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EU Interlaboratory comparison study food-I (2006)

Bacteriological detection of *Salmonella* in minced beef

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Abstract

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The European National Reference Laboratories (NRLs) for *Salmonell*a were able to detect high and low levels of *Salmonell*a in a ring trial using minced beef as matrix, thereby reaching the level of good performance. The Modified Semi-solid Rappaport Vassiliadis (MSRV), a method often used for the detection of *Salmonella* in animal faeces, turned out to be the best method for minced beef.

This was one outcome of the inter-laboratory comparison study organized by the Community Reference Laboratory (CRL) for *Salmonella* on the detection of *Salmonella* spp. in a food matrix in September 2006. The first, and most important goal, was to see if the 25 participating laboratories in this study could detect *Salmonella* in minced beef. The second goal was to compare the different analysis methods for the detection of *Salmonella* in minced beef.

Each laboratory received a package containing minced beef and 35 gelatin capsules containing different levels of *Salmonella*. According to the instructions, the laboratories spiked the meat with the capsules and tested those samples for the presence of *Salmonella*. The laboratories used three methods for running this test: Rappaport Vassiliadis Soya broth (RVS), Mueller Kauffmann Tetrathionate novobiocin broth (MKTTn) and Modified Semi-solid Rappaport Vassiliadis (MSRV). The first two methods are internationally prescribed for the detection of *Salmonella* in food, while the third (MSRV) is prescribed for the detection of *Salmonella* in veterinary faeces.

All laboratories found *Salmonella* in just 88 % of the samples using one of the food methods (MKTTn). The method for the veterinary samples, MSRV, gave the best results, with 99 % of all laboratories detecting *Salmonella* in the spiked samples. The MKTTn food method is therefore not the optimal medium for the detection of *Salmonella* in minced beef.

Key words: *Salmonella;* CRL-*Salmonella;* NRL-*Salmonella;* ring trial; reference materials; *Salmonella* detection methods; minced beef.

Rapport in het kort

EU Ringonderzoek voedsel-I (2006)
Bacteriologische detectie van *Salmonella* in rundergehakt

De Europese Nationale Referentie Laboratoria (NRLs) voor *Salmonella* hebben in een ringonderzoek hoge en lage concentraties *Salmonella* aangetoond in rundergehakt. Hiermee hebben ze laten zien dat ze voldoen aan de gestelde eisen. De Modified Semi-solid Rappaport Vassiliadis (MSRV), een analysemethode die veel gebruikt wordt voor *Salmonella* in dierenmest, bleek de beste methode voor het aantonen van *Salmonella* in rundergehakt.

Vijfentwintig referentielaboratoria deden in september 2006 mee aan een ringonderzoek van het Communautair Referentie Laboratorium (CRL) voor *Salmonella.* Doel was in eerste instantie om na te gaan of de laboratoria *Salmonella* in gehakt goed konden aantonen. In tweede instantie werd ook onderzocht wat de beste analysemethode was voor het aantonen van *Salmonella* in rundergehakt.
Ieder laboratorium kreeg 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*.

Voor het onderzoek gebruikten de laboratoria drie methoden: Rappaport Vassiliadis Soya broth (RVS), Mueller Kauffmann Tetrathionaat met novobiocine (MKTTn) en Modified Semi-solid Rappaport Vassiliadis (MSRV). De eerste twee methoden (RVS en MKKTn) staan bekend als internationaal voorgeschreven voor de analyse van *Salmonella* in levensmiddelen. De derde methode (MSRV) wordt gebruikt om *Salmonella* in dierlijke mest aan te tonen.

Met één van de levensmiddelenmethoden (MKTTn) vonden alle laboratoria in slechts 88 % van de monsters *Salmonella*. De methode voor dierlijke mest (MSRV) bleek de beste resultaten te geven. Hiermee vonden alle laboratoria in 99 % van de besmette monsters *Salmonella*. De levensmiddelenmethode MKTTn blijkt dus niet de meest optimale methode te zijn voor het aantonen van *Salmonella* in rundergehakt.

Trefwoorden: S*almonella;* CRL-*Salmonella;* NRL-*Salmonella;* ringonderzoek*;* referentie materialen; *Salmonella* detectiemethoden; gehakt.

Contents

Summary **9**

List of abbreviations **11**

1 Introduction **13**

2 Participants **15**

3 Materials and methods **17**

3.1 Reference materials *17*

3.2 Minced beef samples *18*
3.2.1 General 18
3.2.2 Total bacterial count in minced beef 18
3.2.3 Number of Enterobacteriaceae count in minced beef 18

3.3 Design of the interlaboratory comparison study *19*
3.3.1 Samples : capsules and minced beef 19
3.3.2 Sample packaging and temperature recording during shipment 19
3.3.3 Methods 20

3.4 Statistical analysis of the data *20*

3.5 Good performance *21*

4 Results **23**

4.1 Reference materials *23*

4.2 Minced beef samples *23*

4.3 Technical data interlaboratory comparison study *24*
4.3.1 Accreditation/certification 24
4.3.2 Transport of samples 24
4.3.3 Media 27

4.4 Control samples *30*
4.4.1 Specificity, sensitivity and accuracy rates of the control samples 33

4.5 Results meat samples artificially contaminated with Sammonella spp *34*
4.5.1 Results per type of capsule and per laboratory 34
4.5.2 Results per selective enrichment, capsule and per laboratory 36
4.5.3 Specificity, sensitivity and accuracy rates of the artificially contaminated samples 42

4.6 PCR *44*

5 Discussion **47**

6 Conclusions **51**

References **53**

Annex 1. History of CRL-*Salmonella* interlaboratory comparison studies on the detection of *Salmonella* **55**

Annex 2. Calculation of T_2 **57**

Annex 3. Information on the media used **59**

Annex 4. Protocol **61**

Annex 5. Standard Operating Procedure **63**

Annex 6. Draft Annex D of ISO 6579 **69**

Summary

In fall 2006 the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*) organised the first interlaboratory comparison study on bacteriological detection of *Salmonella* in a food matrix. Participants were twenty-five National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*) of the EU Member States and of the NRL of Norway.

The main objective of the first interlaboratory comparison study on a food matrix was to compare results obtained with the different levels of contamination and different serotypes of *Salmonella* in the presence or absence of competitive micro-organisms between and within the NRLs. The performance of the laboratories was compared with the agreements as made during the CRL-*Salmonella* workshop of 2005 (Mooijman, 2005). In addition to the performance testing of the laboratories, a comparison was made between the prescribed methods (ISO 6579, 2002) and the requested method (draft Annex D of ISO 6579, 2006). The selective enrichment media were Rappaport Vassiliadis Soya broth (RVS), Mueller Kauffmann Tetrathionate novobiocin broth (MKTTn) and Modified Semi-solid Rappaport Vassiliadis (MSRV). Optionally, a laboratory could also use other, own media or procedures for the detection of *Salmonella* in addition to the prescribed procedures.

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 10 gram of *Salmonella* negative minced beef. The 25 capsules were divided over the following groups: 5 capsules with circa 10 colony forming particles (cfp) of *Salmonella* Typhimurium (STM10), 5 capsules with circa 100 cfp of *S.* Typhimurium (STM100), 5 capsules with circa 100 cfp of *S.* Enteritidis (SE100), 5 capsules with circa 500 cfp of *S.* Enteritidis (SE500) and 5 blank capsules. The other 10 capsules, to which no meat had to be added, were control samples, existing of 3 capsules STM10, 2 capsules SE100, 1 capsule SE500, 2 capsules with circa 5 cfp of *S.* Panama (Span5) and 2 blank capsules.

Significantly more positive isolations were obtained from the artificially contaminated samples (negative minced beef, artificially contaminated with reference materials) after selective enrichment on MSRV or RVS when compared with MKTTn. The accuracy rates for these samples were 99 %, 98 % and 90 % after selective enrichment on respectively MSRV, RVS and MKTTn.

For the MSRV method all NRLs achieved the level of good performance which was defined during the CRL-*Salmonella* workshop of 2005 (Mooijman, 2005). One NRL did not return the test report. One NRL did not perform the requested methods. Four NRLs had small problems with one of the control samples. Two NRLs found positive blanks.

List of abbreviations

BGA	Brilliant Green Agar
BGA mod (+)	Brilliant Green Agar modified (+ Sulphamandelate supplement)
BPLSA	Brilliant Green Phenol-Red Lactose Sucrose Agar
BPW	Buffered Peptone Water
BxLH	Brilliant Green, Xylose, Lysine, Sulphonamide
cfp	colony forming particles
CRL	Community Reference Laboratory
Diassalm	Diagnostic Semi-Solid *Salmonella* Agar
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
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
RV	Rappaport Vassiliadis
RVS	Rappaport Vassiliadis Soya broth
SC	Selenite Cystine
SE	*Salmonella* Enteritidis
SMID2	*Salmonella* Detection and Identification-2
SOP	Standard Operating Procedure
SPan	*Salmonella* Panama
STM	*Salmonella* Typhimurium
TC	Technical Committee
TSA	Tryptone Soya Agar
TSI	Triple Sugar Iron agar
UA	Urea Agar
VRBG	Violet Red Bile Glucose agar
XLD(+nov)	Xylose Lysine Deoxycholate agar (+ novobiocin)
XLT4	Xylose Lysine Tergitol 4 agar

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. Up to 2005 the interlaboratory comparison studies on the detection of *Salmonella* were focused on veterinary samples (e.g. chicken faeces; see Annex 1). However, according to Regulation EC No 882/2004, also food matrices should be dealt with. Therefore a first (pilot) interlaboratory comparison study on the detection of *Salmonella* in minced beef was organised in fall 2006. 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 (draft Annex D of ISO 6579, Anonymous, 2006; Annex 6), participating laboratories were requested also to use MSRV for testing the minced beef samples.

The set-up of this first food study was comparable to earlier interlaboratory comparison studies on the detection of *Salmonella* spp. in veterinary samples. Ten control samples containing different reference materials had to be tested without the addition of minced beef. These reference materials consisted of 3 capsules with circa 10 cfp of *Salmonella* Typhimurium (STM10), 2 capsules with circa 100 cfp of *Salmonella* Enteritidis (SE100),
1 capsule with circa 500 cfp of *Salmonella* Enteritidis (SE500), 2 capsules with circa 5 cfp of *Salmonella* Panama (SPan5) and 2 blank capsules. Twenty-five samples of *Salmonella* negative minced beef spiked with five different reference materials (including blank capsules) had to be examined. The different reference materials consisted of two levels of *Salmonella* Typhimurium (STM10 and STM100) and two levels of *Salmonella* Enteritidis (SE100 and SE500).

2 Participants

Country	City	Institute
Austria	Graz	Institut für Medizinische Mikrobiologie und Hygiene, Nationale Referenzzentrale für Salmonellen (AGES)
Belgium	Brussels	Scientific Institute for Public Health (IPH)
Cyprus	Nicosia	Cyprus Veterinary Services, Laboratory for the Control of Foods of Animal Origin (LCFAO)
Czech Republic	Prague	State Veterinary Institute
Denmark	Copenhagen	Danish Institute for Food and Veterinary Research (DFVF)
Estonia	Tartu	Estonian Veterinary and Food Laboratory, Bacteriology-Pathology Department
Finland	Helsinki	Food Safety Authority (Evira) Department of Animal Diseases and Food Microbiology unit/ Food
Germany	Berlin	Federal Institute for Risk Assessment (BFR) National Veterinary Reference Laboratory for Salmonella
Greece	Halkis	Veterinary Laboratory of Halkis
Hungary	Budapest	National Food Investigation Institute
Ireland	Kildare	Department of Agriculture and Food Central Veterinary Research Laboratory
Italy	Legnaro	Istituto Zooprofilattico Sperimentale delle Venezie, Centro Nazionale di Referenza per le Salmonellosi
Latvia	Riga	Nationaly Diagnostic Centre (NDC) Laboratory of Food and Environmental Investigation
Lithuania	Vilnius	National Veterinary Laboratory
Luxembourg	Luxembourg	Laboratoire de Médecine Vétérinaire de l'Etat, Animal Zoonosis
The Netherlands	Bilthoven	National 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)
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 Nutricion (AESAN) Centro Nacional de Alimentación
Sweden	Uppsala	National Veterinary Institute (SVA), Department of Bacteriology
United Kingdom	Belfast	Agri-Food and Bioscience Institute (AFBI) Veterinary Sciences Divison Bacteriology
United Kingdom	London	Health Protection Agency Local and Regional Services London, Food, Water & Environmental Micorbiology Laboratory (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);
- 10 and 100 colony forming particles (cfp) per capsule for *Salmonella* Typhimurium (STM10 and STM100);
- 100 and 500 colony forming particles (cfp) per capsule for *Salmonella* Enteritidis (SE100 and SE500).

