National Institute
for Public Health
and the Environment

Report 330604010/2008

A.F.A. Kuijpers | C. Veenman | J. Kassteele van de | K.A. Mooijman

EU Interlaboratory comparison study food II (2007)

Bacteriological detection of *Salmonella* in minced beef

rivm

RIVM Report 330604010/2008

EU Interlaboratory comparison study food II (2007)

Bacteriological detection of *Salmonella* in minced beef

A.F.A. Kuijpers
C. Veenman
J. Kassteele van de
K.A. Mooijman

Contact:
A.F.A. Kuijpers
Laboratory of Zoonoses and Environmental Microbiology (LZO)
angelina.kuijpers@rivm.nl

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/330604/07/CS by the Community Reference Laboratory for *Salmonella*

rivm

Abstract

EU Interlaboratory comparison study food II (2007)
Bacteriological detection of *Salmonella* in minced beef

From the 30 European National Reference Laboratories (NRLs) for *Salmonella,* 29 were found to be able to detect high and low levels of *Salmonella* in minced beef. However, five laboratories did require a follow up test to reach this level. One laboratory was still unable to produce satisfactory results in the follow up test. The reasons for this failure are currently being investigated. These results were shown in the second interlaboratory comparison study on food, organized by the Community Reference Laboratory (CRL) for *Salmonella*. The CRL for *Salmonella* is part of the National Institute for Public Health and the Environment (RIVM). The comparison study was conducted in November 2007, with the follow up in February 2008. All European Member States (MS) are obliged to participate in this study.

The study also investigated what the best method for detecting *Salmonella* in minced beef was out of three methods regularly used. One of the detection methods standardised at international level for the detection of *Salmonella* in food was found not to be the most effective. Only 69% of the samples tested with this method were found to be positive. The internationally prescribed method for the detection of *Salmonella* in veterinary samples gave the best results (86%).

In order to improve the performance testing of the laboratories, lower levels of contamination were used in the tests compared to earlier studies. For this study, each laboratory received a package containing minced beef and 35 gelatin capsules containing *Salmonella* spp. at different levels. The laboratories were instructed to spike the minced beef with the capsules and test the samples for the presence of *Salmonella*.

Key words: *Salmonella*; CRL-*Salmonella*; NRL-*Salmonella*; interlaboratory comparison study; minced beef; *Salmonella* detection methods

rivm

Rapport in het kort

EU Ringonderzoek voedsel-II (2007)
Bacteriologische detectie van *Salmonella* in rundergehakt

Van de 30 Europese Nationale Referentie Laboratoria (NRLs) waren er 29 in staat hoge en lage concentraties *Salmonella* in rundergehakt aan te tonen. Vijf laboratoria hadden hiervoor een herkansing nodig. Eén laboratorium kon ook tijdens deze herkansing niet voldoende presteren. Momenteel wordt onderzocht wat de oorzaak hiervan is. Dit blijkt uit het tweede ringonderzoek georganiseerd door het Communautair Referentie Laboratorium (CRL) voor levensmiddelen. Het onderzoek is in november 2007 gehouden, de herkansing in februari 2008. Europese lidstaten zijn verplicht om aan dit onderzoek deel te nemen. Het CRL-*Salmonella* is gevestigd op het Rijksinstituut voor Volksgezondheid en Milieu (RIVM).

Tijdens dit ringonderzoek is ook onderzocht welke van de drie gebruikte analysemethoden de beste was om de *Salmonella*bacterie in rundergehakt aan te tonen. Eén van de internationaal gestandardiseerde methoden voor *Salmonella* in levensmiddelen blijkt niet de optimale methode te zijn. Hiermee werd slechts in 69% van de monsters *Salmonella* aangetroffen. De internationaal voorgeschreven methode om *Salmonella* in dierlijke mest aan te tonen behaalde het beste resultaat (86%).

Om de uitvoering van de laboratoria beter te kunnen testen zijn lagere besmettingsniveaus gebruikt dan in eerdere studies. Voor dit ringonderzoek kreeg ieder laboratorium een pakket toegestuurd met rundergehakt en 35 gelatine capsules met melkpoeder van verschillende besmettingsniveaus *Salmonella*. De laboratoria moesten volgens voorschrift gehakt en capsules samenvoegen en onderzoeken op de aanwezigheid van *Salmonella*.

Trefwoorden: *Salmonella*; CRL-*Salmonella*; NRL-*Salmonella*; ringonderzoek; rundergehakt; *Salmonella* detectie methode

rivm

Contents

rivm

List of abbreviations

BGA (mod)	Brilliant Green Agar (modified)
BPLSA	Brilliant Green Phenol-Red Lactose Sucrose Agar
BPW	Buffered Peptone Water
Cfp	colony forming particles
CRL	Community Reference Laboratory
dPCA	double concentrated Plate Count Agar
dVRBG	double concentrated Violet Red Bile Glucose agar
hcmp	highly contaminated milk powder
ISO	International Standardisation Organisation
LDC	Lysine Decarboxylase
MKTTn	Mueller Kauffmann Tetrathionate novobiocin broth
MLCB	Mannitol Lysine Crystal violet Brilliant green agar
MS	Member States
MSRV	Modified Semi-solid Rappaport Vassiliadis
NRL	National Reference Laboratory
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
RIVM	Rijksinstituut voor Volksgezondheid en het Milieu (National Institute for Public Health and the Environment)
RM	Reference Material
RVS	Rappaport Vassiliadis Soya broth
SE	*Salmonella* Enteritidis
SM ID2 = Chrom ID	*Salmonella* Detection and Identification-2
SOP	Standard Operating Procedure
SPan	*Salmonella* Panama
STM	*Salmonella* Typhimurium
TSI	Triple Sugar Iron agar
UA	Urea Agar
VRBG	Violet Red Bile Glucose agar
XLD	Xylose Lysine Deoxycholate agar
XLT4	Xylose Lysine Tergitol 4 agar

rivm

Summary

In November/December 2007 the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*) organised the second interlaboratory comparison study on bacteriological detection of *Salmonella* in a food matrix (minced beef). Participants were 30 National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*) of the EU Member States and of Norway and candidate country Former Yugoslav Republic of Macedonia (FYROM).

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. For a better testing of the performance of the laboratories the contamination levels in this study were lower than in earlier studies. As a result of this, a new proposal for good performance was made and the performance of the laboratories was compared to this new 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). Optionally a laboratory could also use other, own media or procedures for the detection of *Salmonella*.

Thirty five individually numbered capsules had to be tested by the participants for the presence or absence of *Salmonella.* Twenty five of the capsules had to be examined in combination with each 10 gram of *Salmonella* negative minced beef. These 25 capsules were divided over the following groups: 5 capsules with approximately 5 colony forming particles (cfp) of *Salmonella* Typhimurium (STM5), 5 capsules containing approximately 50 cfp of *S.* Typhimurium (STM50), 5 capsules with approximately 10 cfp of *S.* Enteritidis (SE10), 5 capsules containing 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 SE10, 1 capsule SE100, 2 capsules containing approximately 5 cfp of *S.* Panama (SPan5) and 2 blank capsules.

On average the laboratories found *Salmonella* in only 69% of the (contaminated) samples using one of the food methods (MKTTn). The method for testing veterinary samples (MSRV) gave the best results with 86% of the positive samples, closely followed by the other food method (RVS) with in total 84% positive samples. The MKTTn food method seem to be less optimal for detection of *Salmonella* spp. in minced beef.

Twenty-four out of 30 laboratories achieved the level of good performance for at least one of the used methods, one of them performed the study 3 months later. Five NRLs reached this level of good performance in a follow-up study with extra material. One NRL did not yet achieve the level of good performance.

1 Introduction

An important task of the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*), as laid down in Regulation EC No 882/2004, is the organisation of interlaboratory comparison studies. The history of the interlaboratory comparison studies as organised by CRL-*Salmonella* since 1995 is summarised in Annex 1. Up to 2005 the interlaboratory comparison studies on the detection of *Salmonella* were focused on veterinary samples (e.g. chicken faeces). However, according the regulations also food matrices should be dealt with. In 2006, with success, the first (pilot) interlaboratory comparison study on the detection of *Salmonella* in minced beef was organised. For a better testing of the performance of the laboratories the contamination levels of the samples used in the studies organised since 2007 were lower than in earlier studies. The first and most important objective of the study, organized by the Community Reference Laboratory (CRL) for *Salmonella* in November 2007, was to see if the participating laboratories could detect *Salmonella* at different contamination levels in a food matrix. The second objective was to compare the different methods for the detection of *Salmonella* in minced beef. Furthermore it is important that the examination of samples in the EU Member States is carried out uniformly and comparable results should be obtained by all National Reference Laboratories for *Salmonella* (NRL-*Salmonella*).
The prescribed method for detection of *Salmonella* in a food matrix is ISO 6579 (Anonymous, 2002). However, as good experiences were gained with Modified Semi-solid Rappaport Vassiliadis (MSRV) as selective enrichment medium for the detection of *Salmonella* spp. in animal faeces (Annex D of ISO 6579 Anonymous, 2007), participating laboratories were requested also to use MSRV for testing the minced beef.
The set-up of this study was comparable to earlier interlaboratory comparison studies on the detection of *Salmonella* spp. in veterinary and food samples. The contamination level of the low level capsules was at the detection limit of the method; the level of the high level samples approximately 5-10 times above the detection limit. Ten control samples consisting of different reference materials, had to be tested without the addition of minced beef. These reference materials consisted of 3 capsules with approximately 5 cfp of *Salmonella* Typhimurium (STM5), 2 capsules with approximately 10 cfp of *Salmonella* Enteritidis (SE10), 1 capsule with approximately 100 cfp of *Salmonella* Enteritidis (SE100), 2 capsules with approximately 5 cfp of *Salmonella* Panama (SPan5) and 2 blank capsules. Twenty-five samples of *Salmonella* negative minced beef spiked with 5 different reference materials (including blank capsules) had to be examined. The different reference materials consisted of two levels of *Salmonella* Typhimurium (STM5 and STM50) and two levels of *Salmonella* Enteritidis (SE10 and SE100).

2 Participation

Country	City	Institute
Austria	Graz	Austrian Agency for Health and Food Safety (AGES) Institute for Medical Microbiology and Hygiene
Belgium	Brussels	Scientific Institute of Public Health WIV (IPH) Food microbiology / Institute scientific Louis Pasteur
Bulgaria	Sophia	National Diagnostic and Research Veterinary Institute
Cyprus	Nicosia	Ministry of Agriculture, Natural Resources and Environment Veterinary Services Laboratory for the Control of Foods of Animal Origin (LCFAO)
Czech Republic	Prague	State Veterinary Institute
Denmark	Copenhagen	National Food Institute The Technical University of Denmark Department of Microbiology and Risk Assessment
Estonia	Tartu	Estonian Veterinary and Food Laboratory, Bacteriology-Pathology Department
Finland	Helsinki	Finnish Food Safety Authority Evira Research Department, Microbiology Unit
France	Ploufragan	Agence Francaise De Securtie Santiare Des Aliments (AFSSA)
Germany	Berlin	Federal Institute for Risk Assessment (BFR) National Veterinary Reference Laboratory for Salmonella
Greece	Halkis	Veterinary Laboratory of Halkis Hellenic Republic Ministry of rural development and food
Hungary	Budapest	Central Agricultural Office, Food and Feed Directorate Central Microbiological Laboratory
Ireland	Kildare	Veterinary Research Laboratory CVRL / DAF Department of Agriculture and Food
Italy	Legnaro	Istituto Zooprofilattico Sperimentale delle Venezie, OIE/National Reference Laboratory for Salmonella
Latvia	Riga	Nationaly Diagnostic Centre (NDC) of Food and Veterinary Service
Lithuania	Vilnius	National Veterinary Laboratory
Luxembourg	Luxembourg	Laboratoire de Médecine Vétérinaire de l'Etat, Animal Zoonosis
Macedonia Former Yugoslav Republic of Macedonia (FYROM)	Skopje	Food institute Faculty of veterinary medicine
Malta	Valletta	PHL Evans Building Department of Public Health
Netherlands The	Bilthoven	Nationa Institute for Public Health and the Environment (RIVM)
Norway	Oslo	National Veterinary Institute, Section of Bacteriology
Poland	Pulawy	National Veterinary Research Institute (NVRI) Department of Hygiene of food of animal origin

Country	City	Institute
Portugal	Lisbon	Laboratório Nacional de Investigação Veterinária (LNIV)
Romania	Bucharest	Hygiene and veterinary public health Institute HPVHI
Slovak Republic	Bratislava	State Veterinary and Food Institute Reference Laboratory for Salmonella
Slovenia	Ljubljana	National Veterinary Institute, Veterinary Faculty
Spain	Madrid Majahonda	Agencia Española de Seguridad Alimentaria y Nutrición (AESAN) Centro Nacional de Alimentación Servicio de Microbiología Alimentaria
Sweden	Uppsala	National Veterinary Institute (SVA), Department of Bacteriology
United Kingdom	Belfast	Agri-Food and Bioscience Institute (AFBI) Veterinary Sciences Division Bacteriology
United Kingdom	London	Health Protection Agency London, Food, Water & Environmental Microbiology Laboratory (HPA/ LFWEM)

3 Materials and methods

3.1 Reference materials

Five batches of *Salmonella* reference materials were prepared. For this purpose milk, artificially contaminated with a *Salmonella* strain was spray-dried (In `t Veld et al., 1996). The obtained highly contaminated milk powder (hcmp) was mixed with sterile (γ-irradiated) milk powder (Carnation, Nestlé, the Netherlands) to obtain the desired contamination level. The mixed powder was filled in gelatin capsules resulting in the final reference materials (RMs).
The target levels of the five batches of RMs were:

- 5 colony forming particles (cfp) per capsule for *Salmonella* Panama (SPan5);
- 5 and 50 colony forming particles (cfp) per capsule for *Salmonella* Typhimurium (STM5 and STM50);
- 10 and 100 colony forming particles (cfp) per capsule for *Salmonella* Enteritidis (SE10 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 $T2/(I-1) \leq 2$, where T2 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). Shortly the procedure is as follows:

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

3.2 Minced beef samples

3.2.1 General

Ten kilogram of minced beef was bought at the butcher (in Bilthoven) on 25 October 2007. 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 10 g were each added to 90 ml Buffered Peptone Water (BPW). After pre-enrichment at (37 ± 1) ^{o}C for 16-18 h, selective enrichment was carried out on Modified Semi-solid Rappaport Vassiliadis (MSRV). Next, the suspect plates were plated-out on

Xylose Lysine Deoxycholate agar (XLD) and Brilliant Green Agar (BGA) and confirmed biochemically. The minced beef was stored at - 20 ºC until further use.

