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RIJKSINSTITUUT VOOR VOLKSGEZONDHEID EN MILIEU
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Report on the fourth workshop organised by CRL-*Salmonella*
Bilthoven, the Netherlands, 4-5 November 1999
M. Raes and A.M. Henken (editors)

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Samenvatting

Op 4 en 5 november 1999 is door het Communautair Referentie Laboratorium (CRL) voor *Salmonella* een workshop georganiseerd in Bilthoven, Nederland. Alle Nationale Referentie Laboratoria (NRLs) voor *Salmonella* van de EU lidstaten waren vertegenwoordigd. In totaal waren er 32 deelnemers.
Het programma van de workshop bestond uit verschillende delen. Het eerste deel bestond uit de bespreking van de nieuw zoonose richtlijn. Daarna vond een evaluatie plaats van het bacteriologische ringonderzoek en bacteriologische detectie in de verschillende lidstaten. Verder werd nog gesproken over serologische ringonderzoeken, moleculaire biologie en immunologie.

Summary

At 4 and 5 November 1999 a workshop was organised by the Community Reference Laboratory (CRL) for *Salmonella* in Bilthoven, the Netherlands. All National Reference Laboratories (NRLs) for *Salmonella* of the EU Member States participated (in total 32 participants).
The programme of the workshop was divided according to different subjects. First the new zoonoses directive was discussed. Subsequently an evaluation was held on the bacteriological collaborative study and activities on bacteriological detection in the Member States. Furthermore serological collaborative studies, molecular detection and immunology were discussed.

1. Opening and Introduction of participants

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

First of all I would like to sincerely welcome you all at this workshop. We are with many people, that is, at least 1 to 3 persons of each of the 15 EU Member States. A special word of welcome for Dr. Niemi, who is the representative of the Commission amongst 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 fruitful end, we all have to spent some energy.
During these 2 days I will be your chairman as the head of the CRL-*Salmonella*. I would appreciate it if we all are willing to use the English language during our sessions.
One last remark that I would like to make is that we as CRL-*Salmonella* will treat the information brought forward one way or the other during these days as confidential where it concerns information on performance/functioning of the NRLs. We expect that you among yourself will treat this kind of information gained from other participants as confidential similarly.
With these words the workshop is opened!

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

The aims of the workshop were defined as to discuss:

- The proposed new zoonoses directive
- Results collaborative studies CRL with NRLs
- Organisational aspects of collaborative studies among and within states
- Research activities within Member States
- Whether or not there are specific needs among NRLs
- Activities CRL *Salmonella* 2000

Participating are: the representative of the EU Commission, representatives of NRLs-*Salmonella* and representatives of CRL-*Salmonella*.

2. Review of the presentations

2.1 Current issues on the European Zoonoses Directive

Mr. V.M. Niemi (EU Commission)

Introduction

Council Directive 92/117/EEC (1) (later the Zoonoses Directive) concerns the measures for the protection against specified zoonoses and specific zoonotic agents in animals and products of animal origin in order to prevent outbreaks of food-borne infections and intoxications. The directive seeks to establish a reliable reporting system on the incidence of zoonoses generally, and to bring about also monitoring, control, and ultimately eradication of some invasive serotypes of *Salmonella* in poultry breeding flocks. It also provides for the development of control programmes for other zoonotic agents than *Salmonella*.

Currently the control measures cover only *Salmonella* in poultry breeding flocks since in the late 1980's the increasing number of human salmonellosis caused by *Salmonella* Enteritidis derived from table eggs was regarded as the most alarming issue. A top-down approach was adopted by firstly providing for measures to eradicate *S*. Enteritidis (and *S*. Typhimurium) in breeding flocks to reduce the vertical transmission to commercial flocks. Measures in commercial flocks were foreseen in the future.

However, it was soon realised that not all Member States were able to fully implement the system foreseen by the Zoonoses Directive. Indeed, only four Member States have fully implemented the provisions on *Salmonella* control of breeding flocks. Even though certain deadlines of the Zoonoses Directive were postponed by Directive 97/22/EC (2), a number of Member States still have not submitted their national plans for attaining the objectives of the Zoonoses Directive nor implemented the above provisions. The Commission will take the necessary steps to ensure that the Zoonoses Directive is observed in all Member States, as this is a prerequisite for proposals to improve the measures for controlling zoonoses.

Poultry products, in particular table eggs, remain the main sources of human salmonellosis in most of the Member States. On a national basis some Member States have extended their measures to commercial flocks. Because of the unsatisfactory results in implementing the provisions concerning breeding flocks, it has not been possible to introduce any Community measures to control *Salmonella* in commercial poultry.

The objective of eradicating *Salmonella* from poultry flocks in the Community with a strategy based on ensuring disease freedom from the breeding parent stock, although still sound, needs to be reviewed especially with regards to steps which must be taken to achieve this aim. Compulsory total slaughter and destruction of parent flocks without prior action to reduce the general pathogen prevalence level by other control measures presents serious

difficulties in many Member States. The Commission considers it necessary to review the strategy currently laid down in the Council Directive concerned.

Review of the Zoonoses Directive

With a view to prepare a proposal to improve the measures for controlling zoonoses, the Commission with the experts of the Member States and other interested parties have reflected upon a fundamental change to the actual policy and on the objectives of possible future strategies. As a result, a number of objectives and concerns have been identified such as:

- To give guarantees for the improvement of the safety of consumers by introducing pathogen reduction programmes to be implemented by the Member States;
- To give the flexibility needed for the Member States to achieve common targets;
- To ensure the uniform fulfilment by the Member States;
- To take account of concerns with regard to the spreading of zoonotic agents through trade;
- To be so well scientifically justified that any trade disputes with third countries will be avoided;
- To develop measures according to “farm to table” principle, by producing safe food from safe animals;
- To take account of the level of prevalence of zoonotic agents in the Member States.

The means of attaining these targets, and in particular the pathogen reduction schemes need to be set according to scientifically based risk assessments. In order to allow the Commission to follow-up the progress of such schemes, Member States would need to submit national programmes concerning the achievement of these targets. However, an individual Member State would have the possibility to choose the tools, which it deems necessary to obtain the targets. Since actions to control zoonotic agents start at primary production, the implementation of principles of Good Animal Husbandry Practises will play a key role.

While the current Zoonoses Directive includes only control measures for certain types of *Salmonella*, the new approach would allow for the establishment of control targets for other food-borne pathogens too. With regard to the impact on human health and the feasibility to target the control measures at the farm level, bacteria like *Campylobacter* and cytotoxic *E. coli* should be addressed.

The new approach would mean the improvement of the safety of consumers especially with regard to the pathogens with the greatest importance to health. At the same time consumers have to accept that a zero level of risk is not achievable and even not feasible. Further reflection is needed in order to evaluate the economical impact of the new approach. It might be expected that such impact will be considerable and will depend on the level of the prevalence of the zoonotic agents of concern in the Member States. The possibility to grant financial assistance to those programmes, which fulfil the required options, must be examined.

It should also be noted, that increased safety of foodstuffs will increase the trust of consumers and thus help to avoid food scares. Perhaps most of all, a scientifically based Community level policy on zoonoses and zoonotic agents will also be a tool in trade with third countries.

A critical issue for the optimal implementation of the Community strategy will be the collection of epidemiological data on zoonoses. In the future the measures intended to combat zoonoses should be based on risk assessments. This will increase the importance of accurate information on zoonoses and zoonotic agents from all stages of the food chain. Efforts should be directed to harmonise the reporting system by introducing certain fixed criteria for e.g. the case definitions. At the moment there are several initiatives at Community level, which include also measures on the reporting of zoonoses, like Decision 2119/98/EC (3). Close co-operation within the Commission and between the Commission and the Member States is needed to enable the most efficient use of the data collected and to avoid any duplicate work.

It is clear from the above that establishing a long-term strategy for the control of zoonoses is a complicated issue, for which many aspects must be taken into account. Apart from the paramount objective of public health and consumer protection, aspects such as scientific assessments, practicability of the proposed measures, follow-up of the efficiency of pathogen reduction programmes and the financial impact of such measures play a role in any future policy for controlling zoonoses. It is to be regretted that due to this complicated nature of the problem, the Commission has not been able to come forward with a proposal earlier in time. In view of the above, it is believed however that, before submitting proposals on the subject, more reflection is needed. The Parliament and the Council have therefore through Directive 1999/72/EC (4) granted the Commission time until 31 March 2000 to finalise its ongoing efforts for finding the most appropriate solutions with regard to public health and consumer protection. In the meanwhile, the Commission will endeavour to ensure that Member States apply, with the tools available at present, the highest level of public health and consumer protection possible.

References

(1) Council Directive 92/117/EEC of 17 December 1992 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. Official Journal of European Communities, L 62, 15.3.93, p. 38.
(2) Council Directive 97/22/EC of 22 April 1997 amending 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. Official Journal of European Communities, L 113, 30.4.97, p. 9.
(3) Decision No 2119/98/EC of the European Parliament and of the Council of 24 September 1998 setting up a network for the epidemiological surveillance and control of

communicable diseases in the Community. Official Journal of European Communities, L 268, 3.1.98, p. 1.
(4) Directive 1999/72/EC of the European Parliament and of the Council of 29 July 1999 amending 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. Official Journal of European Communities, L 210, 10.8.1999, p. 12.

2.2 *Salmonella* control measures in poultry production

Mr. M. Madsen (Denmark)

Salmonella infections in poultry production pose a serious threat to the health of consumers within Europe and worldwide.
Within the European Community some member countries have implemented surveillance programmes and control measures in response to the Zoonoses Directive (Council Directive 92/117/EEC) in order to reduce the prevalence of *Salmonella* in poultry production and thereby reducing consumer exposure from poultry products.

The present Zoonoses Directive contains minimum sampling requirements for poultry flocks at the parent level, provides Member States with a structure for confirmation of infection, and provides the legal basis for financial compensation to flock owners. The Zoonoses Directive thus anticipates a situation where Member States adopt a strategy of eradication at the parent level and above.
However, progress within this area has been considerably slower than expected at the time when the Zoonoses Directive was put into effect. At the same time, strategies other than eradication of *Salmonella* in poultry production have been pursued by some Member States.
In 1998, more than 250.000 human cases of salmonellosis were recorded within the European Community, and more than 53% of these were caused by *Salmonella* Enteritidis (1). The implications of this are 1) that human salmonellosis is still one of the most important gastroenteric diseases in the European community causing considerable economic and productivity losses, and 2) that egg-related salmonellosis is the main contributing factor to this picture.
Responding to this problem, and to the fact that a number of Member countries have not yet implemented the present Zoonoses Directive, a revised ”Draft Directive on the monitoring and surveillance of specific biological hazards transmissible from animal to man” (Doc VI/6437/99) is presently under discussion with the purpose of replacing the present Zoonoses Directive probably in the year 2000 to ensure sufficient and continuous progress throughout the Community. An important revision of the Zoonoses Directive is the change into a framework Directive allowing individual Member States to implement monitoring, surveillance and control programmes tailored to meet particular national situations and to progress at different paces according to set target figures.

