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

Bacteriological collaborative study V amongst the National Reference Laboratories for *Salmonella*

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Abstract

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

A fifth bacteriological collaborative study organised by the Community Reference Laboratory for *Salmonella* had as participants the National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*) of the EU Member States. The objectives were:

1. To evaluate the results of the detection of different contamination levels of *Salmonella* Enteritidis (100 and 500 cfp) and *Salmonella* Typhimurium (10 and 100 cfp) in the presence of competitive micro-organisms among and within the NRLs-*Salmonella*, and
2. To evaluate MSRV as selective enrichment medium compared to RV, used as the selective enrichment medium in the standard method.

An adapted ISO 6579 (proposed reference) method was used in combination with MSRV 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 than with the STM10, SE100 and SE500 capsules. The number of positive isolations from the SE100 capsules was also significantly lower than the number of positive isolations from the STM10 and SE500 capsules. Significantly more positive isolations were obtained using MSRV compared to using RV. Significant differences were also found between laboratories. When compared with RV, the use of MSRV led to significantly more positive isolations from the naturally contaminated samples containing *S*. Enteritidis.

Contents

List of Abbreviations

BGA	Brilliant Green Agar
BPW	Buffered Pepton Water
CRL	Community Reference Laboratory
DIASALM	Diagnostic Semi-solid *Salmonella* medium (DIA)
dPCA	double concentrated Plate Count Agar
HCMP	Highly Contaminated Milk Powder
ISO	International Organization of Standardization
MK	Muller Kauffmann broth
MSRV	Modified Semi-solid Rappaport Vassiliadis
MSRV+	MSRV with bromcresolpurpur and saccharose
NRL	National Reference Laboratory
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
Rap	Rappaport broth
RM	Reference Material
RT	Room Temperature
RV	Rappaport Vassiliadis
RVS	Rappaport Vassiliadis Soya broth
SC	Selenite Cystine
SE	*Salmonella* Enteritidis
STM	*Salmonella* Typhimurium
TBG	Tetrathionate-Brilliant-green Bile broth
XLD	Xylose Lysine Deoxycholate agar
XLT-4	Xylose Lysine Tergitol 4 agar

Samenvatting

Het Communautair Referentie Laboratorium voor *Salmonella* (CRL-*Salmonella*) heeft een vijfde bacteriologisch ringonderzoek georganiseerd betreffende de detectie van *Salmonella*. De deelnemers waren de Nationale Referentie Laboratoria voor *Salmonella* (NRLs-*Salmonella*) uit de lidstaten van de Europese Unie en een deelnemer uit Noorwegen.
Dit vijfde ringonderzoek had twee belangrijke doelen. Het ene doel was het vergelijken van de behaalde resultaten met 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 Modified Semi-solid Rappaport Vassiliadis (MSRV) en eventuele andere gebruikte selectieve ophopingsmedia ten opzichte van het ISO medium Rappaport Vassiliadis (RV).
Vijf en dertig individueel genummerde capsules en 20 natuurlijk besmette monsters werden door alle deelnemers onderzocht op de aanwezigheid van *Salmonella*. Vijf en twintig van de capsules moesten onderzocht worden in combinatie met 10 gram kippenfeces. 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 500 kve SE en 5 blanco capsules. De overige 10 capsules, waaraan geen feces moest worden toegevoegd, waren controle capsules.
De capsules werden getest met een van de ISO 6579 afgeleide methode, met daarnaast MSRV als selectief ophopingsmedium en Xylose Lysine Deoxycholaat agar (XLD) als isolatiemedium. Eventueel kon een laboratorium ook de eigen media voor de detectie van *Salmonella* in kippenfeces gebruiken naast de voorgeschreven media.

Detectie van *Salmonella* uit de capsules met 100 kve STM (STM100) gaf significant betere resultaten dan detectie uit de capsules met 10 kve STM (STM10), 100 kve SE (SE100) en 500 kve SE (SE500). Van deze laatste drie werden de significant slechtste resultaten verkregen met de SE100 capsules. Voor alle typen van capsules was de spreiding van positieven 0-5 tussen de laboratoria bij het gebruik van MSRV als selectief ophopingsmedium. Negen van de laboratoria (53%) isoleerden *Salmonella* uit alle vijf de STM100 capsules met MSRV. Voor STM10, SE100 en SE500 waren dit respectievelijk 3 (19%), 2 (13%) en 5 (31%) laboratoria. Echter, bij het gebruik van alleen RV of RVS werden alleen vijf positieven gevonden bij de STM100 en SE500 capsules.
Twee laboratoria isoleerden *Salmonella* uit een blanco capsule met RV als selectief ophopingsmedium. Drie laboratoria konden geen *Salmonella* isoleren uit een *S.* Panama capsule waaraan geen feces hoefde te worden toegevoegd. Eén laboratorium kon geen *Salmonella* isoleren uit één van de drie STM10 controle 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 en zowel tussen als binnen de laboratoria. Tevens werden significante verschillen gevonden tussen laboratoria.

Summary

The Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*) organised a fifth collaborative study on bacteriological detection of *Salmonella*. Participants were the National Reference Laboratories for *Salmonella* (NRL-*Salmonella*) of the EU Member States and a laboratory from Norway.
This fifth collaborative study had two main objectives. First, to compare the results obtained with the different levels of contamination and different serotypes in the presence of competitive micro-organisms between and within laboratories. Second, to compare the results obtained with Modified Semi-solid Rappaport Vassiliadis (MSRV) and other selective enrichments with the results obtained with the ISO medium Rappaport Vassiliadis (RV).
Thirty five individually numbered capsules and 20 naturally contaminated samples 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 chicken faeces. The 25 capsules contained 10 colony forming particles (cfp) of *Salmonella* Typhimurium (STM) (n=5), 100 cfp of STM (n=5), 100 cfp of *Salmonella* Enteritidis (SE) (n=5), 500 cfp SE (n=5) or were blank capsules (n=5). The other 10 capsules, to which no faeces had to be added, were control capsules. The capsules were tested using an adapted ISO6579 method. As additional media MSRV for selective enrichment and Xylose Lysine Deoxycholate agar (XLD) for isolation were used. Optionally, the participants additionally could also use their own media for detection of *Salmonella* in chicken faeces.

Detection of *Salmonella* from capsules containing 100 cfp STM (STM100) revealed significantly more positive isolations than the isolation from capsules containing 10 cfp STM (STM10), 100 cfp SE (SE100) and 500 cfp SE (SE500). From the latter three, significantly less positive isolations were obtained with the SE100 capsules. For all types of capsules the number of positive isolations between laboratories varied from 0-5 with the use of MSRV as selective enrichment. Nine of the laboratories (53%) isolated *Salmonella* from all 5 STM100 capsules with the use of MSRV. For the STM10, SE100 and SE500 capsules respectively 3 (19%), 2 (13%) and 5 (31%) of the participants isolated *Salmonella* from all 5 capsules. However, with the use of RV or RVS only, five positive isolations were obtained only with the STM100 and SE500 capsules.
Two laboratories isolated *Salmonella* from a blank capsule with the use of RV as selective enrichment. Three laboratories could not isolate *Salmonella* from an *S.* Panama capsule to which no faeces was added. One laboratory could not isolate *Salmonella* from one of the STM10 control capsules.
With the use of MSRV as selective enrichment, significant better results were obtained in comparison with RV for the artificially as well as the naturally contaminated samples. Also between as well as within the laboratories, better results for MSRV were obtained. Also between the participants, significant differences were found.

