* participated (in total 35 participants).
The programme of the workshop was divided in four parts. The first part was a bacteriological part in which the bacteriological collaborative studies were evaluated. In the second part of the workshop detection methods for *Salmonella* and the validation of these methods was discussed. The third part was an immunological part. Some immunological methods were discussed. In the last part of the workshop typing methods were discussed.

1. Introduction

1.1 Opening

dr.ir. 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 (see Appendix 2 for participants). At least 1 to 2 persons of each of the 15 member states of the European Union are present, even more than we had expected or hoped for. A special word of welcome I would like to address to mr. V. Niemi, who will be the successor of mr R. Holma from coming Thursday, the first of October, onwards. We appreciate it very much that he is present here among us.

Secondly, I would like to stress 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 energy. 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? (see Appendix 3 for the programme of the workshop) To answer this question let us go back first to the functions and duties of the CRL-*Salmonella* and its activities.

1.1.1 Functions, duties and activities of the CRL-*Salmonella*

The Functions and duties of the Community Reference Laboratory (CRL) for *Salmonella* are:

1. providing national 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 organizing comparative testing;
3. coordinating research into new analytical methods and informing national 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.

These functions and duties were translated into the following yearly activities of the CRL-*Salmonella*:

- the organisation of several collaborative studies;
- the publication of a quarterly newsletter;
- the organisation of a workshop;
- the performance of research
    * on detection methods
    * on stability and homogeneity testing of reference materials;
- ad hoc activities
    * updating manual when necessary
    * responding on incoming requests from e.g. the Commission.

1.1.2 Aims of the workshop

The aims of the workshop can be defined as following (of course our tasks are focussed on analytique techniques and thus these aims must be seen from this point of view):

- to discuss the results of the collaborative studies the CRL-*Salmonella* organised among the NRLs-*Salmonella*;
- to discuss organisational aspects of collaborative studies among and within states;
- to discuss how to coordinate our activities in view of the task given (zoonosis directive); and
- to discuss whether or not there are specific needs among the NRLs-*Salmonella*.

2. Review of the presentations

2.1 Current situation with regard to the zoonosis directive

Mr. V.M. Niemi (EU Commision)

Council Directive 92/117/EEC concerning measures for protection against specified zoonoses and specified zoonotic agents in animals and products of animal origin in order to prevent outbreaks of food-borne infections and intoxications was seen as a first step, in terms of gathering information on the incidence of zoonoses generally, and in monitoring, controlling and ultimately eradicating some invasive serotypes of *Salmonella* in poultry breeding flocks. It was recognised that the prevention of zoonoses was a complex question and that further measures, for example in relation to laying flocks, as well as measures beyond the production stage, would be necessary to bring the problem under control.
It was soon realised that the aims of the Directive and the timetable were too ambitious. Most of the Member States could not respect the time limits in particular regarding the provisions on *Salmonella* control of poultry breeding flocks. The goals regarding the collection of information on trends and sources of zoonotic infections have been achieved to somewhat better extent and currently all 15 Member States submit their annual reports. However, the quality of the data suffers from unharmonized surveillance systems and cannot be compared between the Member States.
Due to the poor implementation it was regarded necessary to postpone certain time limits of the Directive. This was done by adopting Council Directive 97/22/EC. In the meantime the Commission intended to proceed with a fundamental review of the provisions regarding the control of zoonoses and in particular *Salmonella* in poultry.
Directive 97/22/EC introduced Article 15a to Directive 92/117/EEC which provides for the Commission to submit a report to the Council and the Parliament concerning the measures to be implemented for the control and prevention of zoonoses. This report shall refer in particular to:

- the new rules for the reporting system for zoonoses;
- the methods for collecting samples and for examinations in approved national laboratories;
- the control of *Salmonella* in poultry laying flocks;
- the control of *Salmonella* in poultry breeding flocks and in compound feedingstuffs for poultry; and
- any measures to combat zoonoses other than salmonellosis.

The report shall be accompanied by proposals concerning zoonoses, especially in the context of the review of Directive 92/117/EEC.
While preparing its new proposal the Commission shall take into account several general aspects regarding the future development of the EU. In this occasion I shall not go deeply in-

side these political questions, but in my opinion they are greatly influencing the future development of the dossier.
Health protection has gathered more and more importance during recent years. When implementing concrete measures the use of the farm to table principle, which means longitudinally integrated measures throughout the food chain, is the method of choice.
The Union is also preparing itself for the possible enlargement in the next decade. New member states from East and Central Europe need special follow-up. In this respect the policies related to zoonoses are certainly in a key position.
In concrete terms the development referred here is seen in the new Treaty of Amsterdam. The changes in the basic rules of the Community will directly affect the development of such pieces of legislation as the zoonoses directive. The new wording in article 129 paragraph 4 (b) connects the veterinary legislation, which has public health implications, closely to the framework of Community health policies. In practical terms, this is going to mean the use of the co-decision procedure with greater participation of the European Parliament.
As you all are well aware, there exists also, of course, significant development in the field of zoonoses themselves. Directive 92/117 includes concrete measures regarding *Salmonella* control in poultry. Despite the fact that the implementation of the specific measures provided for in the directive has faced certain problems the importance of these policies remains constant. This means that there have to exist Community rules in this field. The experience gained during the implementation of the existing directive should, of course, be taken into account.
Having said that, one has to remind that there are other zoonoses with public health importance, which have emerged over the past few years. These include, e.g., campylobacteriosis and VTEC-infection. The question rises, whether these zoonoses should have as close a look-up in the legislation as salmonellosis has. Also within the domain of salmonellosis there exist new challenges. One of them is inevitably the problem of antibiotic resistant strains of *Salmonella*. Although this is not solely a question of the control of zoonoses, it brings us new topics to debate.
The procedure for the review of the zoonosis directive was set up by Directive 97/22, article 15 a. Although the time limits set up by that directive have already been overtaken, the intention of the Commission is, according to my knowledge, to continue the process. A discussion document was prepared by the Commission last spring and discussed during a working group meeting in April this year. The comments received during that meeting are studied carefully. The next step in the exercise will be the introduction of a strategy paper to the Chief Veterinary Officer's meeting. After that point a more precise preparation, including also technical aspects, will continue at a working group level.
In this workshop I hope that the experts present could express their views about the future control measures on zoonoses and *Salmonella* control in particular. There could also be a technical discussion at general level about the possibilities and needs to harmonise the *Salmonella* testing methods at community level. I am sure that the experts present can give a

valuable contribution to this subject and these comments must be addressed when reviewing the zoonosis directive.

2.2 Evaluation of the second bacteriological collaborative study

detection of *Salmonella* in the presence of competitive micro-organisms[1]

Mrs. N. Voogt (RIVM/CRL-*Salmonella,* the Netherlands)

Introduction

The first bacteriological collaborative study was organized by the CRL-*Salmonella* in 1995. Thirty reference materials (RMs) containing ± 5 colony forming particles (cfp) of *Salmonella* Typhimurium (STM) were examined by all NRLs using the proposed European reference method, ISO 6579, and optionally the method routinely used by a laboratory. No significant difference was found between both methods for the individual laboratories. No information was obtained about the ability of the NRLs to detect *Salmonella* in the presence of competitive micro-organisms. Therefore in the second study *Salmonella* had to be detected in the presence of competitive flora in the form of chicken faeces.

Outline of the study

Fifteen capsules containing 100 cfp STM and 15 containing 1000 cfp STM had to be tested in combination with 1 gram of chicken faeces. Besides that, six control samples had to be examined. Furthermore, a procedure control (no capsule nor faeces added to the media), a negative control (only 1 gram faeces added) and four capsules (two containing 5 cfp *S.* Panama, one containing 100 cfp STM and one blank capsule) to which no faeces had to be added were examined.

Chicken faeces was used to prepare the samples containing competitive micro-organisms. The faeces was tested for the presence/absence of *Salmonella* and the influence of storage at -20 °C on the stability of samples. No *Salmonella* was detected and there was no significant difference in the number of viable bacteria during storage at -20 °C.

All NRLs carried out the ISO 6579 method and optionally the laboratory's own method.

Results

Control samples

No *Salmonella* was detected from the blank capsule, procedure and negative control. All NRLs isolated *Salmonella* from the control capsule containing 100 cfp STM. Both capsules containing *S.* Panama were found negative by three laboratories and two laboratories found one of these two capsules negative.

Reference method
Using the reference method eight laboratories isolated *Salmonella* from all 30 samples and seven NRLs found between 27 and 29 samples positive. Two laboratories reported less samples positive, 21 and 16, respectively.
Nine laboratories did not find differences in the results obtained with capsules containing 100 or 1000 cfp STM. The remaining eight laboratories found between one and five more positives with capsules containing 1000 cfp STM compared to capsules containing 100 cfp STM.
Thirteen laboratories found more positive isolations with the use of Rappaport Vassiliadis (RV) as selective enrichment medium compared to Selenite Cystine (SC). Two laboratories found more positive isolations with the use of SC and one laboratory found no difference.
Own method
Ten laboratories used their own method. The number of laboratories per medium was too small for statistical evaluation. Four laboratories used Rappaport Vassiliadis Soya (RVS) of whom two found identical results and two found better results compared with the reference method. Two other laboratories used the semi-solid medium Modified Semi-solid Rappaport Vassiliadis (MSRV) as selective enrichment; one found the same and the other found better results in comparison with the reference method.

Conclusions
1. *Salmonella* was isolated from most capsules containing 1000 cfp as well as 100 cfp STM;
2. testing capsules in combination with chicken faeces revealed significantly less positive isolations using SC compared to RV as selective enrichment medium; and
3. the results of the routinely used methods makes it worthwhile to test other selective media.

[1] The study is published in report 284500 007: bacteriological detection of *Salmonella* in the presence of competitive micro-organisms (A collaborative study amongst the National Referene Laboratories for *Salmonella*) N. Voogt, P.H. in 't Veld, N. Nagelkerke and A.M. Henken, September 1997

2.3 Evaluation of the third bacteriological collaborative study

Mr. P.H. in 't Veld (RIVM/CLR-*Salmonella*, the Netherlands)

The draft report of the collaborative study was handed out at the workshop and the participants were requested to sent their comments of the report to the CRL-*Salmonella* within four weeks.

For this study two methods were used to test for the presence of *Salmonella* in chicken faeces inoculated by reference materials containing *Salmonella* Typhimurium (STM) or *Salmonella* Enteritidis (SE). The two methods were a reference (REF) method applied by all labo-

ratories based on the ISO 6579 (pre-enrichment in Buffered Pepton Water (BPW) for 16-20 h at 37 °C, followed by selective enrichment in RV for 24 and 48 h at 42 °C and isolation on Brilliant Green Agar (BGA) and second medium of choice) and the laboratory's routine method (OWN method). Three types of reference materials were used to inoculate the chicken faeces, these RMs contained STM at a level of 100 cfp/capsule, STM at a level of 10 cfp/capsule or SE at a level of 100 cfp/capsule. Each type of RMs was tested 14 times in combination with 1 gram of chicken faeces. Besides these 42 samples a number of control samples were tested. These control samples were the following:

- 7 samples consisting of faeces containing antibiotic in combination with RMs containing STM at a level of 100 cfp/capsule;
- 4 samples consisting of RMs containing STM at a level of 10 cfp/capsule;
- 2 samples consisting of RMs containing *Salmonella* Panama at a level of 5 cfp/capsule; and
- 5 samples consisting of RMs containing sterile milk powder (blanks).

So, in total, 60 samples were examined in this study.

The RMs containing SE were used for the first time in a collaborative study and therefore checked for stability. The stability of the material was not as good as expected. The level of contamination decreased from about 200 cfp/capsule to about 100 cfp/capsules at the time of the collaborative study (level based on linear regression of the data obtained with time). As the stability of this material was not as expected more research is needed to improve stability.

The effect of the addition of the antibiotic to the faeces was tested before the collaborative study in order to determine the concentration of antibiotic to be used. Two levels of antibiotic added to faeces were used and tested in combination with the STM 100 capsule. In both cases positive results were obtained, but only few at the higher level of the antibiotic. This higher level was used in the collaborative study to test the feasibility of such a sample to check whether or not participants are actually adding the faeces to the capsules containing *Salmonella*.

Three laboratories found positives using these samples in the collaborative study. Two laboratories found one sample out of 7 positive and one laboratory 5 but only for the REF method.

The control samples used in the collaborative study gave some unexpected results. One laboratory was not able to detect *Salmonella* from both the RMs containing *S.* Panama (5 cfp/capsules) as well as STM (10 cfp/capsule). Also some false positive results were obtained using the procedure control (no RM or faeces added) or for the blanks samples (faeces tested in combination with a RM containing sterile milk powder). All negative controls (only faeces added) were negative.