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 °C. If the test batch fulfilled the pre-set criteria for contamination level and homogeneity, the relevant mixed powders were completely filled into gelatin capsules and stored at -20 °C.

The pre-set criteria were:

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

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

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

3.2 Minced beef samples

3.2.1 General

Ten kilogram *Salmonella* negative minced beef was buyed at the butcher (in Bilthoven) on 14-09-2006. The meat was tested for the absence of *Salmonella* following the procedure as described in draft Annex D of ISO 6579 (Anonymous, 2006; Annex 6). For this purpose 10 portions of 10 g were each added to 90 ml BPW. After pre-enrichment at 37 °C for 16-18 h, selective enrichment was carried out on MSRV. Next, the suspicious colonies were plated-out on XLD and BGA and confirmed biochemical. The meat was stored in portions of 300 g at –20 ºC.

3.2.2 Total bacterial count in minced beef

The total number of aerobic bacteria was investigated in the meat. The procedure of ISO 4833 (Anonymous, 2003) was followed for this purpose. Portions of 20 gram meat were homogenized into 180 ml peptone saline solution in a plastic bag. The content was mixed by using a pulsifier (60 sec). Next tenfold dilutions were prepared in peptone saline solution. Two times one ml of each dilution was brought into two empty Petri-dishes (diameter 9 cm). To these two dishes 25 ml of molten Plate Count Agar (PCA) was added. These plates were incubated at (30 ± 1) °C for (72 ± 3) h and the total number of aerobic bacteria was counted after incubation.

3.2.3 Number of Enterobacteriaceae count in minced beef

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

3.3 Design of the interlaboratory comparison study

3.3.1 Samples: capsules and minced beef

On 18-09-2006 (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 diagnostic specimens (UN 3373) and send by courier service to the participants. After arrival at the laboratory the capsules had to be stored at –20 °C and the meat had to be stored at +5 °C until the start of the study. Details about mailing and handling of the samples and reporting of test results can be found in the Protocol (Annex 4) and Standard Operation Procedure (Annex 5). The test report 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 100 (SE100)	2	5
S. Enteritidis 500 (SE500)	1	5
S. Typhimurium 10 (STM10)	3	5
S. Typhimurium 100 (STM100)	---	5
Blank	2	5

3.3.2 Sample packaging and temperature recording during shipment

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 package contained one logger. 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.

Two biopacks and six cooling devices were placed in one large shipping box. In one of the two biopacks (the one containing the reference materials), the temperature recorder was enclosed. The other biopack contained the minced beef.

3.3.3 Methods

During the CRL-*Salmonella* workshop of May 2006 it was decided that the prescribed method of this interlaboratory comparison study would be ISO 6579 (Anonymous, 2002) and the requested (additional) method, draft Annex D of ISO 6579 (Anonymous, 2006; Annex 6). Beside 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 a Polymerase Chain Reaction based method.

In summary:

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

Selective enrichment on/in:
- Rappaport Vassiliadis Soya broth (RVS)
- Mueller Kauffmann Tetrathionate novobiocin broth (MKTTn)
- Modified semi-solid Rappaport Vassiliadis medium (MSRV) (requested)
- Own selective enrichment medium (not compulsory)

Plating-out medium on:
- Xylose lysine desoxycholate agar (XLD)
- Second plating-out medium for choice (obligatory!)
- Own plating-out medium (not compulsory)

Biochemical confirmation:
- Urea (UA), Triple Sugar Iron agar (TSI) and Lysine Decarboxylase (LDC)

3.4 Statistical analysis of the data

The results of the interlaboratory comparison study were statistically analyzed in order to compare the results of the participating laboratories and the different types of samples and methods (selective enrichment and plating-out media).

The specificity, sensitivity and accuracy rates were calculated for the control samples, and the artificially contaminated samples with minced beef. 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 2.4.1 (R Development Core Team, 2006). Mixed effect logistic regression (Venables and Ripley, 2002) using the lme4 package was used. The lme4 package provides functions for fitting and assessing linear or generalized linear mixed effects models in R (Bates and Sarkar, 2007).
Mixed effect logistic regression allows modeling any possible dependence between the binary outcomes caused by a laboratory effect. In the regression model used here, the fixed part consisted of the capsule, enrichment medium, isolation medium and interactions between these three. Laboratory was the random effect variable in this model.
In order to detect differences among media and capsules, specific contrasts were calculated which are shown as p-values. The differences in performance from one particular laboratory were compared by contrasting the result of this laboratory to the mean of all laboratories, i.e. the outcomes as predicted based on the fixed effects only.

3.5 Good performance

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* (Mooijman, 2005).

The following was suggested:

Control capsules

- Positive control capsules: all should be positive;
  only of Span5 50 % may be negative (1 negative out of 2 capsules).
- Blank control capsules: all negative.

Capsules tested with a matrix

- Blank capsules with ‘matrix’: 80 % negative (4 negative out of 5 capsules) *.
- STM100 and SE500 with ‘matrix’: 80 % positive (4 positive out of 5 capsules).
- STM10 and SE100 with ‘matrix’: 50 % positive (2-3 positive out of 5 capsules).

* 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 (80 % neg.) will still be considered as acceptable.

4 Results

4.1 Reference materials

The level of contamination and the homogeneity of the final batches of capsules are presented in Table 2. 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 2 Level of contamination and homogeneity of SE, SPan and STM capsules

	SE100	SE500	SPan 5	STM10	STM100
Final batch; Test 1					
Date testing capsules	29-06-06	26-6-06	3-8-06	30-7-06	7-8-06
Number of capsules tested	50	50	50	50	50
Mean cfp per capsule	85	564	7	11	101
Min-max cfp per capsule	56-122	390-780	2-13	3-22	59-124
T_2 / (I-1)	2.31	1.95	1.25	1.48	1.55
Final batch; Test 2					
Date testing capsules	26-09-06	27-9-06	27-9-06	26-9-06	26-9-06
Number of capsules tested	25	25	25	25	25
Mean cfp per capsule	74	519	5	9	98
Min-max cfp per capsule	48–96	390-660	1-10	3-15	76-117
T_2 / (I-1)	1.67	1.72	1.36	0.84	1.28

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. At Monday 18 September 2006 the minced beef was mailed to the NRLs, one parcel was sent one day later to the NRL (labcode 18). After receipt the NRLs had to store the minced beef at 5 °C. One laboratory (labcode 18) stored the minced beef at –20 ºC (20 Sept.-5 Oct.).

The number of aerobic bacteria and Enterobacteriaceae was tested twice; firstly 4 days after the meat arrived at the CRL (t = 4 days) and secondly one week after the planned date of the interlaboratory comparison study (t =18 days). The results are shown in Table 3.
Most of the laboratories performed the study on the planned date (25-09-06 t=11 days) and six laboratories (labcodes 4, 5, 6, 13, 18 and 24) one week later (t=18). One laboratory did not perform the study at all (labcode 21).

Table 3 Number of aerobic bacteria and Enterobacteriaceae per gram of minced beef negative for Salmonella

Date	Aerobic bacteria cfp/g	Enterobacteriaceae cfp/g
18 September t = 4 days	$5.2*10^6$	$1.7*10^3$
2 October t = 18 days	$1.4*10^8$	$1.3*10^6$

4.3 Technical data interlaboratory comparison study

4.3.1 Accreditation/certification

Seventeen laboratories mentioned to be accredited for their quality system according to EN-ISO/IEC 17025 (labcodes 2, 5, 6, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22 and 25). Three laboratories (labcodes 3, 4 and 26) are planning to be accredited or certified in the near future. One laboratory (labcode 24) mentioned that they were not accredited or certified to any system and mentioned no planning to do so in the near future. Laboratories 1, 7, 9 and 23 did not answer this question in the test report.

4.3.2 Transport of samples

An overview of the transport time 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 21, who did not return the temperature recorder at all. The temperature recorder of laboratory 8 was broken when it arrived at the CRL, it was therefore not possible to read the results anymore. The majority of the laboratories received the materials within 1 day. The average number of transport time was 27.3 hours (1.1 days). For the majority of the laboratories the temperature of the content of the parcel was below 5 ^{o}C with the exception of the laboratories 2 and 10. In four cases (labcodes 9, 12, 18 and 22) 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.

Table 4 Overview of the temperatures during shipment of the parcels to the NRLs

		Time (h) at			
Labcode	**Transport* total in hours**	**–20 °C - 0 °C**	**0 °C - 5 °C**	**5 °C - 10 °C**	**Additional storage+**
1	26.1	6	20	0	
2	24.12	6	8	10	
3	21.2	7	14	0	
4	25.32	9	16	0	
5	21.55	16	5	0	
6	NA	12	0	0	
7	43.5	6	37	0	
8	21.14	(1)			
9	25.5	11	14	0	18 h at 0 °C
10	67.45	20	32	15	
11	24.58	8	17	0	
12	25.4	6	19	0	19 h at 7 °C
13	21.4	6	15	0	
14	25.28	8	17	0	
15	21.45	9	13	0	
16	24.15	11	13	0	
17	21.2	9	12	0	
18	22.3	12	10	0	46 h at – 20 °C
19	27.1	12	15	0	
20	24.34	12	12	0	
21	26.1	(2)			
22	21.38	6	15	0	24 h at – 20 °C
23	24.14	12	12	0	
24	24.5	12	13	0	
25	27.3	12	15	0	
26	46.2	12	34	0	
Average	27.3 hours	9.9 hours	16.4 hours	1.1 hours	
	(1.1 day)				

*= transport time according to the courier
+ = storage time of the samples at the institute before arriving at the NRL.
NA = not applicable
(1): Temperature recorder broken
(2): Temperature recorder was not returned by the laboratory

Table 5 Media combinations used per laboratory

Labcode	Selective enrichment	Plating-out media
1	RVS MKTTn MSRV	XLD BGA mod
2	RVS MKTTn MSRV	XLD Rambach
3	RVS MKTTn MSRV	XLD BGA mod
4	RVS MKTTn MSRV	XLD SM ID2
5	RVS MKTTn MSRV	XLD BGA XLT4
6	RVS MKTTn MSRV	XLD BGA mod BGA
7	RVS MKTTn MSRV	XLD Rambach
8	RVS Diassalm	XLD SM ID2
9	RVS MKTTn MSRV	XLD BGA mod
10	RVS MKTTn MSRV	XLD BGA mod
11	RVS MKTTn MSRV	XLD BGA
12	RVS MKTTn MSRV	XLD BGA mod
13	RVS MKTTn MSRV	XLD+nov. BGA mod
14	RVS MKTTn MSRV	XLD BPLSA
15	RVS MKTTn MSRV	XLD BGA
16	RVS MKTTn MSRV	XLD Rambach SM ID2
17	RVS MKTTn MSRV	XLD BGA
18	RVS MKTTn MSRV	XLD BGA mod+
19	RVS MKTTn MSRV	XLD BGA
20	RVS MKTTn MSRV	XLD BGA mod Rambach
22	RVS MKTTn MSRV	XLD SM ID2
23	RVS MKTTn MSRV	XLD ONÖZ
24	RVS MKTTn MSRV	XLD BGA MLCB
25	RVS MKTTn MSRV	XLD BGA
26	RVS MKTTn MSRV	XLD Rambach

Explanations of the abbreviations are given in th ‘List of abbreviations’ (page 11)
Descriptions of the media not described in ISO 6579 are given in Annex 3

4.3.3 Media

Each laboratory was asked to test the samples with the prescribed (ISO 6579) and the requested (draft Annex D of ISO 6579) methods. All laboratories except one (labcode 8), 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 8 used only RVS and Diassalm for selective enrichment. The media used per laboratory are shown in Table 5. Five NRLs (labcode 5, 6, 16, 20 and 24) performed besides the prescribed methods also 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.

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 °C (min-max)	Time (minutes)	Incubation temperature in °C (min-max)	Time (h:min)	Incubation temperature in °C (min-max)
SOP & ISO 6579	**overnight**	**36-38**	**45**	**36-38**	**16 – 20**	**36-38**
7	21:45	37	60	25	17:30	37
8	4:00	33.7	45	33.7	18:00	33.7
10	21:00	36.8-37	45	36.8-37	20:30	37
16	-	-	45	37	19:15	37
19	21:05	37	60	37	20:25	37

Deviating times and temperatures are indicated as grey cells.

- = no info

Table 7 Incubation times and temperatures of selective enrichment medium RVS and MSRV

	RVS		MSRV	
Labcode	Incubation time in h:min	Incubation temperature in °C (min-max)	Incubation time in h:min	Incubation temperature in °C (min-max)
ISO 6579 & Annex D	**2 x (24 ± 3) h**	**40.5 – 42.5**	**2 x (24 ± 3) h**	**40.5 – 42.5**
19	47:50	42	47:50	37
20	49:00	37.8-41.9	49:00	41.1-41.9

Deviating times and temperatures are indicated as grey cells.

