rivm

RIVM Report 330604004/2007

EU Interlaboratory comparison study veterinary-X (2006)
Bacteriological detection of *Salmonella* in pig faeces

Kuijpers A.F.A.
Veenman C.
Mooijman K.A.

Contact:
Kuijpers A.F.A.
Laboratory for Zoonoses and Environmental Microbiology
angelina.kuijpers@rivm.nl

This investigation has been performed by order and for the account of European Commission, Health and Consumer Protection Directorate-General and the Laboratory for Zoonoses and Environmental Microbiology (LZO) of the RIVM, within the framework of V/330604/06/CS by the Community Reference Laboratory for *Salmonella*

rivm

Abstract

EU Interlaboratory comparison study veterinary-X (2006)
Bacteriological detection of *Salmonella* in pig faeces

The European National Reference Laboratories (NRLs) for *Salmonella* were able to detect high and low levels of *Salmonella* in pig faeces. This is shown in the 10th interlaboratory comparison study, in which 27 laboratories participated.

The goal of the study, organized by the Community Reference Laboratory (CRL) for *Salmonella* in November 2006, was to see if the participating laboratories could detect *Salmonella* in pig faeces.
Each laboratory received a package containing pig faeces and 35 gelatin capsules displaying different levels of *Salmonella*. The instructions to the laboratories were to spike the pig faeces with the capsules and test these samples for the presence of *Salmonella*, which they did. The laboratories used one prescribed method for running this test, namely, a selective culture step on Modified Semi-solid Rappaport Vassiliadis (MSRV).

The laboratories found *Salmonella* in 97 % of the samples. Three laboratories had problems detecting *Salmonella* in the samples with low levels of contamination. The CRL contacted these laboratories to request an explanation for the deviating results; CRL also offered the possibility of performing extra analyses. These three laboratories too achieved a good performance in the follow-up study in February 2007.

Key words: *Salmonella*; CRL-*Salmonella*; NRL-*Salmonella*; comparison study; pig faeces

rivm

Rapport in het kort

EU Ringonderzoek veterinair-X (2006)
Bacteriologische detectie van *Salmonella* in varkens faeces

De Europese Nationale Referentie Laboratoria (NRLs) voor *Salmonella* zijn erin geslaagd om *Salmonella* in hoge en lage concentraties aan te tonen in varkensmest. Dit hebben de 27 laboratoria laten zien in een studie, die in dit rapport wordt beschreven.

Zevenentwintig referentie-laboratoria deden in november 2006 mee aan het tiende ringonderzoek georganiseerd door het Communautair Referentie Laboratorium (CRL) voor *Salmonella*. Het doel van de studie was om na te gaan of de laboratoria *Salmonella* in varkensfaeces goed konden aantonen.

Ieder laboratorium kreeg een pakket toegestuurd met varkensfaeces en 35 gelatine capsules met melkpoeder van verschillende besmettingsniveaus *Salmonella*. De laboratoria moesten volgens voorschrift faeces en capsules samenvoegen en onderzoeken op de aanwezigheid van *Salmonella*.
Voor het onderzoek gebruikten de laboratoria één voorgeschreven methode met een selectieve kweekstap op Modified Semi-solid Rappaport Vassiliadis (MSRV).

De laboratoria vonden in 97 % van de monsters *Salmonella*. Drie laboratoria hadden problemen met het aantonen van lage concentraties van *Salmonella*. Het CRL heeft met deze laboratoria contact opgenomen voor een verklaring van de afwijkende resultaten en de mogelijkheid geboden om extra analyses uit te voeren. Tijdens een vervolgstudie in februari 2007 behaalden ook deze drie laboratoria het gewenste niveau.

Trefwoorden: *Salmonella*; CRL-*Salmonella;* NRL-*Salmonella;* ringonderzoek; varkensfaeces

rivm

Contents

List of abbreviations

BGA mod	Brilliant Green Agar modified
BGA	Brilliant Green Agar
BPLSA	Brilliant Green Phenol-Red Lactose Sucrose Agar
BPW	Buffered Peptone Water
BxLH	Brilliant Green, Xylose, Lysine, Sulphonamide
cfp	colony forming particles
CRL	Community Reference Laboratory
dPCA	double concentrated Plate Count Agar
dVRBG	double concentrated Violet Red Bile Glucose agar
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
RVS	Rappaport Vassiliadis Soya broth
SE	*Salmonella* Enteritidis
SMID2	*Salmonella* Detection and Identification-2
SOP	Standard Operating Procedure
SPan	*Salmonella* Panama
STM	*Salmonella* Typhimurium
TSI	Triple Sugar Iron agar
UA	Urea Agar
VRBG	Violet Red Bile Glucose agar
XLD(+)	Xylose Lysine Deoxycholate agar (+Novobiocin)
XLT4	Xylose Lysine Tergitol 4 agar

rivm

Summary

In November/December 2006 the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*) organised the tenth interlaboratory comparison study on bacteriological detection of *Salmonella* in animal faeces. For the first time pig faeces was used. Participants were 27 National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*) of the EU Member States and of Norway and candidate country Romania.

The main objective of this tenth study 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). The prescribed method was Annex D of ISO 6579, with selective enrichment on Modified Semi-solid Rappaport Vassiliadis (MSRV). Optionally a laboratory could also use other, own media or procedures for the detection of *Salmonella*.

Thirty five individually numbered capsules had to be tested by the participants for the presence or absence of *Salmonella.* Twenty five of the capsules had to be examined in combination with 10 gram of *Salmonella* negative pig faeces. These 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 faeces 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.

There was no significant difference between the different isolation media after selective enrichment on MSRV. The accuracy rates for the control samples and the artificially contaminated samples were higher than 97 % after selective enrichment on MSRV.

Twenty-four out of 27 laboratories achieved the level of good performance for the MSRV method which was defined during the CRL-*Salmonella* workshop of 2005 (Mooijman, 2005). Three NRLs reached this level later after a good performance in a follow-up study with extra material. One NRL did not return the test report.

1 Introduction

An important task of the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*), as laid down in Regulation EC No 882/2004, is the organisation of interlaboratory comparison studies. The history of the interlaboratory comparison studies as organised by CRL-*Salmonella* since 1995 is summarised in Annex 1.The main objective of this tenth bacteriological interlaboratory comparison study is that the examination of samples in the EU Member States is carried out uniformly and comparable results should be obtained by all National Reference Laboratories for *Salmonella* (NRL-*Salmonella*).
The prescribed method is 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, 2006; see Annex 6).
The set-up of this 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 animal faeces. 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 pig faeces spiked with 5 different reference materials (including blank capsules) had to be examined. The different reference materials consisted of two levels of *Salmonella* Typhimurium (STM10 and STM100) and two levels of *Salmonella* Enteritidis (SE100 and SE500).

2 Participants

Country	City	Institute
Austria	Graz	Institut für Medizinische Mikrobiologie -Veterinärmedizinische Untersuchungen AGES
Belgium	Brussels	Veterinary and Agrochemical Research Center (VAR), General Bacteriology
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	Kuopio	Food Safety Authority (Evira) Department of Animal Diseases and Food Safety/ Kuopio Research unit
France	Ploufragan	Agence Française de Sécurité Sanitaire des aliments, Zoopole - Laboratoire d'études et de recherches avicoles, porcines et piscicoles (AFSSA-LERAPP)
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	National Diagnostic Centre (NDC) Animal diseases diagnostic Laboratory
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 Microbiology

Country	City	Institute
Portugal	Lisbon	Laboratório Nacional de Investigação Veterinária (LNIV)
Romania	Bucharest	Institute for diagnosis and Animal Health, Bacteriology Department
Slovak Republic	Bratislava	State Veterinary and Food Institute Reference Laboratory for *Salmonella*
Slovenia	Ljubljana	National Veterinary Institute, Veterinary Faculty
Spain	Algete Madrid	Laboratorio de Sanidad y Produccion Animal de Algete Central de Veterinaria
Sweden	Uppsala	National Veterinary Institute (SVA), Department of Bacteriology
United Kingdom	Addlestone	Veterinary Laboratories Agency (VLA), Department of food and Environmental Safety
United Kingdom	Belfast	Agri-Food and Bioscience Institute (AFBI) Veterinary Sciences Divison Bacteriology

3 Materials and methods

3.1 Reference materials

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

- 5 colony forming particles (cfp) per capsule for *Salmonella* Panama (SPan5);
- 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 ^{o}C. If the test batches fulfilled the pre-set criteria for contamination level and homogeneity, the relevant mixed powders were completely filled into gelatin capsules and stored at -20 ^{o}C.
The pre-set criteria were:

- mean contamination levels should lie between target level minus 30 % and target level plus 50 % (e.g. between 70 and 150 cfp if the target level is 100 cfp);
- for the homogeneity within one batch of capsules the maximum demand for the variation between capsules should be $T2/(I-1) \leq 2$, where T2 is a measure for the variation between capsules of one batch (see formula in Annex 2) and I is the number of capsules.

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

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

3.2 Pig faeces samples

3.2.1 General

Pig faeces were sampled by the Animal Health Service (Deventer, the Netherlands) at a farm earlier tested negative for *Salmonella*. A larger batch of twenty-five kilogram of faeces from this farm arrived at CRL-*Salmonella* on 2 November 2006. The faeces were 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 Buffered Peptone Water (BPW). After pre-enrichment at (37 ± 1) ^{o}C for 16-18 h, selective enrichment was carried out on Modified Semi-solid

Rappaport Vassiliadis (MSRV). Next, the suspicious colonies were plated-out on Xylose Lysine Deoxycholate agar (XLD) and Brilliant Green Agar (BGA) and confirmed biochemically. The faeces were stored at 5 ºC until further use.