The results obtained by the laboratories using the REF method are presented in the report[2]. One laboratory found all samples negative. The total number of positive isolations ranged between 0 and 39 out of a possible 42, with a mean of 56 % positives. For STM 10 this range was between 0 and 13 (mean 49 % pos.), for STM 100 between 0 and 14 (mean 76 %

pos.) and for SE 100 between 0 and 14 (mean 44 % pos.). The Duncan's multiple range test was carried out to indicate groups of laboratories with similar performance.
Using the OWN method slightly more positives were found. Those laboratories using a semi-solid medium (4 labs used MSRV and 1 Diagnostic Semi-solid *Salmonella* medium (DIA-SALM)) found, on average, significantly more positives for the total number of *Salmonella* isolations.
From this study it was concluded that:

- the detection of STM 10 and SE 100 are near the (overall) detection limit of the reference method for testing chicken faeces;
- there exists a wide spread in sensitivity between laboratories; and
- semi-solid media performed significantly better than the reference method.

[2] The study is published in RIVM report 284500 011 Bacteriological detection of *Salmonella* in the presence of competitive micro-organisms (Bacteriological collaborative study III amongst the National Reference Laboratories for *Salmonella).* M. Raes, N. Voogt, P.H. in 't Veld, N. Nagelkerke and A.M. Henken, December 1998

2.4 National activities with respect to *Salmonella* control and collaborative studies

2.4.1 Plan of approach to combat *Salmonella* and *Campylobacter* in the Dutch poultry sector

Mrs. R. Westendorp and mr. H. Rang (Dutch Product Boards for Livestock, Meat and Eggs)

The Dutch broiler sector has taken new initiatives to combat *Salmonella* and *Campylobacter* infections in chicken meat. A new Plan of Approach came into force on May 1st, 1997.
The plan aims to prevent the introduction, transfer and cross-infection of *Salmonella* and *Campylobacter.* An extended monitoring programme was established to survey the results of the reduction programme. Each lot of chickens is analysed in three different stages:

- one day old chicks: down in the hatchery and chicken box liners at arrival at the broiler farm;
- broilers, prior to slaughter: manure from the caecum in the penhouse; and
- broilers, after slaughter: appendix and neck skin.

The new rules apply to hatcheries, broiler farms and slaughterhouses.
For (grand)parent stock strict rules were established in an earlier stage. Monitoring of these farms was extended to all *Salmonella* types.
For hatcheries, a set of new rules on layout of the plant and the hygiene management was established. In this way, cross infection between lots of eggs contaminated with *Salmonella* and lots free of *Salmonella* is reached. Moreover, the presence of in-house contamination is recognised and transmission routes interrupted.

Broiler farms have to comply with new hygienic measures as well. Periodical cleaning, disinfection and hygiene checks are part of the programme.
The aim of the reduction programme is to reduce *Salmonella* contamination to 10% of the lots of poultry, leaving the slaughterline, in the end of 1999. For *Campylobacter* a goal of 15% is established.
The results are published each quarter, primarily for the broiler sector and the Dutch Ministry of Public Health. Only recently, the report on the second quarter of 1998 was published. The results show a steadily decrease of *Salmonella* contamination rates. For *Campylobacter* it is too early to draw any conclusion yet.

Discussion
Information about the *Salmonella/Campylobacter* status of a broiler flock is given to the slaughterhouse as late as possible during the broiler production period, since especially contamination with *Campylobacter* may occur in the last weeks
Everybody who is part of the production chain participates in the Dutch programme. The costs of the programme amount to Dfl. 16 million in 2.5 years.
The use of Competitive Exclusion flora (CE) is not part of the programme. However, CE is applied by some individual companies. Also, CE is used following antibiotic treatment of SE-positive broiler breeder flocks and (rearing) commercial layer flocks.
To obtain information about the origin of the contamination at each production stage both the materials coming in and the materials going out are tested.
The results obtained at the hatcheries are related to the results found in both the preceding and the next production stage.
Before the implementation of the control programme, the ratio vertical/horizontal transmission was estimated to be 90%/10%. As a consequence of the control programme, the relative contribution of vertical transmission decreases.
There is a programme for control of SE and ST in the egg production chain. The objective is a reduction of SE positive layer flocks from about 10% in the actual situation to less than 5%, within a period of 3 years.

2.4.2 Collaborative studies in the Netherlands

Mr. A.W. van de Giessen (RIVM/NRL-*Salmonella*, the Netherlands)

In the Dutch programme for *Salmonella* control in the poultry sector, sample analyses are carried out by commercial laboratories, which have been provisionally recognized by the Dutch national authorities. On behalf of the Dutch Veterinary Public Health Inspectorate, the Dutch National Reference Laboratory for *Salmonella* regularly organizes collaborative studies amongst these recognized laboratories in order to test their ability to detect *Salmonella* in the absence/presence of competitive micro-organisms. The first collaborative study, conducted in 1997, included 24 commercial laboratories. In this trial, reference materials con-

taining *Salmonella* had to be tested with or without addition of *Salmonella* negative chicken faeces. In total, each laboratory had to test 50 individually numbered capsules/samples. Forty capsules (21 capsules containing 100 colony forming particles (cfp) *Salmonella* Typhimurium (STM), 15 capsules containing 1000 cfp STM and 4 blank capsules) had to be tested in combination with one gram of chicken faeces. Ten capsules (6 capsules containing 5 cfp *Salmonella* Panama (SP) and 4 capsules containing 100 cfp STM) had to be tested without faeces. Additionally, a procedure control (no capsule/no faeces) and a negative control (only faeces) had to be tested. The collaborative study had to be conducted strictly according to a protocol and the laboratories had to use their own method for detection of *Salmonella* in poultry faeces. Fourteen of the 24 laboratories isolated *Salmonella* from all positive capsules tested without addition of faeces. Twelve of the 24 laboratories isolated *Salmonella* from all 36 capsules tested in combination with faeces. In total, 9 different selective enrichment media were used by the 24 participating laboratories, some of them using 2, 3 or 4 media in parallel. Significantly less positive isolations were obtained by using DIASALM compared to RV as selective enrichment medium. This may be due to the fact that most laboratories were not much experienced with the use of the DIASALM medium.

Based on the results obtained in this study and criteria set by the Dutch Product Boards for Livestock, Meat and Eggs, three laboratories had to participate in a supplementary trial, in which they produced satisfactory results. The Dutch NRL for *Salmonella* will continue to regularly organize collaborative studies on *Salmonella* detection amongst the recognized laboratories. In these succeeding trials, each laboratory will have to test 15 samples, which number enables trend analysis. However, in case the results of a laboratory do not meet the specified criteria, this laboratory has to test 50 samples in the next ring trial, which number enables statistical evaluation of the test results.

Discussion

Research is needed to get more information about the difference between adding 1 or 25 grams of faeces to the BPW.

In all probability, different laboratories used different formulas for preparation of the Tetrathionaat bouillon (TBB) selective medium. We intend to find out which media are used exactly.

2.4.3 French legislation and collaborative studies in France

Mrs. F.S. Humbert and Mrs. K. Proux (NRL-*Salmonella,* France)

The legislation for *Salmonella* in poultry in France was originally set up in 1982, but this was a voluntary programme for control of hatcheries, selection and multiplication flocks named COHS (Contrôle Officiel Hygiènique et Sanitaire). *Salmonella* and also *Mycoplasma*

were concerned. For *Salmonella*, samples were mainly for bacteriology, but serological tests were also realised by the Rapid Slide Agglutination (RSA) test with the Pullorum antigen.

This legislation was reviewed in 1991 because of the emergence of *Salmonella* Enteritidis (SE) linked to eggs in order to include, always on a voluntary basis, table egg laying hens. These flocks were submitted to a few sampling periods during production but the surveillance seemed enormous considering the number of flocks (3 500 laying flocks in France) and the fact that the government paid for the analyses. In 1997, nearly all (100%) of pullets but only 33% of layers were voluntary included. This year, the legislation is changing for layers, *i.e.*, move from a voluntary to a compulsory programme and one more sampling period is added during egg production.
Initially, if layers tested positive for SE only, farmers have to slaughter their animals (with compensations). With the new programme, *Salmonella* Typhimurium (STM) will be included and farmers will choose between heat treatment of eggs or slaughtering of all birds. The estimated number of samples generated under this programme is 3,500 X 2 (samples) X 6 (periods) = 42,000 samples/year. This estimation does not take into account all samples that will be needed to confirm any suspicion, and has to be added to what is already done in the breeding sector.
The estimated number of laboratories involved in this surveillance is more than 50 (public and private). Until now, they were only administratively registered by the Ministry of Agriculture.
So the extension of the surveillance programme in table egg laying flocks needs :

1. to introduce Enzyme Linked Immuno Sorbent Assay (ELISA) in order to compare bacteriological and serological testing;
2. to organise ring trials between laboratories as comparable bacteriological and serological results can be obtained;

For the first ring trial we will include 9 laboratories. The work is divided in four steps :

1. last development of our French ELISA method (Kles et al., 1992)[3];
2. organisation of a bacteriological and serological training courses for these 9 laboratories;
3. ring trial; and
4. comparing bacteriological and serological results for 70 flocks in the field.

Our ELISA is an indirect method based on lipopolysaccharids antigens of SE and STM adsorbed with an anti-*E. coli* sera reducing cross reactions. In order to send all pre-coated plates to the participating laboratories, refrigeration has been tested during a month and reference sera and yolks against SE and STM were produced on Special Pathogens Free chickens.
The objectives of the study will be presented to the 9 laboratories and a training course on enrichment media (especially the use of semi-solid media for those who do not use it already) and ELISA will be organised. Because it is the first time diagnostic laboratories will

use *Salmonella* detection by ELISA on a large scale in France, the aim of the ELISA trial is to compare repeatability, reproducibility, correct response to the dose-effect, sensitivity and specificity for each laboratory using plates already coated by ourselves, the same conjugate and the same substrate.

For the field study on layers and meat multiplication, flocks will not be chosen at random. We want to include 30 flocks with a high risk of contamination in order to test sensitivity and speed of bacteriological and immunological response, and 30 flocks that we hope to test negative in order to test specificity. Finally, because of the probable use of killed vaccine in France, discrimination between vaccinated and contaminated animals will be tested in 10 vaccinated flocks. Samples for this field study are 20 sera (in meat multiplication) or yolks (in layers) for serology and one swab and one pool of faecal samples for bacteriology at each time of sampling. For kinetic study layers will be sampled every month and layers every two weeks.

[3] KLES V., MORIN M., HUMBERT F., LALANDE F., GUITTET M and BENNEJEAN G, 1992. Serological diagnostic of avian Salmonellosis : an ELISA development using antigens adsorbed with *E. coli* antisera. Proceeding *Salmonella* Symposium, Ploufragan, France, September 1992, pp 93-94.

2.5 Ideas for future bacteriological collaborative studies

The CRL-*Salmonella* summarised a number of possibilities that can be included in a future study. These possibilities are presented below:

- evaluate the use of semi-solid media (MSRV or DIASALM) by all participants? (define new reference method for detection of *Salmonella* in chicken faeces?)
- evaluate the difference in sensitivity in detecting STM and SE? (CRL-*Salmonella* is currently testing for differences in growth characteristics of both strains)
- include the use of pre-warmed BPW and RVS as in the proposed ISO standard? and
- include more serotypes?

A suggestion of one of the participants was to increase the amount of faeces per sample. The CRL-*Salmonella* will prepare a proposal for a future study as no definite general agreement on a future study could be obtained.

2.6 Review on alternative methods for *Salmonella* detection

Mr. Roy Betts (Campden & Chorleywood Food Research Association)

In the food industry there is a growing need for rapid methods that enable the fast reliable detection of a range of foodborne pathogens and spoilage organisms. Many different procedures have been used to enable rapid detection including chemical, physical, immunological

and genetics based systems. This paper briefly reviews current commercially available rapid methods for the detection of *Salmonella* from foods. The methods are varied and offer the user different positive attributes, but perhaps the most important requirement is that the method is reliable, gives correct results and is acceptable to all those that rely on the result given. The latter points mean that all new methods must be fully validated before use.
The sheets used for this presentation are printed in Appendix 4

2.7 Validation of microbiological methods

2.7.1 Introduction to the Microval-project

Mr. P.H. in 't Veld (RIVM/CRL-*Salmonella*, the Netherlands)

The updated version of an introduction to Microval is printed in Appendix 5.