Salmonella control measures in poultry production may have different levels of ambitions, according to the level of infection in the national poultry population, and according to the willingness of national governments to provide financial support for control programmes. Basically, there are two different control strategies:

1) The *eradication* strategy: this strategy has been used as the basis for a successful test-and-slaughter policy for a number of animal infectious diseases (e.g. foot and mouth disease, fowl typhoid, brucellosis, tuberculosis), and is also the adopted strategy for *Salmonella* control programmes in poultry production in Northern Ireland and the Scandinavian countries.
2) The *reduction* strategy: this strategy has been used successfully for a number of infectious diseases also, and often precedes the implementation of an eradication strategy, aiming at reducing the level of infection in the animal population to a stage, where a test-and-slaughter policy becomes economically feasible. The level of infection may be reduced in a number of ways, including vaccination, antibiotic treatment, alternative feeding strategies, improved hygiene and management practices etc. The reduction strategy is the official strategy for the Danish *Salmonella* control programme in pigs, and is also the adopted strategy for *Salmonella* control in poultry as implemented in e.g. Germany, The Netherlands, Belgium, UK, France a.o.

The experiences with the eradication strategy in Denmark may be summarised to be successful within the broiler sector. A control programme has been operating since 1989 based on a top-down concept with identification and destruction of infected parent flocks, and accompanied by supplementary control measures such as heat treatment of feed, renovation of broiler production facilities and improved hygiene measures at farm and slaughter house levels. The level of infection has been brought down from approx. 80% infected broiler flocks to the present level below 2% infected flocks at slaughter.
Within the Danish table-egg sector it may still be too early to evaluate the success of the eradication strategy. A national surveillance programme based on examination of spent hens at slaughter was initiated by the industry in 1992, and the present public control programme introduced by early 1997. At the present moment, approx. 9 million Euros have been spent, approximately 10% of the table-egg layers have tested positive for *Salmonella* and have been restricted to produce only for pasteurised egg products. Very important, the number of human *S.* Enteritidis cases have been reduced by more than 30% during the period of the public control programme.

Recently, a number of Member States have included vaccination as part of a reduction strategy, due to the development and licensing of more effective *Salmonella* vaccines. The effect of these vaccines are claimed to be good based on data from experimental challenge studies carried out by the vaccine producers, but there appears to be a severe lack of validated data available as regards both the effect in controlled field trials, and as regards the effect on the human health situation when applied as large scale vaccination programmes at the regional or national level.

Table 1 shows the developments in human salmonellosis incidence rates (1) in Member countries that have introduced vaccination of table-egg layers as part of national control programmes.

Table 1 Human salmonellosis incidence rates and fractions attributable to *S.* Enteritidis in some EU member states that have introduced vaccination

Country	Vaccination introduced	Human incidence rate		% S. Enteritidis	
		1995	1998	1995	1998
Belgium	1997	112.7	135.7	55.3	62.2
France	1998	30.9	28.2	32.6	37.4
Germany	1996	141.0	119.0	61.0	58.5
Netherlands	1997	19.9	14.5	48.0	43.0
United Kingdom	1998	57.6	44.9	54.1	69.2

Although fluctuations and trends in human salmonellosis incidence rates may not reflect the effects of vaccination in table egg layers as they are influenced by a number of factors such as changes in habits of food consumption, increased observancy of consumers and medical staff, food import patterns etc. it is interesting to note that some of the countries (Germany, The Netherlands) practising vaccination have observed decreasing incidence rates both overall and due to *S.* Enteritidis. It may, however, also be noted that other countries (France, United Kingdom) despite a decrease in the overall human salmonellosis incidence rates have recorded an increase in the fraction attributable to *S.* Enteritidis. It is apparent that validated data on the effects of vaccination both at the human and egg-layer levels are much needed in order to evaluate and plan future control programmes.

In conclusion, the choice of control strategies for *Salmonella* in poultry production depends to a very large degree on the national situation both in respect of the infection level in poultry flocks and in respect of the willingness and capability of the industry and Government authorities to contribute financially to control programmes. The ambition may well be eradication, but the national situation may dictate reduction for a period of time.

It is the experience at least from the Scandinavian countries that eradication is costly but achievable in broiler production, and for the Scandinavian countries this is also the strategy for the table-egg sector. At the European level eradication may not be achievable in table-egg production in the short-term perspective, and at present vaccination seems to be the only realistic strategy preceding a test-and-slaughter policy. For some countries, vaccination is apparently a useful approach in table-egg production, but valid documentation for the reductive effects of vaccination both in terms of flock infection and excretion rates of

infected eggs is very much needed, as control programmes ideally should be based on detailed risk assessments and cost-efficiency analyses.

(1) Data on human salmonellosis incidence rates kindly provided by Dr Annemarie Kässbohrer, CRL-E Berlin.

2.3 Associations serotypes/products and costs

Mr. W. van Pelt (the Netherlands)

At the Dutch National *Salmonella* Centre (NSC, RIVM) serotyping and phage typing of *Salmonella* isolates takes place derived from humans, (farm) animals, foods and the environment.
Analysis of these national data demonstrates trends in the occurrence and the development of antibiotic resistance among humans consistently in parallel with those in non-human sources especially farm-animals and their products. Of 160,000 isolates collected since 1984 by the NSC, 99 % concerned one of the almost 500 types found in both humans and farm-animals. Multivariate analysis of the data demonstrates that the relationship of *Salmonella* serotypes / phagetypes and “Pig” (piglets and adults), “Cattle” (dairy cattle, veal calves), “Chicken” (broiler-, layer flocks, etc.) and “Eggs” is very specific. The incidence varies strongly dependent on year and season and the prevalence of *Salmonella* amongst diseased animals is much higher than amongst healthy ones. However, the ranking, the relative importance, of the different types is dominated by the species of farm animal whilst differences between subgroups within a species are small in comparison. This allows for a simple procedure to compute the fraction of human salmonellosis derived from each of the species of farm animals per *Salmonella* type and in total. As data on the prevalence of contamination of foods derived from farm animals, the exposure of consumers (a.o. preparation and consumption habits), dose-response relationships of exposure and getting ill, etc. are hardly available these are considered as a “black-box”. Applying the method shows that the gradual decrease of human salmonellosis in the past 15 years in the Netherlands concerns predominantly types that are found mainly among pigs and to a lesser extent those that are mainly found among cattle. Irrespective of the general decrease of human salmonellosis the contribution computed for poultry (chicken and eggs) has remained the same, however, this concerns currently 60 % of all human salmonellosis whilst 15 years ago this was only 25%. In an absolute sense the role of eggs has increased in the past 15 years but is decreasing again in the past few years.
By combining data from laboratory surveillance and results from population- and GP-sentinel studies performed in the past 10 years in the Netherlands, the total number of cases of salmonellosis in the Dutch population can be estimated as a function of the number of isolates received from the public health laboratories. This also allows for an estimation of the fraction of patients that did and did not visit a general practitioner. Estimates from the United States and, recently, from the IID-study in England, besides national data on hospitalisation and mortality, allow the computation of the fraction salmonellosis related to hospitalisation

and mortality. This leads to an estimate of 20,000-85,000 cases per year of salmonellosis for the period 1993-1998 (40,000-155,000 between 1984-1989).
A number of studies in the English speaking countries and in the Netherlands estimated the economic costs in Western countries of visiting a GP, or not, hospitalisation and mortality due to salmonellosis. Hence, using now the assumed / computed fractions of types of patients, the average economic costs of a case of salmonellosis in the general population can be estimated. Discerning the resulting different scenario's for the Netherlands this amounts to 1900-2800 Dutch guilders per average case. For the period 1993-1998 this means an estimated economic loss of 59-163 million Dutch guilders per year over all cases of salmonellosis. Considering the estimates of the fraction of human salmonellosis related to farm animals would mean an economic loss of 36-96 million guilders per year related to contaminated poultry.

2.4 Evaluation of the fourth bacteriological collaborative study

Mr. M. Raes (the Netherlands)

The fourth bacteriological collaborative study was organised for the National Reference Laboratories for *Salmonella* (NRLs) of the Member States of the EU and had two objectives:

1. Evaluation of the results of the detection of *Salmonella* in the presence of competitive micro-organisms among and within the NRLs; and
2. Evaluation of MSRV as selective enrichment compared to the standard method using RV as selective enrichment.

For the detection of *Salmonella* an adapted ISO 6579 and, optionally, the laboratory's own routine method for the detection of *Salmonella* was used. As an additional selective enrichment, the semi solid medium, modified semi solid Rappaport Vassiliadis, MSRV, was used. The MSRV medium is based on the motility which is exhibited by virtually all relevant *Salmonella* strains.
Twenty-five capsules had to be examined in combination with chicken faeces. Furthermore 10 control capsules were examined. Also 20 naturally contaminated samples were examined.
The Rappaport Vassiliadis broth (RV) used in the study differed very much between the participants and manufacturers. According to ISO 6579 the final concentration of the ingredients is: Trypton: 4.5 g/l, Sodium chloride: 7.2 g/l, Potassium dihydrogen phosphate: 1.44 g/l, Magnesium chloride hexahydrate: 36.0 g/l and Malachite green oxalate: 0.036 g/l.
Sometimes RV contains a second buffer salt: Dipotassium hydrogen phosphate which is an ingredient for RVS. This means that some laboratories that assume they use RV broth for selective enrichment, actually use RVS broth.

The capsules used in this study contained *S*. Enteritidis (SE) or *S*. Typhimurium (STM). The SE capsules had a level of contamination of 100 (SE100) and 1000 (SE1000) per capsule.

The STM capsules contained 10 (STM10) and 100 (STM100) cfp per capsule. Each level and serotype was included 5 times in the study and furthermore 5 blank capsules were included.
The naturally contaminated samples originated from a poultry laying flock which was found serologically positive for SE. Samples were taken in several weeks in 1999. When the faeces was found positive for SE, the faeces was mixed and homogenised with glycerol / peptone solution and stored at –20°C in order to stabilise the micro-organisms. The stability of these samples was tested for several weeks. Each time the faeces was tested, two portions were taken from the freezer. From each portion, 25 grams of faeces was tested using RV, MSRV and DIASALM for selective enrichment. Not all samples were found positive after freezing (Table 1). Using RV, *Salmonella* was isolated from the faeces only once. Using MSRV and DIASALM, only two times no *Salmonella* was isolated from the faeces. This occurred both times with faeces from week 16.

Table 1 Stability of naturally contaminated samples at –20°C

	Number of samples positive (n=2)		
Weeks in freezer	RV	MSRV	DIASALM
Faeces week 16 (portion 9 in study)			
2	0	1	2
5	0	0	0
11	0	2	2
14	0	0	0
Faeces week 22 (portions 6, 7 and 10 in study)			
1	0	2	2
6	1	2	1
9	0	2	2
Faeces week 26 (portion 8 in study)			
1	0	2	2
2	0	2	2
4	0	2	2

Artificially contaminated samples

The best results were obtained with the STM100 capsules. The number of positives found with the STM10 and SE1000 capsules was roughly the same. The lowest number of positives was obtained with the SE100 capsules.
The standard method obtained more isolations than MSRV in 4 of the 16 participating laboratories. This was in laboratories 6 and 10 for the capsules containing 1000 cfp SE. Both laboratories do not have any experience using MSRV as selective enrichment. Laboratory 8 found 2 samples positive with the standard method which were found negative using MSRV.

Laboratory 13 found 3 samples positive using the standard method which were found negative using MSRV. Laboratory 8 and 13 both have 2 years experience using MSRV.