1. Introduction

In pursuance of the Council Directive 92/117/EEC the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*) organises bacteriological collaborative studies with the objective that the examination of samples in Member States is carried out uniformly and that comparable results will be obtained.
Earlier studies have shown a significantly reduced number of positive isolations using Selenite/Cystine broth (SC) and significant better results with the use of MSRV compared to the use of Rappaport-Vassiliadis broth (RV) as selective enrichment medium. Therefore, in the fourth study, all laboratories had to use the selective enrichment medium Modified Semi-solid Rappaport Vassiliadis (MSRV), in addition to the reference method.

The fifth study, described in this report, had the same composition of samples to be examined as the fourth study. Also, the prescribed selective enrichment media and one of the isolation media were the same as in the fourth study. A same sample composition and a same method was chosen because it enables the CRL-*Salmonella* to follow the performance of the NRLs-*Salmonella* over time. During the CRL-*Salmonella* workshop (3) it was agreed that laboratories could also perform PCR on the pre-enrichment culture.
In total 4 different types of contaminated capsules had to be examined, containing 2 levels of *Salmonella* Typhimurium and 2 levels *Salmonella* Enteritidis. Furthermore, 20 naturally contaminated faeces samples had to be examined. The prescribed media for selective enrichment were RV(S) and MSRV, for isolation Brilliant Green Agar (BGA) and Xylose Lysine Deoxycholate Agar (XLD) were prescribed.

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	AFSSA Ploufragan Unité Hygiene et Qualitédes Produits avicoles 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
Luxembourg	Laboratoire de Médecine vétérinaire de l'Etat (animal zoonosis) Luxembourg
The Netherlands	National Institute of Public Health and the Environment (RIVM) Bilthoven
Norway	National Veterinary Laboratory Department of Bacteriology Oslo

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 Department of Bacterial Diseases Surrey
Northern Ireland	Department of Agriculture for Northern Ireland Veterinary Sciences Division; Bacteriology Department Belfast

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 Highly Contaminated Milk Powder (HCMP) containing *Salmonella* Typhimurium (STM) and *Salmonella* Enteritidis (SE), of which the low contaminated powders were mixed as described by Voogt et al. (6), was obtained by spray-drying artificially contaminated milk as described by In 't Veld et al. (5).
To obtain the target levels, the HCMP 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. The target levels were 10 and 100 colony forming particles (cfp) STM (STM10 and STM100) and 100 and 500 cfp SE (SE100 and SE500) per capsule.
Sixty capsules of each serotype and each level of contamination were prepared. These capsules were used to determine the number of cfp per capsule and the homogeneity of the batch of powder. After approval of the level of contamination and the homogeneity of the powder, the capsules needed for the collaborative study were prepared.
The contamination levels of the capsules containing 10 and 100 cfp per capsule were determined by dissolving the capsules in 5 ml peptone saline solution in a Petri dish and pouring of 5 ml double concentrated plate count agar (dPCA). The higher contaminated capsules (500 cfp/capsule) were dissolved in 10 ml of peptone saline. Of this solution, two times one millilitre was transferred into a petri dish and 10 ml Plate Count Agar (PCA) was added. After addition of dPCA/PCA the plates were incubated for four hours at 37°C. After incubation, 10 ml double concentrated Violet Red Bile Glucose agar was poured as an overlayer and the plates were incubated overnight at 37°C.

3.1.2 Samples containing competitive micro-organisms

Chicken faeces was tested for presence/absence (p/a) of *Salmonella* according to the routine method (RV and DIASALM as selective enrichment) of the National Institute of Public Health and the Environment, Bilthoven, the Netherlands. In addition to the routine method also MSRV was used to test the faeces. From a poultry laying flock, which was found negative for *Salmonella*, over three kilograms of faeces was used to prepare the samples containing competitive micro-organisms. Faeces of a poultry laying flock found bacteriologically positive for *Salmonella* was used to prepare the naturally contaminated samples.
The faeces was mixed and homogenised with sterilised glycerol/peptone solution (mixing ratio 1:1 g/l) before freezing in order to stabilise the micro-organisms. One litre of glycerol/peptone solution consisted of 300 ml glycerol, 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 (35 individually numbered capsules) and 850 gram of faeces were mailed with cooling devices by cargo freight to the participants. The faeces was divided in 5 individually numbered (1-5) portions of ca 60 gram (*Salmonella* negative) and 5 individually numbered (6-10) portions of ca 110 gram (naturally contaminated).
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.
Twenty-five capsules had to be tested in combination with chicken faeces and 10 capsules had to be tested without faeces as described in the SOP. The content of the capsules is shown in Table 1. The test results and operational details had to be reported to the CRL as proposed in the test report.

Table 1: Overview of the content of the capsules, the number of samples tested and the number of the capsules in the study

Faeces added (n=25)		No faeces added (n=10)	
Capsule	Number of samples tested (numbers in study)	Capsule	Number of samples tested (numbers in study)
STM10	5 (9, 13, 15, 21, 22)	STM10	3 (C2, C8, C9)
STM100	5 (7, 16, 19, 23, 24)	SE100	3 (C1, C3, C7)
SE100	5 (3, 6, 10, 14, 17)	*S.* Panama	2 (C4, C6)
SE500	5 (1, 4, 11, 20, 25)	Blank	2 (C5, C10)
Blank	5 (2, 5, 8, 12, 18)		

Blank: Capsule contained no cfp
S. Panama: Capsule contained 5 cfp *S*. Panama

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 8.1 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 (p/a testing), 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. The laboratories that obtained 0 positive isolations are presented as a dot in the figures.
The variation in observations between different reconstituted capsules of one batch (T_2) were tested. In case of an ideal homogeneous distribution (Poisson distribution), T_2 follows a χ^2 distribution with (I-1) degrees of freedom and a value of $T_2/(I-1)$ of 1 is expected (1).

3.4 Quality assurance participants

Of all participants, seven laboratories (labcode 1, 2, 8, 9, 12, 13 and 16) have a certified quality assurance certificate (Table 2). For statistical evaluation, the presence of a certified quality system was tested, assuming a value of 1 for laboratories that have a certificate and a value of 0 for all other laboratories. Several laboratories are preparing to obtain certification (labcode 5, 6, 7 and 15).

Table 2: Type of certified quality system for bacteriological detection of the six certified NRLs-Salmonella

Name certificate
Cofrac Certification = program no 59
EN45001 (2 laboratories)
EN45001 and ISO/IEC Guide25 (2 laboratories)
ISO9001
UKAS Accreditation

3.5 PCR

The possibility to use PCR was used by 4 laboratories (labcode 1, 7, 10 and 13). All laboratories used different volumes from the pre-enrichment for extraction. Table 3 shows the volumes used, the volume of the DNA-extract and the actual volume of the BPW used in PCR reaction.

Table 3: Dilution factor of the DNA extract compared to the BPW culture

labcode	Volume of BPW (ml)	Volume of DNA extract (ml)	Volume used in PCR reaction (µl)	Actual volume of BPW tested in PCR (µl)
1	1	0.1	5	50
7	1	1	2	2
10	1	0.3	5	17
13	2	0.05	5	200

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 seven weeks before the study, as well as the levels during the study, are presented in Table 4. The values for the homogeneity of the batch are also shown in Table 4. The average level of contamination for the STM10 capsules was 3.8 (1x0, 5x1, 9x2, 11x3, 4x4, 6x5, 8x6, 2x7, 1x8 and 1x9 cfp/capsule) during the study. The STM100 capsules had a level of contamination of 47 cfp per capsule (min. 31 - max. 69). The SE100 and SE500 capsules had a level of contamination of respectively 63 (min. 46 - max. 91) and 450 (min. 390 - max. 550) cfp per capsule.

