National Institute for Public Health
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EU Interlaboratory comparison study veterinary XIII (2010)

Detection of Salmonella in chicken faeces

Report 330604018/2010

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Rijksinstituut voor Volksgezondheid
en Milieu
*Ministerie van Volksgezondheid,
Welzijn en Sport*

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This investigation has been performed by order and for the account of European Health and Consumer Protection Directorate-General, within the framework of V/330604/10/CS by the Community Reference Laboratory for *Salmonella*

List of abbreviations

BGA (mod)	Brilliant Green Agar (modified)
BSA	Brilliance *Salmonella* Agar
BPLSA	Brilliant green Phenol-red Lactose Sucrose Agar
BPW	Buffered Peptone Water
BxLH	Brilliant green, Xylose, Lysine, Sulphonamide
cfp	colony forming particles
CRL	Community Reference Laboratory
dPCA	double concentrated Plate Count Agar
dVRBG	double concentrated Violet Red Bile Glucose agar
EFTA	European Free Trade Association
EU	European Union
Gal	Galactosidase
hcmp	highly contaminated milk powder
ISO	International Standardisation Organisation
LDC	Lysine Decarboxylase
MIC	Minimum Inhibitory Concentration
MKTTn	Mueller Kauffmann Tetrathionate novobiocin broth
MS	Member State
MSRV	Modified Semi-solid Rappaport Vassiliadis
NRL	National Reference Laboratory
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
RIVM	Rijksinstituut voor Volksgezondheid en het Milieu (National Institute for Public Health and the Environment)
RM	Reference Material
RVS	Rappaport Vassiliadis Soya broth
SC	Selenite Cystine broth
SE	*Salmonella* Enteritidis
SM (ID)2	*Salmonella* Detection and Identification-2
SOP	Standard Operating Procedure
SS	*Salmonella Shigella* medium
STM	*Salmonella* Typhimurium
TSI	Triple Sugar Iron agar
UA	Urea Agar
VP	Voges-Proskauer
VRBG	Violet Red Bile Glucose agar
XLD	Xylose Lysine Deoxycholate agar
XLT4	Xylose Lysine Tergitol 4 agar

Abstract

EU Interlaboratory comparison study veterinary XIII (2010)
Detection of *Salmonella* in chicken faeces

Thirty-three National Reference Laboratories (NRLs) for *Salmonella* in the European Union participated in an interlaboratory comparison study in 2010. Of these, thirty-one were able to detect both high and low levels of *Salmonella* in chicken faeces, thereby achieving the desired proficiency level of 'good performance' for the prescribed method. Two laboratories achieved an assessment of only 'moderate performance'. One of these NRLs had difficulty in detecting low levels of *Salmonella* (a sensitivity problem). The second experienced problems in following the prescribed protocol which, in this particular study, included an extra control measure.

These are the results of the thirteenth veterinary interlaboratory comparison study organized by the Community Reference Laboratory (CRL) for *Salmonella*. The study was conducted in March 2010. All NRLs responsible for *Salmonella* detection from all European Member States were required to participate in this study. The CRL for *Salmonella* is part of the Dutch National Institute for Public Health and the Environment (RIVM).

The internationally prescribed method for demonstrating the presence of *Salmonella* in veterinary samples was used during the study. The application thereof resulted in more than 97 percent of the samples being found to be positive for *Salmonella*.

The laboratories were obligated to conduct the study according to the instructions given. Each laboratory received a package containing chicken faeces (free of *Salmonella*) and thirty-five gelatine capsules containing powdered milk infected with different levels of *Salmonella*. The laboratories were instructed to spike samples of chicken faeces with each of the capsules and then test all samples for the presence of *Salmonella*. The extra control measure was included to check whether all participating laboratories added the faeces to the capsules, which was expressly prescribed in the protocol. It consisted of one batch of chicken faeces mixed with an antibiotic to which the *Salmonella* used in this ring trial is susceptible. A negative test result for the presence of *Salmonella* must therefore be obtained in these samples.

Key words: *Salmonella*; CRL; NRL; interlaboratory comparison study; chicken faeces; *Salmonella* detection methods; antibiotic

Rapport in het kort

EU Ringonderzoek veterinair XIII (2010)
Detectie van *Salmonella* in kippenmest

In 2010 waren 31 van de 33 Nationale Referentie Laboratoria (NRL's) in de Europese Unie in staat om hoge en lage concentraties *Salmonella* in kippenmest aan te tonen. Ze behaalden hiermee een goed resultaat. Twee laboratoria werden beoordeeld met een matig resultaat. Een van deze NRL's had moeite de lage concentraties *Salmonella* in kippenmest aan te tonen (een gevoeligheidsprobleem). Het andere NRL had problemen het voorgeschreven protocol te volgen, wat deze keer met behulp van een extra controle werd getoetst.

Dit blijkt uit het dertiende veterinair ringonderzoek dat het Communautair Referentie Laboratorium (CRL) voor *Salmonella* in maart 2010 heeft georganiseerd. Deelname aan dit onderzoek is verplicht voor alle NRL's van de Europese lidstaten die verantwoordelijk zijn voor de detectie van *Salmonella*. Het CRL-*Salmonella* is gevestigd bij het Nederlandse Rijksinstituut voor Volksgezondheid en Milieu (RIVM).

Tijdens de studie wordt de internationaal voorgeschreven methode gebruikt om *Salmonella* aan te tonen in dierlijk mest. Deze methode toonde in meer dan 97 procent van de monsters *Salmonella* aan.

De laboratoria moesten de studie volgens voorschrift uitvoeren. Elk laboratorium kreeg een pakket toegestuurd met kippenmest (vrij van *Salmonella*) en 35 gelatine capsules met melkpoeder dat verschillende besmettingsniveaus van *Salmonella* bevatte. De laboratoria dienden de kippenmest en capsules samen te voegen en vervolgens te onderzoeken of er *Salmonella* aanwezig was. De extra controle was ingelast om te toetsen of de deelnemende laboratoria daadwerkelijk de kippenmest hadden toegevoegd aan de capsules, wat het protocol voorschrijft. Hiervoor werd een partij kippenmest gemengd met een antibioticum waarvoor de *Salmonella* die in dit ringonderzoek werden gebruikt, gevoelig zijn. Met als resultaat dat in deze monsters de *Salmonella* juist niet moest worden aangetroffen.

Trefwoorden: *Salmonella*; CRL; NRL; ringonderzoek; kippenmest; *Salmonella* detectiemethode; antibioticum

Summary

In March 2010 the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*) organised the thirteenth veterinary interlaboratory comparison study on bacteriological detection of *Salmonella* (chicken faeces). Participants were 33 National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*): 28 NRLs from 27 EU Member States, three NRLs from member countries of the European Free Trade Association (EFTA): Switzerland, Norway and Iceland and on request of DG-Sanco two non-Europe NRLs from third countries Israel and Tunisia.

The most important objective of the study was to test the performance of the participating laboratories for the detection of *Salmonella* at different contamination levels in a veterinary matrix. To do so, chicken faeces samples of 10 g each, were analysed in the presence of reference materials (capsules) containing either *Salmonella* (at various contamination levels) or sterile milk powder. A proposal for good performance was made and the performance of the laboratories was compared to this proposal. The prescribed method was Annex D of ISO 6579, with selective enrichment on Modified Semi-solid Rappaport Vassiliadis (MSRV) agar. Optionally a laboratory could also use other, own media or procedures for the detection of *Salmonella*.

Thirty-five individually numbered capsules had to be tested by the participants for the presence or absence of *Salmonella.* Twenty-five of the capsules had to be examined in combination with each 10 grams of *Salmonella*-negative chicken faeces: 5 capsules contained approximately 5 colony forming particles (cfp) of *Salmonella* Typhimurium (STM5), 5 capsules contained approximately 50 cfp of *S*. Typhimurium (STM50), 5 capsules contained approximately 20 cfp of *S*. Enteritidis (SE20), 5 capsules contained approximately 100 cfp of *S*. Enteritidis (SE100) and 5 capsules contained no *Salmonella* at all (blank capsules). Six capsules, to which no faeces had to be added, were control samples, existing of 2 capsules STM5, 2 capsules SE20, 1 capsule SE100 and 1 blank capsule.
An extra control measure was included to check whether all participating laboratories added the faeces to the capsules, which was expressly prescribed in the protocol. It consisted of one batch of chicken faeces mixed with an antibiotic to which the *Salmonella* used in this ring trial is susceptible. A negative test result for the presence of *Salmonella* must therefore be obtained in these samples.

On average the laboratories found *Salmonella* in 97% of the (contaminated) samples when using the prescribed veterinary method, selective enrichment on MSRV.

Thirty-one NRLs fulfilled the criteria of good performance. Two laboratories achieved an assessment of only 'moderate performance'. One of these NRLs had difficulty in detecting low levels of *Salmonella* (a sensitivity problem). The second experienced problems in following the prescribed protocol which, in this particular study, included an extra control measure.

1. Introduction

An important task of the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*), as laid down in Commision Regulation EC No 882/2004, is the organisation of interlaboratory comparison studies. The history of the interlaboratory comparison studies as organised by CRL-*Salmonella* since 1995 is summarised in Annex 1. The first and most important objective of the study, organized by the Community Reference Laboratory (CRL) for *Salmonella* in March 2010, was to see if the participating laboratories could detect *Salmonella* at different contamination levels in animal faeces. This information is important to know whether the examination of samples in the EU Member States is carried out uniformly and comparable results can be obtained by all National Reference Laboratories for *Salmonella* (NRL-*Salmonella*). The second objective was to compare the different methods for the detection of *Salmonella* in animal faeces.
In this study an extra control was included to check whether participants added the faeces to the capsules. Therefore one batch of faeces was mixed with an antibiotic to which the *S*. Enteritides (SE) and *S*. Typhimurium (STM) strains used in the ring trial are susceptible.

The prescribed method is Modified Semi-solid Rappaport Vassiliadis (MSRV) agar as selective enrichment medium for the detection of *Salmonella* spp. in animal faeces (Annex D of ISO 6579, Anonymous 2007).

The set-up of this study was comparable to earlier interlaboratory comparison studies on the detection of *Salmonella* spp. in veterinary, food and feed samples. The contamination level of the low level capsules was close to the detection limit of the method; the level of the high level samples was approximately 5-10 times above the detection limit. Six control samples consisting of different reference materials, had to be tested without the addition of chicken faeces. These latter reference materials consisted of 2 capsules with approximately 5 cfp of *Salmonella* Typhimurium (STM5), 2 capsules with approximately 20 cfp of *Salmonella* Enteritidis (SE20), 1 capsule with approximately 100 cfp of *Salmonella* Enteritidis (SE100) and 1 blank capsule. Twenty-five samples of *Salmonella* negative chicken faeces spiked with 5 different reference materials had to be examined. For the latter samples the different reference materials consisted of two levels of *Salmonella* Typhimurium (STM5 and STM50), 2 levels of *Salmonella* Enteritidis (SE20 and SE100) and blank reference materials. Extra control samples were included; four SE20 capsules had to be tested with the addition of chicken faeces mixed with an antibiotic.

2. Participants

Country	City	Institute
Austria	Graz	Austrian Agency for Health and Food Safety (AGES IVET)
Belgium	Brussels	Veterinary and Agrochemical Research Center (VAR/ CODA) General and Molecular Bacteriology
Bulgaria	Sophia	National Diagnostic and Research Veterinary Institute
Cyprus	Nicosia	Ministry of Agriculture, Natural Resources and Environment Veterinary Services Laboratory for the Control of Foods of Animal Origin (LCFAO)
Czech Republic	Prague	State Veterinary Institute
Denmark	Copenhagen	National Food Institute, Technical University of Denmark Department of Microbiology and Risk Assessment
Estonia	Tartu	Estonia Veterinary and Food Laboratory, Bacteriology-Pathology Department
Finland	Kuopio	Finnish Food Safety Authority Evira Research Department, Veterinary Bacteriology
France	Ploufragan	L'Agence Française de Sécurité Sanitaire des Aliments (AFSSA/ LERAPP)
Germany	Berlin	Federal Institute for Risk Assessment (BfR) National Veterinary Reference Laboratory for *Salmonella*
Greece	Halkis	Veterinary Laboratory of Halkis Hellenic Republic Ministry of rural development and food
Hungary	Budapest	Central Agricultural Office, Food and Feed Safety Directorate Central Food-Microbiological Diagnostic Laboratory
Iceland	Reykjavik	University of Iceland Institute for Experimental Pathology
Ireland	Kildare	Central Veterinary Research Laboratory (CVRL/DAFF) Department of Agriculture, Fisheries and Food
Israel	Kiryat Malachi	Southern Laboratory for poultry health (Beer Tuvia)
Italy	Padova Legnaro	Istituto Zooprofilattico Sperimentale delle Venezie, OIE National Reference Laboratory for *Salmonella*
Latvia	Riga	Institute of Food Safety Animal Health and Environment BIOR Animal Disease Diagnostic Laboratory
Lithuania	Vilnius	National food and veterinary risk assessment institute
Luxembourg	Luxembourg	Laboratoire de Médecine Vétérinaire de l'Etat, Animal Zoonosis
Malta	Valletta	Public Health Laboratory (PHL) Evans Building
Netherlands the	Bilthoven	National Institute for Public Health and the Environment (RIVM/Cib) Centre for Infectious Diseases Control Laboratory for Zoonoses and Environmental Microbiology(LZO)

Country	City	Institute
Norway	Oslo	National Veterinary Institute, Section of Bacteriology
Poland	Pulawy	National Veterinary Research Institute (NVRI) Department of Microbiology
Portugal	Lisbon	Laboratório Nacional de Investigação Veterinária (LNIV)
Romania	Bucharest	Institute for Diagnosis and Animal Health, Bacteriology
Slovak Republic	Bratislava	State Veterinary and Food Institute Reference Laboratory for *Salmonella*
Slovenia	Ljubljana	National Veterinary Institute, Veterinary Faculty
Spain	Madrid Algete	Laboratorio de Sanidad y Produccion Animal de Algete Central de Veterinaria
Sweden	Uppsala	National Veterinary Institute (SVA), Department of Bacteriology
Switzerland	Bern	National Centre for Zoonoses, Bacterial Animal Diseases and Antimicrobial Resistance (ZOBA), Institute of veterinary bacteriology, Vetsuisse faculty Berne
Tunisia	Tunis	Veterinary Research Institute of Tunisia, Bacteriology
United Kingdom	Addlestone	Veterinary Laboratories Agency (VLA) Weybridge Department of Food and Environmental Safety
United Kingdom	Belfast	Agri-Food and Bioscience Institute (AFBI) Veterinary Sciences Division Bacteriology

3. Materials and methods

3.1 Reference materials

Five batches of *Salmonella* reference materials were prepared. For this purpose milk, artificially contaminated with a *Salmonella* strain, was spray-dried (In 't Veld et al., 1996). The obtained highly contaminated milk powder (hcmp) was mixed with sterile (γ-irradiated) milk powder (Carnation, Nestlé, the Netherlands) to obtain the desired contamination level. The mixed powder was filled into gelatine capsules resulting in the final reference materials (RMs).
The target levels of the batches of RMs were:

- 5 and 50 colony forming particles (cfp) per capsule for *Salmonella* Typhimurium (STM5 and STM50);
- 20 and 100 colony forming particles (cfp) per capsule for *Salmonella* Enteritidis (SE20 and SE100).

Before filling all mixed powders into gelatine capsules, test batches of 60 capsules were prepared of each mixture to determine the mean number of cfp per capsule and the homogeneity of the mixture. The remaining mixed powders were stored at –20 °C. If the test batches fulfilled the pre-set criteria for contamination level and homogeneity, the relevant mixed powders were completely filled into gelatine capsules and stored at –20 °C.
The pre-set criteria were:

- mean contamination levels should lie between target level minus 30% and target level plus 50% (e.g. between 70 and 150 cfp if the target level is 100 cfp);
- for the homogeneity within one batch of capsules the maximum demand for the variation between capsules should be $T_2/(I-1) \leq 2$, where T_2 is a measure for the variation between capsules of one batch (see formula in Annex 2) and I is the number of capsules.