For each type of capsule the total number of positive isolations was higher for MSRV than using the standard method. Comparison of the results of RVS and MSRV showed hardly any difference. The total percentage of positives for RVS is 59% (RV 38%) and for MSRV it was 58%. Taking RV and RVS together as one standard method the percentage of positive isolations from the artificially contaminated samples is 46% for the standard method compared to 58% using MSRV.

Naturally contaminated samples

The faeces from week 16, which was not found positive every time tested, was found negative by almost all laboratories. Four times the standard method resulted in more *Salmonella* positive samples than MSRV.

Looking at all 20 naturally contaminated samples, except for laboratory 8, MSRV obtained equally or more positive samples than using the standard method.

The total number of positive isolations using the standard method is 96, which is 32%. Using MSRV the number of positive isolations is 151, which is 50%. In this case MSRV also has more positive isolations compared to RVS. Using RV and RVS a total of 28 and 38% respectively is found positive compared to the 50% found using MSRV.

Preliminary conclusion

The results from MSRV were in line with the expectations. MSRV and RVS seem better suited as selective enrichments for isolation of SE and STM from artificially contaminated faeces than RV.

MSRV seems to be a better selective enrichment than RVS for naturally contaminated samples containing SE.

An advantage of MSRV is that MSRV is easier to use than RV. When MSRV does not show a zone of growth, you do not have to inoculate an isolation medium. When using a broth for selective enrichment, an isolation medium has to be inoculated always.

Discussion:

The Magnesium Chloride concentration according to ISO is not 36 g/l, but 33 g/l because the volume of 400 grams $MgCl_2$ in 1000 ml water is 1260 ml.

An advantage of MSRV is that the composition from different manufacturers is the same.

Even when MSRV does not show a zone of growth, it is possible that *Salmonella* is present in the drop of BPW after incubation.

2.5 Proposed revision ISO 6579

Ms. S.M. Schulten (the Netherlands)

Each year the ISO Technical committee 34 Sub committee 9 (ISO/TC34/SC9) is organising a meeting in which ISO methods are discussed, suggestions for alteration of methods are proposed and resolutions are made. In the annual meeting in July 1997 (Turin), a working group of the European Commission of Normalisation (CEN/275/WG6) requested ISO/TC34/SC9 to launch a revision of ISO 6579:1993, the horizontal method for the detection of *Salmonella* in foods or animal feeding stuffs, for two reasons.

- To include modifications brought by the European method EN 12824:1997; and
- To consider the replacement of the SC broth (selenium is toxic).

In April 1998 (The Hague) a draft revision (Committee Draft) was made and the CD-vote closed on 08/01/99. Many comments were given to these alterations, and in the next meeting in April 1999 (Paris) new modifications have been made to the Committee-Draft. The new changes will be incorporated in the DIS version. At the next annual meeting the DIS can be approved or new alterations can be proposed.

The changes supposed during both meetings are presented below (Fig. 1, 2, and 3).

ISO 6579:1993 General guidance on methods for the detection of *Salmonella*

Scope: detection of all *Salmonella* in foods and animal feeding stuffs

- **Pre-enrichment:**
  - BPW, incubated at 37 °C ± 1 °C for 16 to 20 h.
- **Selective enrichment:**
  - RV, incubated at 42 °C ± 1 °C for 24 h
  - Selenite/Cystine (SC) medium, 37 °C ± 1 °C for 24 h and a further 24 h.
- **Plating out and recognition:**
  - inoculation of two selective solid media: BGA and second of choice
  - incubation at 37 °C, and examined after 24 h and, if necessary after 48 h
- **Confirmation:**
  - 5 colonies from each dish of each selective medium
  - Biochemical confirmation: (Triple Sugar Iron agar (TSI), Urea, L-Lysine decarboxylation,

    β-galactosidase, Voges-Proskauer (VP), indole reaction)
- Serological confirmation

Figure 1

April 1998 (The Hague), adjustments to ISO 6579:1993:
Scope: same, only excluding *S*. Typhi and *S*. Paratyphi

- **Pre-enrichment:** pre-warmed BPW
- **Selective enrichment:**
  - RVS and TTn (thus excluding *S*. Typhi and *S*. Parathyphi from the scope)
  - Incubation at 41,5 °C ± 1 °C for 24 h and if necessary further 24 h (both media)

Figure 2

April 1999 (Paris): ISO/DIS 6579
Scope: detection of all *Salmonella*, including *S*. Typhi and *S*. Paratyphi

- **Pre-enrichment:**
  - BPW at ambient temperature, or pre-warm in case of large volumes.
- **Selective enrichment:**
  - RVS, incubated at 41,5 °C ± 1 °C for 24 h ± 3 h
  - MKTTn, incubated at 37 °C ± 1 °C for 24 h ± 3h
    (as MKTTN can recover *S*. Paratyphi and some *S*. Typhi)
  - delete the additional 24 h in the selective enrichment stage
- **Plating out and recognition:**
  - BGA medium replaced by XLD medium as first mandatory agar
    (XLD today largely used, less dependant on the manufacturer)
  - second agar is left to the choice of the laboratory
  - incubation at 37 °C ± 1 °C for 24 h ± 3h
- **Confirmation:**
  - same as ISO 6579:1993, yet begin with the identification of only 1 of these colonies.
    In case of epidemiological studies: identify systematically at least 5 colonies

Figure 3

2.6 Bacteriological collaborative study with 11 labs in France

Ms. F. Humbert (France)

The objective of this collaborative study was to test the aptitude of veterinary diagnostic laboratories in France to detect *Salmonella* in the presence of competitive micro-organisms. The samples used in this study were (per participating laboratory):

1- 15 capsules « highly » contaminated
2- 25 capsules « moderately » contaminated
3- 5 capsules « weakly » contaminated
4- 4 blank capsules

Highly and moderately contaminated capsules (as well as blank ones) were prepared at AFSSA-Ploufragan (using the same *S.* Typhimurium strain as the one used for the collaborative trials between Member States), while the low level of contamination (c.a. 5 *Salmonella*/capsule) was obtained from SVM-RIVM (Bilthoven, The Netherlands). For the competitive micro-organisms faeces of our SPF poultry flock at Ploufragan was used.

The method used to analyse the samples is the one specified in our national technical standards for veterinary analysis (COFRAC BA-60 and BA-70) and consisted of:

1- Pre-enrichment in BPW;
2- Selective enrichment (MSRV and Tetrathionate are the most often used enrichment media and so they were compulsory for this study, but the laboratories were encouraged to use their own enrichment media, if different, in parallel);
3- Isolation on at least one out of 4 media (Rambach, XLT4, Hektoën and SMID); and
4- Biochemical identification.

Stability tests (15 or 21 capsules analysed every week during 7 weeks) and homogeneity tests (3 batches of 15 or 21 capsules each, analysed on the same day) performed on the reference materials prepared at Ploufragan, gave an average contamination level of 688 cfu/capsule for the high and 61 cfu/capsule for the moderate level of contamination respectively. There was no difference between batches.

The 10 g faecal samples, which had to be added to the capsules, were from an initial sample of 1.5 kg homogenised with glycerol peptone water (mixing ratio: 1/1(w/v)) before freezing. Each laboratory received 49 capsules and had to add faeces (or not) as indicated in the following Table 1.

Table 1 Capsules used in the study with or without faeces

Level of capsules contamination	*Number*	*Presence of faeces (1g)*
Blank	4	yes
Weakly	5	no
Moderately	4	no
Moderately	21	yes
Highly	15	yes

They also had to analyse one control procedure (without capsule and without faeces) and one control faeces (faeces without capsule). So the total samples analysed was 51.

None of the 10 participating laboratories found *Salmonella* in the blank capsules. There was no statistical difference between the participating laboratories (Fischer test and χ^2 test). The level of contamination which gave the greatest difference between laboratories was around 60

cfu per capsule. The numbers of positive capsules detected out of this pool of 21 moderately contaminated capsules analysed with faeces were from 15 to 21 depending on the participating laboratories (Table 2).

Table 2 Number of capsules found positive per laboratory and per type of capsule

Laboratory	Number of capsules found positives				
	4 Blank with faeces	5 weakly without faeces	4 moderately without faeces	21 moderately with faeces	15 Highly with faeces
1	0	5	4	21	15
2	0	5	4	19	15
3	0	5	4	15	15
4	0	5	4	21	15
5	0	5	4	19	15
6	0	5	4	21	15
7	0	5	4	21	15
8	0	5	4	21	15
9	0	4	4	21	15
10	0	5	4	19	15
11	0	5	4	20	15

For the next ring trials, it is expected to have much more participating laboratories because more than 50 private and public veterinary laboratories are now involved in the official *Salmonella* detection programme in poultry productions in France. For practical reasons, this first trial involved only laboratories from the west part of France because that is where poultry production is concentrated.

Discussion

It was discussed whether it is possible for CRL-*Salmonella* to produce and distribute capsules to the NRLs for organising their national bacteriological collaborative studies.

2.7 Bacteriological collaborative study in the Netherlands

Ms. N. Voogt (the Netherlands)

The Dutch National Reference Laboratory for *Salmonella* organises national bacteriological collaborative studies two times a year. The studies are organised by order of the Dutch Veterinary Public Health Inspectorate in the framework of the Dutch programme for *Salmonella* control in the poultry sector. Participants of the studies are (commercial) laboratories, recognised by the Dutch national authorities. The objective of the collaborative

studies is to test the capacity of the laboratories to detect *Salmonella* in the presence of competitive micro-organisms.
Reference capsules containing sublethally injured *Salmonella* Typhimurium (STM) had to be tested for the presence of *Salmonella* with and without the addition of chicken faeces. The reference materials (RM) contained 100 or 1000 cfp STM per capsule. The faecal samples, containing competitive micro-organisms, were prepared from chicken faeces and tested for the presence/absence of *Salmonella*.
As a result of previous collaborative studies and criteria set by the Dutch Product Boards for Livestock, Meat and Eggs, laboratories had to test fifteen or fifty capsules. The contents of these capsules are shown in Table 1. The capsules were tested using the laboratory's routine method for detection of *Salmonella* in chicken faeces. The cumulative results of the study I to IV are presented in Table 2. In that Table, a distinction is made between the results of RV, MSRV and DIASALM.

Table 1 Composition of 15 or 50 samples

15 samples consist of	Faeces	RM
5 samples	1 g	STM 100
5 samples	1 g	STM 1000
2 samples	1 g	*S.* Panama 5
3 samples	1 g	Blank capsules
procedure control	-	-
negative control	1 g	-
50 samples consist of	Faeces	RM
18 samples	1 g	STM 100
18 samples	1 g	STM 1000
4 samples	1 g	Blank capsules
5 capsules	-	*S.* Panama 5
5 capsules	-	STM 100
Procedure control	-	-
Negative control	1 g	-

Table 2 Results of bacteriological study I - IV

STM 100 cfp / capsule				
total no labs	total no samples	RV pos.	DIASALM pos.	MSRV pos.
19	574	418 (72.8%)	393 (68.5%)	
9	173	105 (60.7%)		138 (80%)
STM 1000 cfp / capsule				
total no labs	total no samples	RV pos.	DIASALM pos.	MSRV pos.
19	557	476 (85.5%)	414 (74.4%)	
9	216	157 (72.7%)		180 (83.3%)

A number of preliminary conclusions can be drawn:

1. Testing capsules in combination with chicken faeces gives in comparison with RV
   - ⇨ less positive isolations using DIASALM
   - ⇨ more positive isolations using MSRV;
2. The number of *Salmonella* isolated from capsules containing 100 cfp STM were lower compared to capsules containing 1000 cfp STM; and
3. Using semi-solid media reveals big differences in results between laboratories.