3.2.2 Total bacterial count in minced beef

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

3.2.3 Number of Enterobacteriaceae in minced beef

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

3.3 Design of the interlaboratory comparison study

3.3.1 Samples: capsules and minced beef

On 12 November 2007 (one week before the study) the reference materials (35 individually numbered capsules) and 300 grams of *Salmonella* negative minced beef were packed with cooling devices as biological substance category B (UN 3373) and send by courier service to the participants. After arrival at the laboratory the capsules had to be stored at –20 ^{o}C and the minced beef had to be stored at +5 ^{o}C until the start of the study. Details about mailing and handling of the samples and reporting of test results can be found in the Protocol (Annex 4) and Standard Operation Procedure (Annex 5). The test report which was used during the study can be found at the CRL-*Salmonella* website: http://www.rivm.nl/crlsalmonella/prof_testing/detection_stud/ or can be obtained through the corresponding author of this report.

Ten control capsules had to be tested without meat (numbered C1-C10). Twenty-five capsules (numbered 1 – 25) were each tested in combination with 10 grams of minced beef (negative for *Salmonella*). The types and the number of capsules and meat samples to be tested are shown in Table 1.

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

Capsules	Control capsules (n=10) No meat added	Test samples (n=25) with 10 g *Salmonella* negative minced beef
S. Panama 5 (SPan5)	2	---
S. Enteritidis 10 (SE10)	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 beef were packed in 2 plastic containers firmly closed with screw caps (biopacks). Both biopacks were placed in one large shipping box, together with six frozen (-20 ^{o}C) cooling devices. Each shipping box was sent as biological substances category B (UN3373) by door-to-door courier services. For the control of exposure to abusive temperatures during shipment and storage, so called micro temperature loggers were used to record the temperature during transport. These loggers are tiny sealed units in a 16 mm diameter and 6 mm deep stainless steel case. Each shipping box contained one logger, packed in the biopack with capsules. The loggers were programmed by the CRL-*Salmonella* to measure the temperature every hour. Each NRL had to return the temperature recorder immediately after receipt of the parcel to the CRL. At the CRL-*Salmonella* the loggers were read by means of the computer and all data from the start of the shipment until the arrival at the National Reference Laboratories were transferred to an Excel graphic which shows all recorded temperatures.

3.4 Methods

The prescribed method of this interlaboratory comparison study was 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 based methods.

In summary:

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

Selective enrichment on:
- Rappaport Vassiliadis Soya broth (RVS) (prescribed)

- Mueller Kaufmann Tetrathionate novobiocin broth (MKTTn) (prescribed)
- Modified semi-solid Rappaport Vassiliadis medium (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)

Biochemical confirmation:

- Biochemical confirmation as described in ISO 6579 Annex B.6-B.11 or by reliable, commercial available identification kits.

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

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

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

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

Results were analyzed using the statistical software R 2.6.2 (R Development Core Team, 2008). Mixed effect logistic regression (Venables and Ripley, 2002) using the lme4 package was used. The lme4 package provides functions for fitting and assessing of generalized linear mixed effects models in R (Bates, 2007).
Mixed effect logistic regression allows modelling of 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 among media and capsules are shown as odds ratios and were calculated by stratification by medium. In each stratum, laboratories were taken as a random effect. The overall performance of each laboratory is given as odds ratios compared to the mean of all laboratories, i.e. the outcomes as predicted based on the fixed effects only.

3.6 Good performance

New proposal for definition of "good performance"

During the tenth CRL-*Salmonella* workshop in April 2005 a proposal was made to define "good performance" in interlaboratory comparison studies on detection of *Salmonella*. For a better testing of the performance of the laboratories the contamination level of STM and SE capsules in this study are

lower than in earlier studies. The contamination level of the low level capsules is at the detection limit of the method; the high level samples approximately 5-10 times above the detection limit.

As a result of lowering the contamination levels, a new proposal for "good performance" is necessary:

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 SE10	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	80%	4/5
SE100	80%	4/5
STM5	50%	2-3/5
SE10	20%	1/5

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

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 MSRV/XLD, but no positives with any other selective enrichment medium or isolation medium this was still considered as good result. The opposite was performed for the blank capsules. Here also all combinations of media used per laboratory were 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 as a "no-good" result.
When testing samples with a contamination level close to the detection limit it is expected that approximately 50% of the total number of tested samples will be found positive. For the *Salmonella* Enteritidis samples used in this study the detection limit was close to 10 cfp. As the mean contamination level of the low level SE reference materials was below 10 cfp/capsule (7 cfp), the demand for good performance of this type of RM added to a matrix was amended from 50% to 20% of the total number of samples to be positive.

4 Results

4.1 Reference materials

The level of contamination and the homogeneity of the final batches of capsules are presented in Table 2A and 2B. All batches met the pre-set criteria as stated in section 3.1. The enumerated minimum and maximum levels within each batch of capsules are also given in the table. The final batches were tested twice: firstly immediately after preparing the batch and secondly at the time of the interlaboratory comparison study.

Table 2A Level of contamination and homogeneity of SE, SPan and STM capsules.

	SE10	SE100	SPan5	STM5	STM50
Final batch; Test 1					
Date testing capsules	18-07-07	10-07-07	09-10-07	02-08-07	28-08-07
Number of capsules tested	50	50	50	50	50
Mean cfp per capsule	7	76	7	5	44
Min-max cfp per capsule	2-17	52-110	0-15	1-13	25-69
T_2 / (I-1)	1.19	2.16	1.51	1.25	1.69
Final batch; Test 2					
Date testing capsules	22-11-07	22-11-07	29-11-07	19-11-07	19-11-07
Number of capsules tested	25	25	25	25	25
Mean cfp per capsule	7	71	7	4	40
Min-max cfp per capsule	3-17	50-105	1-17	1-10	28-61
T_2 / (I-1)	1.63	1.95	1.74	1.69	1.53

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

Table 2B Level of contamination and homogeneity of SE capsules used in the follow up study.

	SE10	SE100
Final batch; Test 1		
Date testing capsules	28-11-07	19-11-07
Number of capsules tested	50	50
Mean cfp per capsule	9	90
Min-max cfp per capsule	3-19	63-120
T_2 / (I-1)	1.76	1.88
Final batch; Test 2		
Date testing capsules	12-03-08	12-03-08
Number of capsules tested	25	25
Mean cfp per capsule	7	91
Min-max cfp per capsule	0-17	65-113
T_2 / (I-1)	2.54	1.86

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 beef samples

The minced beef was tested negative for *Salmonella* and stored at -20 °C. On Monday 12 November 2007 the minced beef was mailed to the NRLs. After receipt the NRLs had to store the minced beef at 5 °C.

The number of aerobic bacteria and the number of Enterobacteriaceae were tested twice; firstly at the day the minced beef arrived at the CRL (25/10/2007) and secondly at the planned date (19/11/2007) of the interlaboratory comparison study. The results are shown in Table 3.

Most of the laboratories (twenty-seven) performed the study on the planned date (19/11/2007), one laboratory (labcode 23) performed the study one week earlier (12/11/2007) and one laboratory (labcode 16) one week later (26/11/2007). Laboratory 12 did not return the test report, after further inquiry we were informed they did not perform the study at the planned time finally they performed the study on 6 February 2008. The results from laboratory 12 were not calculated and compared with the other laboratories.

Table 3 Number of aerobic bacteria and the number of Enterobacteriaceae per gram of minced beef.

Date	Aerobic bacteria cfp/g	Enterobacteriaceae cfp/g
25 October 2007 Stored at -20 °C	$7.7*10^7$	$6.8*10^3$
19 November 2007 After one week at 5 °C	$3.6*10^9$	$1.3*10^6$

4.3 Technical data interlaboratory comparison study

4.3.1 General

Laboratory 12 performed the study 3 months later than the other laboratories (6 February 2008). The test report arrived at CRL *Salmonella* on 27 February 2008. Due to this delay it was not possible to report the results of this laboratory together with the results of the other laboratories.

4.3.2 Accreditation/certification

Twenty-four laboratories mentioned to be accredited for their quality system according to ISO/IEC 17025 (Anonymous, 2005) (labcodes 1, 2, 4, 5, 6, 7, 8, 11, 12, 13, 15, 17, 18, 19, 20, 21, 23, 24, 25, 26, 27, 28, 29 and 30). Five laboratories (labcodes 3, 9, 14, 16 and 22) are planning to become accredited or certified in the near future and for one laboratory (labcode 10) the accreditation is already in process.

4.3.3 Transport of samples

An overview of the transport times and the temperatures during transport of the parcels is given in Table 4. The temperature recorders were returned immediately after receipt to CRL-*Salmonella* by all NRLs with the exception of laboratory 12. The majority of the laboratories received the materials within 1 day. However, the parcel of laboratory 1 was brought to another country by the courier and was delayed for 3 days. The total transport time of this parcel was 99 hours. When this latter parcel is not taken into account, the average transport time was 29 hours. For the majority of the parcels the transport temperature did not exceed 5 ^{o}C. Although the parcel of laboratory 1 was delayed for 3 days, most of the time it was stored below 5 ^{o}C. For fourteen NRLs the time of transport recorded on the test report did not correspond with the time reported by the courier. Presumably the parcel arrived at the time reported by the courier at the Institute, but due to internal logistics at the Institute the parcel arrived later at the laboratory of the NRL. The delay varied between 1 to 22 hours. In three laboratories (labcodes 8, 9 and 19) the storage temperature during the delay was higher than 15 ^{o}C. For the other laboratories (labcodes 3, 5, 7, 11, 13, 14, 17, 18, 24, 27 and 30) the storage temperature during the delay was below 5 ^{o}C.

Table 4 Overview of the transport time and of the temperatures during shipment of the parcels to the NRLs.

		Time (h) at			
Labcode	Transport[1] time in hours	- 20 °C - 0 °C	0 °C - 5 °C	5 °C - 10 °C	Additional Storage[2]
1	99	8	88	3	
2	27	21	6		
3	27	15	12		1 hour at 1 °C
4	24	10	14		
5	26	19	7		2 hours at 2 °C
6	28	9	19		
7	23	23			5 hours at 1 °C
8	25	18	7		2 hours at 22 °C
9	47	15	32		1 hour at 16 °C
10	22	12	10		
11	27	12	15		1 hour at 1 °C
12	22	11	11		
13	30	16	14		11 hours at – 15 °C
14	48	44	4		22 hours at 0 °C
15	25	13	12		
16	24	24	0		
17	27	14	32		19 hours at 1 °C
18	25	18	7		1 hour at 1 °C
19	50	14	36		22 hours at 20 °C
20	22	14	8		
21	25	13	12		
22	27	15	12		
23	1			1	
24	46	8	38		1 hour at 2 °C
25	25	12	13		
26	47	14	12	21	
27	24	22	2		1 hour at 1 °C
28	26	13	13		
29	25	25	0		
30	23	15	8		1 hour at 1 °C
Average[3]	29.2				

[1] transport time according the courier

[2] storage time of the samples at the institute before arriving at the laboratory of the NRL

[3] average time without lab no 1 and 23

Table 5 Media combinations used per laboratory.

Labcode	Selective enrichment media	Plating-out Media
1	RVS MKTTn MSRV	XLD BGA
2	RVS MKTTn MSRV	XLD BGA[mod]
3	RVS MKTTn MSRV[*] MSRV[*]	XLD BGA[mod] MLCB
4	RVS MKTTn MSRV	XLD Rambach
5	RVS MKTTn MSRV	XLD SMID2
6	RVS MKTTn MSRV	XLD BPSLA BGA
7	RVS MKTTn MSRV	XLD XLT4
8	RVS MKTTn MSRV	XLD BGA [mod]
9	RVS MKTTn MSRV	XLD BPLS [mod]
10	RVS MKTTn MSRV	XLD BGA [mod]
11	RVS MKTTn MSRV	XLD ChromID
12	RVS MKTTn MSRV	XLD Compass Salmonella
13	RVS MKTTn MSRV	XLD BGA [mod]
14	RVS MKTTn MSRV	XLD Rambach
15	RVS MKTTn MSRV	XLD BGA[mod]
16	RVS MKTTn MSRV	XLD SMID2
17	RVS MKTTn MSRV	XLD SM2 Rambach
18	RVS MKTTn MSRV	XLD Onoz Rambach
19	RVS MKTTn MSRV	XLD Rambach
20	RVS MKTTn MSRV	XLD BGA [mod]
21	RVS MKTTn MSRV	XLD BGA[mod]
22	RVS MKTTn	XLD BGA [mod]
23	RVS MKTTn MSRV	XLD BGA [mod]
24	RVS MKTTn MSRV	XLD Rambach
25	RVS MKTTn MSRV	XLD Rambach
26	RVS MKTTn MSRV	XLD BGA [mod]
27	RVS MKTTn MSRV RV	XLD Hektoen agar Rambach
28	RVS MKTTn MSRV	XLD BGA [mod]
29	RVS MKTTn MSRV	XLD BGA [mod]
30	RVS MKTTn MSRV	XLD BGA [mod]

Explanations of the abbreviations are given in the "List of abbreviations"
Compositions of the media not described in ISO 6579 are given in Annex 3
* laboratory 3 used MSRV from two different manufacturers

4.3.4 Media

Each laboratory was asked to test the samples with the prescribed (ISO 6579) and the requested (AnnexD of ISO 6579) methods. All laboratories except one (labcode 22) used the selective enrichment media RVS, MKTTn and MSRV with the plating out medium XLD and a second plating out medium of own choice. Laboratory 22 used only RVS and MKTTn for selective enrichment. The media used per laboratory are shown in Table 5. Two NRLs (labcodes 3 and 27) used additional to the prescribed and "requested" selective enrichment media another selective enrichment medium. Five NRLs (labcodes 3, 6, 17, 18 and 27) used a third plating out medium. Details on the media which are not described in ISO 6579 are given in Annex 3. In Tables 6-12 information is given on the composition of the media which were prescribed and "requested" and on incubation temperatures and times. In these tables only the laboratories are indicated who reported deviations. Laboratory 11 did not mention the pH and quality control of the media and laboratory 27 did not mention de composition of the media used. Three laboratories incubated the selective enrichment media at deviating temperatures (labcodes 8, 25 and 27). Laboratory 17 incubated the MSRV only for 24 hours.

A second plating-out medium for choice was obligatory. Five-teen laboratories used BGA mod (ISO 6579, 1993) as a second plating-out medium and laboratory 6 used BPLSA (Merck) this is very close to BGA. Eight laboratories used Rambach and four laboratories used SM (ID) 2 agar. The following media were used only by one laboratory : XLT4, Onoz, MLCB, Hektoen and compass agar.

The use of an extra plating agar between the "isolation" and the "confirmation" steps was optional. A total of 18 laboratories performed this extra culture step on many different media (e.g. Nutrient agar (ISO 6579, 2002), Mc Conkey and Bromthymol blue lactose sucrose agar).

Table 6 Incubation time and temperature of BPW.

	Prewarming BPW		**Dissolving capsules in BPW**		**Pre-enrichment in BPW**	
Labcode	**Time (h:min)**	**Incubation temperature in ^{o}C (min-max)**	**Time (min)**	**Incubation temperature in ^{o}C (min-max)**	**Time (h:min)**	**Incubation temperature in ^{o}C (min-max)**
SOP & ISO 6579	**Overnight**	**36-38**	**45**	**36-38**	**16 – 20**	**36-38**
2	20:15	37	45	37	20:30	37
13	-	-	45	37	21:30	37
18	5:30	37	50	36.6-37	18:10	36.6-36.8
26	22:40	37	45	37	22:30	37
29	27:20	36	50	36.7-37.1	18:50	36.6
30	24	37	45	37	21:00	37

Grey cell : deviating times and temperatures

- = no info

Table 7 Composition (in g/L) and pH of BPW medium.

