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EU Interlaboratory comparison study food III (2009)

Bacteriological detection of *Salmonella* in minced chicken meat

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This investigation has been performed by order and for the account of European Commission, Health and Consumer Protection Directorate-General and the Laboratory for Zoonoses and the 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 food III (2009)
Bacteriological detection of *Salmonella* in minced chicken meat

Of the 32 National Reference Laboratories (NRLs) in the European Union, which participated in a comparison study in 2009, 31 were able to detect both high and low levels of *Salmonella* in minced chicken meat. They achieved the desired outcome on the first attempt. During the follow-up study, the CRL *Salmonella* staff visited the NRL that had underperformed, with the aim of providing expert advice. This NRL obtained the desired outcome in the follow-up study. Cross-contamination of samples is the most likely explanation for the initially deviating results.

These are the results of the third interlaboratory comparison study on food, organised by the Community Reference Laboratory (CRL) for *Salmonella*. The comparison study was conducted in October 2009, with the follow-up study in January 2010. The NRLs responsible for *Salmonella* detection from all European Member States were obliged to participate in this study. The CRL for *Salmonella* is part of the Dutch National Institute for Public Health and the Environment (RIVM).

Three different analytical methods for demonstrating the presence of *Salmonella* in chicken meat were used during the study. Two of these are international standardised methods for the detection of *Salmonella* in food, and the third is an internationally prescribed method for the detection of *Salmonella* in veterinary samples. The application of this latter method in the study was not obligatory but requested by the CRL. Using the two methods for testing food, 96 percent of the samples were found to be positive for *Salmonella*. The best results were obtained using the method for veterinary samples, with *Salmonella* detected in 98 percent of the samples.

To perform the study, the laboratories had to follow the instructions given. Each laboratory received a package containing minced chicken meat and 35 gelatin capsules containing powdered milk infected with different levels of *Salmonella* spp. The laboratories were instructed to spike the minced chicken meat with the capsules and then test the samples for the presence of *Salmonella*.

Key words: *Salmonella*; CRL; NRL; interlaboratory comparison study; minced chicken meat; *Salmonella* detection methods

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Rapport in het kort

EU Ringonderzoek voedsel III (2009)
Bacteriologische detectie van *Salmonella* in kippengehakt

In 2009 waren 31 van de 32 Nationale Referentie Laboratoria (NRLs) in de Europese Unie in staat om hoge en lage concentraties van de *Salmonella* bacterie in kippengehakt aan te tonen. Zij behaalden direct het gewenste niveau. Een laboratorium werd tijdens de herkansing bezocht door medewerkers van het CRL *Salmonella*. Met behulp van tips werd uiteindelijk het gewenste resultaat behaald. De oorzaak van hun afwijkend resultaat was waarschijnlijk kruisbesmetting.

Dit blijkt uit het derde voedselringonderzoek dat het Communautair Referentie Laboratorium (CRL) voor *Salmonella* heeft georganiseerd. Het onderzoek is in oktober 2009 gehouden, de herkansing was in januari 2010. Alle NRL's van de Europese lidstaten die ervoor verantwoordelijk zijn *Salmonella* te detecteren, zijn verplicht om aan dit onderzoek deel te nemen. Het CRL-*Salmonella* is gevestigd bij het Nederlandse Rijksinstituut voor Volksgezondheid en Milieu (RIVM).

Tijdens de studie zijn drie analysemethodes gebruikt om de *Salmonella*bacterie in kippengehakt aan te tonen. Twee daarvan zijn internationaal gestandaardiseerde methoden voor *Salmonella* detectie in voedsel. Deze twee methodes toonde in 96 procent van de monsters *Salmonella* aan. De derde, de internationaal voorgeschreven methode om *Salmonella* in dierlijke mest aan te tonen, is niet verplicht maar is op verzoek van het CRL uitgevoerd. Deze methode behaalde het beste resultaat: in 98 procent van de monsters werd *Salmonella* gedetecteerd.

De laboratoria moeten de studie volgens voorschrift uitvoeren. Elk laboratorium kreeg daarvoor een pakket toegestuurd met kippengehakt en 35 gelatinecapsules met melkpoeder dat verschillende besmettingsniveaus *Salmonella* bevatte. De laboratoria moesten vervolgens het kippengehakt en de capsules samenvoegen en onderzoeken of er *Salmonella* in aanwezig was.

Trefwoorden: *Salmonella*; CRL; NRL; ringonderzoek; kippengehakt; *Salmonella* detectiemethode

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Contents

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List of abbreviations

BGA (mod)	Brilliant Green Agar (modified)
BPLSA	Brilliant Green Phenol-Red Lactose Sucrose Agar
BPW	Buffered Peptone Water
BSA	Brilliance *Salmonella* Agar
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
MS	Member State
MSRV	Modified Semi-solid Rappaport Vassiliadis
NRL	National Reference Laboratory
OR	Odds Ratio
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
RIVM	Rijksinstituut voor Volksgezondheid en het Milieu (Dutch National Institute for Public Health and the Environment)
RM	Reference Material
RV(S)	Rappaport Vassiliadis (Soya) broth
SE	*Salmonella* Enteritidis
SM2	*Salmonella* Detection and Identification-2
SOP	Standard Operating Procedure
SPan	*Salmonella* Panama
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 October 2009, the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*) organised the third interlaboratory comparison study on bacteriological detection of *Salmonella* in a food matrix (minced chicken meat). Participants were thirty-two National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*) of the EU-Member States, candidate country Former Yugoslav Republic of Macedonia (FYROM) and countries from the European Free Trade Association (EFTA): Norway, Switzerland and Iceland.

The first and most important objective of the study was to see whether the participating laboratories could detect *Salmonella* at different contamination levels in a food matrix. To do so, minced chicken meat 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. In addition to the performance testing of the laboratories, a comparison was made between the prescribed methods (ISO 6579, 2002) and the requested method (Annex D of ISO 6579, 2007). For the prescribed method, the selective enrichment media were Rappaport Vassiliadis Soya broth (RVS) and Mueller Kauffmann Tetrathionate novobiocin broth (MKTTn). For the requested method, the selective enrichment was Modified Semi-solid Rappaport Vassiliadis (MSRV) agar. Optionally, a laboratory could also use other, own media or procedures for the detection of *Salmonella*.

Thirty-five individually numbered capsules had to be tested by the participants for the presence or absence of *Salmonella.* Twenty-five of the capsules had to be examined in combination with each 10 grams of *Salmonella* negative chicken meat: 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 meat 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 96% of the (contaminated) samples when using selective enrichment in MKTTn and RVS (prescribed food method). The method for testing veterinary samples (MSRV) gave the best results with the detection of *Salmonella* in 98% of the positive samples.

Thirty-one out of 32 laboratories achieved the level of good performance on the first attempt. One NRL achieved an underperformance and was visited by the CRL-*Salmonella* during a follow-up study, thereby reaching the desired outcome. The reason for their initially deviating results was most probably cross-contamination.

1 Introduction

An important task of the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*), as laid down in the Commission Regulation EC No 882/2004, is the organisation of interlaboratory comparison studies. The history of the interlaboratory comparison studies on the detection of *Salmonella*, as organised by CRL-*Salmonella* since 1995 is summarised in Annex 1. In earlier ring trials, the detection of *Salmonella* spp. in veterinary, animal feed and food samples was studied. This was the third study for the detection of *Salmonella* spp. in meat. The organisation of an interlaboratory comparison study on minced chicken meat was discussed with the NRLs for *Salmonella* at the annual CRL-*Salmonella* workshop in May 2009 (Mooijman, 2009). The first and most important objective of the study, organised by the Community Reference Laboratory (CRL) for *Salmonella* in October 2009, was to see whether the participating laboratories could detect *Salmonella* at different contamination levels in minced chicken meat. 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 chicken meat.

The prescribed method for detection of *Salmonella* in a food matrix is ISO 6579 (Anonymous, 2002). However, as good experiences have been gained with selective enrichment on Modified Semi-solid Rappaport Vassiliadis (MSRV) for the detection of *Salmonella* spp. in animal faeces (Annex D of ISO 6579, Anonymous, 2007) but also in food and animal feed samples, participating laboratories were requested also to use MSRV for testing the chicken meat.

The set-up of this study was comparable to earlier interlaboratory comparison studies on the detection of *Salmonella* spp. in veterinary, animal feed and food samples. The contamination level of the low-level capsules was close to the detection limit of the method; the level of the high-level samples approximately five to ten times above the detection limit. Ten control samples, consisting of different reference materials, had to be tested without the addition of chicken meat. These reference materials consisted of 3 capsules containing approximately 5 cfp of *Salmonella* Typhimurium (STM5), 2 capsules containing approximately 20 cfp of *Salmonella* Enteritidis (SE20), 1 capsule with approximately 100 cfp of *Salmonella* Enteritidis (SE100), 2 capsules containing approximately 5 cfp of *Salmonella* Panama (SPan5) and 2 blank capsules. Twenty-five samples of *Salmonella* negative minced chicken meat (10 g each) spiked with five different reference materials had to be examined. For the latter samples, the different reference materials consisted of two levels of *Salmonella* Typhimurium (STM5 and STM50), two levels of *Salmonella* Enteritidis (SE20 and SE100) and blank reference materials.

2 Participation

Country	City	Institute
Austria	Graz	Austrian Agency for Health and Food Safety (AGES) Department of food microbiology
Belgium	Brussels	Scientific Institute of Public Health (WIV) Afd. 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	Esjberg	Danish Veterinary and Food Administration Region South Laboratory
Estonia	Tartu	Estonian Veterinary and Food Laboratory
Finland	Helsinki	Finnish Food Safety Authority Evira Research Department, Microbiology Unit
France	Ploufragan	L'Agence Française de Sécurité Sanitaire des Aliments (AFSSA)
Germany	Berlin	Federal Institute for Risk Assessment (BFR) National 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 Food Microbiological Diagnostic Laboratory
Iceland	Reykjavik	University of Iceland, Keldur Institute for Experimental Pathology
Ireland	Kildare	Central Veterinary Research Laboratory CVRL / DAF Department of Agriculture and Food
Italy	Legnaro (PD)	Istituto Zooprofilattico Sperimentale delle Venezie, OIE National Reference Laboratory for *Salmonella*
Latvia	Riga	National Diagnostic Centre (NDC)
Lithuania	Vilnius	National Food and Veterinary Risk Assessment Institute
Luxembourg	Luxembourg	Laboratoire de Médecine Vétérinaire de l'Etat, LMVE
Macedonia FYROM Former Yugoslav Republic of Macedonia	Skopje	Faculty of veterinary medicine
Malta	Valletta	Public Health Laboratory (PHL) Evans Buildings Dept.
Netherlands, the	Bilthoven	National Institute for Public Health and the Environment (RIVM/Cib) Centre for Infectious Diseases Control Laboratory for Zoonoses and Environmental Microbiology (LZO)
Norway	Oslo	National Veterinary Institute, Section of Bacteriology

Country	City	Institute
Poland	Pulawy	National Veterinary Research Institute (NVRI) Department of Hygiene of Food of animal Origin
Portugal	Lisbon	Laboratório Nacional de Investigação Veterinária (LNIV)
Romania	Bucharest	Hygiene and Veterinary Public Health Institute (IISPV)
Slovak Republic	Bratislava	State Veterinary and Food Institute Reference Laboratory for *Salmonella*
Slovenia	Ljubljana	National Veterinary Institute, Veterinary Faculty
Spain	Madrid Majahonda	Centro National de Alimentacion, agencia Espanola de Seguridad Alimantaria y Nutricion (AESAN)
Sweden	Uppsala	National Veterinary Institute (SVA), Department of Bacteriology
Switzerland	Berne	Institute of veterinary bacteriology, Vetsuisse National Centre for Zoonose (ZOBA)
United Kingdom	Leeds	Health Protection Agency HPA Food, Water & Environmental Microbiology Network
United Kingdom	Belfast	Agri-Food and Bioscience Institute (AFBI) Veterinary Sciences Division Bacteriology

3 Materials and methods

3.1 Reference materials

Five batches of *Salmonella* reference materials were prepared. For this purpose milk, artificially contaminated with a *Salmonella* strain, was spray-dried (In 't Veld et al., 1996). The obtained highly contaminated milk powder (hcmp) was mixed with sterile (γ-irradiated) milk powder (Carnation, Nestlé, the Netherlands) to obtain the desired contamination level. The mixed powder was filled into 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 over layer and, after solidification, the plates were incubated at (37 ± 1) ^{o}C for (20 ± 2) h.

3.2 Minced chicken meat samples

3.2.1 General

A batch of twelve kilograms of *Salmonella* free minced chicken meat was provided by Plukon Royale / De Kuikenaer, Wezep, the Netherlands. The minced chicken meat arrived at CRL-*Salmonella* on 26 June 2009 as frozen portions of 300 grams. The meat was tested for the absence of *Salmonella* following the procedure as described in Annex D of ISO 6579 (Anonymous, 2007). For this purpose,

10 portions of 25 g were each added to 225 ml Buffered Peptone Water (BPW). After pre-enrichment at (37 ± 1) °C for 16–18 h, selective enrichment was carried out in Rappaport Vassiliadis Soya (RVS), Mueller Kaufmann Tetrathionate novobiocin (MKTTn) and on Modified Semi-solid Rappaport Vassiliadis (MSRV). Next, the tubes and suspect plates were plated-out on Xylose Lysine Deoxycholate agar (XLD) and Brilliant Green Agar (BGA) and confirmed biochemically. The minced chicken meat was stored at –20 °C until further use.

3.2.2 Total bacterial count in minced chicken meat

The total number of aerobic bacteria was investigated in the minced chicken meat. The procedure of ISO 4833 (Anonymous, 2003) was followed for this purpose. In summary, a portion of 20 grams of meat was homogenised in 180 ml of 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 two empty Petri-dishes (diameter 9 cm). To each dish 15 ml of molten Plate Count Agar (PCA) was added. After the PCA was solidified an additional 5 ml PCA was added to the agar. The plates were incubated at (30 ± 1) °C for (72 ± 3) h and the total number of aerobic bacteria was counted after incubation.

3.2.3 Number of Enterobacteriaceae in minced chicken meat

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. In summary, a portion of 20 grams of meat was homogenised in 180 ml of 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 two 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. The plates were incubated at (37 ± 1) °C for (24 ± 2) h and the number of typical violet-red colonies was counted after incubation. Five typical colonies were tested for the fermentation of glucose and for a negative oxidase reaction. After this confirmation, the number of *Enterobacteriaceae* was calculated.