Discussion

There is no obvious standard or criteria which the laboratories have to meet. The “poorer” performing laboratories had to test 50 capsules and the “better” performing laboratories had to test 15 capsules. The participating laboratories are obligated to participate in the collaborative studies and obtain good results, in order to get “ster”lab accreditation.

There is a danger in using *Salmonella* Typhimurium as only serotype because the sensitivity of a method is dependent of the serotype found.

2.8 Comparison Tetrathionate and Muller-Kauffman formulas

Ms. F. Humbert (France)

Reviewing the literature on *Salmonella* detection, differences between performances of the different enrichment media can be made and easily related to:

1- The use (or not) of a pre-enrichment step in a non selective media (often BPW but Lactose Broth in the US) at a non selective temperature (37°C but 35°C in the US);

2- The incubation temperature of the enrichment step (37°C or 42°C); and

3- The exact composition of the enrichment media used.

In fact, there are 3 families of enrichment media for *Salmonella*: Selenite, Rappaport and Tetrathionate.

1- Selenite-cystine

There is no problem with the exact formulation of the selenite-cystine: the only difference among commercially available preparations is on the incorporation of either the selenite, either the cystine, either both in the main basal powdered media, or adding them extemporary as fresh separately prepared solutions. But this poorly selective medium is also considered as toxic for the environment and operators, and showed great differences in performances in the 2 first ring trials organised by the CRL (perhaps in relation with different commercial formulations and ways of preparation?). But it seems to be the best medium for *S.* Typhi and Paratyphi.

2- Rappaport-Vassiliadis

This medium is described in ISO 6579 and its preparation needs the mixing of 3 solutions as shown in Table 1. It is recognised that adding one litre of water on 400 g of magnesium chloride gives a total volume of nearly 1260 ml. So the concentration of this selective compound must be adjusted to 28.4 g/l for the hexa-hydrated product or 13.3 g/l for the anhydrous product in the final complete ready to use medium.

In fact there are 3 requirements for a Rappaport-Vassiliadis:

1- A soya peptone in ISO but casein peptone in AOAC standard;

2- An adjusted pH of 5.2; and

3- A malachite green concentration of 28.4 g/l $MgCl_2*6H_2O$ or 13.3 g/l $MgCl_2$.

A number of commercially available formula does not respect these 3 points.

Table 1 Preparation of RV according to ISO 6579 with the concentration of compounds in complete medium

ISO formulation		Composition of complete media g/1110ml	g/l
Solution A (take 1000 ml)			
Soya peptone	5.0 g	5	4.5
NaCl	8.0 g	8	7.2
KH_2PO_4	1.6 g	1.6	1.44
Water	1 000 ml		
Solution B (take 100 ml)			
$MgCl_2$-$6H_2O$	400 g	32	28.4
Water	1 000 ml	(and not 40)	(and not 36)
Þ Total vol. obtained = 1260 ml		or 15 $MgCl_2$ anhydrous	or 13.3 $MgCl_2$ anhydrous
Solution C (take 10 ml)			
Malachite green oxalate	0.4 g	0.04	0.036
Water	100 ml		

3- Tetrathionate

The selectivity of this medium, modified by Muller-Kauffman, is based on the use of tetrathionate by *Salmonella* (and not by other competitive micro-organisms). Tetrathionate may be already present in the medium or may be obtained by the addition of a KI/I_2 solution to thiosulfate. In relation with this main difference or other differences in the concentrations of products like $CaCO_3$ and thiosulfate, Table 2 gives the main formula available.

Table 2 Composition of Tetrathionate according to different prescriptions

	USP (TT)	AOAC BAM	ISO 6579 before 1990 (MK)	commercially available
$CaCO_3$	20	10	38	25
$Na_2S_2O_3$-$5H_2O$	-	30	47.6	40.7
$K_2S_4O_6$	20	-	-	-
Ox bile	8	1	4.7	4.75
I_2	-	6	4	4
KI	-	5	5	5
brilliant green	0.07	0.01	0.01	0.01

But even more minor variations than those indicated in this table are present in commercial media. So it is almost impossible to compare, for tetrathionate, results from different works published, as usually, authors do not give the exact formula they use. It is important to specify supplier, exact reference number and exact composition in order to make comparisons possible. This is of importance because MKTT is going to be used in the new revised version of ISO 6579.

2.9 Overview of current *Salmonella* detection research at VLA Weybridge

Mr. R. Davies (United Kingdom)

As there are no research projects specifically devoted to *Salmonella* detection the presentation described various small studies which have been carried out during the course of other epidemiological research.

Addition of Ferrioxamine E (50 ng/ml) to buffered peptone water did not increase the count of *Salmonellas* achieved after pre-enrichment, but the isolation rate from other naturally contaminated samples was reduced. This was associated with increased growth of Pseudomonas. Addition of Ferrioxamine E to DIASALM medium did not improve the isolation rate from faecal or hatchery environmental samples but there was a marked increase in the isolation rate from water troughs and liquid mud from outdoor pig units, suggesting that Ferrioxamine E may be useful to assist with the recovery of *Salmonella* from nutrient depleted samples.
In order to reduce weekend laboratory work we investigated interruption of culture after pre-enrichment in BPW or selective enrichment in DIASALM, by placing cultures at 4° for 72 hours before continuing. The isolation rate was slightly increased by this procedure, suggesting a cold enrichment effect. Two stage enrichment in RVS followed by MSRV and

delayed secondary enrichment in RVS also increased the isolation rate compared with the standard BPW: DIASALM: Rambach method, but less than the cold enrichment.
In the UK we have encountered several strains of *Salmonella* Dublin which grow poorly in RVS and on BGA. Bulked cattle faeces 'spiked' with a panel of these and other strains were cultured by a variety of methods. BPW: DIASALM: Rambach agar was the most sensitive method, although there was some overgrowth in DIASALM and some strains appeared as pale colonies on Rambach agar. Poorer results were obtained using direct enrichment in selenite cystine broths and plating on BGA and culture in BPW: RVS: BGA only gave half the isolation rate of the BPW: DIASALM: Rambach method.

Similar comparative culture work was carried out with *S.* Pullorum inoculated into bulked poultry faeces. The most sensitive method was Selenite/BGA but even this only detected levels of over 4 log cfu/g faeces. This demonstrates the inefficiency of bacteriological monitoring for *S.* Pullorum. It is also likely that other poorly growing strains of *Salmonella* may also become overgrown in non-selective pre-enrichment, e.g. as in the ISO 6579 method.

A trial is in progress to optimise serological detection of *Salmonella* Pullorum in turkeys, in which unconfirmed positive tests are a significant problem in export consignments. The *S.* Pullorum strains produced a rapid rise in antibody titres in RSA, MAT and TAT tests. *S.* Typhimurium DT104 infection also produced positive reactions to the pullorum test. RSA and MAT tests also gave some positive results in control birds.

An investigation into the relationship between human strains of *S.* Hindmarsh (which is very rare in UK) involved in a localised outbreak and sheep strains from the same area was carried out using plasmid profile analysis and pulsed field gel electrophoresis. There was no similarity between the human outbreak strains and strains from local sheep or between the human strains and a panel of strains from New Zealand, where the infection is more common.

2.10 Ideas for future bacteriological collaborative studies

Some options were given for future bacteriological collaborative studies. It is decided that we will continue using MSRV for selective enrichment. We will not start using DIASALM because laboratories with experience using DIASALM say it is more difficult to work with. For selective enrichment it is also possible to use Tetrathionate. There are however a lot of different manufacturers which all have different compositions of this medium. First some research will be done into the different manufacturers and their media.
The new ISO will prescribe XLD instead of BGA. We will use BGA as well as XLD.
If possible, the next study will again contain naturally contaminated samples and if possible the faeces will contain different serotypes.

The protocols will be mailed earlier and may probably be sent to the laboratories by e-mail or on a diskette.
There will be a postal survey about the interest in capsules containing *Salmonella*.

2.11 Results of collaborative typing study

Ms. W.J. van Leeuwen (the Netherlands)

A fourth collaborative study on serotyping of *Salmonella* was organised in March 1999 by the CRL-*Salmonella.* In this study also the EnterNet Laboratories could participate. For the first time laboratories which were interested had the possibility to perform phage typing of *S.* Enteritidis and *S.* Typhimurium. The phage typing collaborative study, included in this study, was organised in collaboration with the PHLS in London. In this study 16 strains were selected for serotyping and 10 *S.* Enteritidis and 10 *S.* Typhimurium strains for phage typing. For serotyping all (=16) National Reference Laboratories (NRLs) participated and 15 of the EnterNet Laboratories (ENLs). For phage typing 6 of the NRLs and 12 of the ENLs participated (Table 1)

Table 1 Participants in Collaborative study IV on sero and phage typing

Country	NRL	ENL	NRL/ENL
Austria			X/O
Belgium	X	X/O	
Denmark	X/O		
Finland	X	X/O	
France	X	X	
Germany	X/O	X/O	
Greece	X	X	
Ireland	X	X/O	
Northern Ireland	X		
Italy	X/O	X/O	
Luxembourg	X		
The Netherlands			X/O
Portugal	X	X/O	
Scotland		X/O	
Spain	X	X/O	
Sweden	X	X/O	
Switzerland		X/O	
United Kingdom	X/O	X	
Total	14	13	2

X = sero typing
O = phage typing

In this abstract only results of the NRLs will be presented.

Serotyping

Typing methods as routinely performed in the laboratory should be used and if the set of mono specific antisera was incomplete the antigenic formula could be given as far as the antigens were detected.

Eight NRLs typed all the strains correctly and seven strains were typed correctly by all laboratories. In general there were no problems with the identification of the O-antigens. Most problems occurred with typing of the H-antigens (Table 2).

Table 2 Examples of partly correct/incomplete and incorrect detection by NRLs

Group B	*S.* Schwarzengrund 1,4,12,27 : d : 1,7	
	4 : ***l,v*** :7	*S.* Bredeney
Group C2-C3	*S.* Albany 8,20 : z4,z23 : -	
	8 : ***d : 2***	*S.* Virginia
	8 : ***i : z6***	*S.* Kentucky
	6,8 : z4,***z24*** : -	*S.* Duesseldorf
	S. Goldcoast 6,8 : r : l,w	
	6,***7***,8 : l,v : ***z15***	*S.* Edmonton
Group E1	*S.* Weltevreden 3,10,15 : r : z6	
	3,10 : ***d*** :***l,w***	*S.* Birmingham
	3,10 : ***i*** :z6	*S.* Yeerongpilly
Group G	*S.* Cubana 1,13,23 : z29 : -	
	13,23 : -	no serotype
	13,***22*** : z29 : -	*S.* Agoueve
Group O	*S.* Alachua 35 : z4,z23 : -	
	no reaction A-S: z4,z23	No serotype

One of the reasons of incomplete or incorrect results could be the missing of qualified monovalent antisera which are essential for the exact identification of the strains.

Phage typing

In general, the majority of the laboratories did not encounter major problems with the phage typing of the strains.

