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Bacteriological detection of *Salmonella* in the presence of competitive micro-organisms
Bacteriological collaborative study IV amongst the National Reference Laboratories for *Salmonella*, the use of MSRV as selective enrichment

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Abstract

Bacteriological detection of *Salmonella* in the presence of competitive micro-organisms

A fourth bacteriological collaborative study was organised by the Community Reference Laboratory for *Salmonella.* All National Reference Laboratories for *Salmonella* (NRLs) participated. This study had two objectives:

1. Evaluation of the results of the detection of different contamination levels of *Salmonella* Enteritidis (100 and 1000 cfp) and *Salmonella* Typhimurium (10 and 100 cfp) in the presence of competitive micro-organisms among and within the NRLs; and
2. Evaluation of MSRV as selective enrichment compared to the standard method using RV as selective enrichment.

Methods used were an adapted ISO 6579 (proposed reference) method and, optionally, the laboratory's own routine method for the detection of *Salmonella* in chicken faeces. Significantly more positive isolations were obtained with the STM100 capsules compared to the STM10 and SE1000 capsules. These last two capsules showed the same detection level. The number of positive isolations from the SE100 capsules was significantly lower than the number of positive isolations from the STM10 and SE1000 capsules. Significantly more positive isolations were revealed when using MSRV than when using RV.

Contents

Samenvatting

Het Communautair Referentie Laboratorium voor *Salmonella* heeft een vierde bacteriologisch ringonderzoek georganiseerd betreffende de detectie van *Salmonella*. De deelnemers waren de Nationale Referentie Laboratoria (NRLs) voor *Salmonella* uit de lidstaten van de Europese Unie.
Dit vierde ringonderzoek had twee belangrijke doelen. Het ene doel was het vergelijken van de behaalde resultaten betreffende de detectie van verschillende besmettingsniveaus en serotypen van *Salmonella* in de aanwezigheid van stoorflora tussen en binnen de NRLs. Het andere doel was de vergelijking van de resultaten verkregen met het semi-solid medium MSRV voor selectieve ophoping met de resultaten verkregen met het ISO medium RV.
Vijf en dertig individueel genummerde capsules werden door elk van de 16 laboratoria onderzocht op de aanwezigheid van *Salmonella*. Hiervan moesten 25 capsules onderzocht worden in combinatie met 10 gram kippenfeces. De overige 10 capsules, waaraan geen feces moest worden toegevoegd, waren controle capsules. De 25 capsules waren onderverdeeld in 5 capsules met 10 kolonie vormende eenheden (kve) *Salmonella* Typhimurium (STM), 5 capsules met 100 kve STM, 5 capsules met 100 kve *Salmonella* Enteritidis (SE), 5 capsules met 1000 kve SE en 5 blanco capsules.
De capsules werden getest met een van de ISO 6579 afgeleide methode. Daarnaast moest MSRV als selectief ophopingsmedium gebruikt worden. Eventueel kon een laboratorium de eigen methode voor de detectie van *Salmonella* in kippenfeces gebruiken.

Alleen de 5 capsules met 100 kve STM gaven bij 10 van de laboratoria een 100% score. Geen enkel laboratorium isoleerde *Salmonella* uit 100% van de andere positieve capsules.
Vier laboratoria isoleerden geen *Salmonella* uit 1 van de *S*. Panama controle capsules. Eén van deze laboratoria isoleerde wel *Salmonella* uit beide *S*. Panama capsules met SC als selectief ophopingsmedium. Drie laboratoria isoleerden *Salmonella* uit een blanco capsule met MSRV als selectief ophopingsmedium.
Detectie van *Salmonella* uit de STM100 capsules gaf significant betere resultaten dan detectie uit de STM10 en SE1000 capsules. De slechtste resultaten werden verkregen met de SE100 capsules.
Met het selectieve ophopingsmedium MSRV werden significant betere resultaten behaald dan met het ISO medium RV. Dit gold zowel voor de kunstmatig besmette monsters als voor de natuurlijk besmette monsters.

Summary

The Community Reference Laboratory for *Salmonella* organised a fourth bacteriological collaborative study into the methods for the detection of *Salmonella*. Participants were the National Reference Laboratories (NRLs) for *Salmonella* of the Member States of the European Union.
This fourth study had two main objectives. The first was 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. The second objective was the comparison of the results obtained with the semi-solid medium MSRV with the results obtained with the medium prescribed by ISO, RV.
Thirty-five individually numbered capsules had to be examined by each of the 16 laboratories for the presence of *Salmonella*. Twenty-five of these capsules had to be examined in the presence of 10 grams of poultry faeces. The remaining 10, to which no faeces had to be added, were control capsules. The 25 capsules contained 10 colony forming particles (cfp) *Salmonella* Typhimurium (STM) (5x), 100 cfp STM (5x), 100 cfp *Salmonella* Enteritidis (SE) (5x), 1000 cfp SE (5x) and 5 were blank capsules.
The capsules were tested using an adapted ISO 6579 method. In addition to the ISO, MSRV was prescribed in this study. Optionally, the laboratories could use their method routinely used for detection of *Salmonella* in poultry faeces.

Ten laboratories succeeded to isolate *Salmonella* from all 5 capsules containing 100 cfp STM. None of the laboratories succeeded to isolate *Salmonella* from all five of the other types of positive capsules. Four laboratories were not able to isolate *Salmonella* from 1 of the *S.* Panama control capsules. One of these laboratories isolated *Salmonella* from both of the *S.* Panama capsules using SC as selective enrichment medium. Three laboratories isolated *Salmonella* from a blank capsule using MSRV as selective enrichment medium.
Detection of *Salmonella* from the STM100 capsules revealed significantly better results compared to the STM10 and SE100 capsules. The significantly lowest number of positive isolations were obtained with the SE100 capsules.
With the selective enrichment medium MSRV significantly more positive isolations were obtained in comparison with the medium prescribed by ISO, RV. This was true for the artificially as well as the naturally contaminated samples.

1. Introduction

In pursuance of the Council Directive 92/117/EEC the Community Reference Laboratory (CRL) for *Salmonella* organises collaborative studies with the objective that the examination of samples in Member States is carried out uniformly and that comparable results will be obtained. This report describes the fourth bacteriological collaborative study into methods for the detection of *Salmonella*.

Earlier studies have shown a significantly reduced number of positive isolations using Selenite/Cystine broth (SC) compared to Rappaport-Vassiliadis broth (RV) as selective enrichment medium. Therefore it was decided that SC will not be used anymore in future collaborative studies.
In the previous study (bacteriological study III) *Salmonella* had to be isolated from capsules in combination with chicken faeces. Besides capsules containing different levels of *Salmonella* Typhimurium, capsules containing *Salmonella* Enteritidis (SE) were included. From the third study it seemed that semi-solid media are more suitable for isolation of *Salmonella* from chicken faeces, and especially for detection of SE, in comparison with the reference method.

Therefore, in the fourth study, described in this report, all laboratories had to use the selective enrichment medium Modified Semi-solid Rappaport Vassiliadis (MSRV), besides the reference method. In total 4 different types of contaminated capsules had to be examined, containing 2 levels of STM and 2 levels SE. Furthermore, 20 samples of 25 gram of naturally contaminated faeces had to be examined.

2. Participants

Austria	Bundesstaatliche bakteriologisch-serologische Untersuchungsanstalt Graz
Belgium	Veterinary and Agrochemical Research Center (VAR) Bruxelles
Denmark	Danish Veterinary Laboratory Copenhagen
Finland	National Veterinary and Food Research Institute Department of Bacteriology Helsinki
France	Centre National d'Etudes Vétérinaires et Alimentaires Laboratoire central de recherches avicole et porcine Ploufragan
Germany	Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin Berlin
Greece	Veterinary Laboratory of Halkis Halkis
Ireland	Department of Agriculture and Food Veterinary Research Laboratory Dublin
Italy	Istituto Zooprofilattico Sperimentale delle Venezie Legnaro
Luxembourg	Laboratoire de Médecine vétérinaire de l'Etat (animal zoonosis) Luxembourg
The Netherlands	Rijksinstituut voor Volksgezondheid en Milieu (RIVM) Bilthoven

Northern Ireland	Department of Agriculture for Northern Ireland Veterinary Sciences Division; Bacteriology Department Belfast
Portugal	Laboratorio Nacional de Veterindria Lisboa
Spain	Laboratorio de Sanidad Y Produccion Animal de Algete Madrid
Sweden	National Veterinary Laboratory Laboratory of Bacteriology Uppsala
United Kingdom	Central Veterinary Laboratory Bacteriology Department Surrey

3. Materials and Methods

3.1 Preparation of reference materials and faeces samples

3.1.1 Reference materials containing *Salmonella* Typhimurium and *Salmonella* Enteritidis

The reference materials (RMs) were prepared from milk powder containing ±10 and ±100 colony forming particles (cfp) STM per capsule and from Highly Contaminated Milk Powder (HCMP) containing SE. The HCMP containing STM and SE, of which the low contaminated powders were mixed as described earlier by Voogt et al (1) was obtained by spray-drying artificially contaminated milk as described earlier by In 't Veld et al (2).

To obtain the target levels, 2 gram from the HCMP containing SE was diluted in steps (mixing ratio 1:1 g/g) by mixing it with uncontaminated milk powder (Carnation-koffiemelkpoeder Nestlé, the Netherlands) using a mortar and a pestle to a total of 128 gram. From this milkpowder 10 gram was diluted further with uncontaminated milkpowder to a total of 80 gram. The target contamination level of the mixed powders was about 100 and 1000 cfp SE per capsule.

Sixty capsules of each species and each level (0.34 gram per capsule) were prepared. These capsules were to be used to determine the number of cfp per capsule and the homogeneity of the batch. The capsules were stored at –20°C.

For use in the collaborative study and for testing the stability at storage temperature, 240 capsules containing 100 or 1000 cfp SE or 100 cfp STM and 300 capsules containing 10 cfp STM were filled.

The levels of contamination of the capsules were determined two times, i.e. 5 weeks before the study and during the study. The contamination level was determined by pouring plates with 5 ml plate count agar (PCA) and 10 ml Violet Red Bile Glucose agar (VRBG). Four hours after addition of PCA, VRBG was poured as an overlayer. The plates were incubated at 37°C overnight.

3.1.2 Samples containing competitive micro-organisms

Two kilogram of faeces of a poultry laying flock, which was found negative for *Salmonella*, was used to prepare the samples containing competitive micro-organisms. Faeces of a poultry laying flock serologically found positive for SE was used to prepare the naturally contaminated samples. Six portions of 25 gram of chicken faeces were tested for presence/absence (p/a) of SE according to the routine method of the RIVM, Bilthoven, the Netherlands (RV and DIASALM as selective enrichment). In addition to the routine method also MSRV was used to test the faeces. The faeces was mixed and homogenised before freezing with glycerol/peptone solution (mixing ratio 1:1 g/l) and stored at –20°C in order to stabilise the micro-organisms.