3.2.2 Total bacterial count in pig faeces

The total number of aerobic bacteria was investigated in the faeces. The procedure of ISO 4833 (Anonymous, 2003) was followed for this purpose. Portions of 20 gram faeces were homogenized into 180 ml peptone saline solution in a plastic bag. The content was mixed by using a pulsifier (60 sec). Next tenfold dilutions were prepared in peptone saline solution. Two times one ml of each dilution was brought into 2 empty Petri-dishes (diameter 9 cm). To each dish 25 ml of molten Plate Count Agar (PCA) was added. The plates were incubated at (30 ± 1) ^{o}C for (72 ± 3) h and the total number of aerobic bacteria was counted after incubation.

3.2.3 Number of Enterobacteriaceae in pig faeces

In addition to the total count of aerobic bacteria the Enterobacteriaceae count was determined. The procedure of ISO 21528-2 (Anonymous, 2004) was used for this purpose. Portions of 20 gram faeces were homogenized into 180 ml peptone saline solution in a plastic bag. The content was mixed by using a pulsifier (60 sec). Next tenfold dilutions were prepared in peptone saline solution. Two times one ml of each dilution was brought into 2 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) ^{o}C for (24 ± 2) h and the number of typical violet-red colonies was counted after incubation. Five typical colonies were tested to ferment glucose and to give a negative oxidase reaction. After this confirmation the number of Enterobacteriacea was calculated.

3.3 Design of the interlaboratory comparison study

3.3.1 Samples: capsules and pig faeces

On 13-11-2006 (two weeks before the study) the reference materials (35 individually numbered capsules) and 300 grams of *Salmonella* negative pig faeces 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 ^{o}C and the faeces had to be stored at +5 ^{o}C until the start of the study. Details about mailing and handling of the samples and reporting of test results can be found in the Protocol (Annex 4) and Standard Operation Procedure (Annex 5). The test report which was used during the study can be found at the CRL-*Salmonella* website:
http://www.rivm.nl/crlsalmonella/prof_testing/detection_stud/ or can be obtained through the corresponding author of this report.

Ten control capsules had to be tested without faeces (numbered C1-C10). Twenty-five capsules (numbered 1 – 25) were each tested in combination with 10 grams of pig faeces (negative for *Salmonella*). The types and the number of capsules and faeces 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 faeces added	Test samples (n=25) with 10 g *Salmonella* negative pig faeces
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 pig faeces.

3.4 Methods

The prescribed method of this interlaboratory comparison study was draft Annex D of ISO 6579 (Anonymous, 2006; also see 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:
- Modified semi-solid Rappaport Vassiliadis medium (MSRV)
- Own selective enrichment medium (not compulsory)

Plating-out 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)

3.5 Statistical analysis of the data

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

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

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

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

3.6 Good performance

Proposal for definition of 'good performance'

During the tenth CRL-*Salmonella* workshop in April 2005 (Mooijman, 2005) a proposal was made to define 'good performance' in interlaboratory comparison studies on detection of *Salmonella*.

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 'faeces': 80 % negative (4 negative out of 5 capsules) *.
- STM100 and SE500 with 'faeces': 80 % positive (4 positive out of 5 capsules).
- STM10 and SE100 with 'faeces': 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	SPan5	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 Pig faeces samples

The pig faeces were tested negative for *Salmonella* and stored at 5 °C. At Monday 13 November 2006 the pig faeces were mailed to the NRLs. After receipt the NRLs had to store the pig faeces at 5 °C.

The number of aerobic bacteria and Enterobacteriaceae was tested twice; firstly 4 days after the pig faeces arrived at the CRL (t = 4 days) and secondly at the planned date of the interlaboratory comparison study (t = 25 days).The results are shown in Table 3.

Most of the laboratories (twenty-three) performed the study on the planned date (27-11-06, t=25 days), two laboratories (labcodes 4 and 20) performed the study one week earlier (20-11-06) and two laboratories one week later (4-12-06). One laboratory did not perform the study at all (labcode 1).

Table 3 Number of aerobic bacteria and Enterobacteriaceae per gram of pig faeces negative for *Salmonella*

Date	Aerobic bacteria cfp/g	Enterobacteriaceae cfp/g
6 nov t=4	$1.3*10^7$	$2.6*10^5$
27 nov t=25	$1.1*10^8$	$5.8*10^5$

4.3 Technical data interlaboratory comparison study

4.3.1 Accreditation/certification

Twenty laboratories mentioned to be accredited for their quality system according to ISO/IEC 17025 (labcodes 2, 3, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 18, 19, 20, 22, 23, 25, 28). Four laboratories (labcodes 17, 21, 24 and 26) are planning to become accredited or certified in the near future and for one laboratory (labcode 27) the accreditation is already in process. At one laboratory (labcode 4) ISO/IEC 17025 was introduced at the institute but the NRL did not mention a plan or process for accreditation in its laboratory. One laboratory (labcode 9) mentioned that they were not accredited or certified to any system and mentioned no planning to do so in the near future.

4.3.2 Transport of samples

An overview of the transport times and the temperatures during transport of the parcels is given in Table 4. The temperature recorders were returned immediately after receipt to CRL-*Salmonella* by all NRLs with the exception of laboratory 1 and 16, who did not return the temperature recorder. The majority of the laboratories received the materials within 1 day. However, the parcel of laboratory 28 was hold by the customs for 9 days. The total transport time of this parcel was 238 hours. When this latter parcel is not taken into account the average transport time was 26 hours. For the majority of the laboratories the temperature of the content of the parcel was below 5 ^oC with a maximum of 10 ^oC. Although the parcel from laboratory 28 was delayed at the border for 9 days, for most of the time it was stored below 10^oC. In seven cases (labcodes 7, 9, 13, 15, 22, 26 and 27) 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. In general the delay was only a few hours and the parcels were stored at a temperature below 7 ^oC with the exception of laboratory 26 where the parcel was stored at 20 ^oC for 24 hours.

4.3.3 Media

Each laboratory was asked to test the samples with the prescribed (draft Annex D of ISO 6579) method. All laboratories used the selective enrichment medium MSRV with the plating out medium XLD and a second plating out medium of own choice. The media used per laboratory are shown in Table 5. Eight NRLs (labcode 6, 7, 9, 10, 14, 24, 27 and 28) performed besides the prescribed selective enrichment in MSRV also another selective enrichment method. Three NRLs (labcode 6, 9 and 12) performed a third plating out medium. Details on the media which are not described in ISO 6579 are given in Annex 3. In Tables 6-9 information is given on the composition of the media which were

prescribed and on incubation temperatures and times. In these tables only the laboratories are indicated who reported deviations.

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

		Time (h) at			
Lab code	Transport* total in hours	0 °C - 5 °C	5 °C - 10 °C	10 °C - 13 °C	Additional Storage+
1	38	(1)			
2	39	39			
3	17	10	7		
4	20	13	7		
5	18	18			
6	43	22	21		
7	38		38		3h at 5 °C
8	19	13	6		
9	20	20			1h at 1 °C
10	19	3	16		
11	20	20			
12	15	8	7		
13	18	18			3h at 3 °C
14	16	2	14		
15	38	32	6		2h at 2 °C
16	NA	(1)			
17	39	23	16		
18	20	20			
19	18	7	11		
20	19	19			
21	17	17			
22	16	9	7		2h at 7 °C
23	18	7	11		
24	41	2	39		
25	41	41			
26	40	12	28		24h 20 °C
27	16	16			3h at 3 °C
28	238 (2)	127	101	10	
Average	26				

* = transport time according to the courier NA = not applicable

+ = storage time of the samples at the institute before arriving at the NRL.

(1): Temperature recorder was not returned by the laboratory

(2): Delay at the border 10 days (not in the average transport time)

Table 5 Media combinations used per laboratory

Lab code	Selective enrichment	Plating-out media
2	MSRV	XLD Rambach
3	MSRV	XLD BGA mod
4	MSRV	XLD BXLH
5	MSRV	XLD+ BGA mod
6	MSRV RVS	XLD BGA mod Mac Conkey
7	MSRV MKTTn	XLD Rambach
8	MSRV	XLD BGA
9	MSRV RVS	XLD MLCB BGA mod
10	MSRV MKTTn	XLD BGA
11	MSRV	XLD Rambach
12	MSRV	XLD BGA Rapid *Salmonella* Agar
13	MSRV	XLD SM ID2
14	MSRV RVS	XLD BGA

Lab code.	Selective enrichment	Plating-out media
15	MSRV	XLD Onöz
16	MSRV	XLD BGA
17	MSRV	XLD SMID2
18	MSRV	XLD BPLSA
19	MSRV RVS*	XLD BGA mod
20	MSRV	XLD BGA mod
21	MSRV	XLD XLT4
22	MSRV	XLD Rambach
23	MSRV	XLD Rambach
24	MSRV MSRV** MKTTn	XLD SMID2
25	MSRV	XLD BGA mod
26	MSRV	XLD Rambach
27	MSRV RVS	XLD BGA mod
28	MSRV RVS	XLD BGA

Explanations of the abbreviations are given in the 'List of abbreviations' (page 9)
Descriptions of the media not described in ISO 6579 are given in Annex 3
*RVS is only used for the Elisa
** MSRV with 20 mg/L novobiocin

Table 6 Incubation time and temperature of BPW

	Prewarming BPW		Dissolving capsules in BPW		Pre-enrichment in BPW	
Labcode	**Time (h)**	**Incubation temperature in °C (min-max)**	**Time (min)**	**Incubation temperature in °C (min-max)**	**Time (h:min)**	**Incubation temperature in °C (min-max)**
SOP & ISO 6579	**overnight**	**36-38**	**45**	**36-38**	**16 – 20**	**36-38**
6	22	37	50	37	20	37
9	20	37	45	36.75	21:15	37.1-37.75
12	> 24(4 days)	37.3	45	36.9-37.3	22:10	36.9-37.3
19	25	36.4	25	36.5-36.6	22	36.4-36.8
25	18	36.2-36.8	25	36.1-36.2	18	37.8-37.9
28	19	36.3-37	45	36.5-36.9	22:10	36.8

