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EU Interlaboratory comparison study veterinary XII (2009)

Bacteriological detection of Salmonella in chicken faeces

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This investigation has been performed by order and for the account of the European Commission, Health and Consumer Protection Directorate-General and the Laboratory for Zoonoses and Environmental Microbiology (LZO) of the RIVM, within the framework of V/330604/09/CS by the Community Reference Laboratory for *Salmonella*

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Abstract

EU Interlaboratory comparison study veterinary XII (2009)
Bacteriological detection of *Salmonella* in chicken faeces

In 2009, a comparison study of the performance of the 34 National Reference Laboratories (NRLs) for *Salmonella* revealed that all NRLs were able to detect high and low levels of *Salmonella* in chicken faeces. Thirty-three of these laboratories demonstrated a high performance level and compliance with the prescribed method during the first performance testing. One laboratory reached the desired performance level after repeating the tests at a latter date.

These are the results of the twelfth veterinary interlaboratory comparison study organized by the Community Reference Laboratory (CRL) for *Salmonella*. The study was conducted in March 2009, with the follow-up study in July of that year. All European Member States are obliged to participate in this study. The CRL for *Salmonella* is located at the National Institute for Public Health and the Environment (RIVM).

Within the framework of this study, each laboratory received a package containing chicken faeces (free of *Salmonella*) and 35 gelatin capsules containing powdered milk contaminated with different levels of *Salmonella* spp. The laboratories were instructed to spike the chicken faeces with the contents of the capsules and then test the samples for the presence of *Salmonella* in the faeces. For this testing, the laboratories were required to use the internationally prescribed method for the detection of *Salmonella* in veterinary samples.

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

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Rapport in het kort

EU Ringonderzoek veterinair XII (2009)
Bacteriologische detectie van *Salmonella* in kippenmest

In 2009 heeft een vergelijkende studie onder 34 Nationale Referentie Laboratoria (NRL's) uitgewezen dat alle NRL's in staat waren hoge en lage concentraties *Salmonella* in kippenmest aan te tonen. Van deze laboratoria lieten er 33 direct zien dat zij het onderzoek met succes en volgens de voorgeschreven methode konden uitvoeren. Eén laboratorium behaalde het gewenste niveau tijdens een herkansing op een later tijdstip.

Dit zijn de resultaten van het twaalfde veterinair ringonderzoek dat het Communautair Referentie Laboratorium (CRL) voor *Salmonella* heeft georganiseerd. Het onderzoek is in maart 2009 gehouden, de herkansing in juli van dat jaar. Europese lidstaten zijn verplicht om aan dit onderzoek deel te nemen. Het CRL-*Salmonella* is gevestigd bij het Rijksinstituut voor Volksgezondheid en Milieu (RIVM).

Voor dit ringonderzoek kreeg ieder laboratorium een pakket toegestuurd met kippenmest (vrij van *Salmonella*) en 35 gelatine capsules met melkpoeder met verschillende besmettingsniveaus van *Salmonella*. De laboratoria moesten de kippenmest en capsules volgens voorschrift samenvoegen en onderzoeken of de mest *Salmonella* bevatte. De laboratoria gebruikten hiervoor de internationaal voorgeschreven methode om *Salmonella* aan te tonen in dierlijk mest.

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

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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
FYROM	Former Yugoslav Republic of Macedonia
Gal	Galactosidase
hcmp	highly contaminated milk powder
ISO	International Standardisation Organisation
LDC	Lysine Decarboxylase
MKTTn	Mueller Kauffmann Tetrathionate novobiocin broth
MLCB	Mannitol Lysine Crystal violet Brilliant green agar
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
SPan	*Salmonella* Panama
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

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Summary

In March 2009 the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*) organised the twelfth veterinary interlaboratory comparison study on bacteriological detection of *Salmonella* (chicken faeces). Participants were 34 National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*): 28 NRLs from 27 EU Member States, one NRL from candidate country Former Yugoslav Republic of Macedonia (FYROM), 3 NRLs from member countries of the European Free Trade Association (EFTA): Switserland, Norway and Iceland and on request of DG-Sanco 2 non-Europe NRLs from third countries Israel and Tunesia.

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 gram of *Salmonella*-negative chicken faeces. These 25 capsules were divided over the following groups: 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 blank capsules. The other 10 capsules, to which no faeces had to be added, were control samples, existing of 3 capsules STM5, 2 capsules SE20, 1 capsule SE100, 2 capsules containing approximately 5 cfp of *S.* Panama (SPan5) and 2 blank capsules.

On average the laboratories found *Salmonella* in 98 % of the (contaminated) samples when using the prescribed veterinary method, selective enrichment on MSRV.

All NRLs fulfilled the criteria of good performance. Thirty-three out of thirty-four laboratories achieved the desired level at once in the main study. One NRL found a false positive result with a blank sample (without faeces) and did not find a reason for this. The laboratory showed good performance in the follow up study and fulfilled the desired criteria.

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1 Introduction

An important task of the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*), as laid down in 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 2009, 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.

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 at or just above the detection limit of the method; the level of the high level samples was approximately 5-10 times above the detection limit. Ten control samples consisting of different reference materials, had to be tested without the addition of chicken faeces. These latter reference materials consisted of 3 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), 2 capsules with approximately 5 cfp of *Salmonella* Panama (SPan5) and 2 blank capsules. Twenty-five samples of *Salmonella* negative chicken faeces spiked with 5 different reference materials (including blank capsules) had to be examined. The different reference materials consisted of two levels of *Salmonella* Typhimurium (STM5 and STM50) and two levels of *Salmonella* Enteritidis (SE20 and SE100).

2 Participation

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	Nationaly Diagnostic Centre (NDC) of Food and Veterinary Service
Lithuania	Vilnius	National food and veterinary risk assessment institute
Luxembourg	Luxembourg	Laboratoire de Médecine Vétérinaire de l'Etat, Animal Zoonosis
Macedonia (FYROM)	Skopje	Food institute Faculty of veterinary medicine
Malta	Valletta	Public Health Laboratory (PHL) Evans Building
Netherlands the	Bilthoven	National Institute for Public Health and the Environment(RIVM)

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	Zürich	Institute of Veterinary Bacteriology National Reference Centre for Poultry and Rabbit Diseases
Tunesia	Tunis	Veterinary Research Institute of Tunesia, 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 in gelatin capsules resulting in the final reference materials (RMs).
The target levels of the five batches of RMs were:

- 5 colony forming particles (cfp) per capsule for *Salmonella* Panama (SPan5);
- 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 gelatin 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 ^{o}C. If the test batches fulfilled the pre-set criteria for contamination level and homogeneity, the relevant mixed powders were completely filled into gelatin capsules and stored at -20 ^{o}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) ^{o}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) ^{o}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) ^{o}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 10 kilogram from this farm arrived at the CRL-*Salmonella* on 9 February 2009. For the follow-up study 2 kilogram of faeces arrived on 10 June 2009. The faeces was stored at 5 ^{o}C and checked for the absence of *Salmonella* by testing 10 portions of 10 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 10 g were each added to 90 ml Buffered Peptone Water (BPW). After pre-enrichment at (37 ± 1) ^{o}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 biochemically. The chicken faeces was stored at 5 ºC 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 one 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) ^{o}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 was 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 one 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) ^{o}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.

3.3 Design of the interlaboratory comparison study

3.3.1 Samples: capsules and chicken faeces

On 2 March 2009 (two weeks before the study) the reference materials (35 individually numbered capsules) and 300 grams of *Salmonella* negative chicken 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 ^{o}C and the faeces had to be stored at +5 ^{o}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.

Ten control capsules had to be tested without faeces (numbered C1-C10). Twenty-five capsules (numbered 1 – 25) were each tested in combination with 10 grams of faeces (negative for *Salmonella*). 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=10) No faeces added	Test samples (n=25) with 10 g *Salmonella* negative chicken faeces
S. Panama 5 (SPan5)	2	---
S. Enteritidis 20 (SE20)	2	5
S. Enteritidis 100 (SE100)	1	5
S. Typhimurium 5 (STM5)	3	5
S. Typhimurium 50 (STM50)	---	5
Blank	2	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 ^{o}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 graphic 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. This could be different medium combinations and/or investigation of the samples with alternative methods, like Polymerase Chain Reaction 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, commercial available identification kits and 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

Proposal for criteria testing 'good performance'
During the tenth CRL-*Salmonella* workshop in April 2005 a proposal was made to define 'good performance' in interlaboratory comparison studies on detection of *Salmonella* (Mooijman, 2005).
Since 2005, the contamination level of the reference materials has been amended and thus also the definition of good performance has been slightly amended.

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.
In Table 2 the criteria used for testing good performance in the present study are summarised.

Table 2 Used criteria for testing good performance in the veterinary XII study (2009).

Control samples (capsules, no matrix)	Minimum result	
	Percentage positive	No. of positive samples / total no. of samples
SE100	100 %	1/1
STM5	60 %	2/3
SPan5 and SE20	50 %	1/2
Blank control capsules	0 %	0/2

Samples (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.

