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Report on the second workshop organized by CRL *Salmonella*

Bilthoven, the Netherlands, 29 - 30 May 1996

N. Voogt, A.M. Henken (editors)

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This workshop has been organized in order and for the account of the European Commission, Legislation Veterinaire et Zootechnique within the framework of MAP project 284500 by the Community Reference Laboratory for *Salmonella*.

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SUMMARY

On 29 and 30 May 1996 a workshop was organized by the Community Reference Laboratory (CRL) for Salmonella in Bilthoven, the Netherlands. All National Reference Laboratories (NRLs) for Salmonella, except Spain, participated (in total 27 persons).
Various topics were discussed, such as organizational aspects of collaborative studies within Member States, coordination of activities of the CRL and specific needs for training.
The collaborative studies on bacteriological detection and (sero-)typing of *Salmonella* were evaluated and discussed. The preliminary results of the collaborative study for immunological detection of S. Enteritidis were presented.
Furthermore the infrastructure and experiences on collaborative studies within the Member States were presented and discussed. Part of the participants presented research activities on bacteriological and immunological detection and on serotyping of *Salmonella* of their own country.
Within the framework of the use of alternative methods, a representative of the Dutch Standardization Institute held a presentation about the Microval project.
In the near future the CRL will organize several more collaborative studies and publish a book on methods used for *Salmonella* detection as well. The main communication channel between the CRL and NRLs will be the yearly workshop and the quarterly newsletter.

SAMENVATTING

Op 29 en 30 mei 1996 is door het Communautair Referentie Laboratorium (CRL) voor Salmonella een workshop georganiseerd in Bilthoven, Nederland. Alle Nationale Referentie Laboratoria (NRLs) voor Salmonella, met uitzondering van Spanje, waren vertegenwoordigd. In totaal waren er 27 deelnemers.
Verscheidene onderwerpen werden besproken, onder andere de organisatorische aspecten van ringonderzoeken binnen lidstaten, de coördinatie van CRL-activiteiten en de mogelijk bestaande trainingsbehoeften.
De bacteriologische en serotyperings ringonderzoeken werden geëvalueerd en besproken. De voorlopige resultaten van het immunologische ringonderzoek voor de detectie van Salmonella Enteritidis werden gepresenteerd.
Daarnaast werden infrastructuur en ervaring met ringonderzoeken binnen de lidstaten zelf belicht. Een aantal deelnemers vertelde over de onderzoeksactiviteiten die in hun land plaatsvinden op het gebied van bacteriologische en immunologische detectie en serotypering van *Salmonella*.
In het kader van het gebruik van alternatieve methoden was er een gastspreker van het Nationale Normalisatie Instituut, die het Microval project toelichtte.
In de toekomst zal het CRL diverse ringonderzoeken organiseren en een methodenboek voor *Salmonella* detectie uitbrengen. De communicatie tussen CRL en NRLs zal voornamelijk plaatsvinden door middel van een jaarlijkse workshop en een Newsletter, die vier maal per jaar verschijnt.

1. INTRODUCTION

1.1. Opening

by Dr. Ir. A.M. Henken (Director CRL Salmonella)

1.1.1. Functions and duties of CRL Salmonella

The functions and duties of CRL Salmonella can be summarized as:
- providing national laboratories with details of analytical methods and comparative testing;
- coordinating application by national reference laboratories of methods, referred to under the first mentioned point, in particular by organizing comparative testing;
- coordinating research into new analytical methods and informing national laboratories of advances in this field;
- conducting initial and further training courses for the benefit of staff from national reference laboratories and
- providing scientific and technical assistance to the Commission of the European Communities.

1.1.2. Aims of the workshop

The aims of the present workshop are:
- to discuss results of collaborative studies CRL with NRLs;
- to discuss organizational aspects of collaborative studies within Member States;
- to discuss how to coordinate our activities in view of the task given;
- to discuss whether or not there are specific needs for training;
- to discuss how to optimize communication between CRL and NRLs.

2. REVIEW OF THE PRESENTATIONS

2.1. Evaluation of the first collaborative study on bacteriological detection of *Salmonella*

by Ir. P. H. in 't Veld (RIVM/CRL the Netherlands)

A bacteriological collaborative study was organized by the Community Reference Laboratory (CRL) for *Salmonella*. In this study the National Reference Laboratories (NRLs) for *Salmonella* of the Member States of the European Union (EU) participated.
The aim of this study was to compare the results of the proposed reference method for the detection of *Salmonella* (ISO 6579 method) among and within laboratories and the results of the reference and routine method within a laboratory.
Seventeen laboratories examined 30 individually numbered capsules of which 26 contained circa 5 colony forming particles (cfp) per capsule of S. Typhimurium in total and 4 were negative control samples. The number of these capsules was unknown to the participants.
Besides the reference method the laboratories were free to perform the routine method (their own method) in their laboratory.
Because one laboratory did not use the reference method and two laboratories reported positive *Salmonella* isolations from negative control samples, the results of these laboratories were excluded from statistical analysis.
Using the reference method, one laboratory found significantly more capsules negative in comparison with other laboratories. Among the remaining 13 laboratories no significant difference was found in the number of positive *Salmonella* isolations. Eight laboratories performed their own method and no significant difference was found between their own and the reference method for the individual laboratories.
Three laboratories with deviating results carried out the study once again. In that additional study no deviating results were found anymore.

2.2. Proposal for the second collaborative study on bacteriological detection of *Salmonella*

by Ir. P. H. in 't Veld (RIVM/CRL the Netherlands)

The first bacteriological study evaluated the performance of the detection of low numbers of sublethally injured S. Typhimurium without any competitive microorganisms present. In this study the selectivity of the method was, therefore, not evaluated.
A new reference material has become available which was prepared in a comparable manner as the S. Typhimurium reference material. This new reference material contains a S.Enteritidis strain which is resistent to rifampicine.
For the second study it was proposed to evaluate the performance of the laboratories for their ability to detect *Salmonella* in the presence of competitive microorganisms. For *Salmonella* the RM containing S. Enteritidis was proposed. For the competitive microorganisms it was proposed to use poultry faeces.

For the preparation of competitive microoganisms faeces obtained from poultry flock likely to be free from *Salmonella* was obtained. The faeces was homogenized with peptone glycerol (1:1) and frozen at -70 °C. After storage for several days at -70 °C the samples were transferred to -20 °C. Subsequently the faeces was tested for the absence of *Salmonella* using pre enrichment in BPw, selective enrichment in RV and isolation on BGA. Being negative for *Salmonella* the faeces was used to prepare two types of samples that were used to evaluated the homogeneity and stability at -20 °C. A solid type of sample was tested consisting of the faeces frozen at -20 °C. A second type of liquid sample was prepared by suspending faeces in peptone saline solution and separating the solid parts by filtration. The filtrate was frozen at -20 °C. The testing of these types of materials is going on at the moment. After a choice is made between the use of a solid or liquid sample for the competitors the level of inoculation of *Salmonella* has to be established.
The methods proposed to be used for the study are:
- a standard method to be able to compare results using an identical method (proposed ISO 6579);
- own method in addition to standard method (routinely used for testing of faeces).
The proposed number of samples for examination will be in total 30 samples comprising:
- blank samples;
- samples with competitive m.o. and no S. Enteritidis present;
- samples with competitive m.o. and a "low" level of S. Enteritidis and
- samples with competitive m.o. and a "high" level of S. Enteritidis.
After the discussion with the participants it was decided to follow the presented proposal. In addition to this there was a need to use S.Typhimurium besides the S.Enteritidis strain. It was therefore decided to examine in total 60 samples: 30 in combination with S. Enteritidis and 30 in combination with S.Typhimurium.

2.3. Collaborative studies within Member States between the National Reference Laboratory for *Salmonella* and the approved national laboratories

by Ir. A. v.d. Giessen (RIVM/NRL the Netherlands)

2.3.1. Introduction

In Council Directive 92/117/EEC three different bodies, i.e. "approved national laboratories", the "national reference laboratory", and the "competent authority", are defined which should collaborate at a national level within the context of the Zoonoses Directive. In the Directive the following is stated with respect to these bodies:

Competent authority
"means the central authority or authorities of a Member State which is/are responsible for monitoring provisions concerning public health, animal health or other veterinary matters arising from this Directive, or any other authority to which such responsibility has been delegated by the central authority" (Art. 2, point 6)

"collects information on any zoonotic agents.....and on any clinical cases in humans or animals....." (Art. 4, point 1 (d))

"shall evaluate the information collected...." (Art. 5, point 1)

"shall report to the Commission, by 31 March each year, ..." (Art. 5, point 1)

Approved national laboratory
"means a laboratory approved or recognized by the competent authority of a Member State to carry out examinations of official samples in order to detect a zoonotic agent"
(Art. 2, point 3)

National reference laboratory
"... at which the identification of a zoonotic agent or final confirmation of its presence may be carried out." (Art. 3, point 3)

The Directive does not specify the duties and tasks of the national reference laboratories since these are a responsibility of the national authorities and not of the EU Commission. However, it was recommended to define functions and duties of the NRLs parallel to those of the CRL. Especially, it was recommended that a national reference laboratory should pursue harmonization of *Salmonella* detection methods at a national level, in particular by organizing collaborative studies. Furthermore, a national reference laboratory could coordinate research into new analytical methods at a national level.

2.3.2. Infrastructure and collaborative studies in the Netherlands

In the Netherlands a national plan for zoonoses has been established based on the criteria laid down in the Council Directive. Two governmental bodies have been assigned as competent authority: the Dutch Veterinary Service of the Ministry of Agriculture, Nature Management and Fisheries and the Dutch Veterinary Public Health Inspectorate of the Ministry of Public Health, Welfare and Sports. Totally, 48 national laboratories have been assigned including the national reference laboratory, 17 regional Public Health Laboratories, 13 regional Food Inspection Services, the Central Veterinary Institute, 3 regional Animal Health Services, 12 regional Inspection Services for Livestock and Meat, and one commercial laboratory.

Public health sector
All first human *Salmonella* isolates obtained at the 17 regional Public Health Laboratories are sent to the Dutch national reference laboratory (RIVM) for sero- and phagetyping, amounting to 3000-5000 isolates per year. Based on the typing results the frequency distribution of the *Salmonella* types is determined. In addition, surveillance of gastro-enteritis is conducted both at the medical practitioner's level and at population level including microbiological examination of stool samples. Based on these results the annual incidences of *Salmonella* infection in The Netherlands at both levels can be estimated. All *Salmonella* results are reported annually to the competent authority.