3.3 Design of the interlaboratory comparison study

3.3.1 Samples: capsules and minced chicken meat

On Monday 28 September 2009 (one week before the study), the reference materials (35 individually numbered capsules) and 300 grams of *Salmonella* negative minced chicken meat were packed with cooling devices as biological substance category B (UN 3373) and send by courier service to each participant. After arrival at the participant laboratory, the capsules had to be stored at –20 °C and the minced chicken meat had to be stored at +5 °C until the start of the study. Details about mailing and handling of the samples and reporting of test results can be found in the Protocol (Annex 4) and Standard Operation Procedure (Annex 5). The test report used during the study can be found at the CRL-*Salmonella* web site: 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 meat (numbered C1–C10). Twenty-five capsules (numbered 1–25) were each tested in combination with 10 grams of minced chicken meat (negative for *Salmonella*). Table 1 shows the types, the number of capsules and meat samples to be tested.

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 food added	Test samples (n=25) with 10 g *Salmonella* negative minced chicken meat
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 minced chicken meat were packed in two plastic containers firmly closed with screw caps (biopacks). Both biopacks were placed in one large shipping box, together with four frozen (–20 ^{o}C) cooling devices. Each shipping box was sent as biological substances category B (UN3373) by door-to-door courier service. 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 ISO 6579 (Anonymous, 2002) and the requested (additional) method 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 (PCR)-based methods.

In summary:

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

Selective enrichment in/on:
- Rappaport Vassiliadis Soya broth (RVS) (prescribed);
- Mueller Kaufmann Tetrathionate novobiocin broth (MKTTn) (prescribed);
- Modified semi-solid Rappaport Vassiliadis agar (MSRV) (requested);
- 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 or by reliable, commercial available identification kits and serological tests. Follow the instructions of ISO 6579.

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 minced chicken meat (negative for *Salmonella* spp.). The specificity, sensitivity and accuracy rates were calculated according to the following formulae:

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

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

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

Mixed effect logistic regression (Venables and Ripley, 2002) was used for modelling the binary outcomes as a function of a fixed effect part, consisting of the capsules, enrichment media and isolation media and a random effect part, consisting of the different laboratories. Differences between media and capsules are shown as odds ratios and were calculated by stratification by medium. The overall performance of each laboratory is also given as an odds ratio but is compared to the mean of all laboratories, i.e., the outcomes as predicted based on the fixed effects only. 95% confidence limits and p-values are provided as well.
An odds ratio can be interpreted as an effect size and is the ratio of the odds of detecting *Salmonella* in one group to the odds of detecting it in another group. Groups are, for instance, two different media or one laboratory compared to the mean. Results were analysed using the statistical software R (R Development Core Team, 2010). The lme4 package was used for the mixed effect logistic regression (Bates and Maechler, 2010).

3.6 Good performance

Proposal for criteria testing 'good performance'

The criteria used for testing good performance in this study are given in Table 2. For determining good performance per laboratory, all combinations of selective enrichment media and 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 RVS/XLD but no positives with MKTTn or any other selective enrichment or isolation medium, this was still considered a good result. For the blank capsules, all combinations of media used per laboratory were also taken into account. If, for example a laboratory found 2/5 blank capsules positive with MKTTn/BGA but no positives with the other media, this was still considered a 'no-good' result.

Table 2 Used criteria for testing for good performance in the Food-Ill 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, one positive out of five blank samples (20% pos.) will still be considered as acceptable.

4 Results

4.1 Reference materials

Table 3 describes the level of contamination and the homogeneity of the final batches of capsules. The table gives the enumerated minimum and maximum levels within each batch of capsules. The final batches were tested twice: firstly immediately after preparing the batch and secondly, at the time of the interlaboratory comparison study. At the first date of testing the mean contamination level of all batches fulfilled the pre-set-criteria as stated in section 3.1. However, the variation between the SE100 capsules was high. At the second date of testing the mean contamination level of both batches SE capsules were decreased and the variation between the SE100 capsules was increased. The reason for this was not clear. Although the mean contamination level of the SE100 capsules was decreased under the minimum target level and the variation between the capsules was high, it was still considered useful for the purposes of this study.

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	23-09-2009	24-09-2009	24-03-2009	24-09-2009	24-09-2009
Number of capsules tested	25	25	25	25	25
Mean cfp per capsule	12	50	6	6	54
Min-max cfp per capsule	6-18	31-94	1-11	1-14	31-70
T_2 / (I-1)	0.99	5.07	1.55	1.54	1.25

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 Minced chicken meat samples

The minced chicken meat was tested negative for *Salmonella* and stored at –20 °C. On Monday 28 September 2009, the minced chicken meat was mailed to the NRLs. After receipt, the NRLs had to store the chicken meat at 5 °C.

The number of aerobic bacteria and the number of *Enterobacteriaceae* were tested three times; firstly at the day the chicken meat arrived at the CRL (30/6/2009), secondly, after the meat was stored for

one week at 5 °C and thirdly, close to the planned date (6/10/2009) of the interlaboratory comparison study. Table 4 shows the results.

Most of the laboratories (thirty) performed the study in week 41, starting on 6 October 2009.
Two laboratories (lab codes 17 and 18) performed the study one week later.

Table 4 Number of aerobic bacteria and the number of *Enterobacteriaceae* per gram of minced chicken meat

Date	Enterobacteriaceae cfp/g	Aerobic bacteria cfp/g
30 June 2009 after 1 day at 5 °C	$1.5*10^2$	$< 1*10^4$
7 July 2009 after 1 week at 5 °C	$1.2*10^2$	$4*10^6$
21 September 2009 after 1 week at 5 °C	$2*10^1$	$4.2*10^3$

4.3 Technical data interlaboratory comparison study

4.3.1 General

In this study, 32 NRLs participated: 28 NRLs from 27 EU-Member States, one NRL from a European candidate country and three NRLs from countries of the European Free Trade Association.

4.3.2 Accreditation/certification

In total, 31 laboratories indicated to be accredited according ISI/IEC 17025 (Anonymous, 2005). One laboratory (lab 14) mentioned that the application for accreditation had been sent. Twenty-four laboratories are accredited for ISO 6579 and annex D of ISO 6579 for different matrices. Three laboratories (lab codes 1, 5, and 23) are accredited for annex D of ISO 6579 but according to another method for food matrices. Four laboratories (lab codes 16, 22, 24 and 31) are accredited for food and feeding stuffs (ISO 6579) but not for animal faeces and veterinary samples (annex D of ISO 6579). According to EC Regulations No. 882/2004, each NRL should have been accredited for their relevant work field before 31 December 2009 (EC Regulation No. 2076/2005).

4.3.3 Transport of samples

Table 5 presents an overview of the transport times and the temperatures during transport of the parcels. The temperature recorders were returned immediately after receipt to CRL-*Salmonella* by all NRLs. The majority of the laboratories received the materials within 1–2 days. However, the parcel of one (lab code 28, a non EU-MS) was delayed for more than 1 week because of customs problems. If this latter parcel is not taken into account, the average transport time was 37 hours. For 15 parcels the transport temperature did not exceed 5 °C and for nine other parcels the temperature did not exceed 10 °C. Eight parcels were kept below 5 °C for the majority of the transport time but were stored for a few hours above 10 °C. The transport time of the parcel of laboratory 28 was relatively long but most of the time, the temperature did not exceed 5 °C.

Table 5 Overview of the transport time and of the temperatures during shipment of the parcels to the NRLs

		Time (h) at			
Lab code	Transport* total in hours	– 20 °C - 0 °C	0 °C - 5 °C	5 °C - 10 °C	>10 °C
1	46	7	33	6	
2	26	8	10	6	2 hrs at 25–27 °C
3	48	23	25		
4	27	9	15	2	1 h at 13 °C
5	28	8	16	4	
6	74	4	57	13	
7	24	10	14		
8	1	1			
9	46	7	35	2	2 hrs at 23 °C
10	77	7	40	30	
11	27	9	14	4	
12	27	7	17	3	
13	26	9	17		
14	48	8	20		20 hrs 19-20 °C
15	23	9	14		
16	27	9	18		
17	77	9	68		
18	28	25	3		
19	26	23	3		
20	26	10	16		
21	25	9	13		3 hrs at 23 °C
22	27	12	14	1	
23	24	13	11		
24	46	46			
25	26	6	18		2 hrs at 25 °C
26	75	9	66		
27	27	9	17		1 hr at 19 °C
28	8 days	7 days	26		
29	49	8	39		2 hrs at 22 °C
30	48	8	17	21	2 hrs at 11 °C
31	50	21	8	21	
32	22	9	13		
Average*	37				

*Time reported in the test report.

**Average time without lab 28.

Table 6 Media combinations used per laboratory

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

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

Compositions of the media not described in ISO 6579 are given in Annex 3.

*Laboratory 12 used BGA only in combination with MSRV.

For one NRL (lab code 14), 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, the parcel arrived later at the laboratory of the NRL. The delay was 20 hours and the parcel was stored between 19–20 ^{o}C.

4.3.4 Media

Each laboratory was asked to test the samples with the prescribed (ISO 6579) and the requested (Annex D of ISO 6579) methods. Thirty laboratories used the selective enrichment media RVS, MKTTn and MSRV with the plating-out medium XLD and a second plating-out medium of own choice. Table 6 shows the media used per laboratory. Laboratory 1 did not use both prescribed media (RVS and MKTTn) and laboratory 9 did not use RVS. Two NRLs (lab codes 24 and 25) used a third plating-out medium.
Details on the media which are not described in ISO 6579 are given in Annex 3.
Tables 7–13 give information on the composition of the media that were prescribed and 'requested' and on the incubation temperatures. These tables only indicate the laboratories that reported deviations. Two laboratories (lab codes 21 and 31) reported a deviating dissolving time of the capsules. Laboratory 19 incubated the pre-enrichment medium BPW longer than described. Laboratories 11, 18 and 21 did not mention the pH for most of the used media. One laboratory (lab code 16) did not mention the composition of the media used. Two laboratories incubated the selective enrichment medium MKTTn at deviating temperatures (lab codes 10 and 20).

A second plating-out medium for choice was obligatory. Sixteen laboratories used BGA modified (ISO 6579, 1993) or BPLS as a second plating-out medium. Seven laboratories used Rambach, four laboratories SM2 agar, two laboratories Rapid Salmonella and two laboratories BSA. The following media were used only by one laboratory: Hektoen, BGA, BPLSA and Compass.

The use of an extra plating agar between the 'isolation' and the 'confirmation' steps was optional. A total of 20 laboratories performed this extra culture step on many different media (e.g., Nutrient agar: ISO 6579, 2002).

Most of the laboratories used both biochemical and serological tests for confirmation of *Salmonella.* Three laboratories (lab codes 13, 14 and 154) did not use a biochemical test but used serological tests only. Tables 14 and 15 summarise the used confirmation media and serological tests.

Table 7 Incubation time and temperature of BPW

	Dissolving capsules in BPW		Pre-enrichment in BPW	
Lab code	**Time (min)**	**Incubation temperature In ^{o}C (min-max)**	**Time (h:min)**	**Incubation temperature in ^{o}C (min-max)**
SOP & ISO 6579	**45**	**36-38**	**16 – 20**	**36-38**
19	45	37	21:20	37
21	50	36.9	18	36.9
31	35	37	20	37

Grey cell: deviating times and temperatures.

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

Lab code	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**	**5**	**9**	**1.5**	**6.8 – 7.2**
1, 5, 7, 11, 15, 26, 27	10	5	3.5*	1.5	7.3
2	10	4.3	7.2	3.6	7.0
16	-	-	-	-	7.1
21	10	5	9	1.5	-
29	10	5	3.7	1.5	7.2

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 Incubation temperatures of selective enrichment medium RVS, MKTTn and MSRV

	RVS	MKTTn	MSRV
Lab code	**Incubation temperature in ^oC (min-max)**	**Incubation temperature in ^oC (min-max)**	**Incubation temperature in ^oC (min-max)**
ISO 6579 & Annex D	**40.5 – 42.5**	**36–38**	**40.5 – 42.5**
10	41.5–42	41.5–42	41.5–42
20	41.5–42	41.5–42	41.5–42

Grey cell: deviating times and temperatures.

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

Lab code	Enzymatic digest of soya (Peptone)	Sodium Chloride (NaCl)	Potassium Dihydrogen Phosphate* (KH_2PO_4 K_2HPO_4)	Magnesium chloride anhydrous ($MgCl_2$)**	Malachite green oxalate	pH
ISO 6579	**4.5**	**7.2**	**1.44**	**13.4**	**0.036**	**5.0 - 5.4**
1, 4	5	8	1.6	40	0.04	5.4
11	5	8	1.6	40	0.04	-
16	-	-	-	-	-	-
18	4.5	7.2	1.44	28.6	0.04	-
19	5	8	1.4 + 0.2	13.4	0.04	5.2
21	4.5	7.2	1.26 + 0.18	13.4	0.004	-
23	5	8	1.6	40	0.04	5.2
24	4.5	7.2	1.26 + 0.18	28.6	0.04	-
25	4.5	7.2	1.26 + 0.18	13.58	0.04	5.2
32	5	8	1.4 + 0.2	400	0.4	5.2

Grey cell: deviating from ISO 6579. - = no information.

*= 1.4 g/L Potassium dihydrogen phosphate (KH_2PO_4) + 0.2 g/L Di-potassium hydrogen phosphate (K_2HPO_4) gives a final concentration of 1.44 g/L KH_2PO_4 K_2HPO_4.

** = 13.4 g $MgCl_2$ (anhydrous) is equivalent to 28.6 g $MgCl_2$ hexahydrate.

Table 11 Composition (in g/L) and pH of MKTTn

Lab code	Meat extract	Enzymatic digest of casein (Peptone)	Sodium chloride (NaCl)	Calcium Carbonate ($CaCO_3$)	Sodium Thiosulfate Penta hydrate ($Na_2S_2O_3$. $5H_2O$)	Oxbile	Brilliant green	Iodine	Potassium iodide (KI)	Novo-Biocin	pH
ISO 6579	**4.3**	**8.6**	**2.6**	**38.7**	**47.8**	**4.8**	**0.0096 (9.6 mg)**	**4**	**5**	**0.04**	**8.0 – 8.4**
5, 11	4.3	8.6	2.6	38.7	47.8	4.78	0.0096	4	5	0.04	-
7, 15	4.3	8.6	2.6	38.7	30.5*	4.78	0.0096	4	5	0.04	7.8
16	-	-	-	-	-	-	-	-	-	-	-
17	7	2.3	2.3	25	40.7	4.75	0.1g /100ml	20g /100ml	25g /100ml	0	7.85
18, 21	4.23	8.45	2.54	38.04	30.3*	4.75	0.0095	4	5	0.05	-
24	4.3	8.6	2.6	38.7	30.5*	4.78	0.0096	-	-	-	-
25	4.3	8.6	2.6	38.7	30.5*	4.78	0.0096	4	5	0.01	8
26	7	2.4	2.4	25	40.8	4.75	0.0095	3.89	4.75	0.04	-
29	4.3	8.6	2.6	38.7	30.5*	4.78	0.0096	3.9	4.9	0.039	7
31	7	2.3	2.3	25	40.7	4.75	9.5 ml 0.1%	20	25	0.04	7.8
32	4.3	8.6	2.6	38.7	47.8	4.78	0.0096	20	25	0.04	8.2

Grey cell: deviating from ISO 6579. - = no information.