Three laboratories assigned all 10 *S.* Enteritidis strains the correct phage type and 4 laboratories the 10 *S.* Typhimurium strains. Four strains of *S.* Enteritidis and 6 strains of *S.* Typhimurium were assigned correctly by all laboratories. Some laboratories were unable to identify phage types 193 and 208. This situation probably arose because the laboratories were either lacking the full complement of *S.* Typhimurium typing phages, particularly the additional phages necessary for the identification, or the most recent typing chart which identifies reactions of the 193 and 208 phages. Also standardisation of the methods used by

the participating laboratories requires careful monitoring to ensure overall consistency of the results obtained.

It remains worthwhile to organise collaborative studies on serotyping as well as on phage typing and to do this in collaboration with the ENLs.

(Full results of the IV collaborative study will be presented in RIVM Report 284500 013: Test results of *Salmonella* sero and phage typing by the National Reference Laboratories and the EnterNet Laboratories in the Member States of the European Union; Collaborative study IV on sero- and phage typing., *M. Raes, L.R. Ward, H.M.E. Maas, W.J. van Leeuwen and A.M. Henken*, January 2000)

2.12 Activities in Portugal on sero and phage typing

Ms. M. do Rosario Vieira (Portugal)

Due to the increasing number of human salmonellosis in the last years, the first issue of "Informações" 1999 is produced. This brochure is published by:

- The Bacteriology Centre of Instituto Nacional de Saúde, Dr. Ricardo Jorge (INSA) belonging to the Ministry of Health; and
- The Bacteriology Department of Laboratório Nacional de Investigação Veterinária (LNIV) that belongs to the Ministry of Agriculture, Rural Development and Fisheries.

Both institutes are responsible for *Salmonella* serotyping and phage typing. The main objective of this brochure was to publish the available laboratory data at both institutes, from 1995 to 1998 in different areas – animals, food and humans.
The cooperation between INSA and LNIV will continue and for year 2000 a second "Informações" is being prepared, containing, besides most common *Salmonella* serovars and phage types for *Salmonella* Enteritidis and *Salmonella* Typhimurium, also the findings on antimicrobials sensitivity testing.

2.13 *Salmonella* serotyping in poultry in Germany

Ms. C. Dorn (Germany)

Since 1997 to June 1999 2,118 *Salmonella* isolates from poultry were received at the NRL-*Salmonella* in Germany. Most (61%) were isolated from fowl, 23% from turkey, only 10% from ducks and geese and 6% from other poultry.
There is no question that *S.* Enteritidis predominated. It increased from 25% to 36% of all isolates in this period and was mostly found in fowl, in living fowl as well as in meat and eggs.

S. Typhimurium has increased as well to about 7% in 1997, reaching 18% of all isolates in 1999. It was found in fowl, ducks, geese and turkeys, especially in living poultry and poultry meat.
In contrast to this, *S.* Heidelberg (in turkey), *S.* Infantis (in fowl) and *S.* Hadar (in turkey and fowl) were decreasing to 1-4% of isolates. Today they are not as important as in the past.
S. Paratyphi B (d-tartrat positive) was slightly increasing in poultry meat, and its future prevalence should be observed.

2.14 Ideas for future collaborative typing studies

Since only 2 laboratories are interested in phage typing of different serotypes, for example, *S.* Virchow, this possibility will not be included in future typing studies.
In next typing studies, resistance patterns will be made by the laboratories which are able to do this. The laboratories can use their own method and those methods will be compared. It is also possible to prescribe the method which is the standard method of the EnterNet laboratories. This will be further discussed.

2.15 Trends in antimicrobial resistance in *Salmonella* in the United Kingdom

Mr. R. Davies (United Kingdom)

The first *Salmonella* isolate of a particular serotype from each incident is screened against a panel of 16 antimicrobials using a standard disc diffusion method. In general the percentage of *Salmonella* isolates other than *S.* Typhimurium or *S.* Dublin resistant to antimicrobials rose until a peak was reached in the late 1980s or early to mid 1990s. In the last few years resistance has been falling from previous levels, except for chloramphenicol resistance which was as prevalent in 1998 as its peak in 1994. Nalidixic acid resistance is also increasing. In the case of *S.* Typhimurium infection the predominance of DT104 during the 90s has influenced the situation. But again there has been a net reduction in the proportion of resistant strains in the last 2 years, except in the case of trimethoprim and apramycin resistance, which have remained stable, and nalidixic acid resistance, which has increased. Very little resistance is seen in *S.* Dublin and the prevalence of resistant strains has also been reducing. Resistance in *S.* Enteritidis is also rare but apramycin and nalidixic acid resistance is most common.

The pattern of antimicrobial resistance in *Salmonella* varies according to livestock species, with pigs having the lowest proportion of sensitive strains and sheep and poultry the highest. Cattle strain *Salmonella* show the highest levels of resistance to streptomycin, ampicillin, chloramphenicol and cephaperazone. These are all antimicrobials which have been most commonly used in the cattle sector, especially (not chloramphenicol) in the form of routine intra-mammary preparations. The high proportion of DT104 amongst cattle isolates will also

influence the situation however. Pig *Salmonellas* show the highest level of resistance to sulphonamides, tetracycline, neomycin, trimethoprim/sulphonamide and apramycin, which are all antimicrobials currently used as oral preparations for treatment of respiratory and enteric disease. Poultry *Salmonellas* show the highest proportional resistance to furazolidone and nalidixic acid, again reflecting the current and previous (furazolidone is not now permitted for use in the UK) use of these antimicrobials in the industry.

The predominant livestock sector in which nalidixic acid resistance appears to be a problem is the turkey industry. Limited MIC studies have shown that some of these isolates are also fully resistant to fluoroquinolones. In 1998 71.3% of DT104 isolates from turkeys were resistant to nalidixic acid compared with 4.8% in chickens. The problems also appear to be increasing in ducks (14.3%), cattle (11.1%) and pigs (10.7%). Nalidixic acid resistance is not exclusive to DT104 however and of *S.* Typhimurium strains other than DT104 tested 91.7% of these from turkeys, 3.6% from chickens, 6.8% from cattle, 13.6% from pigs and 15.4% from sheep were also resistant. Resistance to nalidixic acid is less common in *Salmonellas* other than *S.* Typhimurium but in 1998 31.2% of those from turkeys, 5.2% from chickens, 2.2% from ducks and 2.6% from pigs were also resistant.

A number of strains have also emerged which show resistance to a wider range of antimicrobials. Thus, as well as the typical penta-resistant resistance pattern in DT104 and isolated strains the addition of cefoperazone, trimethoprim or nalidixic acid resistance can produce strains resistant to 7, 8 or 9 of the 16 antimicrobials in the test panel. The most highly resistant strains appear to be predominantly associated with calves and appear as isolated outbreaks, rather than spreading more widely. This may suggest that highly multiple resistant strains could be either less virulent or less able to survive in the environment.

Discussion
There is no explanation for the decrease of resistance which occurred in 1987.
Increase in nalidixic acid resistance is seen in UK and the rest of Europe.

2.16 Multiresistant *Salmonella* Typhimurium in Finland

Ms. S. Pelkonen (Finland)

Multiresistant *Salmonella* Typhimurium DT104 is not endemic among production animals in Finland. Only five cattle herds have been infected with DT104 during 1995-1997, thereafter no farms have been found infected. In humans, the first case of multiresistant Typhimurium occurred in 1993. There are records for more than 30 years of human *Salmonella* infections contracted by Finns both in their own country (domestic infections) and abroad (foreign infections). In the last years, there have been annually 10 to 50 domestic infections with multiresistant *Salmonella*. The most common phage types have been DT12 and DT104. In addition, there was an extensive human epidemic caused by multiresistant DT12 in Southern

Ostrobothnia in 1997. During this epidemic it appeared that the multiresistant DT12 and DT104 phage types with similar resistance profiles are difficult to distinguish from each other. Therefore, we analysed the multiresistant isolates of phage types DT12, 29, 104, 120, U302, NT and NST, and susceptible isolates of the phage types DT12 and 104 by molecular methods. The isolates from the epidemic were sent to Colindale for confirmatory phage typing.

Altogether 85 isolates were analysed from humans, production animals, imported feedstuffs and environment. Of the isolates 55 were resistant to at least four antibiotics, and 42 isolates were resistant to ampicillin (A), chloramphenicol (C), streptomycin (S), sulphonamide (Su), and tetracyclin (T), having thus the most common DT104 profile ACSSuT. All but two of the multiresistant isolates possessed the two integrons, aadA2/qac/sul and pse-1/qac/sul, typical of the chromosomal multiresistance gene cassette of the major DT104 clone. One multiresistant isolate had only the integron aadA2/qac/sul, and one isolate had two completely different integrons similar to those previously described for multiresistance plasmids of *Salmonella*. One isolate resistant only to SSu possessed only the integron aadA2/qac/sul, suggesting that part of the multiresistance cassette had been deleted.

Macrorestriction profiles obtained with *Xba*I, *Sfi*I and *Bln*I enzymes grouped the multiresistant isolates of the phage types DT12, 29, 104, 104c, 104 var, 120, U302, NT, NST and the sensitive DT104 isolates into one clonal group. One particular combination of *Xba*I, *Sfi*I and *Bln*I macrorestriction profiles contained multiresistant isolates from the phage types DT12, 104, 104 var, 120, U302, NT, NST. All the sensitive DT12 isolates differed clearly from this group. Multiresistant isolates from the epidemic were confirmed to belong to DT12.

All six isolates from the epidemic grouped by all molecular methods as one epidemic strain, but the study did not help in identifying the source of the epidemic. The results demonstrate that Typhimurium strains of a variety of phage types belong to one major clone if they are multiresistant with the resistance cassette integrated in the chromosome. As sensitive DT104 strains belong to the same clone it is tempting to regard them as ancestors of the clone.

2.17 *Salmonella* Typhimurium DT104 in Danish pig and cattle herds

Ms. D.L. Baggesen (Denmark)

In early 1998, major public concern about the occurrence of multiresistant *Salmonella* Typhimurium DT104 in food and especially in pork provoked an interest in a future strategy for control of this organism. Therefore, two studies on the occurrence of *Salmonella* Typhimurium DT104 in the Danish swine and cattle production were planned and performed in 1998 and 1999. The studies were carried out in cooperation between the producer organisations (the Danish Bacon and Meat Council and the Danish Dairy Board) and the

veterinary institutions (Danish Veterinary and Food Administration and Danish Veterinary Laboratory).

The study in the pig production was carried out from June 1998 to February 1999 and comprised (a) all Danish breeding- and multiplying herds (n=366); (b) a random sample of farrow-to-grower herds (n=305); and (c) a random sample of slaughter pig producing herds (n=1962). All herds were examined by 10 samples. For practical reasons the slaughter pig herds were examined by 10 caecum samples (25g) from individual pigs, and the other herd types were examined by 10 pooled pen samples (5g faeces) from pigs 3-9 months of age. Conventional microbiological methods were applied. Herd prevalences (by herd-test cut-off was at least one isolate detected) and the overall prevalences (number of isolates / total number of samples) were calculated.

The study in the cattle production was carried out from September 1998 to March 1999. Included in the study was a random sample of 189 dairy and beef herds, which were investigated by conventional microbiological examination of four pooled faecal samples each representing five individuals and, if possible, a slurry sample. In addition, 76 “high risk herds” selected on the basis of serological examination of a single blood sample were included and examined as the random selected herds.