Veterinary sector
Different *Salmonella* monitoring and control programmes in the veterinary sector actually are carried out. A variety of *Salmonella* isolates from animals, animal feedingstuffs and foods of animal origin obtained at the approved national laboratories is sent to the NRL for typing. However, these typing results do not yield reliable information about the prevalence of *Salmonella* serotypes in different categories of animals or foods. Therefore, additional surveillance of *Salmonella* serotypes in farm animal populations based on statistical principles will be implemented in the end of 1996 and will be continued up to the year 2000. Monitoring of *Salmonella* serotypes in foods of animal origin will be based on similar principles. *Salmonella* isolates obtained in these surveillance studies will be typed at the NRL and results will be reported annually to the competent authority. Reliable epidemiological data obtained in the public health sector and the veterinary sector will provide a basis for an effective intervention strategy.

Collaborative studies
A preliminary collaborative study on immunological detection of Salmonella Enteritidis has been conducted between the Dutch NRL and three approved national laboratories. In this study use was made of reference materials including freeze dried serum samples as well as freeze dried egg yolk samples with different levels of antibodies. Samples were tested by ELISA according to the laboratories' own protocol as well as to the NRL protocol (indirect LPS ELISA). Preliminary results showed that the inter-laboratory results were comparable when the same ELISA protocol was applied and identical materials and reagents were used. A collaborative study on bacteriological detection of *Salmonella* involving all approved national laboratories is scheduled for the second part of 1996. In that study different types of samples with and without competitive microorganisms will be used. National collaborative studies will be organized by the NRL on a regular basis.

2.3.3. Infrastructures/experiences on collaborative studies within other Member States

Abstracts of the contributions of Dr. R. Davies (United Kingdom), Mrs K. Bergström (Sweden) and Dr. D.L. Baggesen (Denmark) are presented on the next pages.

Infrastructure and responsibilities of the National Reference Laboratory and Regional Laboratories in the United Kingdom

Veterinary Division

In England and Wales veterinary diagnostic samples are taken in private veterinary practices and processed in private laboratories or in one of the 14 MAFF Veterinary Investigation Centres (VICs). Other samples taken for monitoring of salmonella under the Zoonoses Order (1989), The Processed Animal Protein Order 1989 and the Poultry Flocks and Hatcheries Order 1993 are largely processed in VICs. Some samples, particularly animal feed monitored under MAFF Codes of practice are also tested at Public Health Laboratories.

Salmonella isolates from these tests are forwarded to the local VIC together with details of the circumstances of the isolation. Isolates are then confirmed as salmonella and typed to 'O' group level. Where appropriate for clinical samples a limited antibiotic sensitivity test is also carried out. When the primary isolation is made in a laboratory with full serotyping capacity, does not result from statutory monitoring, and the serotype is not *S. enteritidis* or *S. typhimurium* then reports of the isolation rather than isolates may be forwarded to VICs.

All salmonella isolates made and confirmed by VICs are sent to the Central Veterinary Laboratory (CVL) for confirmation. CVL and the VICs have recently formed one MAFF executive agency called the Veterinary Laboratories Agency (VLA). CVL carries out full serotyping and antimicrobial sensitivity testing against a range of veterinary and medical antimicrobials on all isolates received. All isolates of *S. enteritidis* and *S. typhimurium* are phage typed at CVL and isolates of *S. hadar, S. virchow, S. pullorum* and *S. thompson* are sent to the Central Public Health Laboratory (CPHL) at Colindale. CPHL also confirms new or non-non-conforming antigenic structures. CVL also has responsibility for epidemiological data collection relating to salmonella isolates from diagnostic and surveillance samples but there is no sentinel surveillance network system. Serological testing for *S. enteritidis* antibodies from official investigations is carried out at CVL using a L.P.S. ELISA and there is also a small amount of serum agglutination testing for various salmonella serotypes for diagnostic and export purposes.

In Scotland the various divisions of the Scottish Agricultural Colleges perform the functions of the VICs and Stobhill Hospital Laboratory has a similar role to CVL. In Northern Ireland the Veterinary Research Laboratory at Stormont is the Central Veterinary Laboratory.

Medical/Public Health Division

Diagnostic samples collected by medical practitioners or by public health or environmental health officers are cultured either in hospital laboratories or public health laboratories. Isolates are then sent to CPHL for serotyping, antibiotic sensitivity testing and phage typing. CPHL also has responsibility for ongoing collation of epidemiological data and has several systems for issuing early warnings of the emergence of new organisms in the human population. Molecular strain differentiation techniques are also used to assist the epidemiological investigation of certain outbreaks.

SALMONELLA CONTROL IN SWEDEN

Karin Bergström
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All "animal-associated" Salmonella is serotyped at The National Veterinary Institute (NVI), all human Salmonellosis is serotyped at The National Bacteriology Laboratory (NBL). Phagetyping is done by the NBL only.

Salmonella surveillance - living animal, slaughter and feedstuff:
- Clinical surveillance
- Slaughter house control
- Autopsies
- Slaughter under special conditions
- Risk herds
- Herds
- Animal feed stuff
- Wild animals
- Pet animals
- Environmental surveillance
- All food for human consumption

All suspected Salmonella isolates from samples mentioned above have to be sent to the National Veterinary Institute (NVI) for verification and serotyping. All positive Salmonella at the NVI has to be reported after the serotyping to.....
- The National Board of Agriculture
- The Regional agricultural board
- The National Bacteriology Laboratory (NBL)
- The Department of Epizootology, NVI

If Salmonella is found in cattle and swine.....
- Ban on animal movement (to and from the herd)
- Slaughter under special conditions
- Prohibiton to visit the herd
- Hygienic measures
- Ban on the use of unpasteurized milk
- Manure - muled down or compost
- Desinfection

Broiler.....
- Stamping out !

A presentation of the Danish National Reference Laboratory on Salmonella and the collaborators

Dorte Lau Baggesen, DVM, Ph.D. Danish Veterinary Laboratory

In Denmark, the activities around *Salmonella* are coordinated within the network of the Danish Zoonosis Centre which include all national institutions working in this area: the Danish Plant Directorate, the Danish Veterinary Service, the Danish Veterinary Laboratory all under the Ministry of Agricultural and Fisheries and the Danish Food Agency, Statens Seruminstitut, the National Board of Health under the Ministry of Health. The magnitude of sampling in the national salmonella control and the authorities and laboratories involved and the methods employed are presented in Table 1.
The area of references of the Danish National Reference Laboratory on *Salmonella* is limited to the official investigation on *Salmonella* in poultry parent flocks with reference to the EEC Zonosis Directive, and further more the Danish Veterinary Laboratory = NRL is the only laboratory approved to perform salmonella examinations in poultry flocks.
At the Danish Veterinary Laboratory and the Danish Zoonosis Centre many additional informations are present. All strains, found within the official control programmes, and isolated from non human sources have to be forwarded to the Danish Veterinary Laboratory for verification and epidemiological characterisation. In addition to the required serotyping, a proportion of all strains are selected for further characterisation which include phage typing of *S.* Typhimurium and *S.* Enteritidis and testing of all serovars for antimicrobial resistance. Molecular epidemiological markers are used "ad hoc" to characterise selected isolates to clarify further problems. Isolates from human patients are serotyped at Statens Seruminstitut and to some extent at decentral hospital laboratories. Per month, 25% of human *S.* Typhimurium and *S.* Enteritidis isolates are phage typed at the Danish Veterinary Laboratory as well as human isolates are included in the molecular investigations with the objective to compare animal-, food and human strains.

Table 1 The Danish *Salmonella* control - 1996

	Estimated no. of samples	Authority	Detection Laboratory	Methods
Feed stuffs	3.500	PD	Commercial/PD	NMKL 71
Herds/flocks:				
Egg prod.*	50.000			ISO 6579
Broilers*	300.000	VD	DVL +	ISO modification
Pig**	1.000.000		Commercial	NMKL 71
Cattle	1.500			
Meat:				
Broilers*	85.000			ISO 6579
Pork	30.000	VD	Commercial	LMKL 71
Beef	3.000			Eia Foss
Manufacture and retails	15.000	LST	LKE	LMKL 71
Humans	120.000	SST	SSI/KMA	direct inoculation selinite broth

*an intensive programme will be established in 1996 including more bacteriological and serological examinations
** mostly serological tests

PD Danish Plant Directorate
DVL Danish Veterinary Laboratory
VD Danish Veterinary Service
LST Danish Food Agency
LKE Municipal Food Control Unit
SST National Board of Health
SSI Statens Seruminstitut
KMA Hospital laboratories

2.4. Evaluation of the first collaborative study on (sero-)typing of *Salmonella*

by Drs. W.J. van Leeuwen and N. Voogt (RIVM/CRL the Netherlands)

2.4.1. Presentation of the report and discussion

A collaborative study on serotyping of *Salmonella* was organized by the Community Reference Laboratory (CRL) for *Salmonella*. In this study the National Reference Laboratories for *Salmonella* (NRLs) of the Member States of the European Union (EU) participated. The aim of this first serotyping study was to be informed about the results of serotyping of *Salmonella enterica* by the NRLs.
Seventeen laboratories each examined 20 individually numbered serovars of *Salmonella enterica*. The participating laboratories were asked to identify these strains with the serotyping method performed routinely in their laboratory. Furthermore, inquiries were made to be informed about the method applied, the origin of the sera and the number of strains typed per year.
Of the 17 participating laboratories 7 laboratories identified the 20 selected strains correctly. One laboratory reported six strains as not typable. Three laboratories identified one strain incorrectly, while 6 laboratories identified two or more strains incorrectly. The main reasons for incorrect results were the incorrect detection of O and/or H antigens, the identification of strains based on an incomplete antigenic formula by means of the Kauffmann-White scheme and an incorrect interpretation of the antigenic formula. It is better to identify strains by giving the antigenic formula as far as detected. Definite conclusions can be based only on agglutination with mono-specific antisera.

The results of the methods, sera etc. were presented on various sheets. Furthermore a short explanation of the main reasons for incorrect typing was given with the help of some examples.
There were no comments on the draft report, so the definite report will be published.
All participants will receive a report.