2.7.2 Validation of a detection kit for *Listeria monocytogenes*

Mr. Roy Betts (Campden & Chorleywood Food Research Association)

With the large number of new microbiological test methods now being commercially produced, the food industry is having to make critical decisions concerning the use of these procedures.
Whilst the reduction in method test times is a great advantage, the user must be confident that the method works and gives results that are accurate. In order for this to occur, new tests must be validated.
Validation involves comparing the method against a recognised reference method, and ensuring that the results are equivalent. In the past ad hoc validations have been done, these have been relevant to some users but were not widely accepted. There is a requirement for a widely accepted method validation scheme, particularly across Europe. This has led to development of a microbiological method validation system that is widely acceptable across Europe.
The MicroVal procedure is now in a draft form and is being progressed through CEN for standardisation as a European standard for method validation.
The sheets used for this presentation are printed in Appendix 6.

2.8 Standardization and ring trial for *Salmonella* detection by PCR

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

Conventional microbiological methods for *Salmonella* detection require about 4 days and intensive laboratory work. As a consequence, there has been an intensive search for faster,

more sensitive, and reliable methods for *Salmonella* detection. In addition to immunological, physical and cultural methods, molecular approaches on the DNA level have been investigated.
Initially, DNA probes for arbitrarily found *Salmonella*-specific DNA sequences, r-RNA genes and sequences of defined virulence genes were used. However, the high costs for the assays, together with the need for specially equipped laboratories and well trained staff prevented their routine use.
Today, in contrast, most of the research concentrates on PCR methodologies. They make use of the possibility to multiply *Salmonella* target DNA sequences by the use of specific primers and a heat stable DNA polymerase. Resulting DNA fragments can be labelled by numerous methods and subsequent detection is easy. Many of the steps involved can be performed by automatic equipment which also lowers the costs entailed.
However, most of these new techniques have not been standardised and officially recognised.
In Germany a committee of the German standardisation organisation DIN has recently adopted and released a "General Method-specific requirements for detection of microorganisms with polymerase chain reaction (PCR)". A second method which specifically elaborates the detection of *Salmonella* has been proposed. It is called "Method for detection of *Salmonella* with polymerase chain reaction (PCR)".
For the evaluation of such methods the NRL-*Salmonella* of Germany has selected 270 cultures of *Salmonella* isolates which represent the 10 dominating serovars and representatives of all subspecies. Until now the PCR systems tested proved positive with all isolates.
In a ring trial involving 11 routine test laboratories and using spiked milk powder the sensitivity turned out to be 98.25% and specificity was 98.17%.

Discussion

The level of contamination of the samples used in the ring trial was 1000 cfp/25 gram milk powder.
At the moment it is still necessary to confirm positive PCR results but in future this might disappear. It is also possible to miss some serotypes by using PCR. The number of strains used to validate the PCR is quite high especially compared to the requirements of the Microval project. However there are at least 2400 different serotypes of *Salmonella* known. The strains used for validation comprises 40-50 strains of the predominant serotypes and for the rest less frequent occurring ones.
The price of the PCR strongly depends on the number of users.

2.9 National activities on immunological detection

2.9.1 Field experiences with an LPS-based ELISA as a tool in the Danish control programme for *Salmonella* in the poultry sector

Mr. M. Madsen (NRL-*Salmonella,* Denmark)

In January 1997 a 3-year National *Salmonella* Surveillance and Control Programme for the Danish poultry sector was initiated.
The programme encompasses the broiler as well as the table egg sector, and constitutes an expansion of the Danish plan which has been in action as from 1994 through 1996 as an implementation of the EU Zoonosis Directive.
The new programme includes as its most important expansions the inclusion of testing of table egg layer flocks, the testing of parent flocks at house as well as at hatchery level, and the introduction of testing for antibodies in serum and egg yolk samples as a surveillance tool. The paper describes the sampling and testing procedures of the Danish *Salmonella* Surveillance and Control Programme.
The serological test employed in the programme is an LPS-based ELISA (the "Mix-ELISA") directed against *Salmonella* Enteritidis and *S.* Typhimurium. The details of the test were presented at the CRL-*Salmonella* Workshop in Bilthoven in 1995. Since then an LPS-based ELISA (the "Inf-ELISA") directed against *S.* Infantis has also been developed. Both ELISA tests have been automated, and are used in the Danish control programme at the parent level where no salmonellas regardless of serovar are allowed. At the table egg layer flocks only the Mix-ELISA is applied.
The routine serological surveillance is performed on serum samples from rearing flocks, and on egg samples from layer flocks, in conjunction with conventional bacteriological culture of faeces samples.
Based on approx. 120,000 samples from broiler parent flocks during August 1997 through July 1998, both the Mix-ELISA and the Inf-ELISA had a calculated specificity of 99.9%. During the period 21 flocks came under suspicion for *Salmonella* infection. Of these, 11 flocks were confirmed by subsequent bacteriological culture of 60 animals, and a further five flocks were slaughtered before verification could be instituted. It should be noted, however, that four flocks were picked up by positive bacteriological routine culture without a corresponding serological response. These flocks were most likely flocks at an early stage of infection before a measurable serological response had been mounted.
In conclusion, the LPS-based ELISAs as applied in the Danish control programme are well suited for large scale screening programmes, and may be applied to both blood and egg yolk samples. Caution should be exerted in the case of fresh infections as it takes 2-3 weeks for a measurable serological response to develop. In *Salmonella* control programmes, as it is employed in the Danish programme, bacteriological culture methods should therefore be used in conjunction with serological testing methods.

2.9.2 Development of monoclonal antibodies against *Salmonella* and their practical application

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

Serotyping of *Salmonella* isolates is generally performed by the use of polyclonal rabbit antisera. They can be obtained by immunisation with killed *Salmonella, Salmonella* LPS or flagelli. However, in immunological terms such antisera are not well defined and unspecified reactions can occur.
Monoclonal antibodies in contrast are highly specific and can be reproduced under highly standardised conditions. In order to obtain monoclonal antibodies against major *Salmonella* serovars two immunisation regimes were established. The first one used Balb/c mice which were immunised i.p. with 5 µg of purified *S.* Typhimurium LPS at an age of 11-13 weeks. The second regime made use of a dose of $5x10^8$ cfu of heat killed bacteria in Balb/c mice at 7-11 weeks of age.
In total 28 hybridoma celllines were obtained. They produce monoclonal antibodies against various antigens, among them O-factors, outer membrane proteins and pili. One monoclonal antibody seems to be specific for the genus Salmonella.
Such monoclonal antibodies can be used for routine serotyping, subtyping, diagnosis and passive protection.

2.10 Ideas for immunological collaborative studies of CRL-*Salmonella*.

An introduction to this agenda item was presented by Mr. A. van de Giessen (RIVM/ NRL-*Salmonella,* the Netherlands).

Discussion
If there is a need for collaborative studies on immunology or molecular detection, we can foresee in this need. When there is a new method which is a good method we shall test it. It is also possible to have the knowledge in different NRLs and the communication via CRL. When the NRL does not have the facilities to perform a collaborative study it should find a laboratory in their country which has got the facilities to perform the study. The CRL organizes especially bacteriological collaborative studies. A few laboratories stated their interest in performing an immunological collaborative study.

2.11 Evaluation of the second and third collaborative study on serotyping

Mrs. N. Voogt (RIVM/CRL-*Salmonella*, the Netherlands)

General data of serotyping

In Table 1 the number of strains typed yearly and the frequency of typing by the NRLs-*Salmonella* are shown.

Laboratories had the possibility to type strains in collaboration with another reference laboratory in their country. In the second study five laboratories sent mostly the strains which do not occur frequently (*S.* Alachua and *S.* Paratyphi B. var Java) to another laboratory. In the third study three laboratories typed strains together with another reference laboratory. Two laboratories took this opportunity in both studies.

Table 1 *Number of strains typed and frequency of typing by the participants*

No of strains typed	no. of laboratories (study II + III)	Frequency of typing	no. of laboratories (study II + III)
7-500	4	Daily	11
500 – 5000	7	Weekly	3
5000 – 10.000	2	Monthly	2
> 10.000	3		

Selected *Salmonella* strains

In study II the Kauffmann-White scheme of 1992 and in study III those of 1997 was used. After discussion at the first workshop of the CRL-*Salmonella* many strains occurring frequently were chosen in both studies. Most of the in total 20 strains belonged to group B (including *S.* Typhimurium, *S.* Derby), C1 (*S.* Infantis, *S.*Virchow), C2-C3 (*S.* Hadar, *S.* Manhattan) or group D1 (*S.* Dublin, *S.* Enteritidis).

Results

In the second and third study 10 and 13 strains, respectively, were typed correctly by all participants. For strains belonging to a group which occurs less frequently, most problems occured at the detection of O antigens. An incorrect detection of H antigens mostly lead to incorrect identification of the serotype of the strain. Examples of partly correct/incomplete/incorrect detection are shown in Table 2.

In study II *S.* Paratyphi B. var. Java was included. The serological detection was mostly correct. Only a few laboratories carried out a biochemical test to make a differentiation in *S.* Paratyphi B. and *S.* Paratyphi B. var. Java. In this study only the results of the serological

typing were evaluated. For surveillance data it is also important to include the biochemical reaction.

Table 2 *Examples of partly correct/incomplete and incorrect detection*

	O antigen	H antigen
S. Muenchen	6,8	**D**:1,2:[z67]
Partly correct		
S. Newport	6,8	**Eh**:1,2
Incomplete		
S. I 6,8:-:1,2	6,8	-:1,2
S. Livingstone	6,7,14	D:**l,w**
Incorrect		
S. Gombe	6,7	**Enz15**

Phagetyping of the strains

In the third study phagetyping was included. Laboratories which had the possibility carried out phagetyping of *S.* Enteritidis and *S.* Typhimurium strains. The results are shown in Table 10a and 10b of report 284500 010[4].

Conclusions

1. better results were obtained using serotypes occurring frequently and with the possibility to confirm the serotyping in another reference laboratory;
2. most problems were found with detection of H antigens; and
3. it remains worthwhile to organize collaborative studies on serotyping as well as phage-typing.

[4]RIVM Report 284500 010, Test results of *Salmonella* serotyping in the Member States of the European Union (Collaborative study III amongst the National Reference Laboratories for *Salmonella*. N. Voogt, H.M.E. Maas, W.J. van Leeuwen and A.M. Henken. September 1999.

2.12 Typing methods

2.12.1 Typing methods in Germany

Mr. A. Schroeter (NRL-*Salmonella,* Germany)

In Germany the federal system entails dividing tasks up between the federal government on the one hand and the individual federal states on the other hand. This also applies to the

health system and therefore also for the field of salmonellosis. Two governmental institutes (Robert Koch-Institut [RKI]) for human isolates and the Federal Institute for Health Protection of Consumers and Veterinary Medicine [BgVV] for isolates from animals, food, feed and the environment get isolated *Salmonella* strains from human and veterinary institutions of each federal state, medical and veterinary centres, universities, zoos, private companies, etc. for confirming the results of serotyping and further differentiation of isolates.
In the National Reference Laboratory for *Salmonella* (NRL-SALM) of the BgVV serotyping, antimicrobial susceptibility testing against 17 antimicrobial agents and phage typing for *S.* Typhimurium and *S.* Enteritidis are routinely performed. Serotyping is done according to the Kauffmann/White scheme with sera produced in the laboratory or bought from companies. Antibiotic resistance determination is done with the agar diffusion assay according to DIN 58940 part 3. The following substances are used under standard conditions:
amikazin (30 mg), ampicillin (10 mg), cefuroxim (30 mg), chloramphenicol (30 mg), colistin (10 mg), enrofloxacin (5 mg), furazolidon (100 mg), gentamicin (10 mg), kanamycin (30 mg), nalidixic acid (30 mg), neomycin (10 mg), polymyxin (300 I.E.), streptomycin (25 mg), trimethoprim (2,5 mg), sulfonamid (300 mg), sulphamethoxazole/trimethoprim (25 mg), tetracycline (30 mg).
For the most common serovars isolated in Germany, namely *S.* Typhimurium and *S.* Enteritidis, phage typing is done according to, respectively, Anderson et al. 1977 (J. Hyg. Camb. 78, 297) for *Salmonella* Typhimurium and Ward et al. 1987 (Epidemiol. Inf. 99, 291-294) for *Salmonella* Enteritidis.
Further differentiation of *Salmonella* isolates can be performed by characterisation of specific components of bacterial cells such as outer membrane proteins, lipopolysaccharides, enzymes in the cytoplasm, the presence of plasmids, the chromosome etc.. Polyacrylamidgelelectrophoresis is used for separation of outer membrane proteins or lipopolysaccharides. It could be shown that different pattern of outer membrane proteins or lipopolysaccharides between different *Salmonella* serovars or within a *Salmonella* serovar could be used for further differentiation.
Plasmidprofiling according to Kado and Liu 1981 (J. Bacteriol. 145, 1365-1373) and plasmid fingerprinting with different restriction enzymes are often used as additional discriminating factors in the characterisation of *Salmonella* serovars.
Multilocus enzyme electrophoresis (MLEE) is widely used for analysing genetic diversity and relationship among *Salmonella* serovars and the development of clonale lines within different *Salmonella* serovars.
Chromosomal DNA can be characterised by a set of different molecular typing methods. In the NRL-SALM fingerprinting of total genomic DNA (RFLP), ribotyping with 16s and 23s rRNA genes, IS200 typing, pulse field gel electrophoresis (PFGE) and random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) with different primer are often used for differentiation of *Salmonella* serovars or clonal lines such as PT4 in *S.* Enteritidis or DT104 in *S.* Typhimurium.