Deviating times and temperatures are indicated as grey cells - = no info

Table 7 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**
5	10	5	3.5*	1.5	7.3
9	10	5	3.7	1.5	-
12	10	5	3.5*	1.5	-
14	10	5	3.5*	1.5	7.35
20	10	5	3.5*	1.5	7.3
21	10	5	3.5*	1.5	-
24	10	5	9	1.5	-
25	10	5	3.5*	1.5	7.28
27	10	5	3.5*	1.5	7.23 - 7.28

grey cells are deviating from ISO 6579 - = no info

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

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

Labcode	Enzymatic digest of casein (Tryptose)	Casein hydro-lysate	NaCl	Potass. Digydrogen phosphate	$MgCl_2$ anhy-drous	Malachite green oxalate	Agar	Novo biocin	pH
Annex D ISO 6579	**4.6**	**4.6**	**7.3**	**1.5**	**10.9**	**0.04**	**2.7**	**0.01 (10 mg/L)**	**5.1-5.4**
4	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.01	5.6
5	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.01	5.5
8	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.2
9	4.6*	4.6	7.3	1.5	10.9	0.04	2.5	0.02	-
10	-	-	-	-	-	-	-	-	5.2
12	9.2**		7.3	1.5	10.9	0.037	2.7	0.02	-
13	4.6	4.6	7.3	1.5	10.9	0.037	2.7	-	5.4-5.5
17	4.6	4.6	7.3	1.5	10.9	0.037	2.7	0.01	5.6
19	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.2
23	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.4
26	4.6	4.6	7.3	1.5	10.9	0.04	2.7	5	5.49
27	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.67
28	4.6	4.6	7.3	1.5	10.9	0.04	2.7	-	5.4-5.46

*4.6 g = 2.3 g Tryptone + 2.3 g meat peptone **= peptone

grey cells are deviating from Draft Annex D of ISO 6579 - = no info

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

Lab Code	Xylose	L-lysine	Lactose	Sucrose (Saccharose)	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**
5	-	-	-	-	-	-	-	-	-	-	-	+ ?	7.2
8	3.75	5.0	7.5	7.5	5.0	3.0	0.08	13	1.0	6.8	0.8	-	-
9	3.75	5.0	7.5	7.5	5.0	3.0	0.08	13	1.0	6.8	0.8	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-	7.4
12	3.5	5	7.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	-	-
17	3.5	5	7.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	-	-
19	3.75	5.0	7.5	7.5	5.0	2.0	0.072	15	1.0	4.34	0.8	-	7.2
21	3.75	5.0	7.5	7.5	5.0	3.0	0.08	12.5	1.0	6.8	0.8	-	-
22	3.75	5.0	7.5	7.5	5.0	3.0	0.08	12.5	2.5	6.8	0.8	-	7.5
24	3.5	5.0	7.5	7.5+7.5*	5.0	3.0	0.08	13.5	2.5	6.8	0.8	-	-
26	3.75	5.0	7.5	7.5	5.0	3.0	0.08	14.5	1.0	6.8	0.8	-	7.06
27	3.75	5.0	7.5	7.5	5.0	3.0	0.08	13	1.0	6.8	0.8	-	5.47

* 7.5 g Sacharose + 7.5 g Sucrose

grey cells are deviating from ISO 6579 - = no info

A second plating-out medium for choice was obligatory. Ten laboratories used BGA mod (ISO 6579, 1993) as a second plating-out medium and laboratory 18 used BPLSA (Merck) this is very close to BGA.

The use of an extra plating agar between the 'isolation' and the 'confirmation' steps was optional. A total of 13 laboratories performed this extra culture step on many different media (e.g. Nutrient agar (ISO 6579, 2002), Colombia, Mc Conkey and Bromthymol blue lactose sucrose agar).

Sixteen 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 10. Laboratory 13 did not mention any confirmation test. Five laboratories (labcode 9, 18, 22, 27 and 28) showed a rather limited confirmation. Laboratory 9 and 27 used only one biochemical test and laboratory 18 only an O antigen test. Three laboratories (labcodes 12, 21 and 24) used a biochemical identification kit and six laboratories (labcodes 6, 14, 18, 20, 22 and 24) performed serotyping.

Table 10 Biochemical and/ or serological confirmation of *Salmonella*

Lab code	Biochemical			Serological			Other
ISO 6579	**UA**	**TSI**	**LDC**	**O antigens**	**Vi antigens**	**H antigens**	
2	+	+	+	-	-	-	Drigalski agar
4	+	-	-	-	-	-	Kligler/indol/Mannitol/nitrate/ONPG/FDA
6	+	+	+	+	-	-	-
9	-	+	-	-	-	-	-
12	-	-	+	-	-	-	Hy Enterotest / Rapid *Salmonella* agar
13	-	-	-	-	-	-	-
14	+	+	+	+	-	-	-
18	-	-	-	+	-	-	-
19	+	+	+	-	-	-	ELISA Transia Plate *Salmonella* Gold
20	-	-	-	+	+	+	
21	-	-	-	-	-	-	API20E
22	-	-	-				Poly O poly H O4, O9
24	-	-	-				Sero agglutination and Biochemical identification strips
27	+	-	-	-	-	-	-
28	+	+	-	-	-	-	-

Grey cell : confirmation is deviating from ISO 6579
-= no info

4.4 Control samples

4.4.1 General

None of the laboratories isolated *Salmonella* from the procedure control (C11: no capsule/no faeces) and from the faeces control (C12: no capsule/negative faeces). Twenty-one laboratories scored correct results for all the control capsules containing *Salmonella*. In Table 11 the results are summarized of all control samples (capsules without faeces) per laboratory and for selective enrichment on MSRV and isolated on XLD.

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

Salmonella Panama 5 capsules (n=2) without addition of faeces
Twenty-five laboratories isolated *Salmonella* from both capsules. One Laboratory (labcode 15) could not detect *Salmonella* Panama (SPan5) in one control capsule. One laboratory (labcode 19) could not detect *Salmonella* Panama in both control capsules. These capsules contained SPan at a low level (circa 5 cfp/capsule). Due to the variation between capsules one out of two capsules containing SPan5 may occasionally be negative. However it is not very likely to find both SPan5 capsules negative.

Salmonella Typhimurium 10 capsules (n=3) without addition of faeces
All participating laboratories tested all the three capsules containing STM10 positive.

Salmonella Enteritidis 100 capsules (n=2) without addition of faeces
Twenty-six laboratories isolated *Salmonella* Enteritidis at a mean level of circa 100 cfp/ capsule from both capsules. One laboratory (labcode 10) could not detect *Salmonella* in one control capsule when using selective enrichment media MRVS and MKTTn.

Salmonella Enteritidis 500 capsules (n=1) without addition of faeces
All participating laboratories tested the capsule containing SE500 positive.

The results are compared with the proposed definition of ‘good performance’ (see Materials and methods). The score for the control samples was too low for two laboratories when using the combination MSRV/XLD (labcode 10 and 19).

Table 11 Number of correct results of the control samples (capsule without faeces) per laboratory

Lab code	MSRV / XLD				
	Blanc n=2	SE100 n=2	SE500 n=1	SPan5 n=2	STM10 n=3
Good Performance	**2**	**2**	**1**	**≥ 1**	**3**
2	0	2	1	2	3
3	0	2	1	2	3
4	0	2	1	2	3
5	0	2	1	2	3
6	0	2	1	2	3
7	0	2	1	2	3
8	0	2	1	2	3
9	0	2	1	2	3
10	0	**1**	1	2	3
11	0	2	1	2	3
12	0	2	1	2	3
13	0	2	1	2	3
14	0	2	1	2	3
15	0	2	1	**1**	3
16	0	2	1	2	3
17	0	2	1	2	3
18	0	2	1	2	3
19	0	2	1	**0**	3
20	0	2	1	2	3
22	0	2	1	2	3
23	0	2	1	2	3
24	0	2	1	2	3
25	0	2	1	2	3
26	0	2	1	2	3
27	0	2	1	2	3
28	0	2	1	2	3

Bold numbers : deviating results

Grey cells : results are below good performance

4.4.2 Specificity, sensitivity and accuracy rates of the control samples

In Table 12 the specificity, sensitivity and accuracy rates for the control capsules without the addition of faeces are shown. The rates are calculated for medium combinations MSRV/XLD and MSRV/second plating out medium. Good results were found with the control samples. The rates were, for all tested media combinations > 94 %.

Table 12 Specificity, sensitivity and accuracy rates for all participating laboratories (n=27) with all control capsules, for the selective enrichment on MSRV and different plating out media

Control capsules		MSRV/XLD	MSRV/2nd medium
Blank (n=2)	No of samples No of negative samples **Specificity in %**	54 54 **100.00**	54 54 **100.00**
Span5 (n=2)	No of samples No of positive samples **Sensitivity in %**	54 51 **94.44**	54 51 **94.44**
STM10 (n=3)	No of samples No of positive samples **Sensitivity in %**	81 81 **100.00**	81 81 **100.00**
SE100 (n=2)	No of samples No of positive samples **Sensitivity in %**	54 53 **98.15**	54 53 **98.15**
SE500 (n=1)	No of samples No of positive samples **Sensitivity in %**	27 27 **100.00**	27 27 **100.00**
All capsules with *Salmonella*	No of samples No of positive samples **Sensitivity in %**	216 212 **98.15**	216 212 **98.15**
All capsules	No of samples No of correct samples **Accuracy in %**	270 266 **98.52**	270 266 **98.52**

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

4.5.1 Results per type of capsule and per laboratory

General

In Table 13 the results are given of the *Salmonella* negative faeces samples artificially contaminated with capsules with selective enrichment on MSRV and isolation medium XLD. Twenty-one laboratories found all the capsules with *Salmonella* positive for the combination MSRV/XLD. In general the results of the samples containing *S.* Typhimurium or *S*. Enteritidis were comparable.