Discussion:

* One of the participants asked whether it was possible to send strains which were not typable in their own institute, to an appropriate institute in their country.
It is a possibility for a laboratory to delegate typing of strains to another laboratory in their country.
* A remark was that more frequently appearing serotypes must be included in the study. During the organization of the next collaborative study this will be done.

2.4.2. Serotyping using agglutination in microtitre plates

Abstracts of the contributions on microtitre plate agglutination given by Dr. R. Davies (United Kingdom) and Drs. W.J. van Leeuwen (the Netherlands) are mentioned on the next pages.

Serotyping using agglutination in microtitre plates at the Central Veterinary Laboratory

This was adopted by CVL following successful institution of the method at CPHL, Colindale and in response to the increased number and serotype range of isolates received after commencement in 1989 of legislation requiring monitoring of processed animal protein and poultry flocks.

The salmonella isolates are cultured in Heart Infusion broth plus 1% glucose overnight and, then boiled to denature flagellae and expose somatic antigens for somatic determination or killed with 1% formal saline to preserve flagellae for flagellar determination. Separate somatic and flagellar microtitre plates are prepared by adding polyvalent sera plus a range of antisera to common antigens diluted to an optimum dilution based on previous titration with the relevant factor. One saline control well per antigen is also included. The antigen is then added and the plate sealed and incubated for two hours at 51°C followed by overnight refrigeration at 4°C. Results are read inside a microagglutination test dark box with an MAT reader and verified by a second worker before recording.

In cases where there is agglutination with polyvalent somatic group antisera (E, G) or flagellar E, G or L complexes follow-up microagglutination tests are carried out. Flagellar phase inversion is carried out by the Craigie tube method after which a second flagellar microtitre plate is used.

Uncommon salmonella serotypes which react with polyvalent 'O' or 'H' antisera but not with individual sera in the microtitre tray are determined by slide agglutination testing supported by tube agglutination when agglutination is weak. Isolates not reacting with polyvalent sera are confirmed as salmonella by biochemical testing before further serotyping is carried out.

The microtitre method detects the common serotypes in one initial test, involving less labour intensive repetitive work and is more readily applied than slide agglutination by less experienced staff. There are disadvantages however, in that the procedure takes longer than slide testing with additional delays when follow-up tests are necessary. It is also not possible to make 'short cuts' in the procedure based on prior knowledge of likely serotypes from a particular source or when there are multiple isolates from a single investigation. In addition the boiling step increases the proportion of autoagglutinating strains and destroys Vi antigens.

Serotyping of *Salmonella* using microtiterplates
W.J. van Leeuwen and H.M.E. Maas

In our laboratory *Salmonella* strains sent for typing are isolated from human as well as non human sources. Yearly about 10.000 strains are received for typing. The mechanized microsystem for serotyping of *Salmonella* strains requires a minimum of reagents and a relatively great output. The antisera used in this system in our laboratory are:
O-antisera: 4,5,7,8 and 9 and H-antisera: i,l(complex),5,r,e(complex), z,10,g(complex),m,s,t,p and f. The choice of the antisera is based on the frequency distribution of the various *Salmonella* serotypes in the Netherlands (most frequently found serotypes: S. Enteritidis, S.Typhimurium, S. Virchow., S. Hadar, S. Infantis, S. Dublin, S. Heidelberg and S. Agona), but the choice of antisera can easily be adapted to the prevalence of serotypes. With this set of sera about 70% of the strains sent for typing are typable with O- as well as H-antisera. More than 90% of the strains are typable with the O-antisera and 25% of the strains needs additional slide agglutination. About 5% of the strains are not typable in this system.

Preparation of the cultures (antigen)
H-tray: - inoculate the strain into 6 ml broth with distilled water;
- incubate o/n at 37 °C in a shaking waterbath;
- add 200 µl drop of gentianviolet/formalin mix.

O-tray: - heat the coloured broth used for H-typing at 100 °C for 45 min;
- add 10 ml of 0.002% gentianviolet solution.

Preparation of antisera
The O- and H-antisera are prepared and absorbed according to the standard methods as described in the Guidelines for the preparation of *Salmonella* antisera by the WHO Collaborating Centre for reference and research on *Salmonella*, Institut Pasteur, Paris. The serum titers are determined by dilution of the antisera and testing the dilutions in trays against 10 different strains harbouring the right antigen. These strains must be received for typing recently. The dilutions used in the microsystem is half of the lowest titer found with any of the tested strains. There is no clear correlation between titers of antisera used in the microsystem and for slide agglutination.

Fill the trays with 50 µl antigen and 50 µl antiserum and read the H-tray after 3h incubation at 45 °C in a waterbath and the O-tray after o/n incubation at 37°C in an incubator.

2.5. Collaborative study for immunological detection of S. Enteritidis and/or S. Typhimurium

2.5.1. Background information as introduction

by Dr. Ir. S.H.W. Notermans (RIVM/CRL the Netherlands)

Sensitive immuno-assays such as the ELISA are very convenient alternatives for demonstrating the presence of invasive S. Enteritidis/S. Typhimurium in production animals, such as poultry. Due to the invasive character of these organisms, antibodies will be produced. Detection of antibodies directed to species-specific epitopes allows specific detection of the organisms.
Background information dealing with the general principles of immunological assays was presented and included among others: the principles of ELISA (including sensitivity, reliability, variations in results), antibody production as a consequence of invasion by *Salmonella*, development of antibodies against LPS, the specificity of detection antibodies against epitope 9 (S. Enteritidis) and 4 or 5 (S. Typhimurium).
Also options for developing more specific ELISA techniques, based among others on the application of synthetic epitopes coupled to a carrier protein, were presented.

2.5.2. Preliminary results of the collaborative study

by Ir. P. H. in 't Veld (RIVM/CRL the Netherlands)

Aim of the study was to investigate whether the examination of samples in Member States is carried out uniformly and that comparable results will be obtained.

For the study 6 ampoules (reference materials) were examined consisting of:
- 4 egg yolk samples (CRL/EY/SE/1/1; CRL/EY/SE/1/2; CRL/EY/SE/1/3; CRL/EY/SE/NC (negative control));
- 2 serum samples (CRL/SOR1/SE/PC; CRL/S1/NC (negative control))

Each ampoule was examined:
- on three different days;
- on each day on three different plates;
- on each plate in duplicate (except for serum negative control (CRL/S1/NC));
- each in dilution series (dilution ratio1:3; dilution range1:100 to 1:24300).

The ampoules were frozen at -20 °C after reconstitution for use on the three days of examination. The method used was the LPS ELISA developed at RIVM. The trays, LPS antigen S. Enteritidis and conjugate were supplied by CRL.

The egg yolk reference materials were prepared from eggs originating from natural infected poultry laying flocks. The faeces of these flocks was found positive for S. Enteritidis by routine bacteriological testing. By means of the LPS ELISA three different titre levels were found and subsequently freeze dried. CRL/EY/SE/1/1 was prepared from egg yolks with titre of 8100-24300, CRL/EY/SE/1/2 from egg yolks with titre of 1800 and CRL/EY/SE/1/3 from egg yolks with titre of 100-200.

The serum reference materials originated from SPF poultry artificially infected with S.Enteritidis by means of oral immunization or, for the negative control, originated from SPF chickens. The sera were freeze dried to obtain the reference materials.

For RM CRL/EY/SE/1/1 the stability and effect of freezing was tested over a period of more than 1 year. For this each month the extinction of a new (fresh) vial was determined besides the extinction of the frozen vial. The results of this test indicated that there was no decrease in average extinction in time for the new and for the frozen vial. The results of the test is presented in Figure 1.
So far only a provisional analysis of the data was performed using the extinction values reported without subtracting blank values. At first the data reported by the participants were screened to eliminate doubtful results. Based on the screening the following (sets of) data were set aside:
- lab 3, day 1, tray 1, replicate 1, CRL/EY/SE/1/1, 1:300 (unlikely low extinction);
- lab 7, day 1, tray 3 (high extinction values for all RMs and dilutions) and
- lab 16, day 1, tray 3 (low extinction values for all RMs and dilutions, possible coating error).
The next step of the analysis was to check whether the variation between the data could be described by a normal distribution. A normal distribution is necessary for performing an analysis of variance (ANOVA) and performing a F-test. The test on normal distribution (normality) of the data was done for EY/SE/1/1 (RM-1) and SOR1/SE/PC (RM-5), before and after screening of results for the dilutions 1:300 and 1:900. At first a visual presentation of the data was made as an indication of normality of the data. An example of such a visual presentation is presented in Figure 2.

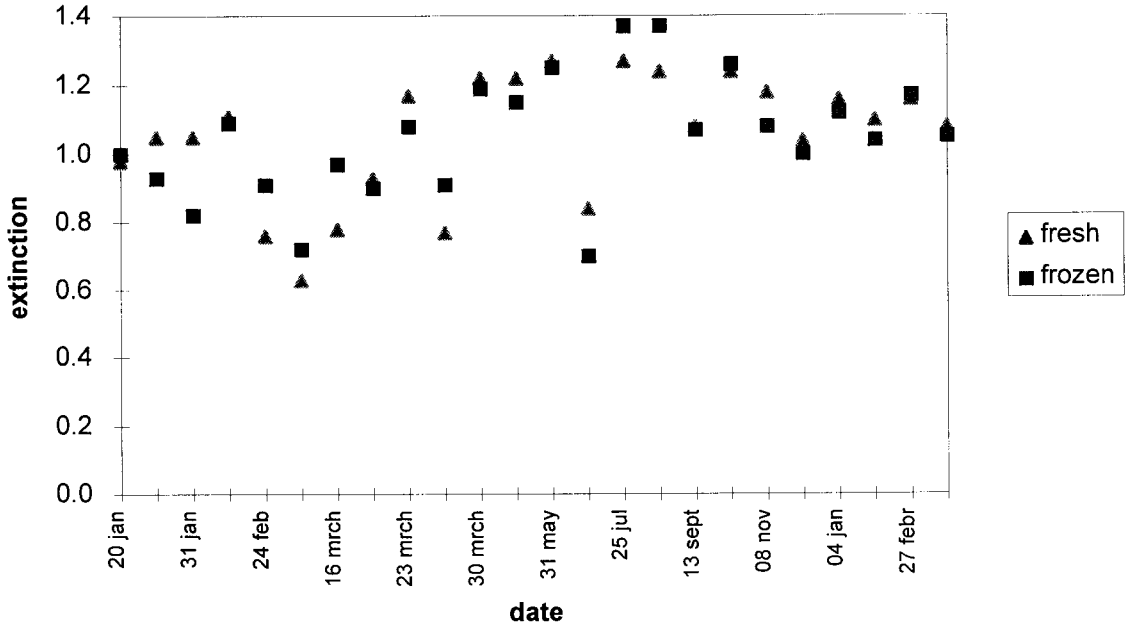

Figure 1: *Extinction values of CRL/EY/SE/1/1 over a period of ca 1 year and the effect of freezing*