4 Results

4.1 Reference materials

The level of contamination and the homogeneity of the final batches of capsules are presented in Table 3.The enumerated minimum and maximum levels within each batch of capsules are also given in this table. 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 the variation between the SE100 capsules was too high. However, at the second date of testing all batches fulfilled the pre-set criteria as stated in section 3.1.

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

	SE20	SE100	SPan5	STM5	STM50
Final batch; Test 1					
Date testing capsules	19-02-2009	29-01-2009	18-02-2009	21-01-2009	07-01-2009
Number of capsules tested	50	50	50	50	50
Mean cfp per capsule	18	67	7	6	62
Min-max cfp per capsule	11-29	45-107	2-14	3-12	39-78
T_2 / (I-1)	0.88	2.70	1.15	1.06	1.55
Final batch; Test 2					
Date testing capsules	19-03-2009	19-03-2009	25-03-2009	19-03-2009	19-03-2009
Number of capsules tested	25	20	25	25	25
Mean cfp per capsule	18	84	7	6	53
Min-max cfp per capsule	8-27	56-115	2-14	2-14	33-66
T_2 / (I-1)	1.29	1.97	1.46	1.35	1.34

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

The faeces was tested negative for *Salmonella* and stored at 5 °C. On Monday 2 March 2009 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 (10/02/2009) and secondly at the planned date of the interlaboratory comparison study (17/03/2009). The results are shown in Table 4.

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

Date	Aerobic bacteria cfp/g	Enterobacteriaceae cfp/g
10 Februari 2009	$2.5*10^8$	$6.2*10^4$
17 March 2009	$5.7*10^8$	$1*10^4$

The majority of the laboratories (thirty-one) performed the study on the planned date (week 16 starting on 12/03/2008). Three laboratories (labcode 4, 5 and 19) performed the study one week earlier.

4.3 Technical data interlaboratory comparison study

4.3.1 General

In this study 34 NRLs participated: 28 NRLs from 27 EU-Member States, 3 NRLs from member countries of the European Free Trade Association State, 1 NRL from a EU-candidate country and, on request of DG-Sanco, 2 NRLs from third countries (non-Europe).

4.3.2 Accreditation/certification

Thirty laboratories mentioned to be accredited for their quality system according to ISO/IEC 17025 (Anonymous, 2005) (labcodes 1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 28, 29, 30, 31, 32, 33 and 34). Four laboratories (labcodes 8, 25, 26 and 27) are planning to become accredited or certified in the near future. The NRLs without accreditation needs to take into account that according to EC Regulations No. 882/2004 each NRL should be accredited for their relevant work field before 31 December 2009 (EC Regulation No. 2076/2005).

4.3.3 Transport of samples

An overview of the transport times and the temperatures during transport of the parcels is given in Table 5. 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 34 was delayed and arrived on Thursday 5 March in the afternoon at the airport/customs and it was stored there until Tuesday 10 March before it was delivered to the institute. The total transport time of this parcel was 195 hours. When this latter parcel and the two parcels from the third countries (non-EU) are not taken into account, the average transport time was 39 hours. For the majority of the parcels the transport temperature did not exceed 5 ^oC. Although the parcel of laboratory 34 was delayed for 8 days, most of the time it was stored below 5 ^oC. For ten 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 28 hours. In five laboratories the storage temperature during the delay was at approximately 20 ^oC or higher. In four laboratories of those latter laboratories (labcodes 4, 9, 12 and 21) this delay was only a few hours. However, in laboratory 13 the information from the temperature recorder showed a variable temperature (10-23 ^oC) during the delay of 24 hours. For the other laboratories the storage temperature during delay was below 7 ^oC.

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

		Time (h) at				
Labcode	Transport time[1] total in hours	< 0 °C	0 °C - 5 °C	5 °C - 10 °C	> 15 °C	Additional Storage[2]
1	26	15	9		2	
2	27	6	21			16 hrs < 7 °C
3	89	61	28			
4	24	6	18			2 hrs at 24 °C
5	77	16	40	21		
6	26	26				
7	47	5	42			
8	144	8	136			
9	23	23				3 hrs at 20 °C
10	75	24	51			
11	24	24				
12	24	3	21			1 hr at 20 °C
13	24	7	17			24 hrs between 10-23 °C
14	24	14	10			
15	52	3	49			
16	26	8	16		2	
17	23	23				
18	1		1			
19	26	5	21			
20	74	2	71		1	
21	43	5	38			1hr at 6 °C and 2 hrs 23 °C
22	27	13	13		1	
23	51	7	44			
24	22	5	17			2 hrs at 3 °C
25	24	23	1			
26	50	1	49			
27	20	5	15			4 hrs at 2 °C
28	22	20	2			28 hrs < 0 °C
29	50	8	42			1 hr at 1 °C
30	49	5	44			
31	48	4	44			
32	21	3	18			
33	50	9	40		1	
34	195	8	182	5		
Average[3]	38.9					

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) and lab 34

Table 6 Media combinations used per laboratory.

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

* Laboratory 3 used not all combinations of MSRV and isolation media.

**Laboratory 12 and 24 used two different MSRV formulations

4.3.4 Media

Each laboratory was asked to test the samples with the prescribed (Annex D of ISO 6579) method. All laboratories used the selective enrichment medium MSRV with the plating out medium XLD and a second plating out medium of own choice. Eleven laboratories used an additional selective enrichment medium: RVS (four laboratories), MKTTn (three laboratories), Selenite Cystine broth (one laboratory), RVS and MKTTn (two laboratories), RVS and another formulation of MSRV (one laboratory). Seven laboratories used more than two isolation media, laboratory 3 used those media only in combination with an own method.
The media used per laboratory are shown in Table 6. Details on the media which are not described in ISO 6579 are given in Annex 3. In Tables 7-11 information is given on the composition of the media which were prescribed and on incubation temperatures and times. In these tables only the laboratories are indicated who reported deviations. Laboratories 4 and 28 did not mention the pH of the media. Two laboratories (labcode 17 and 28) used MSRV without novobiocin and seven laboratories used MSRV with a higher concentration of novobiocin than the prescribed 0.01 g/L. Laboratory 3 used expired batch of medium (MSRV). Laboratory 12 used from one manufacturer two different MSRV formulations (Oxoid CM0910 and CM 1112) but they did not confirm the isolations from the isolation media from the second MSRV formulations. Laboratory 24 used MSRV from two different manufacturers (Oxoid CM 1112 and Merck 1.09878809).

Table 7 Incubation time and temperature of BPW.

	Prewarming BPW		Dissolving capsules In BPW		Pre-enrichment in BPW	
Labcode	**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 & ISO 6579	**Overnight**	**36-38**	**45**	**36-38**	**16 – 20**	**36-38**
3	Overnight	37.2-37.4	55	37.3-37.4	19:39	37.4-37.7
4	Overnight	37.2-37.5	45	35.4-36.6	20:00	35.3-37.4
5	Overnight	37	45	37	24:15	37
26	Overnight	36-38	45	36-38	20:17	36-38
28	Overnight	37	45	37	21:25	37

Grey cell : deviating times and temperatures - = no information

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

Labcode	Enzymatic digest of casein (Peptone)	Sodium Chloride (NaCl)	Disodium hydrogen Phosphate dodecahydrate* ($Na_2HPO_4.12H_2O$)	Potassium dihydrogen phosphate (KH_2PO_4)	pH
ISO 6579	**10.0**	**5.0**	**9.0**	**1.5**	**6.8 – 7.2**
3	10.0	5.0	3.5*	1.5	7.3
4	10.0	5.0	9.0	1.5	-
11	10.0	5.0	3.5*	1.5	-
13	10.0	5.0	3.5*	1.5	7.4
24	10.0	5.0	3.5*	1.5	7.3
28	10.0	5.0	3.5*	1.5	-
29	10.0	5.0	3.5*	1.5	7.3

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.

Labcode	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**
3	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.5
4	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.01	-
10	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.05	5.1
13	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.01	5.5
15	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0	5.1
16	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.04	5.4
17	8.25	0.92	7.3	1.5	12.4	0.04	2.6	0	5.6
21, 27	2.3 + 2.3*	4.6	7.3	1.5	10.9	0.04	2.5	0.01	5.3
22	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.01	5.5
23	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	-
24**	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.4
26	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.1
28	4.5***	-	7.2	1.26+0.18****	13.4	0.04	-	-	-
33	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.01	5.5
34	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.4

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

* 2..3 g Tryptone + 2..3 g Peptone

** laboratory 24 used two different manufacturers MSRV Oxoid CM 112 and Merck 1.09878809

***Enzymatic digest of Soya

**** Potassium Dihydrogen Phosphate K_2HPO_4 1.26 g + DiPotassium hydrogen Phosphate KH_2PO_4 0.18 g

Table 10 Incubation times and temperatures of selective enrichment medium MSRV.