The contamination levels of the capsules were determined following the procedure as described by Schulten et al. (2000). In short the procedure is as follows:

- reconstitution of each capsule in 5 ml peptone saline solution in a Petri dish at (38.5 ± 1) °C for (45 ± 5) min;
- repair of *Salmonella* by the addition of 5 ml molten double concentrated plate count agar (dPCA) to the reconstituted capsule solution, and after solidification, incubation at (37 ± 1) °C for (4 ± ½) h;
- after incubation, 10 ml of molten double concentrated Violet Red Bile Glucose agar (dVRBG) was added as an overlayer and after solidification the plates were incubated at (37 ± 1) °C for (20 ± 2)h.

3.2 Chicken faeces samples

3.2.1 General

Chicken faeces was sampled by the Animal Health Service (GD) Deventer at a *Salmonella* free farm (SPF-farm). A large batch of 24 kilogram from this farm arrived at the CRL-*Salmonella* on 11 January 2010. The faeces was stored at 5 °C and checked for the absence of *Salmonella* by testing 10 portions of 25 g chicken faeces randomly picked from the large batch. For the testing for *Salmonella* Annex D of ISO 6579 (Anonymous, 2007) was followed. For this purpose 10 portions of 25 g were each added to 225 ml Buffered Peptone

Water (BPW). After pre-enrichment at (37 ± 1) °C for 16-18 h, selective enrichment was carried out on Modified Semi-solid Rappaport Vassiliadis (MSRV). Next, the suspect plates were plated-out on Xylose Lysine Deoxycholate agar (XLD) and Brilliant Green Agar (BGA) and confirmed biochemical. Three kilogram of Faeces was mixed with an antibiotic; this was labelled as batch A. The remaining 21 kg of chicken faeces was labelled as faeces batch B. The chicken faeces was stored at 5 °C until further use.

3.2.2 Total bacterial count in chicken faeces

The total number of aerobic bacteria was investigated in the chicken faeces. The procedure of ISO 4833 (Anonymous, 2003) was followed for this purpose. Portions of 20 gram faeces were homogenized into 180 ml peptone saline solution in a plastic bag. The content was mixed by using a pulsifier (60 sec). Next tenfold dilutions were prepared in peptone saline solution. Two times 1 ml of each dilution was brought into 2 empty Petri-dishes (diameter 9 cm). To each dish 15 ml of molten Plate Count Agar (PCA) was added. After the PCA was solidified an additional 5 ml PCA was added to the agar. The plates were incubated at (30 ± 1) °C for (72 ± 3) h and the total number of aerobic bacteria was counted after incubation.

3.2.3 Number of Enterobacteriaceae in chicken faeces

In addition to the total count of aerobic bacteria, the Enterobacteriaceae count was determined. The procedure of ISO 21528-2 (Anonymous, 2004) was used for this purpose. Portions of 20 gram faeces were homogenized into 180 ml peptone saline solution in a plastic bag. The content was mixed by using a pulsifier (60 sec). Next tenfold dilutions were prepared in peptone saline solution. Two times 1 ml of each dilution was brought into 2 empty Petri-dishes (diameter 9 cm). To each dish, 10 ml of molten Violet Red Bile Glucose agar (VRBG) was added. After the VRBG was solidified an additional 15 ml VRBG was added to the agar. These plates were incubated at (37 ± 1) °C for (24 ± 2) h and the number of typical violet-red colonies was counted after incubation. Five typical colonies were tested for the fermentation of glucose and for a negative oxidase reaction. After this confirmation the number of Enterobacteriaceae was calculated.

3.2.4 Development of a stabile mixture of chicken faeces with an antibiotic

It was decided to include extra controls in this study, to check whether the participants would indeed follow the protocol for mixing faeces and capsules. For this purpose chicken faeces was mixed with an antibiotic which had to fulfil the following requirements:

- *Salmonella* Typhimurium and *Salmonella* Enteritidis used in the ring trial had to be susceptible for the antibiotic;
- The antibiotic had to be stabile at the storage temperature of the faeces (5 °C);
- Preferably, some background flora in the chicken faeces had to be resistant for the type and concentration of antibiotic used.

Literature was searched for the antibiotic which fulfilled most of the requirements. Furthermore, information was gained at experts of the Central Veterinary Institute (CVI), the Netherlands (D. Mevius), the Food and Consumer Product Safety Authority (VWA), the Netherlands (P. in 't Veld) and of the laboratory for Infectious Diseases and Perinatal Screening (LIS) of the RIVM, the Netherlands (E. Spalburg). It was concluded that the most suitable antibiotic was Gentamicin (Lorian, 2005).
A susceptibility test (Etest AB Biodisk, Sweden) between MIC 0.064 µg/ml and 1024 µg/ml Gentamicin was performed with the SE and STM strains used in the study. The influence of Gentamicin on the background flora of the faeces was tested by comparing the growth on different agar plates with and without Gentamicin (MIC_{50}). A mixture of 'antibiotic-faeces' was made with different levels of Gentamicin and stored at 5 °C. The growth of *Salmonella*

in the mixture of 'antibiotic-faeces' was tested with reference materials: capsules with different levels of SE and STM. For the detection of *Salmonella*, Annex D of ISO 6579 (Anonymous, 2007) was followed. The stability of the mixture of 'antibiotic-faeces' during storage at 5 °C was tested by repeating the test for growth of *Salmonella* capsules weekly up to 6 weeks. The influence of Gentamicin on the background flora of the faeces was tested by counting the total number of aerobic bacteria and the number of Enterobacteriaceae (as described in section 3.2.2 and 3.2.3) in the 'antibiotic-faeces' mixture during storage.

3.3 Design of the interlaboratory comparison study

3.3.1 Samples: capsules and chicken faeces

On March 8, 2010 (1 week before the study) the reference materials (35 individually numbered capsules) and 2 batches of *Salmonella* negative chicken faeces (60 g faeces mixed with Gentamicin and 300 g non-mixed faeces) were packed with cooling devices as biological substance category B (UN 3373) and sent by door-to-door courier service to the participants. After arrival at the laboratory the capsules had to be stored at –20 °C and the faeces had to be stored at +5 °C until the start of the study. Details about mailing and handling of the samples and reporting of test results can be found in the Protocol (Annex 4) and Standard Operation Procedure (Annex 5). The test report which was used during the study can be found at the CRL-*Salmonella* website: http://www.rivm.nl/crlsalmonella/prof_testing/detection_stud/ or can be obtained through the corresponding author of this report.

Six control capsules had to be tested without faeces (numbered C1-C6). Twenty-five capsules (numbered B1-B25) were each tested in combination with 10 grams of faeces (negative for *Salmonella*) of batch B. Four capsules (numbered A1-A4) were each tested in combination with 10 grams of faeces (negative for *Salmonella*) of batch A. This faeces was mixed with an antibiotic. The types and the number of capsules and faeces samples which had to be tested are shown in Table 1.

Table 1 Overview of the types and the number of capsules tested per laboratory in the interlaboratory comparison study.

Capsules	Control capsules (n=6) No matrix added	Control capsules (n=4) with 10 g *Salmonella* negative chicken faeces mixed with an antibiotic	Test samples (n=25) with 10 g *Salmonella* negative chicken faeces
S. Enteritidis 20 (SE20) batch1	2	-	5
S. Enteritidis 20 (SE20) batch2	-	4	-
S. Enteritidis 100 (SE100)	1	-	5
S. Typhimurium 5 (STM5)batch1	-	-	5
S. Typhimurium 5 (STM5) batch2	2	-	-
S. Typhimurium 50 (STM50)	-	-	5
Blank	1	-	5

3.3.2 Sample packaging and temperature recording during shipment

The capsules and the chicken faeces were packed in 2 plastic containers firmly closed with screw caps (biopacks). Both biopacks were placed in one large shipping box, together with three frozen (-20 °C) cooling devices. Each shipping box was sent as biological substances category B (UN3373) by door-to-door courier services. For the control of exposure to abusive temperatures during shipment and storage, so called micro temperature loggers were used to record the temperature during transport. These loggers are tiny sealed units in a 16 mm diameter and 6 mm deep stainless steel case. Each shipping box contained one logger, packed in the biopack with capsules. The loggers were programmed by the CRL-*Salmonella* to measure the temperature every hour. Each NRL had to return the temperature recorder, immediately after receipt of the parcel, to the CRL. At the CRL-*Salmonella* the loggers were read by means of the computer and all data from the start of the shipment until the arrival at the National Reference Laboratories were transferred to an Excel graph which shows all recorded temperatures.

3.4 Methods

The prescribed method of this interlaboratory comparison study was Annex D of ISO 6579 (Anonymous, 2007). Additional to the prescribed methods the NRLs were also allowed to use their own methods. These could be different medium combinations and/or investigation of the samples with alternative methods, like Polymerase Chain Reaction (PCR) based methods.

In summary:

Pre-enrichment in:
- Buffered Peptone Water (BPW) (prescribed).

Selective enrichment on:
- Modified semi-solid Rappaport Vassiliadis medium (MSRV) (prescribed);
- Own selective enrichment medium (optional).

Plating-out on:
- Xylose Lysine Desoxycholate agar (XLD) (prescribed);
- Second plating-out medium for choice (obligatory);
- Own plating-out medium (optional).

Confirmation of identity:
- Confirmation by means of appropriate biochemical tests (ISO 6579) or by reliable, commercially available identification kits and/or serological tests.

3.5 Statistical analysis of the data

The specificity, sensitivity and accuracy rates were calculated for the control samples, and the artificially contaminated samples with chicken faeces (negative for *Salmonella* spp.). The specificity, sensitivity and accuracy rates were calculated according to the following formulae:

$$\text{Specificity rate: } \frac{\text{Number of negative results}}{\text{Total number of (expected) negative samples}} \times 100\%$$

$$\text{Sensitivity rate: } \frac{\text{Number of positive results}}{\text{Total number of (expected) positive samples}} \times 100\%$$

$$\text{Accuracy rate: } \frac{\text{Number of correct results (positive and negative)}}{\text{Total number of samples (positive and negative)}} \times 100\%$$

3.6 Good performance

The criteria used for testing good performance in this study are given in Table 2. For determining good performance per laboratory, the results found with MSRV together with all combinations of isolation media used by the laboratory were taken into account. For example if a laboratory found for the STM5 capsules with matrix 3/5 positive with MSRV/BGA but no positives with MSRV/XLD, this was still considered as a good result. The opposite was performed for the blank capsules. Here also all combinations of isolation media used per laboratory were taken into account. If for example a laboratory found 2/5 blank capsules positive with MSRV/BGA but no positives with the other isolation media, this was still considered as a 'no-good' result.

Table 2 Used criteria for testing good performance in the veterinary study XIII (2010).

Control samples (capsules, no matrix)	Minimum result	
	Percentage positive	No. of positive samples / total no. of samples
SE100	100%	1/1
STM5 and SE20	50%	1/2
Blank control capsules	0%	0/1

Samples batch B: chicken faeces (capsules with matrix)	Minimum result	
	Percentage positive	No. of positive samples / Total no. of samples
Blank[1]	20% at max[1]	1/5
STM50 and SE100	80%	4/5
STM5 and SE20	50%	2-3/5

1: All should be negative. However, as no 100% guarantees about the *Salmonella* negativity of the matrix can be given, 1 positive out of 5 blank samples (20% pos.) will still be considered as acceptable.

Samples batch A: chicken faeces mixed with Gentamicin (capsules with matrix)	Maximum result	
	Percentage positive	No. of positive samples / Total no. of samples
SE20[2]	25%	1/4 at max[2]

2: All should be negative. However, as no 100% guarantees about the *Salmonella* negativity of the matrix can be given, 1 positive out of 4 samples (25% pos.) will still be considered as acceptable. Furthermore, extensive tests performed at the CRL revealed that approximately 2% of the tested SE20 capsules in combination with the 'antibiotic-faeces' could still be found positive for *Salmonella.*

4 Results

4.1 Reference materials

Table 3 describes the level of contamination and the homogeneity of the final batches of capsules. The table gives the enumerated minimum and maximum levels within each batch of capsules. The final batches were tested twice: firstly immediately after preparing the batch and secondly at the time of the interlaboratory comparison study. At the first date of testing all batches fulfilled the pre-set criteria as stated in section 3.1. Although the variation between the SE100 capsules was increased at the second date of testing the batch was still considered useful for the cause of this study.
Two batches of SE20 capsules and two batches of STM5 capsules had been prepared. One batch of SE20 capsules was prepared to be used for testing with 'antibiotic-faeces' (SE20 batch2). One batch of STM5 capsules was used as control samples (STM5 batch2).

Table 3 Level of contamination and homogeneity of SE and STM capsules.

	SE20 batch1	SE20 batch2	SE100	STM5 batch1	STM5 batch2	STM50
Final batch; Test 1						
Date testing capsules	19-02-09	03-12-09	17-09-09	21-01-09	15-02-10	07-01-09
Number of capsules tested	50	50	50	50	50	50
Mean cfp per capsule	18	22	80	6	6	62
Min-max cfp per capsule	11-29	9-37	48-109	3-12	1-14	40-78
T2 / (I-1)	0.88	1.69	1.69	1.06	1.15	1.55
Final batch; Test 2						
Date testing capsules	16-03-10	16-03-10	23-03-10	16-03-10	16-03-10	23-03-10
Number of capsules tested	25	25	25	25	25	25
Mean cfp per capsule	13	22	78	5	8	56
Min-max cfp per capsule	8-20	14-40	45-112	1-10	1-11	46-70
T2 / (I-1)	0.92	1.64	2.95	0.96	1.03	0.81

cfp = colony forming particles; min-max = enumerated minimum and maximum cfp;
formula T_2 see Annex 2; I is number of capsules; Demand for homogeneity $T_2/(I-1) \leq 2$.

4.2 Chicken faeces samples

4.2.1 General

The faeces was tested negative for *Salmonella* and stored at 5 °C. On Monday March 8, 2010 the faeces was mailed to the NRLs. After receipt, the NRLs had to store the faeces at 5 °C.
The number of aerobic bacteria and the number of Enterobacteriaceae were tested twice; firstly at the day the faeces arrived at the CRL (12/01/2010) and secondly close to the planned date of the interlaboratory comparison study (16/03/2010). Table 4 shows the results.

Table 4 Number of aerobic bacteria and the number of Enterobacteriaceae per gram of chicken faeces.