Blank capsules with negative pig faeces (n=5)
All participating laboratories correctly did not isolate *Salmonella* from these blank capsules with the addition of negative faeces.

S. Typhimurium 10 capsules (STM10) with negative pig faeces (n=5)
Twenty-three laboratories isolated *Salmonella* from the five capsules containing *Salmonella* Typhimurium at a level of circa 10 cfp/capsule in combination with pig faeces. Laboratory 17 and 19 found one capsules negative on MSRV isolated on XLD and also on their second isolation medium (respectively SMID2 and BGA). Laboratory 4 found also one capsules negative with the combination MSRV/XLD but found this capsule positive with on their second isolation medium (BXLH). Laboratory 9 found two capsules negative with selective enrichment on MSRV and in RVS in combination with the isolation media they used (XLD, MLCB and BGA).

S. Typhimurium 100 (STM100) with negative pig faeces (n=5)
All laboratories isolated *Salmonella* from all five capsules containing *Salmonella* Typhimurium at a level of circa 100 cfp/capsule in combination with pig faeces when using MSRV.

S. Enteritidis 100 (SE100) with negative pig faeces (n=5)
Twenty-three laboratories isolated *Salmonella* from all the five capsules containing *Salmonella* Enteritidis at a level of circa 100 cfp/capsule in combination with pig faeces, when using MSRV. Due to a mistake our side laboratory 22 did not receive one capsule (SE100), resulting in n=4 for the artificially contaminated faeces samples of this laboratory. Laboratory 9 found all five capsules negative with selective enrichment on MSRV and in RVS in combination with the isolation media XLD and BGA. Laboratory 10 and 19 found 2 respectively 3 capsules negative with MSRV in combination with both isolation media XLD and BGA. Laboratory 24 found one capsule negative with the MSRV method in combination with both isolation media (XLD and SMID2) but this laboratory found the same capsule positive with the MKTTn and MSRV (20 mg/L novobiocin) selective enrichment media.

S. Enteritidis 500 (SE500) with negative pig faeces (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 pig faeces, when using MSRV. Laboratory 9 found two capsules negative with the MRVS method and three capsule negative with the MKTTn method in contamination with XLD and BGA.

In Figure 1 the number of positive isolations per capsule (n=5) containing *Salmonella* with the addition of 10 g *Salmonella* negative faeces per laboratory is given after pre-enrichment in BPW and selective enrichment 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). The level of good performance is in the figures indicated with a black line. According to this definition the score for the artificially contaminated samples was too low for two laboratories when using MSRV method (lab code 9 and 19).

Table 13 Number of correct results of the artificially contaminated faeces (with capsule) per laboratory

Lab code	MSRV / XLD				
	Blanc N=5	SE100 n=5	SE500 n=5	STM10 n=5	STM100 N=5
Good performance	**≥ 4**	**> 2.5**	**≥ 4**	**> 2.5**	**≥ 4**
2	5	5	5	5	5
3	5	5	5	5	5
4	5	5	5	**4**	5
5	5	5	5	5	5
6	5	5	5	5	5
7	5	5	5	5	5
8	5	5	5	5	5
9	5	**0**	**3**	**3**	5
10	5	**3**	5	5	5
11	5	5	5	5	5
12	5	5	5	5	5
13	5	5	5	5	5
14	5	5	5	5	5
15	5	5	5	5	5
16	5	5	5	5	5
17	5	5	5	**4**	5
18	5	5	5	5	5
19	5	**2**	5	**4**	5
20	5	5	5	5	5
22	5	4*	5	5	5
23	5	5	5	5	5
24	5	**4**	5	5	5
25	5	5	5	5	5
26	5	5	5	5	5
27	5	5	5	5	5
28	5	5	5	5	5

Bold numbers : deviating results

Grey cells: results are below good performance

*= laboratory 22 did not receive one capsule (SE100) n=4

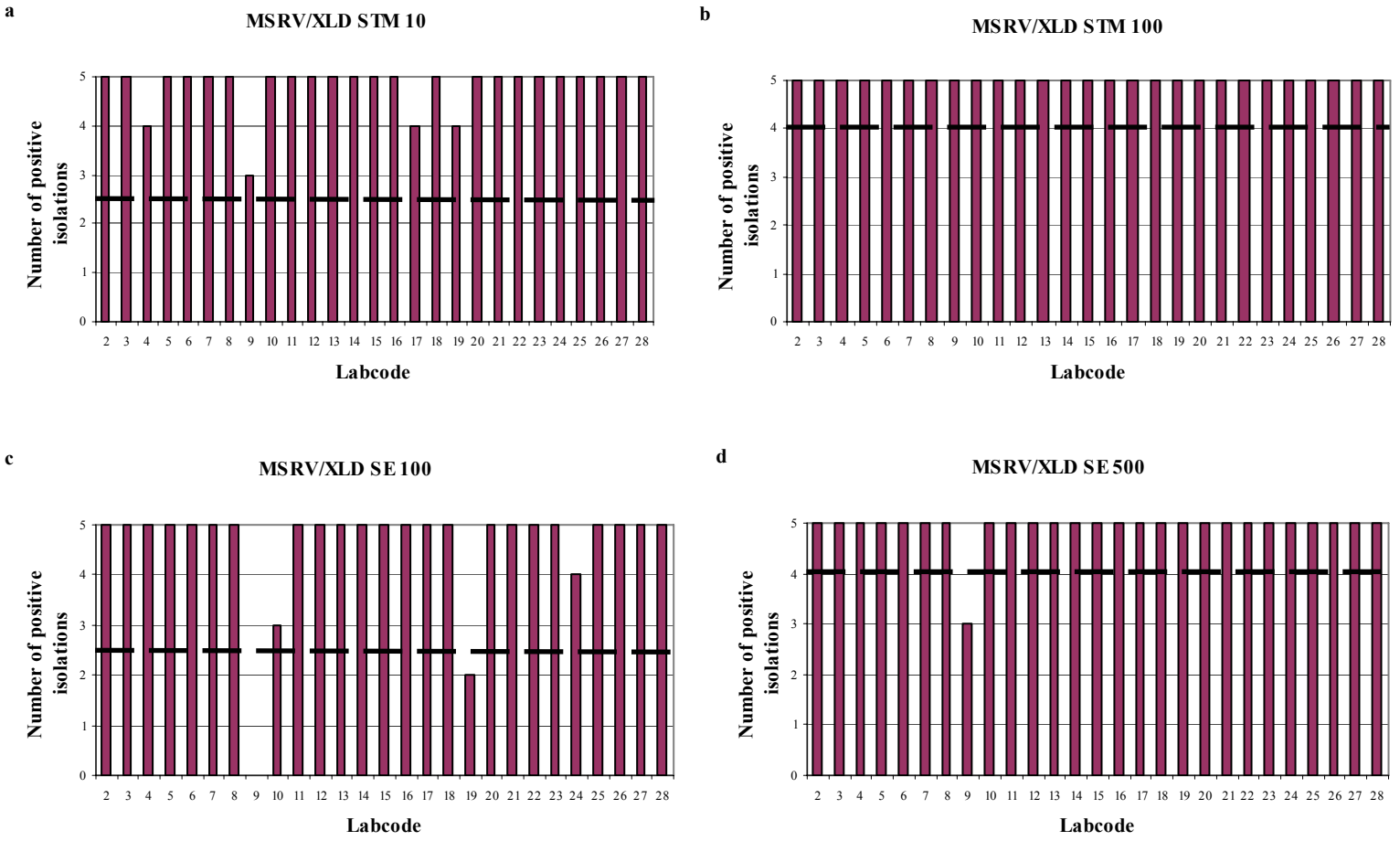

Figure 1 Results artificially contaminated pig faeces analysed with MSRV/XLD for the different capsules - - - = border of good performance

4.5.2 Results for the different selective enrichment and isolation media

Eight laboratories (6, 7, 9, 10, 14, 24, 27 and 28) used beside the prescribed method (MSRV) optionally a second selective enrichment medium RVS or MKTTn. With the exception of laboratory 24 all laboratories found the same or less positive results when using RVS or MKTTn compared to the results found with MSRV.
Beside XLD different isolation media were used by the laboratories: BGA (14 labs), Rambach (5 labs), SMID2 (3 labs), MLCB (1 lab), BPLSA (1 lab), Onöz (1 lab), XLT4 (1 lab) and BXLH (1 lab).The differences in the number of positive isolations after 24 and 48 hours of incubation of the selective enrichment on MSRV followed by plating out on XLD or another plating out medium are given in Table 14. The score on XLD and the second isolation medium is almost the same for all laboratories with the exception of laboratory 4 which scored more with the combination MSRV/BXLH. Half of the laboratories showed only one or two more positive results (5 %) after 48 hours of incubation of the medium combinations MSRV/XLD and MSRV/2nd medium. Only laboratory 8 found seven samples more positive after 48 hours of incubation on MSRV.

Table 14 Mean percentages of positive results of all participating laboratories after selective enrichment on MSRV, incubated for 24 and 48 hours and followed by incubation on different plating out media, when analyzing the artificially contaminated pig faeces samples.