	MSRV	
Labcode	**Incubation time in h:min**	**Incubation temperature in °C (min-max)**
ISO 6579 Annex D	**2 x (24 ± 3) h**	**40.5 – 42.5**
4	47:25	39.5- 41.1

Grey cell: deviating temperatures

Table 11 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**
4	3.5	5.0	7.5	7.5	5.0	3.0	0.08	13.5	2.5	6.8	0.8	-
6	-	-	-	-	-	-	-	-	-	-	-	-
8	3.5	5.0	7.5	7.5	5.0	3.0	0.08	13.5	2.5	6.8	0.8	7.4
11	3.75	5.0	7.5	7.5	5.0	3.0	0.08	12.5	1.0	6.8	0.8	-
21	3.5	5.0	7.5	7.5	5.0	3.0	0.08	13.5	2.5	6.8	0.8	7.5
23	3.5	5.0	7.5	7.5	5.0	3.0	0.08	13.5	2.5	6.8	0.8	-
25	3.5	5.0	7.5	7.5	5.0	3.0	0.08	13.5	2.5	6.8	0.8	-
26	3.5	5.0	7.5	7.5	5.0	3.0	0.08	13.5	2.5	6.8	0.8	7.0-7.4
28	3.75	5.0	7.5	7.5	5.0	3.0	0.08	13.5	1.0	6.8	0.8	-
30	3.5	5.0	7.5	7.5	5.0	3.0	0.08	13.5	2.5	6.8	0.8	7.4
31	3.5	5.0	7.5	7.5	5.0	3.0	0.08	13.5	2.5	6.8	0.8	7.8

Grey cell: deviating from ISO 6579 - = no information

A second plating-out medium for choice was obligatory. Fifteen laboratories used BGA modified (ISO 6579, 1993) as a second plating-out medium. Eight laboratories used Rambach, four laboratories used SM (ID) 2 or BGA agar and two laboratories used XLT4. The following media were used only by one laboratory: BPLS, Onoz, MLCB, BxLH, Rapid *Salmonella* agar, TSI, SS medium, *Salmonella* Brilliance agar and MacConkey.
The use of an extra plating agar between the 'isolation' and the 'confirmation' steps was optional. A total of 17 laboratories performed this extra culture step on many different media (e.g. Nutrient agar (ISO 6579, 2002), Colombia agar, Imuna zivny agar and Bromthymol blue lactose sucrose agar).

Twenty-one laboratories used both biochemical and serological tests for the confirmation of *Salmonella*. Three laboratories (labcode 2, 27 and 32) used a serological test(s) only and ten laboratories (labcodes 1, 3, 4, 9, 13, 22, 25, 26, 29 and 33) used a biochemical test(s) only for the confirmation of *Salmonella.*
The used confirmation media or tests are summarised in Tables 12 and 13.

Table 12 Biochemical confirmation of *Salmonella.*

Labcode	TSI	UA	LDC	Gal	VP	Indole	Kit	Other
1, 20	+	+	+	+	+	+	-	PCR
2	-	-	-	-	-	-	-	PCR
3	+	+	-	-	-	-	Oxoid Salmonella latex	Lysine Iron Agar
4	-	-	-	-	-	-	Oxoid Microbact 12A	
5	+	-	-	-	-	-	GN-ID Panel microgen bioproducts	
6,7, 14, 15	+	+	+	+	-	+	-	
8	-	-	-	-	-	+	HY Enterotest	
9, 22, 33, 34	+	+	+	+	+	+	-	
10	+	+	+	-	-	+	-	Mini VIDAS
11, 21	-	-	-	-	-	-	API 20E Bio Merieux	PCR
12	-	-	-	-	-	-	API 32E Bio Merieux (only by autoagglutinating strains)	
13, 18, 25, 29	+	+	+	-	-	-	-	
16	+	+	+	-	-	+	-	Glucose
17	-	-	-	-	-	-	-	BBL Crystal
18	+	+	+	-	-	-	API 20E, Enterotest 24	
23	+	-	-	-	-	-	API32E Bio merieux	
24	-	+	-	-	-	-	-	
26	+	-	-	-	-	-	API20E	
27, 32	-	-	-	-	-	-	-	
28	+	-	+	-	-	-	-	Sorbitol motility
30	+	+	+	-	+	+	BBL BD : Crystal E/NF	
31	-	+	-	-	-	+		Kigler agar, mannitol & nitrate broth ONPG & FDA medium Motility test

- = Not done/ not mentioned Explanations of the abbreviations are given in the 'List of abbreviations'

Table 13 Serological confirmation of *Salmonella.*

Labcode	Serological		
	O antigens	**Vi antigens**	**H Antigens**
1, 3, 4, 9, 13, 22, 25, 26, 29, 33	-	-	-
2, 7, 10, 11, 12, 15, 16, 18, 20, 21, 24, 31, 32	+	-	+
5	+	+	+
6, 8, 14, 17, 23, 27, 28, 30, 34	+	-	-

- = 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). Seventeen laboratories scored correct results for all the control capsules containing *Salmonella*. The results of all control samples (capsules without faeces) are given in Table 14. In this table the highest number of positive isolations found with MSRV in combination with any isolation medium is given per laboratory. Eleven laboratories used an additional selective enrichment medium (own method), see Table 6. The results found with these own methods were the same as found with the MSRV method.

Blank capsules (n=2) without addition of faeces
The blank capsules contained only sterile milk powder. For the analyses no faeces was added.
Thirty-three participating laboratories correctly analysed the blank capsules negative.
Laboratory 9 found one blank capsule positive on all media used by the laboratory. 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. The relevant laboratory is advised to check their procedures.

Salmonella Enteritidis 20 capsules (SE20) without addition of faeces (n=2)
Thirty laboratories isolated *Salmonella* Enteritidis at a mean level of approximately 20 cfp/ capsule from both capsules. Four laboratories could not detect *Salmonella* in one control capsule with any of the used media. These capsules contained SE at a low level (approx 20 cfp/capsule). Due to change, one out of two capsules containing SE20 may occasionally be negative.

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

Salmonella Panama 5 capsules (SPan5) without addition of faeces (n=2)
Thirty-three laboratories isolated *Salmonella* from both capsules. One laboratory could not detect *Salmonella* Panama (SPan5) in one control capsule on any of the media used by the laboratory. These capsules contained *S*. Panama at a low level (approximately 5 cfp/ capsule). Due to change one out of two capsules containing SPan5 may be negative.

Salmonella Typhimurium 5 capsules (STM5) without addition of faeces (n=3)
Thirty-three laboratories tested all three capsules containing STM5 positive. One laboratory could not detect *Salmonella* (STM5) in one control capsule on all isolation media inoculated from MSRV. These capsules contained STM at a low level (approximately 5 cfp/ capsule). Due to change, one out of two capsules containing STM5 may occasionally be negative

The results of all control samples were compared with the definition of ‘good performance’ (see section 3.6). The score for the control samples was below these criteria for one laboratory (labcode 9).

Table 14 Total number of positive results of the control samples (capsule without faeces) per laboratory.

Labcode	The highest number of positive isolations found with MSRV in combination with any isolation medium				
	Blank n=2	SE20 n=2	SE100 n=1	SPan5 n=2	STM5 n=3
Good Performance	**0**	**≥ 1**	**1**	**≥ 1**	**≥ 2**
1	0	2	1	**1**	3
2	0	2	1	2	3
3	0	2	1	2	3
4	0	2	1	2	3
5	0	2	1	2	3
6	0	2	1	2	3
7	0	**1**	1	2	3
8	0	2	1	2	3
9	**1**	2	1	2	3
10	0	**1**	1	2	3
11	0	2	1	2	3
12	0	2	1	2	3
13	0	2	1	2	3
14	0	2	1	2	3
15	0	2	1	2	3
16	0	**1**	1	2	3
17	0	2	1	2	3
18	0	2	1	2	3
19	0	2	1	2	3
20	0	2	1	2	3
21	0	**1**	1	2	3
22	0	2	1	2	3
23	0	2	1	2	**2**
24	0	2	1	2	3
25	0	2	1	2	3
26	0	2	1	2	3
27	0	2	1	2	3
28	0	2	1	2	3
29	0	2	1	2	3
30	0	2	1	2	3
31	0	2	1	2	3
32	0	2	1	2	3
33	0	2	1	2	3
34	0	2	1	2	3

Bold numbers: deviating results Grey cell: results are below criterion of good performance

4.4.2 Specificity, sensitivity and accuracy rates of the control samples

In Table 15 the specificity, sensitivity and accuracy rates for the control capsules without the addition of faeces are shown. 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). Only small differences (if any) were found between these groups.
The combination MSRV/XLD resulted in general 1 % higher rates than the combination MSRV/non-XLD. As expected the high level control (SE100) showed rates of 100 %. For the low level materials (SPan5, STM5 and SE20) the rates were higher than 94 %.