Labcode	Enzymatic digest of casein (Peptone)	Sodium Chloride (NaCl)	Disodium hydrogen Phosphate dodecahydrate ($Na_2HPO_4.12H_2O$)	Potassium dihydrogen phosphate (KH_2PO_4)	pH
ISO 6579	**10.0**	**5.0**	**9.0**	**1.5**	**6.8 – 7.2**
1	10.0	5.0	3.5*	1.0	7.1
10	10.0	5.0	3.5*	1.5	7.4
11	10	5	9	1.5	-
21	10.0	5.0	3.5*	1.5	7.3
29	10	5	3.5*	1.5	7.3
30	10	5	3.5*	1.5	7.3

Grey cell: deviating from ISO 6579

- = no info

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

Table 8 Incubation times and temperatures of selective enrichment medium RVS and MSRV.

	RVS	MKTTn	MSRV	
Labcode	**Incubation temperature in °C (min-max)**	**Incubation temperature in °C (min-max)**	**Incubation time in h:min**	**Incubation temperature in °C (min-max)**
ISO 6579 & Annex D	**40.5 – 42.5**	**36-38**	**2 x (24 ± 3) h**	**40.5 – 42.5**
8	37	37	47:40	42
17	41.5	37	24:30	41.5
25	41.5-42	41.5-42	48:00	41.5-42
27	36.8-37	36.8-37	47:15	36.7-37

Grey cell: deviating times and temperatures

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

Labcode	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**
4	5.0	8.0	1.4 + 0.2	400***	0.4	5.2
5	4.5	7.2	1.3 + 0.2	13.4	0.036	-
8	5.0	8.0	1.4 + 0.2	13.4	0.036	5.2
10	4.5	7.2	1.3 + 0.2	13.6	0.036	5.3
11	4.5	7.2	1.4	28.5**	0.0036	-
10	4.5	7.2	1.3 + 0.2	13.6	0.036	5.3
12	5	8	1.6	40	0.04	5.2
15	4.5	7.2	1.4	13.4	0.036	-
16	4.5	7.2	1.4	28.6**	0.037	-
20	4.5	7.2	1.4	13.6	0.036	5.3
23, 24, 25, 28	4.5	7.2	1.3 + 0.2	13.6	0.036	5.4
26	5.0	8.0	0.8	40	0.12	6.0
27	-	-	-	100 ml (40%)	-	5.2
30	4.5	7.2	1.3 + 0.2	13.6	0.036	5.2

Grey cell: deviating from ISO 6579
- = no info

*= 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.
*** Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)

Table 10 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**	**4.78**	**9.6 mg**	**4**	**5.0**	**0.04**	**8.0 – 8.4**
2	4.3	8.6	2.6	38.7	30.5*	4.78	9.6	0.8	2.0	0.8	7.7
4	4.3	8.6	2.6	38.7	47.8	4.78	9.6 g	20	25	0.04	8.2
5	4.2	8.5	2.5	38.0	30.3	4.75	9.5	4	5	0.05	-
6	4.3	8.6	2.6	38.7	30.5*	4.78	9.0	4	5.0	5 mg	8
7	4.3	8.6	2.6	38.7	47.8	4.78	9.6	20 ml	-	0.04	8.0
8	4.3	8.6	2.6	38.7	47.8	4.78	9.6	20	25	0.04	8.0
9	4.3	8.6	2.6	38.7	30.3	4.75	9.5	20	25	0.04	7.8
10	4.3	8.6	2.6	38.7	30.5*	4.78	9.6	4.0	5.0	0.04	7.8
11	4.2	8.5	2.54	38.0	30.3*	4.75	9.5	4	5	0.05	-
13	7.0	2.3	2.3	25.0	40.7	4.75	0.1 g/ 100ml	20 g/ 100ml	25 g/ 100ml	0	7.8
14	4.3	8.6	2.6	38.7	30.5*	4.78	9.6	4	5	0.04	7.8
15	4.3	8.6	2.6	38.7	47.8	4.78	9.6	-	-	-	-
16	4.2	8.5	2.5	38.0	30.3*	4.75	9.5	4	5	0.05	-
22	4.3	8.6	2.6	38.7	30.5*	4.78	9.6	20	25	0.04	8.2
26	7.0	2.3	2.3	25.0	40.7	4.75	9.5 ml 0.1%	20	25	0.04	7.8
27	-	-	-	-	-	-	-	-	-	5 ml	8.2
29	-	-	-	-	-	-	-	-	-	0.02	7.9
30	4.3	8.6	2.6	38.7	30.5*	4.7	9.6	20 ml	20 ml	0.04	8.0

Grey cell: deviating from ISO 6579

- = no info

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

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

Labcode	Enzymatic digest of casein (Tryptose)	Casein hydro-lysate	Sodium chloride (NaCl)	Potassium Dihydrogen Phosphate (KH_2PO_4 K_2HPO_4)	Magnesium chloride anhydrous ($MgCl_2$)	Malachite green oxalate	Agar	Novo Biocin	pH
Annex D ISO 6579	**4.6**	**4.6**	**7.3**	**1.5**	**10.9**	**0.04**	**2.7**	**0.01 (10mg/L)**	**5.1-5.4**
2	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.03	5.6
3*	-	4.6	7.3	1.5	10.9	0.04	2.5	0.02	5.3
	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.2
4	-	-	-	-	-	-	-	-	5.2
5	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	-
7	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.2
8	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.05	5.2
10	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.0
11	4.6	4.6	7.3	1.5	10.9	0.04	2.7	2.7 g	-
13	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0	5.5
14	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.3
15	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.6
17	-	-	-	-	-	-	-	-	5.5
19	4.6	4.6	7.3	1.5	10.9	0.04	2.7	-	5.2
26	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.01	5.5
27	-	-	-	-	-	-	2.7	2 ml	5.2
28	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.1	5.5
29	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.01	5.5
30	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.4

Grey cell: deviating from Annex D of ISO 6579

- = no info

* two different manufacturers were used

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

Lab Code	Xylose	L-lysine	Lactose	Sucrose (Saccharose)	Sodium chloride (NaCl)	Yeast Extract	Phenol red	Agar	Sodium deoxy-cholate ($C_{24}H_{39}NaO_4$)	Sodium thio-sulphate ($Na_2S_2O_3$)	Iron (III) Ammonium Citrate ($C_6H_8O_7 \cdot nFe \cdot nH_3N$)	pH
ISO 6579	**3.75**	**5.0**	**7.5**	**7.5**	**5.0**	**3.0**	**0.08**	**9-18**	**1.0**	**6.8**	**0.8**	**7.2 – 7.6**
2	3.75	5.0	7.5	7.5	5.0	3.0	0.008	15	2.5	6.8	0.8	7.3
4	-	-	-	-	-	-	-	-	-	-	-	7.4
5	-	-	-	-	-	-	-	-	-	-	-	-
11	3.5	5.0	7.5	7.5	5.0	3.0	0.08	13.5	2.5	6.8	0.8	-
19	3.5	5.0	7.5	7.5	5.0	3.0	0.08	13.5	2.5	6.8	0.8	7.4
26	3.5	5.0	7.5	7.5	5.0	3.0	0.08	15	2.5	6.8	0.8	7.4
27	-	-	-	-	-	-	-	-	-	-	-	7.4

Grey cell: deviating from ISO 6579
- = no info

Fifteen laboratories used all three required biochemical media (UA, TSI and LDC) for confirmation of *Salmonella.* Laboratories who used additional or less confirmation media or tests are summarised in Table 13. Laboratory 17 did not mention any confirmation test. Two laboratories (labcodes 6 and 10) reported a rather limited confirmation. Laboratory 10 used only one biochemical test and laboratory 6 only an O antigen test. Two laboratories (labcodes 5 and 11) used a biochemical identification kit (which is also allowed). One laboratory (labcode 3) performed a complete serotyping. Laboratory 28 did not mention the details of serotyping and laboratory 20 performed the O antigen test additional to the biochemical confirmation.

Table 13 Biochemical and/ or serological confirmation of *Salmonella*.

Labcode	Biochemical			Serological			Other
ISO 6579	**UA**	**TSI**	**LDC**	**O antigens**	**Vi antigens**	**H antigens**	
3	-	-	-	+	+	+	Chrom. Agar + Mc conkey
4	+	+	+	-	-	-	ONPG
5	-	-	-	-	-	-	Kigler indien + daarna API 20E
6	-	-	-	+	-	-	-
10	+	-	-	-	-	-	-
11	-	-	-	-	-	-	Galerie Microbact GNB 12 A oxoid MB 1132 A
17	-	-	-	-	-	-	-
18	+	+	+	-	-	-	Brolac agar
19	+	+	+	-	-	-	Drigalski agar
20	+	+	+	+	-	-	-
24	+	+	+	-	-	-	Clark et Lubs
27	+	+	+	-	-	-	Simons
28	-	-	-	-	-	-	Serotyping

Grey cell : confirmation is deviating from ISO 6579

-= not done

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 minced beef). Thirteen laboratories scored correct results for all the control capsules containing *Salmonella*. In Annex 6 the results are given 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. In Table 14 the highest number of positive isolations found with all combinations of selective enrichment media and isolation media are given per laboratory.

Blank capsules (n=2) without addition of meat
The blank capsules contained only sterile milk powder. For the analyses no meat was added.
All participating laboratories correctly analysed the blank capsules negative.

Salmonella Panama 5 capsules (Span5) without addition of meat (n=2)
Twenty-seven laboratories isolated *Salmonella* from both capsules. Two Laboratories (labcodes 6 and 7) could not detect *Salmonella* Panama (SPan5) in one control capsule on one selective enrichment medium but they found both positive with the other two selective enrichment media inoculated from the same BPW.

Table 14 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	SE10 n=2	SE100 n=1	SPan5 n=2	STM5 n=3
Good Performance	**0**	**≥ 1**	**1**	**≥ 1**	**≥ 2**
1	0	**1**	1	2	**2**
2	0	**0**	1	2	3
3	0	**1**	1	2	3
4	0	2	1	2	3
5	0	**1**	1	2	3
6	0	**1**	1	2	3
7	0	**0**	1	2	3
8	0	2	1	2	3
9	0	2	1	2	3
10	0	2	1	2	3
11	0	2	1	2	3
13	0	**1**	1	2	3
14	0	2	1	2	3
15	0	2	1	2	3
16	0	2	1	2	3
17	0	**1**	1	2	3
18	0	**1**	1	2	**2**
19	0	**1**	1	2	3
20	0	2	1	2	3
21	0	2	1	2	3
22	0	**1**	1	2	3
23	0	2	1	2	3
24	0	2	1	2	**2**
25	0	**1**	1	2	3
26	0	**1**	1	2	3
27	0	**1**	1	2	3
28	0	2	1	2	3
29	0	2	1	2	3
30	0	2	1	2	**2**

Bold numbers: deviating results
Grey cell: results are below good performance
Laboratory 12 see Annex 6

Salmonella Typhimurium 5 capsules (STM5) without addition of meat (n=3)
Twenty-five laboratories tested all the three capsules containing STM5 positive. Four laboratories (1, 18, 24 and 30) could not detect *Salmonella* (STM5) in one control capsule with all the three selective enrichment media. These capsules contained STM at a low level (approximately 5 cfp/ capsule). Due to the variation between capsules one out of two capsules containing STM5 may occasionally be negative.

Salmonella Enteritidis 10 capsules (SE10) without addition of meat (n=2)
Fifteen laboratories isolated *Salmonella* Enteritidis at a mean level of approximately 10 cfp/ capsule from both capsules. Two laboratories (labcodes 2 and 7) could not detect *Salmonella* Enteritidis in both SE10 control capsules on all the isolation media inoculated from the three enrichment media (RVS, MKTTn and MSRV). These capsules contained SE at a low level (approx 10 cfp/capsule). Due to change one out of two capsules containing SE10 may be negative. However, it is not very likely to find both SE10 capsules negative.
Twelve laboratories could not detect *Salmonella* in one control capsule with all the three selective enrichment media.

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

The results of all control samples were compared with the new definition of "good performance" (see section 3.6). The score for the control samples was below these criteria for two laboratories (labcodes 2 and 7).

4.4.2 Specificity, sensitivity and accuracy rates of the control samples

In Table 15 the specificity, sensitivity and accuracy rates for the control capsules without the addition of meat are shown. The rates are calculated for the different selective enrichment media (RVS, MKTTn and MSRV) and plating-out medium XLD. These rates were as expected. For the high level control sample (SE100) the rates were as expected 100%. For the low level materials (Span5, STM5 and SE10) the rates were expected to lie between 50% and 100%.

Table 15 Specificity, sensitivity and accuracy rates of the control samples (capsules without the addition of meat)[1].

Control capsules		RVS/XLD	MKTTn/XLD	MSRV/XLD[2]
Blank	No. of samples	58	58	56
	No. of negative samples	58	58	56
	Specificity in%	**100.0**	**100.0**	**100.0**
Span5	No. of samples	58	58	56
	No. of positive samples	57	58	55
	Sensitivity in%	**98.3**	**100.0**	**98.2**
STM5	No. of samples	87	87	84
	No. of positive samples	83	83	80
	Sensitivity in%	**95.4**	**95.4**	**95.2**
SE10	No. of samples	58	58	56
	No. of positive samples	42	42	41
	Sensitivity in%	**72.4**	**72.4**	**73.2**
SE100	No. of samples	29	29	28
	No. of positive samples	29	29	28
	Sensitivity in%	**100.0**	**100.0**	**100.0**
All capsules with *Salmonella*	No. of samples	232	232	224
	No. of positive samples	211	212	204
	Sensitivity in%	**91.0**	**91.4**	**91.1**
All capsules	No. of samples	290	290	280
	No. of correct samples	269	270	260
	Accuracy in%	**92.8**	**93.1**	**92.9**

1 The results of laboratory 12 were not taken into account for the calculations

2 Laboratory 22 did not perform selective enrichment on MSRV.

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

4.5.1 Results per type of capsule and per laboratory

General

The results of the *Salmonella* negative minced beef samples artificially contaminated with capsules per selective enrichment medium in combination with the isolation medium that gives the highest number of positives are given in Annex 7. In Table 16 the highest number of positive isolations found with all combinations of selective enrichment media and isolation media are given per laboratory.

Laboratory 22 did not perform selective enrichment on MSRV. In general the number of positive results of the samples containing *S.* Enteritidis were lower than the samples containing *S.* Typhimurium.

Blank capsules with negative minced beef (n=5)

Twenty-four laboratories correctly did not isolate *Salmonella* from these blank capsules with the addition of negative meat. Three laboratories (17, 27 and 30) found more than 1 positive blank with the addition of negative minced beef.