Table 4: Level of contamination and homogeneity of capsules before and during study

	Before collaborative study		At the time of collaborative study	
Capsules	Average no. of cfp per capsule	$T_2/(I-1)$	Average no. of cfp per capsule	$T_2/(I-1)$
STM10	5.5	0.72	3.8	1.14
STM100	90	1.15	47	2.21
SE100	102	3.38	63	1.91
SE500	481	1.54	450	1.58

4.1.2 Naturally contaminated faeces samples

The naturally contaminated faeces used in the collaborative study (week 37 of year 2000) was collected from two different flocks in week 21 and 25 of 2000. Before freezing two out of three samples of week 21 and four out of five samples of week 25 were found positive for *Salmonella*. Serotypes isolated from the faeces of week 21 were *S.* Ruiri, *S.* Tennessee and *S.* Cubana. From faeces of week 25, *S.* Enteritidis PT4, PT35 and *S.* Livingstone were isolated. According to the most recent Kauffman & White scheme (2), the serotypes isolated from faeces of week 21 belong to the groups C1 and D, and the serotypes from faeces of week 25 belong to the groups C1, G and L.

The samples were tested several weeks after freezing (Table 5). Faeces samples from week 21 were found positive with MSRV each time tested. Faeces from week 25 was tested three times and found positive twice using MSRV.

Table 5: Stability of naturally contaminated samples at –20°C

Number of positive samples after several weeks in freezer with different selective enrichments / number of samples tested								
Weeks in freezer	1		3	5		7	9	11
	21[1)]	25[2)]	25	21	25	25	21	21
RV	0/3	1/2	0/2	2/2	0/2	0/4	2/2	¾
DIASALM	3/3	1/2	2/2	0/2	2/2	0/4	1/2	¼
MSRV	3/3	1/2	2/2	2/2	2/2	0/4	2/2	4/4

1) Faeces week 21 (portion 7, 8 and 10 in study)
2) Faeces week 25 (portions 6 and 9 in study)

4.2 Collaborative study

One laboratory (labcode 6) did not examine the samples in their own laboratory, but instead have sent the samples to another laboratory in their country.
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 8).

4.2.1 Control samples

None of the laboratories isolated *Salmonella* from the procedure or faeces control (C11, blank and C12, faeces from portion 3). One laboratory (labcode 7) found all positive control capsules (C1-C10) negative using SC. However, using RV or MSRV this laboratory isolated *Salmonella* from all positive control capsules.

Blank capsules

Two laboratories (labcode 13 and 15) isolated *Salmonella* from a blank capsule using RV. With the other media used, this sample was found negative.

S. Panama

Three laboratories (3, 4 and 9) failed to isolate *Salmonella* from an *S.* Panama capsule with all of the enrichment media used.

STM10

Laboratory 4 failed to isolate *Salmonella* from one of the control capsules containing 10 cfp STM with all media used. From another STM10 control capsule this laboratory isolated *Salmonella* with the use of RV, but did not isolate *Salmonella* with the use of MSRV or Rappaport.

4.2.2 Incubation time and temperature of pre-enrichment

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 10. This laboratory incubated the capsules for 55 minutes. Laboratories 3, 4, 6, 8, 10, 11 and 13 did not preheat the BPW to 37°C as prescribed before adding the capsules.

With the use of RV(S), laboratories that used BPW of 37°C, obtained significantly more positive isolations from the total number of capsules in comparison with the laboratories that used BPW of room temperature (RT) (Table 6). However, evaluating this for each of the type of the capsules separately reveals that this difference is significant for the STM100 capsules only. Using MSRV, no significant differences were found between preheated BPW and BPW of RT for the total results nor for the capsules separately.
For the total number of naturally contaminated samples no significant difference is seen between laboratories preheating or not preheating the BPW. Evaluating the results of the naturally contaminated samples per week, reveals better results if BPW is not preheated for the samples originating from week 25 (containing SE). For the samples originating from week 21, no significant differences were revealed.

Table 6: Percentage of positives obtained with BPW of room temperature or BPW of 37°C

Sample / capsule	**Percentage** of positive isolations per selective enrichment			
	BPW of RT in RV(S)	BPW of 37°C in RV(S)	BPW of RT onto MSRV	BPW of 37°C onto MSRV
Faeces from week 21	23	32	24	38
Faeces from week 25	18	11	38	25
Week 21 + 25	21	29	29	33
STM10	40	38	51	58
STM100	54	67	77	76
SE100	3	16	29	27
SE500	14	24	57	56
Total capsules	28	36	54	54

Significant difference between BPW preheated or room temperature

Except for five laboratories all laboratories incubated the pre-enrichment for the prescribed time (16 - 20 hours). The laboratories 4, 6, 9, 10 and 14 incubated for 21 h 30 min, 23 h 45 min, 20 h 45 min, 22 h and 23 h 45 min, respectively.
For the artificially contaminated samples, significantly less positive isolations were obtained for the STM100 capsules by laboratories incubating >20 hours (Table 7). Also significantly less positive isolations were obtained by laboratories incubating >20 hours for the SE500 capsules with the use of MSRV. No significant difference was found with the use of RV(S) for the SE capsules.
With respect to the naturally contaminated samples, no significant difference was observed for the duration of incubation with either RV(S) or MSRV.

Table 7: Comparison of incubation time of BPW

Sample / capsule	**Percentage** of positive isolations per selective enrichment			
	16-20 hours in RV(S)	> 20 hours in RV(S)	16-20 hours onto MSRV	> 20 hours onto MSRV
Faeces from week 21	30	23	33	27
Faeces from week 25	18	5	40	10
Week 21 + 25	25	16	35	20
STM10	44	28	60	44
STM100	67	48	82	64
SE100	5	20	29	24
SE500	16	28	65	36
Total capsules	33	31	59	42

Significant difference between BPW incubated for 16-20 h or >20 h

4.3 Selective enrichment media

In total 9 different selective enrichment media and the Polymerase Chain Reaction (PCR) were used by the laboratories.

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

The RV(S) and MSRV had to be incubated for two subsequent periods of 24 hours. Six laboratories did not incubate the prescribed selective enrichment media for a total of 48±2 hours. Laboratory 14 and 15 incubated the RV in total for respectively 40 h 10 min and 43 hours. The RVS was incubated for 42 h 45 min, 44 h 55 min, 42 h 55 min and 43 h 30 min by laboratories 2, 5, 7 and 10 respectively. The MSRV was incubated by laboratories 7, 2, 15, 10 and 5 for 43 h 15 min, 44 h 10 min, 45 h 25 min, 43 h 30 min and 43 h respectively.

4.4 Comparison of selective enrichment media for the artificially contaminated samples

4.4.1 Comparison of selective enrichments between laboratories

The cumulative results per selective enrichment used for the artificially contaminated samples are shown in Table 8.

RV

The results obtained with the different selective enrichment media compared to the results obtained with RV (35% positive), revealed significantly better results for MSRV (54%), MSRV+ (80%), DIASALM (52%) and Rappaport (45%). Significantly less positive isolations were obtained using SC (0%) for selective enrichment and PCR (13%) compared to the use of RV.

RVS

Selective enrichment using SC (0%) and the use of PCR (13%) obtained significantly less positive isolations in comparison with RVS (34% positive). Significantly more positive isolations were obtained using MSRV (54%), MSRV+ (80%), DIASALM (52%) and Rappaport (45%).

MSRV

Comparing the results of all selective enrichment media with the results obtained with MSRV (54%) also revealed significant differences. Significantly less positive results were obtained using RV (35%), RVS (34%) and SC (0%) for selective enrichment and using PCR (13%) compared to MSRV. Significantly more positive isolations were obtained using MSRV+. There were no significant differences between MSRV, DIASALM (52%), Rappaport (45%), TBG (40%) and MK(40%).