Date	Aerobic bacteria cfp/g	Enterobacteriaceae cfp/g
January 12, 2010	$9.7*10^7$	$3.7*10^3$
March 16, 2010	$6.5*10^7$	$< 1*10^2$

4.2.2 Testing faeces mixed with Gentamicin

The MIC for Gentamicin was tested on *S*. Enteritides and on *S*. Typhimurium, both isolated from the capsules. For SE the MIC was 0.25 µg/ml and for STM the MIC was 0.38 µg/ml. It was expected that the activity of Gentamicin would decrease because of interference with the faeces and because of storage. It was decided to perform further tests with a higher concentration of Gentamicin than the tested MIC value.
Pure cultures were tested on agar plates containing 2 µg/ml of Gentamicin and both gave negative results.
Tenfold dilutions in BPW of different batches of chicken faeces were inoculated on agar plates with and without the addition of 2 µg/ml Gentamicin. On the agar plates without Gentamicin 30% more growth of the total number of aerobic bacteria was observed in comparison with the agar plates containing Gentamicin.
To test the stability of the Gentamicin, a stock solution of 1000 µg/ml Gentamicin was stored for 10 weeks at 5 °C. The detection of *Salmonella* (Annex D, ISO 6579) was performed with SE and STM capsules in BPW with and without the addition of 2 µg Gentamicin/ml, prepared from the stored stock solution. SE gave a negative result while STM gave a positive result. It was decided to continue the experiments only with SE capsules. From the stored stock solution of Gentamicin dilutions were made, assuming the concentration was still 1000 µg/ml after 10 weeks of storage. In total 8 different concentrations of Gentamicin were added to BPW: varying from 400 µg/ml to 0.02 µg/ml. An SE culture was added to these BPW solutions and cultured at 37 °C for 18 h. no growth of SE was found in BPW containing > 10 µg/ml Gentamicin. This confirmed the assumption that the activity of Gentamicin would decrease during storage at 5 °C.
Different mixtures of 0, 2, 20, 110 and 166 µg Gentamicin per gram grind chicken faeces were made and stored at 5 °C. The growth of *Salmonella* in combination with the different mixtures of antibiotic-faeces was tested weekly with SE20, SE50 and SE100 capsules for up to 6 weeks. For this, 6 portions of 10 g 'antibiotic-faeces' were tested with 6 SE capsules every week. The highest numbers of negative results were found with a concentration of 110 and 166 µg Gentamicin per gram chicken faeces in combination with SE20 capsules. Of in total 42 'SE20-antibiotic-faeces' samples only one was found positive after storage of the mixed 'antibiotic-faeces' of 3 weeks. All other SE20 samples were tested negative, even after 6 weeks of storage of these mixed faeces.
Weekly also the total number of aerobic bacteria and the number of Enterobacteriaceae were tested of the mixed 'antibiotic-faeces' up to 6 weeks of storage. The number of aerobic bacteria remained for all mixtures of 'Gentamicin-faeces' at approximately 10^8 cfp/g. The number of Enterobacteriacea was $5.2*10^5$ cfp/g in fresh faeces (before mixing with Gentamicin and before storage). After mixing with Gentamicin up to a concentration of 166 µg Gentamicin/g faeces, the number of Enterobacteriaceae decreased to $4*10^3$ cfp/g. The number of Enterobacteriacea decreased to zero after 3 weeks of storage of the latter mixed faeces. In the control mixed faeces (only mixed with peptone saline solution without the addition of an antibiotic) the number of Enterobacteriacea decreased only marginally from $5.2*10^5$ cfp/g on day 0 to $3.7*10^5$ cfp/g and $2.3*10^5$ cfp/g after respectively 3 weeks and 6 weeks of storage.

4.3 Technical data interlaboratory comparison study

4.3.1 General

In this study 33 NRLs participated: 28 NRLs from 27 EU-Member States, three NRLs from member countries of the European Free Trade Association State and, on request of DG-Sanco, two NRLs from third countries (outside-Europe).
The majority of the laboratories (thirty) performed the study on the planned date (week 11 starting on 15/03/2008). One laboratory (lab code 23) performed the study a few days earlier and two laboratories (lab codes 12 and 19) a few days later.

4.3.2 Accreditation/certification

All laboratories mentioned to be accredited for their quality system according to ISO/IEC 17025 (Anonymous, 2005). Thirty-one laboratories are accredited for ISO 6579; 12 are also accredited for Annex D of ISO 6579 and one (lab code 3: non-EU) is planning to become accredited for Annex D within 1 year. One laboratory of an EU-MS (lab code 19) is not accredited for samples from primary production (Annex D of ISO 6579) but is accredited for food and feeding stuffs (ISO 6579). One non-EU laboratory (lab code 18) is planning to become accredited for Annex D of ISO 6579 and is currently accredited for a national standard method for food matrices. According to EC Commission Regulations No. 882/2004 each NRL should have been accredited for their relevant work field before December 31, 2009 (EC Commission Regulation No. 2076/2005).

4.3.3 Transport of samples

Table 5 gives an overview of the transport times and the temperatures during transport of the parcels. The NRLs returned the temperature recorders immediately after receipt to the CRL-*Salmonella.* The majority of the laboratories received the materials within 1 day. However, the parcel of laboratory 17 was delayed and arrived after 4 days at the institute. When the two parcels from the third countries (non-EU) are not taken into account, the average transport time was 32 hours. For the majority of the parcels the transport temperature did not exceed 5 ^{o}C. The temperature recorder of laboratory 15 was broken when it arrived at the CRL, it was therefore not possible to read the results. Although the parcel of laboratory 17 was delayed it was stored below 5 ^{o}C for most of the transport time. For eight NRLs the time of transport recorded on the test report did not correspond with the time reported by the courier. Presumably the parcel arrived at the time reported by the courier at the institute, but due to internal logistics at the institute the parcel arrived later at the laboratory of the NRL. The delay varied between 1 to 24 hours. In two laboratories the storage temperature during the delay was between 7 to 23 ^{o}C for up to 24 hours. For the other laboratories the storage temperature during delay was below 5 ^{o}C.

Table 5 Overview of the temperatures during shipment of the parcels to the NRLs.

		Time (h) at				
Lab code	**Transport time[1] total in hours**	**< 0 °C**	**0 °C - 5 °C**	**5 °C - 10 °C**	**> 15 °C**	**Additional Storage[2]**
1	25	17	8			1 hour < 5 °C
2	51	40	11			
3	59	11	48			15 hours < 5 °C
4	52	11	39		2	
5	26	20	6			
6	24	9	15			
7	45	9	36			
8	29	29				
9	27	21	5		1	
10	28	21	7			
11	22	14	8			
12	24	11	13			
13	24	16	8			1 hour < 5 °C
14	2	2				
15	26	Temperature recorder broken				
16	23	18	6			2 hours < 5 °C
17	109	20	75		14	
18	75	75				
19	72	5	45	22		
20	50	26	22	2		
21	28	20	8			
22	48	42	6			1 hour < 5 °C
23	28	25	3			24 hours between 9-19 °C
24	28	24	2		2	21 hours between 7-23 °C
25	48	21	27			
26	28	11	17			
27	27	27				
28	28	10	17		1	
29	26	18	8			
30	27	24	3			1 hour < 5 °C
31	24	12	12			
32	24	12	9		3	
33	24	24				
Average All/EU-MS	35.8					
Average[3]	31.5					

1 = Transport time according to the courier.
2 = Storage time of the samples at the institute before arriving at the laboratory of the NRL.
3 = Average without 2 NRLs of third countries (non-EU).

Table 6 Media combinations used per laboratory.

Lab code	Selective enrichment media	Plating-out Media
1	MSRV MKTTn RVS	XLD BGA^{MOD}
2	MSRV	XLD Rambach
3	MSRV RVS	XLD BGA
4	MSRV RVS	XLD BGA^{MOD} Rambach
5	MSRV	XLD BGA^{MOD}
6	MSRV	XLD Rambach
7	MSRV	XLD BPLS=BGA^{MOD}
8	MSRV SC	XLD BGA^{MOD}
9	MSRV MKTTn	XLD BGA SS
10	MSRV MKTTn RVS	XLD BGA^{MOD}
11	MSRV	XLD SM2
12	MSRV	XLD Rapid *Salmonella*
13	MSRV	XLD BGA^{MOD}
14	MSRV	XLD BGA^{MOD}
15	MSRV	XLD Rambach
16	MSRV	XLD BGA
17	MSRV	XLD BGA^{MOD}
18	MSRV RVS / RV	XLD BSA
19	MSRV	XLD BGA
20	MSRV	XLD Onöz
21	MSRV	XLD BPLS=BGA^{MOD}
22	MSRV MKTTn	XLD Rambach XLT4
23	MSRV	XLD BGA^{MOD}
24	MSRV	XLD BGA^{MOD}
25	MSRV	XLD BxLH
26	MSRV	XLD SM2
27	MSRV	XLD BSA
28	MSRV	XLD BGA Rambach
29	MSRV	XLD SM2
30	MSRV	XLD BPLSA
31	MSRV	XLD XLT4
32	MSRV	XLD BGA^{MOD}
33	MSRV	XLD Rambach

Explanations of the abbreviations are given in the 'List of abbreviations'.
Compositions of the media not described in ISO 6579 are given in Annex 3.

4.3.4 Media

Each laboratory was asked to test the samples with the prescribed method (Annex D of ISO 6579). All laboratories used the selective enrichment medium MSRV the plating-out medium XLD and a second plating-out medium of own choice. Eight laboratories used an additional selective enrichment medium: RVS (lab codes 3 and 4), MKTTn (lab codes 9 and 22), Selenite Cystine broth (lab code 8), RVS and MKTTn (lab codes 1 and 10), RVS and RV (lab code 18). Four laboratories (lab code 4, 9, 22 and 28) used more than two isolation media. Table 6 shows the media used per laboratory. Details on the media which are not described in ISO 6579 are given in Annex 3.
The Tables 7-10 give information on the composition of the media which were prescribed and on incubation temperatures and times. These tables only indicate the laboratories who reported deviations. Five laboratories (lab code 19, 21, 27, 29 and 33) reported a deviating dissolving time of the capsules. Laboratory 19 did not mention the incubation time of the pre-enrichment in BPW and the laboratories 1 and 29 mentioned a longer time than prescribed. Laboratory 12 did not mention the pH of the media. One laboratory (lab code 2) did not mention the composition of the media used. Two laboratories (lab code 2 and 29) used MSRV without novobiocin and 6 laboratories used MSRV with a higher concentration of novobiocin than the prescribed 0.01 g/L. Three laboratories (lab code 13, 28 and 29) reported a higher pH for the MSRV than the described pH of 5.5.

Table 7 Incubation time and temperature of BPW.

	Prewarming BPW		**Dissolving capsules in BPW**		**Pre-enrichment in BPW**	
Lab code	**Time (h:min)**	**Incubation temperature in °C (min-max)**	**Time (min)**	**Incubation temperature in °C (min-max)**	**Time (h:min)**	**Incubation temperature in °C (min-max)**
SOP and ISO 6579	**Overnight**	**36-38**	**45**	**36-38**	**16 – 20**	**36-38**
1	o/n	37	45	37	20:30	37
19	o/n	37	15	37	-	37
21	o/n	37	60	36.7-36.9	18:55	37
26	o/n	36-37	45	**36**	18	36-37
27	o/n	37	35	37	20:05	37
29	o/n	37.1-37.4	30	37.4	21:05	37.4-37.5
33	o/n	36.4-36.9	40	36.9	18:05	36.9

Grey cell: deviating times and temperatures - = no information

Table 8 Composition (in g/L) and pH of BPW medium.

Lab code	Enzymatic digest of casein (Peptone)	Sodium Chloride (NaCl)	Disodium hydrogen Phosphate dode-cahydrate* ($Na_2HPO_4.12H_2O$)	Potassium dihydrogen phosphate (KH_2PO_4)	pH
ISO 6579	**10.0**	**5.0**	**9.0**	**1.5**	**6.8 – 7.2**
12	10	5	3.5	1.5	-
13	10	5	3.5	1.5	7.3
14	10	5	9	1.5	-
21	10	5	3.5	1.5	7.26
24	10	5	3.5	1.5	7.42

Grey cell: deviating from ISO 6579 - = no information

* = 3.5 g Disodium hydrogen phosphate (anhydrous) is equivalent to 9 g disodium hydrogen phosphate dodecahydrate.

Table 9 Composition (in g/L) and pH of MSRV.

Lab code	Enzymatic digest of casein (Tryptose)	Casein hydro-lysate	Sodium chloride (NaCl)	Potassium Dihydrogen Phosphate (KH_2PO_4 K_2HPO_4)	Magnesium chloride anhydrous ($MgCl_2$)	Malachite green oxalate	Agar	Novo Biocin	pH
Annex D ISO 6579	**4.6**	**4.6**	**7.3**	**1.5**	**10.9**	**0.04**	**2.7**	**0.01 (10mg/L)**	**5.1- 5.4**
1	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.05	5.1
2	-	-	-	-	-	-	-	-	5.2
10	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.2
12	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.01	-
13	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.01	5.5
19	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.2
20	4.6*	4.6	7.3	1.5	10.9	0.04	2.5	0.01	5.3
22	2.3	4.6	7.3	1.5	10.9	0.04	2.5	0.01	5.15
27	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.36
28	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.01	5.48
29	8.25**	0.92	7.3	1.5	12.4	0.04	2.6	-	5.6
30	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.35
32	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.1
33	8.25**	0.92	7.3	1.5	12.4	0.04	2.6	0.01	5.35

Grey cell: deviating from Annex D of ISO 6579 - = no information

* 2.3 g Tryptone + 2.3 g Peptone

** Pepton mixture

Table 10 Composition (in g/L) and pH of XLD.

Lab code	Xylose	L-lysine	Lactose	Sucrose (Saccharose)	Sodium chloride (NaCl)	Yeast extract	Phenol red	Agar	Sodium deoxy-cholate ($C_{24}H_{39}NaO_4$)	Sodium thio-sulphate ($Na_2S_2O_3$)	Iron (III) Ammonium Citrate ($C_6H_8O_7 \cdot nFe \cdot nH_3N$)	pH
ISO 6579	**3.75**	**5.0**	**7.5**	**7.5**	**5.0**	**3.0**	**0.08**	**9-18**	**1.0**	**6.8**	**0.8**	**7.2 – 7.6**
2	-	-	-	-	-	-	-	-	-	-	-	7.4
3	3.5	5	7.5	7.5	5	3	0.08	15	2.5	6.8	0.8	7.3
6	3.5	5	7.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	7.4
8, 12, 14, 31	3.75	5	7.5	7.5	5	3	0.08	12.5	1.0	6.8	0.8	-
10	-	5	3.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	7.2
11	3.5	5	7.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	-
17	3.5	5	7.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	7.25
19	-	-	-	-	-	-	-	-	-	-	-	-
26	3.5	5	7.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	-
33	3.5	5	7.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	7.69

Grey cell: deviating from ISO 6579 - = no information

A second plating-out medium for choice was obligatory. Thirteen laboratories used BGA modified (Anonymous, 1993) as a second plating-out medium. Seven laboratories used Rambach, 5 laboratories BGA agar, 3 laboratories used SM(ID)2, 2 laboratories BSA and 2 laboratories used XLT4. The following media were used only by one laboratory: BPLSA, Onoz, BxLH, Rapid *Salmonella* agar and SS medium.
The use of an extra plating agar between the 'isolation' and the 'confirmation' steps was optional and was performed by 19 laboratories. A total of 18 laboratories performed this extra culture step on a Nutrient agar (e.g. Nutrient agar (Anonymous, 2002)) and 1 laboratory (lab code 12) used another agar (Bromthymol blue lactose sucrose agar).

All participating laboratories performed confirmation tests for *Salmonella*: biochemically, serologically or both. Seventeen laboratories used both biochemical and serological tests. Eleven laboratories (lab codes 3, 7, 9, 11, 12, 13, 14, 21, 26, 27 and 28) only used a biochemical test(s), 5 laboratories (lab code 8, 23, 29, 30, 31 and 33) only used a serological test(s) and of 5 laboratories the confirmation was limited. Two laboratories (lab code 12 and 27) performed only 2 biochemical tests and 3 laboratories (lab code 23, 29 and 30) performed only 1 antigen test. The Tables 11 and 12 summarises the confirmation media and tests.

Table 11 Biochemical confirmation of Salmonella.

Lab code	TSI	UA	LDC	Gal	VP	Indole	Kit	Other
1	+	+	+	-	-	+		
2	+	+	+	+	-	+		Semi-solid glucose agar
3	+	-	-	-	-	-	API20E	
4	+	+	+	+	+	+	API20E	PCR
5, 7, 16, 22, 28	+	+	+	+	+	+		
6	+	+	+	+	+	+		PCR (only uncertain results)
8, 23, 29, 30, 33	-	-	-	-	-	-		
9	+	+	+	+	+	+		PCR
10	+	+	+	-	-	+		MacConkey (Lactose)
11	-	-	-	-	-	-	Microbat Oxoid	
12	+	-	+	-	-	-		
13, 14, 21, 26	+	+	+	-	-	-		
15	+	+	+	-	-	-	API20E, Enterotest 24	
17	-	-	-	-	-	+	HY Enterotest	
18	+	+	-	-	-	-		Lysine Iron agar
19	+	-	-	-	-	-	GN-ID panel Microgen	
20	-	-	-	-	-	-	API20E	InvA PCR
24	-	+	-	-	-	-		
25	-	+	-	-	-	+		Kigler agar, manitol, nitrate, ONPG, FDA, motility
27	+	-	-	-	-	-		H_2S, Oxidase
31	-	-	-	-	-	-		PCR
32	+	+	+	-	-	+		Glikose

- = Not done/ not mentioned

Explanations of the abbreviations are given in the 'List of abbreviations'

Table 12 Serological confirmation of Salmonella.