Table 15 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= 34	EU MS n=28	All n= 34	EU MS n=28
Blank	No. of samples	68	56	80	66
	No. of negative samples	67	55	79	65
	Specificity in %	**98.5**	**98.2**	**98.8**	**98.5**
SPan5	No. of samples	68	56	80	66
	No. of positive samples	67	55	78	64
	Sensitivity in %	**98.5**	**98.2**	**97.5**	**97.0**
STM5	No. of samples	102	84	120	99
	No. of positive samples	101	84	118	99
	Sensitivity in %	**99.0**	**100**	**98.3**	**100**
SE20	No. of samples	68	56	80	66
	No. of positive samples	64	52	75	61
	Sensitivity in %	**94.1**	**92.9**	**93.8**	**92.4**
SE100	No. of samples	34	28	41	33
	No. of positive samples	34	28	41	33
	Sensitivity in %	**100**	**100**	**100**	**100**
All capsules with *Salmonella*	No. of samples	272	224	320	264
	No. of positive samples	266	219	311	257
	Sensitivity in %	**97.8**	**97.8**	**97.2**	**97.4**
All capsules	No. of samples	340	280	400	330
	No. of correct samples	333	274	392	322
	Accuracy in %	**97.9**	**97.9**	**98.0**	**97.6**

*Six 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

The results of the *Salmonella* negative chicken faeces samples artificially contaminated with capsules are given in Table 16. The highest number of positive isolations found with MSRV in combination with any isolation medium is given per laboratory. Eleven laboratories used an additional selective enrichment medium (own method), see Table 6. The results found with these own methods were the same as found with the MSRV method, except for laboratory 3 who found less positive results after selective enrichment in RVS in combination with isolation on XLD and BGA.
In general less positive results were found for samples containing *S.* Enteritidis when compared to containing *S.* Typhimurium.

Blank capsules with negative chicken faeces (n=5)

Thirty-three laboratories correctly did not isolate *Salmonella* from the blank capsules with the addition of negative chicken faeces. Only laboratory 29 found one positive blank with the addition of negative chicken faeces for all media used by the laboratory.
All blanks should be tested negative. However, as no 100 % guaranty about the *Salmonella* negativity of the matrix can be given, 1 positive out of 5 blank samples (80 % negative) is still considered acceptable.

S. Enteritidis 20 capsules (SE20) with negative chicken faeces (n=5)

Twenty-seven laboratories were able to isolate *Salmonella* from all the five capsules containing *Salmonella* Enteritidis at a level of approximately 20 cfp/ capsule in combination with chicken faeces. Seven laboratories could not detect *Salmonella* in one or two capsules on all of the used media. These capsules contained SE at a low level (approximately 20 cfp/capsule). Due to change, one out or two capsules out of five containing SE20 may occasionally be negative.

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-one laboratories isolated *Salmonella* from all the five capsules containing *Salmonella* Typhimurium at a level of approximately 5 cfp/ capsule in combination with chicken faeces. Three laboratories found one capsules negative. These capsules contained STM at a low level (approximately 5 cfp/capsule). Due to change, one out of five capsules containing STM5 may be negative.

S. Typhimurium 50 capsules (STM50) with negative chicken faeces (n=5)

All except one laboratory isolated *Salmonella* from all five capsules containing *Salmonella* Typhimurium at a level of approximately 50 cfp/ capsule in combination with chicken faeces.

The results of all artificially contaminated chicken faeces samples were compared with the definition of ‘good performance’ (see section 3.6) and all laboratories fullfilled the criteria.

Table 16 Total number of positive results of the artificially contaminated chicken faeces samples per laboratory.

Labcode	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	5	5	5	5
2	0	**4**	5	5	5
3	0	5	5	5	5
4	0	5	5	5	5
5	0	**4**	5	5	5
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	5	5	5	5
11	0	5	5	5	5
12	0	5	5	5	5
13	0	5	5	5	5
14	0	**4**	5	5	5
15	0	5	5	5	5
16	0	**4**	5	**4**	5
17	0	**4**	5	5	5
18	0	5	5	5	5
19	0	5	5	5	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	5	5	5	5
27	0	5	5	5	5
28	0	5	5	5	5
29	**1**	5	5	**4**	**4**
30	0	5	5	5	5
31	0	5	5	**4**	5
32	0	5	5	5	5
33	0	**3**	5	5	5
34	0	5	5	5	5

Bold numbers: deviating results
Grey cell: results are below good performance

4.5.2 Results per medium, capsule and per laboratory

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

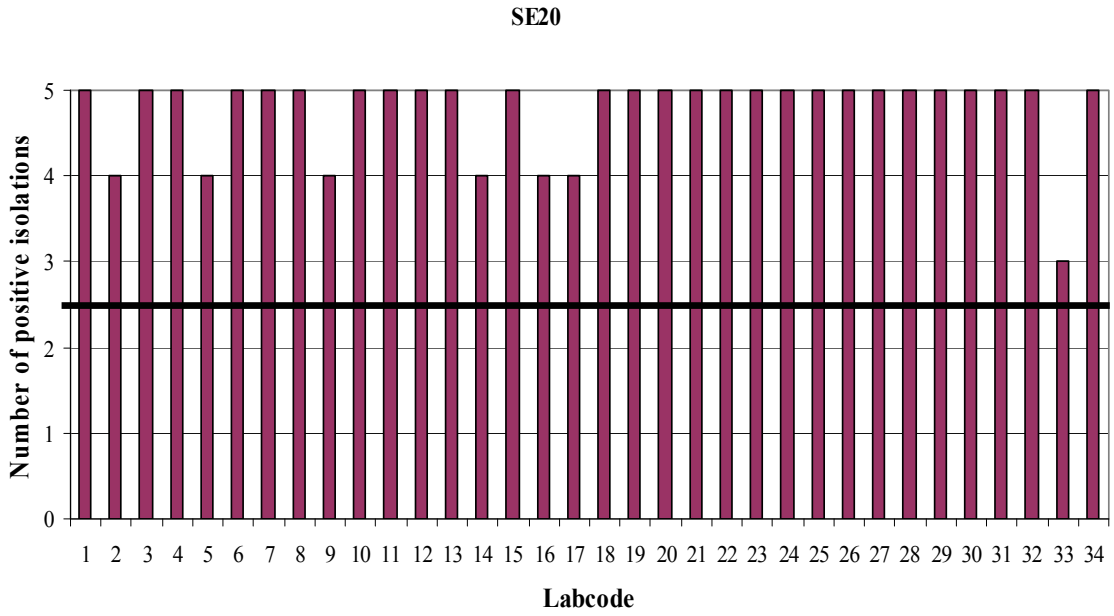
The results of all artificially contaminated chicken faeces samples were compared with the proposed definition of 'good performance' (see section 3.6). In the Figures 1-4 the border of good performance is indicated with a black horizontal line.

All laboratories except one found the same results with MSRV and their own methods (RVS, MKTTn et cetera) in combination with any isolation medium used by the NRL. Laboratory 3 found a lower number of positive results with the combination of selective enrichment in RVS and isolation on BGA or XLD. However, this laboratory scored all samples correctly with the combination MSRV/XLD, RVS/MLCB, MSRV and RVS in combination with *Salmonella* Brilliance.

The difference in the number of positive isolations after 24 and 48 hours of incubation of the selective enrichment MSRV was 2-3 % (see Table 17). The choice of plating out medium does not seem to have a large effect on the number of positive isolations, XLD gave only 2 % more positive results than other plating-out media. The majority of the laboratories used BGA as the second plating out medium (see Table 6).

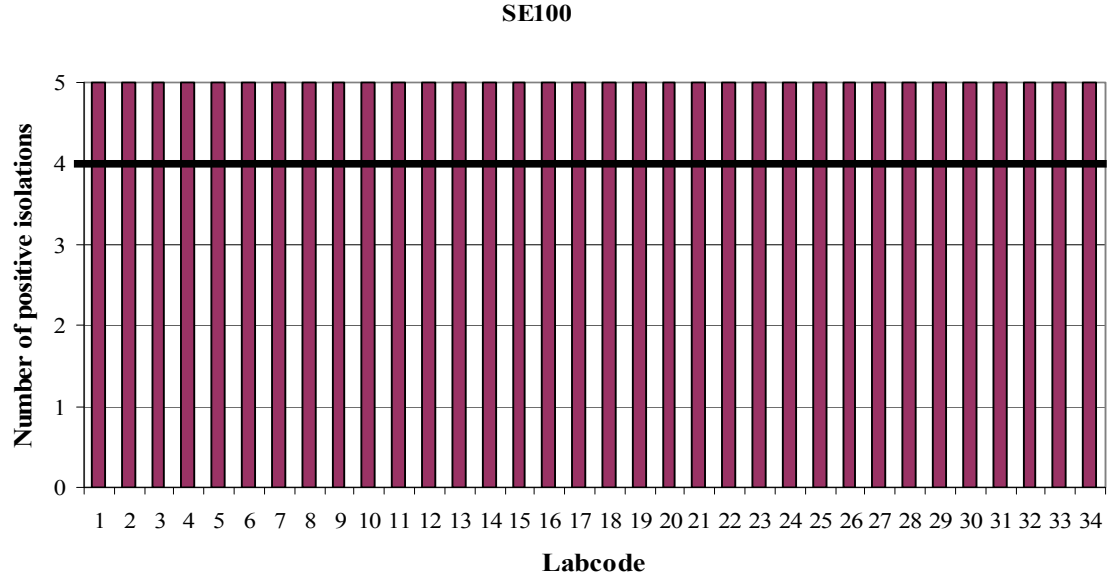
Table 17 Mean percentages of positive results of all participating laboratories after selective enrichment on MSRV, incubated for 24 and 48 hours and followed by isolation on different plating out media, when analyzing the artificially contaminated chicken faeces samples.