Table 8: Cumulative results obtained with different selective enrichment media for the artificially contaminated samples

	Number of laboratories using selective enrichment and number of positive samples obtained with selective enrichment									
	MSRV+	MSRV	DIA	Rap	TBG	MK	RV	RVS	PCR	SC
n laboratories	1	16	3	1	1	1	8	9	4	3
n positive	16	172	31	9	8	8	56	68	10	0
% positive	80	54	52	45	40	40	35	34	13	0

4.4.2 Comparison of selective enrichments within laboratories

The results obtained within the laboratories are shown in Table 9 (prescribed method) and Table 10 (own enrichment).

RV

Five (labcode 1, 3, 4, 8 and 15) out of 8 laboratories using RV (Table 9) obtained significantly more positive isolations with the use of MSRV. In addition to MSRV, laboratories 4 and 8 obtained significantly more positive isolations using Rappaport and

DIASALM respectively. Laboratory 9 obtained significantly less positive isolations using RVS in comparison with RV.

RVS

Five (labcode 2, 5, 9, 11 and 13) out of 9 laboratories using RVS (Table 9) obtained significantly more positive isolations with the use of MSRV. Significantly less positive isolations were obtained using SC by laboratory 2. Significantly more positive isolations were also obtained using MSRV+ (labcode 2), MK and PCR (labcode 13) and RV (labcode 9).

MSRV

No laboratory obtained significantly more positive isolations with an enrichment other than MSRV. Laboratory 8 and 14 obtained more positive isolations using DIASALM, however this difference was not significant.

Table 9: Number of positive results per laboratory obtained with prescribed selective enrichments used for the artificially contaminated samples

	Number of positives with prescribed selective enrichment (n=20) per labcode															
Medium	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
RV	5		0	0				8	18					1	9	15
RVS		8			9	11	5		9	1	10	0	4			
MSRV	11	19	6	13	14	8	11	16	19	1	17	0	8	1	14	14

Laboratory 9: Standard method was RV; RVS also used as selective enrichment

Table 10: Number of positive results obtained with laboratory's own selective enrichments used for the artificially contaminated samples

Medium /	Number of positives with prescribed selective enrichment (n=20) per labcode								
Method	1	2	4	6	7	8	10	13	14
TBG				8					
SC		0			0		0		
MSRV+		16							
MK								8	
DIASALM	11					18			2
Rap			9						
PCR	2				0		0	8	

4.4.3 Comparison of samples between laboratories

The number of positive isolations from the capsules obtained with the prescribed procedure was 0-5 for all types of capsules.

RV

Eight laboratories used RV for selective enrichment as reference method (Table 11). Three of these laboratories (labcode 9, 15 and 16 = 38%) isolated *Salmonella* from all STM100

capsules and two laboratories (labcode 9 and 16 = 25%) isolated *Salmonella* from all SE 500 capsules with the use of RV. For the STM10 and SE100 capsules the number of positives per laboratory was 0-4 with the use of RV.

RVS

Nine laboratories used RVS for selective enrichment as reference method (Table 12). Six of these laboratories (labcode 2, 5, 6, 7, 9 and 11= 67%) isolated *Salmonella* from all 5 STM100 capsules with the use of RVS. No laboratory isolated *Salmonella* from all 5 STM10, SE100 and SE500 capsules. The number of positive isolations from the STM10 and SE100 capsules varied from 0 to 4. From the SE500 capsules the number of positives varied from 0 to 2.

MSRV

Using MSRV, revealed 5 positive isolations by at least one laboratory for each type of capsules (Table 11 + 12). A total of nine laboratories (56%) (labcode 1, 2, 4, 5, 7, 9, 11, 15 and 16) isolated *Salmonella* from all 5 STM100 capsules; Five laboratories (31%) (labcode 2, 8, 9, 15 and 16) isolated *Salmonella* from all 5 SE500 capsules; A positive result for the 5 STM10 capsules was obtained by 3 laboratories (19%) (labcode 1, 2 and 3); And two laboratories (labcode 8 and 9) isolated *Salmonella* from all five SE100 capsules (13%).

Table 11: Number of positive isolations with the use of RV and MSRV per type of capsule.

Labcode	STM10 (n=5)		STM100 (n=5)		SE100 (n=5)		SE500 (n=5)		Total (n=20)	
	RV	MSRV	RV	MSRV	RV	MSRV	RV	MSRV	RV	MSRV
1	2	5	3	5	0	0	0	1	5	11
3	0	1	0	3	0	0	0	2	0	6
4	0	5	0	5	0	1	0	2	0	13
8	3	2	3	4	0	5	2	5	8	16
9	4	4	5	5	4	5	5	5	18	19
14	0	0	1	1	0	0	0	0	1	1
15	4	4	5	5	0	0	0	5	9	14
16	2	3	5	5	3	1	5	5	15	14
Total	15 (38%)	24 (60%)	22 (55%)	33 (83%)	7 (18%)	12 (30%)	12 (30%)	25 (63%)	56 (35%)	94 (56%)

Table 12: Number of positive isolations with the use of RVS and MSRV per type of capsule.

Labcode	STM10 (n=5)		STM100 (n=5)		SE100 (n=5)		SE500 (n=5)		Total (n=20)	
	RVS	MSRV	RVS	MSRV	RVS	MSRV	RVS	MSRV	RVS	MSRV
2	3	5	5	5	0	4	0	5	8	19
5	4	4	5	5	0	1	0	4	9	14
6	3	2	5	4	1	0	2	2	11	8
7	0	3	5	5	0	0	0	3	5	11
9	4	4	5	5	0	5	0	5	9	19
10	0	0	1	1	0	0	0	0	1	1
11	4	4	5	5	0	4	1	4	10	17
12	0	0	0	0	0	0	0	0	0	0
13	2	2	1	3	0	1	1	2	4	8
Total	20 (44%)	24 (53%)	32 (71%)	33 (73%)	1 (2%)	15 (33%)	4 (9%)	25 (56%)	57 (32%)	97 (54%)

For STM10 capsules, the results obtained with RV and RVS are not significantly different compared to MSRV. For the STM100 capsules, the results obtained with MSRV are significantly better than the results obtained with RV. For the SE100 capsules the results obtained with MSRV are significantly better than the results obtained with RVS. For the SE500 capsules the results obtained with MSRV are significantly better than the results obtained with RV and better than the results obtained with RVS.

Significantly more positive isolations were obtained with the STM100 (76%) capsules compared to the STM10 (55%) and SE500 (56%) capsules (Table 13). These latter capsules showed no significant difference in number of positive isolations. The number of positive isolations from the SE100 capsules (23%) was significantly lower compared to the number of positive isolations from the STM10 and SE500 capsules.

Table 13: Number of positives with standard method (RV+RVS) compared to the number of positives using MSRV by all laboratories

	STM10 (n=80)		STM100 (n=80)		SE100 (n=80)		SE500 (n=80)		Total (n=320)	
	n_{pos}	%	n_{pos}	%	n_{pos}	%	n_{pos}	%	n_{pos}	%
Total RV(S)	31	39	49	61	8	10	16	20	104	33
Total MSRV	44	55	61	76	22	23	45	56	172	54

4.4.3.1 Comparison of laboratories using RV

Comparing the results obtained with RV as a standard medium per laboratory with the average of all other laboratories that used RV for selective enrichment as a standard medium revealed significant differences (Figure 1). Laboratories 3, 4 and 14 obtained significantly less positive isolations and laboratory 16 obtained significantly more positive isolations using RV with regard to the average of the other laboratories using RV.