Laboratory 17 had found two blank capsules positive on SM(ID)2 isolated from MKTTn. With the other media they tested 1 blank positive, with the exception of Rambach inoculated from RVS from which they isolated no *Salmonella*.

Laboratory 27 found two blanks positive with all isolation media in combination with MKTTn and they also found one positive blank from RV and RVS isolated on Rambach and XLD.

Laboratory 30 had found two blank capsules positive on BGA isolated from RVS. All other blanks were tested negative.

All blanks should be tested negative. However, as no 100% guaranty about the Salmonella negativity of the matrix can be given, 1 positive out of 5 blank samples (80% negative) is still considered acceptable. Finding more than one blank sample positive is not very likely and may have been caused by cross-contamination or by misinterpretation of the results.

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

Twenty-one laboratories isolated *Salmonella* from all the five capsules containing *Salmonella* Typhimurium at a level of approximately 5 cfp/ capsule in combination with minced beef when using RVS, MKTTn or MSRV. Seven laboratories 3, 4, 7, 13, 18, 19, and 28 found one capsules negative and one laboratory (labcode 24) found two capsules negative with all three selective enrichment media.

S. Typhimurium 50 capsules (STM50) with negative minced beef (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 beef with the selective enrichment media RVS and MSRV. Seven laboratories 2, 7, 9, 17, 22, 23 and 28 found one or more capsules negative when using MKTTn.

S. Enteritidis 10 capsules (SE10) with negative minced beef (n=5)

Twenty-eight laboratories isolated *Salmonella* from at least one capsule containing *Salmonella* Enteritidis at a level of approximately 10 cfp/ capsule in combination with minced beef at least with one of the used selective enrichment media RVS, MKTTn or MSRV. Eight laboratories: 1, 8, 13, 14, 15 19, 24, and 30 isolated *Salmonella* from all the five SE10 capsules with at least one of three

selective enrichment media. Laboratory 9 found none of the capsules positive with either one of the selective enrichment media.

Table 16 Total number of positive results of the artificially contaminated minced beef samples per laboratory.

Labcode	The highest number of positive isolations found with all combinations of selective enrichment media and isolation media				
	Blank n=5	SE10 n=5	SE100 n=5	STM5 n=5	STM50 N=5
Good performance	**≤ 1**	**≥ 1**	**≥ 4**	**≥ 2**	**≥ 4**
1	0	5	5	5	5
2	0	**1**	5	5	5
3	**1**	**1**	**4**	**4**	5
4	0	**2**	5	**4**	5
5	0	**2**	5	5	5
6	0	**4**	5	5	5
7	0	**2**	5	**4**	5
8	0	5	5	5	5
9	0	**0**	**3**	5	5
10	**1**	**4**	5	5	5
11	0	**2**	5	5	5
13	0	5	5	**4**	5
14	0	5	5	5	5
15	0	5	5	5	5
16	0	**1**	5	5	5
17	**2**	**4**	5	5	5
18	**1**	**4**	5	**4**	5
19	0	5	5	**4**	5
20	0	**4**	5	5	5
21	0	**2**	5	5	5
22	0	**2**	5	5	5
23	0	**3**	5	5	5
24	0	5	5	**3**	5
25	0	**3**	5	5	5
26	0	**1**	5	5	5
27	**2**	**2**	5	5	5
28	0	**2**	5	**4**	5
29	0	**3**	5	5	5
30	**2**	5	5	5	5

Bold numbers: deviating results
Grey cell: results are below good performance
Laboratory 12 See Annex 7

S. Enteritidis 100 capsules (SE100) with negative minced beef (n=5)

Twenty-seven laboratories isolated *Salmonella* from all the five capsules containing *Salmonella* Enteritidis at a level of approximately 100 cfp/ capsule in combination with minced beef with at least one of the selective enrichment media RVS, MKTTn or MSRV. Laboratory 3 found one capsule SE100 negative with all used selective enrichment media. Laboratory 9 found only three capsules positive with selective enrichment media RVS and MSRV; in MKTTn they missed all the five SE100 capsules.

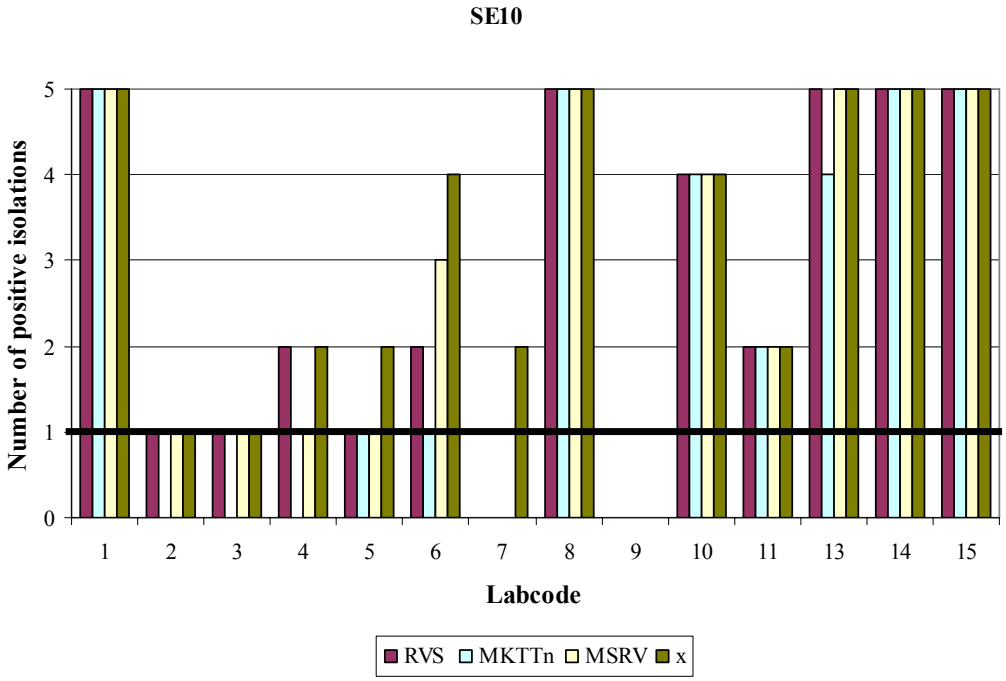
The results of all artificially contaminated minced beef samples were compared with the new definition of "good performance" (see section 3.6). The score for the artificially contaminated samples was below the set criteria for four laboratories (labcodes 9, 17, 27 and 30).

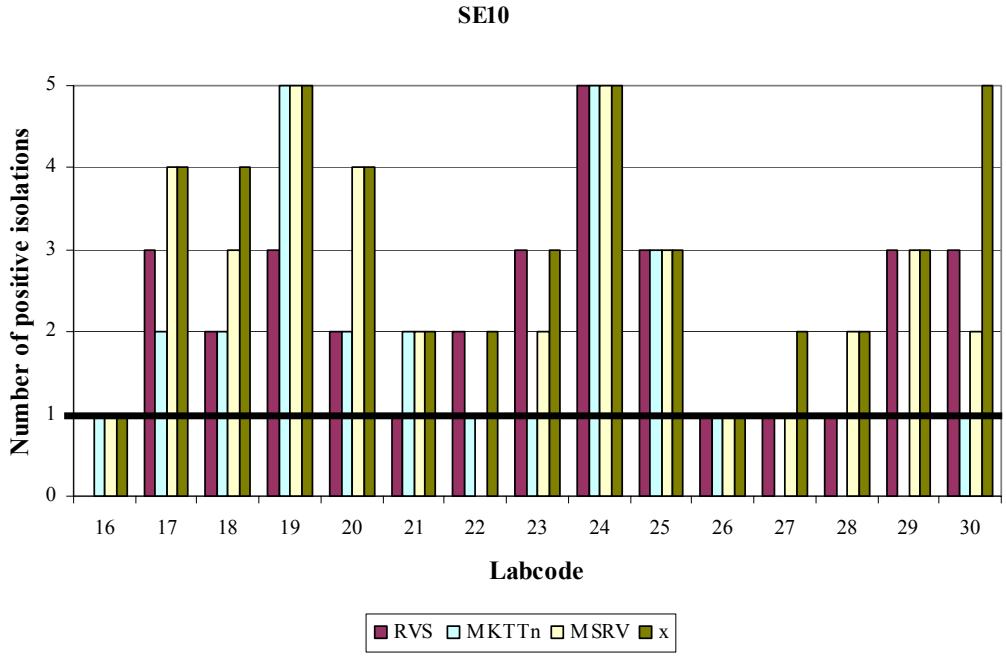
4.5.2 Results per selective enrichment medium, capsule and per laboratory

In the Figures 1, 2, 3 and 4 the number of positive isolations per artificially contaminated meat sample is given per laboratory after pre-enrichment in BPW and selective enrichment in RVS, MKTTn and on MSRV, followed by isolation on selective plating agar XLD. For determining good performance per laboratory, all combinations of selective enrichment media and isolation media used by the laboratory were taken into account. In the figures the most optimal combination of selective enrichment medium and isolation medium is indicated as "x".

The results of all artificially contaminated minced beef samples were compared with the proposed definition of "good performance" (see section 3.6). In the Figures 1-4 the border of good performance is indicated with a black horizontal line. According to this definition the score for the artificially contaminated samples with *Salmonella* was below the set criteria for laboratory 9 and the score for the blank samples without *Salmonella* was above the set criteria for three laboratories (labcodes 17, 27 and 30).

The majority of the laboratories found the highest number of positive isolations when XLD was used as isolation medium. Six laboratories found differently when analysing the meat samples artificially contaminated with SE10 capsules. Laboratories 5, 6, 7 and 30 found a higher number of positive isolations on either BGA, SM2, BPLSA or XLT4 after selective enrichment in RVS. Laboratories 18 and 27 found higher number of positives with the combination MSRV/Rambach and RV/XLD respectively.

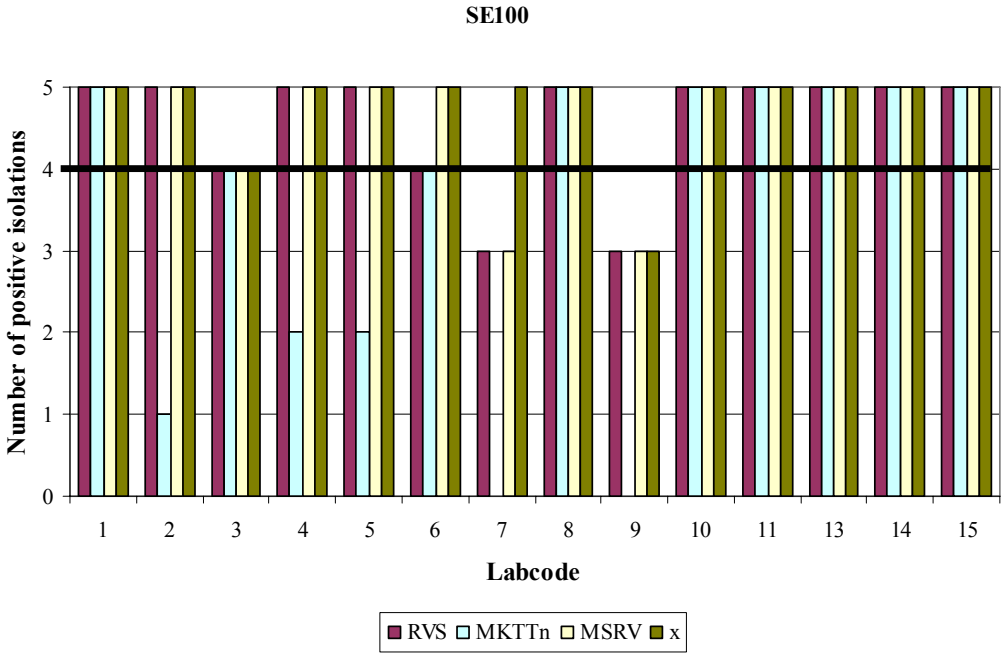




Lab 22 did not use MSRV

= border of good performance

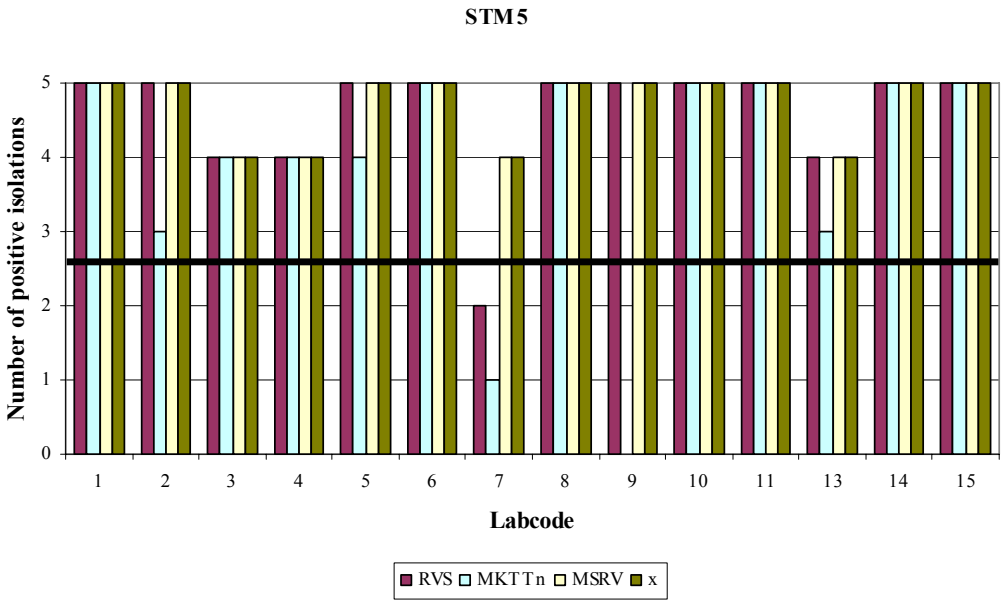
Figure 1 Results minced beef artificially contaminated with SE10 capsules (n=5) after selective enrichment in RVS, MKTTn and on MSRV followed by isolation on selective plating agar XLD. The highest number of positive isolations found with all combinations of selective enrichment media and isolation media used by a laboratory are given as x.