* 30.5 g Sodium thiosulphate (anhydrous) is equivalent to 47.8 g Sodium thiosulphate pentahydrate.

Table 12 Composition (in g/L) and pH of MSRV

Lab code	Enzymatic digest of casein (Tryptose)	Casein hydro-lysate	Sodium chloride (NaCl)	Potassium Dihydrogen Phosphate (KH_2PO_4 K_2HPO_4)	Magnesium chloride anhydrous ($MgCl_2$)	Malachite green oxalate	Agar	Novo Biocin	pH
Annex D ISO 6579	**4.6**	**4.6**	**7.3**	**1.5**	**10.9**	**0.04**	**2.7**	**0.01 (10mg/L)**	**5.1-5.4**
2	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.05	5.2
9	-	-	-	-	-	-	-	-	5.5
11 ?								0.02?	
16, 32	-	-	-	-	-	-	-	-	5.2
17	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0	5.37
19	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.05	5.4
21	4.6	4.6	7.3	1.5	10.9	0.04	2.7	2.7	-
23	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.4
24	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.2
26	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.01	5.6
28	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.34

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

Table 13 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 desoxy-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**	**7.5**	**7.5**	**5**	**3**	**0.08**	**9-18**	**1**	**6.8**	**0.8**	**7.2 – 7.6**
5	3.75	5	7.5	7.5	5	3	0.08	9-18	1	6.8	0.8	-
10	3	5	7.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	7.5
16	-	-	-	-	-	-	-	-	-	-	-	-
17	-	5	3.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	7.48
18	3.5	5	7.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	-
19	3.75	5	7.5	7.5	5	3	0.08	15.5	2.5	6.8	0.8	7.4
21	3.5	5	7.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	-
22	3.5	5	7.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	7.55
32	-	-	-	-	-	-	-	-	-	-	-	7.4

Grey cell: deviating from ISO 6579.
- = no information.

Table 14 Biochemical confirmation of *Salmonella*

Lab code	TSI	UA	LDC	Gal	VP	Indole	Kit	Other
1	+	+	-	-	-	-	Salmonella latex kit (Oxoid)	Lysine Iron Agar
2	+	+	+	-	-	+		Mini VIDAS
3	+	+	+	+	+	+		Malonate
4, 22	+	+	+	+	-	+		
5	-	-	-	-	-	-	API 20E	MacConkey, PCR
6, 12, 28, 30	+	+	+	+	+	+		
7	-	-	-	-	-	-	Entero tube II	PCR
8, 18, 26, 27	+	+	+	-	-	-		
9	-	-	-	-	-	-	Vitek	
10, 19	+	+	+	-	-	+		PCR
11	+	-	-	-	-	-		H2S separately, Oxidase
13	-	-	-	-	-	-		PCR
14	-	-	-	-	-	-		Chromogenic medium
15	-	-	-	-	-	-		Kohns No1 medium (Mast)
16	-	-	-	-	-	-	Rapid 32E	Kligler
17	+	+	+	-	+	+		
20	+	+	+	-	-	+	API 20E	
21	-	-	-	-	-	-	Microbact 12A	
22	+	+	+	+	+	+		PCR
24	-	-	-	-	-	-	ID 32 E	
25	+	+	+	+	+	+		
29	+	+	+	-	-	-	API 20E	
31	+	-	-	-	-	-	Microbact GNB 24E	
32	+	+	+	+	-	+		Semi-solid Glucose

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

Table 15 Serological confirmation of *Salmonella*

Lab code	Serological			Other
	O antigens	H Antigens	Vi antigens	
1	-	-	-	Other, no details
7, 22	-	-	-	Salmonella Poly A-+Vi
21	-	-	-	Salmonella test kit
6, 8, 11, 16, 18, 19, 24, 25, 26, 27, 29	-	-	-	
4, 9, 10, 28, 31, 32	+	-	-	
2, 3, 5, 12, 13, 15, 17, 20, 30	+	+	-	
14	+	+	-	Specific somatic O agglutination
23	+	-	+	

- = Not done/mentioned.

4.4 Control samples

4.4.1 General

None of the laboratories isolated *Salmonella* from the procedure control (C11: no capsule/no meat) nor from the meat control (C12: no capsule/negative meat). Twenty-eight laboratories scored correct results for all the control capsules containing *Salmonella*. Annex 6 gives the results of all control samples (capsules without meat) per laboratory and per selective enrichment medium in combination with the isolation medium that gives the highest number of positives. Laboratory 27 made a mistake during the performance and has no results for the selective enrichment in RVS. Those samples were considered as negative. Table 16 summarises the highest number of positive isolations found with all combinations of selective enrichment media and isolation media per laboratory.

Blank capsules without addition of meat (n=2)
The blank capsules contained only sterile milk powder. For the analyses, no meat was added.
Laboratory 12 found one blank capsule positive on all media used by this 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.

Salmonella Panama 5 capsules (SPan5) without addition of meat (n=2)
Thirty-one laboratories isolated *Salmonella* from both Span5 capsules. One Laboratory (lab code 19) could not detect *Salmonella* Panama (SPan5) in one control capsule with all the three selective enrichment media. These capsules contained Span at a low level (approximately 5 cfp/ capsule). Due to the variation among capsules, one out of two capsules containing Span5 may occasionally be negative.

Salmonella Typhimurium 5 capsules (STM5) without addition of meat (n=3)
Thirty-one laboratories tested 3/3 capsules containing STM5 positive. One laboratory (lab code 6) could not detect *Salmonella* in one control capsule (STM5), with all three selective enrichment media. These capsules contained STM at a low level (approximately 5 cfp/ capsule). Due to the variation among capsules, one out of two capsules containing STM5 may occasionally be negative.

Salmonella Enteritidis 20 capsules (SE20) without addition of meat (n=2)
Thirty-one laboratories isolated *Salmonella* from both SE20 capsules. One Laboratory (lab code 12) could not detect *Salmonella* Enteritidis (SE20) in one control capsule with all three selective enrichment media. These capsules contained SE at a low level (approx 20 cfp/capsule). However, the level was not so low that negative capsules may be expected in the batch of reference materials. It is therefore not very likely that the negative results were caused by negative capsules.

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

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 (lab code 12).

Table 16 Total number of positive results of the control samples (capsule without meat) per laboratory

Lab code	The highest number of positive isolations found with all combinations of selective enrichment media and isolation media				
	Blank n=2	SPan5 n=2	STM5 n=3	SE20 n=2	SE100 n=1
Good Performance	**0**	**≥ 1**	**≥ 2**	**≥ 1**	**1**
1	0	2	3	2	1
2	0	2	3	2	1
3	0	2	3	2	1
4	0	2	3	2	1
5	0	2	3	2	1
6	0	2	**2**	2	1
7	0	2	3	2	1
8	0	2	3	2	1
9	0	2	3	2	1
10	0	2	3	2	1
11	0	2	3	2	1
12	**1**	2	3	**1**	1
13	0	2	3	2	1
14	0	2	3	2	1
15	0	2	3	2	1
16	0	2	3	2	1
17	0	2	3	2	1
18	0	2	3	2	1
19	0	**1**	3	2	1
20	0	2	3	2	1
21	0	2	3	2	1
22	0	2	3	2	1
23	0	2	3	2	1
24	0	2	3	2	1
25	0	2	3	2	1
26	0	2	3	2	1
27	0	2	3	2	1
28	0	2	3	2	1
29	0	2	3	2	1
30	0	2	3	2	1
31	0	2	3	2	1
32	0	2	3	2	1

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

4.4.2 Specificity, sensitivity and accuracy rates of the control samples

Table 17 shows the specificity, sensitivity and accuracy rates found with the control capsules without the addition of meat. The rates are calculated for the different selective enrichment media (RVS, MKTTn and MSRV) and plating-out medium XLD. The calculations were performed on the results of

all participants and on the results of only the EU-MS (without the one candidate country and without countries of the European Free Trade Association). High rates were found. As expected, the high level SE100 capsules showed a rate of 100%. For the low level materials (SPan5, STM5 and SE20) the rates were close to 97%. There was no difference between rates of EU-MSs and the four non-EU-MSs. The rates for the RVS for all participants were slightly lower because of the mistake made by a non EU-MS (laboratory 27).

Table 17 Specificity, sensitivity and accuracy rates found with the control samples (capsules without the addition of meat)

Control capsules		RVS/XLD*		MKTTn/XLD#		MSRV/XLD	
		All n=30	EU n=27	All n=31	EU n=28	All n=32	EU n=28
Blank	No. of samples	60	54	62	56	64	56
	No. of negative samples	59	53	61	55	63	55
	Specificity in%	**98.3**	**98.2**	**98.4**	**98.2**	**98.4**	**98.2**
Span5	No. of samples	60	54	62	56	64	56
	No. of positive samples	57	53	61	55	63	55
	Sensitivity in%	**95**	**98.2**	**98.4**	**98.2**	**98.4**	**98.2**
STM5	No. of samples	90	81	93	84	96	84
	No. of positive samples	86	80	92	83	95	83
	Sensitivity in%	**95.6**	**98.8**	**98.2**	**98.8**	**98.9**	**98.8**
SE20	No. of samples	60	54	62	56	64	56
	No. of positive samples	56	52	60	54	63	55
	Sensitivity in%	**93.3**	**96.3**	**96.8**	**96.4**	**98.4**	**98.2**
SE100	No. of samples	30	27	31	28	32	28
	No. of positive samples	29	27	31	28	32	28
	Sensitivity in%	**96.7**	**100**	**100**	**100**	**100**	**100**
All capsules with *Salmonella*	No. of samples	240	216	248	224	256	224
	No. of positive samples	228	212	244	220	253	221
	Sensitivity in%	**95**	**98.2**	**98.4**	**98.2**	**98.8**	**98.7**
All capsules	No. of samples	300	270	310	280	320	280
	No. of correct samples	287	265	305	275	316	276
	Accuracy in%	**95.7**	**98.2**	**98.4**	**98.2**	**98.8**	**98.6**

*Results without Laboratory 1 (non-EU-MS) and 9 (EU-MS): they did not use RVS.

#Results without Laboratory 1 (non-EU-MS): they did not use MKTTn.

4.5 Results of meat samples artificially contaminated with *Salmonella* spp.

4.5.1 Results per type of capsule and per laboratory

General

Annex 7 summarises the results of the *Salmonella* negative minced chicken meat samples artificially contaminated with capsules per selective enrichment medium in combination with the isolation medium giving the highest number of positives. Twelve laboratories scored correct results for all the samples. Laboratory 27 made a mistake during the performance and had no results for five samples with the selective enrichment in RVS. Those samples were considered as negative. Table 18 summarises the highest number of positive isolations found with all combinations of selective enrichment media and isolation media per laboratory.

Blank capsules with negative minced chicken meat (n=5)

Thirty laboratories correctly did not isolate *Salmonella* from these blank capsules with the addition of negative meat. Two laboratories (2 and 24) found 1 positive blank with the addition of negative minced chicken meat. Laboratory 2 had found one blank capsule positive on all media. Laboratory 24 found only one positive blank after selective enrichment on MSRV. With the other media RVS and MKTTn they correctly found no positive blanks.

All blanks should be tested negative. However, as no 100% guarantee about the *Salmonella* negativity of the matrix can be given, 1 positive out of 5 blank samples (80% negative) is still considered acceptable.

S. Typhimurium 5 capsules (STM5) with negative minced chicken meat (n=5)

All laboratories isolated *Salmonella* from all the five capsules containing *Salmonella* Typhimurium at a level of approximately 5 cfp/ capsule in combination with minced chicken meat when using MSRV. Laboratory 27 missed one capsule because of their mistake with the selective enrichment in RVS.

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

All laboratories isolated *Salmonella* from all five capsules containing *Salmonella* Typhimurium at a level of approximately 50 cfp/ capsule in combination with minced chicken meat with all the selective enrichment media: RVS, MKTTn and MSRV. Laboratory 27 missed one capsule because of their mistake with the selective enrichment in RVS.

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

Twenty-two laboratories isolated *Salmonella* from all the five capsules containing *Salmonella* Enteritidis at a level of approximately 20 cfp/ capsule in combination with minced chicken meat at least with one of the used selective enrichment media RVS, MKTTn or MSRV. Laboratory 12 found four capsules negative when using RVS and MKTTn and they found three capsules negative when using MSRV. These capsules contained SE at a low level (approx 20 cfp/capsule). However, the level was not so low that negative capsules may be expected in the batch of reference materials. It is therefore not very likely that the negative results were caused by negative capsules.

Table 18 Total number of positive results of the artificially contaminated minced chicken meat samples per laboratory

Lab code	The highest number of positive isolations found with all combinations of selective enrichment media and isolation media				
	Blank n=5	STM5 n=5	STM50 n=5	SE20 n=5	SE100 n=5
Good performance	**≤ 1**	**≥ 2**	**≥ 4**	**≥ 2**	**≥ 4**
1	0	5	5	5	5
2	**1**	5	5	**4**	5
3	0	5	5	5	5
4	0	5	5	5	5
5	0	5	5	5	5
6	0	5	5	5	5
7	0	5	5	5	5
8	0	5	5	5	5
9	0	5	5	5	5
10	0	5	5	**4**	5
11	0	5	5	5	5
12	0	5	5	**2**	5
13	0	5	5	**4**	5
14	0	5	5	5	5
15	0	5	5	5	5
16	0	5	5	5	5
17	0	5	5	**4**	5
18	0	5	5	**4**	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	**1**	5	5	**4**	5
25	0	5	5	**4**	5
26	0	5	5	**4**	5
27	0	5	5	5	5
28	0	5	5	5	5
29	0	5	5	5	5
30	0	5	5	**4**	5
31	0	5	5	5	5
32	0	5	5	5	5

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

S. Enteritidis 100 capsules (SE100) with negative minced chicken meat (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 minced chicken meat with the selective enrichment medium MSRV. Laboratory 26 found one capsule SE100 negative with selective enrichment medium MKTTn. Laboratory 27 missed two capsules with selective enrichment in RVS because of their mistake with this medium.