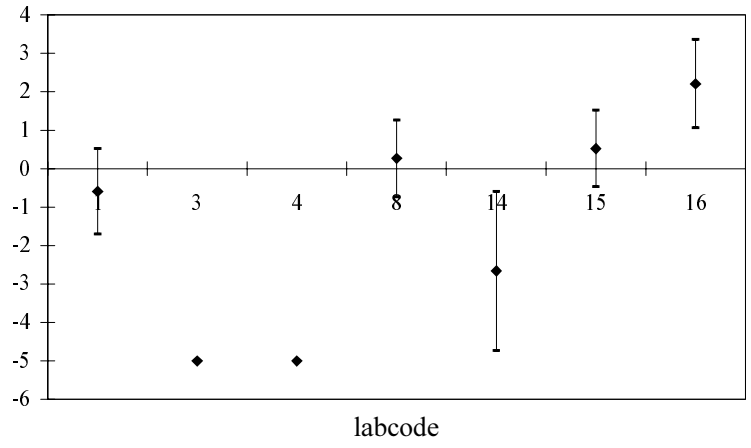

Figure 1: 95% confidence intervals of the results obtained with RV per laboratory compared to the average result of all other laboratories that used RV (0 isolations presented as a dot)

4.4.3.2 Comparison of laboratories using RVS

Comparing the results obtained with RVS as standard medium per laboratory with the average of all other laboratories that used RVS as standard medium for selective enrichment revealed significant differences (Figure 2). Laboratory 10 and 12 obtained significantly less positive isolations and laboratories 6 and 11 obtained significantly more positive isolations using RVS with regard to the average of the other laboratories using RVS.

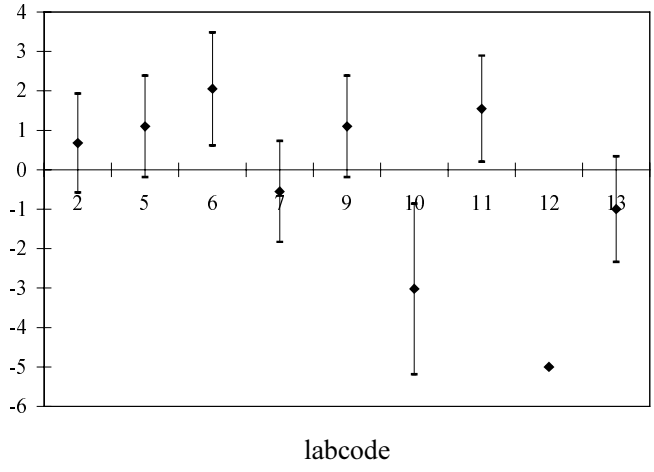

Figure 2: 95% confidence intervals of the results obtained with RVS per laboratory compared to the average result of all other laboratories that used RVS (0 isolations presented as a dot)

4.4.3.3 Comparison of laboratories using MSRV

Comparing the results obtained with MSRV per laboratory with the average of the other laboratories revealed significant differences (Figure 3). Laboratories 3, 10, 12 and 14 obtained significantly less positive isolations and laboratories 2, 8, 9 and 11 obtained significantly more positive isolations compared to the average of all laboratories.

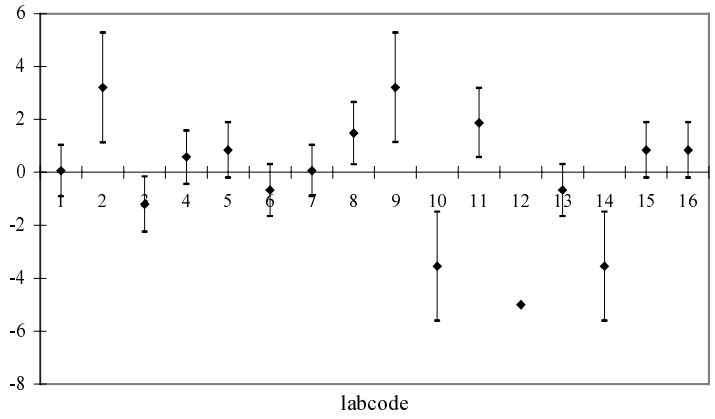

Figure 3: 95% confidence intervals of the results obtained with MSRV per laboratory compared to the average result of all other laboratories (0 isolations presented as a dot)

4.4.3.4 Comparison of laboratories overall

The results were also evaluated for the total score, obtained with RV(S) and MSRV, between laboratories. Five laboratories (labcode 3, 7, 10, 12 and 14) obtained significantly less isolations, compared to the other laboratories. Seven laboratories (labcode 2, 5, 8, 9, 11, 15 and 16) obtained significantly more positive isolations compared to the average of all laboratories (Figure 4).

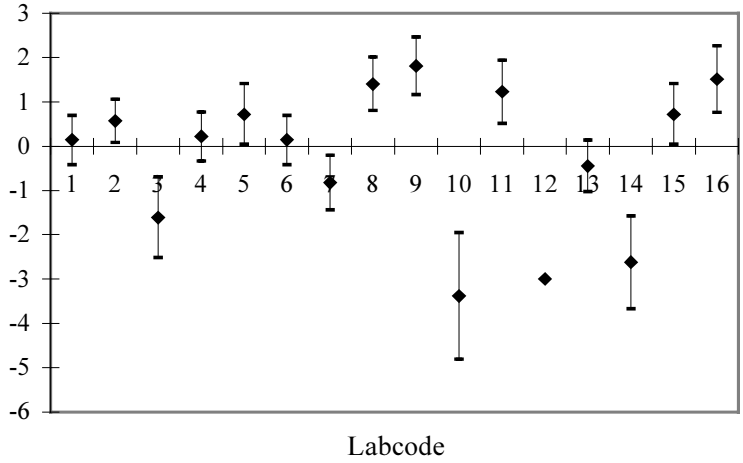

Figure 4: 95% confidence intervals of results per laboratory compared to the average result of all other laboratories

No significant difference could be detected between laboratories having a quality system or not for detection of *Salmonella* from the capsules using RV(S) and MSRV. Laboratories 2, 8 and 16 are laboratories that have a certified quality system and obtain significantly better

results compared to the other laboratories. Laboratories 1 and 13 also have a certified quality system and did not obtain significant better results compared to the other participants, and laboratory 12 even obtained significantly less results compared to the other laboratories. Also laboratories that are working on obtaining a certificate obtain results below average (labcode 7), on average (labcode 6) and above average (labcode 5, 9, 11 and 15).

4.5 Comparison of selective enrichment media for the naturally contaminated samples

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.

4.5.1 Comparison of selective enrichments between laboratories

The cumulative results obtained for the naturally contaminated samples per selective enrichment used are shown in Table 14.

RV

Comparison of the results obtained with RV (18% positive) with the results obtained with other enrichment media revealed significant differences. Four media obtained significantly better results, namely RVS (31%), MSRV (31%), MSRV+ (70%) and TBG (75%). No enrichment revealed significantly less positive isolations in comparison with RV. Although the percentage of positive isolations using MK (35%) is higher than the percentage positives using RVS (Table 14), this is not significantly better, due to the fact that only one laboratory used MK.

RVS

Two selective enrichments (RV (18%) and SC (8%)) and PCR (11%) obtained significantly less positive isolations in comparison with RVS (31%). TBG (75%) and MSRV+ (70%) revealed significantly more positive isolations in comparison with RVS.

MSRV

Significantly less results were obtained using RV and SC for selective enrichment and PCR compared to MSRV. There were no significant differences between MSRV, RVS, MK (35%), DIASALM (22%) and Rappaport (20%). TBG (75%) and MSRV+ (70%) revealed significantly more positive isolations in comparison with MSRV.