2.12.2 Recent work on molecular typing methods

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

Molecular epidemiology is becoming an increasingly important tool in researching the dissemination routes for *Salmonella* and other organisms in animal feed, livestock, human foodstuffs and the human population. Prior to the epidemic spread of *S.* Enteritidis PT4 and *S.* Typhimurium DT104 relatively simple strain differentiation techniques such as phage typing and plasmid profile analysis have provided adequate discrimination in most cases. The epidemic strain appear to be highly clonal however and a high proportion of strains fall into one majority group. An attempt was made to investigate improved differentiation of *S.* Typhimurium DT 104 by a variety of methods. Unfortunately resources for the work were limited so an insufficient number of strains were examined by most laborious techniques.

Biotyping

One hundred and twenty seven confirmed isolates of *S.* Typhimurium 104 were examined using 18 substrates. Five biovars were identified, 1dg (1), 2a (120), 2f (3), 10a (1) and 18f (1). These were discernible using three substrates – Bitters xylose, inositol and trehalose. The level of discrimination by biotyping alone is considered too low to be of practical value.

Plasmid profile analysis

One hundred and twenty-five confirmed isolates of *S.* Typhimurium 104 were examined for their plasmid profile (PP). Sixteen profiles were identified, 0 (1), 1 (1), 2 (96), 3 (1), 4 (1), 6 (1), 7 (8), 8 (1), 9 (4), 10 (1), 11 (1), 13 (4), 14 (1), 15 (2), 16 (1), 17 (1). Four profiles accounted for 90% of strains, the most common, type 2, accounted for 77%. All isolates from feed had the same plasmid profile and biotype.

Antimicrobial sensitivity pattern

A total of 121 isolates were examined. There were broadly three groups; sensitive to all antimicrobials tested (20), those resistant to Tetracycline, Ampicillin, Chloramphenicol, Streptomycin and Sulphonamides (70) and those resistant to the foregoing and in addition Trimethoprim (22).

Pulsed Field Gel Electrophoresis

Twenty isolates of *S.* Typhimurium 104 were examined by PFGE using two enzymes XbaI and SpeI. The panel of isolates consisted of four different plasmid profile types and two biotypes. All strains gave identical banding patterns in PFGE. This technique was less discriminatory than plasmid profile, sensitivity testing and biotyping.

IS200 typing

Ten strains of *S.* Typhimurium 104 representing three types using a combination of plasmid and biotyping were examined for IS200 RFLP. No differences were detected.

16S RNA typing

Technical difficulties prevented the application of ribotyping.

Random Amplified Polymeric DNA (RapD)

This technique was applied to 40 strains of *S.* Typhimurium 104 using two different primers. In each case no differences were detected.

Initial trial of proposed scheme

A combination of plasmid and biotyping was used to examine a total of 17 isolates from three poultry companies;

Owner 1 Three isolates were examined. All had similar profile (PP7) and were not subdivided by biotyping or sensitivity tests (all TPSxCSS).

Owner 2 Three isolates were examined. All had similar profile (PP17) and were not subdivided by biotyping or sensitivity tests (all TPScCSS).

Owner 3 Eleven isolates were examined and divided into four groups by plasmid profile PP2 (5), PP15 (3), PP7 (2) and PP17 (1). They were not subdivided by biotyping. They could be split into two groups by sensitivity pattern (SCT R or S). These isolates originated from five sites within this poultry company.

The same combinations of biotyping and plasmid profile analysis were applied to 37 consecutive *S.* Typhimurium 104 isolates received at CVL for typing.
These isolates were divided into five plasmid profiles; PP2 (26), PP7 (1), PP10 (1), PP13 (5) and PP18 (4). They were not subdivided by biotyping and two groups were discernible by sensitivity pattern (SXT R or S).
A further trial involving plasmid analysis of 153 *S.* Typhimurium 104 strains from 10 pig farms demonstrated 11 profile types of which 80% were the common epidemic type. All the isolated from wildlife, pets and other livestock species on these farms were also of the common type, suggesting that this may be more virulent.

Significance of the trial

These findings suggest that the strains of *S.* Typhimurium DT104 currently circulating are best subdivided by plasmid profile analysis. On the basis of 37 consecutive submissions 70% belonged to type 2, 14% to type 13, 11% to type 8 and 2% each for types 7 and 10. In certain circumstances this could be enough to link sources in an epidemiological investigation. There is some evidence to suggest that the increasing diversity in plasmid profiles could be related to an increase in frequency of Trimethoprim resistance. Several plasmid profiles appear to be linked to Trimethoprim resistance.

It is interesting to note that all feed isolates between 1991 and 1995 when examined had the common plasmid profile (type 2). This may be related to the greater involvement of wildlife with this strain.

2.12.3 Danish typing activities and outbreak investigation

An outbreak of quinolone-resistant, multiresistant *Salmonella* Typhimurium DT104 in Denmark

Mrs. D.L. Baggesen (NRL-*Salmonella,* Denmark)

Infections with the zoonotic *Salmonella* type *S.* Typhimurium DT104 has been recognized since the beginning of the 1990's as a health problem in several industrialized countries. *S.* Typhimurium DT104 has a broad host spectrum and can readily spread to a large proportion of domestic animals as well as to the wild fauna. Because of its extensive reservoir, *S.* Typhimurium DT104 is difficult to control in animal husbandry. It is often resistant to ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline. In addition, the organism readily acquires resistance to other antibacterial agents, including quinolones. As a fluoroquinolone is the drug of first choice for treating extra-intestinal and serious intestinal complications of human salmonellosis, this has the potential of causing therapeutic problems. In England multiresistant *S.* Typhimurium DT104 is the second most common *Salmonella* sero- and phage type after *S.* Enteritidis FT4.

In Denmark the proportion of *S.* Typhimurium DT104 infections has hitherto made up less than 1% of total human *Salmonella* infections, and apart from a small hospital outbreak in 1996, only sporadic cases have been recorded. From 1995 to 1997 there has been no increase in human cases, and no examples showing incipient resistance to quinolones have been isolated[5].

The outbreak

In the summer of 1998 the first community outbreak was registered, the source being pork meat of Danish origin. Fig. 1 shows that most cases occurred in week 23. This coincided with the demonstration of the same type of *Salmonella* in meat at a slaughterhouse on 25 May 1998. The cases from week 27 onwards are presumably due to the use of frozen meat. The organism involved in this outbreak showed the classical resistance pattern, but was also resistant to a quinolone, nalidixic acid. The present outbreak constitutes an example to show that problems with the treatment of human infections can be related to the occurrence of quinolone-resistant bacteria in live animals and food products.

Investigation of the outbreak

The outbreak was recognized at Statens Serum Institut (SSI) on 18 June 1998. Isolates from five patients with *S.* Typhimurium DT104 infection showed the unusual resistance pattern (multiresistance including nalidixic acid resistance) which exactly corresponded to that found in isolates from a slaughterhouse in Zealand, as well as in isolates collected by Food Inspection Agencies in Copenhagen and Roskilde. This resistance profile had not been detected in Danish food animals or food previously and only rarely in humans. Subsequent investigations confirmed that all isolates were of phage type 104 and had the same DNA-

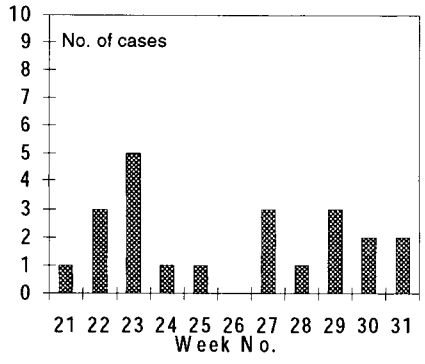
fingerprinting pattern. By DNA sequencing of the gyrase gene a single mutation responsible for the resistance to nalidixic acid was recognized. Compared to the sequence of other nalidixic acid resistant strains the mutation was uncommon and the presence gave therefore a valid indication of epidemiological relationship between strains from patients, food and swine herds.

A connection between the finding of nalidixic acid resistant *S.* Typhimurium DT104 both in fresh pork and in these patients was further supported by patient interviews, which revealed that patients had bought and eaten pork from shops that had received deliveries from the slaughterhouse in question. Furthermore, the swineherd that had provided animals for the slaughterhouse was identified, and *Salmonella* isolates from this herd were identical to isolates from the slaughterhouse, the pork, and the patients. This strain was also found in another swine herd situated near the index stock and connected through shared equipment.

Fig. 1 shows the time of onset of the 22 registered cases of *S.* Typhimurium DT104 infection. All these patients probably form part of the same outbreak, as the resistance profile of the 22 isolates was very unusual. This conjecture has been supported by molecular epidemiological investigations carried out in collaboration with the Danish Veterinary Laboratory. Eighteen of the 22 patients were interviewed, and 9 provided information that directly revealed that they had eaten pork originating from the slaughterhouse concerned. One of the patients was a slaughterhouse employee who was presumably infected at work, and another was a hospital employee who had had contact with one of the other patients. It should be stressed that none of the patients had been abroad. Seven patients were admitted to hospital and six were treated with antibiotics, including fluoroquinolones. Several of the cases have had severe intestinal disease and fluoroquinolone treatment has been reported to lack clinical effect in at least four cases.
A previously healthy 62-year-old woman died from the complications of intestinal perforation. The patient had been treated with fluoroquinolone for five days before operation and was treated per- and postoperatively with ceftriaxone and gentamicin. In addition, *B. fragilis*, which was resistant to the antibiotics given, was isolated from blood culture.

[5] Anonymous. Annual report on zoonoses in Denmark 1997. Copenhagen: Danish Zoonosis Centre, 1998.

Fig. 1. Human cases of multiresistant *Salmonella* Typhimurium DT 104 infection, 11.05.1998-15.08.1998



2.12.4 *Salmonella* serovars in Portugal

Mrs. Amado (NRL-*Salmonella,* Portugal)

Introduction

Laboratório Nacional de Investigação Veterinária (LNIV) is a State Laboratory subordinated directly to the Ministry of Agriculture, Rural Development and Fisheries. It is composed by a main structure in Lisbon and a branch in Oporto (in the northern region of the country).

LNIV is a very important entity in Portugal, with a wide field of activity in Animal Health (as a Diagnostic Laboratory), in Public Health (concerning products of animal origin for human consumption) and Research (concerning the control of animal diseases and improvement of animal health situation).

For all mentioned reasons, LNIV is The National Reference Laboratory for most animal diseases, a.o. salmonellosis.

The Bacteriology Department has been typing *Salmonella* isolates since 1913. Nowadays, not only LNIV isolates are serotyped, but also the isolates from the private and regional laboratories all over the country.

Material and methods

Serotyping is performed following the Kauffmann-White Scheme, with commercially available antisera, namely from Difco, Murex and Sanofi Pasteur.

The searching of Somatic O antigens is made by slide agglutination and the searching of Flagellar H antigens is done by tube agglutination technique.

Concerning the antibiogram techniques, the BioMérieux ATBVET strips for reading with BioMérieuxATB Expression reader were used for the following antimicrobials Gentamycin, Apramioline, Chloramphenicol, Tetracycline, Doxicycline, Kanamycin, Streptomycin , Colistin, Enrofloxacin, Flumequin, Oxolinic Acid, and Nitrofurantoin and the disk diffusion method was used for the remaining antimicrobials tested Nalidixic Acid ,Ampicillin, Sulphamethoxazole+Trimethoprim, Sulphafurazole, Neomycin and Furazolidone.