The results of all artificially contaminated minced chicken meat samples were compared with the definition of 'good performance' (see section 3.6). Only one laboratory (lab code 12) scored below these criteria.

4.5.2 Results per selective enrichment medium, capsule and per laboratory

Figures 1, 2, 3 and 4 show the number of positive isolations per type of artificially contaminated minced meat sample per laboratory after pre-enrichment in BPW, selective enrichment in RVS, MKTTn and on MSRV, followed by isolation on selective plating agar XLD. To determine good performance per laboratory, all combinations of selective enrichment media and isolation media used by the laboratory were taken into account. The highest number of positives is indicated by 'x' in the figures. The results of all artificially contaminated minced chicken meat samples were compared with the definition of 'good performance' (see section 3.6). The black horizontal line in Figures 1–4 indicates the border of good performance.
The majority of the laboratories found the highest number of positive isolations when XLD was used as isolation medium. Laboratory 29 found one sample more positive after isolation on Rambach compared to isolation on XLD, after selective enrichment in both MKTTn and RVS. Laboratory 22 found one sample more positive but another sample more negative after isolation on BGA, compared to isolation on XLD (in both cases after selective enrichment in RVS).
Table 19 gives the differences in the number of positive isolations after 24 and 48 hours of incubation of the selective enrichment media. XLD showed the highest number of positive isolations compared to other plating-out media, independent on the selective enrichment medium used. The majority of the laboratories used BGA as the second plating-out medium (see Table 5).
The choice of plating-out medium does not seem to have a large effect on the number of positive isolations. When MKTTn is used for selective enrichment, XLD gave 3% more positive results than other plating-out media.
The difference in the number of positive isolations after 24 h and 48 h of incubation of the selective enrichment media was the highest for MKTTn (Table 19): 3–4% more positive isolations were found after 48 h of incubation. For RVS and MSRV the difference between the two incubation times was 2–3%.

Table 19 Mean percentages of positive results of all participating laboratories after selective enrichment in RVS, MKTTn and on MSRV, incubated for 24 and 48 hours and followed by incubation on different plating-out media, when analysing the artificially contaminated minced chicken meat samples

Plating-out medium	Selective enrichment medium		
	RVS	MKTTn	MSRV
	24 / 48 h	24 / 48 h	24 / 48 h
XLD	92 / 95%	93 / 96%	95 / 98%
Other (most often BGA)	93 / 95%	90 / 94%	94 / 97%

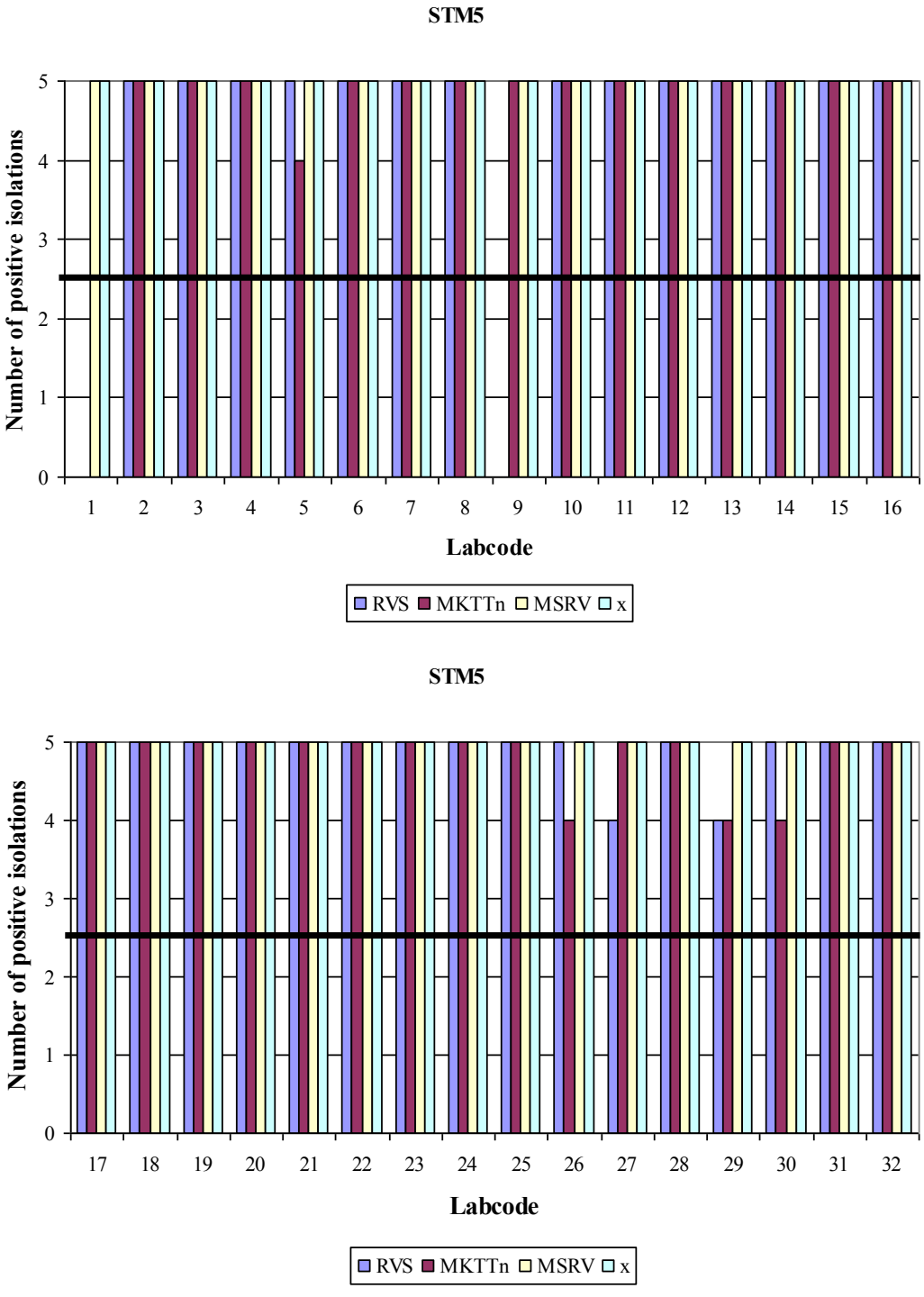


Figure 1 Results of minced chicken meat samples artificially contaminated with STM5 capsules (n=5) after selective enrichment in RVS, MKTTn and on MSRV followed by isolation on selective plating agar XLD. The highest number of positive isolations found with all combinations of selective enrichment media and isolation media used by a laboratory is given as x.

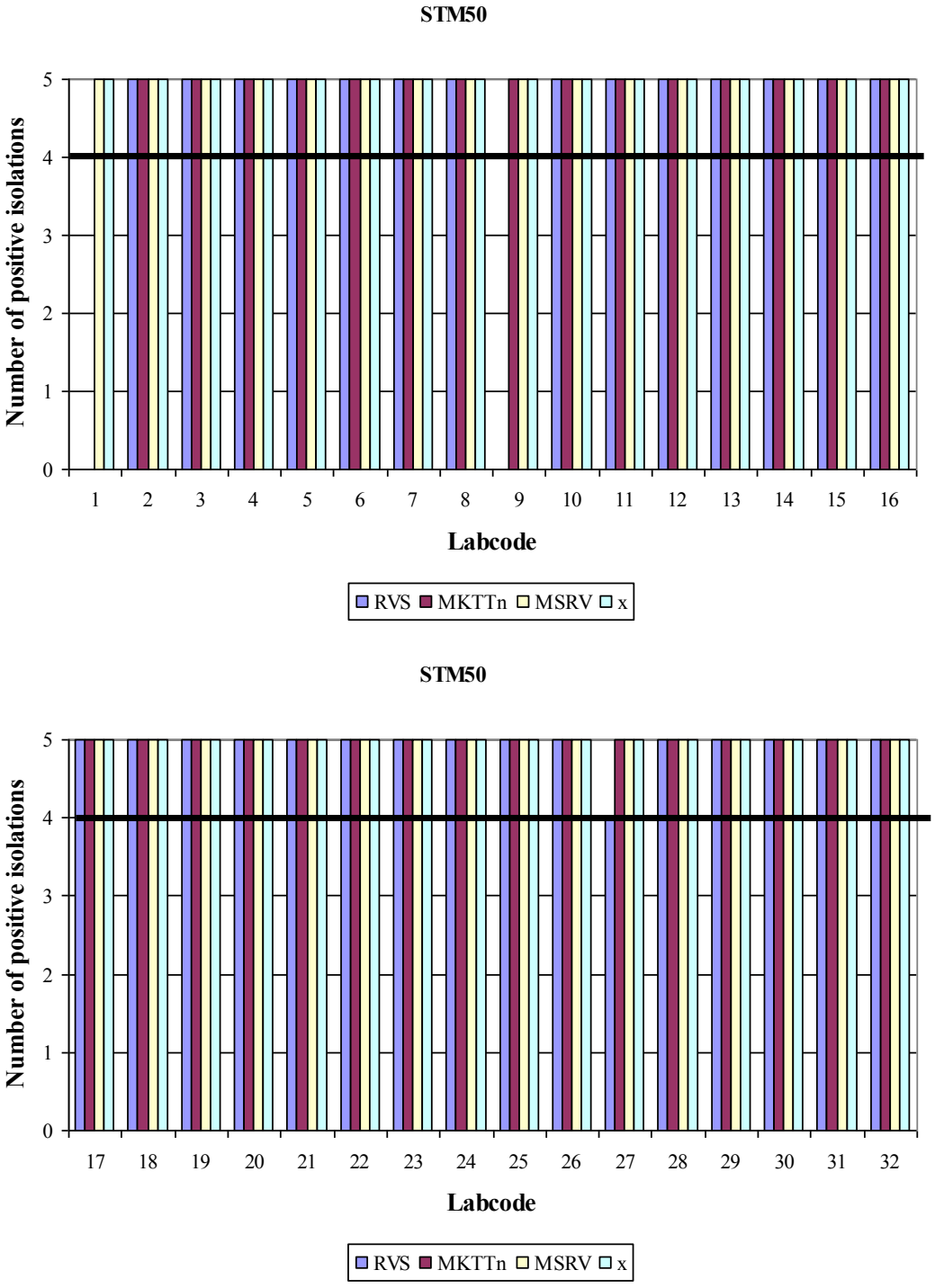
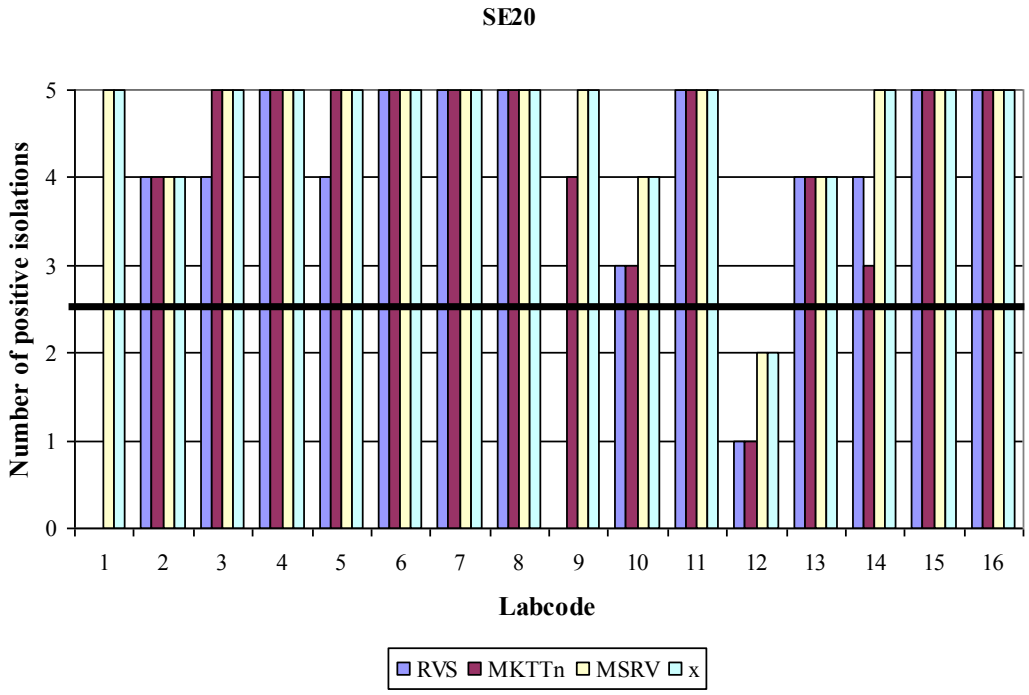
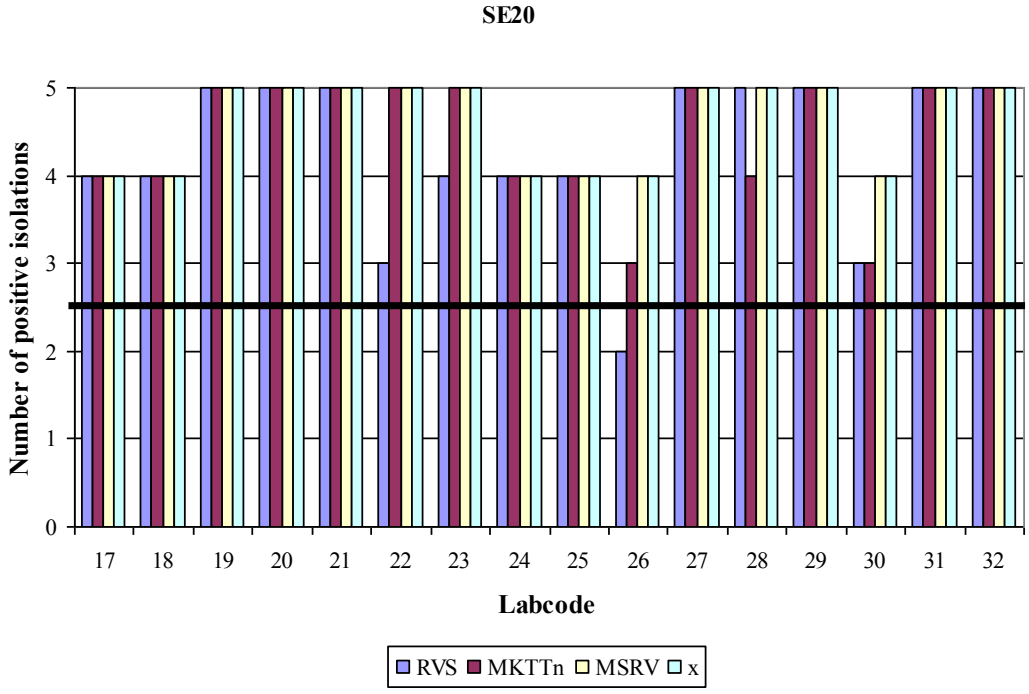


Figure 2 Results of minced chicken meat samples artificially contaminated with STM50 capsules (n=5) after selective enrichment in RVS, MKTTn and on MSRV followed by isolation on selective plating agar XLD. The highest number of positive isolations found with all combinations of selective enrichment media and isolation media used by a laboratory is given as x.