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

Labcode	Enzymatic digest of casein (Peptone)	Sodium Chloride	Disodium hydrogen Phosphate dodecahydrate	Potassium dihydrogen phosphate	pH
ISO 6579	**10.0**	**5.0**	**9.0**	**1.5**	**6.8 – 7.2**
8	-	-	-	-	7.14
12	10.0	5.0	3.5*	1.5	7.3
13	10.0	5.0	3.5*	1.5	7.3
16	-	-	-	-	6.9
22	10.0	5.0	9.0	1.5	-

*= 3.5 g Disodium hydrogen phosphate (anhydrous) is equivalent with 9 g Disodium hydrogen phosphate dodecahydrate.
grey cells are deviating from ISO 6579
- = no info

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

Labcode	Enzymatic digest of casein (Peptone)	NaCl	Potass. Dihydrogen Phosphate* (KH_2PO_4 K_2HPO_4)	$MgCl_2$ anhydrous	Malachite green oxalate	pH
ISO 6579	**4.5**	**7.2**	**1.44**	**13.4**	**0.036**	**5.0 - 5.4**
3	4.5	7.2	1.26 + 0.18	13.58	0.036	5.7
4	4.5	7.2	1.44	28.6**	0.036	-
8	-	-	-	-	-	-
16	-	-	-	-	-	5.3
22	4.5	7.2	1.44	28.4**	0.036	-

*= 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 9 g $MgCl_2$ hexahydrate.
grey cells are deviating from ISO 6579
- = no info

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

Labcode	Meat extract	Enzymatic digest of casein (Peptone)	NaCl	Calcium carbonate	Sodium Thiosulfate pentahydrate	Ox bile for bacterio-logical use	Brilliant green	Iodine	Potas-sium iodide	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**
3	4.3	8.6	2.6	38.7	30.5*	4.78	9.6	4	5.0	0.04	7.8
4	4.23	8.45	2.54	38.04	30.3*	4.75	9.5	4	5.0	0.05	-
5	4.3	8.6	2.6	38.7	30.5*	4.78	9.6	-	-	0.04	-
10	7.0	2.3	2.3	25.0	40.7*	4.75	9.5	4	5.2	0.04	7.8
13	10.7	8.6	2.6	38.7	47.8	4.78	9.6	4	5	0.04	8.0
16	-	-	-	-	-	-	-	-	-	-	8.1
22	4.23	8.45	254	38.04	30.3*	4.75	9.5	4	5	0.05	-
24	4.3	8.6	2.6	38.7	30.5*	4.78	9.6	4	5.0	0.04	-
26	4.3	8.6	2.6	38.7	30.3*	4.75	9.5	200?	250?	8.0	8.0

*Sodium thiosulphate (anhydrous) 30.5 g is equivalent to 47.8 g of Sodium thiosulphate pentahydrate
grey cells are deviating from ISO 6579
- = no info

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

Labcode	Enzymatic digest of casein (Tryptose)	Casein hydro-lysate	NaCl	Potass. Digygrogen phosphate	$MgCl_2$ anhy-drous	Malachite green oxalate	Agar	Novo biocin	pH
Draft Annex D ISO 6579	**4.6**	**4.6**	**7.3**	**1.5**	**10.9**	**0.04**	**2.7**	**0.01 (10 mg/L)**	**5.1-5.4**
1	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.4
2	-	-	-	-	-	-	-	-	5.2
3	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.9
4	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.01	5.6
9	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.6
10	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.01	5.4
11	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.4
13	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.01	5.5
16	-	-	-	-	-	-	-	-	5.6
18	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.2
19	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.0
20	4.6	4.6	7.3	1.5	10.9	0.04	2.7	4 ml	5.28
22	4.6	4.6	7.3	1.5	10.9	0.04	2.7	2.7	-
24	2.3	4.6	7.3	1.5	10.9	0.04	2.5	0.02	-

grey cells are deviating from ISO 6579
- = no info

Table 12 Composition of XLD in g/l

Lab Code	Xylose	L-lysine	Lact ose	Sucrose (Sacchar ose)	NaCl	Yeast extract	Phenol red	Agar	Sodium desoxy-Cholate	Sodium thio-sulphate	Iron (III) Amm. Citrate	Novo-Biocin	pH
ISO 6579	**3.75**	**5.0**	**7.5**	**7.5**	**5.0**	**3.0**	**0.08**	**9-18**	**1.0**	**6.8**	**0.8**	**-**	**7.2 – 7.6**
2	-	-	-	-	-	-	-	-	-	-	-	-	7.4
4	3.5	5.0	7.5	7.5	5.0	3.0	0.08	13.5	2.5	6.8	0.8	-	-
5	3.75	5.0	7.5	7.5	5.0	3.0	0.08	13	1.0	6.8	0.8	-	-
7	3.5	5.0	7.5	7.5	5.0	3.0	0.08	13.5	2.5	6.8	0.8	-	7.4
8	-	-	-	-	-	-	-	-	-	-	-	-	-
13	3.75	5.0	7.5	7.5	5.0	3.0	0.08	13.0	1.0	6.8	0.8	0.015	7.3
16	-	-	-	-	-	-	-	-	-	-	-	-	7.4
20	3.75	5.0	7.5	7.5	5.0	3.0*	0.072	15.0	1.0	4.34	0.8	-	7.11
22	3.5	5.0	7.5	7.5	5.0	3.0	0.08	13.5	2.5	6.8	0.8	-	-
24	3.75	5.0	7.5	7.5	5.0	3.0	0.08	13.0	1.0	6.8	0.8	-	-

- = no info
* 1.0 g peptone included
grey cells are deviating from ISO 6579

The use of an extra plating agar between the ‘isolation’ and the ‘confirmation’ steps was optional. A total of 15 laboratories performed this extra culture step on many different media (e.g. Nutrient agar, TSA, XLD, Colombia, Mc Conkey, Bromkresol purpur and Bromthymol blue lactose sucrose agar).

Twenty laboratories used all three required biochemical media (UA, TSI and LDC) to confirm *Salmonella*. The additional or deviating methods for the confirmation of *Salmonella* are mentioned in Table 13. Six laboratories (lab code 3, 8, 10, 14, 16 and 24) showed a rather limited confirmation. Laboratory 16 did not mention any confirmation test. Laboratory 3, 8 and 24 used only one biochemical test and laboratory 14 only serotyping with O antigen. Three laboratories (lab code 1, 12 and 22) used a biochemical identification kit and two laboratories performed additional serotyping (lab code 12 and 17).

Table 13 Biochemical and serological confirmation of Salmonella

Labcode	Biochemical			Serological			Other
ISO 6579	**UA**	**TSI**	**LDC**	**O antigens**	**Vi antigens**	**H antigens**	
1	+	+	+	-	-	-	API20E
3	+	-	-	-	-	-	-
7	+	+	+	-	-	-	Simmons citrate agar
8	-	+	-	-	-	-	-
10	+	+	-				
12	-	-	-	+	+	+	Biomerieux ID32 E
14	-	-	-	+	-	-	-
16	-	-	-	-	-	-	-
17	+	+	+	+	-	-	-
22	-	-	-	-	-	-	Microbact GNB 12A
24	-	+	-	-	-	-	-

grey cell : confirmation is deviating from ISO 6579
- = not performed/ no info

4.4 Control samples

General

None of the laboratories isolated *Salmonella* from the procedure control (C11: no capsule/no meat) and from the meat control (C12: no capsule/negative meat).). Twenty-one laboratories scored correct results for all the control capsules containing *Salmonella*. In Table 14 the results are summarized of all control samples (capsules without meat) per laboratory and per selective enrichment isolated on XLD. Laboratory 7 did not use XLD after selective enrichment on MSRV. Laboratory 8 did not use the media MSRV and MKTTn.

Blank capsules (n=2) without addition of meat

The blank capsules contained only sterile milk powder. For the analyses no meat was added. Twenty-four participating laboratories correctly analysed the blank capsules negative. Laboratory 20 found one control blank, positive on all the three plating-out media from the same MSRV enrichment. This may have been caused by cross-contamination and the laboratory was advised to check their procedures.

Salmonella Panama 5 capsules (n=2) without addition of meat

Twenty-three laboratories isolated *Salmonella* from both capsules. Two laboratories (labcodes 8 and 14) could not detect *Salmonella* Panama in one control capsule isolated from RVS. Laboratory 14 had good results on the other media, MKTTn and MSRV, from the same BPW enrichment. Laboratory 8 did not use the selective enrichment MKTTn nor MSRV but used an own method Diassalm. The result on Diassalm from the same BPW was also negative. These capsules contained S. Panama at a low level (circa 5 cfp/ capsule). Due to chance one out of two capsules containing SPan 5 may be negative.

Salmonella Typhimurium 10 capsules (n=3) without addition of meat

Twenty-four laboratories tested all the three capsules containing STM10 positive. One laboratory (labcode 23) could not detect STM10 in two control capsules with the MKTTn method. However this laboratory scored 100 % positive with the other methods, RVS and MSRV, from the same BPW enrichment.

Salmonella Enteritidis 100 capsules (n=2) without addition of meat

All the laboratories isolated *Salmonella* Enteritidis at a mean level of circa 100 cfp/ capsule from both capsules.

Salmonella Enteritidis 500 capsules (n=1) without addition of meat

Twenty-four laboratories tested the one SE500 capsule positive. One laboratory (labcode 24) could not detect *Salmonella* in this control capsule with the RVS method and plating-out medium XLD. However, all other medium combinations used by this laboratory and inoculated from the same BPW enrichment, gave a positive result.

Table 14 Number of correct results of the control samples (capsule without meat) per laboratory and per selective enrichment medium.

Labcode	RVS / XLD					MKTTn / XLD					MSRV / XLD				
	Blanc n=2	SE100 n=2	SE500 n=1	SPan5 n=2	STM10 n=3	Blanc n=2	SE100 n=2	SE500 n=1	SPan5 n=2	STM10 N=3	Blanc n=2	SE100 n=2	SE500 n=1	SPan5 n=2	STM10 n=3
Good Performance	**2**	**2**	**1**	**≥ 1**	**3**	**2**	**2**	**1**	**≥ 1**	**3**	**2**	**2**	**1**	**≥ 1**	**3**
1	2	2	1	2	3	2	2	1	2	3	2	2	1	2	3
2	2	2	1	2	3	2	2	1	2	3	2	2	1	2	3
3	2	2	1	2	3	2	2	1	2	3	2	2	1	2	3
4	2	2	1	2	3	2	2	1	2	3	2	2	1	2	3
5	2	2	1	2	3	2	2	1	2	3	2	2	1	2	3
6	2	2	1	2	3	2	2	1	2	3	2	2	1	2	3
7	2	2	1	2	3	2	2	1	2	3	-	-	-	-	-
8	2	2	1	**1**	3	-	-	-	-	-	-	-	-	-	-
9	2	2	1	2	3	2	2	1	2	3	2	2	1	2	3
10	2	2	1	2	3	2	2	1	2	3	2	2	1	2	3
11	2	2	1	2	3	2	2	1	2	3	2	2	1	2	3
12	2	2	1	2	3	2	2	1	2	3	2	2	1	2	3
13	2	2	1	2	3	2	2	1	2	3	2	2	1	2	3
14	2	2	1	**1**	3	2	2	1	2	3	2	2	1	2	3
15	2	2	1	2	3	2	2	1	2	3	2	2	1	2	3
16	2	2	1	2	3	2	2	1	2	3	2	2	1	2	3
17	2	2	1	2	3	2	2	1	2	3	2	2	1	2	3
18	2	2	1	2	3	2	2	1	2	3	2	2	1	2	3
19	2	2	1	2	3	2	2	1	2	3	2	2	1	2	3
20	2	2	1	2	3	2	2	1	2	3	**1**	2	1	2	3
22	2	2	1	2	3	2	2	1	2	3	2	2	1	2	3
23	2	2	1	2	3	2	2	1	2	**1**	2	2	1	2	3
24	2	2	**0**	2	3	2	2	1	2	3	2	2	1	2	3
25	2	2	1	2	3	2	2	1	2	3	2	2	1	2	3
26	2	2	1	2	3	2	2	1	2	3	2	2	1	2	3

- : not performed bold numbers : deviating results grey cells : results are below good performance

4.4.1 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. Good results were found with the control samples. The rates were, for all tested media, > 95 %.

Table 15 Specificity, sensitivity and accuracy rates for all participating laboratories (n=25) with all control capsules, for the different selective enrichment media RVS, MKTTn, MSRV and plating-out medium XLD.