One litre of glycerol/peptone solution consisted of 378 gram of glycerol (300 ml), 7 gram of peptone and 700 ml distilled water. The naturally contaminated samples were to be used for evaluation of media and not for the comparison between laboratories.

3.2 Collaborative study

Two weeks before the study the RMs (in total 35 individually numbered capsules) and 850 gram of faeces were mailed with cooling devices by cargo freight to 15 participants. The 850 gram of faeces was divided in 10 portions (5 individually numbered portions of 60 gram and 5 individually numbered portions of 110 gram).
The content of the 35 capsules was unknown to the participants (Table 1). After arrival at the laboratory the capsules and faeces had to be stored at -20°C until the start of the study. Details about the mailing and storing of the samples are given in the protocol (Appendix 2). The protocol together with the Standard Operating Procedure (SOP) (Appendix 3) and test report (Appendix 4) were mailed to the participants three weeks before the start of the collaborative study.

Table 1: Overview of the contents of the capsules and the number of samples tested

Capsule containing	Number of samples tested	Faeces added
10 cfp STM	5	Yes
100 cfp STM	5	Yes
100 cfp SE	5	Yes
1000 cfp SE	5	Yes
Blank capsule	5	Yes
10 cfp STM	3	No
100 cfp SE	3	No
5 cfp *S.* Panama	2	No
Blank capsule	2	No

Twenty-five capsules had to be tested in combination with chicken faeces. These capsules contained 10 or 100 cfp STM, 100 or 1000 cfp SE and five were blank capsules (0 cfp). The 10 capsules, which had to be tested without faeces, were control capsules. These 10 control capsules contained 10 cfp STM, 100 cfp SE, 5 cfp *S.* Panama or were blank capsules. The content of the capsules as they were used in the collaborative study is presented in Table 2.
The capsules had to be tested as described in the SOP and the test results and operational details had to be reported to the CRL as proposed in the test report.

Table 2: Overview of the capsules as they were used in the collaborative study

Capsule number	Contents Capsules
1	STM 100
2	SE 1000
3	SE 100
4	STM 10
5	STM 10
6	Blank
7	STM 100
8	STM 100
9	SE 1000
10	SE 1000
11	SE 100
12	STM 10
13	STM 10
14	SE 100
15	Blank

Capsule number	Contents Capsules
16	Blank
17	SE 1000
18	STM 100
19	SE 100
20	STM 100
21	Blank
22	SE 1000
23	STM 10
24	SE 100
25	Blank

Capsule number	Contents Capsules
C1	SE 100
C2	STM 10
C3	Blank
C4	STM 10
C5	Blank
C6	STM 10
C7	*S*. Panama
C8	SE 100
C9	SE 100
C10	*S*. Panama

SE 100: Capsule contained 100 cfp *S*. Enteritidis
SE 1000: Capsule contained 1000 cfp *S*. Enteritidis
STM 10: Capsule contained 10 cfp *S*. Typhimurium
STM 100: Capsule contained 100 cfp *S*. Typhimurium
Blank: Capsule contained no cfp

3.3 Statistical analysis of the data

To compare: a) the various methods (selective enrichment and isolation media) used; b) the detection of different *Salmonella* spp.; c) the different participating laboratories; and d) the different types of samples (e.g. cfp per capsule), logistic regression was used (SAS 6.12 PROC GENMOD).
Different categorical variables were used for selective enrichment and isolation media. To identify laboratories that differed in performance compared to other laboratories, dichotomous indicator variables were computed, assuming a value of 1 for the laboratory of interest and a value of 0 for all other laboratories. Approximate 95% confidence intervals for these coefficients associated with laboratories were plotted against labcode to obtain a visual

overview of comparative laboratory performance (Figures 1-4). Two sided p-values of < 0.05 were used to determine statistically significant differences.

4. Results

4.1 Preparation of reference materials and faeces samples

4.1.1 Stability test and homogeneity test of reference materials

The level of contamination of the capsules was determined 5 weeks before the study and once again during the study. The initial levels of contamination, as well as the levels during the study, are presented in Table 3. The values for the homogeneity of the batch are also shown in Table 3. The average level of contamination for the STM 10 capsules was 4.1 (1-5) during the study. The STM 100 capsules had a level of contamination of 210 (152-284) cfp per capsule. The SE 100 and SE 1000 capsules had a level of contamination of 60.6 (42-77) and 220 (140-280) cfp per capsule, respectively.

Table 3 Level of contamination before and during study and the result of homogeneity test

Before collaborative study			At the time of collaborative study		
Capsules	Average no. of cfp per capsule	$T_2/(I-1)$	Capsules	Average no. of cfp per capsule	$T_2/(I-1)$
STM 10	5.7	1.09	STM 10	4.1	4.45
STM 100	277	5.57	STM 100	210	5.05
SE 100	88	1.71	SE 100	61	2.35
SE 1000	189	3.12	SE 1000	220	1.41

4.1.2 Testing stability of naturally contaminated faeces samples

The naturally contaminated faeces used in the collaborative study was collected in week 16, 22 and 26 of 1999. The faeces samples from week 16 originated from three poultry laying flocks. Therefore, before freezing, 18 samples were tested for p/a of *Salmonella*. Nine out of the 18 samples were found positive. Faeces samples taken in week 22 originated from two poultry laying flocks. Nine out of 12 samples tested were found positive for *Salmonella* before freezing. Faeces samples taken in week 26 originated from one poultry laying flock. Before freezing all six tested samples were found positive for *Salmonella*.

The samples were tested several weeks after freezing (Table 4). Faeces samples taken in week 22 and 26 were found positive with MSRV each time tested. Faeces taken in week 16 was found positive twice and also found negative twice using MSRV. With the use of RV as selective enrichment medium, *Salmonella* was isolated from naturally contaminated faeces only once.

It was concluded that it was not sure that all naturally contaminated samples indeed contained *Salmonella*. Therefore, these samples could not be used for evaluation between laboratories, but only for evaluation of the media, as this could be done within samples.

Table 4 Stability of naturally contaminated samples at –20°C

	Number of samples positive (n=2) using		
Weeks in freezer	RV	MSRV	DIASALM
Faeces week 16 (portion 9 in study)			
2	0	1	2
5	0	0	0
11	0	2	2
14	0	0	0
Faeces week 22 (portions 6, 7 and 10 in study)			
1	0	2	2
6	1	2	1
9	0	2	2
Faeces week 26 (portion 8 in study)			
1	0	2	2
2	0	2	2
4	0	2	2

4.2 Collaborative study

4.2.1 Media and incubation time

The media needed to perform the study were prescribed in the SOP. Data about the media used and manufacturers are presented in Appendix 5 (Table 1 to 7). The labcodes for the NRLs used in this study were the same as those in the other studies.

Pre-enrichment

Before adding the chicken faeces to the pre-enrichment medium (BPW), the capsules had to be dissolved at 37°C for 30 minutes. All laboratories dissolved the capsules for the prescribed time and temperature, except laboratory 14. This laboratory incubated the capsules at 35°C.
The prescribed time for the pre-enrichment was 16 - 20 hours. Except for three laboratories all laboratories incubated between 16 and 20 hours. The laboratories 6, 8, 16 incubated for 24 h 30 min, 22 h 45 min and 21 h 20 min, respectively.

Selective enrichment

The prescribed medium for the selective enrichment was Rappaport Vassiliadis broth (RV). Nine laboratories (labcode 2, 6, 9, 10, 11, 12, 14, 15 and 16) used RV for the collaborative study. The $MgCl_2$-concentrations in these media are 16.2 or 16.5 g/l (Table 5). Seven laboratories (labcode 1, 3, 4, 5, 7, 8 and 13) used Rappaport Vassiliadis Soya broth (RVS). The $MgCl_2$-concentrations in the RVS media are 12 or 13.6 g/l (Table 5).

Two of these laboratories (labcode 4 and 8) used RV from LAB M. This medium contains the buffer salt dipotassium hydrogen phosphate which is not prescribed by ISO for preparation of RV and is an ingredient for RVS.

The concentration of dehydrated $MgCl_2$ in the RV from OXOID and Merck is 16.5 g/l. The $MgCl_2$ concentration in the RVS from Merck is 13.58 g/l. The $MgCl_2$ concentration according to ISO is 13.5 g/l.

Table 5 Composition of RV and RVS from different manufacturers

Ingredients	Concentration (g/l) of ingredients in RV and RVS from different manufacturers (code)						
	ISO	Biomerieux	LAB M	Merck		OXOID	
	RV	RV (42073)	RV (LAB86)	RV (1.10236)	RVS (1.07700)	RV (CM669)	RVS (CM866)
Soya Peptone	4.5[1)]	4.5[1)]	4.5	5.0[1)]	4.5	5.0	4.5
Sodium chloride	7.21	7.2	7.2	8.0	8.0	8.0	7.2
KH_2PO_4[2)]	1.44	1.4	1.26		0.6	1.6	1.26
K_2HPO_4[3)]			0.18	0.8	0.4		0.18
$MgCl_2$		16.2	13.58				13.58
$MgCl_2*6H_2O$ ($MgCl_2$)	33.0 (13.5)			40.0 (16.5)	29.0 (12)	40.0 (16.5)	
Malachite green	0.036	0.033	0.033	0.12	0.036	0.04	0.036

1) Casein peptone

2) Potassium dihydrogen phosphate

3) Dipotassium hydrogen phosphate

The RV has to be incubated for two subsequent periods of 24 hours. All laboratories incubated the RV for 18-24 hours except laboratory 1, 2, 5 and 16. The latter 4 laboratories incubated the RV for 24 h 14 min, 14 h 45 min, 24 h 45 min and 24 h 55 min, respectively. The second period of incubation was 18-24 hours for all laboratories except laboratories 1 (24 h 20 min) and 6 (26 h). The total time of incubation was 36-48 hours for all laboratories except laboratory 1 (48 h 35 min).

Isolation

The isolation media, Brilliant Green Agar (BGA), a medium of own choice and optionally the medium routinely used in the laboratory, had to be incubated for 18-24 hours. All laboratories incubated the isolation media for the prescribed time.

4.2.2 Control samples (n=12)

In total 12 control samples were examined per laboratory. Ten capsules to which no faeces had to be added (C1 to C10), one procedure control (C11) to which no faeces or capsule had to be added and a faeces control (C12) to which no capsule had to be added.

Procedure and faeces control

None of the laboratories isolated *Salmonella* from the procedure and the faeces control with any of the methods used.