Plating out medium	**Selective enrichment medium MSRV**
	24 / 48 h
XLD	92 / 97 %
Other (most often BGA)	91 / 96 %

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

The specificity, sensitivity and accuracy rates for all types of capsules added to the pig faeces are shown in Table 15. The results are given for the different medium combinations: BPW followed by selective enrichment on MSRV and isolation on different selective plating agars. The specificity rate (of the blank capsules) was 100 %. For all capsules containing *Salmonella* the sensitivity rate was 97%. The sensitivity rates of the low level contaminated samples were 4-6 percentages lower than for the high level contaminated samples. The sensitivity rates of the samples containing *S*. Enteritidis were 2-4 percentages lower in comparison to the samples containing *S.* Typhimurium.

Table 15 Specificity, sensitivity and accuracy rates for all participating laboratories (n=27) of the artificially contaminated faeces samples (each capsule added to 10 g pig faeces) for the selective enrichment on MSRV and different plating out media

Capsules with pig faeces		MSRV/XLD	MSRV/2nd medium
Blank (n=5)	No of samples	135	135
	No of negative samples	135	135
	Specificity in %	**100.00**	**100.00**
STM10 (n=5)	No of samples	135	135
	No of positive samples	130	131
	Sensitivity in %	**96.30**	**97.04**
STM100 (n=5)	No of samples	135	135
	No of positive samples	135	135
	Sensitivity in %	**100.00**	**100.00**
SE100 (n=5)	No of samples	134	134
	No of positive samples	123	123
	Sensitivity in %	**91.79**	**91.79**
SE500 (n=5)	No of samples	135	135
	No of positive samples	133	133
	Sensitivity in %	**98.52**	**98.52**
All capsules with *Salmonella*	No of samples	539	539
	No of positive samples	521	522
	Sensitivity in %	**96.66**	**96.85**
All capsules	No of samples	674	674
	No of correct samples	656	657
	Accuracy in %	**97.33**	**97.48**

4.6 PCR

Three laboratories (labcodes 6, 10, and 15) applied a PCR method as additional detection technique. These laboratories tested the samples after incubation in BPW. In Table 16 the details are summarized.

Table 16 Details on the Polymerase Chain Reaction method, used as own Method during the interlaboratory comparison study by three laboratories

Lab code	Volume of BPW (µl)	Volume of DNA sample (µl)	Volume of DNA / PCR mix (µl)
6	1000	150	5/50
10	1000	200	5/20.5
15	1000	150	2/7

The 3 laboratories used a commercially available PCR technique. Laboratory 6 used iQ-Check TM *Salmonella* and laboratory 6 Artus TM *Salmonella* RG. Both are real time PCR kits. Laboratory 15 used Inva PCR which is usually used for testing bacterial cultures not from pre-enrichment broths. The PCR techniques of laboratory 15 and 20 are validated for food and the PCR technique of laboratory 6 for faeces, organs and eggs. Laboratory 6 and 10 tested about 50 samples in 2005 using this PCR technique.
The PCR results and the bacteriological culture results found on MSRV are shown in Table 17.
Laboratory 6 scored all samples correct with the PCR method and with the bacteriological culture method. Laboratory 10 scored the same results with the PCR as with the bacteriological culture method. They missed one control sample (SE100) and two tests samples (SE100) with pig faeces with both techniques. Laboratory 15 found one control sample (SPan5) negative with the PCR technique as well as with the bacteriological culture method. However, for the artificially contaminated samples, laboratory 15 found with the PCR technique eleven samples negative (five SE100, four SE500 and one STM10) while with the culture method, from the same BPW, they found correct results.

Table 17 PCR results compared with bacteriological culture results of control capsules and of artificially contaminated pig faeces of laboratories 6, 10, and 15

Capsules	lab 06		lab 10		lab 15	
Cfp/caps.	MSRV	PCR	MSRV	PCR	MSRV	PCR
Controls without faeces (n=10)						
SPan5 (n=2)	2	2	2	2	**1**	**1**
SE100 (n=2)	2	2	**1**	**1**	2	2
SE500 (n=1)	1	1	1	1	1	1
STM10 (n=3)	3	3	3	3	3	3
Blank (n=2)	0	0	0	0	0	0
BPW (n=1)	0	0	0	0	0	0
Pig faeces (n=1)	0	0	0	0	0	0
Test samples with pig faeces (n=25)						
SE100 (n=5)	5	5	**3**	**3**	5	**0**
SE500 (n=5)	5	5	5	5	5	**1**
STM10 (n=5)	5	5	5	5	5	**3**
STM100 (n=5)	5	5	5	5	5	5
Blank (n=5)	0	0	0	0	0	0

Grey cells: unexpected results
Bold numbers: deviating results

4.7 Performance of the NRLs

4.7.1 General

Twenty-four NRLs performed very well and achieved the level of 'good performance' for the tested samples. One NRL did not return the test report (labcode 1). Three laboratories (9, 10 and 19) did not fulfill the requirements of good performance.
Laboratory 19 could not detect *Salmonella* in both SPan5 capsules of the control samples and in three of the five SE100 capsules with negative pig faeces.
Laboratory 10 could not detect *Salmonella* in one SE100 capsule of the control samples (without matrix).
Laboratory 9 could not detect *Salmonella* in all the five SE100 capsules and in two of the five SE500 capsules both with negative pig faeces.

The three laboratories were contacted by the CRL-*Salmonella* in January 2007 to ask for any explanation for the deviating results and the possibility was offered to perform some extra analyses.

4.7.2 Follow-up study

The set-up of this follow-up study was the same as the study in November and the same batch of capsules and pig faeces was used (see Materials and methods). In this follow-up study only the low level capsules and a lower number of capsules were tested. An overview is given in Table 18. The number of aerobic bacteria ($1.8*10^8$ cfu/g) and Enterobacteriaceae ($5.6*10^5$ cfu/g) was tested one week after the planned date of the follow-up study. The numbers were comparable with the counting on 27 November 2006.

Table 18 Overview of the types and the number of capsules tested per laboratory in the follow-up of the interlaboratory comparison study

Capsules	Control capsules (n=5) No faeces added	Test samples (n=12) with 10 g *Salmonella* negative pig faeces
S. Panama 5 (SPan5)	2	-
S. Enteritidis 100 (SE100)	1	5
S. Enteritidis 500 (SE500)	-	-
S. Typhimurium 10 (STM10)	1	5
S. Typhimurium 100 (STM100)	-	-
Blank	1	2

On Monday 19 February 2007 one parcel with only one bio bottle was send to the NRLs containing:
5 control capsules (C1 - C5), 12 capsules (1 – l 2), 150 g pig faeces and 1 temperature recorder.

The performance of this follow up study was on 26 February 2007. Three laboratories (labcode 9, 10 and 19) participated in this follow-up study. The laboratories had to follow the same SOP and Protocol as in the study of November 2006 (see Annexes 4 and 5). The test report was different from the November study. For the media only the differences with the November study had to be indicated. The test report from this follow-up study can be found in Annex 7.
For the media compositions, incubation times and temperatures no differences were observed in comparison with the study of November 2006 with two exceptions. Laboratory 9 changed the concentration of novobiocin in MSRV (20 mg/L in the November study) to the prescribed 10 mg/L. In contrast with the study in November laboratory 19 followed now the pre-scribed incubation time for dissolving the capsules for 45 minutes and kept the incubation time of the BPW to a maximum of 20 hours (see SOP Annex 5).
The three NRLs performed very well and achieved the level of 'good performance' (see Materials and methods) for the tested samples in this follow-up study. Laboratory 10 and 19 could not detect *Salmonella* in only one of the capsules with negative pig faeces: respectively in STM10 and SE100. This was still within the limits of good performance. Laboratory 9 scored all samples correct with the MSRV method.

5 Discussion

Transport of the samples
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 and the temperature of the contents of the packages did not exceed 10 ºC with exception of one NRL. The parcel of this latter NRL was delayed for 9 days but the content of the parcel was for approximately 5 days below 5 ^{o}C and for the rest of the delay time the temperature did not exceed 13 ^{o}C. The results did not seem to have been affected by the transport temperatures.

Performance of the laboratories
The prescribed method (draft Annex D of ISO 6579: MSRV) was used by all the laboratories. One laboratory did not return the test report. The results found with the MSRV method were good. Twenty-four out of 27 laboratories scored a 'good performance'. Three laboratories scored less performance with this study on pig faeces. However, those laboratories scored 'a good performance' with the MSRV method in the study two months earlier (September 2006) with the same batch of capsules but with a different matrix (minced meat). They also showed good performance in the follow-up study.

As the performance of the laboratories was good, the specificity, sensitivity and accuracy rates were high (>92 %) for all samples, tested with the selective enrichment medium MSRV.

Media
The poor performance of laboratory 19 was most probably caused by poor dissolution of the capsules in BPW. This laboratory dissolved the capsules only for 25 minutes while 45 minutes was prescribed. A shorter reconstitution time of the capsules in BPW may result in not completely dissolved gelatine capsules while complete dissolution is essential for the detection of *Salmonella* in the capsules. In this study especially the growth of *Salmonella* capsules with a low contamination level may have been effected as these samples are easier overgrown by disturbing background flora. Laboratory 19 followed the prescribed reconstruction time in the follow-up study and scored a 'good performance'.

According to Annex D of ISO 6579 (see Annex 6; Anonymous, 2006) the concentration of novobiocin in MSRV should be 10 mg/L and the pH between 5.1-5.4. Seven laboratories reported the use of a higher concentration of novobiocin and three laboratories did not mention the use of novobiocin. Nine laboratories reported a higher pH or did not mention the pH. Laboratories 4, 9, 10, 17 and 19 reported a higher pH or a higher concentration of novobiocin or did not mention the composition in MSRV. Those laboratories scored less positive results in this study. Laboratory 9 used a concentration of 20 mg/L novobiocin in MSRV for the study in November and changed this to 10 mg/L in the follow-up study in February. With the lower concentration of novobiocin (10 mg/L) they scored more positive results. A higher concentration of novobiocin in the MSRV can negatively influence the motility of *Salmonella* and may result in less positive results.The higher pH and/or a higher concentration of novobiocin in MSRV could have been an explanation for the lower number of positive results found in the study of November for laboratory 4, 9, 10, 17 and 19. However, no effect on the positive isolations was found for the other laboratories. Other deviations in media compositions or incubation temperatures were reported but no clear effects were found on the results.