Table 14: Cumulative results obtained with different selective enrichment media for the naturally contaminated samples

	Number of laboratories using selective enrichment and number of positive isolations with selective enrichment									
	TBG	MSRV+	MK	MSRV	RVS	DIA	Rap	RV	PCR	SC
n laboratories	1	1	1	16	10	3	1	8	4	3
n positive	15	14	7	99	61	13	4	28	9	5
% positive	75	70	35	31	31	22	20	18	11	8

4.5.2 Comparison of selective enrichments within laboratories

The results obtained within the laboratories with the different selective enrichment media used for the naturally contaminated samples are shown in Table 15 (prescribed media) and 16 (own selective enrichment).

RV

Laboratory 4 and 8 obtained significantly more positive isolations using respectively MSRV and Rappaport; and MSRV and DIASALM in comparison with RV. Laboratory 1 found significantly less positive isolation using DIASALM and PCR in comparison with RV.

RVS

Three laboratories (labcode 6, 9 and 13) found significantly more positive isolations with another selective enrichment than RVS. In laboratory 6 this was TBG, in laboratory 9 MSRV, and in laboratory 13 MSRV, MK and PCR. Laboratory 2 found significantly less positive isolations using SC and MSRV+ in comparison with RVS.

MSRV

One laboratory (labcode 6) obtained significantly more isolations using TBG than using MSRV. Furthermore no media were used that obtained significantly more positive isolations than MSRV. The use of DIASALM and PCR in laboratory 1, RVS in laboratories 9 and 13, SC and MSRV+ in laboratory 2 and the use of RV in laboratory 8, revealed significantly less positive isolations compared to MSRV.

Table 15: Number of positive results obtained with prescribed selective enrichments used for the naturally contaminated samples

	Number of positives with prescribed selective enrichment (n=20) per labcode															
Medium	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
RV	7		0	0				4	8					0	0	9
RVS		18			0	8	0		6	0	17	0	1			11
MSRV	11	20	2	4	0	7	0	11	9	0	17	0	8	0	0	10

Laboratory 16: Standard method was RV; RVS also used as selective enrichment

Laboratory 9: Standard method was RVS; RV also used as selective enrichment

Table 16: Number of positive results obtained with different, not prescribed, selective enrichments used for the artificially contaminated samples

Medium / Method	Number of positives with prescribed selective enrichment (n=20) per labcode								
	1	2	4	6	7	8	10	13	14
TBG				15					
SC		5			0		0		
MSRV+		14							
MK								7	
DIA	2					11			0
Rap			4						
PCR	0				0		0	9	

4.5.3 Comparison of selective enrichments between serotypes

The naturally contaminated samples consisted of two batches (originated from two flocks). Table 17 shows the results of the prescribed media per batch. For laboratories 9 and 16, that also used RVS, only RV was included in the data. The results obtained with RV with the samples from week 25 were significantly different from the results obtained with MSRV. No significant difference was found for the samples from week 21. Also no significant difference was found between the results using RVS or MSRV.

Table 17: Comparison of the results of detection from naturally contaminated samples using the prescribed media

Samples of	Serotypes found in faeces before freezing	Number of positive isolations with combination of	
		RV / MSRV (7 laboratories)	RVS / MSRV (9 laboratories)
Week 21 (12 samples / laboratory)	Ruiri Tennessee Cubana	27 / 34 (n=84) (32.1% / 40.5%)	27 / 26 (n=108) (25.0% / 24.1%)
Week 25 (8 samples / laboratory)	Enteritidis PT4 and 35 Livingstone	1 / 13 (n=56) (1.8% / 23.2%)	17 / 26 (n=72) (23.6% / 36.1%)

Significant difference between selective enrichments used

4.6 Isolation media

The isolation media, Xylose Lysine Deoxycholate Agar (XLD) and Brilliant Green Agar (BGA) 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.

Comparing the results of the different isolation media used reveals differences within some laboratories. Laboratory 7 isolated *Salmonella* from four STM100 capsules with the use of BGA for isolation medium from RV. With the use of XLD for isolation from RV they isolated *Salmonella* one time from the STM100 capsules. For laboratory 7, isolation from MSRV, using BGA resulted in 7 positive isolations for the artificially contaminated samples. Using XLD and XLT-4 for isolation from MSRV results in respectively one and three positive isolations. Laboratory 13 obtained four positive isolations from the artificially contaminated samples on BGA from RV. Using XLD from RV, they isolated no *Salmonella* from the artificially contaminated samples.
Laboratory 4 obtains 11 positive isolations from the artificially contaminated samples on XLD with the use of Rappaport. Using BGA for isolation from Rappaport they isolated *Salmonella* three times.
Laboratory 8 obtained 16 positive isolations from the artificially contaminated samples with the use of BGA and XLD for isolation from MSRV. With the use of Rambach for isolation, they isolated *Salmonella* seven times. From the naturally contaminated samples using MSRV, *Salmonella* was isolated 11 times on BGA and XLD and four times on Rambach.

5. Discussion

Samples

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 500 cfp SE, the (mean) value for homogeneity, expressed as $T_2/(I-1)$, was respectively 1.91 and 1.58 with an average of 63 and 450 cfp per capsule. For the capsules containing ca 10 or ca 100 cfp STM the value for homogeneity was respectively 1.14 and 2.21, with an average of 3.8 and 47 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 (1). As long as the value is not higher than 2, no problems are expected.
The naturally contaminated samples did not contain *Salmonella* consistently when tested. Therefore, these samples could not be used for evaluation between laboratories. They were used for evaluation of the media which was done within samples.

Pre-enrichment

Preheating the pre-enrichment medium to 37°C as prescribed revealed significantly more positive isolations from the artificially contaminated samples with the use of RV(S). This effect was significant for the STM100 capsules only. The capsules containing SE also revealed a higher percentage of positive samples with RV(S) for selective enrichment after pre-heating the BPW. However, this difference was not significant. For the naturally contaminated samples, significantly more positive isolations from the samples containing SE were obtained if the BPW was not preheated. From the other naturally contaminated samples a higher percentage of positive isolations was obtained if BPW was 37°C, but this difference was not significant. It is not clear what temperature is best; For dissolving of the capsules, the BPW must be 37°C. However, more positive isolations are revealed from the naturally contaminated samples containing SE if BPW was not preheated.
Incubating the pre-enrichment for 16-20 hours reveals a higher percentage of positive isolations compared to incubating >20 hours. Significantly better results were obtained for the STM100 capsules with the use of RV(S) and MSRV and for the SE500 capsules with the use of MSRV if BPW was incubated for 16-20 hours. Inoculating RV(S) with the pre-enrichment incubated >20 hours reveals higher percentages of positive isolations for the total number of capsules containing SE (not significant). If however MSRV is inoculated with pre-enrichment incubated >20 hours a lower percentage of positive isolations is obtained for the total number of capsules containing SE. The difference in incubation time of BPW in combination with the use of liquid or semi-solid selective enrichment media for the isolation of SE should be compared between laboratories for naturally contaminated samples. However, the naturally contaminated samples of this study can not be used for this purpose.

Selective enrichment for artificially contaminated samples

The selective enrichment medium MSRV+, used by only one laboratory, revealed significant better results compared to MSRV. This difference is significant if the results of the medium are compared to the average results of all other laboratories. Within the laboratory, the difference between MSRV and MSRV+ is not significant. The laboratory even obtains more positive isolations using MSRV.

For the artificially contaminated samples, MSRV compared to RV and RVS reveals significant differences within 10 laboratories.

Selective enrichment for naturally contaminated samples

For the naturally contaminated samples the use of MSRV reveals significant differences compared to the use of RV or RVS within 4 laboratories. This is however dependent on the serotypes present in the naturally contaminated samples. The serotypes found in faeces of week 21 belong to the groups C1 and D; the serotypes in faeces of week 25 belong to the groups C1, G and L. Previous research (4, 6) revealed the advantage of the use of semi-solid media for *Salmonella* belonging to group D to which SE belongs. The differences between the use of RV or MSRV for the samples from week 25 (containing SE) are significant. The difference between RVS or MSRV for the samples from week 25 is not significant. However the percentage positives using RVS is 23.6% compared to 36.1% using MSRV which is a difference of 12.5%. Over all, these results indicate that MSRV seems to be the best selective enrichment for isolation of *Salmonella* from faeces.