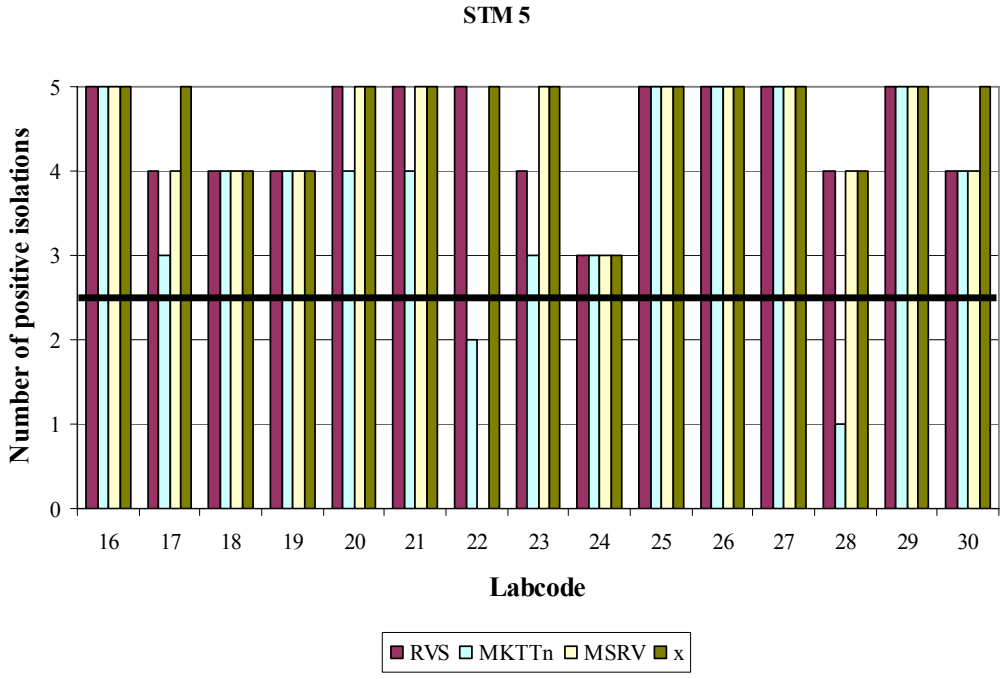


Lab 22 did not use MSRV

= border of good performance

Figure 2 Results minced beef 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 are given as x.

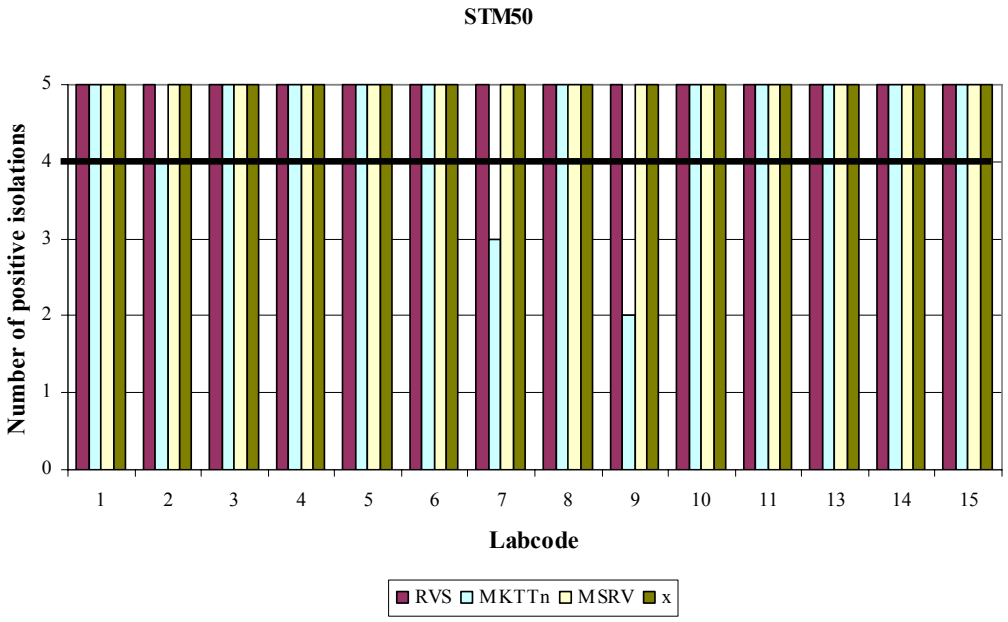


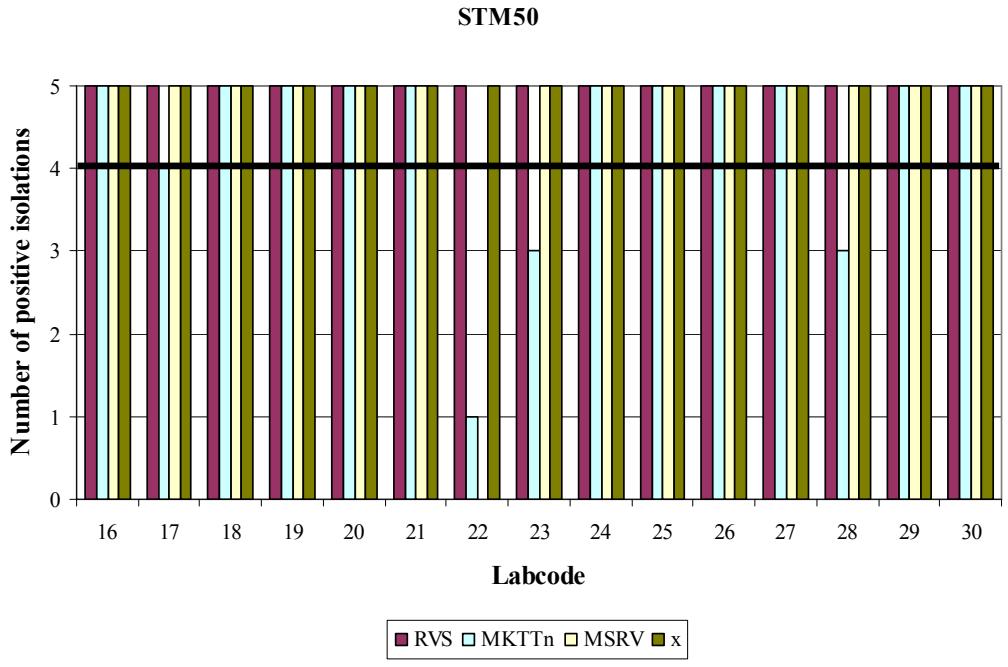


Lab 22 did not use MSRV

= border of good performance

Figure 3 Results minced beef 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 are given as x.





Lab 22 did not use MSRV

= border of good performance

Figure 4 Results minced beef 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 are given as x.

The differences in the number of positive isolations after 24 and 48 hours of incubation of the selective enrichment media are given in Table 17. 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. Only when MKTTn is used for selective enrichment, XLD gave 9% 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 was the highest for MKTTn (Table 17). With MKTTn 12-13% more positive isolations were found after 48 h of incubation. For RVS the difference between the two incubation times was 9-10%. For MSRV this difference was 6-7%.

Table 17 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 analyzing the artificially contaminated minced beef samples.

Plating-out medium	**Selective enrichment medium**		
	RVS	**MKTTn**	**MSRV**
	24 / 48 h	24 / 48 h	24 / 48 h
XLD	58 / 67%	42 / 55%	63 / 69%
Other (most often BGA)	57 / 67%	34 / 46%	58 / 65%

In Tables 18 and 19 differences between selective enrichment media and isolation media per capsule are shown as odds ratios (OR). In addition the 95% confidence intervals and p-values are given.

The interpretation of Table 18 is as follows: given a selective enrichment medium the number of positive isolations found with the different plating out media are compared. For instance the odds of finding *Salmonella* using XLD as isolation medium is a factor OR higher than using an isolation medium other than XLD. If MKTTn is used as selective enrichment medium, XLD shows significantly more positive results. In general for MSRV and RVS the odds are also higher, but not significant.

The interpretation of Table 19 is similar to that of Table 18, except that selective enrichment media are mutually compared given XLD as isolation medium. If MSRV or RVS are used as selective enrichment media, the odds of finding *Salmonella* are significantly higher than in case of MKTTn. RVS compared to MSRV results in lower odds, although this is not significant.

Figure 4 shows the performance of each laboratory as odds ratios compared to the mean of all laboratories for the artificially contaminated samples. The mean is defined as the odds of detecting *Salmonella* based on the fixed effects only (capsule, enrichment medium and isolation medium), hence OR = 1. Laboratories below the mean have a lower odds to detect *Salmonella*. Laboratories 2, 3, 7, 9, 22, 23, 28 and 30 found significant lower number of positive results compared to the mean of all laboratories.

Table 18 Number of positive isolations found with XLD compared to the number of positive isolations found with other isolations found with other isolation media, given a selective enrichment medium. Samples : minced beef, artificially contaminated with *Salmonella* positive capsules.

Selective enrichment medium	Compared isolation media	Capsule	Odds Ratios	95% lower	95% upper	p-value
MKTTn	XLD compared to other than XLD	SE10	2.16	1.16	4.02	0.015
		SE100	3.09	1.62	5.90	0.001
		STM5	2.69	1.46	4.96	0.002
		STM50	5.55	2.22	13.93	0.000
		all SE	2.10	1.42	3.11	0.000
		all STM	3.24	1.97	5.32	0.000
		all capsules	2.10	1.59	2.76	0.000
MSRV	XLD compared to other than XLD	SE10	1.35	0.77	2.36	0.289
		SE100	3.80	0.96	14.99	0.057
		STM5	1.80	0.72	4.50	0.210
		STM50	Inf	0.00	Inf	0.995
		all SE	1.34	0.89	2.01	0.166
		all STM	2.27	0.96	5.36	0.062
		all capsules	1.39	0.99	1.95	0.054
RVS	XLD compared to other than XLD	SE10	0.95	0.56	1.62	0.860
		SE100	2.07	0.72	5.95	0.179
		STM5	0.78	0.33	1.85	0.566
		STM50	1.00	0.00	Inf	1.000
		all SE	1.09	0.75	1.57	0.655
		all STM	0.80	0.35	1.83	0.605
		all capsules	1.03	0.76	1.40	0.854
MKTTn/MSRV/RVS	XLD compared to other than XLD	SE10	1.32	0.96	1.81	0.090
		SE100	1.87	1.27	2.74	0.001
		STM5	1.58	1.07	2.34	0.021
		STM50	3.12	1.60	6.07	0.001
		all SE	1.37	1.11	1.69	0.004
		all STM	1.85	1.34	2.56	0.000
		all capsules	1.42	1.20	1.67	0.000

Grey cells: significant difference $p < 0.05$

Inf. = infinity

Table 19 Number of positive isolations found with a selective enrichment medium compared to the number of positive isolations found with another selective enrichment medium, with the isolation on XLD. Samples : minced beef, artificially contaminated with *Salmonella* positive capsules.

Compared selective enrichment media	Isolation medium	Capsule	Odds Ratios	95% lower	95% upper	p-value
MSRV compared to MKTTn	XLD	SE10	2.73	1.44	5.16	0.002
		SE100	88.73	15.15	519.52	0.000
		STM5	4.73	2.05	10.90	0.000
		STM50	Inf	0.00	Inf	0.994
		All SE	3.70	2.38	5.75	0.000
		All STM	6.13	2.82	13.34	0.000
		All capsules	3.22	2.30	4.50	0.000
RVS compared to MKTTn	XLD	SE10	1.84	1.02	3.31	0.042
		SE100	35.69	10.53	121.00	0.000
		STM5	3.64	1.72	7.69	0.001
		STM50	Inf	0.00	Inf	0.992
		All SE	2.92	1.96	4.35	0.000
		All STM	5.18	2.62	10.23	0.000
		All capsules	2.77	2.04	3.77	0.000
RVS compared to MSRV	XLD	SE10	0.70	0.39	1.26	0.234
		SE100	0.74	0.17	3.31	0.696
		STM5	0.72	0.28	1.83	0.489
		STM50	1.00	0.00	Inf	1.000
		All SE	0.81	0.54	1.23	0.333
		All STM	0.75	0.30	1.84	0.528
		All capsules	0.83	0.59	1.17	0.280

Grey cells: significant difference $p < 0.05$

Inf. = infinity

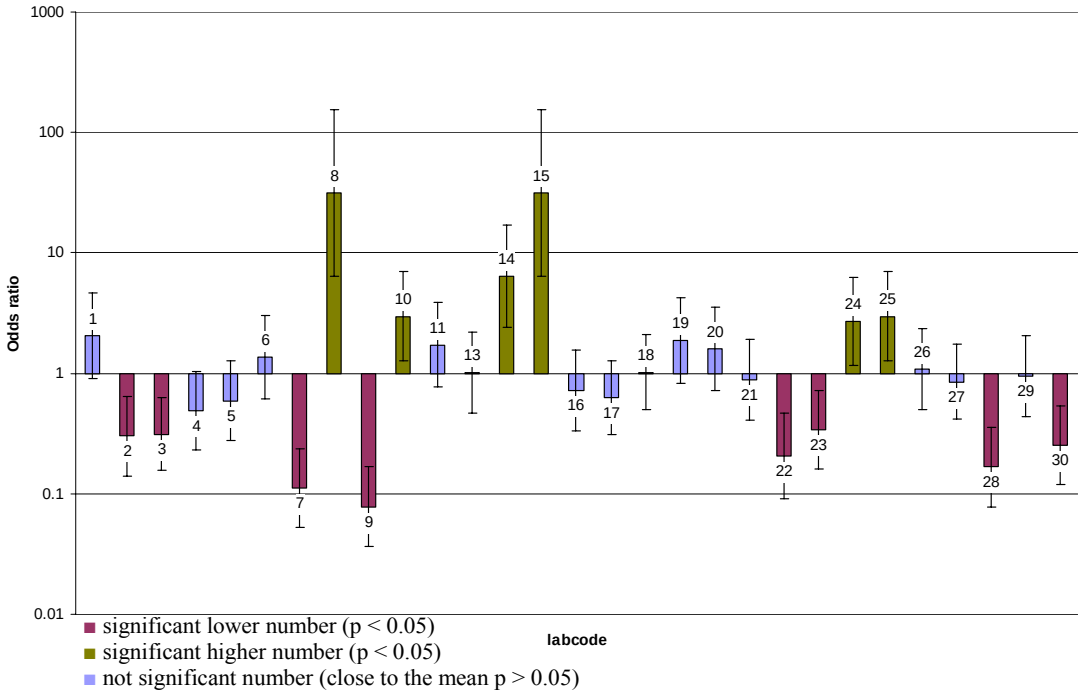


Figure 4 Performance of each laboratory compared to the mean of all laboratories.

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

The specificity, sensitivity and accuracy rates for all types of capsules added to the minced beef are shown in Table 20. The results are given for the different medium combinations: BPW followed by selective enrichment in RVS, MKTTn and on MSRV and isolation on selective plating agar XLD. The specificity rates (of the blank capsules) were for all three selective enrichment media 97-99%. For all capsules containing *Salmonella* the sensitivity rates found with MKTTn were approximately 10% lower than the rates of RVS and MSRV. As the contamination level of the low level reference materials was close to the detection limit the sensitivity rates were expected to be close to 50%. This was indeed the case for the sensitivity rates of SE10 contaminated samples tested with RVS or MSRV (49-54%). However, the low level STM samples were obviously more easy to detect as the sensitivity rates of STM5 were 90-92% (found with RVS or MSRV).

Table 20 Specificity, sensitivity and accuracy rates for all participating laboratories (n=29)[1] of the artificially contaminated minced beef samples (each capsule added to 10 g minced beef) for the selective enrichment in RVS, MKTTn and on MSRV and plating out medium XLD.