PCR

Only three laboratories used a PCR technique additionally to the prescribed methods. For two NRLs the results found with the PCR methods were comparable to the results found with the bacteriological detection method. Laboratory 15 found with the PCR more negative results while with the culture method, inoculated from the same BPW, they found correct results. No explanation was found.

Future studies

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. Besides these low level samples, also high level samples (approximately 5-10 times above the detection limit) should be included, so that laboratories can be sure that *Salmonella* is present. 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*.

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 of 2005. One NRL did not return the test report. Three NRLs reached the level of 'good performance 'after a follow-up study.
- For a good dissolution of the capsules it is important to use the right incubation time of the BPW. Deviating times may influence the growth of *Salmonella* and eventually the number of positive isolations.
- The accuracy, specificity and sensitivity rates for the control samples (without faeces) from MSRV were higher than 94 %.
- The specificity rate of the control samples with blank capsules and the pig faeces samples artificially 'contaminated' with blank capsules was 100 %
- The sensitivity rates for all artificially contaminated pig faeces samples were higher than 92 %.
- The sensitivity rates of the low contaminated samples and the samples containing *S*.Enteritidis were lower in comparison to the highly contaminated samples and the samples containing *S*. Typhimurium.
- The accuracy rate for the artificially contaminated pig faeces samples was 97 %.
- XLD showed (1 %) slightly more positive results than other isolation media.
- 48 hours of incubation of the selective enrichment medium (MSRV) gave 5 % more positive results than 24 hours of incubation independent on the isolation media.
- The temperature recorders in the parcels gave important information about the temperature during transport especially when a parcel was delayed for a few days.

rivm

References

Anonymous, ISO 6579, 1993 (E). Microbiology – General guidance on methods for the detection of *Salmonella*. International Organisation for Standardisation, Geneva, Switzerland.

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

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

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

Anonymous, Draft Amendment of ISO 6579:2002/DAM, 2006 Annex D: Detection of *Salmonella* spp. in animal faeces and in samples from the primary production stage. International Organisation for Standardisation, Geneva, Switzerland.

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

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

Mooijman KA, 2005. The tenth CRL-*Salmonella* workshop; 28 and 29 April 2005, National Institute for Public Health and the Environment, Bilthoven, the Netherlands. RIVM report 330300007.

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

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)

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)
VI	2002	5	STM10	11	10 gram	RVS, MSRV, MKTTn and own	BGA, XLD and own	Korver et al., 2003 (report 330300001)
		5	STM100	139	10 gram			
		5	SE100	92	10 gram			
		5	SE500	389	10 gram			
		5	Blank	0	10 gram			
		3	STM10	11	No			
		3	SE100	92	No			
		2	SPan5	5	No			
		2	Blank	0	No			
		20	None	-	25 gram**			
VII	2003	5	STM10	12	10 gram	RVS, MSRV, MKTTn and own	BGA, XLD and own	Korver et al., 2005 (report 330300004)
		5	STM100	96	10 gram			
		5	SE100	127	10 gram			
		5	SE500	595	10 gram			
		5	Blank	0	10 gram			
		3	STM10	12	No			
		3	SE100	127	No			
		2	SPan5	9	No			
		2	Blank	0	No			
		20	None	-	10 gram**			
VIII	2004	7	STM10	13	10 gram	MSRV and own	XLD and own	Korver et al., 2005 (report 330300008)
		4	STM100	81	10 gram			
		7	SE100	74	10 gram			
		4	SE500	434	10 gram			
		3	Blank	0	10 gram			
		3	STM10	13	No			
		2	SE100	74	No			
		1	SE500	434	No			
		2	SPan5	7	No			
		2	Blank	0	No			
		20	None	-	10 gram**			
IX	2005	5	STM10	9	10 gram[2]	MSRV and own	XLD and own	Berk et al., 2006 (report 330300011)
		5	STM100	86	10 gram			
		5	SE100	122	10 gram			
		5	SE500	441	10 gram			
		5	Blank	0	10 gram			
		3	STM10	9	No			
		2	SE100	86	No			
		1	SE500	441	No			
		2	SPan5	7	No			
		2	Blank	0	No			
		10	None	-	10 gram***			

Table 1.1 (continued)

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

Table 1.2 CRL-*Salmonella* interlaboratory comparison study on the detection of *Salmonella* in food 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	STM10	9	10 gram	RVS, MKTTn, MSRV and own	XLD and own	Kuijpers et al. 2007 (Report 330604003)
		5	STM100	98	10 gram			
		5	SE100	74	10 gram			
		5	SE500	519	10 gram			
		5	Blank	0	10 gram			
		3	STM10	9	No			
		2	SE100	98	No			
		1	SE500	519	No			
		2	SPan5	5	No			
		2	Blank	0	No			

*= 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.

Annex 2 Calculation of T2

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

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

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

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

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

Annex 3 Information on the media used

MKTTn

(Oxoid CM 1048 Hampshire, United Kingdom) (bioMérieux 42114, Marcy l' Etoile, France) (Biokar BK 169 HA, Beauvais, France)
Composition of MKTTn: according ISO 6579, 2002

RVS

(Oxoid CM 0866, Hampshire, United Kingdom) (Difco 218581, Detroit, USA) (AES Laboratoire AEB140862, France) (Biomark B 1252)
Composition of RVS: according ISO 6579, 2002

BGA modified (Oxoid CM 0329B, Hampshire, United Kingdom)
(Difco 218801, Detroit, USA) (Merck 1.10747 Darmstadt, Germany) (AES laboratoire AEB151492, France) (Biomark B439) (Lab M 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

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

BXLH

Home made 12 ingredients, the medium is patented, pH 7.1

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

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, agnesiumsulfate 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

Rapid *Salmonella* Agar (Bio-rad 356 3961, Marnes-La-Coquette, France)
This is a chromogenic detection method validated by AFNOR according to the ISO 16140 requirements, for food and animal feed samples (No. BRD 07/11-12/05)
Composition of Rapid *Salmonella* medium: Casein peptone 5 g, Meat extract 5 g, Selective agents 14g, Chromogenic mixture 310 mg, Agar 12 g, Distilled water qsp 1000 ml, pH = 7,2 ± 0,2

SMID2 (bioMérieux SM2 43621, Marcy l' Etoile, France) (Gelose SMID2 43629)
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 (Oxoid PO5116A, Hampshire, United Kingdo)
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)

rivm

Annex 4 Protocol

INTERLABORATORY COMPARISON STUDY ON THE

DETECTION OF *SALMONELLA* spp. IN ANIMAL FAECES

organised by CRL-*Salmonella*

STUDY X - 2006

Introduction

This is the 10th interlaboratory comparison study on the detection of *Salmonella* spp. in animal faeces amongst the National Reference Laboratories (NRLs for *Salmonella*) in the EU. This study will have a comparable set-up as the earlier studies on the detection of *Salmonella* spp. in veterinary samples. The prescribed method is the procedure as described in 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. 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) pig faeces 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 faeces samples (10g each and negative for *Salmonella* spp.) in combination with a capsule containing STM or SE and 10 control samples (capsules only).
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 faeces). 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 17th of November 2006 (this is after 5 working days after the day of mailing).

Objective

The main objective of the tenth interlaboratory comparison study on the detection of Salmonella in pig faeces is to evaluate the results of the detection of different contamination levels of *Salmonella* in the presence of competitive micro-organisms among and within the NRLs.

Outline of the study

Each participant will receive (in week 46) 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.**
Store parcel 1 at (-20 ± 5)ºC immediately after receipt.

Parcel 2:

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

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

The performance of the study will be in week 48 (starting on 27 November 2006).

The documents necessary for performing the study are:

- Protocol Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in animal faeces X (2006);
- SOP Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in animal faeces X (2006);
- Test report Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in animal faeces X (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.
Please make sure to send your results to CRL--*Salmonella* before 15 December 2006. At the CRL a short report will be prepared to inform all NRLs within 1 to 2 months after the study on the overall results. We will start the first overall analyses immediately after the deadline. Results which will be received after the deadline can not be used in the analyses for the short report.

rivm

If you have questions or remarks about the interlaboratory comparison study please contact:
Angelina Kuijpers (Tel. number: + 31 30 274 2093) or
Kirsten Mooijman (Tel. number: + 31 30 274 3537)
RIVM / MGB Pb 63
P.O. Box 1
3720 BA Bilthoven
The Netherlands
Tel. number: + 31 30 274 2093
Fax. number: + 31 30 274 4434
E-mail : Angelina.Kuijpers@rivm.nl or Kirsten.Mooijman@rivm.nl

Time table of interlaboratory comparison study ANIMAL FAECES X (2006)

Week	Date	Topic
44	30 October – 3 November	Mailing of the protocol, standard operating procedure, test report and draft Annex D of ISO 6579 to the NRLs-Salmonella
46	13-17 November	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 capsules at -20°C ± 5°C** - **Store the faeces at +5°C ± 3°C** **If you did not receive the parcel at 17 November, do contact the CRL immediately.**
47	20-24 November	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)
48	27 November – 1 December	Performance of the study, following the instructions as given in the protocol and the SOP of study Animal faeces X (2006).
50	Before 15 December	Completion of the test report and e-mailing, faxing or mailing it to the CRL. If the test report is e-mailed or faxed to the CRL it is not longer necessary to sent the original test report as well, unless it is not legible (to be indicated by CRL-*Salmonella*).
51	18-22 December	Data input at CRL-*Salmonella* and sending these data by CRL to NRLs for checking
2	8-12 January	Checking the results by the National Reference Laboratories.
	January-February 2007	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.

rivm

Annex 5 Standard Operation Procedure

(SOP)

INTERLABORATORY COMPARISON STUDY ON THE

DETECTION OF *SALMONELLA* spp. IN ANIMAL FAECES

organised by CRL-*Salmonella*

STUDY X - 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 pig faeces. For this purpose Reference Materials (RMs) containing sublethally injured *Salmonella* Typhimurium (STM), *Salmonella* Enteritidis (SE) or *Salmonella* Panama (SPan) as prepared by the Community Reference Laboratory CRL for *Salmonella* are used. As matrix, pig faeces negative for *Salmonella* is used. The application of this SOP is limited to the interlaboratory comparison study for *Salmonella* described in this SOP.