Salmonella enterica was detected in a total of 317 swineherds (51+43+223). The herd prevalences of *Salmonella enterica* in the three herd types (breeding and multiplying, farrow-to-grower, and slaughter pig) examined in 1998 were 11.7%, 16.7%, and 11.4%, respectively (Table 1). *S.* Typhimurium predominated with herd prevalences of 5.2%, 8.2%, and 7.1% in the three herd types. Multiresistant *S.* Typhimurium DT104 was detected in one slaughter pig herd.

Salmonella enterica was detected in 7 of the random selected cattle herds and in 13 of the “high risk herds”. *S.* Typhimurium was the most frequently isolated serotype. Multiresistant *S.* Typhimurium DT104 was not detected in the study. Considering information on serological classification as “high risk herd”, estimates on the prevalences were calculated (Table 1).

Table 1: Results of the bacteriological screenings for *Salmonella* in Danish swine and cattle herds in 1998/1999. The prevalence of *Salmonella enterica* in swine herds based on caecum samples from slaughter pig herds (SP) collected at slaughterhouses and on pooled pen samples collected at the farm from breeding and multiplying (BM) and farrow-to-grower (FG) herds. The prevalence in cattle herds based on 4 pooled faecal samples and a slurry sample.

Herds		*Salmonella enterica*		Multiresistant *S.* Typhimurium DT104	
Type	No.	Prevalence	95% CI	Prevalence	95% CI
Breeding- and multiplying herd	366	11.7 %	-	0.0 %	-
Farrow-to-grower herds	305	16.7 %	12.9 – 20.5 %	0.0 %	0 – 1.0%
Slaughter pig herds	1962	11.4 %	10.1 – 12.6 %	0.051%	0.001 – 0.28%
Pig herds, In total	2633	12.1 %	10.9 – 13.3 %	0. 043%	0.001 – 0.24%
cattle herds, in total	265	3.5 %	1.0 – 6.1 %	0.0 %	0 – 1.7%

The study documented that the prevalence of multiresistant *S.* Typhimurium DT104 in Danish swine and cattle herds was low. Therefore, the decision to eradicate this type of *Salmonella* from the Danish food animal production will also be followed up in the future.

In addition, the study documented that the prevalence of *Salmonella enterica* in Danish slaughter pig herds has decreased with approximately 50% since a similar study was performed in 1993/94 before the Danish Salmonella Control Programme was initiated (Baggesen *et al.*, 1996; Mousing *et al.*, 1997).

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2.18 Molecular detection methods for *Salmonella*

Mr. R. Helmuth (Germany)

Introduction

All DNA-based methods rely on the unique property of the DNA molecule to be double stranded. DNA is mainly composed of the phosphate deoxyribose backbone and the covalently linked bases, where adenine, thymine, guanine and cytosine interact by the formation of hydrogenebonds.

During cell division the single strands of the DNA molecule are separated and doubled by using the old strands as a template for the synthesis of two, new double stranded molecules. An enzyme called DNA-polymerase performs this synthesis.

The double stranded nature of the DNA and the tendency of the single strands to form basepairs, is the basis for all DNA based detection systems in microbiology. Today DNA detection methods have been developed for almost all important bacterial and viral pathogens and are in some cases the only choice for the detection of a micro-organism.

DNA probes

DNA probes are small parts of a genome which can be specifically labelled. They are used for the detection of the complementary DNA sequence in a so called hybridisation assay (for a review see Wolcott 1991). The first step of the method is the denaturation of the target DNA, which results in strand separation. This is generally achieved by heat or an alkaline pH treatment. The resulting single stranded DNA, which is in most of the cases fixed to a matrix, is subsequently used as a target to test if base pairing to the gene probe exists under renaturing conditions. Duplex formation is detected by various staining techniques.

In the case of *Salmonella* several DNA probes have been used (Table 1).

Table 1: DNA-Probes used for the detection of *Salmonella*e

1983	Fitts et al.	Random chromosomal fragment	3.6 Kb
1988	Gopo et al.	Random chromosomal fragment	1.8 Kb
1989	Tsen et al.	Random chromosomal fragment	1.8 Kb
1990	Scholl et al.	Random chromosomal fragment	1.6 Kb
1990	Wilson et al.	RRNA	
1991	Olsen et al.	Random chromosomal fragment	2.3 Kb
1991	Montenegro et al.	Spv Region	3.6 Kb
1992	Aabo et al.	Random chromosomal fragment	2.3 Kb (1.3+0.8Kb)

Initially duplex formation was detected by autoradiography using radioactive labels (Fitts *et al.* 1983). However, the problems involved (special laboratories, training of staff, decontamination etc.) led to the development of alternative, nonradioactive detection systems. Especially the biotin-streptavidin and digoxigenin systems have been widely used.

On a commercial basis the Gene Track *Salmonella* dip stick assay has been put on the market first. It makes use of a 30-40 bp gene probe detecting ribosomal 16 or 23 S RNA (rRNA) which is present in high copy numbers in exponentially growing cells (Wilson *et al.* 1990). The hybridisation assay is based on the use of actually two probes. Both have homology to the rRNA target and are called the capture and detection probes. The capture probe forms the duplex with the rRNA and is used to bind it to the dip stick via a polydeoxiadenyl acid tail. The detector probe in turn is fluorescin labelled and is used for a colorimetric visualisation of the hybridisation product. In 1988, this *Salmonella* assay has been adopted as a first action method by the association of official analytical chemists (AOAC) in the USA. Baily *et al.* (1991) compared this DNA method with cultural methods in 390 broilers with high sensitivity and specificity.

PCR Methods

Today most of the research focuses on methodologies which are based on the so called polymerase chain reaction (PCR). It was discovered in 1985 by Karry Mullis and allows the almost indefinite amplification of a specific DNA sequence (Mullis *et al.* 1987). The basic components of a PCR reaction mixture are given in Table 2.

Table 2 Components in a PCR assay

template DNA	1.5 µg - 1 pg (10^2-10^5 molecules)
specific oligonucleotide primers 1 & 2	1 µM ($6x10^{13}$ molecules)
Deoxyribonucleoside triphosphates (dNTPs)	0.5 mM ($3x10^{16}$ molecules)
Taq DNA polymerase	several units
$MgCl_2$	1-10 mM

First the template DNA is made single stranded by heating it to 95°C. Cooling to temperatures between 37°C and 60°C allows the specific binding of the primers, which mark the start and endpoints of the short DNA fragments generated. At 72°C the Taq polymerase synthesises the double stranded DNA fragments between the primers, and another heating to 95°C generates single stranded fragments again. The repetition of this cycle in a thermocycler can generate about $1x10^9$ (17 pmol) of short fragmented DNA within 30 cycles. They can be detected by gelelectrophoresis, ELISA or real time PCR quantification.
Table 3 lists some of the primers so far used for the detection of *Salmonella*e. The targets used are manifold and allow the detection of the whole genus or specific groups of *Salmonellae* like certain serovars or motile serovars only. The virulence plasmid derived primers allow the detection of only those serovars, which are plasmid carriers, among them are *S.* Typhimurium and *S.* Enteritidis.

Most of the studies use the so called enrichment broth PCR (Table 4). It basically involves a short incubation of the sample in non selective broth (mainly buffered peptone water) and subsequent PCR. Another approach frequently used is the magnetic immuno PCR (MIPA)

which makes use of a magnetic separation technique (Widjojoatmodjo *et al.* 1991). Superparamagnetic latex beads are used to separate *Salmonellae* from inhibiting substances which interfere with PCR.

Almost all of the surveys are qualitative, however, the study performed by Mahone and Lax (1993) is quantitative. They used primers, which detect a 500 bp product of the spvR gene of the *Salmonella* virulence plasmids. By adding an internal competitor, e.g. spvR with a 94bp deletion, which gives rise to a 406 bp product, quantification is possible on the following reasons. The known amount of the competitor in the sample can serve as an internal standard. If the amount of competitor equals the amount of the target, both bands have the same intensity. If the amount of the competitor is higher than the target, its band is more intense. If the amount of competitor is less than the target, its band is less intense.

Table 3 Primers used for the detection of *Salmonellae*

Specificity	Target	Product size	Primer sequences Orientation: 5'-3'	Reference
Salmonella genus	Origin of replication	163 bp.	P1:TTATTAGGATCGCGCCAGGC P2:AAAGAATAACCGTTGTTCAC	Widjojoatmodjo et al. (1991)
Salmonella genus	InvA	284 bp	139:GTGAAATTATCGCCACGTTCGGGCAA 141:TCATCGCACCGTCAAAGGAACC	Rahn et al. (1992)
Salmonella genus	Random genomic fragment	429 bp	ST11:AGCCAACCATTGCTAAATTGGCGCA ST15:GGTAGAAATTCCCAGCGGG TACT	Aabo et al. (1993)
Motile *Salmonella*e	Flagellin genes	236 and 173 bp.	Hin:1750-L:CTAGTGCAAATTGTGACCGCA 1750-R:CCCATCGCGCTACTGGTATC H-li:1788-L:AGCCTCGGCTACTGGTCTTG 1789-R:CCGCAGCAAGAGTCACCTCA	Way et al. (1993)
Salmonella genus	Rfb-genes	Gr. A, D 720 bp Gr. C_2:820 bp. Gr. B: 882 bp	1-rfbl(B):AGAATATGTAATTGTCAG 2-rfbl(B):TAACCGTTTCAGTAGTTC 3-rfbl(C2):ATGCTTGATGTGAATAAG 4-rfbl(C2):CTAATCGAGTCAAGAAAG 5-rfbS(D):TCACGACTTACATCCTAC 6-rfbS(D):CTGCTATATCAGCACAAC	Luk et al. (1993)
Salmonella genus	Fimbriae	261 bp	TAF3:TCCGGCCCGGACTCAACG TAF4:CAGCGCGGCGTTATACCG	Doran et al. (1993)
Salmonella genus	His Operon	496 bp	ACTGGCGTTATCCCTTTCTCTGGTG ATCTTGTCCTGCCCCTGGTAAGAGA	Cohen et al. (1993)
spv *Salmonella*e	SpvR	500 & 406 bp	spvR1:ACAGGTTCCTTCAGTATCGC spvR2:CTGTTGATATCAGGTTT spvR3:TCTGAAATAACCCTGCTCAG	Mahon and Lax (1993)
Salmonella genus *S*. Typhimurium *S*. Enteritidis	Rand. geno. fragment FliC gene SefA gene	429 bp 559 bp 312 bp		Soumet et al. (1999)

Today however the quantification of PCR products is achieved by a sophisticated chemistry of light emitting molecules. In one system the 5`-3` exonuclease activity of the taq polymerase is used to separate a light emitting reporter molecule from a light quencher, which results in a fluorescent signal. Another approach makes use of an increasing light emission provoked by a dye which binds to the minor groove of double stranded DNA. Both systems develop fast and offer a wide variety of possibilities for the sensitive automatic detection of foodborne pathogens.