Capsules with minced beef		RVS/XLD	MKTTn/XLD	MSRV/XLD [2]
Blank	No. of samples	145	145	140
(n=5)	No. of negative samples	143	141	139
	Specificity in%	**98.6**	**97.2**	**99.3**
STM5	No. of samples	145	145	140
(n=5)	No. of positive samples	131	111	129
	Sensitivity in%	**90.3**	**76.6**	**92.1**
STM50	No. of samples	145	145	140
(n=5)	No. of positive samples	145	130	140
	Sensitivity in%	**100.0**	**89.7**	**100.0**
SE10	No. of samples	145	145	140
(n=5)	No. of positive samples	71	58	76
	Sensitivity in%	**49.0**	**40.0**	**54.3**
SE100	No. of samples	145	145	140
(n=5)	No. of positive samples	138	98	134
	Sensitivity in%	**95.2**	**67.6**	**95.7**
All capsules with *Salmonella*	No. of samples	580	580	560
	No. of positive samples	485	397	479
	Sensitivity in%	**83.6**	**68.5**	**85.5**
All capsules	No. of samples	725	725	700
	No. of correct samples	628	538	618
	Accuracy in%	**86.6**	**74.2**	**88.3**

1 The results of laboratory 12 were not taken into account for the calculations.

2 Laboratory 22 did not perform selective enrichment on MSRV.

4.6 PCR

Four laboratories (labcodes 5, 6, 18, and 30) applied a PCR method as additional detection technique. These laboratories tested the samples after incubation in BPW. In Table 21 the details are summarized.

Table 21 Details on the Polymerase Chain Reaction method, used as own Method during the interlaboratory comparison study by four laboratories.

Labcode	Volume of BPW (µl)	Volume of DNA sample (µl)	Volume of DNA / PCR mix (µl)
5	1000	200	5/30
6	1000	300	45/66
18	1000	150	2/10
30	5 ul (BAX)	205	50/one tablet from kit

Laboratory 6 and 18 used a non-commercially available PCR technique. Laboratory 18 used an Inva PCR which is usually used for confirmation of bacterial cultures and not from pre-enrichment broths. Laboratory 5 used Applied system Taqman *Salmonella* enterica detection kit and laboratory 30 used a BAX System *Salmonella*. Both latter PCR methods are real time PCR kits. These PCR techniques are validated for chicken rinse (labcode 6 and 18) and meat and diary products (labcode 6 and 30).

The PCR results and the bacteriological culture results are shown in Table 22. For the control samples (without meat) laboratories 5, 6 and 18 found the same results with the PCR-technique as with the bacteriological culture method. All laboratories found one or two more SE10 samples with minced beef negative with the PCR technique than with the bacteriological culture technique.
Laboratory 18 scored all the blank samples negative with the PCR-technique while with selective enrichment in MKTTn and isolation on Rambach they found one blank positive.
Laboratory 30 scored more deviating results. They found all the three STM5 control samples positive with the PCR technique while with the bacteriological culture method they missed one STM5. They found a positive result with the PCR technique for the sample with only minced beef (C12) while with the bacteriological culture method they found this sample to be negative. Furthermore they scored one blank capsule added to minced beef positive (sample 19) with the PCR technique while with the bacteriological culture technique they found two other blank samples positive (samples 5 and 8).

Table 22 PCR results compared to bacteriological culture results of control capsules and of artificially contaminated minced beef samples of laboratories 5, 6, 18 and 30.

Capsules	Lab 5		Lab 6		Lab 18		Lab 30	
	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR
Controls without meat (n=10)								
Span 5 (n=2)	2	2	2	2	2	2	2	2
SE10 (n=2)	1	1	1	1	1	1	2	2
SE100 (n=1)	1	1	1	1	1	1	1	1
STM5 (n=3)	3	3	3	3	2	2	**2**	**3**
Blank (n=2)	0	0	0	0	0	0	0	0
BPW (n=1)	0	0	0	0	0	0	0	0
Minced beef (n=1)	0	0	0	0	0	0	**0**	**1**
Test samples with minced beef (n=25)								
SE10 (n=5)	**2**	**1**	**4**	**3**	**4**	**2**	**5**	**3**
SE100 (n=5)	5	5	5	5	5	5	5	5
STM5 (n=5)	5	5	5	5	4	4	5	5
STM50 (n=5)	5	5	5	5	5	5	5	5
Blank (n=5)	0	0	0	0	**1**	**0**	**2**	**1**

Grey cells: unexpected results
Bold numbers: different results found with BAC or PCR
BAC: the results of the prescribed or requested selective enrichment medium with the highest number of positives are given.

4.7 Performance of the NRLs

4.7.1 General

Twenty-three NRLs fulfilled the (new) criteria of good performance. Six laboratories scored below these criteria (labcodes 2, 7, 9, 17, 27 and 30). The results from those laboratories are summarised in Table 23 and 24.

Table 23 Number of positive isolations of NRLs with the lower scores, per capsule without the addition of meat for the selective enrichment media (RVS, MKTTn and MSRV) in combination with the isolation media giving the highest number of positive isolations.

.

Medium	RVS, MKTTn and MSRV		
Capsule	SE10	Span5	STM5
No. of capsules	n=2	n=2	n=3
Good performance	≥ 1	≥ 1	≥ 2
Labcode			
2	**0**	2	3
7	**0**	2	3

Bold numbers : deviating results

Grey cell : results are below good performance

Laboratory 2 and 7 could not detect *Salmonella* in both SE10 control samples.
Laboratory 9 could not detect *Salmonella* in all the five SE10 capsules and in two of the five SE100 capsules both with the addition of minced beef.
Laboratory 17, 27 and 30 found more than one blank sample positive with the addition of minced beef.
The six laboratories were contacted by the CRL-*Salmonella* in January 2007 to ask for any explanation for the deviating results and the possibility was offered to perform some extra analyses.
The laboratories gave different explanations: cross contamination, misinterpretation of results by limited confirmation, reconstruction of the laboratory and two laboratories could not give any explanation.

As the results of laboratory 12 are not reported elsewhere in this report they are summarised here. Laboratory 12 found only one minced beef sample artificially contaminated with SE10 negative for *Salmonella* and fulfilled the criteria of good performance. They performed also a real time PCR (BAX system) and the results were identical to the results found with the bacteriological culture method. The technical data of laboratory 12 are reported in clause 4.3 and their results can be found in the tables of Annex 6 and 7.

Table 24 Number of positive isolations of NRLs with the lower scores, per capsule (n=5) with the addition of 10 g *Salmonella* negative minced beef for the different selective enrichment media (RVS, MKTTn and MSRV) in combination with the isolation media giving the highest number of positive isolations.

Capsule	SE10	SE100	STM5	STM50	Blank
No. of capsules	n=5	n=5	n=5	n=5	n=5
Good performance	≥ 1	≥ 4	≥ 2	≥ 4	≤ 1
Labcode					
RVS					
9	**0**	**3**	5	5	0
30	5	5	5	5	**2**
MKTTn					
9	**0**	**0**	**0**	**2**	0
17	2	**3**	5	4	**2**
27	1	5	5	5	**2**
MSRV					
9	**0**	**3**	5	5	0

Bold numbers : deviating results

Grey cell : results are below good performance

4.7.2 Follow-up study

The set-up of the follow-up study was the same as the full interlaboratory comparison study in November, but with another batch of SE10 capsules (see section 4.1 "Reference materials"). In this follow-up study only *S.* Enteritidis and blank capsules were tested, as these samples were causing most of the problems. An overview is given in Table 25. The number of aerobic bacteria ($1.7*10^9$ cfu/g) and Enterobacteriaceae ($2.2*10^5$ cfu/g) were tested on 18 February 2008 after the minced beef was placed at 5 ^oC for one week. The numbers were comparable to the numbers of the minced beef as used for the full study (see Table 3, 19 November 2007).

On Tuesday 12 February 2008 one parcel with only one plastic container was send to the NRLs containing: 5 control capsules (C1 - C5), 15 capsules (1 – l5), 180 g minced beef and 1 temperature recorder.

The performance of this follow up study was on 18 February 2008. Six laboratories (labcodes 2, 7, 9, 17, 27 and 30) participated in this follow-up study. The laboratories had to follow the same SOP and Protocol as in the study of November 2007 (see Annexes 4 and 5). The test report was different from the November study (see Annex 8). For the media only the differences with the November study had to be indicated and special attention and more details were asked for the confirmation of *Salmonella* suspect colonies.

Table 25 Overview of the types and the number of capsules tested per laboratory in the follow-up of the interlaboratory comparison study.

Capsules	Control capsules (n=5) no meat added	Test samples (n=15) with 10 g *Salmonella* negative minced beef
S. Enteritidis 10 (SE10)	3	6
S. Enteritidis 100 (SE100)	1	4
Blank	1	5

For the media compositions, incubation times and temperatures no differences were observed in comparison with the full study of November 2007 with some small exceptions. Laboratory 9 performed the isolation on another medium (Rambach instead of BPLS). Laboratory 7 used an extra isolation medium (BGA additional to XLD and XLT4). All laboratories in this follow up study did pay special attention to the confirmation of the *Salmonella* suspect colonies by the performance of sero (group) typing and/or some extra biochemical tests additional to TSI, UA and LDC.

The results of the follow up study are summarised in Table 26 and 27.

Table 26 Number of positive isolations found by NRLs in the follow up study, per capsule without the addition of meat for the selective enrichment media (RVS, MKTTn and MSRV) in combination with the isolation media giving the highest number of positive isolations.

Medium	RVS, MKTTn and MSRV			
Capsule	SE10	SE100	Blank	C12*
No. of capsules	n=3	n=1	n=1	n=1
Good performance	≥ 1	≥ 1	0	0
Labcode				
2	**2**	1	0	0
7	**1**	1	0	0
9	3	1	0	0
17	3	1	0	0
27	**1**	1	0	**1**
30	3	1	0	0

*sample with only minced beef (C12)
Bold numbers : deviating results
Grey cell : results are below good performance

Table 27 Number of positive isolations found by NRLs in the follow up study, per capsule with the addition of 10 g *Salmonella* negative minced beef for the selective enrichment media (RVS, MKTTn and MSRV) in combination with the isolation media giving the highest number of positive isolations.

Medium	**RVS, MKTTn and MSRV**		
Capsule	SE10	SE100	Blank
No. of capsules	n=6	n=4	n=5
Good performance	≥ 1	≥ 3	≤ 1
Labcode			
2	**2**	4	0
7	6	4	**1**
9	**2**	4	**1**
17	**2**	4	0
27	6	4	**5**
30	**4**	4	0

Bold numbers : deviating results

Grey cell : results are below good performance

Five NRLs fulfilled the criteria of good performance (see section 3.6) for the tested samples in this follow-up study. Laboratory 2, 9 and 17 scored 2/6 and laboratory 30 scored 4/6 positive results with the SE10 samples with minced beef. Laboratory 7 scored 1/3 and laboratory 2 scored 2/3 positive results with SE10 control capsules without meat. Only laboratory 7 and 9 found one blank meat sample positive. Those scores were still within the limits of good performance.

Laboratory 27 scored again underperformance. The sample with only minced beef (C12) and all blank capsules with minced beef were found positive. On the other hand they scored the minimum number of positive results (1/3 positive) with the low level *Salmonella* Enteritidis control samples (capsules tested without meat). The CRL-*Salmonella* contacted this laboratory in March 2008 to ask for any explanation for the deviating results. The NRL performed an audit in their laboratory trying to find any reason for the positive blanks. The most plausible explanation is cross contamination and the laboratory presumes to have found out at what stage the cross contamination likely has occurred. The CRL-*Salmonella* is in contact with this laboratory to check whether their actions taken to prevent cross contamination are successful.

5 Discussion

Transport of the samples
Nor transport time, nor transport temperature seem to have negatively affected the results. The laboratories with the longest transporttimes (labcodes 1 and 19) and/or the highest transport temperatures (labcodes 1, 19, 24 and 26) 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 all the laboratories with the exception of one laboratory. For determining "good performance" per laboratory all combinations of selective enrichment media and isolation media used by each laboratory were taken into account. Twenty-four out of 30 laboratories scored a "good performance". Five laboratories fulfilled these criteria later in the follow up study. The repetitive poor performance of laboratory 27 in this study was most probably caused by cross contamination.
The results found with the MSRV method were very good closely followed by RVS. Although the scope of Annex D of ISO 6579 is detection of *Salmonella* spp. in samples of the primary production, it showed, in this study, 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 even showed the lowest number of positive results.
In this study six laboratories found blank samples with minced beef positive for *Salmonella*. Finding more than one blank meat sample positive is not very likely and may have been caused by cross-contamination or by misinterpretation of the results. The number of background flora in the minced beef was relatively high and may have caused problems with reading of the isolation media. In combination with a limited confirmation, the Enterobacteriaceae present in the minced beef could have been misinterpreted as *Salmonella,* resulting in false positive blank results. The three laboratories who found more than one positive blank capsule during the study were advised to check their procedures, especially for confirmation. In the follow up study these laboratories performed additional confirmation tests and two laboratories did not found any longer positive blanks. The problems of the third laboratory (labcode 27) were attributed to cross contamination.

Specificity, sensitivity and accuracy rates
The rates of the control samples were good. As expected the high level control sample (SE100) showed rates of 100%. For the low level materials (Span5, STM5 and SE10) the rates were expected to lie between 50% and 100%. However, Span5 and STM5 were obviously easier to detect than SE10 as for the first two materials the sensitivity rates were even close to 100%. For SE10 the sensitivity rates were approximately 70%.
For the artificially contaminated meat samples the highest rates were found with MSRV, closely followed by RVS. The rates found with MKTTn were approximately 10% lower than the rates of the two other selective enrichment media. In general the rates showed the expected results. As in this study the contamination level of the low level materials (STM5 and SE10) were close to the detection limit, the sensitivity rates were expected to lie close to 50%. The samples with SE10 indeed showed these expected results. Obviously the low level materials of *S.* Typhimurium (STM5) were easier to detect than the low level materials of *S.* Enteritidis. The sensitivity rates of STM5 with RVS and MSRV were even ≥ 90%.

Media

According to Annex D of ISO 6579 (Anonymous, 2007) the concentration of novobiocin in MSRV should be 10 mg/L and the pH between 5.1-5.4. Thirteen laboratories reported the use of a higher concentration of novobiocin and four laboratories did not mention the use of novobiocin. Ten laboratories reported a higher/lower pH or did not mention the pH. Laboratories 2, 7, 17, 27 and 30 reported a higher pH or a higher concentration of novobiocin or did not mention the composition of MSRV. Those laboratories scored less positive results for the SE10 capsules with matrix in this study. A higher concentration of novobiocin in the MSRV can negatively influence the motility of *Salmonella* and may result in less positive results. A higher pH of MSRV may stimulate the growth of disturbing background flora which can negatively influence the growth of *Salmonella*. The higher pH and/or a higher concentration of novobiocin in MSRV could have been one of the explanations for the lower number of positive results of the minced beef samples artificially contaminated with SE10.
Other deviations in media compositions or incubation temperatures were reported but no clear effects were found on the results.
The selective enrichment media MSRV and RVS showed high percentages of positive results already after 24 hours of incubation. The selective enrichment medium MKTTn showed more positive results after 48 hours of incubation than after 24 hours. Furthermore high amounts of background flora were more disturbing after isolation from MKTTn than from RVS and MSRV.