Control capsules

Laboratory 3 found one STM10 capsule negative with all, except one, selective enrichment media used. Using SC laboratory 3 found one out of three STM10 capsules positive. Laboratory 5 found one STM10 capsule negative using RV that was found positive using MSRV and Muller Kauffmann (MK).

Four laboratories (labcodes 3, 10, 14 and 15) found one *S.* Panama capsule negative with all selective enrichments used by the laboratory except for laboratory 3. Using SC laboratory 3 detected *Salmonella* in both *S.* Panama capsules.

Three laboratories (labcode 4, 9 and 12) found one blank capsules positive using MSRV for selective enrichment.

4.2.3 Comparison of selective enrichments

In total 10 different selective enrichment media were used by the laboratories.

RV: Rappaport Vassiliadis broth
RVS: Rappaport Vassiliadis Soya broth
MSRV: Modified Semi-solid Rappaport Vassiliadis (agar)
TBG: Tetrathionate-Brilliant-green Bile broth
MSRV+: MSRV with bromcresolpurpur and saccharose (agar)
SC: Selenit / Cystine broth
TT: Tetrathionate broth
DIA: Diagnostic Semi-solid Salmonella medium (DIASALM) (agar)
MK: Muller Kauffmann broth
Rap: Rappaport broth

4.2.3.1 Artificially contaminated samples

Results with the different selective enrichment media for the artificially contaminated samples are shown in Table 6.

Table 6 Results obtained with different selective enrichments used for the artificially contaminated samples

Labcode	Positive isolations with different selective enrichments (n=20)									
	RV	RVS	MSRV	TBG	MSRV + add.	SC	TT	DIA	MK	Rap
1		10	13	9						
2	8		8			3				
3			14		14	0	9			
4		14	15					14		
5		0	5						3	
6	13		14							
7		16	16							
8		16	14			4				
9 [1)]	1	4	1							
10	4		4							
11	0		13					13		
12	8		12							12
13		15	12				14			
14	14		16							
15	12		16					16		
16	8		14							
Total	68	75	187	9	14	7	23	43	3	12
% pos.	21	54	58	45	70	12	58	72	15	60

1) Standard method was RV; RVS also used as selective enrichment

Comparison between laboratories

RV

The results obtained with the different selective enrichment media compared to the results obtained with RV, revealed significantly better results for MSRV, DIASALM, RVS, MSRV+, TT and Rappaport. Significantly less positive isolations were obtained using SC for selective enrichment compared to the use of RV.

MSRV

Comparing the results of all selective enrichment media with the results obtained with MSRV also revealed significant differences. Significantly less positive results were obtained using RV, TBG, SC and MK for selective enrichment compared to MSRV. There were no significant differences between MSRV, RVS, TBG, MSRV+, TT, DIASALM and Rappaport.

Comparison within laboratories

Five laboratories (labcode 11, 15 and 16 using RV and labcode 1 and 5 using RVS) obtained significantly more positive isolations using MSRV compared to the use of RV or RVS. Two laboratories (labcode 8 and 13) obtained more positive isolations using RVS compared to using MSRV but this difference was not significant.

Laboratories 11 and 15 obtained significantly more positive isolations using DIASALM compared to RV, laboratory 4 obtained no significant differences between using RVS or DIASALM. Compared with MSRV no significant differences were found using DIASALM.

The use of SC revealed significantly less positive isolations compared to the use of RV(S) and MSRV within laboratories 2, 3 and 8. In laboratory 1, no significant differences were found with the use of TBG compared to the use of RVS. However, compared to MSRV, TBG revealed significantly less positive isolations. Compared to RV and MSRV no significant differences were found using RVS in laboratory 9 and Rappaport in laboratory 12.

Laboratory 3 obtained significantly less positive isolations using TT compared to using MSRV. No significant differences were found between MSRV and MSRV+ in laboratory 3, and between MK and MSRV in laboratory 5.

4.2.3.2 Naturally contaminated samples

The results obtained with different selective enrichment media for the naturally contaminated samples are shown in Table 7.

Comparison between laboratories

RV

Comparison of the results obtained with RV with the results obtained with other enrichment media revealed significant differences. Six media obtained significantly better results compared to RV, namely MSRV, RVS, TBG, MSRV+, DIASALM and MK. SC revealed significantly less positive isolations in comparison with RV and the use of TT revealed no significant difference with RV.

MSRV

Significantly better results were obtained with MK compared to MSRV. Significantly less results were obtained using RV, RVS, SC and TT for selective enrichment compared to MSRV. There were no significant differences between MSRV, TBG, MSRV+, DIASALM and Rappaport.

Comparison within laboratories

Six laboratories (labcode 11, 12, 14 and 15 using RV and labcode 1 and 5 using RVS) obtained significantly more positive isolations using MSRV in comparison with RV(S). Laboratory 8 obtained more positive isolations using RVS in comparison with MSRV, but this difference was not significant.

Laboratories 11 and 15 obtained significantly more positive isolations using DIASALM in comparison with RV. In laboratory 4 no significant difference was seen between the use of RVS or DIASALM. No significant differences were revealed between MSRV and DIASALM in laboratories 4, 11 and 15. The use of MK in laboratory 5 revealed significantly more

positive isolations compared to the use of MSRV. All three laboratories using SC (labcode 2, 3 and 8) obtained significantly less positive isolations in comparison with MSRV. Laboratory 8 obtained significantly more positive isolations using RVS in comparison with SC. No significant differences were found using RV compared to SC in laboratory 2. The use of RVS revealed significantly less positive isolations compared to the use of TBG in laboratory 1. The use of RVS revealed no significant differences in laboratory 4 compared to the use of DIASALM. The use of TT in laboratory 3 revealed significantly less positive isolations compared to the use of MSRV. No significant differences were revealed in laboratory 13 comparing TT with RVS and MSRV. Laboratory 9 did not find significant differences between RV, RVS and MSRV. No significant differences with the use of MSRV were found using MSRV+ in laboratory 3.

Table 7 Results obtained with different selective enrichments used for the naturally contaminated samples

Labcode	Positive isolations with different selective enrichments (n=20)									
	RV	RVS	MSRV	TBG	MSRV + add.	SC	TT	DIA	MK	Rap
1		4	11	12						
2	12		13			5				
3			18		16	1	4			
4		4	6					5		
5		1	9						15	
6	13		13							
7		14	16							
8		9	4			0				
9 [1)]	0	1	1							
10	2		3							
11	0		13					9		
12	1		10							4
13		13	15				13			
14	11		16							
15	8		16					16		
16	4		6							
Total	51	46	170	12	16	6	17	30	15	4
% pos.	28	33	53	60	80	10	43	40	75	20

1) Standard method was RV; RVS also used as selective enrichment

4.2.4 Comparison between laboratories and samples

The numbers of positive isolations from the capsules obtained with the method prescribed are presented in Table 8 per laboratory and per type of capsule. Laboratory 3 made a procedure error with the standard method, but used the MSRV correctly.

Table 8 Number of positive isolations with the use of reference method and MSRV.

Labcode	STM10 (n=5)		STM100 (n=5)		SE100 (n=5)		SE1000 (n=5)		Total (n=20)	
	RV(S)	MSRV	RV(S)	MSRV	RV(S)	MSRV	RV(S)	MSRV	RV(S)	MSRV
1*	3	4	3	4	1	2	3	3	10	13
2	1	1	5	5	0	0	2	2	8	8
3		3		5		3		3		14
4*	4	4	4	5	3	3	3	3	14	15
5*	0	1	0	3	0	0	0	1	0	5
6	4	4	3	4	2	3	4	3	13	14
7*	4	4	5	5	3	3	4	4	16	16
8*	4	3	5	4	3	3	4	4	16	14
9	0	0	1	1	0	0	0	0	1	1
10	0	1	3	3	0	0	1	0	4	4
11	0	1	0	5	0	3	0	4	0	13
12	3	4	3	3	0	2	2	3	8	12
13*	4	4	5	3	3	2	3	3	15	12
14	4	4	4	5	2	3	4	4	14	16
15	4	4	3	5	1	3	4	4	12	16
16	3	4	4	5	1	2	0	3	8	14
Total (percentage excl. lab 3)	38 (51%)	43 (57%)	48 (48%)	60 (80%)	19 (25%)	29 (39%)	34 (45%)	41 (55%)	139 (46%)	173 (58%)

* Reference method is RVS.

The results obtained with the standard method can be split into results obtained with RV and results obtained with RVS (Table 9). This revealed significant differences between the methods. RV gave significantly less positive isolations compared to RVS and MSRV.

Table 9 Percentage of total number of positive isolations obtained with RV, RVS and MSRV

	STM10			STM100			SE100			SE1000			Total		
	M1	M2	M3	M1	M2	M3	M1	M2	M3	M1	M2	M3	M1	M2	M3
Total no. of isolations	19	19	43	26	22	60	6	13	29	17	17	41	68	71	173
Percentage positives	42	63	57	57	73	80	13	43	39	38	57	55	38	59	58

M1: RV
M2: RVS
M3: MSRV

4.2.4.1 Comparison of capsules

Statistical analysis revealed significant differences between capsules. Significantly more positive isolations were obtained with the STM100 capsules (from 0 to 5 positive isolations) compared to the STM10 and SE1000 capsules (both 0 - 4 positive isolations). These latter capsules showed the same detection level. The number of positive isolations from the SE100 capsules (0 - 3 positive isolations) was significantly lower compared to the number of positive isolations from the STM10 and SE1000 capsules.

4.2.4.2 Comparison of laboratories using RV

Nine laboratories used RV for selective enrichment as reference method (Table 8). One of these laboratories (labcode 2) isolated *Salmonella* from all 5 STM100 capsules and one laboratory (labcode 11) could not isolate *Salmonella* from the STM100 capsules. The number of positive isolations varied from 0 to 4 for the STM10 and SE1000 capsules and from 0 to 3 for the SE100 capsules.

Comparing the results per laboratory with the average of all other laboratories that used RV for selective enrichment revealed significant differences (Figure 1 and Appendix 6 Table 1). Laboratories 9, 10 and 11 obtained significantly less positive isolations and laboratories 6, 14 and 15 obtained significantly more positive isolations using RV with regard to the average of the other laboratories using RV.