The difference between laboratories for the number of positive isolations from the naturally contaminated samples can partly be explained by the presence or absence of *Salmonella* in the samples. The spread of the number of positives (0-20) is however to high to completely explain these results. Since these samples can not be used for statistical evaluation, no significant difference is calculated for the results between laboratories.

The use of PCR

The use of PCR did not show better results compared to bacteriology. The best results compared to bacteriology are obtained by the laboratory that used the largest volume of BPW (volume of DNA extract used in comparison with volume of BPW culture used) in the PCR. The laboratory that used the smallest volume in the PCR reaction, obtained the biggest difference between bacteriology and PCR for the artificially contaminated samples.

The use of PCR for detection of Salmonella in faeces needs more attention in the future in order to get results comparable to or better than bacteriology.

Control capsules

None of the laboratories isolated *Salmonella* from the procedure or faeces control (C11, blank and C12, faeces portion 3). One laboratory found all positive control capsules (C1-C10) negative using SC. Using RV and MSRV this laboratory isolated *Salmonella* from all positive capsules.

Two laboratories isolated *Salmonella* from a blank capsule using RV. With the other media used, this sample was found negative. Three laboratories failed to isolate *Salmonella* from an *S.* Panama capsule (no faeces added) with one of the media used. Laboratory 13 failed to

isolate *Salmonella* from one of the STM10 control capsules with all media used. It can not be excluded that this capsule was blank because of the mean level (ca 4) and the variation between the capsules. From another control capsule containing 10 cfp STM they isolated *Salmonella* with RV only.

Isolation media

Within some laboratories, differences between isolation media were revealed. Inoculating isolation media from broths with a low selectivity as selective enrichment, can result in growth of many a-typical colonies by which *Salmonella* is overgrown. A low selectivity of the isolation media can also be the cause of this.

Difficult to explain are the results of different isolation media used for isolation of *Salmonella* from a MSRV plate. If a loop full of agar is taken from the growing zone of a suspect MSRV plate, *Salmonella* should be in the agar and the isolation media should show positive colonies. If the isolation medium does not show suspect colonies after 24 hours of incubation of MSRV, the isolation medium can be inoculated again after 48 hours of incubation. Highly selective media will suppress the growth of *Salmonella*, especially in case the bacteria are sublethally injured.

Faeces

Four laboratories indicated in their test report the absence of growth in the selective enrichment media (broth totally clear, no change of colour) or on the isolation media. All 4 laboratories isolated *Salmonella* from the positive control capsules with all media used after 24 hours of incubation, indicating that possibly a toxic agent was present in the faeces. Since all isolation media were negative and the selective enrichment media hardly showed signs of growth, it is presumed that *Salmonella* and competitive micro-organisms did not grow at all in the pre-enrichment phase of the detection.

The RVS broth of laboratory 7 did not show any growth after incubation (quote: the tubes looked like they were not inoculated). Laboratory 12 noticed hardly any growth of a-typical colonies, neither from the *Salmonella* negative faeces, nor from the naturally contaminated faeces. Laboratory 10 noticed no growth of (a)-typical colonies on the isolation media from RV for almost 90% of the samples. Furthermore over 50% of the MSRV plates of laboratory 10 showed no growth with the negative faeces added to the capsules. Laboratory 13 found almost all their samples positive after 48 hour of incubation, while most positive isolations are seen after 24 hour of incubation of the selective enrichments. This also indicates inhibition of growth of *Salmonella*.

The faeces should be homogenous, resulting in all laboratories having low number of isolations if a toxic agent was present. Unfortunately, all faeces was sent to the participants, so no further analysis could be performed at the CRL-*Salmonella.*

6. Conclusion

The high levels of STM (47 cfp) were easier to isolate compared to the low levels of STM (3.8 cfp) and compared to SE (450 cfp). Over 50% of the laboratories isolated *Salmonella* from all high level STM capsules. The low level of SE (63 cfp) was more difficult to isolate, just over 10% of the laboratories isolated *Salmonella* from all low level SE capsules.
Comparison of selective enrichments between laboratories revealed significantly more positive isolations using MSRV, MSRV+, DIASALM and Rappaport in comparison with the use of RV and RVS for the artificially contaminated samples.
Within 10 laboratories significantly better results in comparison with RV(S) were obtained with the use of MSRV. Within one laboratory, the use of DIASALM revealed significantly better results compared to RV(S). Also the use of Rappaport revealed significantly better results compared to RV(S) within one laboratory.
For the naturally contaminated samples, the use of MSRV and RVS revealed significantly more positive isolations for all the naturally contaminated samples compared to RV. MSRV revealed significantly more positive isolations than RV for the samples containing SE.
Positive isolation strongly depends on a) the selective enrichment medium used; b) the laboratory that used this medium; and c) the experience the laboratory has with using the specific medium. The type of isolation medium used seemed less important.

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Appendix 1 Mailing list

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25	Board of Directors RIVM	dr. H. Pont
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27	Head of Microbiological Laboratory for Health Protection and Director CRL-*Salmonella*	dr. ir. A.M. Henken
28-30	Project Workers	
31-34	Authors	
35	Dutch National Library for Dutch Publications and Bibliography	
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Appendix 2 Protocol

BACTERIOLOGICAL COLLABORATIVE STUDY V
ORGANISED BY CRL *SALMONELLA*

PROTOCOL:

Introduction:
The Community Reference Laboratory (CRL) *Salmonella* organises the fifth 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. Some laboratories used a semi solid medium for selective enrichment. These media seemed to be superior to RV as selective enrichment, especially for the detection of *Salmonella* Enteritidis (SE). Therefore, in the fourth collaborative study all laboratories used Modified Semi solid Rappaport Vassiliadis (MSRV) as selective enrichment next to RV(S). In this study significantly better results were revealed with MSRV compared to RV. Results obtained with RVS were not significantly different from the results obtained with MSRV.
The revised ISO 6579 will prescribe Xylose Lysine Deoxycholate (XLD) for isolation medium. Therefore during the previous CRL-workshop, it was decided to use BGA as well as XLD. Laboratories that are interested can perform PCR on the samples.

For the performance of this study Reference Materials (RMs) produced by the CRL and poultry faeces will be used. The RMs consist 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, which are naturally contaminated with Salmonella, will be examined. The results will be evaluated by the CRL.

Objective:
The main objective of the fifth 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 sensitivity of PCR technique 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 ±110 grams of naturally contaminated frozen faeces.

The performance of the study will be in week 37 (starting on 11 September 2000). 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 V

The performance of the study must be in **week 37 (starting on 11 September 2000)** or one week earlier or later.

7 - 11 August	Mailing the protocol, SOP and test report to the NRLs.
28 August - 1 September	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 an e-mail 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.*

If you did not receive the parcel before or at 1 September, do contact the CRL immediately.

4 - 8 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)

11 - 15 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.

25 - 29 September Completion of the test report and faxing or e-mailing it to the CRL. The original test report will be sent to CRL.

9 - 13 October Checking the results by the National Reference Laboratories.