In general XLD showed (slightly) more positive results than any of the other isolation media used. This effect was most striking when XLD was used after selective enrichment in MKTTn.

PCR

Only four 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. However, all laboratories found one or more SE10 samples with minced beef more negative with the PCR technique. A possible explanation could be the high amount of disturbing background flora in combination with the relatively low amount of *Salmonella* Enteritidis present in the sample.

Evaluation of this study

For a better testing of the performance of the laboratories the contamination levels of the capsules used in this study were lower than in the former (pilot) 2006 Food study (Kuijpers et al., 2007). The contamination levels of the low level reference materials were close to the detection limits. The detection limit of *Salmonella* Typhimurium was obviously lower than the detection limit of *Salmonella* Enteritidis in minced beef and tested with the same methods. From the study it can be concluded that the detection limit of STM in minced beef was close to 1 cfp/10g. Technically it is not possible to prepare reference materials containing only 1 cfp/capsule and therefore the STM5 capsules can be considered as a right choice for the STM low level samples. The detection limit of *Salmonella* Enteritides in minced beef in this study was approximately 10 times higher than that of STM. The contamination level of the SE capsules used in this study was very close to this detection limit and the positivity of the minced beef samples artificially contaminated with these low level SE capsules was sometimes hard to test. This was the reason to amend the criterium for good performance for these samples. For future studies it may be better to adjust the contamination level of the low level SE capsules, so that the level will be somewhat above the detection limit (e.g. 15-20 cfp/capsule).

6 Conclusion

- Twenty-nine out of thirty NRLs for *Salmonella* achieved the level of "good performance" for the detection of *Salmonella* in minced beef. Five laboratories reached this level after a follow up study. One showed repeatedly to have problems with analysing the samples in the full study as well in the follow up study.

- The accuracy, specificity and sensitivity rates for the control samples (without meat) of RVS, MKTTn and MSRV were higher than 91% with the exception of the SE10 capsules which rates were approximately 73%.

- The specificity rate of the minced beef samples artificially "contaminated" with blank capsules was higher than 97%.

- For all artificially contaminated minced beef samples the rates found with MKTTn were approximately 10% lower than the rates of MSRV and RVS.

- The sensitivity rates for artificially contaminated minced beef samples with STM5, STM50 and SE100 capsules were higher than 90% for MSRV and RVS; for MKTTn it was 67%.

- The sensitivity rates for artificially contaminated minced beef samples with SE10 capsules were (as expected) approximately 50% for MSRV and RVS and 40% for MKTTn.

- The low level materials of *S*. Typhimurium (STM5) were easier to detect than the low level materials of *S*. Enteritidis (SE10).

- The accuracy rates for the artificially contaminated minced beef samples were higher than 86% for RVS and MSRV and was 74% for MKTTn.

- When MKTTn is used as selective enrichment medium, plating out on XLD showed significantly more positive results than other plating-out media.

- When MSRV or RVS are used as selective enrichment media, the odds of finding *Salmonella* are significantly higher than when using MKTTn (all in combination with isolation medium XLD).

- XLD showed slightly more positive results than other plating-out media independent on 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 is more important (more positive results after 48h) for selective enrichment in MKTTn than for MSRV, for the matrix used (minced beef).

- MSRV is a good selective enrichment medium for the matrix used (minced beef).

- MKTTn is not the optimal medium for selective enrichment of the matrix used (minced beef with much background flora).

- A complete confirmation of *Salmonella* suspect colonies is very important especially when the number of background flora (Enterobacteriaceae) in a matrix is relatively high and may negatively influence the reading of the isolation media.

- The contamination level of the minced beef samples artificially contaminated with the low level *S*. Enteritidis (SE10) capsules was very close to the detection limit of the method.

rivm

References

Anonymous, 2002, ISO 6579 (E). Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. International Organisation for Standardisation, Geneva, Switzerland.

Anonymous, 2003, ISO 4833. Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of microorganisms – Colony-count technique at 30 degrees C International Organisation for Standardisation, Geneva, Switzerland.

Anonymous, 2004, ISO 21528-2. Microbiology of food and animal feeding stuffs – Horizontal methods for the detection and enumeration of Enterobacteriaceae – Part 2: Colony-count method. International Organisation for Standardisation, Geneva, Switzerland.

Anonymous, 2005, ISO 17025. General requirements for the competence of testing and calibration laboratories. International Organisation for Standardisation, Geneva, Switzerland.

Anonymous, 2007, Amendment of ISO 6579:2002/Amd1 Annex D: Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage. International Organisation for Standardisation, Geneva, Switzerland.

Bates D, 2007. The lme4 Package. Linear mixed-effects models using S4 classes, URL http://cran.r-project.org/web/packages/lme4/lme4.pdf (18 February 2008)

Commision Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules. Official Journal of the European Union L 165 of 30 April 2004.

In `t Veld PH, Strijp-Lockefeer van NGWM, Havelaar AH, Maier EA, 1996. The certification of a reference material for the evaluation of the ISO method for the detection of *Salmonella*. J.Appl.Bacteriol; 80: 496-504

Kuijpers AFA, Veenman C, van de Kassteele J, Mooijman KA, 2007. EU Interlaboratory comparison study food – I (2006) – Bacteriological detection of *Salmonella* in minced beef. RIVM report 330604003

R Development Core Team, 2008. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org (18 February 2008)

Schulten SM, In ’t Veld PH, Ghameshlou Z, Schimmel H, Linsinger T, 2000. The certification of the number of colony forming particles of *Salmonella* Typhimurium and number fraction of negative capsules from artificially contaminated milk powder. Commission of European Communities, Community Bureau of Reference, Brussels, Luxembourg. CRM 507R, EUR 19622 EN.

Venables WN and Ripley BD, 2002. Modern Applied Statistics with S, fourth edition, Springer, New York.

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

Table 1.1 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 1.1 (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 1.1 (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			

Table 1.2 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 [3] (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			
I I	2007	5	STM5	4	10 gram	RVS, MKTTn, MSRV and own	XLD and own	This report
		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			

[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*

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.

rivm

Annex 3 Information on the media used

BGA modified (Oxoid CM 0329, Hampshire, United Kingdom) (BPLS Merck 1.10747 Darmstadt, 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, 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

Chrom ID agar see SM ID2

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 and pH is not provided

Hektoen Enteric (Biokar Diagnostics BK 067HA, Beauvais, France)
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 is not provided, pH 7.5

MLCB (Lab M. Ltd. LAB 116, Bury, United Kingdom)
Inoue T, Takagi S, Ohnishi A, et al. Foodborne disease *salmonella* isolation medium (MLCB). Japanese Journal of Veterinary Science 1968;30(suppl):26.
Composition of MLCB medium: the concentration of the compounds in g/L water: Yeast Extract 5.0, Tryptone 5.0, Meat Peptones 7.0, Sodium Chloride 4.0, Mannitol 3.0, L-Lysine HCL 5.0, Sodium Thiosulphate 4.0, Ferric Ammonium Citrate Green 1.0, Brilliant Green 0.012, Crystal Violet 0.01, Agar No.2 15.0, pH 6.7

Onöz (Merck 115034, Darmstadt, Germany)
Onoz E, Hoffmann K. 1978 [Experience with a new culture medium for *salmonella* diagnosis (author's transl)] Zentralbl Bakteriol [Orig A]. 1978 Jan;240(1):16-21. German.
Composition of Onöz medium: the concentration of the compounds in g/L water: Yeast 3.0, Meat extract 6.0, Pepton from meat 6.8, Lactose 11.5, Sucrose 13.0, Bile salt mixture 3.825, tri-Sodium nitrate 5,5-Hydrate 9.3, Sodium Thiosulfate 5-Hydrate 4.25, L-Phenylalanine 5.0, Iron(III) Citrate 0.5,Magnesiumsulfate 0.4, Brilliant Green 0.00166, Neutral Red 0.022, Aniline Blue 0.25, Metachrome Yellow 0.47, di-Sodium Hydrogen Phosphate2-Hydrate 1.0, Agar-Agar 15, pH 7.1

Rambach (Merck 107500.0001/2, Darmstadt, Germany) (Chromagar RR701 CHROMagar Company, Paris, France)
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 8.0, NaCl 5.0, sodium deoxycholate 1.0, Cromogenic mix 1.5, propylene glycol 10.5, agar-agar 15, Rambach agar supplement 10 ml, pH 7.1-7.3

SMID2 = ChromID Salmonella (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 CM1061, 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 Citratus 0.8, 7-ethyl-2 methyl-4-undecanol hydrogen 4.6 ml, Phenol Red 0.08, Agar 18 pH 7.4

rivm

Annex 4 Protocol

INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN FOOD organised by CRL-*Salmonella*
FOOD STUDY II – 2007

Introduction

This is the second interlaboratory comparison study on the detection of *Salmonella* spp. in a food matrix amongst the National Reference Laboratories (NRLs for *Salmonella*) in the EU. The research of *Salmonella* spp. in food matrices is also an important task for the CRL, as well as for the NRLs-*Salmonella*. This is described in Commission Regulations EC No 882/2004 on official controls.
This second study will have a comparable set-up as the first food study and the earlier studies on the detection of *Salmonella* spp. in veterinary samples.
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 analyse the samples with molecular methods and/or use additional methods (routinely) used in their laboratories.

The samples will consist of minced beef samples (*Salmonella* negative) artificially contaminated with reference materials. The reference materials (RMs) consist of gelatine capsules containing sublethally injured *Salmonella* Typhimurium (STM), *Salmonella* Enteritidis (SE) or *Salmonella* Panama (SPan) at different contamination levels. Each laboratory will examine 25 meat samples (10 g each) in combination with a capsule containing STM or SE and 10 control samples (capsules only).

For a better testing of the performance of the laboratories the contamination levels of the STM and SE capsules in this study are lower than in earlier studies. During the studies in 2006 the STM and SE samples were tested for almost 100% positive. In this study the contamination level of the low level capsules is at the detection limit of the method, and contain circa 5 colony forming particles (cfp) of *S.* Typhimurium (STM5) and 10 cfp of *S.* Enteritidis (SE10) instead of STM10 and SE100 in the first food-study. Besides these low level samples, also high level samples (approximately 5-10 times above the detection limit) are included, so that laboratories can be sure that *Salmonella* is present. The high level capsules contain circa 50 colony forming particles (cfp) of *S.* Typhimurium (STM50) and 100 cfp of *S.* Enteritidis (SE100) instead of STM100 and SE500 in the first food-study.

Like in earlier studies, the parcel will contain an electronic temperature recorder. This will give information on the temperatures and times during transport of the samples. The amount of materials can not be packed in one biobottel and will be divided over two biobottles (one containing capsules and one containing *Salmonella* negative meat). The two biobottles are packed in one box with cooling elements. Only one temperature recorder and will be included only in the biobottle containing the capsules. The recorder will be packed in a plastic bag, which will also contain your lab code. **You are urgently requested to return this complete plastic bag with recorder and lab code to the CRL-*Salmonella*, immediately after receipt of the parcel**. For this purpose a return envelope with a preprinted address label of the CRL-*Salmonella* has been included.

Each box (containing 2 biobottles) 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 15 of November 2007 (this is 4 working days after the day of mailing).

Objectives

The main objective of the second interlaboratory comparison study on the detection of *Salmonella* in a food matrix is to evaluate the results of the detection of different contamination levels of *Salmonella* in the presence of competitive micro-organisms in a food matrix, using different methods, among and within the NRLs.
By comparing the results of this study with the results of the first food-study last year, further information may be obtained on a suitable contamination level of the reference material to test the performance of the laboratories, as the same matrix and methods are used in both studies.

Outline of the study

Each participant will receive (in week 46) one box containing 2 biobottles, packed with cooling elements. The biobottles contain:

Biobottle 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 biobottle 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 biobottle 1 at (-20 ± 5) ºC immediate after receipt.

Biobottle 2:

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

Store biobottle 2 at (5 ± 3) ºC immediate after receipt.

The performance of the study will be in week 47 (starting on 19 November 2007).

The documents necessary for performing the study are:

- Protocol Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in food II (2007);
- SOP Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in food II (2007);
- Test report Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in food II (2007);
- 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 will be reported in the test report and sent to the CRL-*Salmonella* and will be used for (statistical) analysis. Please make sure to send your results to CRL-*Salmonella* before 7 December 2007. At the CRL a short report will be prepared to inform all NRLs within 1 to 2 months after the study on the overall results. We will start the first overall analyses immediately after the deadline. **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 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 II (2007)

Week	Date	Topic
44	29 October – 2 November	Mailing of the protocol, standard operating procedure and test report to the NRLs-*Salmonella*
46	12 – 16 November	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 15 November, 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)
47	19-23 November	Performance of the study, following the instructions as given in the protocol and the SOP of study Food II (2007).
49	Before 7 December	Completion of the test report. Send the test report, preferably by e-mail to the CRL Salmonella (Angelina.kuijpers@rivm.nl)*.
50	10-14 December	Checking the results by the National Reference Laboratories.
	December 2007 January 2008	Sending of the final results to the NRLs together with a short report. As a follow-up, actions will be undertaken for those NRLs which scored below the average results of all NRLs.

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

rivm

Annex 5 SOP

STANDARD OPERATING PROCEDURE
INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN FOOD
organised by CRL-*Salmonella*
FOOD STUDY II - 2007

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 beef (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.
- *Detection of Salmonella*: detection of *Salmonella* from reference materials in the presence of competitive organisms, when the test is 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
LDC	l-Lysine decarboxylation medium
MKTTn	Muller Kaufmann Tetrathionate novobiocin broth
MSRV	Modified semi-solid Rappaport Vassiliadis medium
RM	Reference Material
RVS	Rappaport Vassiliadis medium with Soya
SOP	Standard Operating Procedure
TSI	Triple sugar/iron agar
UA	Urea Agar
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 (Annex D of ISO 6579) it is allowed to use other methods, e.g. the one(s) routinely used in your laboratory [‘Own’ method(s)]. Prepare media for the ‘own’ method(s) according to the relevant instructions. Note all relevant information in the test report.

6.1 Non selective pre-enrichment medium

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

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

6.2 Selective enrichment medium

- 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

- Triple sugar/iron agar (TSI agar) (ISO6579 Annex B.6)
- Urea agar (UA agar) (ISO6579 Annex B.7)
- l-Lysine decarboxylation medium (LDC) (ISO6579 Annex B.8)
- Nutrient agar (optionally) (ISO6579 Annex 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

8.1 General

Below the prescribed and the requested method of the second 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 beef 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 beef to the jars as follows :

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

Do not shake the jars after adding the minced beef.

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 and also incubate for two times 24 h.