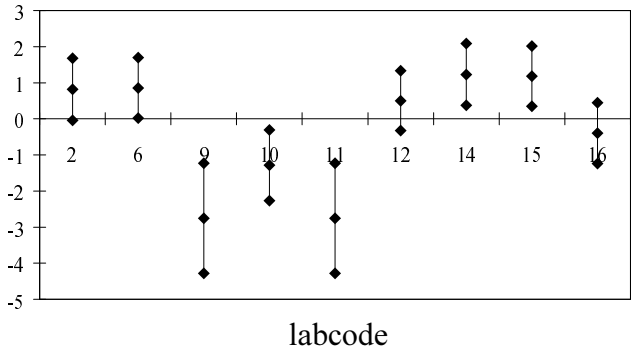

Figure 1 95% confidence intervals of the results per laboratory compared to the average result of all other laboratories that used RV

4.2.4.3 Comparison of laboratories using RVS

Six laboratories used RVS for selective enrichment as reference method (Table 8). Three of these laboratories (labcode 7, 8 and 13) isolated *Salmonella* from all 5 STM100 capsules. One laboratory (labcode 5) could not isolate *Salmonella* from the STM100 capsules using RVS. No laboratory isolated *Salmonella* from all 5 STM10, SE100 and SE1000 capsules. The number of positive isolations varied from 0 to 4 for STM10 and SE1000 capsules and from 0 to 3 for the SE100 capsules.

Comparing the results per laboratory with the average of all other laboratories that used RVS for selective enrichment revealed significant differences (Figure 2 and Appendix 6 Table 2). Laboratory 5 obtained significantly less positive isolations and laboratories 7, 8 and 13 obtained significantly more positive isolations using RVS with regard to the average of the other laboratories using RVS.

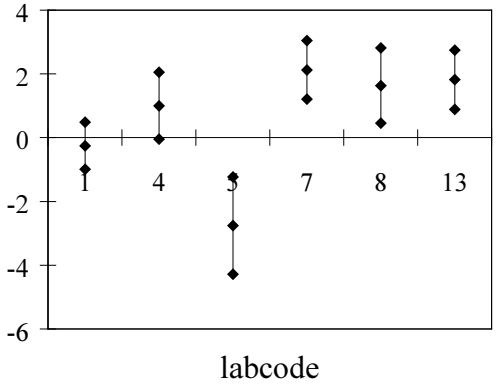

Figure 2 95% confidence intervals of the results per laboratory compared to the average result of all other laboratories that used RVS

4.2.4.4 Comparison of laboratories using MSRV

Using MSRV, 8 laboratories (labcode 2, 3, 4, 7, 11, 14, 15 and 16) isolated *Salmonella* from all 5 STM100 capsules. None of the laboratories isolated *Salmonella* from all 5 STM10, SE100 and SE1000 capsules. The number of isolations varied from 0 to 4 for STM10 and SE1000 capsules and from 0 to 3 for the SE100 capsules.

Comparing the results obtained with MSRV per laboratory with the average of the other laboratories revealed significant differences (Figure 3 and Appendix 6 Table 3). Laboratories 9 and 10 obtained significantly less positive isolations and laboratories 7, 12, 14 and 15 obtained significantly more positive isolations compared to the average of all laboratories.

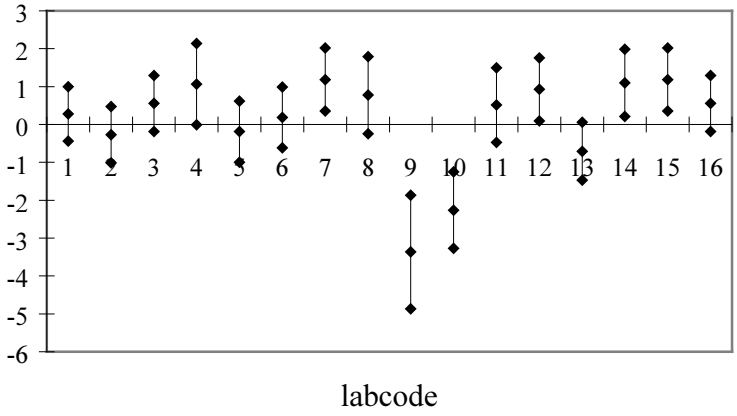

Figure 3 95% confidence intervals of the results per laboratory compared to the average result of all other laboratories

4.2.4.5 Comparison of laboratories overall

The results were also evaluated for the total score, obtained with the standard method and MSRV, between laboratories (Table 10). Three laboratories (labcode 5, 9 and 10) obtained significantly less isolations with both methods, compared to the other laboratories. Seven laboratories (labcode 4, 7, 8, 12, 13, 14 and 15) obtained significantly more positive isolations compared to all laboratories (Figure 4 and Appendix 6 Table 4).

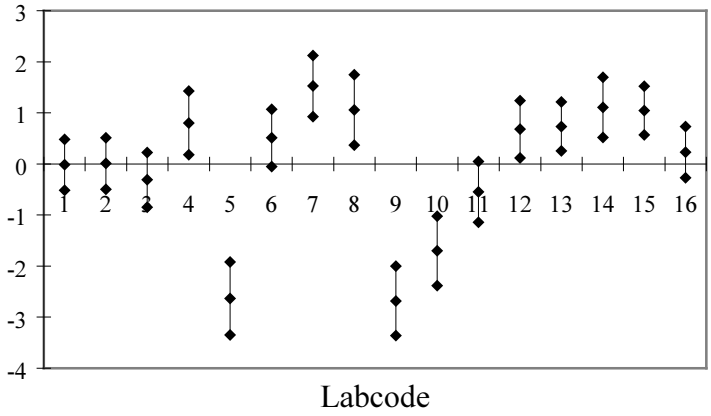

Figure 4 95% confidence intervals of results per laboratory compared to the average result of all other laboratories

Table 10 Comparison of the results per laboratory obtained with the prescribed method, with the average of all other laboratories

Labcode	Results per laboratory compared with the average of other laboratories			
	RV	RVS	MSRV	Total
1		+/-	+/-	+/-
2	+/-		+/-	+/-
3			+/-	+/-
4		+/-	+/-	+
5		-	+/-	-
6	+		+/-	+/-
7		+	+	+
8		+	+/-	+
9	-		-	-
10	-		-	-
11	-		+/-	+/-
12	+/-		+	+
13		+	+/-	+
14	+		+	+
15	+		+	+
16	+/-		+/-	+/-

\+ Significantly more positive isolations compared to the average of all other laboratories using the same selective enrichment

\- Significantly less positive isolations compared to the average of all other laboratories using the same selective enrichment

+/- No significant difference compared to the average of all other laboratories using the same selective enrichment

4.2.4.6 Comparison of second isolation media

No significant differences were found between the different isolation media used for the isolation from RV and RVS. For the isolation from MSRV, only XLT-4 seemed to give significant more positive isolations for both capsules and naturally contaminated samples (data not shown).

5. Discussion

In this study *Salmonella* had to be isolated from capsules in the presence of competitive micro-organisms in the form of chicken faeces. Two different contamination levels of STM and SE were used. For the batch of capsules containing about 100 or 1000 cfp SE, the (mean) value for homogeneity, expressed as $T_2/(I-1)$, was respectively 1.7 and 3.1 with an average of 88 and 189 cfp per capsule. In case of an ideal homogeneous distribution (Poisson distribution) a value of $T_2/(I-1)$ of 1 is expected. However, overdispersion between capsules can be expected and $T_2/(I-1)$ may be larger than 1 (3). For the capsules containing ± 10 or ± 100 cfp STM the value for homogeneity, expressed as $T_2/(I-1)$, was 1.09 and 5.57, respectively, with an average of 5.7 and 277 cfp per capsule. The level of contamination of the capsules containing STM10, STM100 and SE100 decreased during the storage period before the study. As the stability of the capsules containing SE is not optimal more research is needed.
The naturally contaminated samples did not consistently contain *Salmonella* when tested. Therefore, these samples could not be used for evaluation between laboratories. They were used only for evaluation of the media, which was done within samples

None of the laboratories isolated *Salmonella* from the procedure or faeces control. Three laboratories found one blank capsule positive with the use of MSRV.
Using the reference method four laboratories (codes 3, 10, 14 and 15) could not detect *Salmonella* from one of the two *S.* Panama capsules. Using an alternative method these 4 laboratories were not able to isolate *Salmonella* from one of the two *S.* Panama capsules, except for laboratory 3 who found two *S.* Panama capsules positive using SC. The chance for a *S.* Panama capsule to be blank (containing no *Salmonella* at all) is very low ($p<0.2\%$). Laboratory 3 could not detect *Salmonella* from one STM10 control capsule with one of the selective enrichment media used. Laboratory 5 could not isolate *Salmonella* from one STM10 capsule using RV, but isolated *Salmonella* using MSRV and MK. This might be caused by adding the pre-enrichment to RV which was not at room temperature.

Using the reference method two laboratories found all capsules negative. One laboratory used RV for reference method and one laboratory used RVS. The laboratory using RVS could not detect *Salmonella* in one of the control capsules (STM10). The laboratory using RV found all control capsules as expected. It could mean that these laboratories are not able to detect *Salmonella* in combination with chicken faeces using RV(S).
One laboratory using RV and three laboratories using RVS isolated *Salmonella* from all 5 STM10 capsules. None of the laboratories found all capsules containing 10 cfp STM, 100 and 1000 cfp SE in the presence of chicken faeces positive. The number of positive isolations varied from 0 to 4 (STM10 and SE1000) and from 0 to 3 (SE100). It seems that this level of contamination is critical for the detection of *Salmonella* as this may be close to the limit of

detection. As the number of positive isolations varied from laboratory to laboratory, the detection limit seems to differ from laboratory to laboratory.

Significant differences were found between the different types of capsules. Significantly more positive isolations were obtained with the STM100 capsules and significantly less positive isolations were obtained from the SE100 capsules. Therefore, it seems that the limit of detection of SE is higher than the limit for STM.
A relation between the manufacturers of specific media and the number of negative isolations could not be observed.

Selective enrichment
The results obtained with MSRV, DIASALM, RVS and MSRV+ were significantly better compared to the results obtained with RV for both the artificially and naturally contaminated samples. For the artificially contaminated samples, TT and Rappaport also revealed significantly better results compared to RV. For the naturally contaminated samples significantly better results were also obtained with TGB and MK compared to RV. As described by Maijala et al (4) the $MgCl_2$-concentration in the media is important for inhibition of growth of *Salmonella*. The $MgCl_2$-concentration in the RV used by the participants was 16.2 or 16.5 g/l. The concentration in RVS was 12 or 13.6 g/l for the different manufacturers. In MSRV(+) the $MgCl_2$-concentration was 10.93 g/l and in DIASALM the concentration was 11.0 or 9.5 g/l for the different manufacturers. All these selective enrichment media contained a lower concentration of $MgCl_2$ in comparison with RV. This can be a reason for the results obtained with RV.
Using RVS, no significant differences were revealed for the artificially contaminated samples compared to MSRV. However, significantly less positive isolations were obtained for the naturally contaminated samples comparing the results obtained with RVS with the results of MSRV. The use of DIASALM and MSRV+ revealed no significant differences between and within the laboratories compared to MSRV. The better results obtained with TT between the laboratories was not shown in both laboratories that used TT. Within one laboratory, significantly less positive isolations were obtained using TT compared to MSRV. The significantly better results obtained with MK for the naturally contaminated samples were present between and within laboratories. It seems that MK is better for isolation of SE from naturally contaminated samples, however, no *Salmonella* was isolated from the artificially contaminated samples containing SE.