= border of good performance

Figure 3 Results of minced chicken meat samples artificially contaminated with SE20 capsules (n=5) after selective enrichment in RVS, MKTTn and on MSRV followed by isolation on selective plating agar XLD. The highest number of positive isolations found with all combinations of selective enrichment media and isolation media used by a laboratory is given as x.

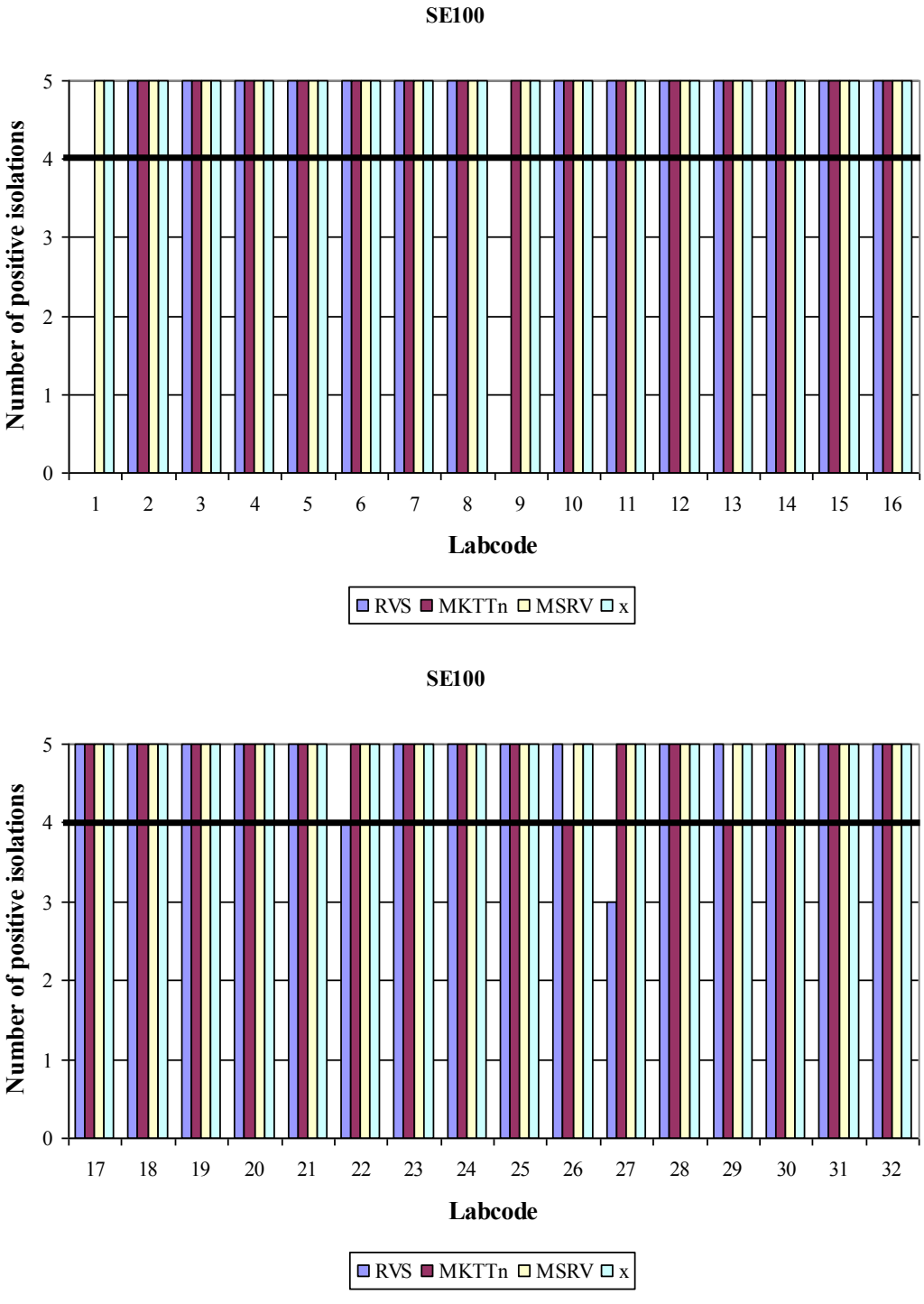


Figure 4 Results of minced chicken meat samples artificially contaminated with SE100 capsules (n=5) after selective enrichment in RVS, MKTTn and on MSRV followed by isolation on selective plating agar XLD. The highest number of positive isolations found with all combinations of selective enrichment media and isolation media used by a laboratory is given as x.

Tables 20 and 21 show the differences between selective enrichment media and isolation media per capsule as odds ratios (OR). In addition the 95% confidence intervals and p-values are indicated. An odds ratio equal to "∞" means that it is very large. Usually, this occurs when the odds of detecting *Salmonella* for the reference medium is zero or near zero. An odds ratio of zero means that the odds of detecting *Salmonella* for the other medium is zero.

The interpretation of Table 20 is as follows: given a selective enrichment medium, the odds of finding a positive isolation with the different plating-out media are compared. For instance the odds of finding *Salmonella* from the SE20 samples after selective enrichment in MKTTn is for XLD as isolation medium a factor 1.49 (OR) higher than for media other than XLD. If MSRV is used as selective enrichment medium, XLD shows higher OR, although this is not significant. In general, for MSRV and MKTTn, the OR are high but not significant. The OR for RVS are in general lower than one; this means that it is more difficult to detect *Salmonella* when RVS is used compared with MKTTn and MSRV.

Table 20 Number of positive isolations found with XLD compared to the number of positive isolations found with other isolation media, given a selective enrichment medium. Samples: minced chicken meat, artificially contaminated with *Salmonella* positive capsules

Selective enrichment medium	Compared isolation media	Capsule	Odds Ratios	95% lower	95% upper	p-value*
RVS	XLD compared to other than XLD	STM5	0.43	0.02	7.99	0.57
		STM50	0.99	0.04	26.83	1.00
		SE20	1.07	0.53	2.16	0.84
		SE100	0.54	0.05	5.34	0.59
		all STM	0.61	0.08	4.85	0.64
		all SE	1.00	0.55	1.83	0.99
		all capsules	0.97	0.56	1.68	0.90
MKTTn	XLD compared to other than XLD	STM5	0.72	0.11	4.60	0.73
		STM50	44380492	0.00	∞	1.00
		SE20	1.49	0.73	3.03	0.27
		SE100	2.21	0.27	18.10	0.46
		all STM	1.52	0.34	6.73	0.58
		all SE	1.47	0.79	2.71	0.22
		all capsules	1.45	0.84	2.49	0.18
MSRV	XLD compared to other than XLD	STM5	77466451	0.00	∞	1.00
		STM50	77466451	0.00	∞	1.00
		SE20	1.02	0.40	2.61	0.97
		SE100	1.00	0.00	∞	1.00
		all STM	77465883	0.00	∞	1.00
		all SE	1.02	0.42	2.47	0.97
		all capsules	1.37	0.61	3.07	0.44
All enrichment media	XLD compared to other than XLD	STM5	0.99	0.27	3.62	0.99
		STM50	6.91	0.67	71.18	0.10
		SE20	1.20	0.77	1.86	0.42
		SE100	1.20	0.30	4.79	0.79
		all STM	1.76	0.63	4.91	0.28
		all SE	1.16	0.80	1.70	0.44
		all capsules	1.21	0.86	1.71	0.26

* significant difference $p < 0.05$.

Table 21 Number of positive isolations found with a selective enrichment medium compared to the number of positive isolations found with another selective enrichment medium, given that the isolation is on XLD. Samples: minced chicken meat, artificially contaminated with *Salmonella* positive capsules

Compared selective enrichment media	Isolation medium	Capsule	Odds Ratios	95% lower	95% upper	p-value
RVS compared to MKTTn	XLD	STM5	2.02	0.25	16.17	0.51
		STM50	1.61E-07	0.00	∞	1.00
		SE20	0.77	0.37	1.60	0.49
		SE100	0.63	0.06	6.29	0.70
		all STM	1.32	0.21	8.18	0.77
		all SE	0.79	0.42	1.48	0.46
		all capsules	0.86	0.49	1.51	0.59
RVS compared to MSRV	XLD	STM5	2.95E-08	0.00	∞	1.00
		STM50	1.61E-07	0.00	∞	1.00
		SE20	0.40	0.17	0.90	0.03
		SE100	1.95E-07	0.00	∞	0.99
		all STM	1.22E-07	0.00	∞	0.99
		all SE	0.40	0.19	0.84	0.02
		all capsules	0.37	0.18	0.77	0.01
MKTTn compared to MSRV	XLD	STM5	1.63E-08	0.00	∞	1.00
		STM50	1.00	0.00	∞	1.00
		SE20	0.49	0.21	1.15	0.10
		SE100	3.45E-08	0.00	∞	1.00
		all STM	1.21E-08	0.00	∞	1.00
		all SE	0.49	0.22	1.07	0.07
		all capsules	0.43	0.20	0.89	0.02

Grey cells: significant difference $p < 0.05$.

The interpretation of Table 21 is similar to that of Table 20, except that selective enrichment media are mutually compared, given XLD as isolation medium. The odds of finding *Salmonella* for the SE20 capsules are significant higher when MSRV is used compared to RVS.

Figure 5 shows the performance of each laboratory as odds ratios compared to the mean of all laboratories for the artificially contaminated samples. The blank capsules are not used in this calculation. The mean (= 1) is defined as the odds of detecting *Salmonella* based on the fixed effects only (capsule, enrichment medium and isolation medium). Laboratories below the mean have a lower odds to detect *Salmonella*. Twelve laboratories 4, 6, 7, 8, 11, 15, 16, 19, 20, 21, 31 and 32 found a higher number of positive results compared to the mean of all laboratories; they scored all samples correctly for all used media. In general, the laboratories performed very well. There is only a small difference between the performance of the laboratories just above the mean (laboratory 3) or just below the mean (laboratory 25). Laboratory 3 missed only one capsule on one isolation medium and laboratory 25 missed only one capsule on all used media. Fourteen laboratories scored a significant lower performance. Laboratory 12 showed the lowest performance.
The majority of the laboratories scored very well in this study, resulting in a high mean of all laboratories. The performance of laboratory 23 is closest to the mean of all laboratories; they missed just two isolates, both on different selective enrichment media and different isolation media. As for

determining the performance of a laboratory, all used media were taken into account, lab 23 showed correct results for all capsules in Table 18 and Annex 7.

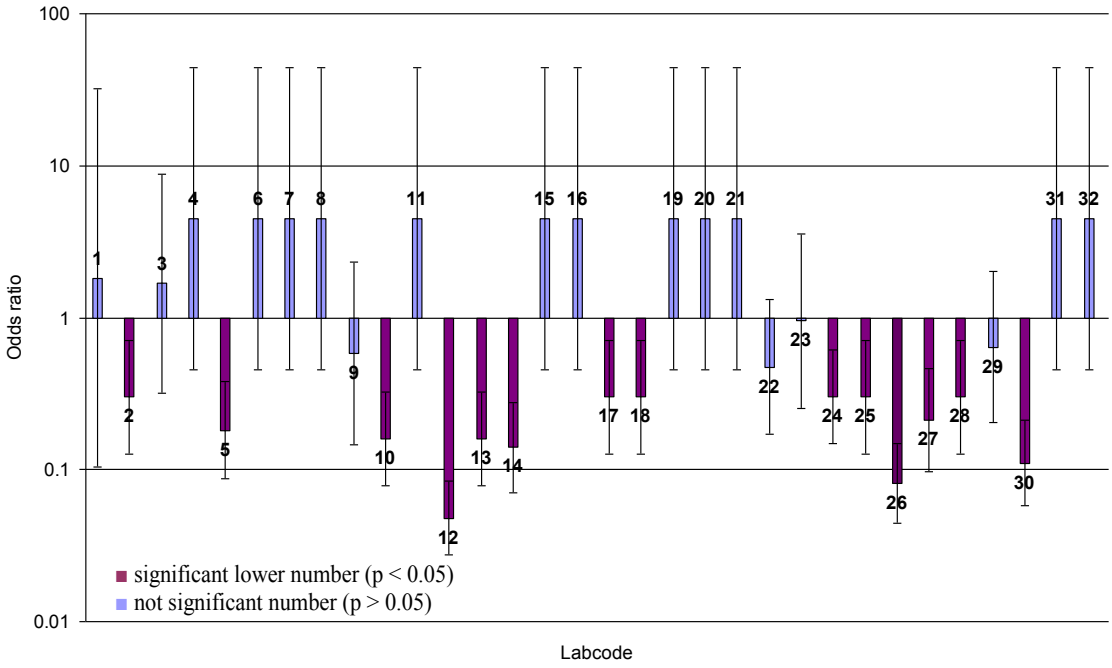


Figure 5 Performance of each laboratory compared to the mean of all laboratories for the artificially contaminated minced chicken meat samples (without blanks).

4.5.3 Specificity, sensitivity and accuracy rates of the artificially contaminated minced chicken meat samples

Table 22 shows the specificity, sensitivity and accuracy rates for all types of capsules added to the minced chicken meat. The results are given for the different medium combinations: pre-enrichment in BPW, followed by selective enrichment in RVS, MKTTn and on MSRV and isolation on selective plating agar XLD. The calculations were performed on the results of all participants and on the results of only the EU-MS (without candidate EU-MS and countries of the European Free Trade Association). It was expected that the blank capsules and the high-level materials would score close to 100% and the low-level materials a minimum of 75–80%. The rates showed the expected or even better results. The specificity rates (of the blank capsules) were for all three selective enrichment media 99%. The highest rates were found with MSRV. The lowest sensitivity rates were found for the SE20 capsules with RVS (85%) closely followed by MKTTn (87%). With MSRV a sensitivity rate of 93% was found for the SE20 capsules. The sensitivity rates for the other capsules were close to 100%. There was no difference between rates of EU-MSs and the four non-EU-MSs. The rates for the RVS for all participants were a little lower because of the mistake made by a non EU-MS (laboratory 27).