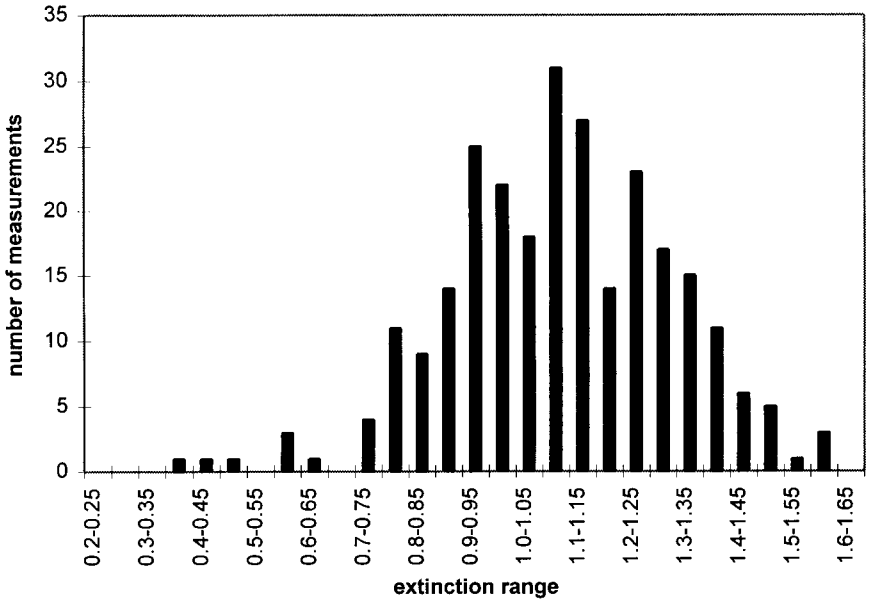

Figure 2: *Histogram of the distribution of the extinction values after screening of the results for CRL/EY/SE/1/1 at the 1:300 dilution*

Furthermore the data were tested for kurtosis and skewness. These two parameters indicate whether the data have a normal distribution. Skewness indicates the presence of tails (asymmetric distribution), kurtosis means that there is an excess of values near the mean or far from it. In some cases the normality of the data was confirmed, for others it did not. Therefore more information needs to be evaluated before a definite decision on normality of the data can be taken.
The next step in the analysis of the data was the ANOVA. This was tested after screening of results for the dilution and RMs mentioned above. In all cases highly significant differences between laboratories existed. For the 1:900 dilutions also a significant effect between days was observed. No other significant effects (such as trays and replicates) were found.
As significant differences between laboratories existed it was tested whether there were laboratories that could be identified as an outlier by means of the Grubbs test. Only for RM-5 at the 1:900 dilution an outlier could be detected.

The results presented so far are just the preliminary results, further analysis of the data is necessary before definite conclusions can be made on the comparability of the results.

2.6. Need for training

by Dr. Ir. S.H.W. Notermans (RIVM/CRL the Netherlands)

One of the tasks of the CRL is to provide technical support to the NRLs, including training and education if necessary. The support concerns analytical methodology and comprises among others detection of S. Enteritidis/S. Typhimurium by cultural and/or immunological techniques and identification and typing of isolates. Training in the above mentioned subjects can be done on an individual basis, groupwise, by participation in ringtrials, etc.
From the enquiry held it revealed that individual training, especially to become introduced in new techniques, is highly appreciated. An additional appreciated aspect was the extension of the workshop with a scientific programme. In such a workshop new developments in analytical techniques and experiences with new techniques should be exchanged as well as research activities of the CRL and the NRLs.

2.7. Research activities

2.7.1. Bacteriological research activities

2.7.1.1. Overview of results survey: methods used in relation to sample type

by Ir. P. H. in 't Veld (RIVM/CRL the Netherlands)

For this the CRL prepared a questionnaire that was sent to the NRL of the Member States. The questionnaire sent was directed towards the traditional detection methods, other non traditional methods had to be reported separately. The questionnaire was sent to 16 NRL. Responses were obtained from 14 NRL. A summary of the results is presented below.

Salmonella detection methods for raw meat.
Of the 14 NRL nine examined raw meat samples, the others did not examine this type of sample in their laboratory.
- *Homogenization and pre-enrichment*:
Eight laboratories homogenized the samples before the addition to Buffered Peptone water (BPw), one did not. All labs incubated the BPw according to ISO 6579 (16-20 h at 37 °C).
- *Selective enrichment:*
A range of selective enrichment broths was used, Rappaport-Vassiliadis broth with or without soya peptone (RV(S)) was the most commonly used (six out of nine labs) selective enrichment broth. Three labs did not use a second selective enrichment broth. About half of the labs incubated the selective enrichment broths for 24 h only, the others used an additional 24 h incubation as well.
- *Isolation:*
Again here a wide range of isolation media were used. Brilliant Green Agar (BGA) was the most commonly used (six labs). All media were incubated at 37 °C and in most cases for 24 h only. Only two labs used only one isolation medium.

Salmonella detection methods for poultry meat.
Again here the same nine laboratories who examined raw meat also examined poultry meat. Eight of these nine labs used identical methods for raw meat and poultry meat. The one laboratory having a different procedure for poultry meat used a rinse method for inoculation of the selective enrichment broth instead of homogenization and pre-enrichment. Selective enrichment was done in Selenite Cysteine only for 24 h at 37 °C.

Salmonella detection methods for faeces.
Thirteen laboratories had a procedure for the detection of *Salmonella* in faeces. One laboratory used two procedures, so in total 14 responses are summarized. Of the nine labs who also examined meat and poultry samples, six reported that they used another procedure for the detection of *Salmonella* in faeces than for meat and poultry.
- *Homogenization and pre-enrichment:*
Nine laboratories did not homogenize, the remaining five labs homogenized their samples. Eight labs used pre-enrichment in BPw, the others did not use pre-enrichment but direct selective enrichment. Pre-enrichment in BPw was done for 16-20 h at 37 °C as for the other types of samples. Four of the eight labs who used pre-enrichment did not homogenize the samples.
- *Selective enrichment:*
A range of selective enrichment broths was used, Rappaport-Vassiliadis broth with or without soya peptone (RV(S)) was the most commonly used (10 out of 14) selective enrichment broth. Seven labs did not use a second selective enrichment broth. About half of the labs incubated the selective enrichment broths for 24 h only, the others used an additional 24 h incubation as well.
- *Isolation:*
Again here a wide range of isolation media was used. Brilliant Green Agar (BGA) was the most commonly used (nine times). All media were incubated at 37 °C and in most cases for 24 h only. Five labs used only one isolation medium.
One lab used in addition to their procedure using direct selective enrichment also direct isolation of *Salmonella* on McConkey agar

2.7.1.2. Presentation of laboratories on their research activities

Abstracts of contributions on bacteriological research activities are presented on the next pages. These were from Mrs S.L.Kivelä (Finland), Mr. A. Ferrarese (Italy), Dr. M. Madsen (Denmark) and Dr. R. Davies (United Kingdom).

Comparison of a method of pooling of faeces samples with a method of individual faeces samples for *Salmonella* investigation

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National Veterinary and Food Research Institute,Helsinki,Finland
Olli Ruoho, Karijoki,Finland

Introduction
Large surveys has been made on the occurrence of *Salmonella* in cattle in Finland. It has been voluntary to participate in the survey. To make the participation less expensive to the farmers, pooling samples of faeces has been examined instead of individual faeces samples. To investigate the reliability of pooling sample a research was performed where both pooling samples (a tablespoon/animal) and individual samples were taken at the same time.

Samples
Altogether 3295 individual faeces samples and 160 pooling samples were collected from 130 herds. 25 salmonella positive herds were found. *Salmonella* Infantis was the only serovar to be found.

Culturing procedure
Faeces samples were enriched in RVS-broth (LAB M 86) at the same day they arrived to the laboratory. The broths were incubated at 42° C for 18 to 24 hours. The selective enrichment broths were streaked onto bromtymol-blue lactose agar (BROLAC, Merck 1639) and brilliant green agar (BRG; LAB M 34) which were incubated at 37°C for 20-24 hours. Typical colonies were confirmed biochemically (triple sugar iron agar, urea agar and BROLAC) and serologically.

Results
160 pooling samples and 160 groups of individual samples were collected.. Each group contained samples from the same animals than the corresponding pooling sample (10-48 animals). 100 pooling samples yielded negative results with both method. 54 samples were positive for *Salmonella* with both method.
Six pooling samples were negative even if they contained faeces from animals which had found positive for *Salmonella* in individual samples.
5 of 6 false negative pooling samples contained samples from more than 20 animals. The ratio of *Salmonella* positive animals was low in four cases.
Kappa has been used to measure the agreement between these two methods. The material has been divided in two groups: pooling samples which have less/more than 20 samples of faeces.

Table 1.

Number of samples	Kappa	Sensitivity	Predictive value(-)
<20	0.98	96.88 %	98.33 %
>20	0.85	82.14 %	89.13%

EFFECT OF PRE-ENRICHMENT OF FAECES SAMPLES FOR *SALMONELLA* INVESTIGATION

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INTRODUCTION

Modified ISO 6579 method is used in routine isolation of *Salmonella* from organs and faeces in NRL in Finland. Pre-enrichment procedure is used only in cases when it is suspected that samples include only small amounts of *Salmonella*-bacteria. The effect of pre-enrichment in isolation of *Salmonella* from faecal samples of cattle was examined. The amount of positive isolations of pre-enrichment and direct selective enrichment procedures were compared.