Results

The results are shown in Appendix 7

Conclusions

- the most common serovar found over the past 3 years, was *S.* Enteritidis (44.6% of the isolates belonged to this serovar), followed by *S.* Typhimurium (16.3%) and *S.* Anatum (6.7%);
- *S.* Anatum has been persistently isolated since 1995, in poultry, feed and food of poultry origin;
- new serovars as *S.* Hadar/Istanbul, *S.* Blockley/Haardt and *S.* Infantis turned up on 1996 for the first time, and persisted on 1997. On the other hand, serovars like *S.* Kentucky and *S.* Lindenburg were no longer found;

- S. Mbandaka emerged on 1997 also for the first time, twice in poultry samples and more often on feed; and
- the antimicrobial performances for the main serovars are presented in the last table of Appendix 7. All strains belonging to *S.* Hadar/Istanbul serovar were resistant to Enrofloxacin, while the remaining isolates showed high sensitivity to ENR.

2.13 Report on phagetyping meeting in Berlin

Mr. A. Schroeter (NRL-*Salmonella,* Germany)

Phage-typing is a useful method for further differentiation of frequently isolated *Salmonella* serovars. Advantages of phage-typing are:

1. typing of a large number of strains per day is possible;
2. the cost for of equipment is relative low in comparison with other methods;
3. two different components of a bacterial cell (cell wall, chromosome) are characterised by one method; and
4. phage-typing is one possibility to get more complex epidemiological data in order to develop strategies to solve global problems in respect to salmonellosis in the world.

Among the EU member states phage-typing is used only by some NRL's on a routine basis. Furthermore, different phage-typing systems were developed and are used in different countries. Therefore it was the aim of the workshop to bring together members of those NRL's presently using phage-typing in order to discuss the scientific and practical aspects of their experience.

The programme of the workshop focused on two parts:

1. epidemiological data of phage-typing in the countries of participants; and
2. practical aspects of phage-typing.

In Austria, Denmark, England and Wales, Germany and the Netherlands *Salmonella* Enteritidis (SE) and *Salmonella* Typhimurium (ST) could be frequently isolated from human and nonhuman sources. The dominating phage types are PT4, PT8, PT6 for SE and DT104, DT12, DT193, DT120 for ST. All data presented by the participants are published in the Newsletter of the CRL-*Salmonella* Vol. 2, No. 4 in 1998.
The phage-typing systems developed by the PHLS in London UK for *S.* Typhimurium (Anderson et al. 1977, J. Hyg. Camb. 78, 297) and *S.* Enteritidis (Ward et al. 1987, Epidemiol. Inf. 99, 291-294) are routinely used in different countries and are widely accepted. Other EU member states should be encouraged to use both systems in order to get comparable result. This would allow to draw better fundamental epidemiological conclusions on salmonellosis in the EU. Using both systems according to the recommendations of the PHLS there is a need for exchange of information (to the methodology, reading of phage patterns, etc.) and

harmonisation. The workshop in Berlin gave first results in this respect especially to the expanded systems and basic problems of phage typing. Furthermore, it was concluded that ring trials are necessary and should be organised by the CRL-*Salmonella* in collaboration with the PHLS in London.

2.14 Discussion on future collaborative studies on serotyping

The results of the second and third collaborative study were discussed. In the last study also 4 *S.* Typhimurium (STM) and *S.* Enteritidis (SE) strains were included for phage typing. It was concluded that these ring trials are necessary and very helpful to the quality testing of the method. In case of discrepancy between results of a NRL and the CRL it was proposed to send back the strains to the CRL for further analysis. The next study will include 20 strains for serotyping with more variation in serotypes and at the same time 10 STM and 10 SE for phage typing.
Harmonisation and standardisation of phage typing was also discussed during a workshop organised by the NRL-*Salmonella*, Germany, in Berlin in june 1998. A need for ring trials on phage typing was clear and it was proposed to organize these studies together with the Enter-Net. This will be discussed during the workshop of Enter-Net in November. The CRL-*Salmonella* will contact Linda Ward from the PHLS in London about cooperation.
Different other typing methods such as molecular methods and antibiotic resistance determination were presented by Davies and Schroeter. Many different methods are in use. May be in future there will be also a need for comparison and inter-laboratory standardisation of such additional methods.

2.14.1 Report on five important meetings on antibiotic resistance

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

In the last two years the discussion on the agricultural use of antimicrobial agents especially in animal husbandry has received more scientific, public and political attention. The reason for this are the increasing problems with resistant micro-organisms in human and veterinary medicine. In *Salmonella* the spread of multiresistant *Salmonella* Typhimurium DT104 is a good example.
Consequently international and national organisations have intensified their search for a scientifically based definition of the problem's magnitude and the development of corrective and preventive measures.
In order to reach this goal, the World Health Organisation WHO initiated two major meetings on the subject. The first one was called „The Medical Impact of The Use of Antimicrobials in Food Animals". The meeting was held in Berlin in October 13-17 1998 and it concentrated on all aspects of the agricultural use of antimicrobial agents.

The second meeting however focused on just one group of antimicrobial agents, the fluorquinolones. It was entitled „On the Use of Quinolones in Food Animals and Potential Impact on Human Health", Geneva, June 2-5 1998. Both meetings stressed the view, that „Bacteria and genes, including resistance genes, can pass between humans, animals and other ecosystems". However „ the magnitude of the medical and public health impact of antimicrobial use in food animals is not known".
These views were reinforced by a third conference called „The Microbial Threat" held in Copenhagen, September 6-10 1998. It was initiated and attended by the chief medical officers of the EU. At the end of this meeting the Copenhagen recommendations were laid down. Among other conclusions they call for the prudent use of antimicrobial agents. During the conference a theme issue of the British Medical Journal on antibiotic resistance was released.
Details of the conferences and their conclusions and recommendations can be read in the references below.

References

The Medical Impact of the Use of Antimicrobials in Food Animals, Report of a WHO Meeting, Berlin, Germany, 13-17 October 1997; WHO/EMC/ZOO/97.4.
Use of Quinolones in Food Animals and Potential Impact on Human Health, Report of a WHO Meeting, Geneva, Switzerland, 2-5 June 1998; WHO/EMC/ZDI/98.10.
Antimicrobial Resistance: Theme Issue BMJ 1998; 317 609-690.

2.15 Evaluation, plans and closing remarks

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

Evaluation

It was discussed whether or not the aims of the workshop were reached. It was agreed upon that that was the case. Questions with respect to needs for specific training can be addressed directly to the CRL-*Salmonella* or the other NRLs-*Salmonella*. The CRL-*Salmonella* is in the position to organise such individual and group training or can function as an intermediate to find a suitable training place in case of specific questions.
In general the NRLs-*Salmonella* express that there is no need for the CRL-*Salmonella* to change her way of working. However, the NRLs-*Salmonella*, and also other institutes in various member states, increasingly ask for deliverance of reference material or additional collaborative studies. The collaborative studies of the CRL-*Salmonella* are used for quality assurance purposes in some countries. Such questions increase the workload of the CRL-*Salmonella* even further beyond the financial threshold. Questions from institutes within a member state should be dealt with in principle by the NRL-*Salmonella* of that specific member state. An NRL-*Salmonella* has within her country a similar responsibility as the CRL-*Salmonella* has at the European level.

Plans

The plans of the CRL-*Salmonella* for the near future, i.e. 1999, are:

- to organise a bacteriological collaborative study in order to test semi solid media to improve on the standard method;
- to organise collaborative typing studies, that is a serotyping study and a phagetyping study for those who do phagetyping. The CRL-*Salmonella* will discuss and communicate with the NRLs-*Salmonella* whether there is sufficient support and need for molecular typing studies and/or typing studies with respect to antibiotic resistance;
- it will be investigated what the pro's and contra's are for a new immunological collaborative study. A few member states are interested in this;
- next year a fourth workshop will be organised; and
- the other regular activities will be, the newsletter, manual updates, etc.

The CRL-*Salmonella* will discuss with the Commission the new document on zoonosis when it is available, the use of analytique techniques mentioned for that purpose and the long term view of the Commission on the taks of the CRL-*Salmonella* and NRLs-*Salmonella*.

Also, the CRL-*Salmonella* will try to increase the communication among the various European players on the *Salmonella* front, like CRL-Epidemiology, Enternet and the CRL-*Salmonella*.

Closing remarks

Thank you:

- participants, for contributing one way or the other to make this workshop a success;
- Commission, for making this workshop possible via financial support;
- the organising team, for making such a nice programme and time-schedule; and
- the CRL-*Salmonella* team, for all the work done that could be presented here.

From January the first, 1999, dr.ir. P.H. in't Veld from the RIVM will be the new head of the CRL-*Salmonella*. Dr.ir. A.M. Henken will step back as head of CRL-*Salmonella* at that date and will remain as head of the Microbiological Laboratory for Health protection that encompasses the CRL-*Salmonella*.

3. Appendices

3.1 Appendix 1: Mailing list

01	European Commission	A. Checchi Lang
02	European Commission	B. Hogben
03	European Commission	V. Niemi
04	Veterinary Public Health Inspector	drs. H. Verburg
05-40	Participants of the workshop	
41	Board of Directors RIVM	dr. G. Elzinga
42	Director Sector Publich Health Research	prof. dr. ir. D. Kromhout
43	Head of Microbiological Laboratory for Health Protection and Director CRL-*Salmonella*	dr. ir. A.M. Henken
44	Editors	
45	Dutch National Library for Publications and Bibliography SBD/Information and Public Relations	
46	Registration agency for Scientific Reports	
47	Library RIVM	
48-57	Sales department ot RIVM Reports	
58-67	Spare copies	

3.2 Appendix 2: Participants of the workshop

European Commission, Legislation Veterinaire et Zootechnique	Mr. V. Niemi (from October 1st 1998 onwards)

from the National Reference Laboratories for Salmonella:

Austria	Mrs. I. Neckstaller Mr. W. Thiel
Belgium	Mrs. M.L. Chasseur Mrs. I. d'Hooghe
Denmark	Mrs. D.L. Baggesen Mr. M. Madsen
Finland	Mrs. S. Kivela Mrs. E. Seuna
France	Mrs. F.S. Humbert Mrs. K. Proux
Germany	Mr. R. Helmuth Mr. A. Schroeter
Greece	Mrs. M. Passiotou-Gavala
Ireland	Mrs. A. Murphy Mr. J. Ward
Italy	Mrs. A. Ricci
Luxembourg	Mr. J. Schon
The Netherlands	Mr. A. van de Giessen Ms. N. Voogt
Portugal	Mrs. A. Amado Mrs. M. Do Rosario Vieira

Spain	Mr. F.J. Garciapena
	Mrs. C. Rubiomontejano
Sweden	Mrs. K.E. Bergstrom
	Mr. A. Gunarsson
United Kingdom	Mr. R. Davies
	Mr. S. McDowell
from CRL-Epidemiology Berlin	Mrs. A. Kaesbohrer
from CRL-*Salmonella*	Mr. A. Henken
	Ms. N. van Leeuwen
	Mrs. H. Maas
	Mr. M. Raes
	Ms. S. Schulten
	Mr. P. in 't Veld
Invited speakers:	Mr. R. Betts
	Mrs. R. Westendorp
	Mr. F. van Zijderveld

3.3 Appendix 3: Programme of the workshop

Sunday 27 September 1998

20.00 - 21.00 Social get together in the bar of hotel Mitland

Monday 28 September 1998

08.30 - 09.00 Opening and introduction of participants

09.00 - 09.30 Current situation with regard to the zoonosis directive

09.30 - 10.00 Evaluation of the second bacteriological collaborative study
Detection of *Salmonella* in the presence of competitive micro-organisms

10.00 - 10.45 Evaluation of the third bacteriological collaborative study
Detection of SE and ST

10.45 - 11.15 Coffee/tea

11.15 - 12.30 National activities with respect to *Salmonella* control and collaborative studies
performed by NRLs within Member States
- *Salmonella* and the Dutch poultry sector (25 min)
- collaborative studies in the Netherlands (25 min)
- French legislation and collaborative studies in France (25 min)

12.30 - 13.00 Ideas for future bacteriological collaborative studies of CRL-Salmonella

13.00 - 14.00 Lunch

14.00 - 14.35 Review on alternative methods for *Salmonella* detection

14.35 - 15.05 Presentation about validation of microbiological methods
- introduction to Microval project

15.05 - 15.35 Coffee/tea (photo)

15.35 - 16. 20 Presentation about validation of microbiological methods
- validation of a detection kit for L. monocytogenes (as an example)

16.20 - 16.50 Discussion about requirements of validation of *Salmonella* methods

16.50 - 17.30 Standardization and ring trial for *Salmonella* detection by PCR

19.30 - 21.00 Dinner

21.00 - ? Free (bowling (22.00 - 23.00)/visit Utrecht/ SALMOVA meeting)