Plating out medium	**Selective enrichment medium MSRV**
	24 / 48 h
XLD	95 / 98 %
Other (most often BGA)	94 / 96 %



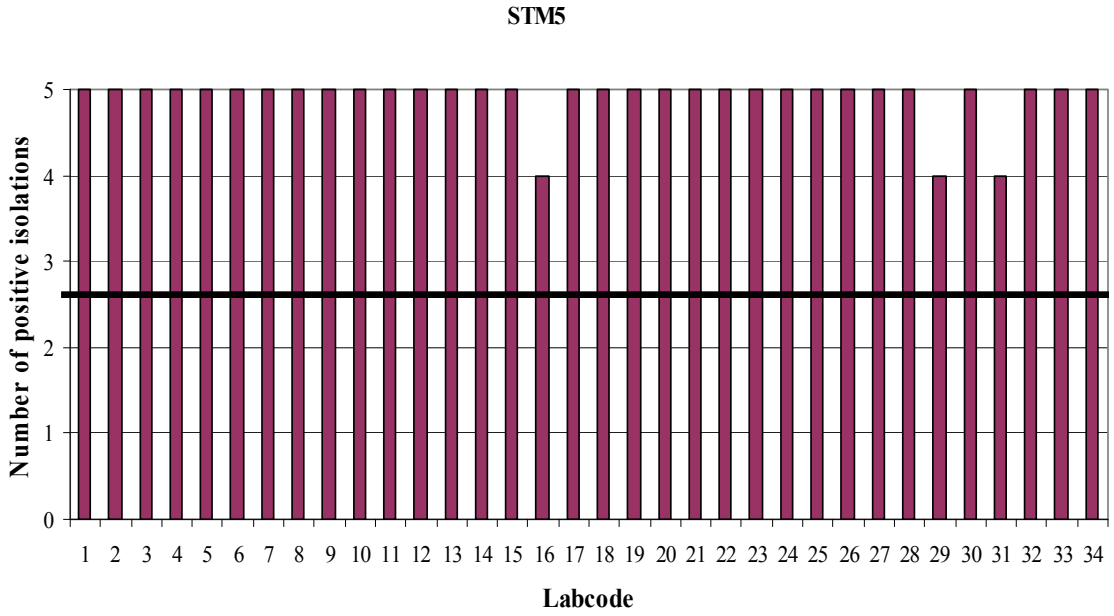
— = border of good performance

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



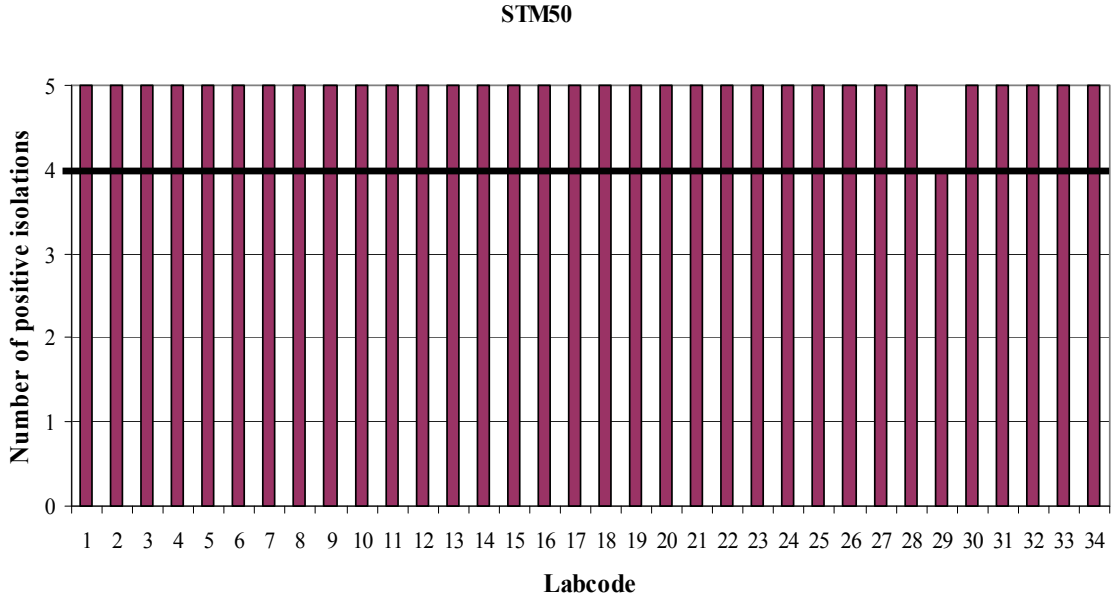
— = border of good performance

Figure 2 Results chicken faeces 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 chicken faeces 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 chicken faeces artificially contaminated with STM50 capsules (n=5) after selective enrichment on MSRV followed by isolation on selective plating agar XLD.

Table 18 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=34	EU MS n=28	All n=34	EU MS n=28
Blank (n=5)	No. of samples	170	140	200	165
	No. of negative samples	169	140	199	165
	Specificity in %	**99.4**	**100**	**99.5**	**100**
STM5 (n=5)	No. of samples	170	140	200	165
	No. of positive samples	167	138	197	163
	Sensitivity in %	**98.2**	**98.6**	**98.5**	**98.8**
STM50 (n=5)	No. of samples	170	140	200	165
	No. of positive samples	169	140	199	165
	Sensitivity in %	**99.4**	**100**	**99.5**	**100**
SE20 (n=5)	No. of samples	170	140	200	165
	No. of positive samples	162	132	190	155
	Sensitivity in %	**95.3**	**94.3**	**95.0**	**93.9**
SE100 (n=5)	No. of samples	170	140	200	165
	No. of positive samples	170	140	200	165
	Sensitivity in %	**100**	**100**	**100**	**100**
All capsules with *Salmonella*	No. of samples	680	560	800	660
	No. of positive samples	668	550	786	648
	Sensitivity in %	**98.2**	**98.2**	**98.3**	**98.2**
All capsules	No. of samples	850	700	1000	825
	No. of correct samples	837	690	985	813
	Accuracy in %	**98.5**	**98.6**	**98.5**	**98.6**

* Six 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

The specificity, sensitivity and accuracy rates for all types of capsules added to the chicken faeces are shown in Table 18. The results are given for the different medium combinations: BPW followed by selective enrichment on MSRV and isolation on selective plating agar XLD and on other selective isolation agar medium (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 100 % for EU-MS with MSRV. There was not much difference between the rates for MSRV/XLD and MSRV/non-XLD of the capsules containing *Salmonella.*
As expected the high level SE100 and STM50 showed rates of 100 % or very close to 100 %. For the low level materials STM5 and SE20 the rates were respectively higher than 98 % and 94 %.

4.6 PCR

Five laboratories (labcodes 1, 2, 11, 20 and 21) applied a PCR method as additional detection technique. In Table 19 the details are summarized.

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

Labcode	Volume of BPW (µl)	Volume of DNA sample (µl)	Volume of DNA / PCR mix (µl)
1	1500	50	5/-
2	-	300	5/40
11	10000	100	3/-
20	1000	150	5/50
21	1000	150	5/11

Four laboratories tested the samples after incubation in BPW. Laboratory 2 started the extraction after selective enrichment on MSRV.
Laboratory 1 used a not-commercially available real time PCR which has not been validated.
Laboratory 2 (Malorny et al., 2004) and 11 (Hein et al., 2006) used a not commercial available real time PCR technique used for the matrices: chicken rinse or meat, minced meat and raw milk.
Laboratory 20 used a commercial available real time PCR (Biorad iQ-Check *Salmonella* kit) which has been validated for food, feed and environmental matrices (AFNOR, 2004).
Laboratory 21 used an InvA-PCR which is not commercial available and normally is used for confirmation of bacterial cultures and not from pre-enrichment broths. InvA-based PCR method is originally described by Rahn et al., 1992.

The PCR results and the bacteriological culture results are shown in Table 20. Laboratories 11 and 20 found the same results with the PCR-technique as with the bacteriological culture method.
Laboratories 1 and 2 found one capsule more negative with the PCR technique. Laboratory 21 found four different results between the PCR technique and the culture method.

Table 20 PCR results compared to bacteriological culture (BAC) results of control capsules and of artificially contaminated chicken faeces samples.

Capsules	Lab 1		Lab 2		Lab 11		Lab 20		Lab 21	
	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR
Controls without faeces (n=10)										
SPan 5 (n=2)	**1**	**1**	2	2	2	2	2	2	2	2
SE20 (n=2)	**2**	**1**	2	2	2	2	2	2	**1**	**2**
SE100 (n=1)	1	1	1	1	1	1	1	1	1	1
STM5 (n=3)	3	3	3	3	3	3	3	3	3	3
Blank (n=2)	0	0	0	0	0	0	0	0	0	0
BPW (n=1)	0	0	0	0	0	0	0	0	0	0
Faeces (n=1)	0	0	0	0	0	0	0	0	0	0
Test samples with faeces (n=25)										
SE20 (n=5)	5	5	**4**	**4**	5	5	5	5	**5**	**4**
SE100 (n=5)	5	5	**5**	**4**	5	5	5	5	**5**	**3**
STM5 (n=5)	5	5	5	5	5	5	5	5	5	5
STM50 (n=5)	5	5	5	5	5	5	5	5	5	5
Blank (n=5)	0	0	0	0	0	0	0	0	0	0

Bold numbers: deviating result
Grey cells: different results found with BAC or PCR
BAC: Bacteriological results found with the prescribed selective enrichment medium MSRV (and isolation on XLD)

4.7 Performance of the NRLs

4.7.1 General

Thirty-three NRLs fulfilled the criteria of good performance. One laboratory scored below these criteria. Laboratory 9 found one blank control capsule (without faeces) positive on all media used by the laboratory.