Control capsules		RVS/XLD	MKTTn/XLD[1]	MSRV/XLD [1, 2]
Blank (n=2)	No of samples No of negative samples **Specificity in %**	50 50 ***100.00***	48 48 ***100.00***	46 45 ***97.83***
SPan 5 (n=2)	No of samples No of positive samples **Sensitivity in %**	50 48 ***96.00***	48 48 ***100.00***	46 46 ***100.00***
STM10 (n=3)	No of samples No of positive samples **Sensitivity in %**	75 75 ***100.00***	72 70 ***97.22***	69 69 ***100.00***
SE100 (n=2)	No of samples No of positive samples **Sensitivity in %**	50 50 ***100.00***	48 48 ***100.00***	46 46 ***100.00***
SE500 (n=1)	No of samples No of positive samples **Sensitivity in %**	25 24 ***96.00***	24 24 ***100.00***	23 23 ***100.00***
All capsules with *Salmonella*	No of samples No of positive samples **Sensitivity in %**	200 197 ***98.50***	192 190 ***98.96***	184 184 ***100.00***
All capsules	No of samples No of correct samples **Accuracy in %**	250 247 ***98.80***	240 238 ***99.16***	230 229 ***99.57***

1 = Laboratory 8 did not perform selective enrichment in MKTTn and on MSRV
2 = Laboratory 7 did not perform selective enrichment on MSRV/XLD

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

4.5.1 Results per type of capsule and per laboratory

General

In Table 16 the results are given of the *Salmonella* negative minced beef samples artificially contaminated with capsules per selective enrichment method and plating-out medium XLD. Laboratory 7 did not use XLD in combination with MSRV; laboratory 8 did not perform selective enrichment on MSRV and in MKTTn. Eleven laboratories found all the capsules with *Salmonella* positive for all the three selective enrichment media RVS, MKTTn and MSRV. In general the results between the samples with *S*. Typhimurium or *S*. Enteritidis were comparable.

Blank capsules with negative minced beef (n=5)

Twenty three participating laboratories correctly did not isolate *Salmonella* from the blank capsules with the addition of negative meat. Two laboratories (20 and 26) found some blank capsules, with the addition of negative minced beef, positive. Laboratory 20 found one blank capsule with the MKTTn method in combination with BGA positive. Laboratory 26 found one blank capsule positive when using MSRV.

S. Typhimurium 10 capsules (STM10) with negative minced beef (n=5)

All laboratories, except one, isolated *Salmonella* from the five capsules containing *Salmonella* Typhimurium at a level of circa10 cfp/capsule in combination with minced beef when using RVS and MSRV. Laboratory 9 found two capsules negative with both the RVS and MSRV. With MKTTn less positive results were found. Eight laboratories (labcode 6, 7, 9, 15, 18, 20, 23 and 26) found one to four capsules with this selective enrichment medium negative.

S. Typhimurium 100 capsules (STM100) with negative minced beef (n=5)

All laboratories, except one, isolated *Salmonella* from all five capsules containing *Salmonella* Typhimurium at a level of *ca.*100 cfp/capsule in combination with minced beef when using RVS or MSRV. Laboratory 9 found one capsule negative with the RVS method. Six laboratories (labcode 6, 7, 9, 15, 20, and 26) found one to three capsules negative with the MKTTn method.

Table 16 Number of correct results of the artificially contaminated meat (with capsule) per laboratory and per selective enrichment medium.

Labcode	RVS / XLD					MKTTn / XLD					MSRV / XLD				
	Blanc n=5	SE100 n=5	SE500 n=5	STM10 n=5	STM100 n=5	Blanc n=5	SE100 n=5	SE500 n=5	STM10 n=5	STM100 n=5	Blanc n=5	SE100 n=5	SE500 n=5	STM10 n=5	STM100 n=5
Good performance	**≥ 4**	**> 2.5**	**≥ 4**	**> 2.5**	**≥ 4**	**≥ 4**	**> 2.5**	**≥ 4**	**> 2.5**	**≥ 4**	**≥ 4**	**> 2.5**	**≥ 4**	**> 2.5**	**≥ 4**
1	5	5	5	5	5	5	**3**	5	5	5	5	5	5	5	5
2	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
3	5	5	5	5	5	5	**4**	5	5	5	5	5	5	5	5
4	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
6	5	5	5	5	5	5	**2**	**4**	**1**	**3**	5	5	5	5	5
7	5	5	5	5	5	5	**4**	5	**3**	**4**	-	-	-	-	-
8	5	**3**	5	5	5	5	-	-	-	-	-	-	-	-	-
9	5	**3**	**2**	**3**	**4**	5	5	5	**4**	**4**	5	**3**	**4**	**3**	5
10	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
11	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
12	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
13	5	5	5	5	5	5	**1**	**2**	5	5	5	5	5	5	5
14	5	5	5	5	5	5	**3**	**4**	5	5	5	5	5	5	5
15	5	5	5	5	5	5	5	**4**	**1**	**2**	5	5	5	5	5
16	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
17	5	5	5	5	5	5	**4**	5	5	5	5	5	5	5	5
18	5	5	5	5	5	5	5	5	**4**	5	5	5	5	5	5
19	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
20	5	**4**	5	5	5	5	**2**	5	**4**	**4**	5	5	5	5	5
22	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
23	5	5	5	5	5	5	5	5	**1**	5	5	5	5	5	5
24	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
25	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
26	5	**2**	5	5	5	5	**1**	**2**	**2**	**4**	**4**	5	5	5	5

- : not performed bold numbers : deviating results grey cells : results are below good performance

S. Enteritidis 100 capsules (SE100) with negative minced beef (n=5)
Twentyone laboratories isolated *Salmonella* from all the five capsules containing *Salmonella* Enteritidis at a level of circa 100 cfp/capsule in combination with minced beef, when using RVS or MSRV. Four laboratories (labcode 8, 9, 20 and 26) found one to three capsules negative with the RVS method and laboratory 9 found also two capsules negative with the MSRV method. Nine laboratories (labcode 1, 3, 6, 7, 13, 14, 17, 20, and 26) found one to four capsules negative with the MKTTn method.

S. Enteritidis 500 capsules (SE500) with negative minced beef (n=5)
All laboratories, except one, isolated *Salmonella* from all the five capsules containing *Salmonella* Enteritidis at a level of circa 500 cfp/capsule in combination with minced beef, when using RVS or MSRV. Laboratory 9 found two capsules negative with the RVS method and one capsule negative with the MSRV method. Five laboratories (labcode 6, 13, 14, 15 and 26) found one to three capsules negative with the MKTTn method.

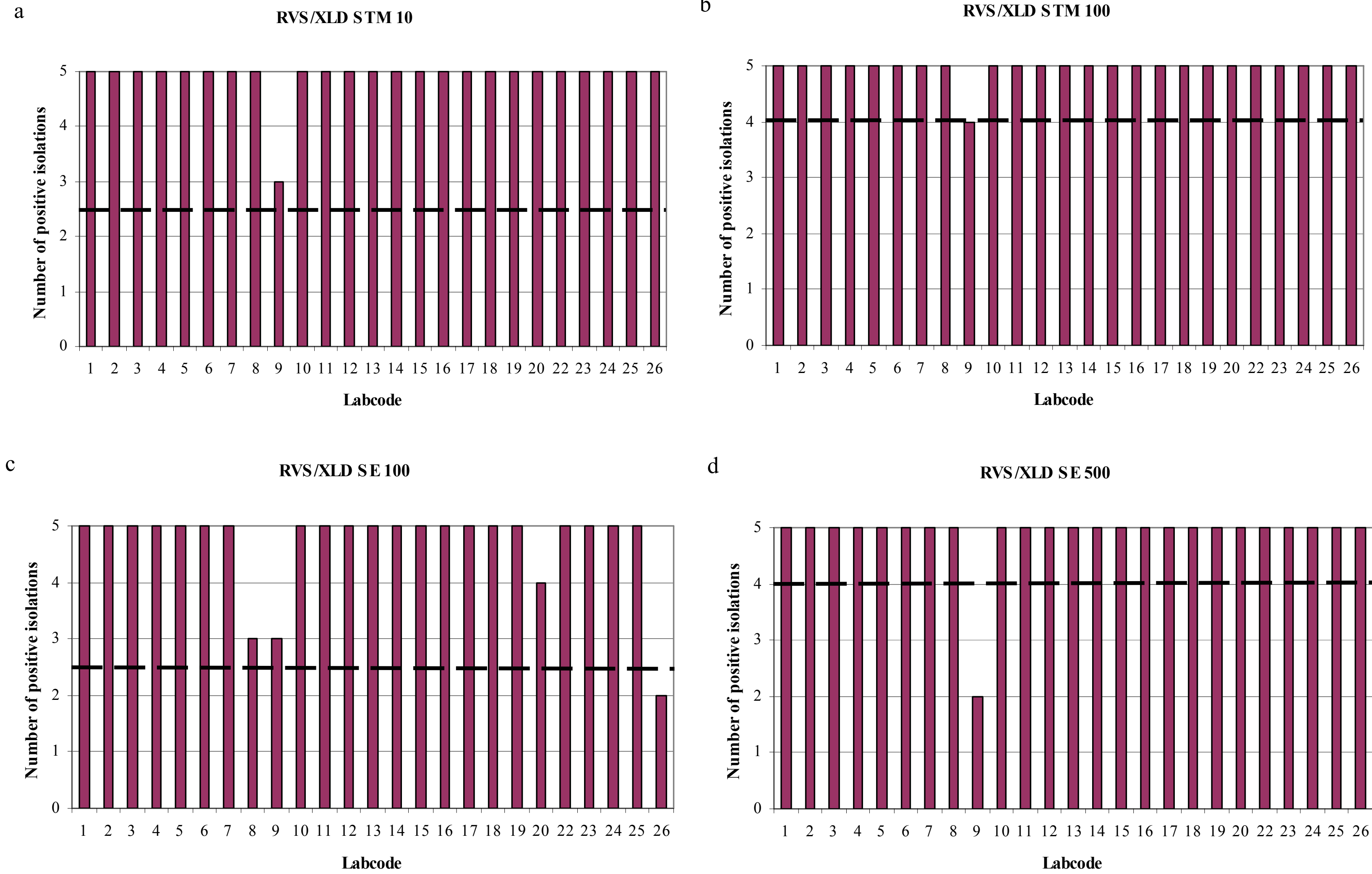
4.5.2 Results per selective enrichment, capsule and per laboratory

In Figures 1, 2 and 3 the number of positive isolations per capsule (n = 5) containing *Salmonella* with the addition of 10 g *Salmonella* negative minced beef and per laboratory is given after pre-enrichment in BPW and selective enrichment in respectively RVS, MKTTn and on MSRV followed by isolation on selective plating agar XLD. The results are compared with the proposed definition of ‘good performance’ (see Materials and methods) for the different methods and capsules. The level of good performance is in the figures indicated with a black line. According to this definition the score was too low for five laboratories when using MKTTn (labcode 6, 13, 15, 20 and 23) for one laboratory when using RVS (labcode 9) and for one laboratory when using both MKTTn and RVS (labcode 26). All laboratories showed good performance when using MSRV. On MSRV all the laboratories scored 100 % positive results, except laboratory 9 who found 75 % of the samples positive. Laboratory no 8 did not perform analyses on MSRV, but still found ‘good performance’ when using RVS. The number of positive isolations found by all laboratories on XLD and a second plating-out medium in combination with the different selective enrichment media RVS, MKTTn and MSRV are given in Table 17. In general 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).

Table 17 Mean percentages of positive results of all participating laboratories using different plating-out media and different selective enrichment media after 24 and 48 hours of incubation when analyzing the artificially contaminated minced beef samples.