Isolation media
XLT-4 revealed significantly better results compared to BGA for isolation of *Salmonella* from MSRV for the artificially and naturally contaminated samples. For isolation from RV no significant differences were found for the isolation media.

The method prescribed by ISO 6579 is Rappaport Vassiliadis broth. Comparison of the different formulas of the medium used by the different laboratories learnt that six of the 16 laboratories used RVS instead of RV for the standard selective enrichment. The use of RVS revealed significantly better results compared to using RV and therefore seems to be a better selective enrichment medium for isolation of *Salmonella* from faeces than RV.

In this study capsules containing two levels of STM and SE were used. This seems to be a good combination for evaluation of laboratories and methods for detection of *Salmonella.* Further studies are needed to evaluate the difference in the ability to detect *Salmonella* in naturally contaminated samples.

6. Conclusion

The high levels of STM (±210 cfp) were easier to isolate compared to the low levels of STM (±4.1 cfp) and compared to SE (±220 cfp). The low level of SE (±61 cfp) was even more difficult to isolate.

The use of MSRV revealed significantly more positive isolations in comparison with the use of RV for the artificially and the naturally contaminated samples. Using RVS, no significant differences were revealed for the artificially contaminated samples compared to MSRV. However, significantly less positive isolations were obtained for the naturally contaminated samples comparing the results obtained with RVS with the results of MSRV. Compared to RV, the use of RVS revealed significantly more positive isolations for the naturally contaminated samples.

Positive isolation strongly depended on a) the selective enrichment medium used; and b) the laboratory that used this medium. The type of isolation medium used seemed less important. The isolation medium XLT-4 obtained significantly more positive isolations after selective enrichment on MSRV.

References

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2. In ’t Veld PH, van Strijp-Lockefeer NGWM, Havelaar AH, Maier EA. The certification of a reference material for the evaluation of the ISO method for the detection of *Salmonella*. J Appl Bacteriol 1996; 80: 496-504.

3. Heisterkamp, Hoekstra JA, van Strijp-Lockefeer NGWM, Havelaar AH, Mooijman KA, in ‘t Veld PH, Notermans SAW, Maier EA, Griepink B. Statistical analysis of certification trials for microbiological reference materials. Commission of the European Communities; Community Bureau of Reference, Brussel; 1993: EUR 15008 EN

4. Maijala R, Johansson T, Hirn J. Growth of Salmonella and competing flora in five commercial Rappaport-Vassiliadis (RV)-media. Int J Food Microbiol; 1992; 17(1); 1-8

Appendix 1 Mailing list

1	European Commission	A. Checchi Lang
2	European Commission	B. Hogben
3	European Commission	V. Niemi
4	President of the Council of Health, the Netherlands	prof. dr. J. J. Sixma
5	Veterinary Public Health Inspector	drs. H. Verburg
6-21	Participants of the study (National Reference Laboratories)	
22	Board of Directors RIVM	dr. G. Elzinga
23	Director Sector Public Health Research	prof. dr. ir. D. Kromhout
24	Head of Microbiological Laboratory for Health Protection and Director CRL-*Salmonella*	dr. ir. A.M. Henken
25-27	Project Workers	
28-31	Authors	
32	Dutch National Library for Publications and Bibliography	
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35	Library RIVM	
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52-61	Spare copies	

Appendix 2 Protocol

BACTERIOLOGICAL COLLABORATIVE STUDY IV
ORGANIZED BY CRL *SALMONELLA*

PROTOCOL:

Introduction:
The Community Reference Laboratory (CRL) *Salmonella* organises the fourth bacteriological collaborative study on the methods for the detection of *Salmonella* amongst the National Reference Laboratories (NRLs). In the first and second collaborative study (October 1995 and October 1996 respectively) no significant difference was found between the routine method and the reference method for the individual laboratories. The number of positive isolations was on average significantly lower with selenite/cystine compared to Rappaport-Vassiliadis (RV) as selective enrichment medium. In the third study the ability of the laboratories to detect different contamination levels of *Salmonella* in the presence of competitive organisms was tested. The NRLs had to use the principles of the adapted method of the ISO 6579 for the detection of *Salmonella* (excluding selenite/cystine). Some laboratories used a semi solid medium for selective enrichment. Those media seemed to be superior to RV as selective enrichment, especially for the detection of *Salmonella* Enteritidis (SE). Therefore, in this collaborative study all laboratories use Modified Semi solid Rappaport Vassiliadis (MSRV) as selective enrichment next to RV.
For the performance of this study Reference Materials (RMs) produced by the CRL and poultry faeces will be used. The RMs consists of gelatine capsules containing sublethally injured *Salmonella* Typhimurium (STM) or SE.
Each laboratory will examine 25 faeces samples in combination with a capsule containing STM or SE and 10 control samples (no faeces added to the capsule). Next to the capsules, 20 samples that are naturally contaminated with Salmonella will be examined. The results will be evaluated by the CRL.

Objective:
The main objective of the fourth bacteriological collaborative study 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.
Evaluate the ability of the laboratories to use Modified Semi solid Rappaport Vassiliadis (MSRV) for the detection of *Salmonella*.

Outline of the study:

The study will be carried out according to the adapted ISO 6579 method and optionally the routine method of a laboratory.

Each participant will receive a parcel containing:

- 25 numbered vials; each containing one *Salmonella* Typhimurium or *Salmonella* Enteritidis capsule;
- 10 control vials; each containing one capsule with or without *Salmonella*;
- 6 portions of 60 gr. frozen poultry faeces.
- 5 portions of 100 grams of naturally contaminated frozen faeces.

The performance of the study will be in week 36 (starting on 6 September 1999). All data will be reported on the test report to the CRL Salmonella and will be used for (statistical) analysis.

The media used for the collaborative study will not be supplied by the CRL.

Time table of bacteriological collaborative study IV

The performance of the study must be in **week 36 (starting on September 6th 1999)** or one week earlier or later.

9 - 13 August	Mailing the protocol, SOP and test report to the NRLs.
23 - 27 August	Mailing the parcel to the NRLs. CRL will mail the parcel by cargo freight from the Dutch airport (Schiphol) to the airport of destination. The participants have to collect the parcel at the airport. Please collect the parcel directly after arrival at the airport. For this you need the airway bill number. This number and other necessary information will be indicated in a fax in the week before mailing. **The transport costs from the airport of destination to the laboratory can't be paid by the CRL, so this will be at the expense of the NRL**. The parcel will be mailed with cooling devices to keep the temperature low during transport and storage at the airport. A cold chain monitor is included in the parcel to check the temperature during shipment. For collecting the parcel at the airport take your own cooling box with cooling devices or ice with you. Open the parcel at the airport and check the contents for damage. Put the contents of the parcel into your own cooling box. Check the cold chain monitor and *note on test report (a copy of the concerning page is enclosed in the parcel) the date, time, the colour of the different compartments and whether the complete compartment has become blue.* Place the cold chain monitor in the cooling box with the reference materials. Immediately after arrival at the laboratory **store the materials at -20 °C**. Check the cold chain monitor again and *note on test report date, time, the colour of the different compartments and whether the complete compartment has become blue.*

	For a small number of laboratories the CRL will mail the parcel by courier service. The parcel will be mailed with cooling devices to keep the temperature low during transport. A cold chain monitor is included in the parcel to check the temperature during shipment. Check the cold chain monitor upon arrival as described above. Immediately after arrival at the laboratory **store the materials at -20 °C.** **If you did not receive the parcel before or at 27 August, do contact the CRL immediately**.
30 August - 3 September	Adjustment, if necessary, of the temperature setting of the incubators. Preparation of 1. Non selective pre-enrichment medium (see SOP 5.1) 2. Selective enrichment media (see SOP 5.2) 3. Solid selective plating media (see SOP 5.3) 4. Confirmation media (see SOP 5.4)
6 - 9 September	Performance of the study. **Note**: Each laboratory is free to determine when they want to examine the samples as long as it will be done in the scheduled weeks.
27 September - 1 October	Completion of the test report and faxing it to the CRL. The original test report will be sent to CRL.
11 - 15 October	Checking the results by the National Reference Laboratories.

If you have questions or remarks about the bacteriological collaborative study please contact:

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Appendix 3 Standard Operating Procedure

BACTERIOLOGICAL COLLABORATIVE STUDY IV
ORGANIZED BY CRL SALMONELLA

SOP/CRL/05
Version no.1
200799

STANDARD OPERATING PROCEDURE:

Detection of *Salmonella* in the presence of competitive micro-organisms.

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. For this purpose Reference Materials (RMs) containing sublethally injured *Salmonella* Typhimurium or *Salmonella* Enteritidis as prepared by the Community Reference Laboratory (CRL) *Salmonella* and poultry faeces are used. Its application is limited to the bacteriological collaborative study for *Salmonella* described is this SOP.

2 References

International Organisation of Standardisation.
Microbiology - General guidance on methods for the detection of *Salmonella*. ISO 6579 (Third edition 1993-09-01).

Beckers, H.J., Van Leusden, F.M., Meijssen, M.J.M., Kampelmacher, E.H. 1985. Reference material for the evaluation of a standard method for the detection of *Salmonella* in foods and feeding stuffs. J. Appl. Bacteriol., 59, 507-512.

3 Definitions

For the purpose of this SOP, the following definitions apply:

3.1 *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.

3.2 *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.

3.3 *Reference Material*: a gelatine capsule containing a quantified amount artificially contaminated spray dried milk.

4. Principle

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

5. Culture media

Composition and preparation of the media and reagents are described in Annex B of the ISO 6579 that is part of the manual CRL-Salmonella supplied by the CRL. Preparation of MSRV is described in paragraph 6.