The laboratory was contacted by the CRL-*Salmonella* in April 2009 to ask for any explanation for the deviating results.
Laboratory 9 checked their procedures and reviewed the information relevant for this trial but they were not able to find a reason for their false positive blank control. They performed confirmation of isolates by using all biochemical tests prescribed by the ISO method and they were quite sure that it has not been a problem of exchange of capsules. The most plausible explanation would be cross-contamination during the phase of samples preparation. However, the laboratory was not able to find out how cross contamination might have happened. Laboratory 9 participated in a follow up study organised by the CRL-*Salmonella* in June 2009.

4.7.2 Follow-up study

The lay-out of the follow-up study for laboratory 9 was the same as the full interlaboratory comparison study in March. However, the number of samples was different, though the batches of these capsules were the same (see section 4.1 'Reference materials'). An overview on the type and number of samples for this follow-up study is given in Table 21. A new batch of chicken faeces was sampled by the Animal Health Service (GD) Deventer and arrived at the CRL-*Salmonella* on 10 June 2009 (see section 3 'Chicken faeces samples').

Table 21 Overview of the types and the number of capsules tested by laboratory 9 in the follow-up of the interlaboratory comparison study.

Capsules	Control capsules no faeces added	Test samples with 10 g *Salmonella* negative chicken faeces
S. Enteritidis 20 (SE20)	3	4
S. Enteritidis 100 (SE100)	---	1
S. Typhimurium 5 (STM5)	---	4
S. Typhimurium 50 (STM50)	1	---
Blank	3	6
Total number of capsules	n = 7	n = 15

The laboratory had to follow the same Protocol and SOP as in the study of March 2009 (see Annexes 4 and 5). The test report was different from the March study (see Annex 6). For the media only the differences with the March study needed to be indicated.

On Monday 15 June 2009 a parcel was send to laboratory 9 containing:
7 control capsules (C1 – C7), 15 capsules (1 – 15), 200 g chicken faeces and 1 temperature recorder.
On 10 June 2009, the number of aerobic bacteria ($3.8*10^9$ cfu/g) and Enterobacteriaceae ($4.3*10^7$ cfu/g) in the faeces were tested at the CRL-*Salmonella*.
During the follow up study laboratory 9 did not mention differences in the media they used. In the main study they confirmed the *Salmonella* suspected colonies only with biochemical tests. While in the follow up study they performed both biochemical and serological confirmation of *Salmonella* suspected isolates.
Laboratory 9 scored all the seven control samples and fourteen of the fifteen test samples correct. They could not detect *Salmonella* in one out of the 4 SE20 capsules with the addition of faeces. With this result, laboratory 9 fulfilled the criteria of good performance (see section 3.6) for the test samples in this follow-up study.

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 (labcodes 8 and 34) and highest transport temperature (labcodes 5 and 13) still found good results. In some laboratories a delay was noticed between the delivery time of the courier and the receipt time by the laboratory. It is not always clear whether this delay concerns the parcel including the contents or only the temperature recorder (labcodes 13 and 28).

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 in RVS in combination with isolation on BGA and XLD but they scored all samples correctly with the combinations MSRV/XLD, RVS/MLCB, MSRV and RVS in combination with Brilliance *Salmonella* Agar (BSA). This laboratory is a non-EU MS and participated for the first time. They do not use the prescribed method (MSRV) as a routine method and they had problems with the ordering of medium. Therefore they used expired batch of MSRV medium. Still they found good results with the combination MSRV/XLD and MSRV/BSA. The laboratory used all the isolation media mentioned in Table 6 only in combination with their own selective enrichment medium RVS. They used the ringtrial as an opportunity to compare all the media. After the ringtrial they added the MSRV to their routine procedure. Furthermore they changed some other details in their routine like pre-warming of the BPW.

For determining 'good performance' per laboratory the best performing isolation medium after selective enrichment on MSRV was taken into account. Thirty-three out of thirty-four laboratories scored 'good performance'. One laboratory scored under the level of 'good performance'. Laboratory 9 found one blank control capsule (without faeces) positive on all media used by the laboratory. They could not give an explanation for this underperformance. The laboratory showed good results in a follow-up study organised in June 2009.

Some laboratories could not detect *Salmonella* in one of the two (SPan5 and SE20) or one of the three (STM5) low level control capsules on all the used media. These capsules contained a low level *Salmonella* (approximately 5-20 cfp/ capsule). Due to the variation between capsules one may occasionally be negative.

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.
Almost no differences between the rates for MSRV/XLD and MSRV/non-XLD of the different capsules containing *Salmonella* were found.
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 (SPan5, STM5 and SE20) the sensitivity rates were higher than 93 % while at least 75-80 % was expected.

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 94 and 99 %.

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. Seven laboratories reported the use of a higher concentration of novobiocin and three laboratories did not use or did not mention the use of novobiocin. Eight laboratories reported a higher pH or did not mention the pH. A higher concentration of novobiocin in the MSRV can negatively influence the motility of *Salmonella* and may result in less positive results. A higher pH of MSRV may stimulate the growth of disturbing background flora which can negatively influence the growth of *Salmonella*.
According to Annex D of ISO 6579 (Anonymous, 2007) the incubation temperature of MSRV should be between 40.5 and 42.5 ^{o}C. One laboratory (labcode 4) mentioned a somewhat lower incubation temperature. This lower incubation temperature may stimulate the growth of disturbing background flora, and especially low numbers of *Salmonella* can than easily become overgrown.
Deviations in media compositions or incubation temperatures were reported but no clear effects were found on the results.

PCR
Five laboratories used a PCR technique additional to the prescribed method and four of them found the same or only one deviation compared with the bacteriological detection methods. One laboratory found more deviations. 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 contamination levels of the capsules used in this veterinary study were comparable with the last veterinary, food and feed studies (Kuijpers et al., 2008a, 2008b and 2009). The contamination level of the low level *Salmonella* Enteritidis reference material used during this study was slightly higher (15-20 cfp/capsule) than of the materials used in the former veterinary and food studies (approximately 5-10 cfp/capsule). This higher level was chosen as during the former studies it was shown that the low level *S*. Enteritides materials were just at or below the detection limit of the method, resulting in many negative results. Therefore it was decided to use in the follow up study of the veterinary ringtrial of 2008 a batch of SE20 capsules, which gave good results. Good results with the SE20 capsules were also observed in the feed study of 2008.
From this study it can be concluded that the level of approximately 15-20 cfp *Salmonella* Enteritidis per capsule (SE20) is suitable for testing whether an NRL is able to detect a low level of *Salmonella* in a veterinary matrix.

6 Conclusion

- All thirty-four NRLs for *Salmonella* were able to detect high and low levels of *Salmonella* in chicken faeces. Of these, thirty-three laboratories achieved the level of 'good performance' at once. One laboratory achieved the level of 'good performance' after a follow up study.

- The accuracy, specificity and sensitivity rates for the control samples (without faeces) of MSRV were higher than 94 %

- 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 higher than 94 % 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 higher than 86 % for MSRV.

- The difference between the results of the prescribed method (MSRV) and an own method(s) were nil.

- The level of approximately 15-20 cfp *Salmonella* Enteritidis per capsule (SE20) is suitable for testing whether an NRL is able to detect a low level of *Salmonella* in a veterinary matrix.

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

Table A.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	STM5	6	No	RV and SC	BGA and own	Voogt et al., 1996 (report 284500003)
		4	Blank	0	No			
II	1996	15	STM100	116	1 gram	RV, SC and own	BGA and own	Voogt et al., 1997 (report 284500007)
		15	STM1000	930	1 gram			
		2	SPan5	5	No			
		1	STM100	116	No			
		1	Blank	0	No			
III	1998	14	STM10	11	1 gram	RV and own	BGA and own	Raes et al., 1998 (report 284500011)
		14	STM100	94	1 gram			
		7	STM100	94	1 gram*			
		14	SE100	95	1 gram			
		4	STM10	11	No			
		2	SPan5	5	No			
		5	Blank	0	No			
IV	1999	5	STM10	4	10 gram	RV or RVS, MSRV and own	BGA and own	Raes et al., 2000 (report 284500014)
		5	STM100	210	10 gram			
		5	SE100	60	10 gram			
		5	SE500	220	10 gram			
		5	Blank	0	10 gram			
		3	STM10	5	No			
		3	SE100	60	No			
		2	SPan5	5	No			
		2	Blank	0	No			
V	2000	5	STM10	4	10 gram	RV or RVS, MSRV and own	BGA and XLD	Raes et al., 2001 (report 284500018)
		5	STM100	47	10 gram			
		5	SE100	63	10 gram			
		5	SE500	450	10 gram			
		5	Blank	0	10 gram			
		3	STM10	4	No			
		3	SE100	63	No			
		2	SPan5	5	No			
		2	Blank	0	No			
		20	None	-	25 gram**			

Table A.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 A.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	This report
		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			

[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 A.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			

Table A.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 0.1/100mL, Iodine 4, Potassium iodide 5, Novobiocine 0, pH ?