Lab code	Serological			
	O antigens	**H antigens**	**Vi Antigens**	**Other**
1, 4, 6, 8, 10, 15, 16, 19, 20, 22, 24, 31, 32, 33	+	+	-	
2, 5, 17, 23, 30	+	-	-	
3, 7, 9, 11, 12, 13, 14, 21, 26, 27, 28	-	-	-	
18	-	-	-	Latex Agglutination Test Oxoid
29	-	-	-	Anti-*Salmonella* Gr. B, D

- = Not done/ not mentioned

4.4 Control samples

4.4.1 General

None of the laboratories isolated *Salmonella* from the procedure control (C11: no capsule/no faeces) nor from the faeces control (C12: no capsule/negative chicken faeces). Thirty-two laboratories scored correct results for all the control capsules containing *Salmonella*. Table 13 gives the results of all control samples (capsules without faeces). This table gives the highest number of positive isolations found with MSRV in combination with any isolation medium per laboratory. Eight laboratories used an additional selective enrichment medium (own method see Table 6). Annex 6 Table A6.1 gives the results found with these own methods, which are the same as found with the MSRV method.

Blank capsules (n=1) without addition of faeces
The blank capsule contained only sterile milk powder. For the analyses no faeces was added. All participating laboratories correctly analysed the blank capsule negative for all used media.

Salmonella Enteritidis 20 capsules (SE20) without addition of faeces (n=2)
Thirty-two laboratories isolated *Salmonella* Enteritidis at a mean level of approximately 20 cfp/capsule from both capsules. One laboratory could not detect *Salmonella* in 1 control capsule with any of the used media. These capsules contained SE at a low level (approx 20 cfp/capsule). However, the level was not so low that negative capsules may be expected in the batch of reference materials. It is therefore not very likely that the negative result was caused by a negative capsule. Still it was considered acceptable to find at least 1/2 SE20 capsules positive.

Salmonella Enteritidis 100 capsules (SE100) without addition of faeces (n=1)
All participating laboratories tested the capsule containing SE100 positive.

Salmonella Typhimurium *5 capsules (STM5) without addition of faeces (n=2)*
All thirty-three laboratories tested both capsules containing STM5 positive.

The results of all control samples were compared with the definition of 'good performance' (see section 3.6) and all laboratories fulfilled the pre-set criteria.

Table 13 Total number of positive results of the control samples (capsule without faeces) per laboratory.

Lab code	The highest number of positive isolations found with MSRV in combination with any isolation medium			
	Blank n=1	SE20 n=2	SE100 n=1	STM5 n=2
Good Performance	**0**	**≥ 1**	**1**	**≥ 1**
1	0	**1**	1	2
2	0	2	1	2
3	0	2	1	2
4	0	2	1	2
5	0	2	1	2
6	0	2	1	2
7	0	2	1	2
8	0	2	1	2
9	0	2	1	2
10	0	2	1	2
11	0	2	1	2
12	0	2	1	2
13	0	2	1	2
14	0	2	1	2
15	0	2	1	2
16	0	2	1	2
17	0	2	1	2
18	0	2	1	2
19	0	2	1	2
20	0	2	1	2
21	0	2	1	2
22	0	2	1	2
23	0	2	1	2
24	0	2	1	2
25	0	2	1	2
26	0	2	1	2
27	0	2	1	2
28	0	2	1	2
29	0	2	1	2
30	0	2	1	2
31	0	2	1	2
32	0	2	1	2
33	0	2	1	2

Bold number: deviating result

4.4.2 Specificity, sensitivity and accuracy rates of the control samples

Table 14 shows the specificity, sensitivity and accuracy rates for the control capsules without the addition of faeces. The rates are calculated for the selective enrichment MSRV with plating-out medium XLD and 'non-XLD media'. The calculations were performed on the results of all participants and on the results of only the EU Member States (without the results of the European Free Trade Association States, candidate and third countries). No differences were found between these groups.

Table 14 Specificity, sensitivity and accuracy rates of the control samples (capsules without the addition of (faeces) for the selective enrichment on MSRV and plating-out on XLD or non-XLD.

Control capsules		**MRVS/ XLD**		**MSRV/ non-XLD***	
		All n= 33	EU MS n=28	All n= 33	EU MS n=28
Blank	No. of samples	33	28	36	31
	No. of negative samples	33	28	36	31
	Specificity in%	**100**	**100**	**100**	**100**
STM5	No. of samples	66	56	72	62
	No. of positive samples	66	56	72	62
	Sensitivity in%	**100**	**100**	**100**	**100**
SE20	No. of samples	66	56	72	62
	No. of positive samples	65	55	71	61
	Sensitivity in%	**98.5**	**98.2**	**98.6**	**98.4**
SE100	No. of samples	33	28	36	31
	No. of positive samples	33	28	36	31
	Sensitivity in%	**100**	**100**	**100**	**100**
All capsules with *Salmonella*	No. of samples	165	140	180	155
	No. of positive samples	164	139	179	154
	Sensitivity in%	**99.4**	**99.3**	**99.4**	**99.4**
All capsules	No. of samples	198	168	216	186
	No. of correct samples	197	167	215	185
	Accuracy in%	**99.5**	**99.4**	**99.5**	**99.5**

*Three laboratories used more than one non XLD isolation medium

All = results/of all laboratories

EUMS = results of only the laboratories of the EU Member States

4.5 Results faeces samples artificially contaminated with *Salmonella*

4.5.1 Results per type of capsule and per laboratory

General
Table 15 gives the results of the *Salmonella* negative chicken faeces samples artificially contaminated with capsules. This table gives the highest number of positive isolations found with MSRV in combination with any isolation medium per laboratory. Eight laboratories used an additional selective enrichment medium (own method see Table 6). Table A6.2 in Annex 6 gives the results found with these own methods.
In general less positive results were found for samples containing *S*. Enteritidis when compared to the ones containing *S.* Typhimurium.

Blank capsules with negative chicken faeces (n=5)
Thirty-one laboratories correctly did not isolate *Salmonella* from the blank capsules with the addition of negative chicken faeces. 1 laboratory (lab code 5) found 1 positive blank with the addition of negative chicken faeces for all the media used by the laboratory. Laboratory 3 correctly found negative blanks for the prescribed method MSRV but found 2 positive results with their own method, RVS.
All blanks should be tested negative. However, as no 100% guaranty about the *Salmonella* negativity of the matrix can be given, 1 positive result out of 5 blank samples (80% negative) is still considered acceptable. Finding more than 1 blank positive is not very likely. Possible causes for finding a blank sample positive may be cross contamination, mixing up positive and negative samples or limited confirmation or misinterpretation of confirmation results.

S. Enteritidis 20 capsules (SE20) with negative chicken faeces (n=5)
Twenty-one laboratories were able to isolate *Salmonella* from all the 5 capsules containing *Salmonella* Enteritidis at a level of approximately 20 cfp/capsule in combination with chicken faeces. Ten laboratories could not detect *Salmonella* in 1 or 2 capsules on all of the used media. One laboratory (lab code 26) found 3 capsules negative for all the media used. These capsules contained SE at a low level (approximately 20 cfp/capsule). However, the level was not so low that negative capsules may be expected in the batch of reference materials. It is therefore not very likely that the negative results were caused by negative capsules.

S. Enteritidis 100 capsules (SE100) with negative chicken faeces (n=5)
All laboratories isolated *Salmonella* from all the five capsules containing *Salmonella* Enteritidis at a level of approximately 100 cfp/capsule in combination with chicken faeces.

S. Typhimurium *5 capsules (STM5) with negative chicken faeces (n=5)*
Thirty laboratories isolated *Salmonella* from all the 5 capsules containing *Salmonella* Typhimurium at a level of approximately 5 cfp/capsule in combination with chicken faeces. Three laboratories found 1 capsules negative. Laboratory 1 correctly found all 5 STM5 capsules positive with the prescribed method MSRV but found only 1 positive result with their own method, MKTTn. These capsules contained STM at a low level (approximately 5 cfp/capsule). Due to the variation between capsules, 1 out of 5 capsules containing STM5 may be negative.

S. Typhimurium *50 capsules (STM50) with negative chicken faeces (n=5)*
All laboratories except laboratory 5, isolated *Salmonella* from all 5 capsules containing *Salmonella* Typhimurium at a level of approximately 50 cfp/capsule in combination with chicken faeces.

Laboratory 5 tested 1/5 sample negative. Laboratory 1 correctly found all 5 STM50 capsules positive with the prescribed method MSRV but found only 3 positive results with their own method, MKTTn.

Table 15 Total number of positive results of the artificially contaminated chicken faeces samples per laboratory.

Lab code	The highest number of positive isolations found with MSRV in combination with any isolation medium				
	Blank n=5	SE20 n=5	SE100 n=5	STM5 n=5	STM50 n=5
Good performance	**≤ 1**	**> 2**	**≥ 4**	**> 2**	**≥ 4**
1	0	**4**	5	5	5
2	0	**4**	5	5	5
3	0	**3**	5	5	5
4	0	**4**	5	**4**	5
5	**1**	5	5	5	**4**
6	0	5	5	5	5
7	0	5	5	5	5
8	0	5	5	5	5
9	0	**4**	5	5	5
10	0	**4**	5	5	5
11	0	5	5	5	5
12	0	5	5	5	5
13	0	**4**	5	5	5
14	0	**4**	5	5	5
15	0	5	5	5	5
16	0	**4**	5	**4**	5
17	0	5	5	5	5
18	0	5	5	5	5
19	0	**4**	5	**4**	5
20	0	5	5	5	5
21	0	5	5	5	5
22	0	5	5	5	5
23	0	5	5	5	5
24	0	5	5	5	5
25	0	5	5	5	5
26	0	**2**	5	5	5
27	0	5	5	5	5
28	0	5	5	5	5
29	0	5	5	5	5
30	0	5	5	5	5
31	0	5	5	5	5
32	0	5	5	5	5
33	0	5	5	5	5

Bold number: deviating result
Grey cell: result is below good performance

The results of the artificially contaminated chicken faeces samples were compared with the definition of 'good performance' (see section 3.6) and 32 laboratories fulfilled these criteria for the prescribed method MSRV. Laboratory 26 scored below the level of good performance with the SE20 samples. Two laboratories (lab codes 1 and 3) found different results between the prescribed and the 'own' method. If the same criteria as used for MSRV are followed for testing the performance of the 'own' method, these results would not fulfil the criteria of good performance.

4.5.2 Results per medium, capsule and per laboratory

Figures 1, 2, 3 and 4 show the number of positive isolations per artificially contaminated chicken faeces sample, per laboratory after pre-enrichment in BPW and selective enrichment on MSRV followed by isolation on selective plating agar XLD.

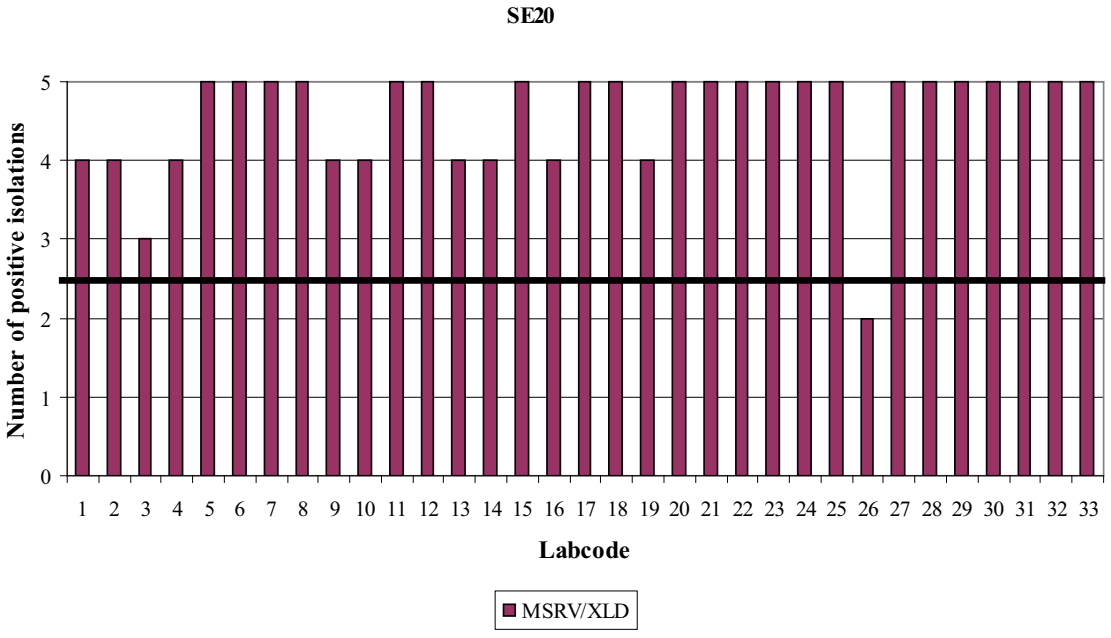
The results of all artificially contaminated chicken faeces samples were compared with the proposed definition of 'good performance' (see section 3.6). In Figures 1-4 the border of good performance is indicated with a black horizontal line.

31/33 laboratories who used an additional 'own method' found the same results with their own methods as with MSRV. Laboratory 1 found less STM samples positive after selective enrichment in RVS and MKTTn. Laboratory 3 found 2 positive blanks with RVS and more positive results with the SE20 samples when compared to MSRV. Still both laboratories scored all samples correctly with the prescribed method (MSRV).

Table 16 presents the results of the number of positive isolations after 24 and 48 hours of incubation of the selective enrichment MSRV. A longer incubation time did not give more positive results. The choice of plating-out medium does not seem to have any effect on the number of positive isolations, XLD and other plating-out media gave the same results. The majority of the laboratories used BGA as the second plating-out medium (see Table 6).

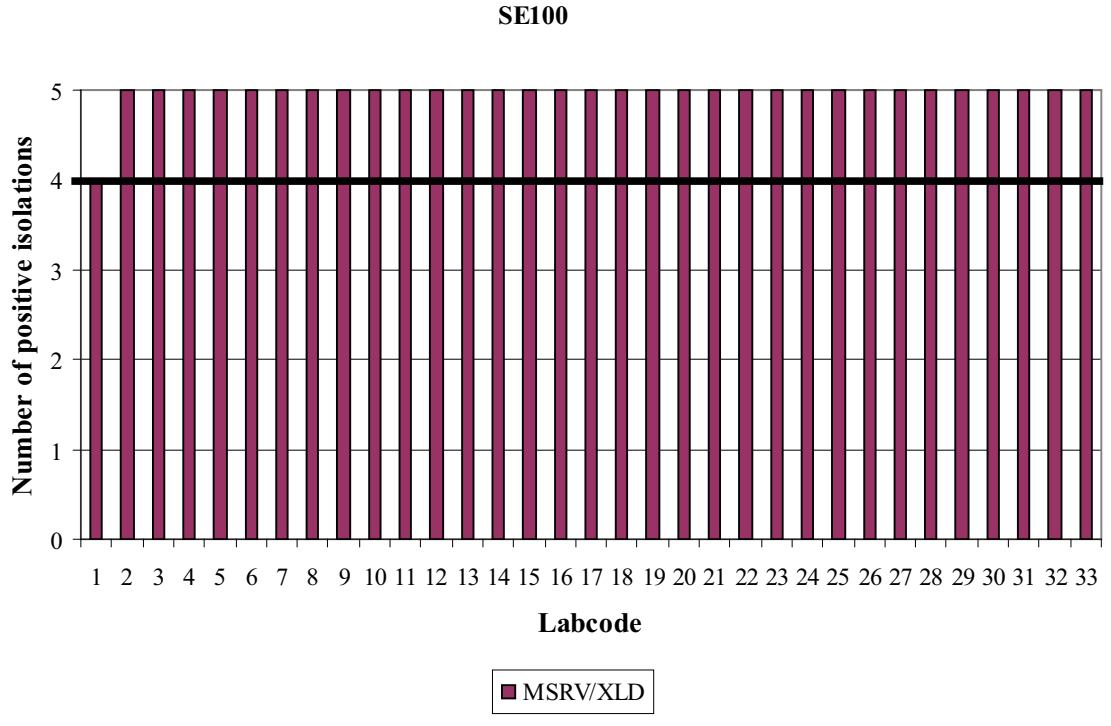
Table 16 Mean percentages of positive results of all participating laboratories after selective enrichment on MSRV, incubated for 24 and 48 hours and followed by isolation on different plating-out media, when analyzing the artificially contaminated chicken faeces samples.