5.1 Non selective pre-enrichment medium

5.1.1 Buffered Peptone water (Annex B.1)

5.2 Selective enrichment medium

5.2.1 Rappaport Vassiliadis magnesium chloride/malachite green medium (RV medium) (Annex B.2)

5.2.2 Modified Semi solid Rappaport Vassiliadis (Paragraph 6)

5.3 Solid selective media

5.3.1 Phenol red/brilliant green agar (Annex B.4)

5.3.2 Second medium (ISO 6579; 4.3)

5.4 Confirmation media

Selection of colonies for confirmation (optionally)

5.4.1 Nutrient agar (Annex B.5)

Biochemical confirmation

5.4.2 Triple sugar/iron agar (TSI agar) (Annex B.6)

5.4.3 Urea agar (Annex B.7)

5.4.4 l-Lysine decarboxylation medium (Annex B.8)

6 Preparation of MSRV

The medium must be boiled to dissolve (instructions manufacturer). After boiling the medium must be transparent blue. After cooling down to 50°C the supplement or the novobiocine has to be added. The final concentration of the novobiocine in the medium should be 0.01 gr/l. Plates should be poured with a volume of 15 to 20 ml.

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

7.1.1 Oven (for dry sterilisation) or autoclave (for wet sterilisation);
7.1.2 Incubator, capable of operating at 37 °C ± 1 °C;
7.1.3 Water bath, capable of operating at 42 °C ± 0.1 °C or incubator, capable of operating at 42 °C ± 0.5 °C;
7.1.4 Water bath, capable of operating at 37 °C ± 0.1 °C;
7.1.5 Loops;
7.1.6 pH-meter; having an accuracy of calibration of ± 0.1 pH unit at 25 °C.

7.2 Glassware

Disposable labware is an acceptable alternative to reusable glassware if it has suitable specifications.

7.2.1 Culture bottles or jars, capacity = 250 ml;
7.2.2 Culture tubes; 8 mm in diameter and 160 mm in length;
7.2.3 Micro-pipettes; nominal capacity 0,1 ml.
7.2.4 Petri dishes; small size (diameter 90 mm to 100 mm) and/or large size (diameter 140 mm).

8 Procedure

Thaw the portions frozen faeces **overnight** at 5 °C. Take the frozen faeces out of the freezer at the end of the working day.

8.1 Pre-enrichment

Allow the BPW to equilibrate to room temperature, if it was stored at a lower temperature. Record on test report (page 3) the requested data of the BPW. 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. Label 25 jars containing 225 ml of BPW from 1 to 25. For the naturally contaminated samples number 20 jars of BPW from N1 to N20. Also label 12 jars of BPW from C1 to C12 (control capsules). One jar is a procedure control (= C11) to which no capsule/faeces is added and one jar is a negative faeces control to which only 10 gr. faeces is added (= C12). These control jars should be handled in the same way as the other jars from then on.

After equilibration add to 35 labelled jars a gelatine capsule from the vial with the corresponding label number. Do not open the gelatine capsule and/or do not shake the BPW to dissolve the capsule more rapidly. Place the jars in the 37 °C incubator for **30 minutes** for dissolving of the capsules. Note the temperature and time at the start and at the end of this period on test report (page 3). After 30 minutes add the thawed faeces to the jars according to the following scheme, except to the 11 control jars;

- **add 10 gr. faeces from portion 1 to jars labelled 1-5,**
- **add 10 gr. faeces from portion 2 to jars labelled 6-10,**
- **add 10 gr. faeces from portion 3 to jars labelled 11-15 and C12,**
- **add 10 gr. faeces from portion 4 to jars labelled 16-20,**
- **add 10 gr. faeces from portion 5 to jars labelled 21-25,**
- **add no faeces to jars labelled C1 - C11,**
- **add 25 gr. faeces from portion 6 to jars labelled N1-N4,**
- **add 25 gr. faeces from portion 7 to jars labelled N5-N8,**
- **add 25 gr. faeces from portion 8 to jars labelled N9-N12,**
- **add 25 gr. faeces from portion 9 to jars labelled N13-N16,**
- **add 25 gr. faeces from portion 10 to jars labelled N17-N20.**

Do not shake the jars after adding the faeces.

Place all jars in the 37 °C incubator for 16 h to 20 h. Note the temperature and time at the start and at the end of the incubation period and other requested data on test report (page 3).

8.2 Selective enrichment

Allow the selective enrichment broths to equilibrate to room temperature, if they were stored at a lower temperature and dry the surface of the MSRV plates in a Laminair Air Flow cabinet. Record on test report (page 4-6) the requested data of the selective enrichment broths and MSRV plates. Label 25 jars/tubes/plates of each selective enrichment broth from 1 to 25. Also label 20 selective enrichment broths/plates from N1 to N20 and 12 jars/tubes/plates from C1 to C12 (control jars).

After equilibration:

- Transfer 0.1 ml of homogenised BPW culture to a tube containing 10 ml RV medium with the corresponding label number. Incubate at 42 °C for 24 h and a further 24 h;
- Inoculate the MSRV plates with three drops of homogenised BPW culture, with a total volume of 0.1 ml. Incubate **(not upside down)** at 42 °C for 24 h and a further 24 h;
- Inoculate the routinely used selective medium/media (other than the one mentioned above), with the corresponding label number, from the homogenised 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 in the appropriate incubator(s)/water bath(s) and note the temperature and time for the different enrichment media at the start and at the end of the incubation period and other requested data on test report (page 4-6).

8.3 Isolation on media (first and second isolation)

Note: In the case that you do not have large dishes (140 mm) at your disposal use two small dishes, one after the other, using the same loop.
Record on test report (page 7-9) the requested data of the isolation media used. Label 25 large petri dishes of the isolation media from 1 to 25, label 20 large petri dishes from N1 to N20 and label 12 large petri dishes from C1 to C12 (these petri dishes are inoculated with the control selective enrichment broths).

8.3.1 First isolation

Inoculation:
Inoculate, by means of a loop, from all selective enrichment cultures and from suspect MSRV plates the surface of a large size petri dish with the corresponding label number (see also *note* at the beginning of section 8.3) containing:

1) phenol red/brilliant green agar;
2) selective isolation medium of your own choice (using a new loop);
3) selective isolation medium/media routinely used in your laboratory (using a new loop). Only if media are used other than those mentioned above.

Incubation:
ad 1/2: Place the petri dishes with the bottom up in the incubator set at 37 °C (note the temperature and time at the start and at the end of the incubation and other requested data on test report, page 7-8).
ad 3: Incubate the medium/media at the temperature routinely used (note temperature and time and other requested data on test report, page 9).

After incubation for 18 h to 24 h, examine the petri dishes for the presence of typical colonies of *Salmonella*. Typical colonies of *Salmonella* grown on phenol red/brilliant green agar cause to change the colour of the medium from pink to red.
If growth is slight or no typical colonies of *Salmonella* are present, re-incubate at 37 °C for 18 h to 24 h. Re-examine for the presence of typical colonies of *Salmonella*.

8.3.2 Second isolation
After a total incubation time of 48 h of the selective enrichment media, repeat the procedure described above (**8.3.1 First isolation**).

8.4 Confirmation of colonies from first and/or second isolation

For confirmation take from each petri dish of each selective medium at least 1 colony considered to be typical or suspect (only use well isolated colonies).
Before biochemical confirmation, 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 10) the requested data of the nutrient agar. Incubate the inoculated plates at 37 °C for 18 h to 24 h.
Otherwise directly biochemical confirm the suspected colonies. If the selected colony is not confirmed as *Salmonella*, test 5 typical colonies at most. Report the number of colonies tested and the number of colonies confirmed as *Salmonella* for each dish in Table 1 (first isolation) and 2 (second isolation) on test report (page 12-17).

8.4.1 Biochemical confirmation

By means of a loop, inoculate the media specified below with the colony selected as described above. Optionally inoculate other media that are routinely used for biochemical confirmation. Record on test report (page 10) the requested data of the media.

8.4.1.1 TSI agar

8.4.1.2 Urea agar

8.4.1.3 l-Lysine decarboxylation medium

8.4.2 Interpretation of the biochemical tests

Salmonella generally show the reactions given in table 1 (ISO 6579; page 5+6).

8.4.2.1 TSI agar:

Butt: yellow by fermentation of glucose
black by formation of hydrogen sulfide
bubbles or cracks due to gas formation from glucose

Slant: red or unchanged

8.4.2.2 Urea agar: coloured yellow

8.4.2.3 l-Lysine decarboxylation medium: coloured purple

9. Test report

The test report will contain all information that might influence the result and is not mentioned in this SOP. Some incidents or deviations from the specified procedures will also be recorded. The test report will include the names of the persons, who are carrying out the work and will be signed by these persons.

Schedule of the adapted ISO 6579 method

day 1	**Pre-enrichment**	1 capsule to 225 ml BPW 30 min. at 37 °C add 10 or 25 gr faeces to BPW 16-20 h at 37 °C
day 2	**Selective enrichment**	0.1 ml BPW culture in 10 ml RV 0.1 ml BPW culture on MSRV plate 24 h at 42 °C
day 3	**First isolation**	inoculate from RV and suspect MSRV plates a. phenol red/brilliant green agar b. other selective medi(um)(a) incubate both 18-24 h at the specified temperature
	Continue sel. enrichment	incubate RV and MSRV medium (see day 2) another 24 hours at 42 °C
day 4	**Second isolation**	inoculate from RV and suspect MSRV plates a. phenol red/brilliant green agar b. other selective medi(um)(a) incubate both 18-24 h at the specified temperature
	Biochemical confirmation	inoculate the media from first isolation media (day 3) for biochemical identification and incubate 18-24 h at the specified temperature
day 5	**Biochemical confirmation**	inoculate the media from second isolation media (day 4) for biochemical identification and incubate 18-24 h at the specified temperature

Appendix 4 Test Report

TEST REPORT
OF THE FOURTH BACTERIOLOGICAL COLLABORATIVE STUDY
ORGANIZED BY CRL *SALMONELLA*

Detection of *Salmonella* in the presence of competitive micro-organisms
The use of Modified Semi solid Rappaport Vassiliadis as selective enrichment

Laboratory code	:
Laboratory name	: .....
Date of receipt of the parcel	: ..... - ..... - 1999
Starting date for detection	: ..... - ..... - 1999

Shipment

Cold chain monitor:

1. Check at airport:

date :..... - 1999
time :..... h ..... min

Parcel damaged □ YES
□ NO

Colour of compartment

A □ completely coloured □ partly coloured
□ white □ light blue □ dark blue

B □ completely coloured □ partly coloured
□ white □ light blue □ dark blue

C □ completely coloured □ partly coloured
□ white □ light blue □ dark blue

D □ completely coloured □ partly coloured
□ white □ light blue □ dark blue

2. Check at laboratory:

date :..... - 1999
time :..... h ..... min

Parcel damaged □ YES
□ NO

Colour of compartment

A □ completely coloured □ partly coloured
□ white □ light blue □ dark blue

B □ completely coloured □ partly coloured
□ white □ light blue □ dark blue

C □ completely coloured □ partly coloured
□ white □ light blue □ dark blue

D □ completely coloured □ partly coloured
□ white □ light blue □ dark blue

Pre-enrichment

Manufacturer of the BPW:

- name : .....
- code number : .....
- batch number : .....
- expire date : .....
- pH of the BPW : .....