RVS (Oxoid CM 0866, Hampshire, United Kingdom) (AES Laboratoire AEB140862, France) (Himedia Laboratories M1491, Mumbai, India) (Biokar BK 148HA, Beauvais, France)
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/Hampshire, United Kingdom) (BPLS Merck 1.10747 Darmstadt, Germany) (Biomark B439) (Lab M, lab 34 Bury, United Kingdom) (HImedai Laboratories M971, Mumbai, India) (Hy Laboratories Ltd. PD083, Rehovot, Israel) (BLSF 50534 Staten Serum Institute)
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: according ISO 6579, 1993

BGA (Conda laboratories, 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 6.9

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 (SIFIN TN 1111, Berlin, Germany) (Biokar BK07HA, Beauvais, France)
Brillant Green Phenol Red Agar acc. to EP/USP
Composition of BGA medium: the concentration of the compounds in g/L water: Peptones (meat and casein) 10, Yeast extract 3, Lactose 10, Sucrose 10, Sodium chloride 5, Phenol red 0.08, Brilliant green 0.0125, Agar 12, pH 6.7-7.1

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 (previously 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 25 pH 7.2

BxLH
Home made 12 ingredients, the medium is patented, pH 7.2

MacConkey (Oxoid CM0115, Hampshire, United Kingdom)
http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0115&org=124&c=UK&lang=EN (21-08-2009)
Composition of MacConkey Agar No. 3 : the concentration of the compounds in g/L water: Peptone 20, Lactose 10, Bile Salts No3. 1.5, Sodium Chloride 5, Neutral red 0.03, Crystal violet 0.0001, Agar 15 pH 7.1

MLCB (Oxoid CM0783, Hampshire, United Kingdom)
Inoue T, Takagi S, Ohnishi A, et al. Foodborne disease *salmonella* isolation medium (MLCB). Japanese Journal of Veterinary Science 1968;30 (suppl):26.
Composition of MLCB medium: the concentration of the compounds in g/L water: Yeast Extract 5, Lab-Lemco powder 2, Peptones 10, Sodium Chloride 4, Mannitol 3, L-Lysine HCL 5, Sodium Thiosulphate 4, Ferric Ammonium Citrate Green 1, Brilliant Green 0.0125, Crystal Violet 0.009, Agar 15, pH 6.9-7.0

Onöz (Merck 115034, Darmstadt, Germany)
Onoz E, Hoffmann K. 1978 [Experience with a new culture medium for *salmonella* 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 3.0, Meat extract 6.0, Pepton from meat 6.8, Lactose 11.5, Sucrose 13.0, Bile salt mixture 3.825, tri-Sodium nitrate 5,5-Hydrate 9.3, Sodium Thiosulfate 5-Hydrate 4.25, L-Phenylalanine 5.0, Iron(III) Citrate 0.5,Magnesiumsulfate 0.4, Brilliant Green 0.00166, Neutral Red 0.022, Aniline Blue 0.25, Metachrome Yellow 0.47, di-Sodium Hydrogen Phosphate2-Hydrate 1.0, Agar-Agar 15, pH 7.1-7.2

Rambach (Merck 107500.0001/2/3, 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, Cromogenic 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) http://www3.bio-rad.com/B2B/BioRad/product/br_category.jsp?BV_SessionID=@@@@1946217959.1250839688@@@@&BV_EngineID=ccchadeidlglfldcfngcfkmdhkkdfll.0&categoryPath=%2fCatalogs%2fFood+%7c+Animal+%7c+Environment+Testing%2fFood+Testing%2fChromogenic+Media+%2fRAPID+Salmonella+Medium+&divName=Food+%7c+Animal+%7c+Environment+Testing&loggedIn=false&lang=English&country=NL&catLevel=5&catOID=-40019&isPA=false&serviceLevel=Lit+Request&searchStr=3563961&cateName=Ordering+Information (21-08-2009)
Casein Peptone 5, Meat extract 5, Selective agents 14, Chromogenic mixture 310 mg, Agar 12, pH 7.2

Salmonella Shigella SS medium (Oxoid CM0099, Hampshire, United Kingdom)
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: Lab-Lemco Powder 5, Peprtone 5, Lactose 10, Bile salts 8.5, Sodium citrate 10, Sodium thiosulphate 8.5, Ferric citrate 1, Brilliant green 0.00033, Neutral red 0.025, Agar 15, pH 7

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, Cromogenic mix 9.6, Sodium chloride 5, Selective mix 0.03, Agar 14 pH 6.7- 7.3

TSI (bioMérieux D1037, Marcy l' Etoile, France)
ISO 6579, 2002
Composition of TSI medium: the concentration of the compounds in g/L water: Casein and meat peptone 20, Lactose 10, Glucose 1, Sucrose 10, Sodium chloride 5, Ferric ammonium citrate o.2, Sodium thiosulfate 0.2, Phenol red 0.025, Agar 15

XLT4 (Oxoid PO5116A, Hampshire, United Kingdom) lab 11 (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 4.6 ml, Phenol Red 0.08, Agar 18 pH 7.4

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Annex 4 Protocol

INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN CHICKEN FAECES
organised by CRL-*Salmonella*
STUDY XII - 2009

Introduction

This protocol describes the procedures for the 12th 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* Typhimurium (STM), *Salmonella* Enteritidis (SE) or *Salmonella* Panama (SPan) at different contamination levels. Each laboratory will examine 25 faeces samples (10 g each) in combination with a capsule containing STM or SE and 10 control samples (capsules only).

The samples will be packed in 2 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 preprinted address label of the CRL-*Salmonella* has been included.

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 5th of March 2009 (this is 4 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 2009) one box containing 2 plastic containers, packed with cooling elements. The containers contain:

Container 1:

- 25 numbered vials; each containing one *Salmonella* Typhimurium, one *Salmonella* Enteritidis or a blank capsule (numbered 1-25);
- 10 control vials; each containing one capsule with or without *Salmonella* (numbered C1-C10).

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:

- 300 g of chicken faeces (free from *Salmonella*).

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

The performance of the study will be in week 12 (starting on 16 March 2009).
The documents necessary for performing the study are:

- Protocol Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in animal faeces XII (2009) (this document);
- SOP Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in animal faeces XII (2009);
- Test report Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in animal faeces XII (2009);
- 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 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 **3 April 2009.** 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 summary report.

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

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RIVM / LZO (internal Pb 63)
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Time table of interlaboratory comparison study
ANIMAL FAECES XII (2009)

Week	Date	Topic
7	9 - 13 February	Mailing of the protocol, standard operating procedure and test report to the NRLs-*Salmonella*
10	2 - 6 March	Mailing of the parcels to the NRLs as biological substance category B (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 5 March, do contact the CRL immediately.**
11	9 - 13 March	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)
12	16 - 20 March	Performance of the study, following the instructions as given in the protocol and the SOP of study Animal faeces XII (2009).
14	Before 3 April	Completion of the test report. Send the test report, preferably by e-mail to the CRL *Salmonella* (Angelina.Kuijpers@rivm.nl)* .
15	6 -10 April	Data input at CRL-*Salmonella* and sending these data to NRLs. Checking these results by the National Reference Laboratories.
	May - June 2009	Sending of the final results to the NRLs together with a short report. As a follow-up, actions will be undertaken for those NRLs which scored below the average results of all NRLs.

* 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*).

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Annex 5 Standard Operating Procedure

(SOP)

INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN CHICKEN FAECES
organised by CRL-*Salmonella*
STUDY XII - 2009

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	XLD and a second medium for choice (obligatory!)

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. Control the quality of the media before use.

Beside 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. Note 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 for choice (obligatory)
- Own medium (optionally)

6.4 Confirmation media

- Biochemical confirmation as described in ISO 6579 Annex B.6-B.11 or by reliable, commercial 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 twelfth 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. Beside 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 25 jars, each containing 90 ml of BPW, from 1 to 25. Also label 12 jars of BPW from C1 to C12 (control capsules). One jar is a procedure control (= C11) to which no capsule or faeces is added and one jar is a negative faeces control to which only 10 g faeces is added (= C12). These control jars should further be handled in the same way as the other jars. 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 & 3) the requested data of 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 to jars labelled 1-25 and C12,**
- **Add no faeces to jars labelled C1 - C11,**

Do not shake the jars after adding the faeces.

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 & 21 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 of the MSRV and own selective enrichment media (if used) in the test report. Label 25 MSRV plates from 1 to 25. Also label 12 MSRV plates from C1 to C12. Incubate the MSRV plates for 24 h and later on for another 24 h. If own selective enrichment media are used, label them in the same way as described for MSRV.

After equilibration:
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).

8.4 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 25 (standard size) Petri dishes of each isolation medium from 1 to 25 and label 12 (standard size) Petri dishes from C1 to C12.

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 & 9).