After equilibration:
Prescribed methods:

- Transfer 0.1 ml of BPW culture to each tube containing 10 ml RVS medium. Incubate at 41,5 °C ± 1 °C for 24 h ± 3 h and later on another 24 h ± 3 h;
- Transfer 1 ml of BPW culture to each tube containing 10 ml MKTTn medium. Incubate at 37 °C ± 1 °C for 24 h ± 3 h and later on another 24 h ± 3 h;

Requested method:

- Inoculate the MSRV plates with three drops of BPW culture, with a total volume of 0.1 ml. Incubate **(not upside down)** at 41,5 °C ± 1 °C for 24 h ± 3 h and if negative for another 24 h ± 3 h;

Optional method:

- Inoculate the routinely used selective medium/media (other than those mentioned above), with the corresponding BPW culture (note the inoculation volume of BPW used and the volume of the selective medium/media on test report). Incubate at the temperature 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 the case that you do not have large dishes (140 mm) at your disposal use two standard (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 test report, page 12-13).
2) Second isolation medium. Follow the instructions of the manufacturer (record temperature and time and other requested data in 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 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 biochemical 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 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.

Biochemical confirmation

By means of a loop, inoculate the media specified below with the colony selected as described above (either directly from the isolation medium, or from nutrient agar). For each of the mentioned media follow the instructions in 9.5 of ISO 6579 (2002). Optionally inoculate other media which are routinely used for biochemical confirmation. Record in the test report (page 19) the requested data of the media.

- TSI agar
- UA agar
- LDC

Interpretation of the biochemical tests

Salmonella generally show the reactions given in Table 1 of ISO 6579:2002 on page 9.

- TSI agar:
  Butt:-yellow by fermentation of glucose (+);
  -black by formation of hydrogen sulphide (+);
  -bubbles or cracks due to gas formation from glucose (+);
  Slant: -red or unchanged: lactose and sucrose are not used (-);
- Urea agar: yellow: no splitting of ammonia (UA -);
- l-Lysine decarboxylation medium: turbidity and purple colour (LDC +);

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 II(2007)
On detection of *Salmonella* spp. in minced beef (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 beef 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 (24 h) 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	**Biochemical confirmation**	Inoculate the media from first isolation media (day 3) for biochemical identification and incubate 24 (± 3)h at the specified temperature
5	**Biochemical confirmation**	Inoculate the media from second isolation media (day 4) for biochemical identification and incubate 24 (± 3)h at the specified temperature

Annex 6 Number of positive results of the control samples (capsule without meat) 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	SE10 n=2	SE100 n=1	SPan5 n=2	STM5 n=3	Blank n=2	SE10 n=2	SE100 n=1	SPan5 n=2	STM5 n=3	Blank n=2	SE10 n=2	SE100 n=1	SPan5 n=2	STM5 n=3
Good Performance	**0**	**≥ 1**	**1**	**≥ 1**	**≥ 2**	**0**	**≥ 1**	**1**	**≥ 1**	**≥ 2**	**0**	**≥ 1**	**1**	**≥ 1**	**≥ 2**
1	0	**1**	1	2	**2**	0	**1**	1	2	**2**	0	**1**	1	2	**2**
2	0	**0**	1	2	3	0	**0**	1	2	3	0	**0**	1	2	3
3	0	**1**	1	2	3	0	**1**	1	2	3	0	**1**	1	2	3
4	0	2	1	2	3	0	2	1	2	3	0	2	1	2	3
5	0	**1**	1	2	3	0	**1**	1	2	3	0	**1**	1	2	3
6	0	**1**	1	**1**	3	0	**1**	1	2	3	0	**1**	1	2	3
7	0	**0**	1	2	3	0	**0**	1	2	3	0	**0**	1	2	3
8	0	2	1	2	3	0	2	1	2	3	0	2	1	2	3
9	0	2	1	2	3	0	2	1	2	3	0	2	1	2	3
10	0	2	1	2	3	0	2	1	2	3	0	2	1	2	3
11	0	2	1	2	3	0	2	1	2	3	0	2	1	2	3
12*	0	2	1	2	3	0	2	1	2	3	0	2	1	2	3
13	0	**1**	1	2	3	0	**1**	1	2	3	0	**1**	1	2	3
14	0	2	1	2	3	0	2	1	2	3	0	2	1	2	3
15	0	2	1	2	3	0	2	1	2	3	0	2	1	2	3
16	0	2	1	2	3	0	2	1	2	3	0	2	1	2	3
17	0	**1**	1	2	3	0	**1**	1	2	3	0	**1**	1	2	3
18	0	**1**	1	2	**2**	0	**1**	1	2	**2**	0	**1**	1	2	**2**
19	0	**1**	1	2	3	0	**1**	1	2	3	0	**1**	1	2	3
20	0	2	1	2	3	0	2	1	2	3	0	2	1	2	3

rivm

Labcode	RVS					MKTTn					MSRV				
	Blank n=2	SE10 n=2	SE100 n=1	SPan5 n=2	STM5 n=3	Blank n=2	SE10 n=2	SE100 n=1	SPan5 n=2	STM5 n=3	Blank n=2	SE10 n=2	SE100 n=1	SPan5 n=2	STM5 n=3
Good Performance	**0**	**≥ 1**	**1**	**≥ 1**	**≥ 2**	**0**	**≥ 1**	**1**	**≥ 1**	**≥ 2**	**0**	**≥ 1**	**1**	**≥ 1**	**≥ 2**
21	0	2	1	2	3	0	2	1	2	3	0	2	1	2	3
22	0	**1**	1	2	3	0	**1**	1	2	3	**-**	**-**	**-**	**-**	**-**
23	0	2	1	2	3	0	2	1	2	3	0	2	1	2	3
24	0	2	1	2	**2**	0	2	1	2	**2**	0	2	1	2	**2**
25	0	**1**	1	2	3	0	**1**	1	2	3	0	**1**	1	2	3
26	0	**1**	1	2	3	0	**1**	1	2	3	0	**1**	1	2	3
27	0	**1**	1	2	3	0	**1**	1	2	3	0	**1**	1	2	3
28	0	2	1	2	3	0	2	1	2	3	0	2	1	2	3
29	0	2	1	2	3	0	2	1	2	3	0	2	1	2	3
30	0	2	1	2	**2**	0	2	1	2	**2**	0	2	1	2	**2**

-: not performed

*: laboratory 12 performed the study 3 months later

bold numbers: deviating results

grey cells : results are below the criteria of good performance

Annex 7 Number of positive results of the artificially contaminated meat (with capsule) 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=5	SE10 n=5	SE100 n=5	STM5 n=5	STM50 n=5	Blank n=5	SE10 n=5	SE100 n=5	STM5 n=5	STM50 n=5	Blank n=5	SE10 n=5	SE100 n=5	STM5 n=5	STM50 n=5
Good Performance	≤ 1	≥ 1	≥ 4	≥ 2	≥ 4	≤ 1	≥ 1	≥ 4	≥ 2	≥ 4	≤ 1	≥ 1	≥ 4	≥ 2	≥ 4
1	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
2	0	**1**	5	5	5	0	**0**	**1**	**3**	**4**	0	**1**	5	5	5
3	**1**	**1**	**4**	**4**	5	0	**0**	**4**	**4**	5	0	**1**	**4**	**4**	5
4	0	**2**	5	**4**	5	0	**0**	**2**	**4**	5	0	**2**	5	**4**	5
5	0	**2**	5	5	5	0	**1**	**2**	**4**	5	0	**1**	5	5	5
6	0	**4**	**4**	5	5	0	**1**	**4**	5	5	0	**3**	5	5	5
7	0	**2**	5	**3**	5	0	**0**	**0**	**2**	5	0	**0**	**3**	**4**	5
8	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
9	0	**0**	**3**	5	5	0	**0**	**0**	**0**	**2**	0	**0**	**3**	5	5
10	0	**4**	5	5	5	**1**	**4**	5	5	5	0	**4**	5	5	5
11	0	**2**	5	5	5	0	**2**	5	5	5	0	**2**	5	5	5
12*	0	**4**	5	5	5	0	**4**	5	5	5	0	**4**	5	5	5
13	0	5	5	**4**	5	0	**4**	5	**3**	5	0	5	5	**4**	5
14	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
15	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
16	0	**0**	5	5	5	0	**1**	**4**	5	5	0	**1**	**4**	5	5
17	**1**	**3**	5	**4**	5	**2**	**2**	**3**	5	**4**	**1**	**4**	5	**4**	5
18	0	**3**	5	**4**	5	**1**	**3**	**4**	**4**	5	0	**4**	5	**4**	5
19	0	**3**	**4**	**4**	5	0	5	5	**4**	5	0	5	5	**4**	5
20	0	**2**	5	5	5	0	**2**	**4**	**4**	5	0	**4**	5	5	5

rivm

Labcode	RVS					MKTTn					MSRV				
	Blank n=5	SE10 n=5	SE100 n=5	STM5 n=5	STM50 n=5	Blank n=5	SE10 n=5	SE100 n=5	STM5 n=5	STM50 n=5	Blank n=5	SE10 n=5	SE100 n=5	STM5 n=5	STM50 n=5
Good Performance	**≤ 1**	**≥ 1**	**≥ 4**	**≥ 2**	**≥ 4**	**≤ 1**	**≥ 1**	**≥ 4**	**≥ 2**	**≥ 4**	**≤ 1**	**≥ 1**	**≥ 4**	**≥ 2**	**≥ 4**
21	0	**2**	5	5	5	0	**2**	**3**	**4**	5	0	**2**	5	5	5
22	0	**2**	5	5	5	0	**1**	**0**	**2**	**1**	-	-	-	-	-
23	0	**3**	5	5	5	0	**1**	**0**	**3**	**3**	0	**2**	5	5	5
24	0	5	5	**3**	5	0	5	5	**3**	5	0	5	5	**3**	5
25	0	**3**	5	5	5	0	**3**	5	5	5	0	**3**	5	5	5
26	0	**1**	5	5	5	0	**1**	5	5	5	0	**1**	5	5	5
27	**1**	**1**	5	5	5	**2**	**1**	5	5	5	0	**1**	5	5	5
28	0	**1**	5	**4**	5	0	**0**	**0**	**1**	**3**	0	**2**	5	**4**	5
29	0	**3**	5	5	5	0	**0**	5	5	5	0	**3**	5	5	5
30	**2**	5	5	5	5	0	**1**	5	**4**	5	0	**2**	5	**4**	5

-: not performed

*: laboratory 12 performed the study 3 months later

bold numbers: deviating results

grey cells : results are below the criteria of good performance

rivm

Annex 8 Test report follow-up study

INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN FOOD
organised by CRL-*Salmonella*
FOOD STUDY II FOLLOW UP
February 2008

Laboratory code This is the same code as in FOOD II 2007	
Laboratory name	
Address	
Country	
Date of arrival of the parcels	…… - ……. – 2008
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	…… - ……. – 2008

PRE-ENRICHMENT – Buffered Peptone Water (BPW)	
Medium information BPW	
Was the composition of BPW the same as used in BRO FOOD II 2007 ?	
☐ Yes	
☐ No please give more details in an annex :	
Preparation of BPW	
Date of preparation	…… - ……. – 2008
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: …… - ……. – 2008 time: ……... h ……..…… min temperature incubator: ……..…… °C
At the end	Date: …… - ……. – 2008 time: …… h …… min temperature incubator: ……..…… °C
Incubation time and temperature for dissolving the capsules (45 min)	
At the start	Date: …… - ……. – 2008 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: …… - ……. – 2008 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end	Date: …… - ……. – 2008 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 II 2007 ?	
☐ Yes	
☐ No please give more details in an annex :	
Preparation of RVS	
Date of preparation	…… - ……. - 2008
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: …… - ……. – 2008 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the first period (24 h)	Date: …… - ……. – 2008 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the start of the second period (48 h)	Date: …… - ……. – 2008 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the second period (48 h)	Date: …… - ……. – 2008 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 II 2007 ?	
☐ Yes	
☐ No please give more details in an annex :	
Preparation of MKTTn	
Date of preparation	…… - ……. – 2008
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: …… - ……. – 2008 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the first period (24 h)	Date: …… - ……. – 2008 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the start of the second period (48 h)	Date: …… - ……. – 2008 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the second period (48 h)	Date: …… - ……. – 2008 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 II 2007 ?	
☐ 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	…… - ……. - 2008
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: …… - ……. – 2008 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the first period (24 h)	Date: …… - ……. – 2008 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the start of the second period (48 h)	Date: …… - ……. – 2008 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the second period (48 h)	Date: …… - ……. – 2008 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 II 2007 ?
☐ 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 II 2007 ?	
☐ Yes	
☐ No please give more details in an annex :	
Preparation of XLD	
Date of preparation	…… - ……. – 2008
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: …… - ……. - 2008 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the first period (24 h)	Date: …… - ……. - 2008 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the start of the second period (48 h)	Date: …… - ……. - 2008 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the second period (48 h)	Date: …… - ……. - 2008 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 II 2007 ?	
☐ Yes	
☐ No please give more details in an annex :	
Preparation of the second isolation medium	
Date of preparation	…… - ……. – 2008
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: …… - ……. - 2008 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the first period (24 h)	Date: …… - ……. - 2008 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the start of the second period (48 h)	Date: …… - ……. – 2008 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the second period (48 h)	Date: …… - ……. – 2008 time: …….. h ……..…… min temperature incubator: ……..…… °C

rivm

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 II 2007 ?
☐ 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 II 2007 ?	
☐ Yes	
☐ No please give more details in an annex :	
Preparation of the nutrient agar	
Date of preparation	…… - ……. – 2008
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control of agar ?	☐ Yes ☐ No

CONFORMATION 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	
General questions	
Did you use PCR ?	☐ Yes ☐ No
If yes and when different from PCR-technique used during food BRO 2007, please give more information in an annex .	

Table 1: Results of isolation using RVS (dish numbers 1-15, C1-C5, C11 and C12)

sample no.	RVS 24 hours						RVS 48 hours					
	XLD		Second		Own		XLD		Second		Own	
	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												
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-C5, C11 and C12)

	MKTTn 24 hours						MKTTn 48 hours					
sample no.	XLD		Second		Own		XLD		Second		Own	
	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												
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-C5, C11 and C12)

sample no.	MSRV 24 hours						MSRV 48 hours					
	XLD		Second		Own		XLD		Second		Own	
	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												
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-C5, C11 and C12)

sample no.	OWN 24 hours						OWN 48 hours					
	XLD		Second		Own		XLD		Second		Own	
	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												
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-C5, C11 and C12)

sample no.	PCR + or -		
		no.	
1		C1	
2		C2	
3		C3	
4		C4	
5		C5	
6		C11	
7		C12	
8			
9			
10			
11			
12			
13			
14			
15			

rivm

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

Name of person (s) carrying out the follow up food II interlaboratory Comparison study.	
Is the person(s) carrying out the follow up food II 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 :. When not NRL (see page 1) mention also the name of the laboratory.	
Date and signature	

Please send the completed test report before 7 March 2008 preferable by email to CRL-*Salmonella*. If the test report is e-mailed to the CRL it is not longer necessary to sent 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 Pb 63
P.O. Box 1
3720 BA Bilthoven
The Netherlands

E-mail : Angelina.Kuijpers@rivm.nl
Tel. number: + 31 30 274 2093
Fax. number: + 31 30 274 4434

RIVM

National Institute
for Public Health
and the Environment

P.O. Box 1
3720 BA Bilthoven
The Netherlands
www.rivm.com