2 References

International Standard – ISO 6579: 2002(E)
Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.

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 of a test organism in spray dried milk.

4 Principle

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

5 List of abbreviations

BPW	Buffered Peptone Water
MSRV	Modified semi-solid Rappaport Vassiliadis medium
RM	Reference Material
SOP	Standard Operating Procedure
TSI	Triple sugar/iron agar
UA	Urea Agar
XLD	Xylose Lysine Deoxycholate agar

6 Culture media

For this study the prescribed method is the procedure as described in draft Annex D of ISO 6579 (2006-09-12).

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

Composition and preparation of the media and reagents are described in Annex B, and in 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 method (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

- Modified Semi solid Rappaport Vassiliadis (MSRV) (ISO6579 Draft Annex D 2006-09-12)
- Selective enrichment medium routinely used in your laboratory (optionally)

6.3 Solid selective media for first and second isolation

6.4 Xylose-Lysine-Desoxycholat (90 mm plates) (ISO6579 Annex B.4)

- Second isolation medium for choice (obligatory)
- Own medium (optionally)

6.5 Confirmation media

6.6 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;
- pH-meter; having an accuracy of calibration of ± 0.1 pH unit at 25 °C.

7.2 Glassware

- Culture bottles or jars with nominal capacity of 200 ml;
- Culture tubes with approximate sizes: 8 mm in diameter and 160 mm in length;
- Micro-pipettes; nominal capacity 0,1 ml;
- Petri dishes; standard size (diameter 90 mm to 100 mm)
-

8 Procedure

8.1 General

Below the requested method of the tenth interlaboratory comparison study in pig faeces of CRL-*Salmonella* is described. The different steps in the procedure are also summarized in Annex A of this SOP. Beside these method it is also allowed to use one or more own methods. Please record all relevant data in the test report. Details of the method can be found in ISO 6579 and 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 faeces is added and one jar is a negative faeces control to which only 10 g faeces is added (= C12). These control jars should further be handled in the same way as the other jars. Place all jars (at least) **overnight** at 37 °C (± 1 °C). Also place some extra non-labelled jars containing 90 ml of BPW at 37 °C in case some jars might have been contaminated. Record in the test report (page 2 & 3) the requested data of BPW.

8.2 Pre-enrichment (day 1)

Take the numbered vials with the *Salmonella* capsules and the control capsules out of the freezer one hour before they are added to the BPW, to allow them to equilibrate to room temperature.
Shortly before adding the capsules, take the jars with BPW from the 37 °C incubator and inspect them for visual growth. Discard infected jars.
Add to 35 labelled jars a gelatine capsule from the vial with the corresponding label number. Do not open the gelatine capsule and do not shake the BPW to dissolve the capsule more rapidly. Place the jars with the capsules in the 37 °C incubator for **45 minutes** for dissolving of the capsules. Record the temperature and time at the start and at the end of this period in the test report (page 3). After 45 minutes add the faeces to the jars according to the following scheme:

- **Add 10 g of faeces to jars labelled 1-25 and C12,**
- **Add no faeces to jars labelled C1 - C11,**
- ***Do not shake the jars after adding the faeces.***

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

If PCR is performed, fill in all requested data on page 16 & 21 of the test report.

8.3 Selective enrichment (day 2)

Allow the selective enrichment media to equilibrate to room temperature, if they were stored at a lower temperature. Dry the surface of the MSRV plates in a Laminair Air Flow cabinet if necessary. Record (page 4-7) the requested data of the MSRV and own selective enrichment media (if used) in the test report. Label 25 MSRV plates from 1 to 25. Also label 12 MSRV plates from C1 to C12. Incubate the MSRV plates for 24 h and later on for another 24 h. If own selective enrichment media are used, label them in the same way as described for MSRV and also incubate for two times 24 h.

After equilibration:
Prescribed method:

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

Optional method:

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

Place the jars/tubes/plates in the appropriate incubator(s)/water bath(s) and record the temperature and time for the different enrichment media at the start and at the end of the incubation period and other requested data in the test report (page 4-7).

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

Record in the test report (page 8-13) the requested data of the isolation media used. Label 25 (standard size) Petri dishes of each isolation medium from 1 to 25 and label 12 (standard size) Petri dishes from C1 to C12.

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

1) Xylose Lysine Desoxycholate agar (XLD)
   Place the Petri dishes with the bottom up in the incubator set at 37 °C (record temperature and time and other requested data in test report, page 8 & 9).
2) Second isolation medium. Follow the instructions of the manufacturer (record temperature and time and other requested data in test report, page 10 & 11).
3) Optionally: selective isolation medium/media routinely used in your laboratory. Incubate the medium/media at the temperature routinely used (record temperature and time and other requested data in test report, page 12 &13).

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

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

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

For confirmation take from each Petri dish of each selective medium at least 1 colony considered to be typical or suspect (use only well isolated colonies). Store the plates at 5^{o} C ± 3^{o} 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 14) 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 MSRV) and Table 2 (isolation using own enrichment) on the test report pages 17-20. For the results of detection of *Salmonella* using PCR fill in Table 3 on the test report page 21.

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 15) 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 name of the NRL, the person in charge for the NRL, and the names of the persons who are carrying out the work and will be signed by these persons. If the study was carried out by another laboratory than the NRL, please also give the details of this laboratory in the test report.

Scheme of Bacteriological Interlaboratory Comparison Study ANIMAL FAECES X (2006)
on the detection of *Salmonella* spp. in pig faeces

Day	Topic	Description
0	**Prewarming BPW**	Place at least at the end of the day sufficient jars, each containing 90 ml BPW, at 37 °C ± 1 °C.
1	**Pre-enrichment**	Add 1 capsule to 90 ml (prewarmed) BPW Do not shake Incubate 45 min. at 37 °C ± 1 °C Add 10 g faeces to BPW Incubate 18 h ± 2 h at 37 °C ± 1 °C
2	**Selective enrichment**	0,1 ml BPW culture on MSRV plate, incubate at (41.5 ± 1) °C for (24 ± 3) h Own selective enrichment medi(um)(a)
3	**First isolation after 24 h**	Inoculate from suspect MSRV (24h) plates and own medi(um)(a) ➢ 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 MSRV medium another 24 (± 3) hours at the relevant temperatures
4	**Second isolation after 48 h**	Inoculate from suspect MSRV (48h) plates if first isolation was negative) and own medi(um)(a) ➢ Xylose Lysine Desoxycholate agar ➢ Second isolation medium ➢ Own selective medi(um)(a)
4	**Biochemical confirmation**	Inoculate the media from first isolation media (day 3) for biochemical identification and incubate 24 (± 3)h at the specified temperature .
5	**Biochemical confirmation**	Inoculate the media from second isolation media (day 4) for biochemical identification and incubate 24 (± 3)h at the specified temperature

rivm

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

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

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

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.

Bibliography

[1] De Smedt, J.M., Bolderdijk, R.F., Rappold, H. and Lautenschlaeger, D. Rapid *Salmonella* detection in foods by motility enrichment on a modified semi-solid Rappaport-Vassiliadis medium. Journal of Food Protection, 49, 7, 1986, 510-514.

[2] Soutourina, O.A., Semenova, E.A., Parfenova, V.V., Danchin, A. and Bertin, P. Control of bacterial motility by environmental factors in polarly flagellated and peritichous bacteria isolated from lake Baikal. Applied and Environmental Microbiology, 67, 9, 2001, 3852-3859.

[3] Veenman, C., Korver, H. and Mooijman, K.A. Research acitivities CRL-*Salmonella*. National Institute for Public Health and the Environment, Bilthoven, the Netherlands. RIVM report 330300 010, 2006.