Table 22 Specificity, sensitivity and accuracy rates for all participating laboratories of the artificially contaminated meat samples (each capsule added to 10 g minced chicken meat) for the selective enrichment in RVS, MKTTn and on MSRV and plating out medium XLD

Capsules with minced chicken meat		RVS/XLD*		MKTTn/XL#		MSRV/XLD	
		All n=30	EU n=27	All n=31	EU n=28	All n=32	EU n=28
Blank (n=5)	No. of samples	150	135	155	140	160	140
	No. of negative samples	149	134	154	139	158	138
	Specificity in%	**99.3**	**99.3**	**99.4**	**99.3**	**98.8**	**98.6**
STM5 (n=5)	No. of samples	150	135	155	140	160	140
	No. of positive samples	148	134	151	136	160	140
	Sensitivity in%	**98.7**	**99.3**	**97.4**	**97.1**	**100**	**100**
STM50 (n=5)	No. of samples	150	135	155	140	160	140
	No. of positive samples	149	135	155	140	160	140
	Sensitivity in%	**99.3**	**100**	**100**	**100**	**100**	**100**
SE20 (n=5)	No. of samples	150	135	155	140	160	140
	No. of positive samples	127	112	135	121	148	128
	Sensitivity in%	**84.7**	**83.0**	**87.1**	**86.4**	**92.5**	**91.4**
SE100 (n=5)	No. of samples	150	135	155	140	160	140
	No. of positive samples	147	134	153	138	160	140
	Sensitivity in%	**98**	**99.3**	**98.7**	**98.6**	**100**	**100**
All capsules with *Salmonella*	No. of samples	600	540	620	560	640	560
	No. of positive samples	571	515	594	535	628	548
	Sensitivity in%	**95.2**	**95.4**	**95.8**	**95.5**	**98.1**	**97.9**
All capsules	No. of samples	750	675	775	700	800	700
	No. of correct samples	720	649	748	674	786	686
	Accuracy in%	**96**	**96.2**	**96.5**	**96.3**	**98.3**	**98**

*Results without Laboratory 1 (non-EU-MS) and 9 (EU-MS): they did not use RVS.

#Results without Laboratory 1 (non-EU-MS): they did not use MKTTn.

4.6 Own method

PCR

Six laboratories (lab codes 5, 7, 10, 13, 23 and 26) applied a PCR method as an additional detection technique. Five laboratories tested the samples after pre-enrichment in BPW and one NRL (lab 13) after selective enrichment on MSRV. Table 23 summarises further details on the volumes used in the PCR techniques.

Table 23 Details on real time Polymerase Chain Reaction procedures, used as own method during the interlaboratory comparison study by six laboratories

Lab code	Volume of BPW (µl)	Volume of DNA sample (µl)	Volume of DNA / PCR mix (µl)
5	400	500	30/ kit
7	5	200	50/ unknown
10	1000	50	5/ 50
13	-	300	5/ 25
19	1000	100	5/ 50
23	10	200	50/ tablet

Laboratory 10 used a non commercial and non validated PCR. Laboratory 13 used a non-commercially available Real-time PCR validated for the matrices: chicken rinse, minced meat and raw milk (Malorny et al., 2004). The other laboratories used a commercially available PCR. Laboratory 5 used a non validated Real time PCR, Laboratory 7 an endpoint PCR (BAX) validated for food (Nordval 030), laboratory 23 a PCR (BAX) validated for food and water (AFNOR 100201) and laboratory 19 a real-time PCR (iQ check, Biorad) validated for food (Nordval 2004-30-5408-00035).

All the laboratories found the same results with the PCR technique as with the bacteriological culture methods. Laboratories 5, 7 and 23 scored all samples correctly. Laboratories 10 and 13 missed one SE20 sample, which was comparable with selective enrichment on MSRV. Laboratory 19 missed one control capsule of Span5, which was comparable to the bacteriological culture method; they missed this capsule with all selective enrichment media.

4.7 Performance of the NRLs

4.7.1 General

Thirty-one NRLs fulfilled the criteria of good performance.

Laboratory 12 scored below the criteria of good performance with the prescribed and requested method. The difficulties were found with the blank control capsules and SE20 capsules with the addition of meat. They found one blank control capsule (without matrix) positive, missed one of the two SE20 capsules (without matrix) on all used media, and for the minced chicken meat samples artificially contaminated with SE20 they found four capsules negative when using RVS and MKTTn and three capsules negative when using MSRV.
This laboratory was contacted by the CRL-*Salmonella* in November 2009 to ask for any explanations for the deviating results. The NRL investigated possible reasons and checked their procedures. They found the same sero-type and phage-type for the false positive sample as for the samples artificially contaminated with *S.* Typhimurium. This supported the idea that cross-contamination among the samples may have happened. Furthermore, the proficiency test was carried out by two laboratories in the institute (one food and one veterinary laboratory). The pre-enrichment step was performed by one laboratory and both laboratories next analysed the pre-enrichment samples separately. Since the same false positive sample was identified by both laboratories, it was very likely that the contamination happened during the first phase of the protocol (preparation of the samples or pre-enrichment). The

sensitivity problem (low numbers of positives with low contaminated SE samples) was most likely caused by problems with the media.
The NRL took some measures to solve the problems, amongst others, an intensive cleaning and disinfection of the laboratory. To reduce the risk of cross-contamination, some new rooms were refurbished to separate different phases of analysis and the plastic bags used for the pre-enrichment of BPW were changed into plastic jars. Furthermore, negative controls were added to the start and the end of the daily routine *Salmonella* analysis as well as sensitivity control of new batches of media. To check whether the actions taken have been successful, laboratory 12 participated in a follow-up study organised by the CRL-*Salmonella* in January 2010.

Two laboratories (lab codes 1 and 9) did not use both prescribed selective enrichment media, according to ISO 6579 (2002). Both laboratories were contacted by the CRL-*Salmonella* in November 2009 to ask why they did not use the prescribed method ISO 6579 procedure for the detection of *Salmonella* in food samples in EU legislation.
Laboratory 1, a non-EU-MS, did not use either one of the prescribed methods (RVS and MKTTn). The laboratory indicated that they routinely use only RV for selective enrichment of *Salmonella* from food samples and have no experience with MKTTn or MSRV. For this study they used the requested selective enrichment medium MSRV as an opportunity to test the method but they are not planning to use MSRV routinely.
Laboratory 9 (EU-MS) did not use the RVS method. It was decided by the ministry of the country of this NRL that all official food laboratories will use the same microbiological methods (MSRV and MKTTn) in the framework of their official food control. The laboratories have much experience with the use of MSRV for the detection of *Salmonella* in food and veterinary samples and they kept the MKTTn medium to conform with the ISO 6579. Unfortunately, the CRL-*Salmonella* did not get the validation data asked for from this NRL.
Both laboratories used Annex D of ISO 6579 (MSRV) to analyse the chicken meat samples. The results found with this procedure fulfilled the criteria of good performance and no further action was taken.

Laboratory 27 fulfilled the criteria of good performance with MKTTn and MSRV but they made a mistake with the RVS method. The NRL did not have results for the control samples and a part of the artificially contaminated samples. This laboratory was contacted by the CRL-*Salmonella* in November 2009 to ask the reason for this mistake and what they did to prevent this in the future. The NRL investigated a possible reason. The ring trial is a substantial workload upon the lab staff, the samples are not similar to routine samples and the message given about this particular work step was not properly received and executed. The person who transferred the incubated BPW to the selective enrichment media oversaw the racks with RVS-tubes that were ready to use. The incident was reported through their quality system as a non conformance, discussed with the persons who failed in their communication and at their staff meeting. It was concluded that this was not a systemic error and no further actions were necessary.

4.7.2 Follow-up study

The set-up of the follow-up study was the same as the full interlaboratory comparison study in October 2009, only with another number of samples (see section 4.1 ‘Reference materials’). In this follow-up study, more blank and SE20 capsules were tested, as these samples were causing most of the problems. Table 24 gives an overview.

Table 24 Overview of the types and the number of capsules tested by laboratory 12 in the follow-up of the interlaboratory comparison study

Capsules	Control capsules (n=7) no meat added	Test samples (n=15) with 10 g *Salmonella* negative minced chicken meat
S. Enteritidis 20 (SE20)	3	4
S. Enteritidis 100 (SE100)	-	2
S. Typhimurium 5 (STM5)	-	3
S. Typhimurium 50 (STM50)	1	-
Blank	3	6

Laboratory 12 showed repeatedly deviating results in ring trials since 2007 (veterinary as well as food studies). Two staff members of the CRL-*Salmonella* visited this laboratory while they performed the follow-up for this study (starting on 25 January 2010). During a two-day visit (25 and 26 January 2010) the procedures were checked for possible (technical) problems explaining the deviating results.

On Monday 18 January 2010, one parcel with two plastic containers was sent to laboratory 12 containing: 7 control capsules (numbered C1 – C7), 15 capsules (numbered 1 – l5), 400 g minced chicken meat and 1 temperature recorder.
The performance of this follow-up study started on 25 January 2010. The laboratory had to follow the same SOP and protocol as in the study of October 2009 (see Annexes 4 and 5). The test report was different from the October study (see Annex 8). For the media only, the differences with the October study needed to be indicated.
During the visit at the veterinary, food and media (preparation) department of laboratory 12, some technical deviations were observed. These deviations were seen during the handling of the samples and during several steps in the *Salmonella* detection procedure. At the end of the visit, a report with observations and advice for possible improvements was made by the staff members of the CRL-*Salmonella* and handed over to the staff members of laboratory 12.
For the media compositions, incubation times and temperatures, no differences were observed in comparison with the full study.
Laboratory 12 correctly scored all blanks negative but could not detect *Salmonella* Enteritidis (SE20) in two to three (depending on the medium used) of the four SE20 capsules with matrix (meat). The NRL also could not detect one of the three SE20 control capsules (without meat).
Still, the visit was considered successful, as laboratory 12 fulfilled the criteria of good performance (see section 3.6) for the test samples in this follow-up study, be it at the minimum level for the sensitivity result.

5 Discussion

Transport of the samples
Neither transport time nor transport temperature seem to have negatively affected the results. The laboratories with the longest transport times and/or the highest transport temperatures (lab codes 6, 10, 14, 26 and 28) still found good results.

Performance of the laboratories
The prescribed method (ISO 6579: RVS and MKTTn) and the requested method (Annex D of ISO 6579: MSRV) were used by 30 laboratories. Two laboratories (lab codes 1 and 9) used different methods (deviating from EU legislation).
Laboratory 1, a non-EU-MS, did not use RVS and MKTTn but used RV. The laboratory did use MSRV in this ring trial as a test but mentioned that they are not planning to use MSRV routinely. The CRL-*Salmonella* requested all participants to use MSRV in addition to the prescribed methods for food and feed matrices because good experiences have been gained with this method. Although the scope of Annex D of ISO 6579 is detection of *Salmonella* spp. in samples of the primary production, MSRV showed, in this and earlier comparison studies, better results when compared to the results found with the prescribed method for food analyses (RVS and MKTTn: ISO 6579).
Laboratory 9 (EU-MS) did not use RVS but MSRV in addition to MKTTn. The NRL indicated this to be the nationally prescribed method for the detection of *Salmonella* in food and animal feed samples. Unfortunately, no validation data could be shown to the CRL.
The results of both laboratories fulfilled the criteria of good performance and no further action was taken.

To determine 'good performance' per laboratory all combinations of selective enrichment media and isolation media used by each laboratory were taken into account. Thirty-one out of 32 laboratories scored a 'good performance'. One laboratory (lab 12) scored under the level of 'good performance'.

In a time frame of three years, laboratory 12 scored three times below the level of good performance in different ring trials, therefore it was decided to visit this laboratory at the time they performed a follow-up study. It was important that the follow-up, in combination with the visit of two members of the CRL-*Salmonella*, took place before the next detection study (planned in February/March 2010). The visit was focused on possible technical deviations and advise was given on possible improvements. In the full study, this NRL found positive blanks with all methods, which was most probably caused by cross-contamination in the first steps of the protocol. The laboratory also showed to have a sensitivity problem (low numbers of positives with low contaminated SE samples), which was most likely caused by problems with the media in combination with a low start temperature of the incubators. The laboratory planned some measures to solve the problems but not all actions could be performed before the start of the follow-up study in January 2010. The incubator was at too low a temperature before the start of the dissolving of the capsules and the incubation of the BPW. The temperature during the dissolving step was between 35.5–36.4 ^{o}C and the temperature during the pre-enrichment in BPW was between 35.4–37 ^{o}C. According to the Standard Operating Procedure of the ring trial for the reconstitution of the capsules, the BPW with the capsules should be placed at 37 ± 1 ºC. A lower temperature of the BPW may result in not completely dissolved gelatin capsules and this is essential for the detection of *Salmonella* in the capsules. In this study, especially the growth of S. Enteritidis may have been affected, as this serovar grows slower than S. Typhimurium. The low temperatures of the BPW are most likely explanations for the low number of positive SE20 samples found by the laboratory in the follow-up study.

Despite the remaining sensitivity problems, the laboratory scored good performance in the follow-up study. This time, the specificity rates were 100% for the control samples as well as for the artificially contaminated samples. The NRL improved their performance but will continue with their measures to further improve their sensitivity rates.

Good results were found with MSRV. Although the scope of Annex D of ISO 6579 is detection of *Salmonella* spp. in samples of the primary production, in this study it showed better results when compared to the results found with the prescribed method for food analyses (ISO 6579). The selective enrichment medium MKTTn of ISO 6579 showed the lowest number of positive results.

Specificity, sensitivity and accuracy rates
The rates of the control samples were good. For the high-level SE capsules the rates were 100%. For the low-level materials SE20, SPan5 and STM5, the rates were close to 97%.
Although the mean contamination level of the SE100 capsules was decreased under the minimum target level, the sensitivity was still 98–100%. All laboratories were able to find all SE100 capsules positive after selective enrichment in MSRV.

Media
Deviations in media compositions or incubation temperatures were reported but no clear effects were found on the results.
The increase in the number of positive results after 48 h of incubation of the selective enrichment media was only small when compared to 24 h of incubation: 2–3% for MSRV and RVS; 3–4% for MKTTn.