Tuesday 29 September 1998

08.30 - 09.15 Theory on immunological detection

09.15 - 10.00 A comparison between immunological methods

10.00 - 10.30 Coffee/tea

10.30 - 11.10 Presentations by Member States of their national activities on immunological detection, be it collaborative studies or research into method development
- Field experiences with an LPS-based ELISA as a tool in the Danish Control programme for *Salmonella* in the poultry sector (25 min)
- Development of monoclonal antibodies against *Salmonella* and their practical application (15 min)

11.10 - 11.30 Ideas for immunological collaborative studies of CRL-Salmonella

11.30 - 12.15 Evaluation of the second and third collaborative study on serotyping

12.15 - 13.15 Lunch

13.15 - 14.30 Typing methods
- Typing methods in Germany (15 min)
- Recent work on molecular typing methods (15 min)
- Danish typing activities and outbreak investigation (30 min)
- *Salmonella* serovars in Portugal (15 min)

14.30 - 14.45 Report on the phagetyping meeting in Berlin and suggestions for ring trials (10 min)

14.45 - 15.00 Discussion on future collaborative serotyping studies

15.00 - 15.30 Coffee/tea

15.30 - 16.00 Report on five important meetings on antibiotic resistance

16.00 - 16.30 Closing remarks

17.00 Departure to railway station and airport

3.4 Appendix 4: Review on alternative methods for *Salmonella* detection

Phases of the test

- Recovery/repair of injured targets
- Increase in numbers of target cells to achieve numbers that can be detected with the detection system to be used
- Application of a suitable detection system that is specific enough for our requirements

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Rapid Methods

- The rapid detection of Salmonella has been the goal of methods developers for many years
- This has resulted in the development of many different methods and techniques
- Before looking at the different methods, we should understand what we are trying to achieve
- Low levels of injured target organisms
- Different food matrices
- Large numbers of similar organisms that we do not want to detect

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Rapid Methods for the Detection of *Salmonella*

- The Challenge - to detect 1 *Salmonella* within 25 g of sample
- The organism can be injured/damaged
- In the presence of great numbers of competing organisms
- The sample matrix can have antimicrobial / growth limiting factors
- We may wish to consider a larger sample size

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Current Methods

- Can detect Salmonella with all of the constraints of the food
- Can do this simply, with no complex equipment or reagents
- Test time 3 to 7 days
- Microbiologists want the simplicity (and economy) of current methods, but faster.

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Rapid Methods

Alternative enrichment systems
Alternative detection systems
Confirmation

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Concentration

- Immunomagnetic separation (IMS)
  Magnetic beads coated with suitable antibodies (Dynal, Foss)
- Immuno-extraction
  Solid phase coated with antibody (Tecra Unique, bioMerieux Vidas)
- Various research techniques
  Ultrasound
  Biphasic separation
  etc

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Enrichments

- Currently--pre and selectively enrich
  Time 48h to 72h
  Dispose of most of the cells we culture
- Concentration
- Dual enrichments
- Enrich & detect together

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Enrichment

- Dual enrichment- ie do pre & selective enrichment together
- Salmocyst (Merck)- a tablet of selective agent added to a preenrichment after a predetermined time period
- Sprint (Oxoid)- a timed release capsule added to a preenrichment that releases selective agents after a predetermined time (6h)
- These methods are faster because they combine or semi combine the 2 enrichment periods

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Enrich and Detect Together

- By combining a detection system into the enrichment we can reduce manual operations and perhaps detect sooner–detection occurs immediately the detection threshold of the test is passed
- Impedance / conductance systems
- Preenrich
- Place sample of the completed preenrichment into a selective broth{s}, place in conductance instrument (Bactometer, Malthus, Rabit, Bactrak) & monitor

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Immunoassay

- Microplate ELISA (Organon Teknika, Tecra, Bioline, RDT, Transia)
  - Well know & widely used--test time 50h
- Dipstick ELISA (Tecra)
  - UNIQUE-dipstick ELISA-a concentration / detection system (24h)
- Automated ELISA
  - bioMerieux VIDAS-automates all washing steps (50h)
  - EiaFoss system-automates washing-uses IMS (24h)
- Immunochromatography
  - Celsis-Lumac, etc (50h)
  - No equipment required, no washing required--very

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Detection Systems

- Most rapid methods for Salmonella are really only rapid detection systems-ie they only replace the agar plate
- Immunoassay
  - Microplate ELISA
  - Dipstick ELISA
  - Automated ELISA
  - Immunochromatography
- Nucleic acid hybridisation probes
- Polymerase Chain Reaction (PCR)

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Nucleic acid hybridisation probes

- Gene-Trak
- ^{32}P labelled probes-little used (Chromosomal DNA)
- Colorimetric probes (rRNA)
- 50h test time

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Polymerase Chain Reaction (PCR)

- Biochemical amplification using DNA polymerase
- Very sensitive (10^9 fold amplification in 3h)
- Too sensitive--detection of dead cells ?
- Interference by food constituents
  - Qualicon BAX, electrophoresis-gel detection
  - Sanofi Probelia, microplate format, colorimetic detection
  - PE Taqman, fluorescent detection

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The Future

Wide range of test types, large number of manufacturers

Work well in most cases

Rapid ?----- what do we mean

Cost--a big factor

Valid

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Confirmation

- Few people currently use the "standard" method
- Wide use of biochemical kits (API, Micro ID etc)
- Rapid systems
  - Immuno methods
  - Nucleic acid probes
  - PCR-detect & confirm

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The Future

- PCR-further development-better detection systems
- Viable cell PCR (RT-PCR of mRNA)
- Other amplification systems (NASBA from Organon Teknika)
- Biosensors ?
- Lab on a chip technology ?

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3.5 Appendix 5: Introduction to Microval

MicroVal secretariat
NEDERLANDS NORMALISATIE-INSTITUUT
P.O. Box 5059
NL-2600 GB Delft, The Netherlands
tp: +31 15 2 690 112
tf: +31 15 2 690 190
e.mail: microval@nni.nl

MICROVAL
A EUROPEAN APPROACH TO THE CERTIFICATION OF NEW MICROBIOLOGICAL METHODS

SUMMARY

A description is given of MicroVal, a European project for the validation and approval of alternative methods for the microbiological analysis of food and beverages. The general rules and certification procedures are described as well as how the certification is organised. MicroVal aims at certifying alternative methods, i.e. methods which perform as well as internationally standardised methods. When ever possible, an ISO standard method is used as the reference.

Certification of alternative methods should ensure its acceptance by governmental inspection laboratories and laboratories in the food trade, thus facilitating the commerce within the European Union.

INTRODUCTION

Numerous and diverse alternative methods for microbiological research are currently being offered to the market as a result of recent developments, in particularly in the field of biotechnology, automation and micro-electronics. These alternative, often more rapid and or convenient, methods are of great interest to the food industry since they can provide better means to monitor raw materials, processes and products (according to hazard analysis and critical control points [HACCP] principles). Microbiological tests are also very important in the governmental food inspection, in international trade, in commercial relationships between trade partners and in product-liability matters. The results of these tests should be reliable and it is therefore very important that all parties involved agree with and accept the methodology employed.

Before a new microbiological method can be widely accepted not only must its intrinsic technical quality be established objectively, but, as mentioned above, there must also be a guarantee that interested parties will accept the results obtained using such a method. It should be pointed out in this respect that in the commercial interest of the suppliers and users of novel test methods, such a situation should be realised within an acceptable period,

preferably within a year - as opposed to, for example, the approval programme of the AOAC (formerly the Association of Official Analytical Chemists) which takes about three years. At present there is no European mechanism to achieve general acceptance. This is not in the interest of the development of a European food market and does not stimulate the development of a European test kit industry.

"MicroVal" is a "Eureka" project (the Eureka programme was set upon 1985 to stimulate cross-border technological cooperation and advancement throughout Europe). It started in June 1993 with the aim of setting up a European validation procedure in four years' time and furthermore of creating such conditions that the results of the procedure will be accepted as far as possible by all interested parties in Europe. Standardisation and certification play important roles in this respect. MicroVal started off as a Dutch-French collaboration at a Dutch initiative (Unilever Research Laboratory). The project has ended with 21 full partners from the United Kingdom, Denmark, the Netherlands, France, Germany, Spain and Portugal. (Annex 1).

ORGANISATION OF THE PROJECT

Management of the project

The project was managed by a project manager (Dr. Mike van Schothorst, Nesté Switzerland) who was supported by the Steering Committee MicroVal, (SCOM) of which committee representatives of the various project partners were the members. The project administration was carried out by the Nederlands Normalisatie-instituut (Dutch Standards Institute).

The Steering Committee received support from a "Scientific Advisory Board" consisting of

- Dr. R. Lodi of the Centro Studi Latte di Milano, Italy,
- Dr. P. Teufel of the Bundesinstitut fhr gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin, Germany
- Dr. N. Skovgaard, Royal Veterinary College, Denmark

Working Groups

Four working groups had been set up by the Steering Committee. The task of the fourth working group comprised the financial aspects of the project. The tasks of the other working groups are described in the next paragraphs.

Working Group 1

It was the task of the first working group to draw up a survey of all existing documents and procedures, to compare them and to make a recommendation concerning the procedure that was the most suitable to serve as a starting point for the "MicroVal project". To this end, the working group had studied information on the validation procedures used by or proposed by l'Association Française de Normalisation (AFNOR), AOAC International, the AOAC

Research Institute, the European Community (EC), the International Dairy Federation (IDF) and the International Union of Pure and Applied Chemistry (IUPAC). The final report of the first working group was presented at the first Annual Meeting in Paris, June 1994.

Working Group 2

The assignment of the second working group was to draw up the **general rules** for the organisation of the validation procedure and the MicroVal Certification Scheme.

Working Group 3

The third working group had started in parallel with the second, and was focusing on the drawing up of the **technical rules** for the validation, the test protocol and the organization of the trial validations in the second stage of the project.

The following alternative methods have been used to perform trial validations:

- Salmonella-Tek (Organon Teknika)
- Listeria immunoenzymatic detection (Transia Diffchamb)
- Genetrak, hybridisation Assay for Listeria monocytogenes (R-Biopharm)
- 3 M- Petrifilm high sensitivity Coliforms (3 M)
- Bactrac 4000 Series (SY-LAB)

As a result of the experience from these studies, the technical rules have been implemented and precised.

THE PRINCIPLES OF THE MICROVAL VALIDATION

The **General rules** describe the methodology and the organization to be used for the European certification of alternative microbiological methods for the food and drink industry by an independent organization: MicroVal Certification.

The term "alternative" is used to refer to the entire "test procedure and reaction system". This term includes all ingredients whether material or otherwise, required for implementing the method.

The MicroVal certification procedure is based on three principles.

The **first** principle of the certification is to perform a method comparison study of the alternative method against the reference method, followed by a interlaboratory method performance study of the both the alternative and the reference method .

The **second** principle is that the quality organization of the manufacturer where the materials are produced must be in conformity with quality assurance requirements. The frame of these requirements is the EN 29002 (1) standard (ISO 9002).

The **third** principle is a regular control of the quality of the certified methods is made after the certification is granted.

THE EUROPEAN CERTIFICATION ORGANIZATION

The European certification organization is constituted of a Group of MicroVal Certification Bodies (secretariat held by NNI) and headed by the MicroVal General Committee (secretariat held by NNI) which has a European composition and is in charge of:

- the elaboration/modification of the rules and the Certification Scheme
- the elaboration of the list of reference methods, reference materials, culture collections, expert laboratories, methods and laboratory reviewers, technical auditors
- the creation of specific Expert Committees for each technical need (qualification of reviewers, reference methods ....)
- the admission of new MicroVal Certification Bodies and if necessary exclusion,
- the examination of the reports of the audits of the MicroVal Certification Bodies,
- the supervision of all of the exceptions to the Rules and the MicroVal Certficiation Scheme,
- the supervision of the efficiency of MicroVal Certification Bodies.
- the appeal by the applicant if the appeal to the MicroVal Certification Body has failed.

For the time being, the SCOM will function as a preliminary MicroVal General Committee.

The MicroVal Certification Bodies are responsible for handling the applications and evaluating the reports of the expert laboratories and the auditors. The certification bodies shall comply with requirements in EN 45011 (2) and shall be accredited. Up to day, 3 certification bodies have declared their willingness to become MicroVal Certification Bodies : AFNOR, DIN and TNO.

The procedure is described in Annex 2.
The manufacturer presents his request for the certification of a alternative method to a MicroVal Certification Body. Each MicroVal Certification Body, must establish a MicroVal Committee. In this committee all interested parties shall be represented. The certification body checks the administrative conformity of the request and selects one method reviewer, one laboratory reviewer (if necessary) and one quality auditor. Three additional reviewers (one method and two laboratory reviewer) and one technical auditor are selected by the MicroVal General Committee.