2) Second isolation medium. Follow the instructions of the manufacturer (record temperature and time and other requested data in test report, page 10 & 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 &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 5 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 17-20).
If a PCR method has been used, report the results in Table 3 of the test report (page 21).

Confirmation of identity
The identity from 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.

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 shall also be recorded. The test report shall include the name of the person in charge for the NRL, and the names of the persons who are carrying out the work and has to be signed by these persons. 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 XII (2009) 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 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
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 the second isolation media (day 4).

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Annex 6 Test report follow up study

INTERLABORATORY COMPARISON STUDY ON THE
DETECTION OF *SALMONELLA* spp. IN CHICKEN FAECES
organised by CRL-*Salmonella*
STUDY XII- FOLLOW UP
June 2009

Laboratory code This is the same code as in FAECES XII March 2009	
Laboratory name (NRL)	
Address	
Country	
Date of arrival of the parcels	Date: …… - ……. – 2009 time: ……... h ……..…… min
Start time of storage at - 20ºC (capsules) Start time of storage at +5ºC (faeces)	Date: …… - ……. – 2009 time: ……... h ……..…… min Date: …… - ……. – 2009 time: ……... h ……..…… min
Parcels damaged?	☐ Yes ☐ No
Starting date testing	…… - ……. – 2009

PRE-ENRICHMENT – Buffered Peptone Water (BPW)	
Medium information BPW	
Was the composition of BPW the same as used in Faeces XII March 2009 ?	
☐ Yes	
☐ No, please give more details in an annex :	
Preparation of BPW	
Date of preparation	…… - ……. – 2009
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control of BPW?	☐yes ☐no
Prewarming time and temperature of the BPW (overnight)	
Start at	Date: …… - ……. – 2009 time: ……... h ……..…… min temperature incubator: ……..…… °C
End at	Date: …… - ……. - 2009 time: …… h …… min temperature incubator: ……..…… °C
Incubation time and temperature for dissolving the capsules (45 min)	
Start at	Date: …… - ……. - 2009 time: ……... h ……..…… min temperature incubator: ……..…… °C
End at	time: …… h …… min temperature incubator: ……..…… °C

Incubation time and temperature for pre-enrichment (18 ± 2) hrs after adding the faeces	
Start at	Date: …… - ……. - 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C
End at	Date: …… - ……. - 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C

SELECTIVE ENRICHMENT - (MSRV)	**Modified Semi solid Rappaport Vassiliadis medium**
Medium information MSRV	
Was the composition of MSRV the same as used in BRO Faeces XII March 2009 ?	
☐ Yes	
☐ No, please give more details in an annex :	
Specific data of composition of MSRV medium. What is the concentration of novobiocin in 1000 ml water:	
Novobiocin	☐ 0.01 g/L ☐ 0.02 g/L ☐ Other : …g/L
Preparation of MSRV	
Date of preparation	…… - ……. - 2009
pH after preparation	……., measured at ……. °C
pH at the day of use	……., measured at ……. °C
Did you perform quality control of MSRV?	☐yes ☐no
Incubation time and temperature for selective enrichment	
Start of the first period (first 24 h)	Date: …… - ……. - 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C
End of the first period (first 24 h)	Date: …… - ……. - 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C
Start of the second period (48 h)	Date: …… - ……. - 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C
End of the second period (48 h)	Date: …… - ……. - 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C

OWN SELECTIVE ENRICHMENT - Own Selective enrichment medium, routinely used in your laboratory (optional)
Name of medium :
Was the composition of the Own selective the same as used in BRO FAECES XII March 2009 ?
☐ Yes ☐ No Please give more details in an annex :

FIRST AND SECOND ISOLATION - Xylose Lysine Desoxycholate medium (XLD)		
Medium information XLD		
Was the composition of XLD the same as used in BRO FAECES XII March 2009 ?		
☐ Yes		
☐ No please give more details in an annex :		
Size of petri dishes		
Size of petri dishes used	☐90 mm ☐100 mm ☐140 mm	
Preparation of XLD		
Date of preparation	…… - ……. - 2009	
pH after preparation	……., measured at ……. °C	
pH at the day of use	……., measured at ……. °C	
Did you perform quality control of XLD ?	☐yes ☐no	
Incubation time and temperature for isolation		
Start incubation of XLD, inoculated from 24 h MSRV	Date: …… - ……. - 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C	
End incubation of XLD, inoculated from 24 h MSRV	Date: …… - ……. - 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C	
Start incubation of XLD,	Date: …… - ……. - 2009	

inoculated from 48 h MSRV	time: …….. h ……..…… min temperature incubator: ……..…… °C
End incubation of XLD, inoculated from 48 h MSRV	Date: …… - ……. - 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C

FIRST AND SECOND ISOLATION – Second Isolation medium.		
Medium information second isolation medium :		
Name of second isolation medium :	…….	
Was the composition of the second medium the same as used in BRO FAECES XII March 2009 ?		
☐ Yes		
☐ No please give more details in an annex :		
Size of petri dishes		
Size of petri dishes used	□90 mm	□100 mm □140 mm
Preparation of the second isolation medium		
Date of preparation	…… - ……. - 2009	
pH after preparation	……., measured at …… °C	
pH at the day of use	……., measured at …… °C	
Did you perform quality control ?	□yes □no	
Incubation time and temperature for isolation		
Start incubation of second medium, inoculated from 24 h MSRV	Date: …… - ……. - 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C	
End incubation of second medium, inoculated from 24 h MSRV	Date: …… - ……. - 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C	
Start incubation of second medium, inoculated from 48 h MSRV	Date: …… - ……. - 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C	
End incubation of second medium, inoculated from 48 h MSRV	Date: …… - ……. - 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C	

FIRST AND SECOND ISOLATION – Own Isolation medium routinely used in your lab. (optional)
Name of medium :
Was the composition of the Own isolation medium the same as used in BRO FAECES XII March 2009? ☐ Yes ☐ No Please give more details in an annex :
CONFIRMATION – Nutrient agar
Did you streak the colonies on Nutrient agar before starting confirmation?
□Yes If yes give further information on nutrient agar below □No
Medium Nutrient agar
Name of Nutrient agar :
Was the composition of Nutrient agar the same as used in BRO FAECES XII March 2009 ?
☐ Yes
☐ No please give more details in an annex :
Preparation of the nutrient agar

Date of preparation	…… - ……. – 2009
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control of agar ?	☐ Yes ☐ No

CONFIRMATION of *Salmonella* suspected colonies

What media/test did you use for confirmation ?

☐ Biochemical: ☐ Triple sugar/iron agar (TSI)
☐ Urea Agar (UA)
☐ L-Lysine decarboxylation medium (LDC)
☐ Galactosidase
☐ Voges-Proskauer (VP)
☐ Indole
☐ Identification kit name of the kit : ……..
☐ Other : …….

☐ Serotyping: ☐ O antigen ☐ H antigen ☐ Vi antigen
☐ Other : ……..

☐ Other confirmation test : …….

DETECTION BY PCR

General questions

Did you use PCR ?	☐ Yes ☐ No
If yes and when different from PCR-technique used during faeces BRO March 2009, please give more information in an annex .	

Table 1: Results of isolation using **MSRV** (dish numbers 1-15 and C1-C7,C11 and C12)

sample no.	MSRV 24 hours						MSRV 48 hours					
	XLD		Second isolation medium		Own isolation		XLD		Second isolation medium		Own isolation	
	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												
13												
14												
15												
C1												
C2												
C3												
C4												
C5												
C6												
C7												
C11												
C12												

Col [a] = **number** of colonies used for confirmation
Sal [b] = **number** of colonies confirmed as *Salmonella*

Table 2: Results of isolation using **OWN** selective enrichment (dish numbers 1-15, C1-C7, C11 and C12)

sample no.	Own * 24 hours						Own * 48 hours					
	XLD		*		*		XLD		*		*	
	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												
13												
14												
15												
C1												
C2												
C3												
C4												
C5												
C6												
C7												
C11												
C12												

Col [a] = **number** of colonies used for confirmation
Sal [b] = **number** of colonies confirmed as *Salmonella*
* = fill in the name of the medium used

Table 3: Results of detection using PCR (dish numbers 1-23 & C1-C8, C11 and C12)

Sample no.	PCR + or -		
		no.	
1		C1	
2		C2	
3		C3	
4		C4	
5		C5	
6		C6	
7		C7	
8			
9		C11	
10		C12	
11			

12	
13	
14	
15	

Comment(s) on operational details that might have influenced the test results:

Name of person (s) carrying out the follow up Faeces XII interlaboratory Comparison study (2009).	
Is the person(s) carrying out the follow up Faeces XII interlaboratory Comparison study (2009) working in the laboratory of NRL mentioned on page 1 ?	□YES □NO give more information of the laboratory carrying out the study : Laboratory name ……. Address …… Is this laboratory accredited for the determination of *Salmonella*. □YES □NO
Date and signature	

Name of person in charge of the NRL	
Date and signature	

Please send the completed test report **before 6 July**, preferably by email, to CRL-*Salmonella*. If the test report is e-mailed to the CRL, it is not longer necessary to send the original test report as well, unless it is not legible (to be indicated by CRL-*Salmonella*).
Use the address below:

Angelina Kuijpers
CRL *Salmonella* (Pb 63)
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