Plating out medium	**Selective enrichment Medium MSRV**
	24 / 48 h
XLD	96 / 97%
Other (most often BGA)	96 / 97%



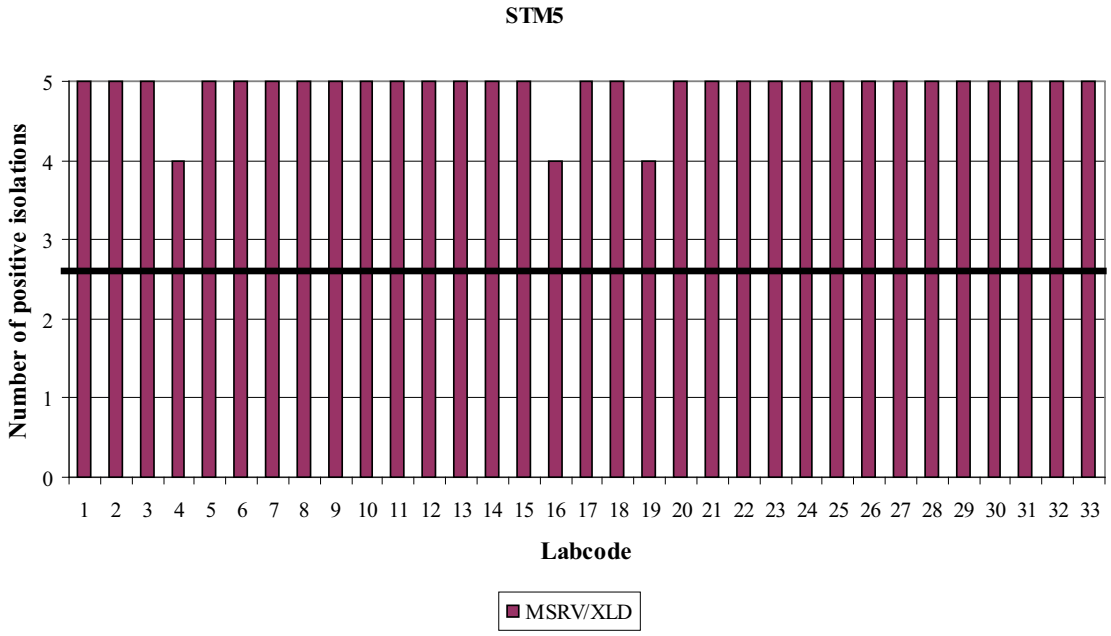
- = border of good performance

Figure 1 Results per laboratory of chicken faeces samples artificially contaminated with SE20 capsules (n=5) after selective enrichment on MSRV followed by isolation on selective plating agar XLD.



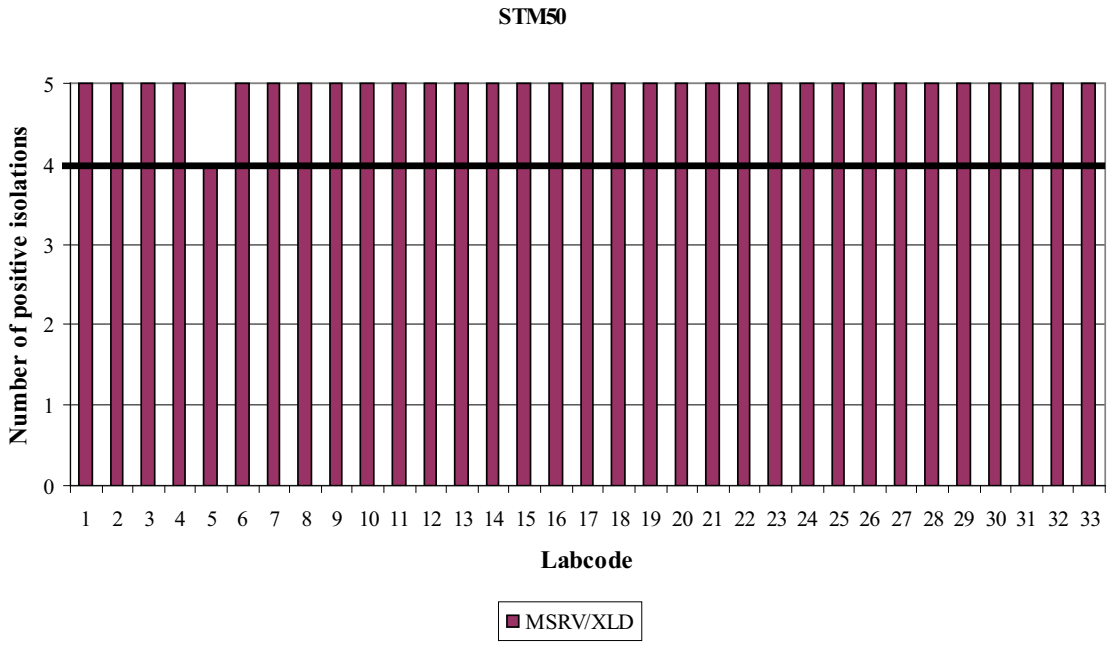
- = border of good performance

Figure 2 Results per laboratory of chicken faeces samples artificially contaminated with SE100 capsules (n=5) after selective enrichment on MSRV followed by isolation on selective plating agar XLD.



- = border of good performance

Figure 3 Results per laboratory of chicken faeces samples artificially contaminated with STM5 capsules (n=5) after selective enrichment on MSRV followed by isolation on selective plating agar XLD.



- = border of good performance

Figure 4 Results per laboratory of chicken faeces samples artificially contaminated with STM50 capsules (n=5) after selective enrichment on MSRV followed by isolation on selective plating agar XLD.

Table 17 Specificity, sensitivity and accuracy rates of the artificially contaminated chicken faeces samples (each capsule added to 10 g chicken faeces) for the selective enrichment on MSRV and plating out on XLD or non-XLD.

Capsules with Chicken faeces		**MRVS/ XLD**		**MSRV/ non-XLD***	
		All n=33	EU MS n=28	All n=33	EU MS n=28
Blank (n=5)	No. of samples No. of negative samples **Specificity in%**	165 164 **99.4**	140 139 **99.3**	180 179 **99.4**	155 154 **99.4**
STM5 (n=5)	No. of samples No. of positive samples **Sensitivity in%**	165 162 **98.2**	140 137 **97.9**	180 176 **97.8**	155 151 **97.4**
STM50 (n=5)	No. of samples No. of positive samples **Sensitivity in%**	165 164 **99.4**	140 139 **99.3**	180 178 **98.9**	155 153 **98.7**
SE20 (n=5)	No. of samples No. of positive samples **Sensitivity in%**	165 151 **91.5**	140 128 **91.4**	180 164 **91.1**	155 141 **91**
SE100 (n=5)	No. of samples No. of positive samples **Sensitivity in%**	165 164 **99.4**	140 139 **99.3**	180 180 **100**	155 155 **100**
All capsules with *Salmonella*	No. of samples No. of positive samples **Sensitivity in%**	660 641 **97.2**	560 543 **97**	720 698 **96.9**	620 600 **96.8**
All capsules	No. of samples No. of correct samples **Accuracy in%**	825 805 **97.6**	700 682 **97.4**	900 877 **97.4**	775 754 **97.3**

* Three laboratories used more than one non XLD isolation medium.
All = results/of all laboratories.
EUMS = results of only the laboratories of the EU Member States.

4.5.3. Specificity, sensitivity and accuracy rates of the artificially contaminated samples

Table 17 shows the specificity, sensitivity and accuracy rates for all types of capsules added to the chicken faeces. This table gives the results for the different medium combinations: pre-enrichment in BPW, followed by selective enrichment on MSRV and isolation on selective plating agar XLD and on other selective isolation agar media (non-XLD). The calculations were performed on the results of all participants and on the results of only the EU Member States (without the results of the European Free Trade Association States, candidate and third countries). Only small differences (if any) were found between these groups.
The specificity rates (of the blank capsules) were 99% for EU-MS with MSRV.
As expected the high level SE100 and STM50 showed sensitivity rates of 100% or very close to 100%. For the low level materials STM5 and SE20 the rates were respectively 97 - 98% and 91 - 92%.

4.5.4 Results faeces samples mixed with Gentamicin

Twenty-nine laboratories found, as expected, no positive results in the 4 samples of SE20 capsules in combination with faeces mixed with Gentamicin. Four laboratories isolated *Salmonella* from these capsules A1-A4. Two laboratories (lab code 4 and 14) isolated *Salmonella* only once. One laboratory (lab code 9) found only 1 positive with the prescribed method (MSRV) but 3 positives with their own method MKTTn in combination with the isolation medium SS. One laboratory (lab code 15) found all samples positive with the prescribed method (MSRV). Table 18 shows the results of the laboratories who found *Salmonella* in the 'Gentamicin-faeces' artificially contaminated with SE20 capsules.
In prior investigations it was shown that only a small number of SE20 capsules were found positive after testing in combination with the 'Gentamicin-faeces' (< 2%). It was therefore not very likely to find more than one SE20 capsule positive in combination with Gentamicin-faeces by the participating laboratories. Possible explanations for finding more than one sample positive could be:

- no 'Gentamicin-faeces' or less than the prescribed amount of faeces was added to the BPW;
- faeces of batch B (non-mixed) was added to the BPW;
- cross-contamination with a resistant *Salmonella* serovar;
- limited confirmation or misinterpretation of confirmation results.

Table 18 Number of positive results found with chicken faeces mixed with Gentamicin artificially contaminated with SE20 capsules.

Lab code	number of positive isolations	
	SE 20 (n=4)	SE 20 (n=4)
Good performance	≤ **1**	≤ **1**
	MSRV	**Own method**
4	1	1 RVS/XLD,BGA,Rambach
9	1	**3** MKTTn/SS, 1 MKTTn/XLD and 1 MSRV/BGA
14	1	-
15	**4**	-

- = Not done/ not mentioned

In an attempt to clarify the positive results with the 'Gentamicin-faeces' samples, the relevant laboratories were asked for additional information. They were asked to perform additionally, when possible, serotyping, phagetyping and to test the antimicrobial resistance for Gentamicin of some samples (the positive A-samples and some B- and C-samples).
Laboratory 4 and 14 were not able to perform extra tests. Laboratory 9 found deviating confirmation test results for two samples isolated from MKTTn/SS. Laboratory 15 performed several extra tests: serotyping, phagetyping and antimicrobial resistance testing for Gentamicin. The results did not show deviations from the SE strain included in the capsules of the interlaboratory comparison study.

4.6 PCR

Five laboratories (lab codes 4, 6, 9, 20 and 31) applied a PCR method as additional detection technique. In Table 19 the details are summarized. Laboratoy 6 performed a PCR on only five the samples (A1-A4 and B1).

Table 19 Details on the Polymerase Chain Reaction method, used as own method during the interlaboratory comparison study by five laboratories.

Lab code	Volume of BPW (µl)	Volume of DNA sample (µl)	Volume of DNA / PCR mix (µl)
4	1000	150	5/50
6	1000	50	5/50
9	1500	75	5/20
20	1000	150	5/10
31	10000	100	3/?

All laboratories tested the samples after incubation in BPW. Four laboratories used a not commercially available PCR. Laboratory 4 used a commercially available real time PCR (Biorad iQ-Check *Salmonella* kit) which has been validated by AFNOR, 2004. Laboratory 6 used a PCR described by Aabo et al. (1993). Laboratory 20 used an InvA-PCR normally used for confirmation of bacterial cultures and not from pre-enrichment broths. InvA-based PCR method is originally described by Rahn et al. (1992). Laboratory 31 used a real time PCR technique described by Hein et al., 2006. Laboratory 9 did not give more details or references about the used PCR.

Four laboratories found the same results with the PCR-technique as with the bacteriological culture methods. Laboratory 20 found three samples more negative (two times SE20 and once STM5) with the PCR technique.

4.7 Performance of the NRLs

Thirty-one NRLs fulfilled the criteria of good performance for the prescribed method MSRV.
For 2 laboratories (lab code 15 and 26) the performance was considered 'moderate' without a need for a follow up study.

Laboratory 26 scored below the criteria of good performance for the prescribed method. The difficulty was found with the SE20 capsules with the addition of faeces. They mentioned in their test report to have problems to dissolve the capsules completely. The laboratory was contacted by the CRL-*Salmonella* in April 2010 to ask for any explanations for the deviating results. The NRL could not explain the deviation except for the problem with reconstitution of the capsules. They treated all the samples the same: batch of media, operators, procedure and equipment. They asked the CRL about possibility of lack of capsules viability.
The CRL concluded that there was no lack of capsule viability for the following reasons:

- The CRL laboratory tested the level of contamination and homogeneity of 75 SE20 capsules from the batch used in the ring trial and the min-max values we found were
  8-29 cfp per capsule. From the statistical calculation this means that the fraction of negative capsules in this batch is < 0.01%.
- The temperature during transport of the sample to laboratory 26 was below 2 ºC. From earlier experiments it is known that at this temperature *Salmonella* remains stable in the capsules.
- All other participants did not have problems with the SE20 capsules: occasionally a laboratory missed only one out of five SE20 capsules.

Laboratory 26 found three SE20 capsules negative out of five. For all other samples the laboratory showed 100% correct results and also in previous studies this laboratory had shown good results. Furthermore, they mentioned in their test report (before knowing the outcome of the study) that the capsules did not dissolve completely. The CRL advised this NRL for a next interlaboratory comparison study to pay extra attention to the complete reconstitution of the capsules in BPW. The temperature of their incubator was at the lower limit for reconstruction of the capsules (36 ºC). For a better reconstruction it may help to increase the temperature of the incubator to approximately 37-38 ºC.
It was considered that a follow-up study was not necessary and the results of laboratory 26 were indicated as a 'moderate performance'.

For two laboratories (lab code 1 and 3) the results found with the prescribed and the 'own' method were not always comparable. If the same criteria as used for MSRV were followed for the performance of the 'own' method, these results would not have fulfilled the criteria of good performance. The relevant laboratories were contacted by the CRL-*Salmonella* in April 2010 to ask for any explanations for the deviating results.
Laboratory 1 had difficulties with the detection of STM in the artificially contaminated samples after selective enrichment in MKTTn. This NRL mentioned to have financial problems and therefore problems with obtaining good quality media.
Laboratory 3 found two positive blanks with RVS. The following explanations were given by the NRL. At the time of the ring trial there were some changes of staff members and problems existed with the availability of personnel. The workload was high, which may have caused a mixing up of positive and negative samples. Another possibility of contamination may have been related to the use of a platinum loop (for economic reasons) for the isolation from RVS. For the isolation from MSRV disposable loops were used. Unfortunately laboratory 3 did not store the 'false positive' blanks of RVS so that additional testing was not possible (like serotyping and phagetyping). The CRL advised the NRL to use extra controls (negative and positive) together with their routine samples.