The independent expert laboratory is in charge of the coordination and the supervision of the two phases of the validation procedure. The expert laboratory must comply with the requirements of EN 45001 (3) and be accredited .
The expert laboratory is preselected by the manufacturer or the MicroVal Certification Body from the database of laboratories to be established by the MicroVal General Committee. The

qualification "expert laboratory" is only valid for the MicroVal certification of the alternative method it was selected for.

The collaborative laboratories are selected by the expert laboratories. There must be a minimum of 8 (giving valid results) from 3 different countries. These laboratories must comply with EN 45001 but do not need to be accredited.

The MicroVal Committee advises the MicroVal Certification Body on whether or not certification should he granted. This is done on basis of the report of the 2 method reviewers. The final decision is taken by the MicroVal Certification Body after endorsement of the decision by the Group of MicroVal Certification Bodies. The secretariat of the MicroVal General Committee is informed of this decision.

The Secretariat of the MicroVal General Committee keeps a record of all MicroVal applications and all (positive and negative) decisions taken.

The manufacturer can give information about the MicroVal certification on the package through a logo. This logo must however always be accompanied by written information included in the packages. The information must include at least the date of certification, the reference method used, field of application (including the food groups tested), restrictions etc.. The logo will be under the responsibility of NNI.

THE MICROVAL TECHNICAL RULES

The technical rules have a scope which covers both qualitative and quantitative alternative methods.

Qualitative alternative methods

As noted previously, validation will proceed via a method comparison study and an interlaboratory method performance study. The method comparison study will be done by the expert laboratory and will consist of experimental determination of:

- Specificity (for both target and non-target organisms) - a defined test set of target and competing organisms has been defined and will be held in a culture collection for use in MicroVal studies.
- Limit of detection - to show the lowest concentration of analyte that can be detected in a range of foods, depending on the scope of the test system.
- Relative accuracy - a comparison or results of analyses done by the new method with analyses of identical samples done with the reference method.

The samples used should be naturally contaminated, if that were not possible, artificially contaminated materials could be employed.

It is accepted that the definition of the reference method is critical to the validation procedure, a hierarchy of acceptable reference methods has been produced and centres around the use of ISO or CEN methods, although other methods can be used if no relevant ISO or CEN method exists.

The interlaboratory performance study can be done in parallel of in tandem to the comparison study, and is organized by the expert laboratory. There is no limit to the number of collaborating laboratories, however, a minimum of eight sets of acceptable results are required for the study to be valid. The study is centred on the use of at least 300 samples, with a minimum of three contamination levels and at least 5 replicates per level. Samples are analysed by the alternative and the reference method. Each participant must complete a detailed questionnaire of their testing details, if any critical factor falls outside the predetermined test parameters, then that laboratory results are excluded from the study. The results are returned to the expert laboratory for analysis.

On completion of the comparison and the interlaboratory study, the expert laboratory completes a final report which is passed to the method reviewers for assessment.

Quantitative alternative methods

Again, both comparison and interlaboratory studies are involved, and are organized by the expert laboratory. The comparison study will consist of determination of:

- Linearity - to show that increases in analyte cause a proportional increase in the test results.
- Relative accuracy
- Specificity (for both target and non-target) organisms
- Sensitivity - the ability of a method to detect slight variations in the amount of analyte in a matrix.

The rest of the procedure of the validation is the same as for qualitative methods.

It is understood that some alternative methods will already have been evaluated by either individual laboratories or by other validation/assessment schemes. In such cases in order to reduce unnecessary work, this existing data can be incorporated into the MicroVal validation, as long as the independent laboratory used a reference method and procedures that conform to MicroVal requirements.

THE MICROVAL STANDARD

The first goal to establish an international accepted protocol for the validation of alternative microbiological methods through standardization has been achieved. In the spring of 1996 the MicroVal Steering committee started the procedure for European standardization by forwarding a proposal for a European standard based on the criteria developed by

MicroVal for the validation of alternative microbiological methods to the European Standardization Committee (CEN).

In June 1996 CEN/TC 275 "Food analysis- Horizontal methods" accepted the new work item for a European standard "Protocol for the validation of alternative microbiological methods" based on the two MicroVal documents "General" and "Technical" rules for validation criteria". After adoption this European standard will form the basis of European certification of alternative microbiological methods. Through the CEN/ISO "Vienna agreement" this European standard will also be adopted as ISO standard.
The new work item was allocated to a task force under responsibility of its Working Group 6 "Microbial contamination" with Roy Holbrook from Unilever Research, UK, as convenor and the secretariat handled by Bertrand Lombard from AFNOR.

CONCLUSION

Although the MicroVal certification procedure is not yet implemented, we thought it would be interesting to inform you about our aims and how alternative methods will be validated in Europe.

The word alternative is chosen on purpose to indicate that internationally standardised reference methods, such as those published by ISO, will keep their importance in international trade. Reference methods are often laborious expensive, and time consuming. More convenient, cheaper and more rapid methods are therefore often preferred for daily use. However, results obtained with such methods should be accepted by official control laboratories and trade partners, MicroVal has been set up to achieve this

In the last phase of the project the second goal of the project has been reached. This was to set up a European organization that would perform an independent certification of alternative microbiological methods based on the European standard. In this last phase the MicroVal project has dealt with setting up this European certification structure and the cost aspects.

The near future will be devoted to finalize the European Standard within CEN, and to implement the certification organization (especially between the certification bodies involved).

Although certification of methods by MicroVal will certainly help the unification of the food trade in Europe, we hope that the MicroVal certificate will also be appreciated in countries outside Europe. Especially industries that are exporting food products to Europe may wish to use such methods to facilitate their commerce.

REFERENCES

1. EN 29002 (ISO 9002) - Quality systems - Model for quality assurance in production, installation and servicing
2. EN 45011 - General criteria for certification bodies operating products certification.
3. EN 45001 - General criteria for the operation of testing laboratories

Annex 1

FULL PARTNERS IN THE MICROVAL PROJECT

Method manufacturers

- R-Biopharm GmbH, Germany
- Merck, Germany
- Organon Teknika, Netherlands
- Sanofi Diagnostics Pasteur, France
- 3M SantJ, France
- Diffchamb S.A., France
- Riedel-de Haën, Germany

Research institutes

- ADRIA, France
- Alfred Jørgensen Laboratory, Denmark- Campden & Chorley Food Research Ass., UK
- Escola Superior de Biotecnologia University, Portugal
- Gaiker (non profit association of companies), Spain
- INETI (Instituto Nacional de Engenharia e Tecnologia Industrial), Portugal
- Laboratory of the Government Chemist (LGC), UK
- Leatherhead Food research Ass., UK,

Food processing industry

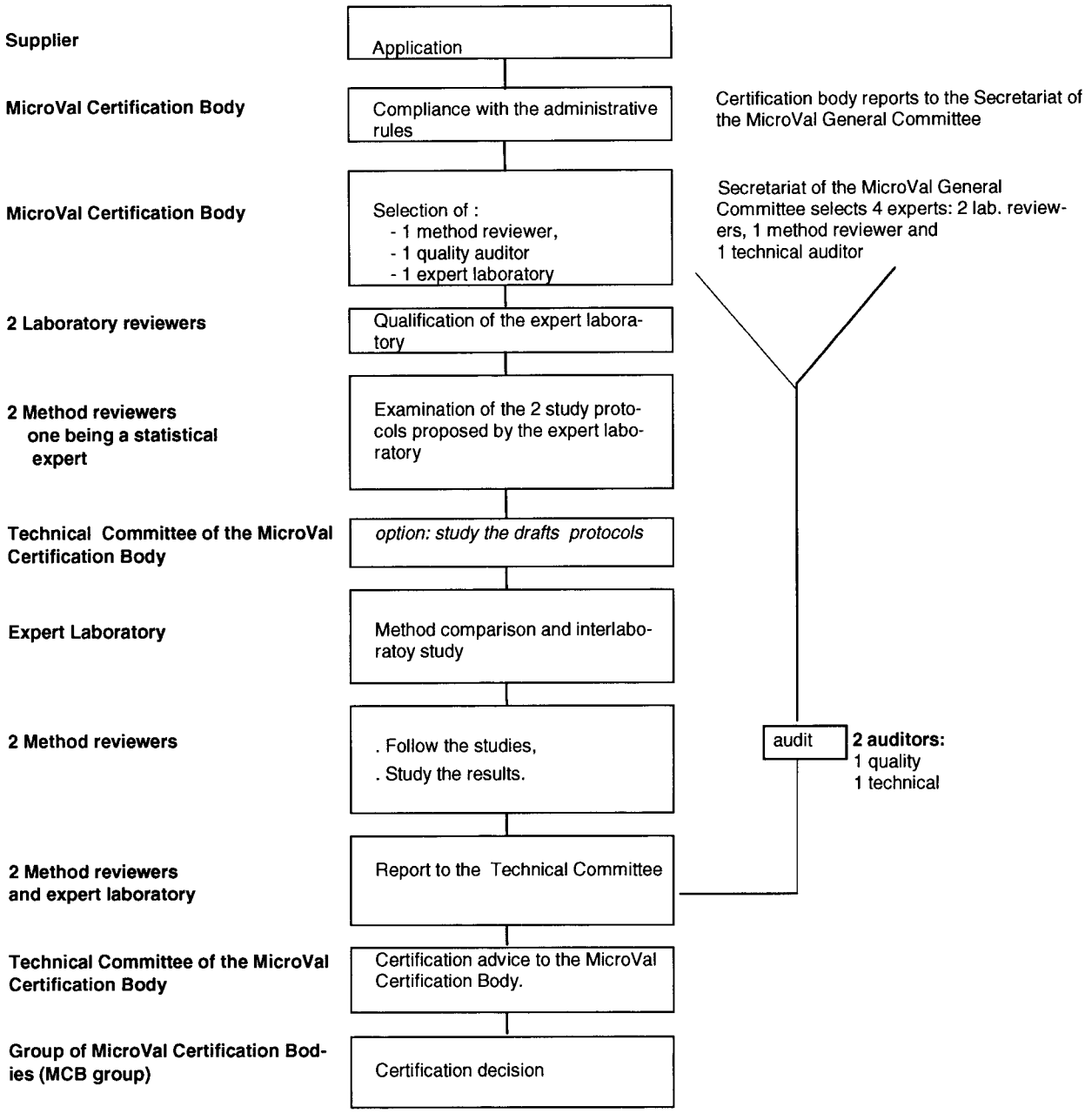
- Kraft Jacobs Suchard, Germany
- Nestlé, Netherlands
- Unilever, Netherlands
- UNIR (Ultra Propre Nutrition Industrie Recherche), France

Standardization institutes

- AFNOR - (Association Française de Normalisation), France
- NNI - (Nederlands Normalisatie-instituut), the Netherlands

Annex 2

Flow chart of the certification process



3.6 Appendix 6: Validation of microbiological methods

Qualitative Tests

- Comparative Study
  - Specificity - range of organisms
  - Limit of De.ection
  - Relative Accuracy - reference method critical
- Collaborative Study
  - 10 laboratories
  - 5 replicates, one food, 3 contamination levels

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Comparative Study

- Specificity MicroVal requirements
  30 positive pure strains (full enrichment used)
  50 negative pure strains (enrichment only used if result is +ive)
- Actually used 30 +ive and 81-ive
- BUT which strains to test ?
- 30 *L.mono,* 81 *L.other spp.,*+ related organisms & those likely to come through enrichment

CCFRA

Quantitative Tests

- Comparative Study
  - Linearity
  - Relative accuracy
  - Specificity
  - Limit of determination
  - Sensitivity
- Collaborative Study
  - 12 laboratories
  - 3 replicates, one food, 3 contamination levels

CCFRA

Collaborative Study

- To consider repeatability and reproducibility
- 1 food category
- 8 samples (min)
- 3 levels (0, limit of detection, above limit of detection)
- Blind duplicates
- Min 10 labs with results (12 used)

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MicroVal

- Several trials were done during the project
  - 2 ELISA
  - 1 Nucleic acid probe
  - 1 Agar plate alternative
  - 1 Conductance method

CCFRA

Instructions

- The instructions to collaborating labs must be exact
- Questionnaire-get labs to list everything they do
  - date & condition of sample arrival
  - storage
  - media codes
  - incubation conditions
- No such thing as a wrong answer !!