Table 4 Studies describing the detection of *Salmonella*e by PCR

MIPA[1]	10^2	Pure culture	Luk and Lindberg (1991)
MIPA[1]	10^5cfu/ml	Humans	Widjojoatmodjo et al. (1991)
Multiplex	1 cfu/assay	Filtered water	Way et al. (1993)
Enrichment Broth	10^2 cfu/g	Equine faeces	Cohen et al. (1994)
Enrichment Broth	10^2	Pig, bovine, equine	Stone et al. (1994)
Enrichment Broth	not given	Diff. meat	Aabo et al. (1995)
Enrichment Broth	not given	Dog faeces	Stone et al. (1995a)
Enrichment Broth[2]	20 cfu/assay	Veterinary clinical specimens	Stone et al. (1995b)
Sandwich[3]	50cfu/assay	Pure culture	Summit et al. (1995)
Enrichment Broth[4]	<=10cfu/750g	Diff. food	SCE et al. (1998)
Rough colonies	not given	Pure culture	Hoofer et al. (1999)

1 Magnetic Immuno PCR
2 PCR Oligonucleotide Libation Assay
3 Solid Phase Chemiluminescent Assay
4 PCR-ELISA

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Discussion

A lot of development will be done to the detection of *Salmonella* by PCR. A lot of companies are working on a method for detection by PCR for Salmonella. PCR will be used routinely soon.

2.19 Validation diagnostic PCR for food pathogens (EU project)

Ms. A. Miko (Germany)

In the last couple of years there has been a great interest in the standardisation and harmonisation of PCR assays. In Germany a standardisation protocol for the detection of micro-organisms and especially *Salmonella* has been released recently (Anonymous 1999). Their adoption by the European CEN has been suggested, which would lead to a European PCR standard.

In this line the EU has funded a thematic network on “Validation and standardisation of diagnostic Polymerase Chain Reaction (PCR) for detection of foodborne pathogens. Starting in 1999 under the leadership of Dr. Jeffrey Hoorfar from the Danish Veterinary Laboratory it has the overall objective to facilitate implementation of diagnostic Polymerase Chain Reaction for both verification and detection of foodborne pathogens in Europe. To this end a

research group was established whose goal will be the development, harmonisation and validation of PCR methodology throughout Europe. The work will be limited to five major zoonotic pathogens: *Salmonella* spp., Campylobacter spp., enterohemorrhagic Escherichia coli (EHEC), Listeria monocytogenes and Yersinia enterocolitica. Naturally contaminated and spiked samples from pig carcass swabs and poultry carcass rinse, and two major types of food (raw meat and milk) will be included as models in the validation trials.
The study involves 34 veterinary institutes, food laboratories, diagnostic companies and universities from all EU Member States (except Luxembourg) and, additionally, from Poland, the Czech Republic, Slovakia, Estonia, Norway and Switzerland (21 countries).

The information given here shows, that PCR offers the possibility to detect *Salmonellae* with high sensitivity and specificity. Comparative studies show the use and reliability of PCR in field surveys. The costs can be competitive with cultural examinations. For this reason the near future will bring major progress in its applications.

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2.20 A collaborative study for *Salmonella* detection by PCR

Six NRLs replied positively on a postal survey in the CRL-*Salmonella* Newsletter about a collaborative study on PCR detection of *Salmonella*. We now have to find out how to organise such a study. Some options are discussed and the following decisions are made:

1. All laboratories which are interested can use their PCR method during the fifth bacteriological collaborative study. All results can then be compared.
2. When necessary a training course might be organised after the next workshop.

2.21 Immunological collaborative study with 11 labs in France

Ms. K. Proux (France)

As it is indicated in the Zoonoses Directive 92/117/EEC serology may, after agreement of the Scientific Veterinary Committee, be accepted. Provided that it offers guarantees equivalent to bacteriological culture methods. At the present time bacteriology is used in the national surveillance system dated 26 October 1998 which concerns measures against *Salmonella* Enteritidis (SE) and Typhimurium (ST). But serological methods are rapid and cheap and may be used as an initial screening of flocks. When serological positive flocks are identified, they are confirmed by bacteriological culture.

Our aim is to compare serology to bacteriology. The project is divided into 2 steps. During the first step presented here our serological method was transferred to the diagnostic laboratories and a ring trial was organised in order to validate labs to analyse samples collected on conventional flocks. For the second step serology and bacteriology will be compared on meat breeders and layers.

Our indirect ELISA is based on lipopolysaccharide antigens of SE and ST. Sera are diluted 1:300 and prediluted yolks (1:10) are diluted 1:30. This test has already been tested experimentally on birds reared in a restricted area (K. Proux et al. 1998; V. Kles et al. 1992).

At first control sera and yolks were produced on 60 SPF hens, 20 weeks of age. The hens were inoculated with 10^8 cfu SE or 10^7 cfu ST. Sera and yolks were collected on animals sacrificed at the peak of seroconversion and selected for the ring trial. Moreover the same reactives were sent to the 11 laboratories (same lot numbers). The following criteria were studied on several samples: accuracy, repeatability, reproducibility, dose-effect, sensitivity and specificity (Table 1).

Table 1 Criteria and samples studied for the ring-trial

Criteria	Samples
Accuracy	22 positive samples (OD>0.150) -2 sera repeated 10 times -2 yolks repeated 10 times -18 sera and yolks repeated 2 times
Repeatability	2 sera and 2 yolks (SE and ST) repeated 10 times
Reproducibility	2 plates tested on 2 different days
Dose-effect	Serial dilution of 2 sera (SE and ST) from 1:2 to 1:32
Sensitivity	4 weakly positive sera and yolks
Specificity	Negative samples: 3 sera and 2 yolks

About accuracy an interval was calculated for each sample, it was equal to the mean value of all laboratories +/- x times standard deviation SD, with ‘x SD’ given in the table of Student depending on the number of analyses on the sera. For example the yolk sample against ST was tested 100 times (10 times by 10 labs) and the x value is equal to 1.66 and the interval was the mean value +/- 1.66 SD. It was expected that for each lab the mean value of this sample was belonging to the interval. In fact only one lab presented mean values of all samples above the upper limits of the intervals.

A variation coefficient (VC) was calculated to test repeatability and for all labs VC was under 20% for all samples and these results were always correct on the second day. For reproducibility, results on 18 positive samples were compared when tested on day 1 and 2

using a Wilcoxon test. No significant difference was observed between day 1 and 2 on the mean value of all labs ($p=0.145 > 0.05$) so this method seems to have a good reproducibility. When results were studied for each lab a significant difference was seen between day 1 and 2 for 4 labs ($p<0.05$), but in fact results were improved on the second day because labs were more used to this ELISA (Table 2).

Table 2 Reproducibility, comparison between day 1 and 2 of 18 positive samples

	Lab number										
	1	2	3	4	5	6	7	8	9	10	11
p	0.157	0.000	0.879	0.022	0.006	0.948	0.557	0.002	0.267	0.356	0.145

About dose-effect serial dilutions of 2 sera (ST and SE) were tested and results were correctly ranged because the more the sera were diluted, the more the COD were lower for all labs. Concerning sensitivity it was observed that 4 weakly positive sera were sometimes classified as negative or positive by one or more labs. According to this remark it was decided to include a new class of sera. Sera with COD between 0.150 (cut-off) and 0.400 are considered as inconclusive. At last, all laboratories classified all the 5 negative samples as seronegative when tested on day 1 and 2, proving a correct specificity.

To conclude with the ring trial lab 2 was excluded because all mean values of all samples were above the upper limit of tolerance of our interval statistically calculated. Furthermore this ring trial was the first step of our project and now 10 labs have been selected for the next step of the study. This comparison will occur on 70 flocks meat breeding and laying types: 30 potentially positive to test sensitivity, 30 potentially negative to test specificity and 10 vaccinated flocks in order to follow immunity stimulation and so to assess efficacy of the killed vaccine allowed in our country. Moreover, for serological analysis 20 samples (sera on breeding flocks and yolks on layers) and for bacteriological analysis one environmental swab and one pooled faeces sample will be collected monthly in each flock, and these flocks will be followed from 20 to 55 weeks of age.

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Discussion
The hens were infected at an age of 20 weeks.
Pooling of samples was tested. Some of the weakly positive samples were found negative when pooled with other, negative, samples. Therefore it was decided not to pool samples.

2.22 Application of Transia Elisamatic II for *Salmonella* detection in poultry samples

Mr. J.C. Jorgensen (Denmark)

The purpose of this study was to evaluate to which degree the Transia Elisamatic II for *Salmonella* detection (SEP) method without modifications is applicable to 'sock' samples as used in the Danish *Salmonella* Control Programme for Poultry.

Transia Elisamatic II for *Salmonella* detection (SEP) was developed for use in the food industry. The kit was performance tested by AOAC Research Institute, AFNOR and NMKL (DanVal) and was found to perform to the manufacturer's specifications.
The SEP (Short Enrichment Protocol) version is based on a traditional pre-enrichment in buffered peptone water and a selective enrichment in Rappaport-Vassiliadis bouillon with soya, followed by a sandwich ELISA method.

Methods
All samples in the study were examined by the routine cultivation method used by the laboratory, i.e. pre-enrichment in buffered peptone water and a selective enrichment in Rappaport-Vassiliadis bouillon with soya, followed by streaking on Rambach agar, and the Transia Elisamatic II for *Salmonella* detection (SEP).

Results
A total of 1812 field samples from 366 flocks were tested. The results are shown in Table 1.

Table 1 Field samples. Sensitivity, specificity and predictive values

		Cultural method		
		Positive	Negative	Total
Diffchamb method	Positive	97	5	102
	Negative	13	1697	1710
	Total	110	1702	1812

Sensitivity 97/110 = 0.882
Specificity 1697/1702 = 0.997
Predictive value, positive test 97/102 = 0.951
Predictive value, negative test 1697/1710 = 0,992

Conclusions
The Diffchamb method is substantially equivalent to the culture method used at present. It does however fail to discover some samples positive by the culture method.
Before the study is fully published an investigation remains of the strains not detected by the Diffchamb method.
Considering that the method was developed to food that normally is not as heavily contaminated as faecal samples the results are very promising.

Discussion
Each pair of socks is examined separately and diluted 1:10 in Buffered Pepton Water.

2.23 Vidas as selective enrichment or detection system

Ms. N. Voogt (the Netherlands)

Introduction
The VIDAS (= Vitek Immuno Diagnostic Assay System) is a method, for the detection of *Salmonella*, based on an immunological technique. The method is developed to detect *Salmonella* in food, food ingredients and environmental samples. The VIDAS is an automated immunoanalyser in which 12 (mini-VIDAS) or 60 samples can be tested at the same time. It is a multi-task instrument that can perform both classical techniques like immunoanalysis and more original techniques like automated immunoconcentration. In addition to *Salmonella* it is also possible to test other organisms like *E. coli O157, Staphylococcus, Campylobacter* and *Listeria*.
In our laboratory the VIDAS was tested twice; the first time the immunoanalysis (VIDAS SLM) and the second time the automated immuno-concentration *Salmonella* (VIDAS ICS) was carried out. In both cases you need a strip containing all necessary reagents and a tip coated with anti-*Salmonella* antibodies.
The principle of the VIDAS is simple and based on a specific reaction between an antigen and an antibody. A pipette, the Solid Phase Receptacle (SPR), serves as the solid phases (coated with anti-*Salmonella* antibodies) as well as the pipette for the assay.

VIDAS SLM
Principle
The VIDAS SLM is developed as an enzyme-linked fluorescent immuno-assay (ELFA) for the detection of pathogens in food industry. There is a completely automated analysis from sample insertion to result print-outs. An aliquot of the pre-enrichment medium is placed into the reagent strip and after starting the analyser the test is carried out automatically. After some washing cycles *Salmonella* antigens, if present in sample, will be captured by the antibodies. Then conjugate and substrate are added to the system and the intensity of the reaction is measured and interpreted by the system. The values of the samples are compared

with values of standards stored in the computer. The final report contains, among others, the results given as either ‘positive’ or ‘negative’.