Both laboratories fulfilled the criteria of good performance for the prescribed method MSRV and no further action was taken.

Four laboratories (lab code 4, 9, 14 and 15) found one or more positive results for the control samples with 'antibiotic-faeces' (A-samples) which were included for a check on the application of the protocol.
Laboratory 4 was not able to perform phagetyping or an antimicrobial resistance test for Gentamicin. The laboratory performed PFGE on sample A4 but was not possible to compare the outcome with a PFGE profile of the SE strain used in the ring trial.
Laboratory 9 found deviating confirmation results for two strains isolated from their own method MKTTn in combination with isolation medium SS. These two 'false positive' results were most probably caused by a misinterpretation of the confirmation test. There was no doubt about the positive result of sample A4, isolated from MSRV and MKTTn in combination with all used isolation media.
Laboratory 14 did not store the isolated strains and did not give further information.
The laboratories 4, 9 and 14 found only one positive result (out of four samples) which was still considered as acceptable.
Laboratory 15 found deviating results for all four control samples with 'antibiotic-faeces'. The NRL indicated for each sample what results were found with the biochemical and serological tests (see Tables 11 and 12). No deviations were observed in these results. The additionally performed serotyping, phagetyping and antimicrobial resistance testing for Gentamicin did not show deviations from the *Salmonella* Enteritides strain as included in the capsules. It was not very likely to find all four 'antibiotic-faeces' samples positive, meaning that laboratory 15 must have had a problem with following the protocol. Either no (or less) faeces were added to the A capsules or the wrong batch of faeces (batch B) was added. The NRL could not find any explanation. As the problem with following the protocol may have existed for the whole study, the results of laboratory 15 were indicated as moderate performance. However, a follow-up study was not considered to be necessary.

5 Discussion

Transport of the samples
In general the transport time or the transport temperature of the parcels does not seem to have negatively affected the results. The laboratory with the longest transport time (lab codes 17, 18 and 19) and highest transport temperature (lab codes 17, 19, 23 and 24) still found good results.

Performance of the laboratories
The prescribed method (Annex D of ISO 6579: MSRV) was used by all laboratories. Eleven laboratories used additionally an 'own' selective enrichment medium (RVS, MKTTn, SC or another MSRV formulation). For all laboratories except one, the results with MSRV and the own selective enrichment media in combination with all used isolation media gave the same scores. Laboratory 3 found a lower number of positive results with selective enrichment on MSRV in comparison with RVS. This laboratory is a non-EU MS and participated for the second time. The laboratory does not use the prescribed method (MSRV) as a routine method.

For determining 'good performance' per laboratory, the best performing isolation medium after selective enrichment on MSRV was taken into account. Thirty-one out of in total 33 laboratories scored 'good performance'. 2 laboratories scored a 'moderate performance'.
One laboratory (lab code 15) found deviating results for all control samples with antibiotic which were included to check whether the participants followed the protocol for adding faeces to the capsules. Laboratory 15 may have had some problems with following the protocol. As this may have been the case for the full study the results of this NRL were indicated as moderate.
One laboratory (lab code 26) showed a sensitivity problem (low number of positives with low level *Salmonella* Enteritidis capsules). The laboratory had a problem with the reconstitution of the capsules in BPW, which was most probably caused by the fact that the temperature in the incubator was at the lower limit for reconstitution. A lower temperature of the BPW may result in a not complete dissolution of the gelatine capsules which is essential for the detection of *Salmonella* in the capsules. In this study especially the growth of *S*. Enteritidis may have been affected as this serovar grows slower than *S*. Typhimurium.
Two laboratories scored an 'underperformance' for their own method but they fulfilled the criteria for the prescribed method MSRV.

Specificity, sensitivity and accuracy rates
The calculations were performed on the results of all participants and on the results of only the EU Member States (without the results of the European Free Trade Association States, candidate and third countries). Only small differences (if any) were found between these groups.
The rates of the control samples were high. As expected the high-level control sample (SE100) showed a sensitivity rate of 100%. For the low level materials (STM5 and SE20) the sensitivity rates were also high: > 98%.
For the artificially contaminated faeces samples the sensitivity of the high level materials showed as expected rates of 99 - 100%. For the low level materials the sensitivity was still between 92 and 98%.

Media
According to Annex D of ISO 6579 (Anonymous, 2007) the concentration of novobiocin in MSRV should be 10 mg/L and the pH between 5.1 and 5.4. Six laboratories reported the use of a higher concentration of novobiocin and two laboratories did not use or did not mention the use of novobiocin. The CRL contacted these laboratories as a high concentration of novobiocin can negatively influence the motility of *Salmonella* and may result in less positive results. Laboratory 1 mentioned that the used concentration of novobiocin was

10 mg/L and that they reported this incorrect in their test report. Laboratory 10 and 27 mentioned to change the concentration of novobiocin in their protocol to fulfil the criteria of ISO 6579.
Three laboratories reported a higher pH or did not mention the pH of the MSRV. A higher pH of MSRV may stimulate the growth of disturbing background flora which can negatively influence the growth of *Salmonella*.
According to the Standard Operating Procedure of the ring trial for the reconstitution of the capsules, the BPW with capsules should be placed at 37 ^{o}C for 45 minutes. A few laboratories reported a shorter reconstitution time. The CRL contacted these laboratories as a complete reconstitution of the gelatine capsules in BPW is essential for the detection of *Salmonella*. Laboratory 27 mentioned that the reconstitution time was 45 minutes and that the reported time in their test report was incorrect.
The reported deviations in media compositions and incubation temperatures did not likely affect the results.

PCR
Five laboratories used a PCR technique additional to the prescribed method and four of them found the same results as with the bacteriological detection methods. One laboratory found more negative results with the PCR method. A possible explanation for this could be that this latter laboratory routinely uses this technique for confirmation of *Salmonella* and not for isolation from pre-enrichment broths (as they did for this study).

Evaluation of this study
The chicken faeces in this study arrived earlier at CRL *Salmonella* because of experiments that were necessary to make and test the mixture of chicken faeces with an antibiotic. The longer storage time of chicken faeces affected the background flora of the faeces. The lower disturbance of background flora in the present veterinary study may have positively influenced the detection of *Salmonella* and thus the outcome of this study.
The procedure for the handling of the ring trial samples (the addition of the capsules and matrix to the BPW) is different from routine samples. Further research will be performed at the CRL-*Salmonella* to improve the set up of the interlaboratory comparison studies for the use of test samples more comparable to routine samples.

6 Conclusions

- Thirty-one NRLs for *Salmonella* were able to detect high and low levels of *Salmonella* in chicken faeces with the prescribed method MSRV. Two laboratories scored a 'moderate performance'.

- The accuracy, specificity and sensitivity rates for the control samples (without faeces) of MSRV were > 98%.

- The specificity rate of the chicken faeces samples artificially 'contaminated' with blank capsules was 99% when tested with the prescribed method (MSRV).

- The sensitivity rates for artificially contaminated chicken faeces with STM and SE capsules were > 91% for the prescribed method MSRV.

- The low level materials of *S*. Typhimurium (STM5) were easier to detect than the low level materials of *S*. Enteritidis (SE20).

- The accuracy rates of the artificially contaminated chicken faeces samples were > 97% for MSRV.

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Annex 1 History of CRL-*Salmonella* interlaboratory comparison studies on the detection of *Salmonella*

Table A1.1 History of CRL-Salmonella interlaboratory comparison studies on detection of Salmonella in veterinary samples.

Study	Year	Number of samples	Capsules	Actual number of cfp/capsule	*Salmonella* negative faeces[1] added	Selective enrichment medium	Plating-out medium	Reference [3] (RIVM report)
I	1995	26 4	STM5 Blank	6 0	No No	RV and SC	BGA and own	Voogt et al., 1996 (Report 284500003)
II	1996	15 15 2 1 1	STM100 STM1000 SPan5 STM100 Blank	116 930 5 116 0	1 gram 1 gram No No No	RV, SC and own	BGA and own	Voogt et al., 1997 (Report 284500007)
III	1998	14 14 7 14 4 2 5	STM10 STM100 STM100 SE100 STM10 SPan5 Blank	11 94 94 95 11 5 0	1 gram 1 gram 1 gram* 1 gram No No No	RV and own	BGA and own	Raes et al., 1998 (Report 284500011)
IV	1999	5 5 5 5 5 3 3 2 2	STM10 STM100 SE100 SE500 Blank STM10 SE100 SPan5 Blank	4 210 60 220 0 5 60 5 0	10 gram 10 gram 10 gram 10 gram 10 gram No No No No	RV or RVS, MSRV and own	BGA and own	Raes et al., 2000 (Report 284500014)
V	2000	5 5 5 5 5 3 3 2 2 20	STM10 STM100 SE100 SE500 Blank STM10 SE100 SPan5 Blank None	4 47 63 450 0 4 63 5 0 -	10 gram 10 gram 10 gram 10 gram 10 gram No No No No 25 gram**	RV or RVS, MSRV and own	BGA and XLD	Raes et al., 2001 (Report 284500018)

Table A1.1 (continued)

Study	Year	Number of samples	Capsules	Actual number of cfp/capsule	*Salmonella* negative faeces[1] added	Selective enrichment medium	Plating-out medium	Reference [3] (RIVM report)
VI	2002	5	STM10	11	10 gram	RVS, MSRV, MKTTn and own	BGA, XLD and own	Korver et al., 2003 (Report 330300001)
		5	STM100	139	10 gram			
		5	SE100	92	10 gram			
		5	SE500	389	10 gram			
		5	Blank	0	10 gram			
		3	STM10	11	No			
		3	SE100	92	No			
		2	SPan5	5	No			
		2	Blank	0	No			
		20	None	-	25 gram**			
VII	2003	5	STM10	12	10 gram	RVS, MSRV, MKTTn and own	BGA, XLD and own	Korver et al., 2005 (Report 330300004)
		5	STM100	96	10 gram			
		5	SE100	127	10 gram			
		5	SE500	595	10 gram			
		5	Blank	0	10 gram			
		3	STM10	12	No			
		3	SE100	127	No			
		2	SPan5	9	No			
		2	Blank	0	No			
		20	None	-	10 gram**			
VIII	2004	7	STM10	13	10 gram	MSRV and own	XLD and own	Korver et al., 2005 (Report 330300008)
		4	STM100	81	10 gram			
		7	SE100	74	10 gram			
		4	SE500	434	10 gram			
		3	Blank	0	10 gram			
		3	STM10	13	No			
		2	SE100	74	No			
		1	SE500	434	No			
		2	SPan5	7	No			
		2	Blank	0	No			
		20	None	-	10 gram**			
IX	2005	5	STM10	9	10 gram[2]	MSRV and own	XLD and own	Berk et al., 2006 (Report 330300011)
		5	STM100	86	10 gram			
		5	SE100	122	10 gram			
		5	SE500	441	10 gram			
		5	Blank	0	10 gram			
		3	STM10	9	No			
		2	SE100	86	No			
		1	SE500	441	No			
		2	SPan5	7	No			
		2	Blank	0	No			
		10	None	-	10 gram***			

Table A1.1 (continued)

Study	Year	Number of samples	Capsules	Actual number of cfp/capsule	*Salmonella* negative faeces added[2]	Selective enrichment medium	Plating-out medium	Reference [3] (RIVM report)
X	2006	5	STM10	9	10 gram	MSRV and own	XLD and own	Kuijpers et al., 2007 (Report 330604004)
		5	STM100	98	10 gram			
		5	SE100	74	10 gram			
		5	SE500	519	10 gram			
		5	Blank	0	10 gram			
		3	STM10	9	No			
		2	SE100	98	No			
		1	SE500	519	No			
		2	SPan5	5	No			
		2	Blank	0	No			
XI	2008	5	STM5	6	10 gram	MSRV and own	XLD and own	Kuijpers et al., 2008 (Report 330604011)
		5	STM50	47	10 gram			
		5	SE10	9	10 gram			
		5	SE100	90	10 gram			
		5	Blank	0	10 gram			
		3	STM5	6	No			
		2	SE10	9	No			
		1	SE100	90	No			
		2	SPan5	5	No			
		2	Blank	0	No			
XII	2009	5	STM5	6	10 gram	MSRV and own	XLD and own	Kuijpers et al., 2009 (Report 330604014)
		5	STM50	53	10 gram			
		5	SE20	18	10 gram			
		5	SE100	84	10 gram			
		5	Blank	0	10 gram			
		3	STM5	6	No			
		2	SE20	18	No			
		1	SE100	84	No			
		2	SPan5	7	No			
		2	Blank	0	No			

Table A1.1 (continued)

Study	Year	Number of samples	Capsules	Actual number of cfp/capsule	*Salmonella* negative faeces added[2]	Selective enrichment medium	Plating-out medium	Reference [3] (RIVM report)
XIII	2010	5	STM5	5	10 gram	MSRV and own	XLD and own	This report
		5	STM50	56	10 gram			
		5	SE20	13	10 gram			
		5	SE100	78	10 gram			
		5	Blank	0	10 gram			
		4	SE20	22	10 gram*			
		2	STM5	8	No			
		2	SE20	13	No			
		1	SE100	78	No			
		1	Blank	0	No			

[1]Faeces mixed (1:1) with a solution of peptone/glycerol. Final concentration glycerol in the faeces mixture was 15%(v/v).
[2] Faeces not mixed with any preservation medium.
[3] The report of each study can be found at the CRL-*Salmonella* website: http://www.rivm.nl/crlsalmonella/publication/ or can be obtained through the corresponding author of this report.
* = With antibiotics.
** = Naturally contaminated chicken faeces with *Salmonella.*
*** = Naturally contaminated dust with *Salmonella.*

Table A1.2 CRL-Salmonella interlaboratory comparison study on the detection of Salmonella in food samples.

Study	Year	Number of samples	Capsules	Actual number of cfp/capsule	*Salmonella* negative meat	Selective enrichment medium	Plating-out medium	Reference [1] (RIVM report)
I	2006	5	STM10	9	10 gram	RVS, MKTTn, MSRV and own	XLD and own	Kuijpers et al., 2007 (Report 330604003)
		5	STM100	98	10 gram			
		5	SE100	74	10 gram			
		5	SE500	519	10 gram			
		5	Blank	0	10 gram			
		3	STM10	9	No			
		2	SE100	98	No			
		1	SE500	519	No			
		2	SPan5	5	No			
		2	Blank	0	No			
II	2007	5	STM5	4	10 gram	RVS, MKTTn, MSRV and own	XLD and own	Kuijpers et al., 2008 (Report 330604010)
		5	STM50	40	10 gram			
		5	SE10	7	10 gram			
		5	SE100	71	10 gram			
		5	Blank	0	10 gram			
		3	STM5	4	No			
		2	SE10	7	No			
		1	SE100	71	No			
		2	SPan5	7	No			
		2	Blank	0	No			
III	2009	5	STM5	6	10 gram	RVS, MKTTn, MSRV and own	XLD and own	Kuijpers et al., 2008 (Report 330604017)
		5	STM50	54	10 gram			
		5	SE20	12	10 gram			
		5	SE100	50	10 gram			
		5	Blank	0	10 gram			
		3	STM5	6	No			
		2	SE20	12	No			
		1	SE100	50	No			
		2	SPan5	6	No			
		2	Blank	0	No			

[1] The report of each study can be found at the CRL-*Salmonella* website: http://www.rivm.nl/crlsalmonella/publication/ or can be obtained through the corresponding author of this report.

Table A1.3 CRL-Salmonella interlaboratory comparison study on the detection of Salmonella in animal feed samples.