[4] Voogt, N., Raes, M., Wannet, W.J.B., Henken, A.M. and van de Giessen, A.W. Comparison of selective enrichment media for the detection of *Salmonella* in poultry faeces. Letter in Applied Microbiology, 32, 2001, 89-92.

rivm

Annex 7 Test report follow-up study

FOLLOW UP
TEST REPORT

INTERLABORATORY COMPARISON ON THE

DETECTION OF *SALMONELLA* spp. IN ANIMAL FAECES

organised by CRL-*Salmonella*

STUDY X – 2006 FOLLOW UP

Laboratory code This is the same code as in the FAECES BRO 2006	
Laboratory name (NRL)	
Address	
Country	
Date of arrival of the parcels	Date: …… - ……. – 2007 time: ……... h ……..…… min
Start time of storage at - 20°C (capsules) Start time of storage at +5°C (faeces)	Date: …… - ……. – 2007 time: ……... h ……..…… min Date: …… - ……. – 2007 time: ……... h ……..…… min
Parcels damaged?	□ YES □ NO
Starting date testing	…… - ……. – 2007

PRE-ENRICHMENT – Buffered Peptone Water (BPW) (I)

Medium information BPW
Did you use the same BPW as mentioned in the test report from FAECES BRO 2006 ?
☐ Yes
☐ No please give more details in an annex :

Preparation of BPW	
Date of preparation	…… - ……. – 2007
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control of BPW?	☐ yes ☐ no

Prewarming time and temperature of the BPW	
Start at	Date: …… - ……. – 2007 time: ……... h ……..…… min temperature incubator: ……..…… °C
End at	Date: …… - ……. – 2007 time: …… h …… min temperature incubator: ……...…… °C

Incubation time and temperature for dissolving the capsules	
Start at	Date: …… - ……. - 2007 time: ……... h ……..…… min temperature incubator: ……..…… °C
End at	time: …… h …… min temperature incubator: ……...…… °C

Incubation time and temperature for pre-enrichment (18 ± 2) hrs after adding the faeces	
Start at	Date: …… - ……. – 2007 time: …….. h ……..…… min temperature incubator: ……..…… °C
End at	Date: …… - ……. – 2007 time: …….. h ……..…… min temperature incubator: ……..…… °C

SELECTIVE ENRICHMENT - Modified Semi solid Rappaport Vassiliadis medium (MSRV) (I)
Medium information MSRV
Did you use the same MSRV as mentioned in the test report from FAECES BRO 2006 ?
□ Yes
□ No please give more details in an annex :

Preparation of MSRV	
Date of preparation	…… - ……. – 2007
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control of MSRV?	□ yes □ no

Incubation time and temperature for selective enrichment	
Start of the first period (first 24 h)	Date: …… - ……. – 2007 time: …….. h ……..…… min temperature incubator: ……..…… °C
End of the first period (first 24 h)	Date: …… - ……. – 2007 time: …….. h ……..…… min temperature incubator: ……..…… °C
Start of the second period (second 24 h)	Date: …… - ……. – 2007 time: …….. h ……..…… min temperature incubator: ……..…… °C
End of the second period (second 24 h)	Date: …… - ……. – 2007 time: …….. h ……..…… min temperature incubator: ……..…… °C
OWN SELECTIVE ENRICHMENT - Selective medium, routinely used in your laboratory (I)	
If you use more selective media, please write these on an annex.	
Medium:	

Medium information
Did you use the same medium as mentioned in the test report from FAECES BRO 2006 ?
□ Yes
□ No please give more details in an annex :

Preparation of the medium	
Date of preparation	…… - ……. – 2007
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control of the medium?	□ yes □ no

Further details concerning the medium	
Volume of the medium per jar/tube in ml	
Inoculation volume of BPW	
Prescribed incubation temperature in ^{o}C	

Incubation time and temperature for own selective enrichment	
Start of the first period (first 24 h)	Date: …… - ……. – 2007 time: …….. h ……..…… min temperature incubator: ……..…… °C
End of the first period (first 24 h)	Date: …… - ……. – 2007 time: …….. h ……..…… min temperature incubator: ……..…… °C
Start of the second period (second 24 h)	Date: …… - ……. – 2007 time: …….. h ……..…… min temperature incubator: ……..…… °C
End of the second period (second 24 h)	Date: …… - ……. – 2007 time: …….. h ……..…… min temperature incubator: ……..…… °C

FIRST AND SECOND ISOLATION - Xylose Lysine Desoxycholate medium (XLD) (I)

Medium information XLD
Did you use the same XLD as mentioned in the test report from FAECES BRO 2006 ?
□ Yes
□ No please give more details in an annex :

Preparation of XLD	
Date of preparation	…… - ……. – 2007
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control of XLD ?	□ yes □ no

Incubation time and temperature for isolation	
Start incubation of XLD, inoculated from 24 h MSRV	Date: …… - ……. - 2007 time: …….. h ……..…… min temperature incubator: ……..…… °C
End incubation of XLD, inoculated from 24 h MSRV	Date: …… - ……. – 2007 time: …….. h ……..…… min temperature incubator: ……..…… °C
Start incubation of XLD, inoculated from 48 h MSRV	Date: …… - ……. - 2007 time: …….. h ……..…… min temperature incubator: ……..…… °C
End incubation of XLD, inoculated from 48 h MSRV	Date: …… - ……. - 2007 time: …….. h ……..…… min temperature incubator: ……..…… °C

FIRST AND SECOND ISOLATION – Second Isolation medium. (I)

Give information on the second isolation medium.	
Name of the medium	
Prescribed incubation temperature in ^{o}C	

Medium information of the second isolation medium
Did you use the same medium as mentioned in the test report from FAECES BRO 2006 ?
□ Yes
□ No please give more details in an annex :

Preparation of the second isolation medium	
Date of preparation	…… - ……. – 2007
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control ?	□ yes □ no

Incubation time and temperature for isolation	
Start incubation of second medium, inoculated from 24 h MSRV	Date: …… - ……. - 2007 time: …….. h ……..…… min temperature incubator: ……..…… °C
End incubation of second medium, inoculated from 24 h MSRV	Date: …… - ……. - 2007 time: …….. h ……..…… min temperature incubator: ……..…… °C
Start incubation of second medium, inoculated from 48 h MSRV	Date: …… - ……. - 2007 time: …….. h ……..…… min temperature incubator: ……..…… °C
End incubation of second medium, inoculated from 48 h MSRV	Date: …… - ……. - 2007 time: …….. h ……..…… min temperature incubator: ……..…… °C

FIRST AND SECOND ISOLATION – Own Isolation medium routinely used In your lab. (I)	
If you use more selective media, please write these on an annex.	
Name of the medium	
Prescribed incubation temperature in ^{o}C	

Medium information of your own medium
Did you use the same medium as mentioned in the test report from FAECES BRO 2006 ?
□ Yes
□ No please give more details in an annex :

Preparation of your own medium	
Date of preparation	…… - ……. – 2007
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control ?	□ yes □ no

Incubation time and temperature for isolation	
Start incubation of own medium, inoculated from 24 h selective enrichment medium	Date: …… - ……. - 2007 time: …….. h ……..…… min temperature incubator: ……..…… °C
End incubation of own medium, inoculated from 24 h selective enrichment medium	Date: …… - ……. - 2007 time: …….. h ……..…… min temperature incubator: ……..…… °C
Start incubation of own medium, inoculated from 48 h selective enrichment medium	Date: …… - ……. - 2007 time: …….. h ……..…… min temperature incubator: ……..…… °C
End incubation of own medium, inoculated from 48 h selective enrichment medium	Date: …… - ……. - 2007 time: …….. h ……..…… min temperature incubator: ……..…… °C

CONFIRMATION – Nutrient agar (I)
Did you streak the colonies on Nutrient agar before starting confirmation?
□ yes □ no If yes give further information on nutrient agar below

Medium information Nutrient agar
Did you use the same medium as mentioned in the test report from FAECES BRO 2006 ?
□ Yes
□ No please give more details in an annex :

Preparation of the nutrient agar	
Date of preparation	…… - ……. – 2007
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control of agar ?	□ yes □ no

BIOCHEMICAL CONFIRMATION
Medium information :
Did you use the same media as mentioned in the test report from FAECES BRO 2006 ?
☐ Yes
☐ No please give more details in an annex :

DETECTION BY PCR	
General questions	
Did you use PCR ?	☐ Yes ☐ No
If yes, when different as mentioned in the test report from faeces BRO 2006 give more information in an annex	

Table 1: Results of isolation using **MSRV** (dish numbers 1-12)

sample no.	MSRV 24 hours						MSRV 48 hours					
	XLD		Second * isolation medium		Own * isolation medium		XLD		Second * isolation medium		Own * isolation medium	
	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												

Table 1 (continued): Results of isolation using **MSRV** (dish numbers C1-C5)

sample no.	MSRV 24 hours						MSRV 48 hours					
	XLD		Second * isolation medium		Own * isolation medium		XLD		Second * isolation medium		Own * isolation medium	
	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]
C1												
C2												
C3												
C4												
C5												

Col [a] = number of colonies used for confirmation

Sal [b] = number of colonies confirmed as *Salmonella*

* = fill in the isolation medium used

Table 2: Results of isolation using own selective enrichment (dish numbers 1-12)

	Own selective enrichment 24 hours						Own selective enrichment 48 hours					
sample no.	*		*		*		*		*		*	
	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												

Table 2 (continued):Results of isolation using own selective enrichment(dish numbersC1-C5)

	Own selective enrichment 24 hours						Own selective enrichment 48 hours					
sample no.	*		*		*		*		*		*	
	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]
C1												
C2												
C3												
C4												
C5												

Col [a] = number of colonies used for confirmation

Sal [b] = number of colonies confirmed as *Salmonella*

* = fill in the isolation medium used

Table 3: Results of detection using PCR (dish numbers 1-12 and C1-C5)

Sample no.	PCR + or -		
		no.	
1		C1	
2		C2	
3		C3	
4		C4	
5		C5	
6			
7			
8			
9			
10			
11			
12			

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

Name of person (s) carrying out the tenth interlaboratory Comparison study (2006).	
Is the person(s) carrying out the tenth interlaboratory	□ YES □ NO give more information of the laboratory carrying

Comparison study (2006) working in the laboratory of NRL mentioned on page 1 ?	out the study : Laboratory name ……. Address …… Is this laboratory accredited or certified for the determination of *Salmonella*. □ YES □ NO
Date and signature	

Name of person in charge When not NRL (see page 1) mention also the name of the laboratory.	
Date and signature	

Please send the completed test report before 12 March by email

Use the address below:

Angelina Kuijpers E-mail : Angelina.Kuijpers@rivm.nl

RIVM / LZO (MGB) Pb 63

P.O. Box 1

3720 BA Bilthoven

The Netherlands

Tel. number: + 31 30 274 2093

Fax. number: + 31 30 274 4434