Protocol

In our study faecal samples of poultry layer flocks, broiler flocks, veal calves and dairy cows were tested. These samples were collected in the framework of a Dutch surveillance programme of zoonotic agents in farm animals. The standard method used in the surveillance programme to detect *Salmonella* is a modified ISO 6579 method with the use of one selective enrichment medium (RV), one selective solid medium (BGA) and three biochemical tests for confirmation (TSI, urea and LDC).

Protocol SLM: after pre-enrichment in buffered peptone water, there was a selective enrichment step for 6 hours in RV. After these 6 hours one ml was transferred into 10 ml M-broth, supplied by the manufacturer. The M-broth was incubated for 18 hours at 42 °C. After incubation one ml from the M-broth was brought into a tube and heated for 15 minutes in a water bath. Half a ml of the sample was dispensed into the strip of the VIDAS that contains all the ready-to-use reagents required. The strip was inserted into the system along with the SPR and in approximately 45 minutes the assay was completed. If the VIDAS was positive a sample was taken from both the M-broth and RV for further confirmation on plate.

Results

In total 645 faecal samples were tested for *Salmonella* (277 of poultry layer flocks, 150 of broiler flocks, 88 of veal calves and 130 of dairy cows). Thirty-three faecal samples were found positive using both methods. Using the VIDAS instrument, 4 of these 33 samples could not be confirmed as *Salmonella*. Four of the 645 faecal samples were only positive using the modified ISO. In total 21 faecal samples were only positive using the VIDAS instrument. Five of these 21 samples could be confirmed as *Salmonella.*

<u>VIDAS ICS</u>

Principle

The principle of the VIDAS ICS is based on an automated immunological enrichment. It replaces the successive steps of selective enrichment using RV or other selective enrichment broths and it is performed in three steps. The pipette, coated with antibodies, captures the *Salmonella* present in the pre-enrichment broth. After different washing steps only *Salmonella* antigens were adsorbed on the SPR surface. Through a specific release process an immuno-concentrated solution of *Salmonella* could be collected from the strip and used for further detection.

Protocol

In our study faecal samples of only poultry layer flocks and broiler flocks were tested. In the standard method DIASALM was used as selective enrichment medium besides RV (see protocol VIDAS SLM). The protocol used for the ICS was identical to this method with exception of the selective enrichment step.

Results

In total 308 faecal samples were tested for *Salmonella*. Twenty-six of these samples were found positive with both methods. Eleven of the 308 samples were only found positive using

the ISO method and in total four faecal samples were only positive with the use of the VIDAS ICS.

Preliminary conclusions

(These are preliminary conclusions, because the results were not yet statistically analysed)

- The results achieved with the SLM assay show a lot of false-positive samples; about 40% of the positive results could not confirmed on plate.
- The results achieved with the ICS assay show a lot of false-negative samples; about 30% of the positive results with the standard method were negative with the ICS.

Discussion

There are laboratories that have experience using an ELISA as enrichment. It revealed better results compared to standard method.

2.24 CRL-*Salmonella* activities in 2000

Mr. A.M. Henken (the Netherlands)

The regular yearly working programme of CRL-*Salmonella* contains the following activities:

- The organisation of two collaborative studies;
- The publication of 4 newsletters, one at the end of each quarter;
- The organisation of a workshop with the NRLs for *Salmonella* of the Member States;
- Performance of ad hoc activities when necessary (e.g. on request of EC); and
- To some extend performance of research on detection methods and reference materials in relation to the collaborative studies organised.

These activities were discussed at the workshop. The participants agreed on some specific issues as stated below:

With respect to the next bacteriological collaborative study

The participants proposed not to include DIASALM for the selective enrichment step in the next study, but to continue with MSRV. Furthermore it was proposed to adapt the standard method to the new ISO 6579 meaning that RVS will be used for selective enrichment instead of RV and that XLD as well as BGA will be used for isolation. The contamination levels of the capsules used in the last study seem to be right and acceptable. However, if logistically possible, the participants would appreciate it to include also faeces containing other naturally occurring serotypes for detection in the study.

With respect to the next collaborative typing study

The next typing study will be held in March 2000. This study will be organised again in co-operation with PHLS and the EnterNet laboratories. In the study 20 serotypes frequently occurring in Europe will be selected. Laboratories interested can perform antibiotic resistance pattern typing on these strains using their own methods. For phage typing again 10 *S.*

Typhimurium and 10 *S.* Enteritidis strains will be included. No phage typing on other serotypes, like *S.* Virchow, will be included.

With respect to a possible collaborative study using PCR

Some participants are interested in a collaborative study on detection of *Salmonella* by PCR. Laboratories that are interested in performing PCR can do so in addition to the next bacteriological study that will be organised in autumn 2000. The results will be evaluated and if necessary a training session will be organised, details of which will be discussed next year.

With respect to some other points

1. Questions have arisen about the composition of Tetrathionate enrichment broth available from different manufacturers. There seems to be quite some variation. It was decided to make an inventory on this through the CRL-*Salmonella*.
2. The question was raised whether the CRL-*Salmonella* could provide NRLs that are interested with capsules to facilitate their national activities with respect to the organisation of bacteriological collaborative studies. At present the CRL-*Salmonella* does only provide these capsules for its own collaborative studies. Capsules can be bought at SVM (Bilthoven), but the participants consider these expensive while also the variation in choice of concentration is limited. However, the CRL-*Salmonella* promises to make an inventory among the NRLs to see what exactly the need is and when this need proves to be substantial shall discuss with SVM what options are available.
3. A participant brought a question forward whether for the next workshop the report could be ready before the start. This will be discussed by the CRL team when organising the next workshop.
4. E-mail is proposed as a way that should be used more frequently to facilitate communication on SOPs, protocols, test reports and other things. A list of e-mail addresses shall be made by the CRL and published in the newsletter.
5. At each workshop an issue is chosen for in-depth presentation and discussion. For next year two issues are brought forward: 1. Issues on (pooled) sampling; and 2. Antibiotic resistance. With respect to antibiotic resistance it was proposed to exchange information with the EnterNet laboratories as they are already standardising antibiotic resistance typing. The possibility of a shared workshop was mentioned.

2.25 Closing remarks

Mr. A.M. Henken (the Netherlands)

Evaluation

The workshop was evaluated by posing the following three questions:

1. Were the objectives of the workshop met?
2. Are the plans of the CRL-*Salmonella* for the near future clear?
3. Are there things in general that should change?

The participants agreed that the objectives of the workshop were met and that the plans for the year 2000 were clear. The participants asked whether it would be possible for the CRL-

Salmonella to provide the NRLs with capsules for their use in national trials. The CRL-*Salmonella* promised to investigate this matter further.

Closing remarks

The workshop was closed by thanking the participants for their contribution in the presentations and discussions, the Commission for making the workshop possible by providing the necessary budget, the CRL team for the work done presented at the workshop and, last but not least, the workshop team for organising the workshop.

Appendix 1 Mailing list

01.	European Commission	A. Checchi Lang
02.	European Commission	B. Hogben
03.	European Commission	V. Niemi
04.	President of the Council of Health, the Netherlands	prof. dr. J. J. Sixma
05.	Veterinary Public Health Inspector	drs. H. Verburg
06-41	Participants of the workshop	
42.	Dutch National Library for Publications and Bibliography	
43.	Board of Directors RIVM	dr. G. Elzinga
44.	Head of Microbiological Laboratory for Health Protection and Director CRL-*Salmonella*	dr. ir. A.M. Henken
45.	Authors	
46.	SBD/Information and Public Relations	
47.	Registration agency for Scientific Reports	
48	Library RIVM	
49-65	Sales department of RIVM Reports	
66-75	Spare copies	

Appendix 2 Participants of the workshop

European Commission, Legislation Veterinaire et Zootechnique	Mr. V. Niemi

From the National Reference Laboratories for *Salmonella*:

Austria	Mr. W. Thiel
Belgium	Ms. I. d'Hooghe
Denmark	Ms. D.L. Baggesen Mr. J. C. Jorgensen Mr. M. Madsen
Finland	Ms. T. Johansson Ms. S. Pelkonen
France	Ms. F.S. Humbert Ms. K. Proux
Germany	Ms. C. Dorn Mr. R. Helmuth Ms. A. Miko
Greece	Ms. M. Passiotou-Gavala
Ireland	Mr. J. Egan Mr. J. Ward
Italy	Ms. C. Polera
Luxembourg	Mr. J. Schon
The Netherlands	Ms. N. Voogt
Portugal	Ms. A. Amado Ms. M. Do Rosario Vieira
Spain	Ms. C. Rubio Montejano

Sweden	Ms. A. Aspen
	Mr. E. Eriksson
United Kingdom	Mr. R. Davies
	Mr. S. McDowell
CRL-*Salmonella*	Mr. A. Henken
	Ms. N. van Leeuwen
	Mr. M. Raes
	Ms. S. Schulten
	Mr. W. Wannet
Invited speaker:	Mr. W. v. Pelt (The Netherlands, RIVM)

Appendix 3 Programme of the workshop

Wednesday 3 November 1999

21.00 - 22.00	Social get together in the bar of hotel Mitland in Utrecht

Thursday 4 November 1999

08.30 - 09.00	Opening and introduction of participants
09.00 - 10.00	The new zoonoses directive
10.00 - 11.00	Discussion about the new zoonoses directive with 3 presentations: -Salmonella control measures in poultry production: ambitions and realities -associations serotypes/products and costs
11.00 - 11.30	Coffee/tea (photo)
11.30 - 12.00	Evaluation of the fourth bacteriological collaborative study
12.00 - 12.15	Proposed revision ISO 6579
12.15 - 13.15	Lunch
13.15 - 14.45	National activities with respect to bacteriological detection: -bacteriologic collaborative study with 11 labs in France -bacteriologic collaborative study in the Netherlands -comparison tetrathionate and Muller-Kauffman -overview current research S detection at VLA Weybridge
14.45 - 15.00	Ideas for future bacteriological collaborative studies
15.00 - 15.30	Coffee/tea
15.30 - 16.00	Results of collaborative typing study
16.00 - 16.45	National activities with respect to sero- and phage typing: -activities Portugal on sero- and phage typing -report about Salmonella serotyping in poultry in Germany
16.45 - 17.00	Ideas for future collaborative sero- and phage typing studies
17.00 - 18.00	Antibiotic resistance: -trends in antimicrobial resistance in S in the UK -research on multiresistant ST in Finland -screening for ST DT104 in Danish pig and cattle herds
19.30 - 21.00	Dinner
21.00 - ?	Free (bowling (22.00 - 23.00)/visit Utrecht/ SALMOVA meeting)

Friday 5 November 1999

08.30 - 09.30	Molecular detection
09.30 - 09.50	Validation diagnostic PCR for food pathogens (EU-project)
09.50 - 10.15	A collaborative study for Salmonella detection by PCR?
10.15 - 10.45	Coffee/tea
10.45 - 11.45	Immunological detection: -immunological collaborative study with 11 labs in France -application of Transia Elisamatic II for S detection in poultry samples -VIDAS
11.45 - 12.15	Discussion about activities CRL-Salmonella 2000
12.15 - 12.45	Closing remarks
13.00 - 14.00	Lunch
14.00	Departure to railway station and airport/CRL-Salmonella team meeting