Study	Year	Number of samples	Capsules	Actual number of cfp/capsule	*Salmonella* negative feed	Selective enrichment medium	Plating-out medium	Reference [1] (RIVM report)
I	2008	5	STM5	5	25 gram	RVS, MKTTn, MSRV and own	XLD and own	Kuijpers et al., 2009 (Report 330604012)
		5	STM50	43	25 gram			
		5	SE20	15	25 gram			
		5	SE100	48	25 gram			
		5	Blank	0	25 gram			
		3	STM5	5	No			
		2	SE20	15	No			
		1	SE100	48	No			
		2	SPan5	5	No			
		2	Blank	0	No			

[1] The report of each study can be found at the CRL-*Salmonella* website: http://www.rivm.nl/crlsalmonella/publication/ or can be obtained through the corresponding author of this report.

Annex 2 Calculation of T_2

The variation between capsules of one batch of reference materials is calculated by means of the so-called T_2 statistic (Heisterkamp et al., 1993)*.

$$T_2 = \sum_i [(z_i - z_+ / I)^2 / (z_+ / I)]$$

where, z_i = count of one capsule (*i*)
z_+ = sum of counts of all capsules
I = total number of capsules analysed

In case of a Poisson distribution, T_2 follows a χ^2 -distribution with (I-1) degrees of freedom. In this case, the expected T_2-value is the same as the number of degrees of freedom and thus $T_2/(I-1)$ is expected to be equal to one. For the variation between capsules of one batch, the Poisson distribution is the theoretical smallest possible variation which could be achieved. However, over-dispersion is expected and $T_2/(I-1)$ will mostly be larger than 1 (Heisterkamp et al., 1993)*. An acceptable variation for a batch of capsules will be $T_2/(I-1) \leq 2$.

*Heisterkamp SH, Hoekstra JA, van Strijp-Lockefeer NGWM, Havelaar A, Mooijman KA, In `t Veld PH, Notermans SHW, 1993. Statistical analysis of certification trials for microbiological reference materials. Commission of European Communities, Community Bureau of Reference, Brussels, Luxembourg. EUR Report; EUR 15008 EN.

Annex 3 Information on the media used

MKTTn (Oxoid CM 1048 Hampshire, United Kingdom) (Biokar BK 169 HA, Beauvais, France) (Himedia Laboratories M1496I, Mumbai, India)
Composition of MKTTn: according ISO 6579, 2002

MKTTn (Oxoid CM343 Hampshire, United Kingdom)
Composition of MKTTn medium: the concentration of the compounds in g/L water:
Meat extract 7, Enzymatic digest of casein 2.3, Sodium chloride 2.3, Calcium carbonate 25, Sodium thiosulfate pentahydrate 40.7, Ox bile for bacteriological use 4.75, Brilliant green 0.1/100 ml, Iodine 4, Potassium iodide 5, Novobiocine 0, pH 7.8

RVS (Oxoid CM 0866, Hampshire, United Kingdom) (Himedia Laboratories M1491, Mumbai, India) (Merck VM540500601, Darmstadt, Germany) (Scharlau Chemie SA 02-379, Barcelona, Spain)
Composition of RVS: according ISO 6579, 2002

RV (Oxoid CM 0669, Hampshire, United Kingdom)
Vassiliadis P., Pateraki E., Papaiconomou N., Papadakis J.A. and Trichopoulos D. 1976 Annales de Microbiologie (Institut Pasteur) 127B. 195-200
Composition of RV medium: the concentration of the compounds in g/L water: Soya Peptone 5, Sodium chloride 8, Potassium dihydrogen phosphate 1.6, Magnesium chloride 40, Malachiet green 0.04 pH 5.3- 5.4

SC (Merck 1.07709, Darmstadt, Germany)
Composition of SC medium: according ISO 6579, 1993

BGA modified (Oxoid CM 0329; PO5033A, Hampshire, United Kingdom) (BPLS, Merck 1.10747, Darmstadt, Germany) (Biomark B439) (Lab M, lab 34 Bury, United Kingdom) (HImedia Laboratories M971, Mumbai, India) (Hy Laboratories Ltd. DD074, Rehovot, Israel) (Staten Serum Institute BGA, Copenhagen, Denmark) (Scharlau 01-309, Barcelona, Spain) (SIFIN TN 1110, Berlin, Germany)
Watson and Walker 1978 A modification of brilliant green agar for improved isolation of *Salmonella*. J. Appl.Bact. 45 195-204
Composition of BGA modified: Edel and Kampelmacher; according ISO 6579, 1993

BGA (Conda laboratories 136600, Madrid, Spain)
Composition of BGA medium: the concentration of the compounds in g/L water: Yeast extract 3, Tryptone 5, Peptic digest of animal tissue 5, Lactose 10, Saccharose 10, Sodium chloride 5, Phenol red 0.08, Sulfadiazine 0.08, Agar 20, pH 7.4

BGA (Oxoid CM 0263, Hampshire, United Kingdom)
Composition of BGA medium: the concentration of the compounds in g/L water: Proteose peptone 10, Yeast extract 3, Lactose 10, Sucrose 10, Sodium chloride 5, Phenol red 0.08, Brilliant green 0.0125, Agar 12, pH 6.8-7.0

BGA (Biokar BK071HA, Beauvais, France)
Kristensen, M., V. Lester, and A. Jurgens. 1925. On the use of trypsinized casein, brom-thymol-blue, bromcresol-purple, phenol-red and brilliant-green for bacteriological nutrient media. Brit. J. Exp. Pathol. 6:291-299

Composition of BGA medium: (Kristensen) the concentration of the compounds in g/L water: Tryptone 5, Peptic digest of meat 5, Yeast extract 3, Lactose 10, Sucrose 10, Sodium chloride 5, Phenol red 0.08, Brilliant green 0.0125, Agar 13.5, pH 6.7-7.1

BGA (AES 004235 Cranbury, USA)
Composition of BGA medium: not mentioned

BPLSA (Merck 107237.0500, Darmstadt, Germany)
Adam D., Zusatz von Natriumdesoxycholat zum Brilliantgrün-Phenolrot-Agar nach Kristensen-Kauffmann zur Hemmung des Schwärmvermögens von Proteuskeimen, 1966 Ärztl. Lab. 12, 245
Composition of BPLSA medium: the concentration of the compounds in g/L water: Peptone from meat 5, Peptone from casein 5, Meat extract 5, Sodium chloride 3, di-sodium hydrogen phosphate 2, Lactose 10, Sucrose 10, Phenol red 0.08, brilliant green 0.0125, Agar agar 12, pH 7

Brilliance *Salmonella* Agar BSA (previous OSCM) (Oxoid CM 1092, Hampshire, United Kingdom)
Schönenbrücher V, Mallinson ET, Bülte M. A comparison of standard cultural methods for the detection of foodborne *Salmonella* species including three new chromogenic plating media. Int J Food Microbiol. 2008 Mar 31;123(1-2):61-6
Composition of BSA agar: the concentration of the compounds in g/L water: Salmonella Growth mix 14, Chromogen mix 25, Agar 15, Cefsulodin 0.012, novobiocin 0.05, pH 7.2

BxLH
Composition of BxLH: not mentioned
Home made 12 ingredients, the medium is patented, pH 7.2

Onöz (Merck 115034, Darmstadt, Germany)
Onoz E, Hoffmann K. 1978 [Experience with a new culture medium for S*almonella* diagnosis (author's transl)] Zentralbl Bakteriol [Orig A]. 1978 Jan;240(1):16-21. German
Composition of Onöz medium: the concentration of the compounds in g/L water: Yeast 3, Meat extract 6, Pepton from meat 6.8, Lactose 11.5, Sucrose 13, Bile salt mixture 3.825, Tri-Sodium nitrate 5,5-Hydrate 9.3, Sodium Thiosulfate 5-Hydrate 4.25, L-Phenylalanine 5, Iron(III) Citrate 0.5, Magnesiumsulfate 0.4, Brilliant Green 0.00166, Neutral Red 0.002, Aniline Blue 0.25, Metachrome Yellow 0.47, di-Sodium Hydrogen Phosphate2-Hydrate 1, Agar-Agar 15, pH 7.1-7.2

Rambach (Merck 107500.0002, Darmstadt, Germany)
Rambach, A.: New Plate Medium far Facilitated Differentiation of *Salmonella* spp. from Proteus sac. and Other Enteric Bacteria». - Appl. Environm. Microbiol., 56; 301-303 (1990).
Composition of Rambach medium: the concentration of the compounds in g/L water: Peptone 8, NaCl 5, Sodium deoxycholate 1.0, Chromogenic mix 1.5, Propylene glycol 10.5, Agar-agar 15, Rambach agar supplement 10 ml, pH 7.1-7.3

Rapid Salmonella agar (Biorad 3563961, Marnes-La-Coquette, France)
Composition of Rapid Salmonella agar: the concentration of the compounds in g/L water: Casein Peptone 5, Meat extract 5, Selective agents 14, Chromogenic mixture 0.31, Agar 12, pH 7.2

Salmonella Shigella SS medium (Becton Dickinson 211597, Ontario, Canada)
Rose, H. M., and M. H. Kolodny. 1942. The use of SS (*Shigella-Salmonella*) Agar for the isolation of Flexner Dysentery bacilli from the feces. J. Lab. Clin. Med. 27:1081-1083
Composition of SS agar: the concentration of the compounds in g/L water: Beef extract 5, Pancreatic digest of casein 2.5, peptic digest annual tissue 2.5, Lactose 10, Bile salts 8.5,

Sodium citrate 8.5, Sodium thiosulphate 8.5, Ferric citrate 1, Brilliant green 0.00033, Neutral red 0.025, Agar 13.5, pH 7.1

SM(ID)2 = Chrom ID (bioMérieux SM2 43621, Marcy l' Etoile, France)
Pignato, S., G. Giammanco, and G. Giammanco. 1995 Rambach agar and SM-ID medium sensitivity for presumptive identification of *Salmonella* subspecies I to VI. J. Med. Microbiol., Vol 43, Issue 1 68-71
Composition of SM ID2 medium: the concentration of the compounds in g/L water: Peptones (swine and bovine) 6.3, Tris 0.2, Lactose 6, Ox bile (bovine and swine) 1.5, Chromogenic mix 9.6, Sodium chloride 5, Selective mix 0.03, Agar 14 pH 6.7- 7.3

XLT4 (Oxoid PO5116A, Hampshire, United Kingdom) (Biokar Diagnostics BK 156 HA Beauvais, France)
Miller, R.G., C.R. Tate. 1990. XLT4: A highly selective plating medium for the isolation of *Salmonella*. The Maryland Poultryman, April: 2-7 (1990)
Composition of XLT4 medium: the concentration of the compounds in g/L water: Peptone 1.6, Yeast Extract 3, L-Lysine 5, Lactose 7.5, Saccharose 7.5, Xylose 3.75, Sodium Chloride 5, Sodium Thiosulphate 6.8, Ferric Ammonium Citrate 0.8, 7-ethyl-2 methyl-4-undecanol hydrogen (Tergitol 4) 4.6 ml, Phenol Red 0.08, Agar 18 pH 7.4

Annex 4 Protocol

INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN CHICKEN FAECES organised by CRL-*Salmonella*

STUDY XIII - 2010

Introduction

This protocol describes the procedures for the 13th interlaboratory comparison study on the detection of *Salmonella* spp. in animal faeces amongst the National Reference Laboratories (NRLs) for *Salmonella* in the EU. This study will have a comparable set-up as the earlier studies on the detection of *Salmonella* spp. in veterinary samples. The prescribed method is the procedure as described in Annex D of ISO 6579 (EN-ISO 6579:2002/Amd1: 2007: Amendment 1: Annex D: Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage). Furthermore, laboratories who are interested can also perform PCR on the samples and/or use additional methods (routinely) used in their laboratories.

The samples will consist of chicken faeces samples (*Salmonella*-negative) artificially contaminated with reference materials. The reference materials (RMs) consist of gelatine capsules containing sublethally injured *Salmonella* strains at different contamination levels. Each laboratory will examine twenty-nine faeces samples (10 g each) in combination with a *Salmonella* capsule and six control samples (capsules only). Two different batches of chicken faeces have to be tested: twenty-five samples with one batch and four samples with another batch of faeces.

The samples will be packed in two plastic containers in one large box together with cooling elements. One container will contain the capsules, the other container will contain the chicken faeces. The container with the capsules will also contain a temperature recorder to measure the temperature during transport of the samples. The recorder will be packed in a plastic bag, which will also contain your lab code. **You are urgently requested to return this complete plastic bag with recorder and lab code to the CRL-*Salmonella*, immediately after receipt of the parcel**. For this purpose a return envelope with a pre-printed address label of the CRL-*Salmonella* will be included. Do not forget to note your lab code before returning it to the CRL.
Each box will be sent as biological substance category B (UN3373) by door-to-door courier service. Please contact CRL-Salmonella when the parcel has not arrived at your laboratory at March 11, 2010 (this is four working days after the day of mailing).

Objective

The main objective of the interlaboratory comparison study is to evaluate the performance of the NRLs for *Salmonella* for their ability to detect *Salmonella* spp. at different contamination levels in poultry faeces.

Outline of the study

Each participant will receive (in week 10 of 2010) one box containing two plastic containers, packed with cooling elements. The containers contain:
Container 1:
contains one plastic bag with 35 numbered vials each containing one capsule with or without *Salmonella*

- 4 vials numbered **A**1-A4;
- 25 vials numbered **B**1-B25;
- 6 vials numbered **C**1-C6.

This container will also contain the small electronic temperature recorder in a plastic bag with your lab code. **This recorder (in the plastic bag) should be returned to the CRL-*Salmonella* as soon as possible.**
Store container 1 at (-20 ± 5) ºC immediately after receipt.

Container 2:
contains two plastic bags
- 60 g of chicken faeces (free from *Salmonella*) marked with **A**;
- 300 g of chicken faeces (free from *Salmonella*) marked with **B**.

Store container 2 at (5 ± 3) ºC immediately after receipt.

The performance of the study will be in week 11 (starting on March 15, 2010).

The documents necessary for performing the study are:

- Protocol Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in animal faeces XIII (2010) (this document);
- SOP Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in animal faeces XIII (2010);
- Test report Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in animal faeces XIII (2010);
- ISO 6579 (2002). Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.;
- ISO 6579:2002/Amd 1: 2007 Amendment 1 Annex D: Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage.

The media to be used for the collaborative study will not be supplied by the CRL.

All data have to be reported in the test report and sent to the CRL-*Salmonella* before **April 2, 2010.** The CRL will prepare a summary report soon after the study to inform all NRLs on the overall results.
Results which will be received after the deadline can not be used in the analyses for the interim summary report.

If you have questions or remarks about the interlaboratory comparison study please contact:

Angelina Kuijpers (Tel. number: + 31 30 274 2093)
Kirsten Mooijman (Tel. number: + 31 30 274 3537)
RIVM / LZO (internal Pb 63)CRL *Salmonella*
P.O. Box 1 3720 BA Bilthoven, The Netherlands
http://www.rivm.nl/crlsalmonella
Fax. number: + 31 30 274 4434
E-mail : Angelina.Kuijpers@rivm.nl or Kirsten.Mooijman@rivm.nl

Time table of interlaboratory comparison study
ANIMAL FAECES XIII (2010)

Week	Date	Topic
7	February 15 - 19	Mailing of the protocol, standard operating procedure and test report to the NRLs-*Salmonella*
10	March 8 – 12	Mailing of the parcels to the NRLs as biological substance (UN3373) by door-to-door courier service Immediately after arrival of the parcels at the laboratory: - Check for any serious damages (**do not accept damaged packages)**; - Check for completeness; - Remove the electronic temperature recorder from the container (leave it in the plastic bag with lab code) and return it to CRL-*Salmonella* using the return envelope; **Store the capsules at -20°C ± 5 °C** - **Store the faeces at +5°C ± 3 °C** - **If you did not receive the parcel at 11 March, do contact the CRL immediately.** Preparation of: 1. Non selective pre-enrichment medium (see SOP 6.1) 2. Selective enrichment media (see SOP 6.2) 3. Solid selective plating media (see SOP 6.3) 4. Confirmation media (see SOP 6.4)
11	March 15 – 19	Performance of the study, following the instructions as given in the protocol and the SOP of study Animal faeces XIII (2010).
13	Before April 2	Completion of the test report. Send the test report, preferably by e-mail to the CRL-*Salmonella* (Angelina.Kuijpers@rivm.nl)* .
14	April 5 - 9	Data input at CRL-*Salmonella* and sending these data to NRLs these results by the National Reference Laboratories.
	May - June 2010	Sending of the final results to the NRLs together with a short summary. As a follow-up, actions will be undertaken in case of poor performance.