MATERIALS AND METHODS

Samples
Altogether 613 faecal samples were collected from 21 herds of cattle. Only herds which were *Salmonella* positive were investigated. The number of animals in herds ranged from 5 to 55 and was in average 29.

Culturing procedures
Faecal samples of about 1 g were pre-enriched in 9 ml of 1 % buffered peptone water (BPW; LAB M 46). The broths were incubated at 37 °C for 16 - 20 hours. 0,1 ml of pre-enriched sample was transferred to Rappaport-Vassiliadis soya peptone broth (RVS; LAB M 86). The broths were incubated at 42 °C for 18 - 24 hours.

In direct selective enrichment method faecal samples of about 10 µl were enriched in 10 ml of RVS-broths. The broths were incubated at 42 °C for 18 - 24 hours.

The selective enrichment broths were streaked onto bromthymol-blue lactose agar (BROLAC; Merck 1639) and brilliant green agar (BRG; LAB M 34) which were incubated at 37 °C for 18 - 24 hours. Typical or suspect colonies (2 - 3 colonies from each plate) were confirmed biochemically using triple sugar iron agar (TSI; LAB M 53), urea agar (LAB M 130) and BROLAC agar. The final identification was done with slide agglutination test according to Kaufmann-White -scheme.

RESULTS

237 (38,7 %) *Salmonella* positive samples were yieldid in at least one procedure (Table 1). 234 positive samples were found with pre-enrichment and 220 with direct selective enrichment. Enrichment procedure missed 17 positive samples. 10 of these samples had been positive with selective enrichment procedure 1- 4,5 months ago. Pre-enrichment procedure missed 3 positive samples.

The sensitivities and negative predictive values were calculated (Table 2). Using the chi-square test no significant difference ($p = 0,41$) was found between the methods.

Table 1. *Salmonella* positive results from faecal samples of cattle obtained by selective enrichment and pre-enrichment procedures

	RVS 42 °C	BPW + RVS 37 °C + 42 °C	Total
Number of positive samples	220 (92,8 %)	234 (98,7 %)	237

Table 2. Sensitivities and negative predictive values of results of the procedures (95 % confidence)

	RVS 42 °C	BPW + RVS 37 °C + 42 °C
Sensitivity (%)	92,8	98,7
Pred. val.- (%)	95,9	99,2

Evaluation of immunomagnetic separation (I.M.S.) as selective enrichment for *Salmonella* detection in poultry feed and egg-products samples.

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Summary

Immunomagnetic separation technique is based on the employment of magnetic particles on whose surface a selected combination of purified polyclonal and monoclonal antibodies specific for *Salmonellae* are bound (Dynabeads).
A magnetic particle concentrator (MPC-M), with a removable magnetic strip, is used to perform the test.
This procedure replaces the traditional selective enrichment, since *Salmonellae*, that could be present in the pre-enriched sample, are selectively bound by Dynabeads antibodies and so physically separated by means of a magnetic field.
This step is followed by isolation on selective agars and biochemical and serological confirmation of suspect colonies, as in traditional methods.
The first advantage of this technique is the shortening of the length of time necessary to achieve *Salmonella* isolation, because the selective enrichment step lasts about 30 minutes, instead of the 24 hours necessary for the enrichment in liquid selective media.
Moreover, for some kind of samples, some Authors have demonstrated that the employment of I.M.S. corresponds to an increment in bacteriological method sensitivity (Soncini and Colombi, 1995; Mansfield and Forsythe, 1993).
For this reason we have chosen to perform the immunomagnetic separation technique, and to compare it with the conventional ISO methodology, on samples generally characterized by a scarce *Salmonella* contamination, as feed and raw materials, as well as egg-products.
Our experience with this method brought to a long period of "training" to acquire the correct procedure and handling.
This is particularly due to the high viscosity of this kind of samples, which creates many problems in particles magnetic separation, so that a ten fold dilution becomes necessary.
Following this standardization phase, we processed 109 samples (53 eggs and egg-products, 56 feed and raw materials) both with I.M.S. and with traditional method, and we obtained identical results (see table 1).

So we confirmed that this method is effective in reducing the time necessary to get to *Salmonella* isolation, whereas no improvement in test sensitivity was found.

This can be due to the not sufficiently high number of examined samples as well as to the nature of samples themselves, which does not allow to achieve the best performances of this technique, as reported also by other Authors (Cudjoe et al., 1994).

The low contamination level of the examined samples must also be considered, because it could be lower than the one necessary for isolation, which is about 10^4 cells/ml for *S.enteritidis* (Holt et al., 1995).

References

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Table 1: comparative performances of selective enrichment procedures

		R.V.S.		I.M.S.	
kind of sample	**n°**	**+**	**-**	**+**	**-**
finished feed	6	0	6	0	6
raw materials					
meals	16	1	15	1	15
others	42	1	41	1	41
egg-products					
raw	35*	19	16	19	16
pasteurized	10	0	10	0	10
TOTAL	**109**	**21**	**88**	**21**	**88**

* among these samples, one was positive only with I.M.S., and another one only with R.V.S.

Danish research projects on *Salmonella* detection

Mogens Madsen, DVM, Ph.D., Danish Veterinary Laboratory

1. *Comparative study of bacteriological and immunological detection of S. Enteritidis (SE) and S. Typhimurium (ST) in experimentally infected poultry*
In this study, three flocks (orally infected with SE, ST and a control) of 50 SPF chickens are followed from day-old to end-of-lay (60 weeks) by bacteriological (ISO method) and immunological (mix-ELISA) testing. The study is concluded mid-1997; objectives is an evaluation of the mix-ELISA on experimentally infected animals and the provision of base-line negative immunological values as well as life-long immunological and bacteriological values for SE and ST.
2. *Comparative study of bacteriological and immunological detection of SE, ST and other serotypes in naturally infected parent flocks and table-egg layers*
A three year intensive national surveillance and control programme for salmonella in poultry has been approved in principle by the Danish government and is expected to start in the latter half of 1996. The programme focuses on elimination of all *Salmonella* serotypes at parent level, and on reduction/elimination of SE and ST from table-egg producing flocks. The programme provides the opportunity to compare a new immunological method (the tandem-ELISA) applied to serum and egg yolk antibodies to bacteriological detection (ISO method).
3. *Effects of pooling, and evaluation of various sampling techniques and material, on detection of salmonella in broiler flocks*
All broiler flocks in Denmark are subject to an ante-mortem salmonella testing and control programme, based on the examination of 60 faecal samples (12 pools of 5 samples) two weeks before slaughter, detecting theoretically a flock prevalence level of 5% or above. There is an industry demand of examination of 300 samples for export and certified products. The project, which commences in late 1996, focuses on optimal pool sizes and alternative collection methods for bacteriological culture.
4. *Population growth kinetics of salmonella under stress conditions and in combination with natural competitive flora, in the presence of selective/inhibitive substances*
A Ph.D. study commencing October 1996 focuses on growth kinetics of salmonella under simulated natural conditions in order to optimise recovery procedures for bacteriological culture.
5. *Detection of salmonella in poultry meat and pig faeces/pork*
As part of an EU project (SALINPORK) an evaluation of culturing methods for salmonella is carried out on poultry meat, pork and pig faeces. The overall aim is to optimise the procedure for pre-enrichment and selective enrichment to achieve a level of ca. 100.000 cfu/g within 24 h to obtain rapid detection of salmonella. In addition, the project develops and evaluates the application of rapid methods, i.e. PCR with solid phase in NUNC plates(possibility of automated testing), and immunological antigen detection by the Delphia system based on lantanids with different extinction frequencies (possibility for test of more antibodies within a sample).
6. *In vivo examination of the effect of tylosin as growth promoter on the colonisation and establishment of salmonella infections in pigs*
In the public debate in Denmark on growth promoters it has been claimed that the widespread use of growth promoters, in particular tylosin, is partly responsible for the increase of salmonella infections in food animals like pigs and poultry. The project investigates this aspect of growth promoters by examining the colonisation and establishment of salmonella in experimentally infected pigs.
7. *Bacteriological resistance typing*
In 1995 a national surveillance programme for resistance to antibiotics and growth promoters obtained funding for a three year period. Among several bacterial genera, salmonella isolates are tested for resistance to antibiotics and growth promoters, the results of which may form the basis for resistance profiles useful as epidemiological markers.
8. *Serotyping by PCR*
As a future project the possibility of using PCR for serotyping will be investigated, an approach that would overcome present problems with serotyping of rough isolates.

Salmonella Methodology Research at the Central Veterinary Laboratory

Bacteriology

In-house evaluation of various salmonella culture media and test kits has been carried out by Rob Davies. The most successful single regime for all samples has been Buffered Peptone Water/Diasalm/Rambach agar which has high sensitivity and selectivity and also avoids much of the time spent on checking false positive colonies and confirmatory tests for salmonella. Most commercial rapid test kits were lacking in sensitivity when use on naturally contaminated faeces and environmental samples and antibody based tests often missed specific serotypes, even in pure culture. Immunomagnetic separation direct from pre-enrichment to plating agar lacked sensitivity when applied to faeces samples without selective enrichment. Introduction of a selective enrichment stage produced equivalent sensitivity to conventional culture but IMS was unsuitable for use with semi-solid media. SM-ID agar was shown to be an extremely effective (but expensive) supplementary agar but XLT-4 was disappointing because of the high prevalence of H_2S negative or late H_2S producing strains.

A simple one-step salmonella test which gives relatively sensitive presumptive identification of salmonella within 24 hours was developed and antibody coated cellulose sponges have been used to enhance the sensitivity of isolation of salmonella from pre-enrichment culture compared with a standard liquid inoculum. Work on using IMS for rapid selection of second flagellar phase antigens in serotyping and improved methods for serotyping autoagglutinating strains of salmonella was also carried out.

Work in the molecular genetics unit, run by Martin Woodward, has concentrated on production of single and multiple defined flagellar and surface protein deficient salmonella mutants for diagnostic and pathogenesis studies. Other projects have involved molecular strain differentiation of *S. typhimurium* DT104 by plasmid profile analysis, pulsed field gel electrophoresis, IS 200 typing, Ribotyping and RAPD typing. The unit has also produced a PCR test based on the SEF14 gene for rapid detection of *S. enteritidis* after a short period of pre-enrichment culture.