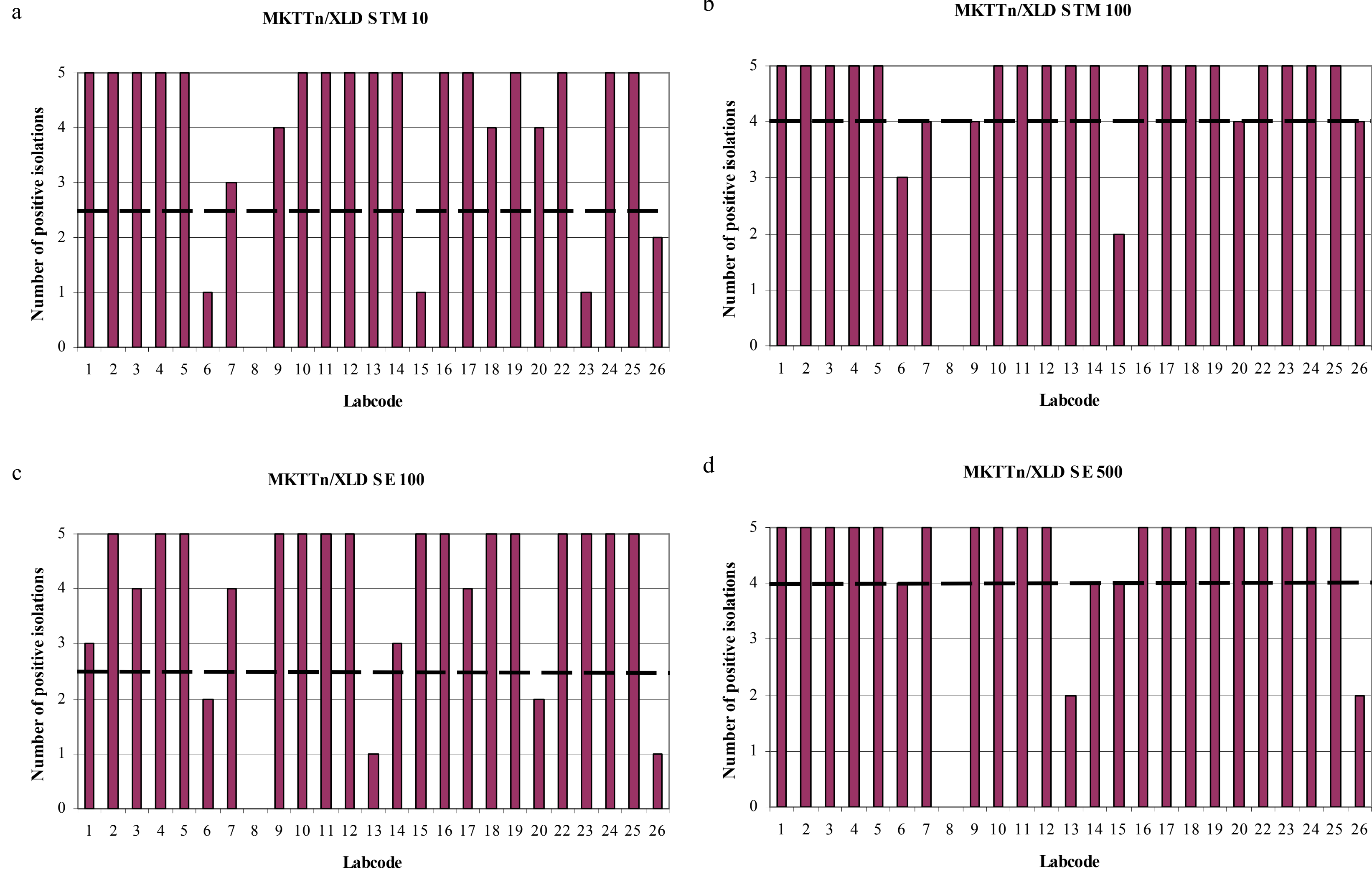
Plating-out medium	**Selective enrichment medium**		
	RVS	**MKTTn**	**MSRV**
	24 / 48 u	24 / 48 u	24 / 48 u
XLD	95 / 97 %	82 / 89 %	95 / 98 %
Other (most often BGA)	94 / 97 %	60 / 71 %	92 / 97 %

The choice of the 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 17 % 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 the combination MKTTn/XLD 7 % more positive isolations were found after 48 h of incubation. When another plating-out medium was used, the difference in the number of positive isolations was even more striking: 11 % more positives after 48 h of incubation of MKTTn. For RVS and MSRV the differences between the two incubation times were smaller: 2-5 %.



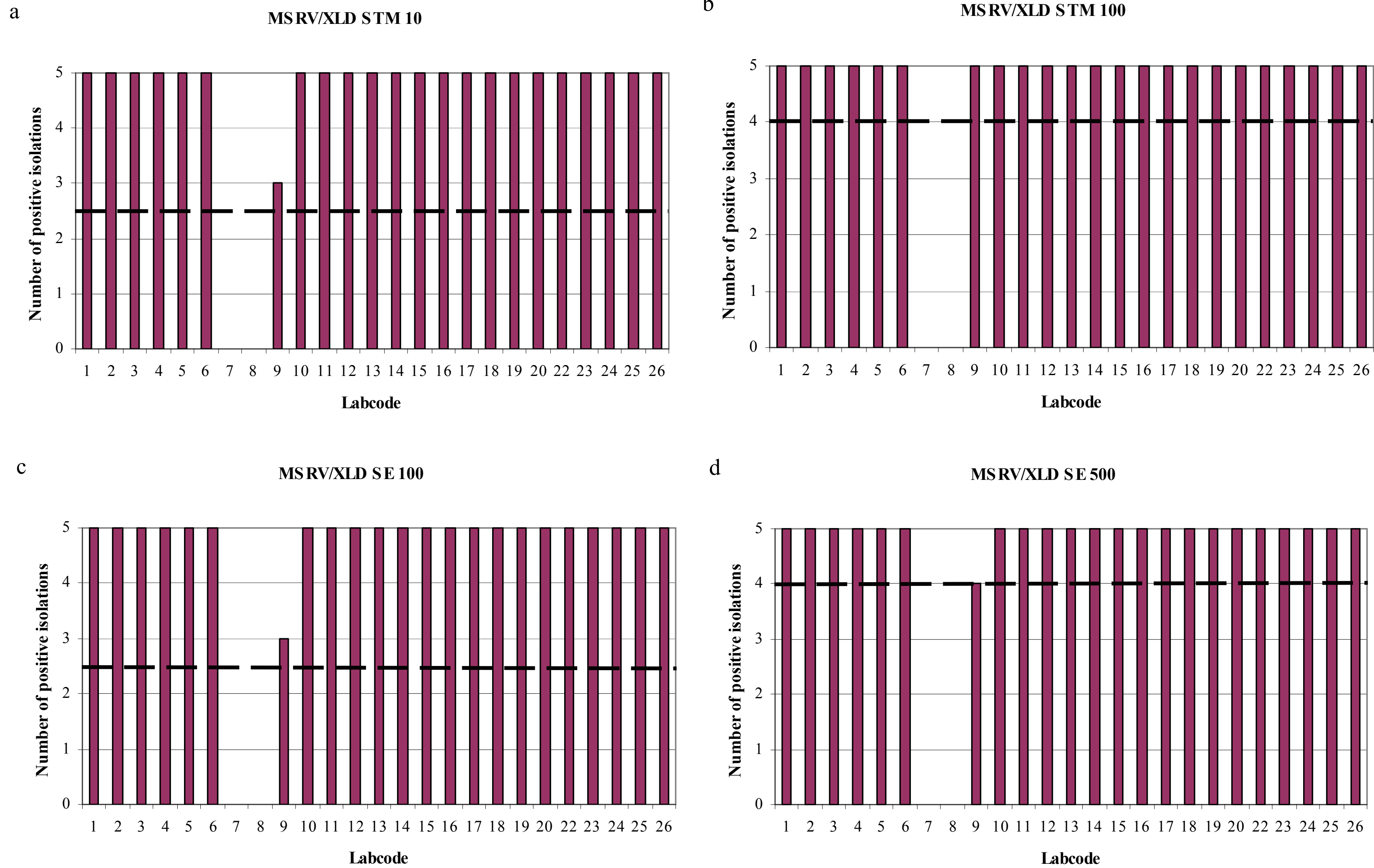
- - - = border of good performance

Fig. 1 Results artificially contaminated minced beef analyzed with RVS/XLD for the different capsules



- - - = border of good performance

Fig. 2 Results artificially contaminated minced beef analyzed with MKTTn/XLD for the different capsules



- - - = border of good performance

Fig. 3 Results artificially contaminated minced beef analyzed with MSRV/XLD for the different capsules

Differences between the selective enrichment media per capsule are shown as p-values in Table 18 and 19 (significant differences indicated in grey cells). Because of the multiple comparisons, 49 in total, the significance level of 0.05 was divided by 49 (Bonferroni correction, Lyman Ott and Longnecker, 2001). In the tables, a medium indicated on the left side in a row gave more positive results than the one indicated on the right side.
When MKTTn was used as selective enrichment medium, XLD showed significantly more positive results than BGA. There was no significant difference between the isolation media when RVS or MSRV was used for the selective enrichment. The combinations RVS/XLD and MSRV/XLD scored significantly more positive results when compared to MKTTn/XLD. There was no significant difference between RVS and MSRV for any capsule.

In Figure 4 the performance of each laboratory is compared to the mean of all laboratories for the artificially contaminated samples for all medium and capsule (positive for *Salmonella*) combinations. The laboratories 3, 6, 8, 9, 13, 15, 20 and 26 scored a significant lower number of positive outcomes for all medium and capsule combinations compared to the mean of all laboratories. Those are marked in the figure (p-value < 0.05).

Table 18 Comparison of results (p-values) for different selective enrichment media and different isolation media per capsule type added to Salmonella negative minced beef

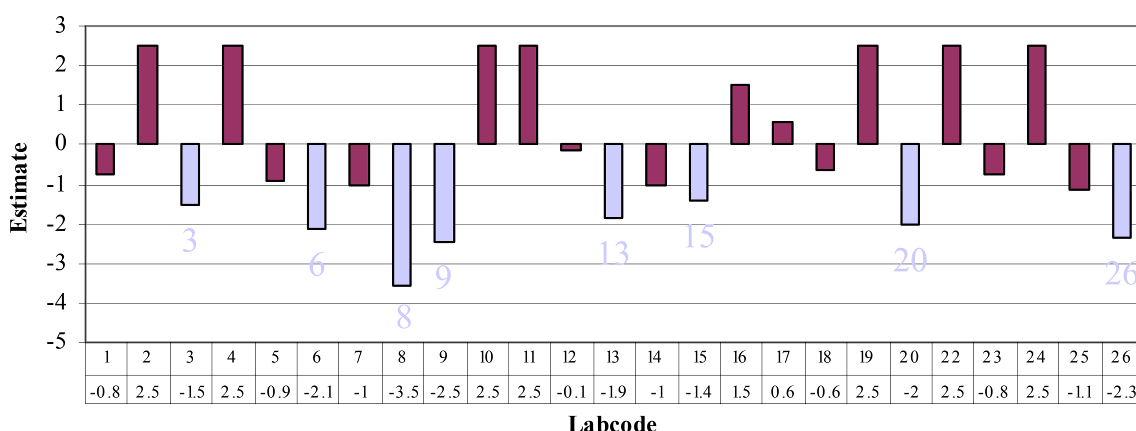
Selective enrichment medium	Compared isolation media	SE 100	SE 500	STM 10	STM 100	All SE	All STM	All Capsules
MKTTn	XLD vs BGA	0.0002	0.0010	0.0007	0.0001	0.0000	0.0000	0.0000
MSRV		0.2884	0.5926	0.0767	0.9715	0.2962	0.9681	0.9644
RVS		0.7856	0.6955	0.3995	1.0000	0.6337	0.6550	0.5329
RVS/MKTTn/MSRV		0.0276	0.1161	0.0071	0.9674	0.0108	0.9595	0.9484

grey cells are: significant different ($p < 0.05/49$)

Table 19 Comparison of results (p-values) for the plating-out medium XLD and different selective enrichment media per capsule type added to Salmonella negative minced beef.

Plating-out medium	Compared selective enrichment media	SE 100	SE 500	STM 10	STM 100	All SE	All STM	All Capsules
XLD	MSRV vs MKTTn	0.0005	0.0329	0.0006	0.9677	0.0002	0.9616	0.9500
	RVS vs MKTTn	0.0011	0.0288	0.0001	0.0128	0.0003	0.0000	0.0000
	RVS vs MSRV	0.1670	0.5319	0.6892	0.9740	0.1946	0.9750	0.9706

grey cells are : significant different (p < 0.05/49)



The labcodes with a significant lower score (p-value < 0.05) are marked in the figure.

Fig. 4 The performance of each laboratory compared to the mean of all laboratories for all medium and capsules combinations.

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

The specificity, sensitivity and accuracy rates for all types of capsules with the addition of *Salmonella*-negative minced beef are shown in Table 20. The results are given for the different medium combinations: BPW followed by selective enrichment in RVS or MKTTn or on MSRV and isolation on selective plating agar XLD. The specificity rates (of the blank capsules) were for all three selective enrichment media 99-100 %. For all capsules containing

Salmonella the sensitivity rates found with MKTTn was circa 10 % lower than the sensitivity rates of RVS and MSRV.