* If the test report is e-mailed to the CRL, it is not necessary to send the original test report as well, unless it is not legible (to be indicated by CRL-*Salmonella*).

Annex 5 Standard Operating Procedure (SOP)

INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN CHICKEN FAECES organised by CRL-*Salmonella*

STUDY XIII - 2010

1 Scope and field of application

This standard operating procedure (SOP) describes the procedure for the detection of *Salmonella* in the presence of competitive micro-organisms in chicken faeces. For this purpose Reference Materials (RMs) containing sublethally injured *Salmonella* Typhimurium (STM), *Salmonella* Enteritidis (SE) or *Salmonella* Panama (SPan) as prepared by the Community Reference Laboratory (CRL) for *Salmonella* are used. As matrix, chicken faeces negative for *Salmonella* is used. The application of this SOP is limited to the interlaboratory comparison study for *Salmonella* described in this SOP.

2 References

International Standard – ISO 6579: 2002(E)
Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.
ISO 6579:2002/Amd 1 2007. Amendment 1 Annex D: Detection of *Salmonella* spp. in animal faces and in environmental samples from the primary production stage.

3 Definitions

For the purpose of this SOP, the following definitions apply:
- *Salmonella*: micro-organisms which form typical colonies on isolation media for *Salmonella* and which display the serological and/or biochemical reactions described when tests are carried out in accordance with this SOP.
- *Reference Material*: a gelatine capsule containing a quantified amount of a test organism in spray dried milk.

4 Principle

The detection of *Salmonella* involves the following stages:
a) Pre-enrichment
b) Selective enrichment
c) Isolation
d) Confirmation of typical colonies as *Salmonella*.

5 List of abbreviations

BPW Buffered Peptone Water
MSRV Modified semi-solid Rappaport Vassiliadis medium
RM Reference Material
SOP Standard Operating Procedure
XLD Xylose Lysine Deoxycholate agar

6 Culture media

For this study the prescribed method is the procedure as described in Annex D of ISO 6579, for which the following media are needed.

Non selective pre-enrichment medium	BPW
Selective enrichment medium	MSRV
Selective plating medium for first and second isolation medium for choice (obligatory!)	XLD and a second

Composition and preparation of the media and reagents are described in Annex B, and in Annex D of the ISO 6579: 2002(E). In the list of media given in 6.1 up to 6.4, reference is made to the relevant part of ISO 6579. Complete ready-to-use media or dehydrated media are also allowed to be used, as long as the composition is in accordance with the information given below. Check the quality of the media before use.

In addition to the prescribed method (Annex D of ISO 6579) it is allowed to use other methods, e.g. the one(s) routinely used in your laboratory ['Own' method(s)]. Prepare media for the 'own' method(s) according to the relevant instructions. Record all relevant information in the test report.

6.1 Non selective pre-enrichment medium

- Buffered Peptone water (BPW) (ISO6579 Annex B.1)

Mind to distribute the BPW in portions of 90 ml into suitable flasks before sterilisation.

6.2 Selective enrichment medium

- Modified Semi solid Rappaport Vassiliadis (MSRV) (ISO6579 Annex D)
- Own selective enrichment medium routinely used in your laboratory (optionally)

6.3 Solid selective media for first and second isolation

- Xylose-Lysine-Desoxycholat (90 mm plates) (ISO6579 Annex B.4)
- Second isolation medium of choice (obligatory)
- Own medium (optionally)

6.4 Confirmation media

- Biochemical confirmation as described in ISO 6579 Annex B.6-B.11 or by reliable, commercially available identification kits.
- Nutrient agar (optionally) (ISO6579 Annex B.5)

7 Apparatus and glassware

The usual microbiological laboratory equipment. If requested, note specifications of the apparatus and glassware on the test report.

7.1 Apparatus

- Oven (for dry sterilisation) or autoclave (for wet sterilisation);
- Water bath or incubator, capable of operating at 37 °C ± 1 °C;
- Water bath or incubator, capable of operating at 41.5 °C ± 1 °C;
- Sterile loops of 1 μl;
- pH-meter; having an accuracy of calibration of ± 0.1 pH unit at 25 °C.

7.2 Glassware

- Culture bottles or jars with nominal capacity of 200 ml;
- Culture tubes with approximate sizes: 8 mm in diameter and 160 mm in length;
- Micro-pipettes; nominal capacity 0.1 ml;
- Petri dishes; standard size (diameter 90 mm to 100 mm).

8 Procedure

Below the prescribed method of the thirteenth interlaboratory comparison study in chicken faeces of CRL-Salmonella is described. The different steps in the procedure are also summarized in Annex A of this SOP. In addition to this method it is also allowed to use one or more own methods. Please record all relevant data in the test report. Details of the method can be found in ISO 6579 and Annex D of ISO 6579.

8.1 Prewarming BPW (day 0)

Label 38 jars, each containing 90 ml of BPW as follow:
4 jars from A1 to A4;
25 jars from B1 to B25;
9 jars from C1 to C9 (control capsules).
Place all jars (at least) overnight at 37 °C (± 1 °C). Also place some extra non-labelled jars containing 90 ml of BPW at 37 °C in case some jars might have been contaminated. Record in the test report (page 2 and 3) the requested data on BPW.

8.2 Pre-enrichment (day 1)

Take the numbered vials with the *Salmonella* capsules and the control capsules out of the freezer one hour before they are added to the BPW, to allow them to equilibrate to room temperature.
Shortly before adding the capsules, take the jars with BPW from the 37 °C incubator and inspect them for visual growth. Discard infected jars.
Add to 35 labelled jars a gelatine capsule from the vial with the corresponding label number. Do not open the gelatine capsule and do not shake the BPW to dissolve the capsule more rapidly. Place the jars with the capsules in the 37 °C incubator for 45 minutes for dissolving of the capsules. Record the temperature and time at the start and at the end of this period in the test report (page 3).
After 45 minutes add the faeces to the jars according to the following scheme:
Add 10 g of faeces from batch A to each jar labelled A1-A4 and C8;
Add 10 g of faeces from batch B to each jar labelled B1-B25 and C9;
Add no faeces to jars labelled C1 – C7;
Do not shake the jars after adding the faeces.
One jar is a procedure control (= C7) to which no capsule or faeces is added and two jars are negative faeces controls to which only 10 g faeces is added (C8 with faeces batch A and C9 with faeces batch B). These control jars should be handled in the same way as the other jars.

Place all jars in the 37 °C (± 1 °C) incubator for 18 h ± 2 h. Record the temperature and time at the start and at the end of the incubation period and other requested data on page 3 of the test report.
If PCR is performed, fill in all requested data on page 16 and 23 of the test report.

8.3 Selective enrichment (day 2)

Allow the MSRV plates to equilibrate to room temperature, if they were stored at a lower temperature. Dry the surface of the MSRV plates in a Laminair Air Flow cabinet if necessary. Record (page 4-7) the requested data on the MSRV and own selective enrichment media (if used) in the test report.
Label 38 MSRV plates as follow:
4 plates from A1 to A4;
25 plates from B1 to B25;
9 plates from C1 to C9 (control).
If other selective enrichment media are used, label them in the same way as described for MSRV.

After equilibration of the media:
Prescribed method:
- Inoculate the MSRV plates with three drops of BPW culture, with a total volume of 0.1 ml. Incubate (not upside down) at 41.5 °C ± 1 °C for 24 h ± 3 h and if negative for another 24 h ± 3 h.

Optional method:
- Inoculate the routinely used selective medium/media (other than those mentioned above), with the corresponding BPW culture (note the inoculation volume of BPW used and the volume of the selective medium/media on test report). Incubate at the temperature and for the time routinely used.

Place the jars/tubes/plates in the appropriate incubator(s)/water bath(s) and record the temperature and time for the different enrichment media at the start and at the end of the incubation period and other requested data in the test report (page 4-7).
Isolation media (first and second isolation) (day 3 and 4)
Record in the test report (page 8-13) the requested data of the isolation media used. Label 38 (standard size) Petri dishes of each isolation medium from A1 to A4, B1 to B25 and C1 to C9.

First isolation after 24 h
Inoculation:
Inoculate from suspect MSRV plates, the surface of an isolation medium in one standard size Petri dish with the corresponding label number in such a way that well isolated colonies will be obtained. The following isolation media will be used:

1) Xylose Lysine Desoxycholate agar (XLD)
   Place the Petri dishes with the bottom up in the incubator set at 37 °C (record temperature and time and other requested data in test report, page 8 and 9).

2) Second isolation medium. Follow the instructions of the manufacturer (record temperature and time and other requested data in test report, page 10 and 11).

3) Optionally: selective isolation medium/media routinely used in your laboratory. Incubate the medium/media at the temperature and for the time routinely used (record temperature and time and other requested data in test report, page 12 and 13).

After incubation for 24 h ± 3 h, examine the Petri dishes for the presence of typical colonies of *Salmonella.*

Second isolation after 48 h
After a total incubation time of 48 h ± 3 h of the selective enrichment media, repeat the procedure described above **(First isolation after 24 h)**. Repeat the full procedure only when the First isolation after 24 h on selective enrichment media is negative.

8.5 Confirmation of colonies from first and second isolation (day 4 and day 5)

For confirmation take from each Petri dish of each isolation medium at least 1 colony considered to be typical or suspect (use only well isolated colonies). Store the plates at 5 °C ± 3 °C.
Before confirmation (see below), optionally, streak the typical colonies onto the surface of nutrient agar plates with the corresponding label numbers, in a manner which allows to develop well isolated colonies. Record the requested data of the nutrient agar on the test report (page 14). Incubate the inoculated plates at 37 °C ± 1 °C for 24 h ± 3 h.
If the selected colony is not confirmed as Salmonella, test at maximum another five typical colonies from the original isolation medium (stored at 5 °C). Report the number of colonies tested and the number of colonies confirmed as Salmonella for each dish in Table 1 (isolation using MSRV) and Table 2 (isolation using own enrichment) on the test report (pages 17-22).
If a PCR method has been used, report the results in Table 3 of the test report (page 23).

Confirmation of identity
The identity of the colony selected as described above (either directly from the isolation medium, or from nutrient agar) is confirmed by means of appropriate biochemical and serological tests. Follow the instructions of ISO 6579. The interpretation of the biochemical tests is given in Table 1 of ISO 6579:2002 on page 9.
Conserve one positive isolate (Salmonella strain) from each sample (one Salmonella confirmed colony from one of the two isolation media from the samples A1-A4, B1-B25 and C1-C9).

9 Test report

The test report should contain all information that might influence the results and is not mentioned in this SOP. Incidents or deviations from the specified procedures should also be recorded. The test report should include the name of the person in charge for the NRL, and the names of the persons who are carrying out the work. If the study was carried out by another laboratory than the NRL, please also give the details of this laboratory in the test report.

Scheme of Bacteriological Interlaboratory Comparison Study ANIMAL FAECES XIII (2010)on the detection of Salmonella spp. in chicken faeces		
Day	**Topic**	**Description**
0	**Prewarming BPW**	Place at least at the end of the day sufficient jars, each containing 90 ml BPW, at 37 °C ± 1 °C.
1	**Pre-enrichment**	Add 1 capsule to 90 ml (prewarmed) BPW Do not shake Incubate 45 min. at 37 °C ± 1 °C Add 10 g faeces to BPW Incubate 18 h ± 2 h at 37 °C ± 1 °C
2	**Selective enrichment**	0.1 ml BPW culture on MSRV plate, incubate at (41.5 ± 1) °C for (24 ± 3) h Own selective enrichment medi(um)(a)
3	**First isolation after 24 h**	Inoculate from suspect MSRV (24h) plates and from Own selective medi(um)(a) ➢ XLD agar, incubate at (37 ± 1) °C for (24 ± 3) h ➢ Second isolation medium* ➢ Own selective medi(um)(a)* *= Incubate for specified time at the specified temperature
3	**Continue selective enrichment**	Incubate MSRV medium and if necessary Own med(ium) (a) another 24 (± 3) hours at the relevant temperatures.
4	**Second isolation after 48 h**	If the first isolation was negative, inoculate from suspect MSRV (48h) plates and Own medi(um)(a) ➢ XLD agar, incubate at (37 ± 1) °C for (24 ± 3) h ➢ Second isolation medium* ➢ Own selective medi(um)(a)* *= Incubate for specified time at the specified temperature
4	**Confirmation of identity**	Confirm the identity of the *Salmonella* suspect colonies from the first isolation media (day 3).
5	**Confirmation of identity**	Confirm the identity of the *Salmonella* suspect colonies from isolation media (day 4).

Annex 6 Results found with 'own methods'

Table A6.1 Results control samples, analysed with an 'own method'

The highest number of positive isolations found with the given selective enrichment medium in combination with any isolation medium								
Lab code	**Other than MSRV 'own method'**				**MSRV**			
	Blank n=1	**SE20 n=2**	**SE100 n=1**	**STM5 n=2**	**Blank n=1**	**SE20 n=2**	**SE100 n=1**	**STM5 n=2**
Good Performance	**0**	**≥ 1**	**1**	**≥ 1**	**0**	**≥ 1**	**1**	**≥ 1**
	MKTTn				**MSRV**			
1	0	**1**	1	2	0	**1**	1	2
9	0	2	1	2	0	2	1	2
10	0	2	1	2	0	2	1	2
22	0	2	1	2	0	2	1	2
	RVS				**MSRV**			
1	0	**1**	1	2	0	1	1	2
3	0	2	1	2	0	2	1	2
4	0	2	1	2	0	2	1	2
10	0	2	1	2	0	2	1	2
18*	0	2	1	2	0	2	1	2
	SC				**MSRV**			
8	0	2	1	2	0	2	1	2

Table A6.2 Results faeces samples artificially contaminated with Salmonella, analysed with an 'own method'

The highest number of positive isolations found with the given selective enrichment medium in combination with any isolation medium										
Lab code	**Other than MSRV ' own method'**					**MSRV**				
	Blank n=5	**SE20 n=5**	**SE100 n=5**	**STM5 n=5**	**STM50 n=5**	**Blank n=5**	**SE20 n=5**	**SE100 n=5**	**STM5 n=5**	**STM50 n=5**
Good Performance	**≤ 1**	**> 2**	**≥ 4**	**> 2**	**≥ 4**	**≤ 1**	**> 2**	**≥ 4**	**> 2**	**≥ 4**
	MKTTn					**MSRV**				
1	0	**4**	5	**1**	**3**	0	**4**	5	5	5
9	0	**4**	5	5	5	0	**4**	5	5	5
10	0	**4**	5	5	5	0	**4**	5	5	5
22	0	5	5	5	5	0	5	5	5	5
	RVS					**MSRV**				
1	0	**4**	5	5	**4**	0	**4**	5	5	5
3	**2**	5	5	5	5	0	**3**	5	5	5
4	0	**4**	5	**4**	5	0	**4**	5	**4**	5
10	0	**4**	5	5	5	0	**4**	5	5	5
18*	0	5	5	5	5	0	5	5	5	5
	SC					**MSRV**				
8	0	5	5	5	5	0	5	5	5	5

* Laboratory 18 also analysed the samples with RV which showed the same results as RVS and MSRV
Bold numbers: Deviating results Grey cells: Results below the level of good performance

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