2.7.2. Research activities on serotyping

The abstract of the presentation given by Mrs A. Aspán (Sweden) is mentioned below.

Current research activities on Salmonella at the National Veterinary Institute in Sweden

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Epidemiological analyses of out-breaks of Salmonella are an important tool for evaluating the causes and taking measurments for future prevention of Salmonella infections. All reported cases of Salmonella in domestic animal production in Sweden are beeing registred by the National Veterinary Institute (NVI), and strains are sent to the NVI for serotyping. However, serotyping is not a sufficient sensitive tool to fully evaluate an out-break situation. New, often DNA-based, methods to "fingerprint" bacteria have been developed and are presently evalutated in our laboratory. To date pulsed-field gel electrophoresis (PFGE) has given us valuable information concerning several Salmonella serotypes. This information is used in the dialogue between NVI and Swedish animal feedingstuffs and food producers in the process of keeping, and hopefully even improving, the present Salmonella situation in Sweden. However, PFGE is a time consuming and rather expensive method, and there is a need of new, faster methods, with the same accuracy as PFGE .

2.7.3. Immunological research activities

Abstracts of the presentations of Dr. R. Davies (United Kingdom) and Mr. A. Ferrarese (Italy) are presented on the next pages.

2.7.4. Discussion on coordination of research efforts

It was discussed whether or not there is a need to coordinate our research efforts. It was mentioned that already other networks exist through which some coordination takes place. On top of that the NRL's specifically can inform the CRL and each other of research initiatives they consider relevant to mention. This can be done via the existing channels, i.e. at the yearly workshop and in the quarterly newsletter. At present, no additional activity is needed.

Salmonella Methodology Research at the Central Veterinary Laboratory

Serology

Serological research carried out by Chris Thorns' group has concentrated on characterisation of surface antigens on salmonellas, particularly *S. enteritidis* and *S. typhimurium*, using monoclonal antibodies, immunogold labelling and genetic probes. The aim of the work is to identify novel antigens which can be used in diagnostic tests and pathogenicity and environmental survival studies. This work has led to the identification of the SEF14 fimbrial antigen on *S. enteritidis*. Antibodies to SEF14 have been used in the 'SEFEX' latex agglutination test for rapid confirmation of *S. enteritidis* colonies after culture and an attempt has been made to produce an ELISA for rapid detection of SEF14 during the broth culture stage of salmonella isolation. SEF14 has also been used to produce an ELISA system for highly specific detection of antibodies to *S. enteritidis* in poultry serum.

Similar work is underway to identify antigens specific to all salmonellas and for certain serotypes such as *S. typhimurium* and *S. dublin* for use in diagnostic tests.

False positive reactions to the *S. enteritidis* ELISA in individual serum samples have been a problem and there have also been difficulties with disputed positive serological results. Gerry Cooper has been carrying out work using SEF14 fimbrial dot blotting and flagella (gm) Western blotting for further confirmation of the presence of antibodies to *S. enteritidis* in sera which should help resolve these difficulties in future.

First experience with an ELISA test for detecting antibodies to *Salmonella enteritidis* in chicken flocks

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Sezione diagnostica di Vicenza

Introduction

In order to achieve a practical and effective diagnostic tool to control *Salmonella enteritidis* infection in poultry flocks we set up an LPS based ELISA test which was then proved on a first field experiment.

Preparation of LPS antigen

The lipopolysaccharide (LPS) antigen was prepared by the hot phenol-water extraction method of Westphal and others (1952). A *Salmonella enteritidis* strain (a field isolate) was grown in Tryptone soya agar at 37°C for 8 hours and then harvested and washed two times with saline.The bacterial suspension was brought at a concentration of approximately 10 mg/ml dry weight with distilled water and then mixed with an equal volume 90% phenol at 65°C. The mixture was kept at a temperature of 65°C for 15 minutes in a waterbath under continuous stirring, then cooled and centrifuged at 6000 rpm for 30 minutes at 0°C. The water phase was removed and dialysed against running tap water for 48 hours.

ELISA procedure

Microtitre plates (Polysorb, Nunc) were coated with 100 µl of *Salmonella enteritidis* LPS diluted at 1/250 in coating buffer (0.05 M carbonate/bicarbonate buffer, ph 9.6), incubated for one hour at 37° and then washed three times with phosphate buffered saline containing 0.05% Tween 20 (PBST). 100 µl of test and control sera/egg yolks were added to the wells at a dilution of 1/400 in PBST and incubated for one hour at 37°. Following three other washings, 100 µl of rabbit anti-chicken IgG peroxidase coniugate (Sigma) diluted 1/3000 in PBST with 1% skim-milk powder was added to each well and incubated for one hour at 37°. After five other washing, 100 µl af ABTS peroxidase substrate (Kirkegaard & Perry) were added and the reaction was allowed to proceed for 30 minutes after which the optical density was read at 405 nm using a Titertek Multiskan Plus MK II ELISA plate reader.

Field experience

During a one mounth period 60 eggs and 6 fecal samples were randomly collected from each production unit of 18 commercial layers flocks of variyng age in our region. Egg yolks were serologically tested with the ELISA test described above whilst 10 grams of each fecal samples were bacteriologically tested for the presence of Salmonellas: after a pre-enrichment period of 18-20 hours at 37°C in buffered peptone water, 0.1 ml was seeded in Rappaport Vassiliadis soya peptone broth, incubated for 24 hours at 43° C and than plated in XLT4 agar plates which, after 24 hours of incubation at 37° C, were controlled for the presence of bacterial growth referable to Salmonellas.

Results

Egg yolks from 12 flocks of the 20 tested showed low OD values (highest OD 0.263) and the bacteriological examination of the respective fecal samples resulted negative. Although with OD values uniformly low, 4 flocks showed one egg yolk with OD values from low to moderate (0.309, 0.326, 0.650 and 0.795) among the 60 examined. Also in this case the bacteriology resulted negative. Finally, two flocks showed high OD values: 22 and 10 samples each were found to have an OD value higher than 0.900 (the highest were respectively 1.857 and 1.532) and 2 and 6 egg yolks higher than 0.300.
The bacteriological examination of 6 fecal samples from the second flock resulted in the isolation of a *Salmonella typhimurium* strain. No further examinations were attempted.
As the bacteriological examination of 12 fecal samples from the first pools was found negative, the second flock was resampled and 6 other fecal samples togheter with livers, ovaries and ceca from 2 dead layers hens were recultured . The fecal samples resulted again negatives as well as livers and ceca; only from the ovary of one hens (with obvious signs of ovarites) *Salmonella enteritidis* was isolated.

References

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2.8. The use of alternative methods

by Ir. P. H. in 't Veld (RIVM/CRL the Netherlands)

2.8.1. Microval project summary of the presentation by Ms Irene Rentenaar (Dutch Standardization Institute)

The Microval is aiming to produce guidelines for validation and approval of alternative methods for the microbiological analysis of food, animal feeding stuffs and beverages. In this Eureka project 21 partners from various European countries are involved from industry, test kit manufacturers, research institutes and standardization bodies. The project started in June 1993 with a feasibility study whether it was possible to arrive at a uniform concept for a European validation procedure of alternative microbiological test methods for food products. The second stage of the project involved five validation studies to work out the procedure developed in stage 1. The final stage is of the project will be the reporting and publication of the results and to submit the general and technical rules for validation for European standardization by CEN.

The text of the presentation and some of the overheads presented are enclosed as an annex.

3. PLANS AND EVALUATION

3.1. Plans for the near future of the CRL

The CRL will organize several collaborative studies. The first one will be a bacteriological study with 60 samples, each containing faecal competitive flora and one of two *Salmonella* subspecies, i.e. Enteritidis or Typhimurium.
Secondly, immunological collaborative studies are considered, although definite decisions will be made after the results of the first study have be fully analysed. The specific content will depend on progress made, but emphasis will of course be on invasive types. Attention should be given to fast methods.
Thirdly, a second serotyping study will be organized. The study will focuss on typing of the more important serotypes and will have an option to let the NRLs delegate some of the actual typing to other relevant institutes in their countries.
The CRL will be publishing (in 1996) a book on methods used for *Salmonella* detection. The methods will focuss on (variations in) bacteriological methods. The relevant information will be obtained by questionnaire from the NRLs.

3.2. Communication

The CRL and NRLs communicate by:
- the yearly spring workshop;
- the quarterly newsletter; and from now onwards also
- with letters from each NRL to the CRL when the NRL considers initiatives in her country worthwhile to communicate via the CRL (newsletter) to other NRLs.

The yearly workshop is much appreciated as means to communicate directly with other NRLs and with the CRL. It should be considered to discuss also some subjects really in depth to bring in understanding from a more theoretical point of view. Also the newsletter is appreciated although it might be considered to change the format a little bit to make it easier to handle. Each NRL can use the information in the newsletter to inform her national approved laboratories. However, this is to the judgement of the NRL. The target group of the CRL newsletter remains to be the NRLs. In the newsletter also e-mail addresses will be given, at least from CRL members. When in the future the need for direct communication will be increasing, internet might be a tool to consider provided that all have access to it.

3.3. Evaluation and closing remarks

3.3.1. Evaluation

It was concluded that the aims of the workshop as defined during the opening session were reached. The participants were asked whether the workshop met with their expectations. This was indeed the case as their expectations coincided with the aims as formulated.

3.3.2. Closing remarks

- the participants were thanked for their contribution. The presentations given by some of the NRLs were much appreciated. It should be realized that the success of a workshop very much depends on the active participation of each attendant;
- the Commission was thanked for the (financial) support given;
- the former CRL team, with dr. Notermans as leader, was thanked for its initiatives with respect to the activities of the CRL;
- the present CRL team, with dr. Henken as leader, was thanked for organizing the workshop.