Table 20 *Specificity, sensitivity and accuracy rates for all participating laboratories (n=25) of the artificially contaminated meat samples (all capsules with the addition of 10 g minced beef), for the different selective enrichment media RVS, MKTTn, MSRV and plating-out medium XLD.*

Capsules with minced beef		RVS/XLD	MKTTn/XLD[1]	MSRV/XLD [1,2]
Blank (n=5)	No of samples No of negative samples **Specificity in %**	125 125 ***100.00***	120 120 ***100.00***	115 114 ***99.13***
STM10 (n=5)	No of samples No of positive samples **Sensitivity in %**	125 123 ***98.40***	120 100 ***83.33***	115 113 ***98.26***
STM100 (n=5)	No of samples No of positive samples **Sensitivity in %**	125 124 ***99.20***	120 111 ***92.50***	115 115 ***100.00***
SE100 (n=5)	No of samples No of positive samples **Sensitivity in %**	125 117 ***93.60***	120 99 ***82.50***	115 113 ***98.26***
SE500 (n=5)	No of samples No of positive samples **Sensitivity in %**	125 122 ***97.60***	120 111 ***92.50***	115 114 ***99.13***
All capsules with *Salmonella*	No of samples No of positive samples **Sensitivity in %**	500 486 ***97.20***	480 421 ***87.71***	460 455 ***98.91***
All capsules	No of samples No of correct samples **Accuracy in %**	625 611 ***97.76***	600 541 ***90.17***	575 569 ***98.96***

1 = Laboratory 8 did not perform selective enrichment in MKTTn and on MSRV
2 = Laboratory 7 did not perform selective enrichment on MSRV/XLD

4.6 PCR

Four laboratories (labcodes 1, 8, 14 and 19) 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)
1	1000	100	5/15
8	1000	100	1/5
14	1000	300	5/20
19	100	200	5/45

Two laboratories (labcodes 14 and 19) used a validated (for meat, milk, fish, eggs, chickenrins) PCR and tested > 400 samples in 2005 using this PCR technique. Laboratory 19 used a commercially available PCR (real-time PCR iQ-Check ™ *Salmonella*).
The PCR results are compared with the bacteriological culture results (BAC) as shown in Table 22. For the bacteriological results only the results of the prescribed or requested selective enrichment medium giving the highest number of positives are given. As laboratory 8 used only one prescribed selective enrichment medium (RVS), also the own selective enrichment results (Diassalm) are given.
Laboratory 14 and 19 scored all samples correct with the PCR method and with the bacteriological culture method.
Laboratory 1 found one control sample without capsule with meat and one blank capsule with meat positive, while with the bacteriological culture method, from the same BPW, they found correct results (negative).
Laboratory 8 found with the PCR method one control sample with Span5 negative and nine capsules (three SE500, five SE100 and one STM10) with the addition of meat negative. Some of these results were also tested negative with the bacteriological culture technique.

Table 22 PCR results compared with bacteriological culture results with control capsules and artificially contaminated minced beef samples of laboratories 1, 8, 14 and 19.

Capsules	lab 01		lab 08			lab 14		lab 19	
Cfp/caps.	BAC	PCR	BAC Dias -salm	BAC RVS	PCR	BAC	PCR	BAC	PCR
Controls without meat (n=10)									
Span 5 (n=2)	2	2	1	1	1	2	2	2	2
SE100 (n=2)	2	2	2	2	2	2	2	2	2
SE500 (n=1)	1	1	1	1	1	1	1	1	1
STM10 (n=3)	3	3	3	3	3	3	3	3	3
Blank (=2)	0	0	0	0	0	0	0	0	0
BPW (n=1)	0	0	0	0	0	0	0	0	0
Minced beef (n=1)	0	1	0	0	0	0	0	0	0
Test samples with minced beef (n=25)									
SE100 (n=5)	5	5	2	3	0	5	5	5	5
SE500 (n=5)	5	5	3	5	2	5	5	5	5
STM10 (n=5)	5	5	3	5	4	5	5	5	5
STM100 (n=5)	5	5	5	5	5	5	5	5	5
Blank (n=5)	0	1	0	0	0	0	0	0	0

grey cells = unexpected results
BAC = the results of the prescribed or requested selective enrichment medium giving the highest number of positives are given.
Laboratory 8 the results of both RVS and Diassalm are given.

5 Discussion

Transport of the samples

For this study the samples were shipped by courier service from door-to-door as diagnostic specimens, resulting in short transport times. All NRLs received the packages within 1-2 days. The temperature of the contents of the packages did not exceed 5 ºC for all the laboratories except two. The temperature of these two packages did not exceed 10 ºC. The results did not seem to be affected by the transport temperatures.

Performance of the laboratories

The prescribed method (ISO 6579: RVS and MKTTn) and the requested method (draft Annex D of ISO 6579: MSRV) were used by all the laboratories with the exception of one laboratory while another laboratory did not return the test report. The score was below the criteria of good performance when using MKTTn, one laboratory when using RVS and one laboratory for both when using the MKTTn and RVS methods. The results found with the MSRV method were very good; all the laboratories showed a good performance on MSRV. As all laboratories found good performance on MSRV, it was not necessary to send any laboratory extra materials.
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, superior results to ISO 6579. The selective enrichment medium MKTTn of ISO 6579 showed the lowest number of positive results.
In each interlaboratory comparison study (blind) control samples are added, which was also the case in this study. In general the results of the control samples are good, although occasionally, blank samples were found positive for *Salmonella*. In this study two laboratories found positive blank samples. Theoretically there is a small chance to find a positive blank with the addition of meat because it is not possible to give 100% guarantees about the *Salmonella* negativity of the matrix. It is also possible that this may have been caused by cross-contamination and the relevant laboratories were advised to check their procedures.

Media

The capsules had to be dissolved in pre-warmed BPW. Laboratory 8 pre-warmed, dissolved the capsules and cultured the BPW at 33.7 ^{o}C instead of 37 ^{o}C. A lower temperature of the BPW may result in not completely dissolved gelatin capsules and this is essential for the detection of *Salmonella* in the capsules. A low incubation temperature during pre-enrichment of the sample in BPW may result in less positive isolations of *Salmonella*. In this study especially the growth of *S.* Enteritidis may have been affected as this serovar grows slower than *S.* Typhimurium. The low dissolving and the low incubation temperature of the BPW are most probably explanations for the low number of positives found by laboratory 8. 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. Only four laboratories found more positive results after 48 hours of incubation on MSRV. The selective enrichment medium MKTTn showed more positive results after 48 hours of incubation than after 24 hours. Here the high selectivity of MKTTn may have influenced the growth rate of *Salmonella*.

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 additionally to the prescribed and requested methods. The results found with the PCR methods were comparable to the results found with the bacteriological detection methods. Except for the positive result of one blank and a positive control sample with only minced beef by laboratory 1. No direct explanation was found.

Specificity, sensitivity and accuracy rates
The specificity, sensitivity and accuracy rates were very high (>94 %) for all samples tested with the selective enrichment media RVS and MSRV. The selective enrichment medium MKTTn however, showed less positive results, resulting in sensitivity rates which were circa 10% lower than the sensitivity rates of the other selective enrichment media.
For a better testing of the performance of the laboratories during ring trials it would be more interesting to use, low level samples of which the contamination level is at the detection limit of the method and also to use samples with a contamination level 5-10 times above the detection limit. With these samples it is expected that circa 50 % of the low level samples will be tested positive. In this study even the low level samples were tested for almost 100% positive. It may therefore be necessary to adjust the contamination level of the samples in future studies. Further research will be performed at the CRL-*Salmonella.*

Evaluation of this pilot study
At the workshop of CRL-*Salmonella* in May 2006 the detection of *Salmonella* in food was discussed (Mooijman, 2006) and it was agreed to perform an interlaboratory comparison study on a food matrix in September 2006 (week 39). In June 2006 the NRLs were informed with the time table of this study through a CRL-*Salmonella* newsletter which was published on the website of CRL *Salmonella.* Unfortunately not all the NRLs were present at the workshop, read the newsletter or looked at the CRL *Salmonella* website. Problems arose when in the first week of September (week 36) the timetable, protocol, SOP and test report were send to all NRLs. Seven NRLs indicated not to be the right laboratory for performing the food analyses. These NRLs informed the CRL to send the samples to another laboratory

in their institute or country which is more acquainted to the microbiological analyses of food samples. Eventually the mailing of the parcels to the right laboratories in week 38 went very well but the e-mail with the protocol, testreport, SOP etc. did not always arrive at the right person. This was for some NRLs the reason that the performance of the study was delayed. Furthermore, it was not always clear for the NRLs that they needed to return the temperature recorders.
Twenty-five out of 28 NRLs participated, one NRL (EU member) did not send the results from this study to the CRL and two NRLs (one EU member and one candidate EU member) did not participate.
All the participated NRLs performed very well and showed a good performance.

To prevent problems in a next study with a non veterinary matrix, CRL- *Salmonella* will send timely information and timetables by e-mail to the NRLs.
Finally, the CRL-*Salmonella* will do the outmost to keep the list with addresses and contact persons of all NRLs up-to-date. For this the CRL will need information from DG-Sanco as well as from the NRLs itself.

6 Conclusions

- All NRLs performed very well with the MSRV method and achieved the level of 'good performance' as was suggested during the CRL-*Salmonella* workshop 2005 for the tested samples. One laboratory did not perform all the requested methods but still found good performance on RVS. One NRL did not return the test report.
- For a good dissolution of the capsules it is important to use the right incubation temperature of the BPW.
- Deviating temperatures of the BPW during pre-warming and enrichment may influence the growth of *Salmonella* and eventually the number of positive isolations.
- The accuracy, specificity and sensitivity rates for the control samples (without meat) from RVS, MKTTn and MSRV were higher than 98 %.
- The specificity rate of the minced beef samples artificially 'contaminated' with blank capsules was higher than 99 %.
- For all artificially contaminated minced beef samples the rates found with MKTTn were circa 10 % lower than the rates of MSRV and RVS.
- The sensitivity rates for all artificially contaminated minced beef samples were higher than 94 % for MSRV and RVS; for MKTTn it was 88 %.
- The accuracy rates for the artificially contaminated minced beef samples were higher than 98 % for RVS and MSRV; for MKTTn it was 90 %.
- 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 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 or RVS for the matrix used (minced beef).
- MSRV is a good selective enrichment medium for the matrix used (minced beef).
- The MKTTn method is not the optimal medium for selective enrichment of the matrix used (minced beef).
- This first (pilot) food study showed to have been successful for the number of participants as well as for the good results found by the participants.

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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 animal faeces and in samples of the primary production stage

Study	Year	Number of samples	Capsules	Actual number of cfp/capsule	Salmonella negative faeces[1] added	Selective enrichment Medium	Plating-out medium	Reference ● (RIVM report)
I	1995	26 4	STM5 Blank	6 0	No No	RV and SC	BGA and own	Voogt et al., 1996 (report 284500003)
II	1996	15 15 2 1 1	STM100 STM1000 SPan5 STM100 Blank	116 930 5 116 0	1 gram 1 gram No No No	RV, SC and own	BGA and own	Voogt et al., 1997 (report 284500007)
III	1998	14 14 7 14 4 2 5	STM10 STM100 STM100 SE100 STM10 SPan5 Blank	11 94 94 95 11 5 0	1 gram 1 gram 1 gram* 1 gram No No No	RV and own	BGA and own	Raes et al., 1998 (report 284500011)
IV	1999	5 5 5 5 5 3 3 2 2	STM10 STM100 SE100 SE500 Blank STM10 SE100 SPan5 Blank	4 210 60 220 0 5 60 5 0	10 gram 10 gram 10 gram 10 gram 10 gram No No No No	RV or RVS, MSRV and own	BGA and own	Raes et al., 2000 (report 284500014)
V	2000	5 5 5 5 5 3 3 2 2 20	STM10 STM100 SE100 SE500 Blank STM10 SE100 SPan5 Blank None	4 47 63 450 0 4 63 5 0 -	10 gram 10 gram 10 gram 10 gram 10 gram No No No No 25 gram**	RV or RVS, MSRV and own	BGA and XLD	Raes et al., 2001 (report 284500018)
VI	2002	5 5 5 5 5 3 3 2 2 20	STM10 STM100 SE100 SE500 Blank STM10 SE100 SPan5 Blank None	11 139 92 389 0 11 92 5 0 -	10 gram 10 gram 10 gram 10 gram 10 gram No No No No 25 gram**	RVS, MSRV, MKTTn and own	BGA, XLD and own	Korver et al., 2003 (report 330300001)

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 ● (RIVM report)
VII	2003	5 5 5 5 5 3 3 2 2 20	STM10 STM100 SE100 SE500 Blank STM10 SE100 SPan5 Blank None	12 96 127 595 0 12 127 9 0 -	10 gram 10 gram 10 gram 10 gram 10 gram No No No No 10 gram**	RVS, MSRV, MKTTn and own	BGA, XLD and own	Korver et al., 2005 (report 330300004)
VIII	2004	7 4 7 4 3 3 2 1 2 2 20	STM10 STM100 SE100 SE500 Blank STM10 SE100 SE500 SPan5 Blank None	13 81 74 434 0 13 74 434 7 0 -	10 gram 10 gram 10 gram 10 gram 10 gram No No No No No 10 gram**	MSRV and own	XLD and own	Korver et al., 2005 (report 330300008)
IX	2005	5 5 5 5 5 3 2 1 2 2 10	STM10 STM100 SE100 SE500 Blank STM10 SE100 SE500 SPan5 Blank None	9 86 122 441 0 9 86 441 7 0 -	10 gram[2] 10 gram 10 gram 10 gram 10 gram No No No No No 10 gram***	MSRV and own	XLD and own	Berk et. al., 2006 (report 330300011)

* = with antibiotics; ** = Naturally contaminated chicken faeces with *Salmonella*
*** = Naturally contaminated dust with *Salmonella*
[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
● = The report of each study can be found at the CRL-*Salmonella* website: http://www.rivm.nl/crlsalmonella/publication/ or can be obtained through the corresponding author of this report

Table 1.2 CRL-Salmonella interlaboratory comparison study and detection of Salmonella in food products

Study	Year	Number of samples	Capsules	Actual number of cfp/capsule	Salmonella negative meat	Selective enrichment medium	Plating-out medium	Reference (RIVM report)
I	2006	5 5 5 5 5 3 2 1 2 2	STM10 STM100 SE100 SE500 Blank STM10 SE100 SE500 SPan5 Blank	9 98 74 519 0 9 98 519 5 0	10 gram 10 gram 10 gram 10 gram 10 gram No No No No No	RVS, MKTTn, MSRV and own	XLD and own	This report

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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, overdispersion is expected and $T_2/(I-1)$ will mostly be larger than 1 (Heisterkamp et al., 1993). An acceptable variation for a batch of capsules will be $T_2/(I-1) \leq 2$.