The choice of plating-out medium does not seem to have a large effect on the number of positive isolations. When MKTTn is used for selective enrichment medium, XLD gave 3% more positive results than other plating-out media. For MSRV and RVS the difference between XLD and another plating-out medium was only 1%.

PCR
Six laboratories used a PCR technique additional to the prescribed and requested methods. The results found with the PCR methods were comparable to the results found with the bacteriological detection methods.

Evaluation of this study
The chosen matrix in this study, minced chicken meat, contained more background flora than the matrices used in earlier food, feed and veterinary studies. The lower disturbance of background flora in the present food study may have positively influenced the detection of *Salmonella* and thus, the outcome of this study.
The procedure for the handling of the ring trial samples (the addition of the capsules and matrix to the BPW) is different from routine samples. Further research will be performed at the CRL-*Salmonella* to improve the set-up of the ring trial with test samples more comparable to routine samples.

6 Conclusions

- All participants achieved the level of 'good performance' for the detection of *Salmonella* in minced chicken meat. One laboratory was visited by the CRL during a follow-up study and reached the desired level.
- The accuracy, specificity and sensitivity rates for the control samples (without matrix) found after selective enrichment in RVS, MKTTn and on MSRV were between 93–100%.
- The specificity rate of the minced chicken meat samples artificially 'contaminated' with blank capsules was 99%.
- For all artificially contaminated minced chicken meat samples with *Salmonella*, the rates found with MSRV were higher than the rates of MKTTn and RVS.
- The sensitivity rates for artificially contaminated minced chicken meat samples with *Salmonella* were 98% after selective enrichment in MSRV. After selective enrichment in MKTTn and RVS the sensitivity rate was 96%.
- The sensitivity rates for minced chicken meat samples artificially contaminated with *Salmonella* Typhimurium were 99–100% and approximately 94–95% for *Salmonella* Enteritidis.
- The low-level materials of *S*. Typhimurium (STM5).were easier to detect than the low-level materials of *S*. Enteritidis (SE20).
- The accuracy rates for the artificially contaminated minced chicken meat samples were 98% for MSRV and 96% for MKTTn and RVS.
- XLD showed slightly more positive results (3%) than other plating-out media independent of the selective enrichment medium used.
- The number of positive isolations is more influenced by the choice of the selective enrichment medium than by the choice of the plating-out medium.
- A longer incubation time than 24 hours gives only 2–4% more positive results after 48h for all selective enrichment media.
- MSRV is a good selective enrichment media for the detection of *Salmonella* in the matrix used (minced chicken meat).

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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	Kuijpers et al., 2009 (Report 330604014)
		5	STM50	53	10 gram			
		5	SE20	18	10 gram			
		5	SE100	84	10 gram			
		5	Blank	0	10 gram			
		3	STM5	6	No			
		2	SE20	18	No			
		1	SE100	84	No			
		2	SPan5	7	No			
		2	Blank	0	No			

[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			
III	2009	5	STM5	6	10 gram	RVS, MKTTn, MSRV and own	XLD and own	This report
		5	STM50	54	10 gram			
		5	SE20	12	10 gram			
		5	SE100	50	10 gram			
		5	Blank	0	10 gram			
		3	STM5	6	No			
		2	SE20	12	No			
		1	SE100	50	No			
		2	SPan5	6	No			
		2	Blank	0	No			

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

Table 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

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

BGA modified (Oxoid CM 0329, Hampshire, United Kingdom) (BPLS Merck 1.10747 Darmstadt, Germany) (Lab M Ltd., lab 34 Bury, United Kingdom) (Biomark Laboratories B439, Maharashtra, India) (Himedia Laboratories M971-500G, Mumbai, India) (Biorad 3564464, Marnes-La-Coquette, France) (SIFIN TN 1110, Berlin, Germany)
Watson and Walker 1978 A modification of brilliant green agar for improved isolation of *Salmonella*. J. Appl. Bact. 45 195-204
Composition of BGA modified: according ISO 6579, 1993

BGA (Oxoid CM 0263, Hampshire, United Kingdom)
Composition of BGA medium: the concentration of the compounds in g/L water: Proteose peptone 10.0, Yeast extract 3.0, Lactose 10.0, Sucrose 10.0, Sodium chloride 5.0, Phenol red 0.08, Brilliant green 0.0125, Agar 12.0, pH 6.9

BPLSA (Merck 107237.0500, Darmstadt, Germany)
Composition of BPLSA medium: the concentration of the compounds in g/L water: Peptone from meat 5.0, Peptone from casein 5.0, Meat extract 5.0, Sodium chloride 3.0, di-sodium hydrogen phosphate 2.0, Lactose 10, Sucrose 10, Phenol red 0.08, Brilliant green 0.0125, Agar agar 12.0, pH 7

Brilliance Salmonella Agar BSA (previously OSCM II) (Oxoid CM 1092, Hampshire, United Kingdom)
Schönenbrücher V, Mallinson ET, Bülte M. A comparison of standard cultural methods for the detection of food-borne 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, Cefsulodin 0.012, Novobiocin 0.005, Agar 25 pH 7.2.

Compass Salmonella : (Biokar Diagnostics BM 06608, Beauvais, France)
Perez JM et al., Comparison of four chromogenic media and Hektoen agar for detection and presumptive identification of Salmonella strains in human stools, J Clin. Microbiol., 2003 Mar., 41(3), 1130 – 4.
Composition of Compass agar : the concentration of the compounds in g/L water: Pepton 10, Sodium chloride 5, Phosphate Buffer 7, Inhibitory agents 9, Chromogenic mixture 1.4, Bacteriological agar 15 pH 7.

Hektoen Enteric (Oxoid CM 0419B, Hampshire, United Kingdom, King, S. a. Metzger, W.J.: A new plating medium for the isolation of enteric pathogens. I. Hektoen Enteric Agar. - Appl. Mikrobiol.,1968: 16; 557-578.
Composition of Compass agar : the concentration of the compounds in g/L water: Proteose peptone 12, Yeast extract 3, Lactose 12, Sucrose 12, Salicilin 2, Bile salts No.3 9, Sodium chloride 5, Sodium thiosulphate 5, Ammonium ferric citrate 1.5, Acid fuchin o.1, Bromothymol blue 0.065, Agar 14, pH7.5.

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, Cromogenic mix 1.5, propylene glycol 10.5, agar-agar 15, Rambach agar supplement 10 ml, pH 7.0-7.3.

Rapid *Salmonella* agar (Biorad 356 4705, Marnes-La-Coquette, France)
Composition of Rapid Salmonella medium: the concentration of the compounds in g/L water Casein Peptone 5, Meat extract 5, Selective agents (Sodium citrate, sodium deoxycholate, ferrammonium citrate) 14, Chromogenic mixture 310 mg, Agar 12, pH 7-7.2.

SM2 = SM ID2 (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) 6.25, Tris 0.16, Lactose 6.0, Ox bile (bovine and swine) 1.5, Cromogenic mix 9.63, Sodium chloride 5.0, Selective mix 0.03, Agar 14, pH not mentioned.

XLT4 (Oxoid CM 1061, Hampshire, United Kingdom)
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 (or niaproof 4 4.6 g), 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 FOOD
organised by CRL-*Salmonella*
FOOD STUDY III - 2009

Introduction

This protocol describes the procedures for the third interlaboratory comparison study on the detection of *Salmonella* spp. in a food matrix amongst the National Reference Laboratories (NRLs for *Salmonella*) in the EU. This study will have a comparable set-up as the earlier studies (food, veterinary and feed) on the detection of *Salmonella* spp. The prescribed method is the procedure as described in ISO 6579 (Microbiology of food and feeding stuffs – Horizontal method for the detection of *Salmonella* spp. Fourth edition, 2002.) Beside ISO 6579 it is requested also to use 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). The method in this annex is especially intended for the detection of *Salmonella* spp. in animal faeces and environmental samples from the primary production stage, but is also applicable for the analyses of food samples. 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 minced chicken meat (*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 meat 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 will contain the minced chicken meat. 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. Do not forget to note your lab code before returning it to the CRL.
Each box with samples 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 3th of October 2009** (this is 4 working days after the day of mailing).

Objectives

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 a food matrix using different methods.

Outline of the study

Each participant will receive (in week 40 of 2009) one box containing 2 biopacks, packed with cooling elements. The containers contain:

Biopack 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 biopack will 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 biopack 1 with capsules at -20 ºC immediate after receipt.

Biopack 2:

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

Store biopack 2 with meat at 5 ºC immediate after receipt.

The performance of the study will be in week 41 (starting on 5 October 2009).

The documents necessary for performing the study are:

- Protocol Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in food III (2009) (this document);
- SOP Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in food III (2009);
- Test report Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in food III (2009);
- ISO 6579 (2002). Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.;
- Amendment 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 23 October 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 short report.**

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

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

Time table of interlaboratory comparison study FOOD III (2009)

Week	Date	Topic
38	14 -18 September	Mailing of the protocol, Standard Operating Procedure and test report to the NRLs-*Salmonella*
40	28 September – 3 October	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 parcel (leave it in the plastic bag with lab code) and return it to CRL-*Salmonella* using the return envelope; - **Store the capsules at -20 ± 5 °C** - **Store the meat at +5 ± 3 °C** **If you did not receive the parcel at 3 October, do contact the CRL immediately.** Preparation of: 1. Non selective pre-enrichment medium (see SOP 6.1) 2. Selective enrichment media (see SOP 6.2) 3. Solid selective plating media (see SOP 6.3) 4. Confirmation media (see SOP 6.4)
41	5 – 9 October	Performance of the study, following the instructions as given in the protocol and the SOP of study Food III (2009).
43	Before 23 October	Completion of the test report. Send the test report by e-mail to the CRL *Salmonella* Angelina.kuijpers@rivm.nl *.
44	26 - 30 October	Checking the results by the National Reference Laboratories.
	December 2009	Sending of the final results to the NRLs together with a short report. A follow-up will be discussed with NRLs who showed no good performance, according to pre-defined criteria.

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

rivm

Annex 5 Standard Operating Procedure

SOP
INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN FOOD
organised by CRL-*Salmonella*
FOOD STUDY III- 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 a food matrix. 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, minced chicken meat (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.

Amendment 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.

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
MKTTn	uller Kaufmann Tetrathionate novobiocin broth
MSRV	Modified semi-solid Rappaport Vassiliadis medium
RM	Reference Material
RVS	Rappaport Vassiliadis medium with Soya
SOP	Standard Operating Procedure
XLD	Xylose Lysine Deoxycholate agar

6 Culture media

For this study the prescribed method is ISO 6579, with an extra incubation step of 24 h of the selective enrichment media. Additional to ISO 6579 it is requested also to apply Annex D of ISO 6579.

Non selective pre-enrichment medium	BPW
Selective enrichment medium	MKTTn & RVS (prescribed)
	MSRV (requested)
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 (ISO 6579) and requested 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) O6579 Annex B.1)

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

6.2 Selective enrichment medium

- Rappaport Vassiliadis medium with soya (RVS broth) (ISO6579 Annex B.2)
- Muller Kauffmann tetrathionate-novobiocin broth (MKTTn) (ISO6579 Annex B.3)
- Modified Semi solid Rappaport Vassiliadis (MSRV) (requested) (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-Desoxycholate (140 mm and 90 mm plates) (ISO6579 Annex B.4)
- Second isolation medium for choice (obligatory)
- Own medium routinely used in your laboratory (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) (ISO6579Annex B.5)

7 Apparatus and glassware

The usual used 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 and of 10 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 and 1 ml;
- Petri dishes; standard size (diameter 90 mm to 100 mm) and/or large size (diameter 140 mm).

8 Procedure

Below the prescribed and the requested method of the interlaboratory comparison study in a food matrix of CRL-*Salmonella* is described. The different steps in the procedure are also summarized in Annex A of this SOP. Beside these methods it is also allowed to use one or more own methods. Please record all relevant data in the test report. Details of the prescribed method can be found in ISO 6579. Details of the requested method can be found in Annex D of ISO 6579 (2007).

8.1 Prewarming BPW (day 0)

Label 25 jars 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 meat is added and one jar is a negative meat control to which only 10 g minced chicken meat 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 minced chicken meat to the jars as follows :

- **Add 10 g of minced chicken meat to jars labelled 1-25 and C12,**
- **Add no meat to jars labelled C1 - C11,**

Do not shake the jars after adding the minced chicken meat.

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 20 & 29 of the test report.

8.3 Selective enrichment (day 2)

Allow the selective enrichment broths RVS and MKTTn (prescribes method) to equilibrate to room temperature, if they were stored at a lower temperature. Dry the surface of the MSRV plates (requested method) in a Laminair Air Flow cabinet if necessary. Record (page 4-11) the requested data of the selective enrichment broths (RVS and MKTTn), MSRV plates and own selective enrichment media (if used) in the test report. Label 25 jars/tubes/plates of each selective enrichment medium from 1 to 25. Also label 12 jars/tubes/plates from C1 to C12. All selective media are incubated 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 above.

After equilibration:
Prescribed methods:

- Transfer 0.1 ml of each BPW culture to each tube with a corresponding label containing 10 ml RVS medium. Incubate at 41.5 °C ± 1 °C for 24 h ± 3 h and later on for another 24 h ± 3 h;
- Transfer 1 ml of each BPW culture to each tube with a corresponding label containing 10 ml MKTTn medium. Incubate at 37 °C ± 1 °C for 24 h ± 3 h and later on for another 24h ± 3 h;

Requested method:

- Inoculate each MSRV plate with three drops of each BPW culture with a corresponding label. Inoculate a MSRV plate 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 the test report). Incubate at the temperature 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 4-11).

8.4 Isolation media (first and second isolation) (day 3 and 4)

Record in the test report (page 12-17) the requested data of the isolation media used. Label two times 25 large size Petri dishes and 25 standard size Petri dishes of the isolation media from 1 to 25 and label two times 12 large size Petri dishes and 12 standard size Petri dishes from C1 to C12.

Note:
In case you do not have large dishes (140 mm) at your disposal use two standard size (90 -100 mm) dishes, one after the other, using the same loop.

First isolation after 24 h
Inoculation:
Inoculate, by means of a 10 µl loop, from MKTTn and RVS cultures the surface of isolation media in large size Petri dishes (or two standard size Petri dishes) with the corresponding label numbers. Use a 1 µl loop to inoculate from suspect MSRV plates, the surface of isolation media in one standard size Petri dish with the corresponding label number. The following isolation media will be used:

1) Xylose Lysine Desoxycholate agar (XLD)
   Place the inoculated plates with the bottom up in the incubator set at 37 °C (record temperature and time and other requested data in the test report, page 12-13).
2) Second isolation medium. Follow the instructions of the manufacturer (record temperature and time and other requested data in the test report, page 14-15).
3) Optionally: selective isolation medium/media routinely used in your laboratory. Incubate the medium/media at the temperature routinely used (record temperature and time and other requested data in the test report, page 16-17).