CCFRA

Trial Evaluation

- Gene Trak *L. monocytogenes* Kit
- DNA hybridisation test using *L.mono* specific DNA probes directed towards rRNA
- Detection is colorimetric and is achieved after enrichment (24h half Fraser, 24h Oxford)
- Part of a comparative study had already been performed in an AFNOR study
- CCFRA trial "filled gaps" in the comparative study and did a full collaborative study

CCFRA

Samples

- Samples must be transported in conditions which minimise growtn
- How can this be achieved
- Use powders (milk powder)
- Each lab received 16 x 3 samples of milk powder preweighed as 25g
- How to inoculate
- Use of BCR reference materials
- Each lab received 3 capsules
  - 1 blank
  - 1 to give detection limit
  - 1 to give above detection limit.
- Labs inoculated themselves

CCFRA

Results

- Lab 11 did not complete the trial
- Various other non-conformities were noted
- Results analysed initially using stats of McClure(1990) JAOAC 73(6)
- Sensitivity-probability method classifies a +ive as a +ive
- Specificity-probability method classifies a -ive as a -ive
- False +ive-probability method classifies a -ive as a +ive
- False -ive- probability method classifies a +ive as a -ive

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Comments concerning the study

- The comparative study is straightforward for a research lab used to handling method evaluations
- The collaborative study requires good co-ordination & good external lab contacts
  - do not assume labs will do as they are instructed
  - do not assume labs will do as they say
  - use a good non-conformance questionnaire
  - plan for every eventuality
  - give very thorough instructions & a contact number & name that is manned throughout the trial

CCFRA

MicroVal - The Future

The MicroVal Project Finished in Dec 1997

The Certification rules are being used as a basis for a full certification procedure

The Technical rules have gone forward to CEN for standardisation

CEN are rewriting the rules using comments from experts on CEN TC275 WG6

The rules will then become a CEN standard (and eventually an ISO standard)

CCFRA

3.7 Appendix 7: Results of serotyping of Portugal

Most of the typed serovars used in this study, were isolated from diseased animals, with exception of poultry breeder samples sent in for *Salmonella* control.
The following serovars were found in 1995, 1996 and 1997.

Serovars of *Salmonella* strains typed in 1995

Serovars	Poultry	Caged birds	Cattle	Pigs	Horses	Dogs and Cats	Rabbits	Feed	Food#	Total
S. Alachua	1	-	-	-	-	-	-	-	-	1
S. Ardwick	1	-	-	-	-	-	-	2	2	5
S. Anatum	11	-	-	-	-	-	-	-	4	15
S. Augustenborg	1	-	-	-	-	-	-	-	-	1
S. Enteritidis	65	-	4	-	-	1	1	2	3	76
S. Havana	3	-	-	-	-	-	-	-	-	3
S. Lindenburg	16	-	-	-	-	-	-	-	1	17
S. Kentucky	15	-	-	-	-	-	-	-	2	17
S. Papuana	1	-	-	-	-	-	-	-	1	2
S. Regent	4	-	-	-	-	-	-	-	-	4
S. Senftenberg	8	-	-	-	-	-	-	-	-	8
S. Typhimurium	12	1	2	1	1	-	-	2	25	44
S. Harrisonburg	-	-	-	-	-	-	-	1	-	1
S. not typable	-	1	-	1	-	-	-	2	-	4
Total	138	2	6	2	1	1	1	9	38	198

Legend: Caged birds = Canaries and Parrots

Food of animal origin, for human consumption

Serovars of *Salmonella* strains typed in 1996

Serovars	Poultry	Caged birds	Cattle	Pigs	Horses	Dogs and Cats	Rabbits	Feed	Food #	Total
S. Agona	1	-	-	-	-	-	-	-	-	1
S. Anatum	10	-	-	-	-	-	-	4	8	22
S. Ardwick	-	-	-	-	-	-	-	-	2	2
S. Assinie	-	-	-	-	-	-	-	1	-	1
S. Bardo	-	-	-	-	-	-	-	-	1	1
S. Blockley/ *	8	-	-	-	-	-	-	-	-	8
S. Brandenburg	1	-	-	-	-	-	-	-	5	6
S. Derby	1	-	1	-	-	-	-	-	7	9
S. Enteritidis	144	-	-	3	-	-	1	-	13	161
S. Fyris	-	-	-	-	-	-	-	-	1	1
S. Gloucester	1	-	-	-	-	-	-	-	2	3
S. Goettingen	-	-	-	-	-	-	-	-	1	1
S. Hadar/ **	7	-	-	-	-	-	-	-	-	7
S. Havana	24	-	-	-	-	-	-	-	1	25
S. Heidelberg	-	-	-	-	-	-	-	-	1	1
S. Infantis	17	-	-	1	-	-	1	-	5	24
S. Kentucky	1	-	-	-	-	-	-	-	-	1
S. Kouka	-	-	-	-	-	-	-	1	-	1
S. Madelia	1	-	-	-	-	-	-	-	-	1
S. Manhattan	1	-	-	-	-	-	-	-	-	1
S. Muenchen	3	-	-	-	-	-	-	-	-	3
S. Putten	1	-	-	-	-	-	-	-	-	1
S. Newport	1	-	-	-	-	-	-	1	-	2
S. Regent	1	-	-	-	-	-	-	-	-	1
S. Senftenberg	6	-	-	-	-	-	1	2	-	9
S. Shubra	-	-	1	-	-	-	-	-	-	1
S. Typhimurium	23	2	8	5	-	1	1	-	5	45
S. Virchow	-	-	-	-	-	-	-	-	1	1
*S.*1,4,[5],12:i:?	1	-	-	-	-	-	-	-	-	1
S. not typable	4	-	-	-	-	1	-	-	5	10
Total	257	2	10	9	-	2	4	9	58	351

Legend: Caged birds = Canaries and Parrots
* *S.* Blockley/Haardt
** *S.* Hadar/Istanbul
# Food from animal origin, for human consumption

Were also typed on 1996 from other animal species:
2 *S.* Typhimurium
3 *S.* Enteritidis
1 *S.* Ndolo

Serovars of *Salmonella* strains typed in 1997

Serovars	Poultry	Caged birds	Cattle	Pigs	Horses	Dogs and Cats	Rabbits	Feed	Food #	Total
S. Anatum	21	-	-	-	-	-	-	1	-	22
S. Blockley/ *	8	-	-	-	-	-	-	-	1	9
S. Brandenburg	-	-	-	-	-	-	-	-	2	2
S. Bredeney	-	-	-	-	-	-	-	-	1	1
S. Coeln	1	-	-	-	-	-	-	-	-	1
S. Derby	1	-	-	1	-	-	-	-	1	3
S. Enteritidis	129	-	1	1	-	1	3	-	14	149
S. Give	-	-	-	-	-	-	-	-	1	1
S. Hadar/ **	7	-	-	-	-	-	1	-	2	10
S. Havana	2	-	-	-	-	-	-	1	1	4
S. Heidelberg	2	-	-	-	-	-	-	-	2	4
S. Indiana	2	-	-	-	-	-	-	-	-	2
S. Infantis	6	-	-	-	-	-	-	-	2	8
S. Lexington	-	-	-	-	-	-	-	-	2	2
S. Liverpool	-	-	-	-	-	-	-	-	2	2
S. Orion	2	-	-	-	-	-	-	-	-	2
S. Mbandaka	2	-	-	-	-	-	-	4	-	6
S. Mikawasima	-	-	1	-	-	-	-	-	-	1
S. Panama	-	-	-	1	-	-	-	-	-	1
S. Norwich	1	-	-	-	-	-	-	-	-	1
S. Rissen	1	-	-	-	-	-	-	-	-	1
S. Saintpaul	1	-	-	-	-	-	-	-	-	1
S. Senftenberg	1	-	-	-	-	-	-	-	-	1
S. Typhimurium	17	3	5	6	-	4	1	-	8	44
S. Virchow	3	-	-	-	-	-	-	-	1	4
S. Wyldegreen	1	-	-	-	-	-	-	-	-	1
S. Wentworth	-	-	-	-	-	-	-	-	2	2
S. Westhampton	-	-	-	-	-	-	-	1	-	1
*S.*1,4,[5],12:i:?	1	-	1	4	-	-	-	-	1	7
*S.*1,4,[5],12:eh:en?	2	-	-	-	-	-	-	-	-	2
S. 1,9,12:-:-	4	-	-	-	-	-	-	-	-	4
S. II 48:z10:-	-	-	1	-	-	-	-	-	-	1
S..not typable	1	-	-	2	-	-	-	-	-	3
Total	216	3	9	15	-	5	5	7	43	303

Legend: Caged birds = Canaries and Parrots
* *S.* Blockley/Haardt
** *S.* Hadar/Istanbul
# food from animal origin, for human consumption

Were also typed on 1997 from other animal species:
1 *S.* Abortusovis
8 *S.* Typhimurium
1 *S.* Senftenberg
2 *S.* Heidelberg
1 *S.* 1,4,[5],12:i:?
1 *S.* II 50:b:z6
1 *S.* Enteritidis

Main serovars of *Salmonella* strains, typed in 1995-1997

Serovars	1995		1996		1997		Total	
S. Anatum	15	7.5%	22	6.1%	22	6.8%	59	6.7%
S. Blockley/ *	0	-	8	2.2%	9	2.8%	17	1.9%
S. Enteritidis	76	38.3%	164	45.9%	150	47%	390	44.6%
S. Hadar/ **	0	-	7	1.9%	10	3.1%	17	1.9%
S. Havana	3	1.5%	25	7%	4	1.2%	32	3.6%
S. Infantis	0	-	24	6.7%	8	2.5%	32	3.6%
S. Kentucky	17	8.5%	1	0.2%	0	-	18	2%
S. Lindenburg	17	8.5%	0	-	0	-	17	1.9%
S. Senftenberg	8	4%	9	2.5%	2	0.6%	19	2.1%
S. Typhimurium	44	22.2%	47	13.1%	52	16.3%	143	16.3%

Main serovars of *Salmonella* strains isolated from Poultry (1995-1997)

Serovars	1995		1996		1997		Total	
S. Anatum	11	7.9%	10	3.8%	21	9.7%	42	6.8%
S. Blockley/ *	0	-	8	3.1%	8	3.7%	16	2.6%
S. Enteritidis	65	47.1%	144	56%	129	59.7%	338	55.3%
S. Hadar/ **	0	-	7	2.7%	7	3.2%	14	2.2%
S. Havana	3	2.1%	24	9.3%	2	0.9%	29	4.7%
S. Infantis	0	-	17	6.6%	6	2.7%	23	3.7%
S. Kentucky	15	10.8%	1	0.3%	0	-	16	2.6%
S. Lindenburg	16	11.5%	0	-	0	-	16	2.6%
S. Senftenberg	8	5.7%	6	2.3%	1	0.4%	15	2.4%
S. Typhimurium	12	8.6%	23	8.9%	17	7.8%	52	8.5%

ANTIMICROBIAL TESTING OF *SALMONELLA* SEROVARS ISOLATED ON 1996-1997

Serovars	n° tested	Antimicrobials Resistance		Antimicrobials Sensitivity	
S. Anatum	37	100% 95-99% 80-94%	SF Na SxT	100% 95-99% 80-94%	Oa, ENR, AR Apr, Fr, CN, CT K, F
S. Blockley/*	17	100% 95-99% 80-94%	C, S, K, F, SF - Na, D, SxT, Te	100% 95-99% 80-94%	Apr, CT - Oa, Amp, D, AR, CN
S. Enteritidis	188	100% 95-99% 80-94%	- SF -	100% 95-99% 80-94%	- Oa, ENR, AR, FR, CN, K Apr, CT, C, S
S. Hadar/**	17	100% 95-99% 80-94%	Na, D, ENR, S, AR, SF, Te - Oa	100% 95-99% 80-94%	CT, Fr, K, F - C, CN
S. Infantis	30	100% 95-99% 80-94%	- - SF, Na	100% 95-99% 80-94%	CT, CN, K Oa, ENR, AR Apr, C, D, Fr, F, Te
S. Typhimurium	73	100% 95-99% 80-94%	- SF -	100% 95-99% 80-94%	AR Oa Apr, CT, ENR, Fr, CN, K

Legend: * *S.* Blockley/Haardt

** *S.* Hadar/Istanbul

CN – Gentamycin	CT – Colistin	K – Kanamycin
F – Nitrofurantoin	Amp – Ampicillin	D - Doxicillin
Na – Nalidixic Acid	Oa – Oxolinic Acid	ENR - Enrofloxacin
S – Streptomycin	Te - Tetracycline	Apr - Apramycin
SF – Sulphafurazole	AR – Flumequin	Fr – Furazolidone

SxT – Sulphametoxazole+Trimethoprim

The results obtained with Neomycin were not included in this study because this antimicrobial was tested only for 6 months. Although, good results were achieved in all isolates, except for *S.*Blockley/Haardt.