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

Annex 3. Information on the media used

Diassalm
van Netten P, Van der Zee H, Van der Moosdijk A,1991 The use of diagnostic selective semi-solid medium for the isolation os *Salmonella* enteritidis from poultry. Proceedings of the 10th Symposium on the quality of poultry meat, Spelderholt Beckbergen, pp. 59-67.

Composition of Diassalm medium: the concentration of the compounds in g/L water::Tryptose 20, PeptonAcid hydrolysate of casein 6.1, Ferrous ammonium sulphate 0.2, Sodium thiosulphate 5.0, Sucrose 7.5, Lactose 0.5, Bromocresol purple 0.08, Magnesium chloride anhydrous ($MgCl_2$) 11, Malachite green oxalate 0.037, Agar 2.8, Novobiocin 10 mg, pH 5.5 +/- 0.2

BGA modified (Oxoid CM 0329B, Hampshire, United Kingdom)
(Difco 218801, Detroit, USA) (Merck 1.10747 Darmstadt, Germany) (HIMEDIA M971) (Biomark B439) (Lab 34)
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

Sulphamandelate supplement (BGA mod(+))
(Oxoid CM SR0087, Hampshire, United Kingdom)

BGA (Biogenetics BM 730, Padua, Italy)
Composition of BGA medium: the concentration of the compounds in g/L water:
Yeast Extract 3, Triptone 5, Animal Peptone 5, Sodium Chloride 5, Lactose 10, Saccharose 10, Brillant Green 0.0125, Phenol Red 0.08, Sulphadiazyne 0.08, Agar 20

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 6.9

MLCB (Lab M. Ltd. LAB 116, Bury, United Kingdom)
Inoue T, Takagi S, Ohnishi A, et al. Foodborne disease salmonella plating-out 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

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 7.12

Rambach (Merck 107500.0001/2, Darmstadt, Germany)
Rambach, A.: New Plate Medium far Facilitated Differentiation of Salmonella spp. from Proteus sac. and Other Enteric Bacteria». - Appl. Environm. Microbiol., 56; 301-303 (1990).
Composition of Rambach medium: the concentration of the compounds in g/L water:
Peptone 8.0, 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 (bioMérieux SM2 43621, Marcy l' Etoile, France) (Gelose SMID2 801882601)
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

XLT4 (Biokar BK156HA, Beauvais, France)
Miller, R.G., C.R. Tate. 1990. XLT4: A highly selective plating medium for the isolation of Salmonella. The Maryland Poultryman, April: 2-7 (1990).
Composition of XLT4 medium: the concentration of the compounds in g/L water:
Peptone 1.6, Yeast Extract 3, Lisine 5, Lactose 7.5, Saccarose 7.5, Xilose 3.75, Sodium Chloride 5, Sodium Tiosolphate 6.8, Ferrum Ammonium Citratus 0.8, Phenol Red 0.08, Agar 18.

Annex 4. Protocol

INTERLABORATORY COMPARISON ON THE
DETECTION OF *SALMONELLA* spp. IN FOOD
organised by CRL-*Salmonella*
FOOD STUDY I - 2006

Introduction

This is the first 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 a 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 food-study will have a comparable set-up as the earlier studies on the detection of *Salmonella* spp. in veterinary samples. At the workshops of CRL *Salmonella* in 2005 and 2006 the detection of *Salmonella* in a food matrix was discussed and it was decided to start with meat.
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 recommended also to use draft Annex D of ISO 6579. The method in this annex is especially intended for the detection of *Salmonella* spp. in animal faeces and samples of the primary production stage, but is also applicable to food samples. A copy of the latest version of draft Annex D (12 September 2006) will be provided with this study. Furthermore laboratories who are interested can also perform PCR on the samples and/or use additional methods (routinely) used in their laboratories.
Artificially contaminated (*Salmonella* negative) minced beef samples are tested by using 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 and negative for *Salmonella* spp.) in combination with a capsule containing STM or SE and 10 control samples (no meat is added to the capsule).
Finally, to obtain more detailed information on the temperatures and times during transport of the samples we will include an electronic temperature recorder in the parcel. The amount of materials can not be packed in one parcel and will be divided over two parcels (one containing capsules and one containing *Salmonella* negative meat). The two parcels are packed in one box with cooling elements. We will include only one temperature recorder and only in the parcel 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 parcels) will be sent as diagnostic specimens by door-to-door courier service. Please contact CRL-*Salmonella* when the parcel has not arrived at your laboratory at 22nd of September 2006 (this is after 5 working days after the day of mailing).

Objectives

The main objective of the first 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.

Outline of the study

Each participant will receive (in week 38) one box containing 2 parcels, packed with cooling elements. The parcels contain:

Parcel 1:

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

Parcel 2:

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

Parcel 1 should be stored at (-20 ± 5)ºC immediate after receipt.
Parcel 2 should be stored at (5 ± 3)ºC immediate after receipt.

The performance of the study will be in <u>week 39</u> (starting on 25 September 2006).

The documents necessary for performing the study are:

- Protocol Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in food I (2006);
- SOP Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in food I (2006);
- Test report Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in food I (2006);
- ISO 6579 (2002). Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.;
- Draft Amendment ISO 6579:2002/amendedDAmd 1 (2006-09-12) Amendment 1 Annex D: Detection of *Salmonella* spp. in animal faeces and in 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.
In the time table of the interlaboratory comparison study Food I (see next page) on the bacteriological detection of *Salmonella*, a **strict deadline** for sending the results to the CRL-*Salmonella* is indicated (15 October 2006). We will prepare a short report 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. It may still be possible to use late results in the analyses for the final report but results received after publishing the short report can not be incorporated in the final report.

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

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Time table of interlaboratory comparison study FOOD I (2006)

Week	Date	Topic
36	4 – 8 September	Mailing of the protocol, standard operating procedure, test report and draft Annex D of ISO 6579 to the NRLs-Salmonella
38	18 – 22 September	Mailing of the parcels to the NRLs as diagnostic specimens 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 meat at +5°C ± 3°C** - **Store the capsules at -20°C ± 5°C**. **If you did not receive the parcel at 22 September, 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)
39	25-29 September	Performance of the study, following the instructions as given in the protocol and the SOP of study Food I (2006).
41	Before 15 October	Completion of the test report and faxing or e-mailing it to the CRL. The original test report will be sent to CRL.
42	16-20 October	Checking the results by the National Reference Laboratories.
	November/ December 2006	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.

Annex 5. Standard Operating Procedure

(SOP)

INTERLABORATORY COMPARISON ON THE
DETECTION OF *SALMONELLA* spp. IN FOOD
organised by CRL-*Salmonella*
FOOD STUDY I - 2006

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 for *Salmonella* (CRL) are used. As food 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.

Draft Amendment ISO 6579:2002/amendedDAmd 1 (2006-09-12). Amendment 1 Annex D:
Detection of *Salmonella* spp. in animal faces and in 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 artificially contaminated spray dried milk.

4 Principle

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

5 List of abbreviations

BPW	Buffered Peptone Water
MKTTn	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. Besides ISO 6579 it is requested also to apply draft 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 draft 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 methods (ISO 6579) and requested (draft 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 (ISO657 Draft Annex D (MSRV) (requested) 2006-09-12)
- Selective enrichment medium routinely used in your laboratory (optional)

6.3 Solid selective media for first and second isolation

- Xylose-Lysine-Desoxycholate (ISO6579 Annex B.4)
  This medium must be boiled to dissolve (instructions manufacturer). After boiling the medium must be transparent red. Plates should be poured with a volume of 15-20 ml in 90 mm plates and / or 30-40 ml in 140 mm-plates
- Second isolation medium for choice (obligatory)
- Own medium (optionally)

6.4 Confirmation media

Biochemical confirmation

- Triple sugar/iron agar (TSI agar) (ISO6579 Annex B.6)
- Urea agar (ISO6579 Annex B.7)
- l-Lysine decarboxylation medium (ISO6579 Annex B.8)
- Nutrient agar (optional) (ISO6579 Annex B.5)

7 Apparatus and glassware

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

7.1 Apparatus

- Oven (for dry sterilisation) or autoclave (for wet sterilisation);
- Water bath or incubator, capable of operating at 37 °C ± 1 °C ;
- Water bath or incubator, capable of operating at 41,5 °C ± 1 °C
- Loops 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 First interlaboratory comparison study in a food matrix of CRL-*Salmonella* are 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 draft Annex D of ISO 6579 (version 120906).

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 gr. 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 according to the following scheme:

- **Add 10 grams 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 and MSRV plates 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.

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)

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.

Record in the test report (page 12-17) the requested data of the isolation media used. Label 2 times 25 large size Petri dishes and 25 standard size Petri dishes of the isolation media from 1 to 25 and label 2 times 12 large size Petri dishes and 12 standard size Petri dishes from C1 to C12.

First isolation after 24 h
Inoculation:
Inoculate, by means of a loop, from MKTTn and RVS cultures the surface of an isolation medium in a large size Petri dish (or two standard size Petri dishes) and from suspect MSRV plates, the surface of an isolation medium 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 Petri dishes with the bottom up in the incubator set at 37 °C (record temperature and time and other requested data in test report, page 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 ± 3h of the selective enrichment media, repeat the procedure described above (**First isolation after 24 h**). Only repeat the full procedure 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 (only use 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 test report pages 21-28. For the results of detection of *Salmonella* using PCR fill in Table 5 on 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
- Urea agar
- l-Lysine decarboxylation medium

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 (-)
- l-Lysine decarboxylation medium: turbidity and purple colour (+)

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.

Scheme of Bacteriological Interlaboratory Comparison Study FOOD I (2006)
on detection of *Salmonella* spp. in minced beef

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**	1 capsule to 90 ml (prewarmed) BPW Do not shake 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 and other 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 and MSRV medium another 24 (± 3) hours at the relevant temperatures
4	**Second isolation after 24 h**	If the first isolation was negative, inoculate from RVS, MKTTn, suspect MSRV plates and Other 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. Draft Annex D of ISO 6579

ISO TC 34/SC 9

Date: 2006-09-12
Amended ISO 6579:2002/DAM

Annex D

Detection of Salmonella spp. in animal faeces and in samples of the primary production stage

D.1 Introduction

ISO 6579 is primarily intended for the isolation of *Salmonella* spp. from food and feeding stuffs and is not always suitable for the detection of *Salmonella* spp. from other matrices.
This annex is applicable to the detection of *Salmonella* spp. in:
animal faeces (like poultry, pigs, cattle);

environmental samples in the area of the primary production stage (like dust).

The method in this annex is based upon ISO 6579, with a different selective enrichment. Therefore, where possible reference will be made to the full text of ISO 6579.
The selective enrichment medium as described in this annex (being Modified Semi-solid Rappaport Vassiliadis: MSRV) is intended for the detection of motile Salmonellae and is not appropriate for the detection of non-motile Salmonellae.
NOTE The non-motile Salmonella serovars *Salmonella* Gallinarum and *Salmonella* Pullorum do not seem to survive long in environmental samples and will therefore rarely be detected in faecal or environmental (like dust) samples (regardless of the method). The number of other non-motile Salmonella serovars in faecal samples seems to be generally low. For example, in a study of Voogt *et al* (2001) in which *ca* 1 000 faecal samples of poultry layer flocks and *ca* 900 faecal samples of broiler flocks were analysed, less than 1 % of the total number of samples were positive in a selective broth and at the same time negative on MSRV (and likely to be non-motile). Similar results were found in a Dutch study with *ca* 3 200 faecal samples of pigs (non-published data). On the other hand, in the case of the Voogt study, up to almost 40 % of positive samples would not have been detected (i.e. false negatives) if only a selective broth (in this case Rappaport Vassiliadis) had been used instead of a semi-solid medium.