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 selective 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 on the test report (page 18) the requested data of the nutrient agar. 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 RVS), Table 2 (isolation using MKTTn), Table 3 (isolation using MSRV) and Table 4 (isolation using own enrichment) on the test report pages 21-28. For the results of detection of *Salmonella* using PCR fill in Table 5 on the test report page 29.

Confirmation of identity
The identity from the colony selected 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. Note in the test report (page 19) which media/tests have been used for confirmation. The interpretation of the biochemical tests is given in Table 1 of ISO 6579:2002 on page 9. Optionally inoculate other media which are routinely used for confirmation. Record in the test report (page 19) the requested data.

9 Test report

The test report will contain all information that might influence the results and is not mentioned in this SOP. Some incidents or deviations from the specified procedures will also be recorded. The test report will include the names of the persons, who are carrying out the work and will 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 FOOD III (2009)
On detection of Salmonella spp. in minced chicken meat (see Annex A)

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 minced chicken meat to BPW Incubate 18 h ± 2 h at 37 °C ± 1 °C
2	**Selective enrichment**	0.1 ml BPW culture in 10 ml RVS, incubate at (41.5 ± 1) °C for (24 ± 3) h 1 ml BPW culture in 10 ml MKTTn, incubate at (37 ± 1) °C for (24 ± 3) h 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 RVS, MKTTn, suspect MSRV plates (24h) and own medi(um)(a): ➢ Xylose Lysine Desoxycholate 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 RVS, MKTTn, MSRV and own medium another 24 (± 3) hours at the relevant temperatures
4	**Second isolation after 48 h**	If the first isolation was negative, inoculate from RVS, MKTTn, suspect MSRV plates (48 h) and Own medi(um)(a): ➢ Xylose Lysine Desoxycholate agar ➢ Second isolation medium ➢ Own selective medi(um)(a)
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 first isolation media (day 4).

Annex 6 Number of positive results of the control samples (capsule without food) per laboratory and per selective enrichment medium in combination with the isolation medium that gives the highest number of positive isolations

Labcode	RVS					MKTTn					MSRV				
	Blank n=2	SPan5 n=2	STM5 n=3	SE20 n=2	SE100 n=1	Blank n=2	SPan5 n=2	STM5 n=3	SE20 n=2	SE100 n=1	Blank n=2	SPan5 n=2	STM5 n=3	SE20 n=2	SE100 n=1
Good Performance	**0**	**≥ 1**	**≥ 2**	**≥ 1**	**1**	**0**	**≥ 1**	**≥ 2**	**≥ 1**	**1**	**0**	**≥ 1**	**≥ 2**	**≥ 1**	**1**
1*	0	2	3	2	1	-	-	-	-	-	0	2	3	2	1
2	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
3	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
4	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
5	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
6	0	2	**2**	2	1	0	2	**2**	2	1	0	2	**2**	2	1
7	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
8	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
9	-	-	-	-	-	0	2	3	2	1	0	2	3	2	1
10	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
11	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
12	**1**	2	3	**1**	1	**1**	2	3	**1**	1	**1**	2	3	**1**	1
13	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
14	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
15	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
16	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
17	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
18	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
19	0	**1**	3	2	1	0	**1**	3	2	1	0	**1**	3	2	1
20	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1

Lab code	RVS					MKTTn					MSRV				
	Blank n=2	SPan5 n=2	STM5 n=3	SE20 n=2	SE100 n=1	Blank n=2	SPan5 n=2	STM5 n=3	SE20 n=2	SE100 n=1	Blank n=2	SPan5 n=2	STM5 n=3	SE20 n=2	SE100 n=1
Good Performance	**0**	**≥ 1**	**≥ 2**	**≥ 1**	**1**	**0**	**≥ 1**	**≥ 2**	**≥ 1**	**1**	**0**	**≥ 1**	**≥ 2**	**≥ 1**	**1**
21	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
22	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
23	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
24	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
25	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
26	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
27**	0	0	0	0	0	0	2	3	2	1	0	2	3	2	1
28	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
29	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
30	0	2	3	**1**	1	0	2	3	**1**	1	0	2	3	2	1
31	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
32	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1

-: not performed.

bold numbers: deviating results.

grey cells : results are below the criteria of good performance.

*Laboratory 1 did not use RVS but used RV.

**Laboratory 27 made a mistake when using RVS.

Annex 7 Number of positive results of the artificially contaminated chicken meat samples

(with capsule) per laboratory and per selective enrichment medium in combination with the isolation medium that gives the highest number of positive isolations.

Lab code	RVS					MKTTn					MSRV				
	Blank n=5	STM5 n=5	STM50 n=5	SE20 n=5	SE100 n=5	Blank n=5	STM5 n=5	STM50 n=5	SE20 n=5	SE100 n=5	Blank n=5	STM5 n=5	STM50 n=5	SE20 n=5	SE100 n=5
Good Performance	**≤ 1**	**≥ 2**	**≥ 4**	**≥ 2**	**≥ 4**	**≤ 1**	**≥ 2**	**≥ 4**	**≥ 2**	**≥ 4**	**≤ 1**	**≥ 2**	**≥ 4**	**≥ 2**	**≥ 4**
1*	0	5	5	5	5	-	-	-	-	-	0	5	5	5	5
2	**1**	5	5	**4**	5	**1**	5	5	**4**	5	**1**	5	5	**4**	5
3	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
4	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
5	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
6	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
7	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
8	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
9	-	-	-	-	-	0	5	5	**4**	5	0	5	5	5	5
10	0	5	5	**3**	5	0	5	5	**3**	5	0	5	5	**4**	5
11	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
12	0	5	5	**1**	5	0	5	5	**1**	5	0	5	5	**2**	5
13	0	5	5	**4**	5	0	5	5	**4**	5	0	5	5	**4**	5
14	0	5	5	**4**	5	0	5	5	**3**	5	0	5	5	5	5
15	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
16	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
17	0	5	5	**4**	5	0	5	5	**4**	5	0	5	5	**4**	5
18	0	5	5	**4**	5	0	5	5	**4**	5	0	5	5	**4**	5
19	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
20	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5

Lab code	RVS					MKTTn					MSRV				
	Blank n=5	STM5 n=5	STM50 n=5	SE20 n=5	SE100 n=5	Blank n=5	STM5 n=5	STM50 n=5	SE20 n=5	SE100 n=5	Blank n=5	STM5 n=5	STM50 n=5	SE20 n=5	SE100 n=5
Good Performance	**≤ 1**	**≥ 2**	**≥ 4**	**≥ 2**	**≥ 4**	**≤ 1**	**≥ 2**	**≥ 4**	**≥ 2**	**≥ 4**	**≤ 1**	**≥ 2**	**≥ 4**	**≥ 2**	**≥ 4**
21	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
22	0	5	5	**4**	5	0	5	5	5	5	0	5	5	5	5
23	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
24	0	5	5	**4**	5	0	5	5	**4**	5	**1**	5	5	**4**	5
25	0	5	5	**4**	5	0	5	5	**4**	5	0	5	5	**4**	5
26	0	5	5	**2**	5	0	**4**	5	**3**	**4**	0	5	5	**4**	5
27**	0	**4**	**4**	5	**3**	0	5	5	5	5	0	5	5	5	5
28	0	5	5	5	5	0	5	5	**4**	5	0	5	5	5	5
29	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
30	0	5	5	**3**	5	0	**4**	5	**3**	5	0	5	5	**4**	5
31	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
32	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5

-: not performed.

bold numbers: deviating results.

grey cells : results are below the criteria of good performance.

*Laboratory 1 did not use RVS but used RV.

**Laboratory 27 made a mistake when using RVS.

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Annex 8 Test report follow-up study

INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN FOOD
organised by CRL-*Salmonella*
FOOD STUDY III
FOLLOW UP January 2010

Laboratory code This is the same code as in FOOD III 2009	
Laboratory name	
Address	
Country	
Date of arrival of the parcels	…… - ……. – 2010
Start time of storage at - 20 °C (capsules) Start time of storage at + 5 °C (meat)	Date:……. Time:…… Date:……. Time:……
Parcels damaged?	☐ Yes ☐ No
Starting date testing	…… - ……. – 2010

PRE-ENRICHMENT – Buffered Peptone Water (BPW)	
Medium information BPW	
Was the composition of BPW the same as used in BRO FOOD III 2009 ?	
☐ Yes	
☐ No please give more details in an annex :	
Preparation of BPW	
Date of preparation	…… - ……. – 2010
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 (at least overnight)	
At the start	Date: …… - ……. – 2010 time: ……... h ……..…… min temperature incubator: ……..…… °C
At the end	Date: …… - ……. – 2010 time: …… h …… min temperature incubator: ……..…… °C
Incubation time and temperature for dissolving the capsules (45 min)	
At the start	Date: …… - ……. – 2010 time: ……... h ……..…… min temperature incubator: ……..…… °C
At the end	time: …… h …… min temperature incubator: ……..…… °C
Incubation time and temperature for pre-enrichment (18 ± 2) hrs after adding the meat	
At the start	Date: …… - ……. – 2010 time: ……... h ……..…… min temperature incubator: ……..…… °C
At the end	Date: …… - ……. – 2010 time: ……... h ……..…… min temperature incubator: ……..…… °C

SELECTIVE ENRICHMENT - Rappaport Vassiliadis Soya medium (RVS)
Medium information RVS

Was the composition of RVS the same as used in BRO FOOD III 2009 ?	
☐ Yes	
☐ No please give more details in an annex :	
Preparation of RVS	
Date of preparation	…… - ……. – 2010
pH after preparation	……., measured at ……. °C
pH at the day of use	……., measured at ……. °C
Did you perform quality control of RVS?	☐ Yes ☐ No
Incubation time and temperature for selective enrichment	
At the start of the first period (24 h)	Date: …… - ……. – 2010 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the first period (24 h)	Date: …… - ……. – 2010 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the start of the second period (48 h)	Date: …… - ……. – 2010 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the second period (48 h)	Date: …… - ……. – 2010 time: …….. h ……..…… min temperature incubator: ……..…… °C

SELECTIVE ENRICHMENT - Muller Kauffmann Tetra Thionate + novobiocin (MKTTn)	
Medium information MKTTn	
Was the composition of MKTTn the same as used in BRO FOOD III 2009 ?	
☐ Yes	
☐ No please give more details in an annex :	
Preparation of MKTTn	
Date of preparation	…… - ……. – 2010
pH after preparation	……., measured at ……. °C
pH at the day of use	……., measured at ……. °C
Did you perform quality control of MKTTn?	☐ Yes ☐ No
Incubation time and temperature for selective enrichment	
At the start of the first period (24 h)	Date: …… - ……. – 2010 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the first period (24 h)	Date: …… - ……. – 2010 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the start of the second period (48 h)	Date: …… - ……. – 2010 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the second period (48 h)	Date: …… - ……. – 2010 time: …….. h ……..…… min temperature incubator: ……..…… °C

SELECTIVE ENRICHMENT - Modified Semi solid Rappaport Vassiliadis medium (MSRV)	
Medium information MSRV	
Was the composition of MSRV the same as used in BRO FOOD III 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	…… - …….. - 2010
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	
At the start of the first period (24 h)	Date: …… - ……. – 2010 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the first period (24 h)	Date: …… - ……. – 2010 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the start of the second period (48 h)	Date: …… - ……. – 2010 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the second period (48 h)	Date: …… - ……. – 2010 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 FOOD III 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 FOOD III 2009 ?	
☐ Yes	
☐ No please give more details in an annex :	
Preparation of XLD	
Date of preparation	…… - …….. – 2010
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	
At the start of the first period (24 h)	Date: …… - ……. - 2010 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the first period (24 h)	Date: …… - ……. - 2010 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the start of the second period (48 h)	Date: …… - ……. - 2010 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the second period (48 h)	Date: …… - ……. - 2010 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 FOOD III 2009 ?
☐ Yes

☐ No please give more details in an annex :	
Preparation of the second isolation medium	
Date of preparation	…… - …….. – 2010
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	
At the start of the first period (24 h)	Date: …… - ……. - 2010 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the first period (24 h)	Date: …… - ……. - 2010 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the start of the second period (48 h)	Date: …… - ……. – 2010 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the second period (48 h)	Date: …… - ……. – 2010 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 FOOD III 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 FOOD III 2009 ?	
☐ Yes	
☐ No please give more details in an annex :	
Preparation of the nutrient agar	
Date of preparation	…… - …….. – 2010
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/tests did you use for confirmation ?
☐ Biochemical: ☐TSI ☐UA ☐ 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

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

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

	RVS 24 hours						RVS 48 hours					
sample no.	XLD		Second isolation medium		Own isolation medium		XLD		Second isolation medium		Own isolation medium	
	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 MKTTn (dish numbers 1-15, C1-C7, C11 and C12

	MKTTn 24 hours						MKTTn 48 hours					
sample no.	XLD		Second isolation medium		Own isolation medium		XLD		Second isolation medium		Own isolation medium	
	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 3: Results of isolation using MSRV (dish numbers 1-15, C1-C7, C11 and C12)

	MSRV 24 hours						MSRV 48 hours					
Sample no.	XLD		Second isolation medium		Own isolation medium		XLD		Second isolation medium		Own isolation medium	
	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 4: Results of isolation using OWN selective enrichment medium (dish numbers 1-15, C1-C7, C11 and C12)

	OWN 24 hours						OWN 48 hours					
sample no.	XLD		Second isolation medium		Own isolation medium		XLD		Second isolation medium		Own isolation medium	
	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 5: Results of detection using PCR (dish numbers 1-15, C1-C7, C11 and C12)

sample no.	PCR + or -		
		no.	
1		C1	
2		C2	
3		C3	
4		C4	
5		C5	
6		C6	
7		C7	
8		C11	
9		C12	
10			
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 food III interlaboratory Comparison study.	
Is the person(s) carrying out the follow up food III interlaboratory Comparison study 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 or certified 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 12 February 2010 preferable by email to CRL-*Salmonella*. 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*).
Use the address below:

Angelina Kuijpers
RIVM / LZO internal Pb 63
P.O. Box 1
3720 BA Bilthoven
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Tel. number: + 31 30 274 2093
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