Incubation time and temperature for dissolving the capsules

- at the start : time: ..... h ..... min
  : temperature incubator: ..... °C
- at the end : time: ..... h ..... min
  : temperature incubator: ..... °C

Incubation time and temperature for pre-enrichment

- at the start : time: ..... h ..... min
  : temperature incubator: ..... °C
- at the end : time: ..... h ..... min
  : temperature incubator: ..... °C

Type of incubator: □ vented incubator
□ nonvented incubator

Thermometer used for reading the incubation temperature:

- calibrated □ YES
  □ NO
- scale division :.....

(If the temperature is recorded automatically, please provide printout of the temperatures)

Place of thermometer in the incubator :.....
Place of jars in the incubator :.....

Selective enrichment

1. Rappaport Vassiliadis medium

Manufacturer of the RV medium:

- name : .....
- code number : .....
- batch number : .....
- expire date : .....
- pH of the broth : .....

Specific data of composition of RV medium. What is the concentration of the following compounds:

- Soya Peptone: : .....
- Sodium chloride: : .....
- Potassium dihydrogen phosphate : .....
- Dipotassium hydrogen phosphate : .....
- Magnesium chloride anhydrous : .....
- Magnesium chloride.$6H_2O$: .....
- Malachite green : .....

Incubation time and temperature for selective enrichment:

- at the start first period : time: ..... h ..... min
  : temperature incubator: ..... °C
- at the end first period : time: ..... h ..... min
  : temperature incubator: ..... °C
- at the start second period : time: ..... h ..... min
  : temperature incubator: ..... °C
- at the end second period : time: ..... h ..... min
  : temperature incubator: ..... °C

Type of incubator: □ vented incubator
□ nonvented incubator
□ waterbath

Thermometer used for reading the incubation temperature:

- calibrated □ YES
  □ NO
- scale division :.....

(If the temperature is recorded automatically, please provide printout of the temperatures)

Place of thermometer in the incubator:.....

Place of jars in the incubator :.....

2. Modified Semi solid Rappaport Vassiliadis medium

How much experience do you have using MSRV as selective enrichment:

Manufacturer of the MSRV medium:

- name : .....
- code number : .....
- batch number : .....
- expire date : .....
- pH of the broth : .....

Incubation time and temperature for selective enrichment:

- at the start first period : time: ..... h ..... min
  : temperature incubator: ..... °C
- at the end first period : time: ..... h ..... min
  : temperature incubator: ..... °C
- at the start second period : time: ..... h ..... min
  : temperature incubator: ..... °C
- at the end second period : time: ..... h ..... min
  : temperature incubator: ..... °C

Type of incubator: □ vented incubator
□ nonvented incubator
□ waterbath

Thermometer used for reading the incubation temperature:

- calibrated □ YES
  □ NO
- scale division :.....

(If the temperature is recorded automatically, please provide printout of the temperatures)

Place of thermometer in the incubator:.....

Place of plates in the incubator :.....

3. **Selective medium, routinely used in your laboratory**

If you use more selective media, please write these on an annex.

Medium : .....

Manufacturer of the medium:

- name : .....
- code number : .....
- batch number : .....
- expire date : .....
- pH of the broth : .....

Volume of the medium per jar/tube : ..... ml
Inoculation volume of BPW : ..... ml

Incubation temperature : ..... °C

Incubation time and temperature for selective enrichment:

- at the start first period : time: ..... h ..... min
  : temperature incubator: ..... °C
- at the end first period : time: ..... h ..... min
  : temperature incubator: ..... °C
- at the start second period : time: ..... h ..... min
  : temperature incubator: ..... °C
- at the end second period : time: ..... h ..... min
  : temperature incubator: ..... °C

Type of incubator: □ vented incubator
□ nonvented incubator
□ waterbath

Thermometer used for reading the incubation temperature:

- calibrated □ YES
  □ NO
- scale division :.....

(If the temperature is recorded automatically, please provide printout of the temperatures)
Place of thermometer in the incubator:.....
Place of jars/plates in the incubator :.....

First and second isolation

1. Phenol red/brilliant green agar

Manufacturer of the phenol red/brilliant green agar:

- name : .....
- code number : .....
- batch number : .....
- expire date : .....
- pH of the medium : .....

Size of petri dishes used : □ 90 mm □ 100 mm □ 140 mm

Incubation time and temperature for **first** isolation:

- at the start : time: ..... h ..... min
  : temperature incubator: ..... °C
- at the end : time: ..... h ..... min
  : temperature incubator: ..... °C

Incubation time and temperature for **second** isolation:

- at the start : time: ..... h ..... min
  : temperature incubator: ..... °C
- at the end : time: ..... h ..... min
  : temperature incubator: ..... °C

Type of incubator: □ vented incubator
□ nonvented incubator

Thermometer used for reading the incubation temperature:

- calibrated □ YES
  □ NO
- scale division :.....

(If the temperature is recorded automatically, please provide printout of the temperatures)

Place of thermometer in the incubator:.....
Place of plates in the incubator :.....

2. <u>Isolation medium of your own choice</u>

Medium : .....

Manufacturer of your own medium:

- name : .....
- code number : .....
- batch number : .....
- expire date : .....
- pH of the medium : .....

Size of petri dishes used : □ 90 mm □ 100 mm □ 140 mm

Incubation time and temperature for **first** isolation:

- at the start : time: ..... h ..... min
  : temperature incubator: ..... °C
- at the end : time: ..... h ..... min
  : temperature incubator: ..... °C

Incubation time and temperature for **second** isolation:

- at the start : time: ..... h ..... min
  : temperature incubator: ..... °C
- at the end : time: ..... h ..... min
  : temperature incubator: ..... °C

Type of incubator: □ vented incubator
□ nonvented incubator

Thermometer used for reading the incubation temperature:

- calibrated □ YES
  □ NO
- scale division :.....

(If the temperature is recorded automatically, please provide printout of the temperatures)

Place of thermometer in the incubator:.....

Place of plates in the incubator :.....

3. <u>Isolation medium routinely used in your laboratory</u>

If you use more isolation media, please write these on an annex.

Medium : .....

Manufacturer of this medium:

- name : .....
- code number : .....
- batch number : .....
- expire date : .....
- pH of the medium : .....

Size of petri dishes used : □ 90 mm □ 100 mm □ 140 mm

Incubation temperature : ..... °C

Incubation time and temperature for **first** isolation:

- at the start : time: ..... h ..... min
  : temperature incubator: ..... °C
- at the end : time: ..... h ..... min
  : temperature incubator: ..... °C

Incubation time and temperature for **second** isolation:

- at the start : time: ..... h ..... min
  : temperature incubator: ..... °C
- at the end : time: ..... h ..... min
  : temperature incubator: ..... °C

Type of incubator: □ vented incubator
□ nonvented incubator

Thermometer used for reading the incubation temperature:

- calibrated □ YES
  □ NO
- scale division :.....

(If the temperature is recorded automatically, please provide printout of the temperatures)

Place of thermometer in the incubator:.....

Place of plates in the incubator :.....

Confirmation

Optionally

Manufacturer of the nutrient agar:

- name : .....
- code number : .....
- batch number : .....
- expire date : .....
- pH of the medium : .....

Size of petri dishes used : □ 90 mm □ 100 mm □ 140 mm

Incubation time and temperature for confirmation:

- at the start : time: ..... h ..... min
  : temperature incubator: ..... °C
- at the end : time: ..... h ..... min
  : temperature incubator: ..... °C

Type of incubator: □ vented incubator
□ nonvented incubator

Thermometer used for reading the incubation temperature:

- calibrated □ YES
  □ NO
- scale division :.....

(If the temperature is recorded automatically, please provide printout of the temperatures)

Place of thermometer in the incubator:.....

Place of plates in the incubator :.....

Biochemical confirmation

If you use more biochemical confirmation tests, please write these on an annex.

Manufacturer of TSI agar:

- name : .....
- code number : .....
- batch number : .....
- expire date : .....

Manufacturer of urea agar:

- name : .....
- code number : .....
- batch number : .....
- expire date : .....

Manufacturer of l-Lysine decarboxylation medium:

- name : .....
- code number : .....
- batch number : .....
- expire date : .....

*Table 1: Results of **first isolation** and confirmation tests (dish numbers 1-25)*

	RV [a]				MSRV [b]				Own enrichment broth			
	BGA [c]		second [d] medium		BGA		second medium		first [e] medium		second medium	
no.	col [f]	Sal [g]	col	Sal	col	Sal	col	Sal	col	Sal	col	Sal
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												
13												
14												
15												
16												
17												
18												
19												
20												
21												
22												
23												
24												
25												

[a] RV = Rappaport Vassiliadis medium
[b] MSRV = Modified Semi solid Rappaport Vassiliadis
[c] BGA = phenol red/brilliant green agar
[d] second = second isolation medium of your own choice
[e] first = first isolation medium that you routinely use
[f] col = number of colonies used for confirmation
[g] Sal = number of colonies confirmed as *Salmonella*

*Table 1 (continued): Results of **first isolation** and confirmation tests (dish numbers N1-N20)*

	RV [a]				MSRV [b]				Own enrichment broth			
	BGA [c]		second [d] medium		BGA		second medium		first [e] medium		second medium	
no.	col [f]	Sal [g]	col	Sal	col	Sal	col	Sal	col	Sal	col	Sal
N1												
N2												
N3												
N4												
N5												
N6												
N7												
N8												
N9												
N10												
N11												
N12												
N13												
N14												
N15												
N16												
N17												
N18												
N19												
N20												

[a] RV = Rappaport Vassiliadis medium
[b] MSRV = Modified Semi solid Rappaport Vassiliadis
[c] BGA = phenol red/brilliant green agar
[d] second = second isolation medium of your own choice
[e] first = first isolation medium that you routinely use
[f] col = number of colonies used for confirmation
[g] Sal = number of colonies confirmed as *Salmonella*

*Table 1 (continued): Results of **first isolation** and confirmation tests (dish numbers C1-C12)*

	RV [a]				MSRV [b]				Own enrichment broth			
	BGA [c]		second [d] medium		BGA		second medium		first [e] medium		second medium	
no.	col [f]	Sal [g]	col	Sal	col	Sal	col	Sal	col	Sal	col	Sal
C1												
C2												
C3												
C4												
C5												
C6												
C7												
C8												
C9												
C10												
C11												
C12												