ANNEX 1
PARTICIPANTS OF THE WORKSHOP

European Commission, Legislation Veterinaire et Zootechnique	Dr. R. Vanhoorde
The Netherlands	
RIVM/CRL	Dr. Ir. A.M. Henken Dr. Ir. S.H.W. Notermans Ir. P. H. in 't Veld Mrs Ing. N. Voogt
RIVM/NRL	Ir. A. v.d. Giessen Mrs Drs. W.J. v. Leeuwen
Austria	
Bundesstaatliche bakteriologisch-serologische Untersuchungsanstalt Graz	Dr. W. Thiel
Belgium	
Nationaal Instituut Diergeneeskundig Onderzoek	Dr. H. Imberechts
Denmark	
Danish Veterinary Laboratory	Dr. D.L. Baggesen Dr. M. Madsen
Finland	
National Veterinary and Food Research Institute, Dep. of Food Microbiology	Mrs T. Johansson Mrs S.L. Kivelä
France	
Central National d'Etudes Vétérinaires et Alimentaires	Mrs Dr. F. Humbert
Germany	
CRL-Epidemiology of Zoönoses	Mrs Dr. A. Käsbohrer
Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin	Dr. M. Hartung
Institut für Epidemiologische Diagnostics	Dr. J. Geue
Greece	
Ministry of Agriculture, Veterinary Institute Microbiology Department	Dr. J. Papanikolauo

Ireland Department of Agriculture and Food, Veterinary Research Laboratory	Mr. J.Ward
Italy Istituto Zooprofilattico di Vicenza	Mr. A. Ferrarese
Luxembourg Laboratoire de Médecine vétérinaire	Dr. J. Schon
Portugal Laboratorio Nacional de Veterindria	Dra. A. Amado Mrs M. do Rosario Vieira
Spain Laboratorio de Sanidad Y Produccion Animal de Algete	not represented
Sweden National Veterinary Institute	Mrs A. Aspan Mrs K. Bergström
National Food Administration	Dr. C. Wiberg
United Kingdom Central Veterinary Laboratory, Bacteriology Department	Dr. R. Davies

ANNEX 2
PROGRAMME OF THE WORKSHOP

Wednesday 29 May, 1996

08.30 - 09.00 Opening — Henken

09.00 - 09.45 Evaluation of the first collaborative study on bacteriological detection of *Salmonella* — In 't Veld
* Objectives and method used
* Analysis and results
* Results additional study
* Conclusions and restrictions when interpreting the results
* Discussion

09.45 - 10.15 Proposal for the second collaborative study on bacteriological detection of *Salmonella* — In 't Veld
* Discussion

10.15 - 10.45 Coffee/tea

10.45 - 12.00 Collaborative studies within Member States between the National Reference Laboratory for *Salmonella* and the approved national laboratories — Van de Giessen

* Introduction: Van de Giessen
* Infrastructure and collaborative studies in the Netherlands: Van de Giessen
* Infrastructures/Experiences on collaborative studies within other Member States
Speakers: Davies, Bergström and Baggesen
* Criteria for organizing collaborative bacteriological studies: In 't Veld
* Discussion on organization of collaborative studies: Henken

12.00 - 12.45 Evaluation of the first collaborative study on (sero-)typing of *Salmonella* — Van Leeuwen + Voogt
* Introduction: Van Leeuwen
* Presentation of the report and discussion: Voogt
* Serotyping using agglutination in microtitre plates
Speakers: Davies and Van Leeuwen

12.45 - 14.00 Lunch

14.15 - 17.00 Visit to RIVM laboratories

17.00 - 17.30 Coffee/tea with a snack

17.30 - 18.30 Collaborative study for immunological detection of S. Enteritidis/S. Typhimurium: the present situation Notermans
* Introduction: Notermans
  - back ground information
* Presentation of the preliminary results of the collaborative study: In 't Veld
  - objectives and method
  - data on reference materials
  - statistical analysis and preliminary results
* Discussion: Henken

18.30 - 19.30 Need for training Notermans

20.30 - 23.00 Dinner in 'The White Swan'

Thursday 30 May, 1996

08.30 - 10.30 Information on research activities on new methods
- bacteriological In 't Veld
* Overview on results survey methods used in relation to sample material (raw meat, chicken meat and faeces)
* Presentations of laboratories on their research activities
  Speakers: Kivelä, Ferrarese, Baggesen and Davies
- typing Van Leeuwen + Voogt
  Speakers: Ferrarese and Aspan
- immunological Notermans
  Speakers: Notermans and Davies
* Discussion on how to achieve coordination of research efforts: Henken

10.30 - 11.00 Coffee/tea

11.00 - 12.00 The use of alternative methods In 't Veld
* Summary of discussion of subgroup of the scientific veterinary comittee and its recommendations
* Presentation of objectives of Microval and an overview of activities and present situation (Rentenaar (NNI)).

12.00 - 13.00 Plans, Communication CRL/NRL's, evaluation and closing remarks Henken

13.00 - 14.00 Lunch

14.30 Shuttle to airport for workshop participants

ANNEX 3

MICROVAL, A CHALLENGING EUREKA PROJECT

Validation and approval of alternative methods for the microbiological analysis of food, animal feedingstuffs and beverages.

Irene M.F. Rentenaar, project secretary MicroVal
NEDERLANDS NORMALISATIE-INSTITUUT
P.O. Box 5059
NL-2600 GB Delft, The Netherlands

INTRODUCTION

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

Before a new microbiological method can be widely accepted not only must its intrinsic technical quality be established objectively, but, as mentioned above, there must also be a guarantee that interested parties will accept the results obtained using such a method. It should be pointed out in this respect that in the commercial interest of the suppliers and users of novel test methods, such a situation should be realised within an acceptable period, preferably within a year - as opposed to, for example, the approval programme of the AOAC (formerly the Association of Official Analytical Chemists) which takes about three years. At present there is no European mechanism to achieve general acceptance. This is not in the interest of the development of a European food market and does not stimulate the development of a European test kit industry.

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

PROJECT PLANNING

The project design consisted of three stages, as described in detail below.

Stage 1: Feasibility (1 July 1993 - 1 July 1994)

In this stage a decision had to be made as to whether it was possible to arrive at a uniform concept for a European validation procedure of alternative microbiological test methods for food products that is acceptable to all project partners. On the one hand one could envision a body that takes charge of the organisation of the validation for the whole of Europe and on the other hand one could think of a mutual recognition of procedures that were to be carried out strictly on a national level. In view of the complexity and diversity of the many new test methods that have originated from bio-technology, there was a preference for a super-national mechanism (i.e. an international network of laboratories and experts). This did not however preclude placing the administration and management in the hands of one or more well-equipped national institutes. It was clear that many practical matters had to be considered as regards the precise details and interpretation of the procedure. Examples of this include specific requirements for the

laboratories involved in the validation, a group of experts involved at the start and completion of a validation, the method of reporting, etc. In this first year these aspects required thought, discussion and many meetings. The information available on existing procedures from AOAC International was considered as well.
In June 1994 the Steering Committee decided that there was sufficient consensus and foundation to proceed to the second stage.

Stage 2: (1 July 1994 - 1 July 1996)

Five separate validations have been planned for in this stage; two were carried out in the first year, three will be carried out in the second year.

The experiences gained as a result will be used to focus the procedure further. The aim will be to perform the procedure as realistically as possible, which should eventually lead to a consensus about the best mechanism for the validation of microbiological methods in Europe. In this stage it will also become clear whether the execution of an entire validation procedure within one year is feasible. The test kits to be validated will have to reflect a wide diversity, so that a test kit in this context can also be a specific apparatus or instrument.

Parallel to the technical work that must be done in the form of testing the proposed procedure in practical situations, there is also work to be done on several prerequisites that have to be fulfilled in order to arrive at a general acceptation of the validation procedure in Europe.

Discussions with other parties (such as AOAC, NMKL, CEN) involved in validation (and/or standardisation and certification) have been started with a view to investigating the opportunities for the exchange of data or for a mutual acknowledgement of each other's results.

Stage 3: Reporting (1 July 1996 - 1 July 1997)

This year will be used for the finalization of the project thus reporting and publication of the results of the project taking place. Because of the intended acceptance by various European and national institutions all aspects of the validation procedure will have to be documented in great detail.

ORGANISATION OF THE PROJECT

Management of the project

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

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

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

Working Groups

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

Working Group 1

It was the task of the first working group to draw up a survey of all existing documents and procedures, to compare them and to make a recommendation concerning the procedure that is the most suitable to serve as a starting point for the "MicroVal project". To this end, the working group has studied information on the validation procedures used by or proposed by l'Association Française de Normalisation (AFNOR), AOAC International, the AOAC Research Institute, the European Community (EC), the International Dairy Federation (IDF) and the International Union of Pure and Applied Chemistry (IUPAC). The final report of the first working group was presented at the first Annual Meeting in Paris, June 1994.

Working Group 2

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

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

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

The MicroVal certification procedure is based on three principles.
The **first** principle of the certification is to perform a comparative study of the alternative method against the reference method, followed by a collaborative study of the alternative method .
The **second** principle is that the quality organization of the manufacturer where the materials are produced must be in conformity with quality assurance requirements. The frame of these requirements is the EN 29002 standard (ISO 9002).
The **third** principle is a regular control of the quality of the certified methods is made after the certification is granted.

The intended European certification organization is constituted of a network of MicroVal Certification Bodies and headed by the European MicroVal Committee (for the moment the MicroVal Steering Committee) which has a European composition and is in charge of:

- the elaboration/modification of the rules
- the elaboration of the list of reference methods, reference materials, culture collections, expert laboratories, methods and laboratory reviewers, technical auditors
- the admission of MicroVal Certification Bodies,
- the control of the activity of MicroVal Certification Bodies.

The MicroVal Certification Bodies are responsible for handling applications and evaluating the reports of the expert laboratories and the auditors. Each MicroVal Certification Body, must establish a MicroVal Committee. All interested parties shall be represented on this committee. The certification bodies shall comply with requirements in EN 45011 and shall be accredited. It is still under discussion whether the European MicroVal Committee or the MicroVal Certification Bodies will grant the certification licences.

The intended procedure is described below.
The manufacturer presents his request for the certification of a alternative method to a MicroVal Certification Body. This body checks the administrative conformity of the request and selects one method reviewer, one laboratory reviewer (if necessary) and one quality auditor. Three additional reviewers (one method and two laboratory reviewer) and one technical auditor are selected by the European MicroVal Committee.

The expert laboratory is in charge of the coordination and the supervision of the two phases of the certification procedure. The expert laboratory must comply with the requirements of EN 45001 and be accredited .
It is preselected by the manufacturer or the MicroVal Certification Body from the database of laboratories established by the European MicroVal Committee. The qualification "expert laboratory" is only valid for the MicroVal certification of the alterative method it was selected for.