D.2 Normative references

See Ch. 2 of ISO 6579
Additional:
ENV ISO 11133-1: 2000, Microbiology of food and animal feeding stuffs – Guidelines on preparation and production of culture media – Part 1: General guidelines on quality assurance for the preparation of culture media in the laboratory
ISO/TS 11133-2: 2003, Microbiology of food and animal feeding stuffs – Guidelines on preparation and production of culture media – Part 2: Practical guidelines on performance testing of culture media.

D.3 Terms and definitions

See Ch. 3 of ISO 6579.

D.4 Principle

D.4.1 General

The detection of *Salmonella* in animal faeces and in samples of the primary production stage necessitates four stages, as described in Ch. 4 of ISO 6579.

D.4.2 Pre-enrichment in non-selective liquid medium

Buffered Peptone Water (BPW) is inoculated at ambient temperature with the test portion, then incubated at 37 °C ± 1 °C for 18 h ± 2 h.

D.4.3 Enrichment on selective semi-solid medium

Modified Semi-solid Rappaport Vassiliadis (MSRV) agar plates are inoculated with the culture obtained in D.4.2.
The MSRV is incubated at 41,5 °C ± 1 °C for 24 h ± 3 h. If a plate is negative after 24 h it is incubated for a further 24 h ± 3 h.

D.4.4 Selective plating and identification

From the culture obtained in D.4.3, two selective solid media are inoculated:
Xylose Lysine Deoxycholate (XLD) agar;

Any other solid selective medium complementary to XLD agar (see 4.4 of ISO 6579).

The XLD agar is incubated at 37 °C ± 1 °C and examined after 24 h ± 3 h.
The second selective medium is incubated in accordance with the manufacturer's instructions.

D.4.5 Confirmation of identity

Colonies of presumptive *Salmonella* are subcultured, then plated-out as described in D.4.4, and their identity is confirmed by means of appropriate biochemical and serological tests.

D.5 Culture media, reagents and sera

D.5.1 General

For current laboratory practice, see ISO 7218
All media and reagents needed for this annex are described in Annex B of ISO 6579, except for Modified Semi-solid Rappaport Vassiliadis (MSRV) medium, which is described in D.5.2. Alternatively, dehydrated complete media or diluents may be used. Follow, in that respect, the manufacturer's instructions.
NOTE The composition of MSRV as described by De Smedt *et al.* (1986), contained 20 mg/L novobiocin. However, from a scientific point of view, 10 mg/L novobiocin is preferred. In studies performed at the CRL-*Salmonella,* more *Salmonella* positive results were found in pig faeces samples when tested with MSRV containing 10 mg/L than with MSRV containing 20 mg/L novobiocin (Veenman *et al.,* 2006). Furthermore, when testing different animal faeces (pigs, chicken, cattle) and naturally contaminated dust, the migration zones on MSRV containing 10 mg/L novobiocin were (much) larger than on MSRV containing 20 mg/L novobiocin (Veenman *et al.,* 2006). Influence of novobiocin on bacterial motility is earlier described by Soutourina *et al.* (2001).

For the preparation of the selective plating agar media (see B.4, XLD-agar) standard size Petri dishes can be used (90 mm or 100 mm) instead of large size Petri dishes (140 mm).

D.5.2 Modified Semi-solid Rappaport Vassiliadis medium (MSRV)

D.5.2.1 Base medium

Composition

Ingredient	Amount	Unit
Enzymatic digest of casein	4,6	g
Acid hydrolysate of casein	4,6	g
Sodium chloride (NaCl)	7,3	g
Potassium dihydrogen phosphate (KH_2PO_4)	1,5	g
Magnesium chloride anhydrous ($MgCl_2$)	10,9	g
Malachite green oxalate	0,04	g
Agar	2,7	g
Water	1 000	ml

Preparation

Suspend the ingredients into the water.
Heat to boiling with agitation. **Do not autoclave**.
Do not hold the medium at high temperatures longer than necessary.
Cool the medium to 47-50 °C.

D.5.2.2 Novobiocin solution

Composition

Ingredient	Amount	Unit
Novobiocin sodium salt	0,05	g
Water	10	ml

Preparation

Dissolve the novobiocin sodium salt in the water.
Sterilize by filtration through a filter with a pore size of 0,22 μm.
The solution can be stored for up to 4 weeks at 5 °C ± 3 °C or in small portions (e.g. of 2 ml) at –20 °C for up to one year.

D.5.2.3 Complete medium

Composition

Ingredient	Amount	Unit
Base medium (D.5.2.1)	1 000	ml
Novobiocin solution (D.5.2.2)	2	ml

Preparation

Aseptically add 2 ml of the novobiocin solution (D.5.2.2) to 1000 ml of base medium (D.5.2.1) at 47-50 °C. Mix carefully.
The final pH should be 5,2 (5,1 – 5,4) at 20-25 °C.
Pour carefully into plates up to a final volume of 15-20 ml in Petri dishes with a diameter of 90 mm.
Allow the medium to solidify before moving and handle with care.
Store the plates, **with surface upwards**, for up to 2 weeks at 5 °C ± 3 °C in the dark.
Do not invert the plates, as the semi-solid agar is too sloppy to do so.
Any plates in which the semi-solid agar has liquefied or fragmented should not be used.
Immediately before use, and only if necessary, dry the surface of the agar plates carefully, for example by placing them with the lids off and the agar surface **upwards** in a Laminar Air Flow cabinet. Mind not to overdry the medium.

D.6 Apparatus and glassware

See Ch. 6 of ISO 6579.
Additional:
Sterile loops of 1 μl

D.7 Sampling

See Ch. 7 of ISO 6579.

D.8 Preparation of test sample

See Ch. 8 of ISO 6579.
Generally an amount of sample is added to a quantity of BPW to yield a 1/10 dilution (e.g. 25 g of sample added to 225 ml of BPW). However, for some type of samples it may be necessary to use another ratio.

D.9 Procedure

D.9.1 Non-selective pre-enrichment

Pre-warm the BPW to room temperature before use.
Mix samples well by the most suitable means for the sample type.
Weigh the sample and add it to the appropriate quantity of BPW (see D.8). Incubate the jars at 37 °C ± 1 °C for 18 h ± 2 h.

D.9.2 Selective enrichment

Allow the MSRV plates to equilibrate at room temperature if they were stored at a lower temperature.
Inoculate the MSRV plates with 3 drops of incubated BPW culture. The 3 drops should total 0,1 ml and be placed separately and equally spaced on the surface of the medium.
NOTE When taking a subculture from BPW, it is very important not to disturb particulate samples. Therefore, containers should be moved carefully, and not mixed, shaken or swirled. Aim to extract an inoculum from the largest volume of free fluid nearest the interface between container and surface of culture, but it is advisable to go deeper if there are particulates floating on the surface.
Incubate the inoculated MSRV plates at 41,5 °C ± 1 °C for 24 h ± 3 h.
Do not invert the plates.
Positive plates will show a grey-white, turbid zone extending out from the inoculated drop. The turbid zone is characterized by a white halo with a clearly defined edge.
If the plates are negative after 24 h, reincubate for a further 24 h ± 3 h.

D.9.3 Selective plating

Allow the Xylose Lysine Deoxycholate agar (XLD) plates and the second selective plating medium (see 5.2.4.2 of ISO 6579) to equilibrate at room temperature if they were stored at a lower temperature. If necessary dry the surface of the plates before use.
Observe the MSRV plate (if necessary on a clear white surface or light box). Determine where the furthest point of spread of opaque growth from the inoculation points is and dip a loop of 1 µl just inside the border of the opaque growth. Withdraw the loop ensuring that no large lumps of MSRV are extracted. Inoculate the surface of an XLD plate so that well-isolated colonies will be obtained. Proceed in the same way with the second selective plating medium using a new sterile loop.
NOTE 1 By plating-out little material from MSRV (using a 1 µl loop), well isolated colonies can be obtained by using only one standard size Petri dish (90-100 mm) with selective plating agar. The use of large dishes (140 mm) will therefore not be necessary.
Incubate the XLD plates inverted at 37 °C ± 1 °C for 24 h ± 3 h.
Incubate the second selective plating medium in accordance with the manufacturer’s instructions.
Return negative MSRV plates to the 41,5 °C incubator and incubate for a further 24 h ± 3 h. Repeat the selective plating procedure after 48 h of incubation of MSRV.
Typical colonies of *Salmonella* grown on XLD-agar have a black centre and a lightly transparent zone of reddish colour due to the colour change of the indicator.
NOTE 2 *Salmonella* H_2S negative variants (e.g. *Salmonella* Paratyphi A) grown on XLD agar are pink with a darker pink centre. Lactose-positive *Salmonella* grown on XLD agar are yellow with or without blackening (also see 9.4.4 of ISO 6579).
Check the second selective plating medium after the appropriate incubation time for the presence of colonies which, from their characteristics, are considered to be presumptive *Salmonella*.

D.9.4 Confirmation

For confirmation of the typical colonies, isolated on the selective plating media, follow the instructions as given in Ch. 9.5 of ISO 6579. In 9.5.2. of ISO 6579 it is prescribed to streak isolated colonies from the selective plating media onto nutrient agar before performing the biochemical confirmation. However, this extra cultural step is not necessary if well-isolated colonies (of a pure culture) are available on the selective plating media. If this is the case perform the biochemical confirmation directly on a typical (suspect), well-isolated colony of each selective plating medium.

D.10 Expression of results

See Ch. 10 of ISO 6579.

D.11 Test report

See Ch. 11 of ISO 6579.

D.12 Quality assurance

See Ch. 12 of ISO 6579.

For the performance testing of media, the information as described in ENV ISO 1133-1 and in ISO/TS 11133-2 is followed. However, in these ISO documents, procedures are given for selective broths as well as for selective agar media for the detection of *Salmonella,* but not for semi-solid media like MSRV. The procedure given below can be used for testing the performance of MSRV and is based upon the procedure and test strains as described for selective (enrichment) media for the detection of *Salmonella* (like MKTTn and RVS, see B.2 and B.3 of ISO 6579) in ISO/TS 11133-2.
The procedure given below has been extracted from ISO/TS 11133-2, 5.4.2.1, but with an adapted concentration of the test strains. The references given are references to the chapters of ISO/TS 11133-2. The procedure, test strains and criteria are summarised in Table 1.

- Inoculation of target microorganisms: Inoculate MSRV for each test organism with *ca* 10^4 cfu/ 0,1 ml (for preparation of the inoculum see 5.2.1);

- Inoculation of non-target microorganisms: Inoculate MSRV for each test organism with 10^5 – 10^6 cfu/ 0,1 ml (for preparation of the inoculum see 5.2.1);

- Inoculation of target and non-target microorganisms as a mixed culture: Inoculate MSRV with a mixed culture containing *ca* 10^4 cfu/ 0,1 ml of target microorganisms and 10^5 – 10^6 cfu/ 0,1 ml of non-target microorganisms (for preparation of the inoculums see 5.2.1).

Incubate the MSRV plates at 41,5 °C ± 1 °C and assess the plates after 24 h ± 3 h and after 48 h ± 6 h.

Table 1 Performance testing of MSRV

Function	Control strains	Final concentration in the inoculum of 0,1 ml	Incubation of MSRV	Criteria
Specificity	*S.* Typhimurium ATCC 14028 or *S.* Enteritidis ATCC 13076	10^4 cfu	41,5 °C ± 1 °C, 2x 24 h ± 3 h	Grey-white, turbid zone extending out from the inoculated drop. After 48 h, the turbid zones of the 3 drops will be (almost) fully migrated over the plate
Selectivity	*E. coli* ATCC 25922 or ATCC 8739 *E. faecalis* ATCC 29212 or ATCC 19433	$10^5 - 10^6$ cfu	41,5 °C ± 1 °C, 2x 24 h ± 3 h	Possible growth at the place of the inoculated drop without a turbid zone
Productivity	*S.* Typhimurium ATCC 14028 or *S.* Enteritidis ATCC 13076 + *E. coli* ATCC 25922 or ATCC 8739 + *P. aeruginosa* ATCC 27853	10^4 cfu $10^5 - 10^6$ cfu $10^5 - 10^6$ cfu	41,5 °C ± 1 °C, 2x 24 h ± 3 h	Grey-white, turbid zone extending out from the inoculated drop. After 48 h, the turbid zones of the 3 drops will be (almost) fully migrated over the plate Possible extra: subculture with 1 µl loop just inside the border of the opaque growth and spread onto XLD. Incubate at 37 °C ± 1 °C for 24 h ± 3 h. Criteria: growth of characteristic colonies in majority

Remark: In general *S.* Typhimurium will show faster growth and larger migration zones than *S.* Enteritidis.

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