[a] RV = Rappaport Vassiliadis medium
[b] MSRV = Modified Semi solid Rappaport Vassiliadis
[c] BGA = phenol red/brilliant green agar
[d] second = second isolation medium of your own choice
[e] first = first isolation medium that you routinely use
[f] col = number of colonies used for confirmation
[g] Sal = number of colonies confirmed as *Salmonella*

*Table 2: Results of confirmation tests of the **second isolation** (dish numbers 1-25)*

	RV [a]				MSRV [b]				Own enrichment broth			
	BGA [c]		second [d] medium		BGA		second medium		first [e] medium		second medium	
no.	col [f]	Sal [g]	col	Sal	col	Sal	col	Sal	col	Sal	col	Sal
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												
13												
14												
15												
16												
17												
18												
19												
20												
21												
22												
23												
24												
25												

[a] RV = Rappaport Vassiliadis medium
[b] MSRV = Modified Semi solid Rappaport Vassiliadis
[c] BGA = phenol red/brilliant green agar
[d] second = second isolation medium of your own choice
[e] first = first isolation medium that you routinely use
[f] col = number of colonies used for confirmation
[g] Sal = number of colonies confirmed as *Salmonella*

*Table 2 (continued): Results of **second isolation** and confirmation tests (dish numbers N1-N20)*

	RV [a]				MSRV [b]				Own enrichment broth			
	BGA [c]		second [d] medium		BGA		second medium		first [e] medium		second medium	
no.	col [f]	Sal [g]	col	Sal	col	Sal	col	Sal	col	Sal	col	Sal
N1												
N2												
N3												
N4												
N5												
N6												
N7												
N8												
N9												
N10												
N11												
N12												
N13												
N14												
N15												
N16												
N17												
N18												
N19												
N20												

[a] RV = Rappaport Vassiliadis medium
[b] MSRV = Modified Semi solid Rappaport Vassiliadis
[c] BGA = phenol red/brilliant green agar
[d] second = second isolation medium of your own choice
[e] first = first isolation medium that you routinely use
[f] col = number of colonies used for confirmation
[g] Sal = number of colonies confirmed as *Salmonella*

*Table 2 (continued): Results of **second isolation** and confirmation tests (dish numbers C1-C12)*

	RV [a]				MSRV [b]				Own enrichment broth			
	BGA [c]		second [d] medium		BGA		second medium		first [e] medium		second medium	
no.	col [f]	Sal [g]	col	Sal	col	Sal	col	Sal	col	Sal	col	Sal
C1												
C2												
C3												
C4												
C5												
C6												
C7												
C8												
C9												
C10												
C11												
C12												

[a] RV = Rappaport Vassiliadis medium
[b] MSRV = Modified Semi solid Rappaport Vassiliadis
[c] BGA = phenol red/brilliant green agar
[d] second = second isolation medium of your own choice
[e] first = first isolation medium that you routinely use
[f] col = number of colonies used for confirmation
[g] Sal = number of colonies confirmed as *Salmonella*

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

Date: ..... - ..... - 1999

Name of technician/technologist carrying out the third bacteriological collaborative study:

.....

signature:.....

Name of person in charge:

.....

signature:.....

Appendix 5 Data about media used

Table 1 Manufacturer of BPW from participants

Labcode	Manufacturer BPW	Code
1	OXOID	CM 509
2	OXOID	CM 509
3	Merck	1.07228
4	LAB M	LAB 46
5	AES	AEB 140 302
6	Merck	1.07228
7	Merck	1.07228
8	LAB M	LAB 46
9	Own preparation	
10	Biomerieux	42043
11	SVM	E4900z
12	Merck	1.07228
13	Own preparation	
14	OXOID	CM 509
15	Merck	1.07228
16	OXOID	CM 509

Table 2 Manufacturer of Standard selective enrichment

Labcode	Manufacturer RV	Code
1	Merck	1.0770 (=RVS)
2	OXOID	CM 669
3	OXOID	CM 866 (=RVS)
4	LAB M	LAB 86 (=RVS)
5	OXOID	CM 866 (=RVS)
6	OXOID	CM 669
7	Merck	1.0770 (=RVS)
8	LAB M	LAB 86 (=RVS)
9	OXOID	CM 669
10	Biomerieux	42073
11	OXOID	CM 669
12	OXOID	CM 669
13	OXOID	CM 866(=RVS)
14	Own preparation	
15	OXOID	CM 669
16	OXOID	CM 669

Table 3 Manufacturer of MSRV from participants

Labcode	Manufacturer MSRV	Code
1	OXOID	CM 910
2	LAB M	LAB 150
3	OXOID	CM 910
4	LAB M	LAB 150
5	BIOKAR	BK 137
6	OXOID	CM 910
7	Merck	1.09878.0500
8	LAB M	LAB 150
9	OXOID	CM 910
10	OXOID	CM 910
11	Difco	1868-17
12	Merck	1.09878.0500
13	OXOID	CM 910
14	OXOID	CM 910
15	LAB M	LAB 150
16	OXOID	CM 910

Table 4 Manufacturer of optional further selective enrichments

Labcode	Name medium	Manufacturer	Code
1	TBG Bouillon (Muller Kaufmann)	Merck	1.05178
2	Selenite Lodox broth	OXOID	TW50051
3	MSRV + additions[1)] Selenite Cystine Tetrationat novobiocin[2)]	OXOID Merck OXOID	CM 910 1.07709 CM 671
4	DIASALM + supplement	LAB M	LAB 537
5	Muller Kauffman	AES	AEB 140702
6			
7			
8	Selenite Cystine		
9	RVS	LAB M	LAB 86
10			
11	DIASALM + novobiocine	LAB M	LAB 537
12	Rappaport broth	Biomerieux	42091
13	Tetrationat broth + additions[2)]	OXOID	CM 671
14			
15	DIASALM + novobiocine	Merck	109803
16			

1) additions: Bromcresolpurpur, saccharose and novobiocine
2) Tetrationat, novobiocine, iodine and potassium-iodine

Table 5 Manufacturer of BGA

Labcode	Manufacturer BGA	Code
1	OXOID	CM329
2	OXOID	CM329
3	OXOID	CM329
4	LAB M	LAB 34
5	AES	AEB 151492
6	Merck	1.10747
7	Merck	7447.0500
8	LAB M	LAB 34
9	OXOID	CM329
10	OXOID	CM329
11	SVM	E1800y
12	Difco	0285-17-7
13	Merck	1.07237
14	OXOID	CM329
15	OXOID	CM329
16	OXOID	CM329

Table 6 Manufacturer of second isolation medium

Labcode	Name medium	Manufacturer	Code
1	XLD [1)]	Becton Dickinson	4311838
2	XLT-4 [2)] + supplement	Difco	0234 17
3	Rambach	Merck	1.07500
4	Önöz	Merck	1.15037
5	Rambach	Merck	1.07500
6	XLD	Merck	1.05287
7	Rambach	Merck	1.07500
8	MLCVBG[3)]	LAB M	LAB 116
9	XLT-4	Difco	0234 17
10	SMID	Biomerieux	432 91
11			
12	SMID	Biomerieux	432 91
13	XLT-4	Merck	113919
14	XLD + novobiocine	BBL	4311838
15	Rambach	Merck	1.07500
16	Rambach	Merck	1.07500

1) Xylose Lysine Deoxycholaat
2) Xylose Tergitol
3) Mannitol Lysine Crystal Violet Brilliant Green

Table 7 Manufacturer of isolation medium routinely used

Labcode	Name medium	Manufacturer	Code
1	Rambach Agar	Merck	13078
2			
3			
4	Rambach	Merck	1.07500
5	XLT-4	Difco	0234-17
6	Gassner	Merck	1282
7			
8			
9			
10			
11			
12	SMID	Biomerieux	43291
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15			
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Appendix 6 Calculated coefficients for statistical analysis

Table 1 Estimated value of laboratories using RV with regard to average of all other laboratories that used RV

Labcode	Estimate (E)	Standard Error (StE)	E - 1.96*StE	E + 1.96*StE
2	0.82	0.44	-0.05	1.68
6	0.86	0.43	0.02	1.69
9	-2.75	0.78	-4.28	-1.23
10	-1.29	0.50	-2.26	-0.31
11	-2.75	0.78	-4.28	-1.23
12	0.50	0.42	-0.33	1.33
14	1.23	0.44	0.37	2.09
15	1.18	0.43	0.35	2.02
16	-0.40	0.43	-1.24	0.44

Table 2 Estimated value of laboratories using RVS with regard to average of all other laboratories that used RVS

Labcode	Estimate (E)	Standard Error (StE)	E - 1.96*StE	E + 1.96*StE
1	-0.25	0.38	-0.99	0.48
4	1.00	0.53	-0.05	2.05
5	-2.75	0.78	-4.28	-1.23
7	2.13	0.47	1.21	3.04
8	1.64	0.60	0.46	2.81
13	1.82	0.47	0.89	2.75

Table 3 Estimated value of laboratories using MSRV with regard to average of all other laboratories

Labcode	Estimate (E)	Standard Error (StE)	E - 1.96*StE	E + 1.96*StE
1	0.28	0.37	-0.44	0.99
2	-0.27	0.38	-1.01	0.48
3	0.56	0.38	-0.19	1.29
4	1.07	0.55	-0.01	2.14
5	-0.19	0.41	-1.00	0.62
6	0.19	0.41	-0.62	0.99
7	1.19	0.42	0.36	2.02
8	0.77	0.52	-0.25	1.79
9	-3.36	0.77	-4.86	-1.86
10	-2.26	0.52	-3.27	-1.25
11	0.51	0.50	-0.47	1.50
12	0.93	0.42	0.09	1.76
13	-0.71	0.39	-1.47	0.06
14	1.10	0.45	0.21	1.99
15	1.19	0.43	0.35	2.02
16	0.55	0.38	-0.19	1.29

Table 4 Estimated value of laboratories (all methods) with regard to average of all other laboratories

Labcode	Estimate (E)	Standard Error (StE)	E - 1.96*StE	E + 1.96*StE
1	-0.02	0.25	-0.52	0.48
2	0.01	0.26	-0.50	0.51
3	-0.31	0.27	-0.85	0.23
4	0.80	0.32	0.18	1.42
5	-2.63	0.36	-3.35	-1.92
6	0.51	0.29	-0.05	1.07
7	1.53	0.31	0.93	2.13
8	1.06	0.35	0.37	1.75
9	-2.68	0.35	-3.36	-2.00
10	-1.70	0.35	-2.38	-1.02
11	-0.55	0.30	-1.14	0.05
12	0.68	0.29	0.12	1.24
13	0.73	0.25	0.25	1.21
14	1.11	0.30	0.52	1.69
15	1.05	0.24	0.57	1.52
16	0.23	0.26	-0.27	0.73