The collaborative laboratories are selected by the expert laboratories There must be a minimum of 8 from 3 different countries. These laboratories must comply with EN 45001.

The MicroVal Committee advises the MicroVal Certification Body and/or the European MicroVal Committee on whether or not certification should he granted. This is done on basis of the report of the 2 method reviewers. The final decision is taken by the MicroVal Certification Body or the European MicroVal Committee. This is still under discussion. The Secretariat of the European MicroVal Committee is informed of this decision .

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

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

A further aim of Working Group 2 is to write a **MicroVal Certification Scheme** which is the complete and detailed procedure of certification.

Working Group 3

The third working group has started in parallel with the second, and is focusing on the drawing up of the **technical rules** for the validation, the test protocol and the organization of the trial validations in the second stage of the project. The subjects that will be dealt with here concern the determination of the obligatory and voluntary criteria (precision, specificity, determination limit, etc.) that the alternative methods will have to meet and the definitions of the technical criteria.

The trial validations of 2 test-kits "Salmonella-Tek of Organon Teknika" and "Listeria immunoenzymatic detection of Transia Diffchamb" have already taken place. As a result of the experience from these two studies, the technical rules have been implemented and precised.

The second round of trial validation will give more attention to quantitative methods, also a quantitative instrumental method will be included. The following test-kits have been selected:

- Genetrak, hybridisation Assay for Listeria monocytogenes (R-Biopharm)
- 3 M- Petrifilm high sensitivity Coliforms (3 M)
- Bactrac 4000 Series (SY-LAB)

Comparative and collaborative studies are on the way.

The Working Group is also considering how data from other sources (e.g. AOAC, manufacturers, research establishments) can be incorporated into the mechanism.

CONCLUSION

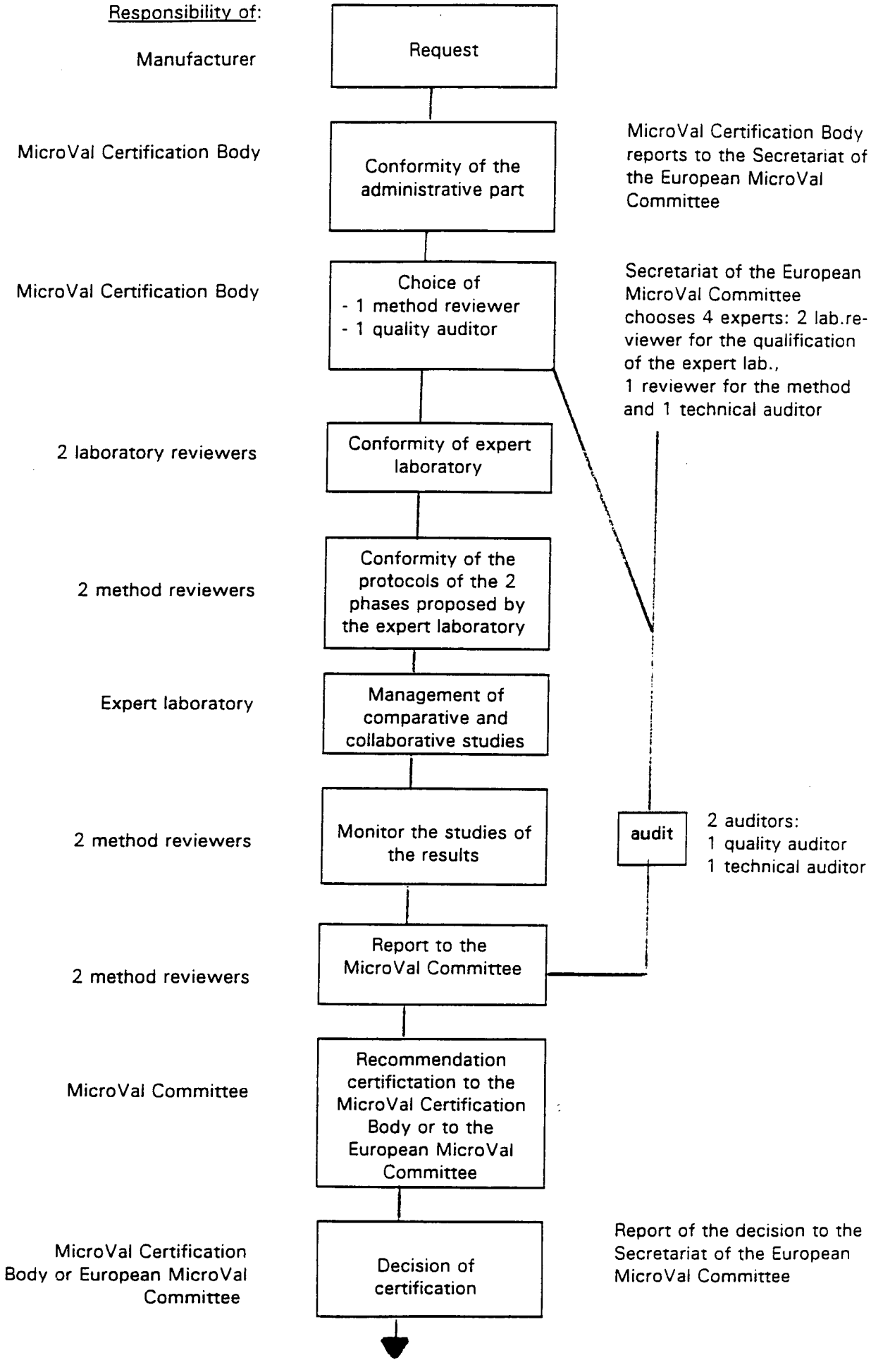
Although the MicroVal certification procedure is still under development, we thought it would be interesting to inform you about our aims and how we think alternative methods will be validated in Europe in the future.

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

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

INTENDED

FLOWCHART OF THE CERTIFICATION PROCESS



TECHNICAL RULES AND VALIDATION CRITERIA (1)

Two categories of methods are distinguished:

* QUALITATIVE METHOD. The responds of the method is absence of presence of the analyte

* QUANTITATIVE METHOD. The method enables determination of the amount of the analyte (semi-quantitative methods are included)

TECHNICAL RULES AND VALIDATION CRITERIA (2)

Parameters to be determined:

1. Qualitative methods

A. Comparative study

- Relative trueness
- Limit of detection
- Specificity

B. Collaborative study

- Precision
- Repeatability
- Reproducibility

TECHNICAL RULES AND VALIDATION CRITERIA (3)

Parameters to be determined:

2. Quantitative methods

A. Comparative study

- Linearity
- Relative trueness
- Specificity
- Limit of determination
- Sensitivity

B. Collaborative study

- Repeatability
- Reproducibility

TECHNICAL RULES AND VALIDATION CRITERIA (4)

For each parameter the following points are described:

- Definition
- Measurement protocol
- Calculation
- Interpretation

TECHNICAL RULES AND VALIDATION CRITERIA (5)

Other items addressed in the technical rules:

- Categories of food to be tested per microorganism for evaluating the relative trueness
- Assessment of negative control and low levels of contamination
- Guidelines for the organizations of the collaborative studies (including the preparation of samples)
- Minimum number of samples for the measurement protocol of the precision for qualitative methods
- Flow chart for the acceptance of external results (e.g. AOAC, NMKL)
- Design of a spiking protocol (absence of naturally contaminated samples)

TECHNICAL RULES AND VALIDATION CRITERIA (6)

Categories of food to be tested per microorganism for evaluating the relative trueness

Salmonella

- Raw meat products and raw minced products of poultry (e.g. chicken/turkey), pork, beef
- Processed meat products and seafood
- Dairy products: cheese powder, milk powder
- Egg and egg products: raw egg, processed egg products
- Animal feed
- Chocolate and spices

TABLE: VALIDATION PROCEDURES INVENTORY

	AFNOR	AOAC RI	AOAC INT	IDF	IUPAC
1 SYSTEM OF REFERENCE					
1.1 REFERENCE METHOD					
1.1.1 USE	YES	RECOMMENDED	YES	YES	YES
1.1.2 DEFINITION	YES		YES	NO	NO
1.1.3 LIST	NO			NO	NO
1.2 VALIDATION PROTOCOL					
1.2.1 STEPS	PRELIMINARY + COLLABORATIVE	PERFORMANCE-TESTING	PRELIMINARY + COLLABORATIVE	PRELIMINARY + COLLABORATIVE	PRELIMINARY + COLLABORATIVE
1.2.2 CRITERIA					
1.2.2.1 DEFINITION	YES*	YES	YES	YES**	YES***
1.2.2.2 LIST	YES	YES	YES	YES	NO
1.3 REQUIREMENTS FOR METHOD MANUFACTURERS	YES	YES	-	NO	NO
1.4 REQUIREMENTS FOR LABORATORIES AND CERTIFICATION BODIES	NO	YES/LABS	-		NO
2 CERTIFICATION					NO
2.1 BODIES INVOLVED	AFNOR	AOAC RI	AOAC INT	NO	
2.2 FLOW CHART	YES	YES	YES	NO	
2.3 VALIDATION METHOD STATUS	ROUTINE ANALYSIS ANALYSIS BY CERTIFIED LABS				
2.4 INFORMATION	YES	YES	YES		
2.5 VALIDATION DURATION	4 YEARS		1ST ACTION; 2 YEARS MIN.		

* AFNOR STANDARD: V03-100

** IDF 128:1965 - IDF 161:1992

*** Pure and Appl. Chem. 1966 Vol 60 N° 6 and 1990 Vol. 62 N° 1

10 STEPS OF THE AOAC RI PERFORMANCE TESTING PROGRAM

1 Application and data submission

2 Preliminary data review

3 Identify expert reviewers (two)

4 Identify independent laboratory

5 Develop and approval testing protocol

6 Testing by independent laboratory

7 Independent laboratory to complete evaluation

8 Expert reviewers assess lab results

9 Kit producer submits revision of claims, if any

10 Report on approval (1 year) or denial status

CRITERIA FOR GRANTING "PERFORMANCE TESTED" STATUS:

1 The applicant's performance data supports and confirms all claims made in the kit's descriptive insert, and

2 The independent laboratory's performance data corroborates the applicants' performance data within the statistical limits specified in the protocol.

The selection of the most appropriate parameters to be compared will be determined by the expert reviewers panel on an application by application basis depending on the technology of the particular test kit model.