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

Maurice Raes (research assistant CRL)
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Appendix 3 Standard Operating Procedure

BACTERIOLOGICAL COLLABORATIVE STUDY V
ORGANISED BY CRL *SALMONELLA*

SOP/CRL/06
Version no.1
070800

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 in 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, which is part of the manual CRL-Salmonella supplied, by the CRL. Preparation of MSRV and XLD 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 6.1)

5.3 Solid selective media

5.3.1 Phenol red/brilliant green agar (Annex B.4)
5.3.2 Xylose-Lysine-Deoxycholate (Paragraph 6.2)
5.3.3 Third 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. Extra media

6.1 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.

6.2 Preparation of XLD

The medium must be boiled to dissolve (instructions manufacturer). After boiling the medium must be transparent red. Plates should be poured with a volume of 30-40 ml (140 mm-plates).

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 37°C for better dissolving of the capsules.
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 or 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 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:

- **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 page 3 of the test report.

If PCR is performed, fill in all requested data on test report page 12. Results of PCR can be written in the test report Table 4 (page 22).

8.2 Selective enrichment

Allow the selective enrichment broths to equilibrate to room temperature, if they were stored at a lower temperature. Dry the surface of the MSRV plates in a Laminar 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 jars/tubes/plates from N1 to N20 and 12 jars/tubes/plates from C1 to C12.

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 those 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.

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) Xylose Lysine Deoxycholate agar; and optionally
3) selective isolation medium/media routinely used in your laboratory. Only if media used are 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*.

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 biochemically 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 (isolation using RV), 2 (isolation using MSRV) and 3 (isolation using own enrichment) on test report page 13-21. For the results of detection of *Salmonella* using PCR fill in Table 4 on test report page 22.

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 which are routinely used for biochemical confirmation. Record on test report (page 11) 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; and
-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 other selective enrichment medi(um)(a)
day 3	**First isolation**	inoculate from RV, suspect MSRV plates and other medi(um)(a) a. phenol red/brilliant green agar b. Xylose Lysine Deoxycholate agar c. other selective medi(um)(a) incubate 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, suspect MSRV plates and other medi(um)(a) a. phenol red/brilliant green agar b. Xylose Lysine Deoxycholate agar c. other selective medi(um)(a) incubate 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 FIFTH BACTERIOLOGICAL COLLABORATIVE STUDY
ORGANISED BY CRL *SALMONELLA*

Detection of *Salmonella* in the presence of competitive micro-organisms
The use of PCR technique for detection of *Salmonella* in faeces

Laboratory code	:
Laboratory name	:
.....	
Date of receipt of the parcel	: ..... - ..... - 2000
Starting date for detection	: ..... - ..... - 2000

Shipment

Cold chain monitor:

1.Check at airport:

date : …… - …… 2000

time : …… h …… min

Parcels damaged □ YES

□ NO

Package containing capsules			Package containing faeces		
Colour of compartment			Colour of compartment		
A	□ completely coloured	□ partly coloured	A	□ completely coloured	□ partly coloured
	□ white □ light blue	□ dark blue		□ white □ light blue	□ dark blue
B	□ completely coloured	□ partly coloured	B	□ completely coloured	□ partly coloured
	□ white □ light blue	□ dark blue		□ white □ light blue	□ dark blue
C	□ completely coloured	□ partly coloured	C	□ completely coloured	□ partly coloured
	□ white □ light blue	□ dark blue		□ white □ light blue	□ dark blue
D	□ completely coloured	□ partly coloured	D	□ completely coloured	□ partly coloured
	□ white □ light blue	□ dark blue		□ white □ light blue	□ dark blue

2.Check at laboratory:

date : …… - …… 2000

time : …… h …… min

Parcels damaged □ YES

□ NO

Package containing capsules			Package containing faeces		
Colour of compartment			Colour of compartment		
A	□ completely coloured	□ partly coloured	A	□ completely coloured	□ partly coloured
	□ white □ light blue	□ dark blue		□ white □ light blue	□ dark blue
B	□ completely coloured	□ partly coloured	B	□ completely coloured	□ partly coloured
	□ white □ light blue	□ dark blue		□ white □ light blue	□ dark blue
C	□ completely coloured	□ partly coloured	C	□ completely coloured	□ partly coloured
	□ white □ light blue	□ dark blue		□ white □ light blue	□ dark blue
D	□ completely coloured	□ partly coloured	D	□ completely coloured	□ partly coloured
	□ white □ light blue	□ dark blue		□ 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
  : temperature of BPW at the start: □ Room temperature
  □ 37°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. <u>Rappaport Vassiliadis (Soya) medium</u>

Manufacturer of the RV(S) medium:

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

Specific data of composition of RV(S) 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. <u>Modified Semi solid Rappaport Vassiliadis medium</u>

How much samples did you test in 1999 and 2000 using MSRV as selective enrichment:

1999:…… 2000:……

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

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>Selective medium, routinely used in your laboratory</u>

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

Prescribed 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 broth : ……

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>Xylose Lysine Deoxycholate medium</u>

Manufacturer of XLD medium:

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

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 broth : ……

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

Prescribed 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 broth : ……

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

Manufacturer of TSI agar:

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

Manufacturer of urea agar:

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

Manufacturer of l-Lysine decarboxylation medium:

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

Other biochemical confirmation tests:

Manufacturer of ……:

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

Manufacturer of ……:

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

Manufacturer of ……:

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

Detection by PCR

Is the PCR used commercially available:

□ Yes

□ No

If yes, name of PCR, manufacturer and batch used in the study:

……

Is the PCR validated:

□ Yes

□ No

How much samples did you test for *Salmonella* using this PCR in 1999: ……

At what moment did you start with the extraction/detection?
(before or after incubation of BPW)

……

Volume of pre-enrichment used for extraction : ……
Volume of DNA-sample obtained from extraction : ……
Volume of DNA-sample added to PCR-mixture : ……

Composition of PCR-mixture:

Compound	volume per sample	manufacturer and batch of specific compound
……	……	……
……	……	……
……	……	……
……	……	……
……	……	……
……	……	……
……	……	……
……	……	……

Name of thermocycler: ……
Add a print out of the cycles used, or write down the cycles.
What kind of detection system is used: ……
Add a print out of the results of the detection system (i.e. a photograph of the gel).

Table 1: Results of isolation using RV (dish numbers 1-25)

	RV 24 hour						RV 48 hour					
	BGA		XLD		third medium		BGA		XLD		third medium	
no.	col [a]	Sal [b]	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												

col [a] = number of colonies used for confirmation
Sal [b] = number of colonies confirmed as *Salmonella*

Table 1 (continued): Results of isolation using RV (dish numbers N1-N20)

	RV 24 hour						RV 48 hour					
	BGA		XLD		third medium		BGA		XLD		third medium	
no.	col [a]	Sal [b]	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												

col [a] = number of colonies used for confirmation
Sal [b] = number of colonies confirmed as *Salmonella*

Table 1 (continued): Results of isolation using RV (dish numbers C1-C12)

	RV 24 hour						RV 48 hour					
	BGA		XLD		third medium		BGA		XLD		third medium	
no.	col [a]	Sal [b]	col	Sal	Col	Sal	col	Sal	col	Sal	col	Sal
C1												
C2												
C3												
C4												
C5												
C6												
C7												
C8												
C9												
C10												
C11												
C12												

col [a] = number of colonies used for confirmation
Sal [b] = number of colonies confirmed as *Salmonella*

Table 2: Results of isolation using MSRV (dish numbers 1-25)

	MSRV 24 hour						MSRV 48 hour					
	BGA		XLD		third medium		BGA		XLD		third medium	
no.	col [a]	Sal [b]	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												

col [a] = number of colonies used for confirmation

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

Table 2 (continued): Results of isolation using MSRV (dish numbers N1-N20)

	MSRV 24 hour						MSRV 48 hour					
	BGA		XLD		third medium		BGA		XLD		third medium	
no.	col [a]	Sal [b]	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												

col [a] = number of colonies used for confirmation
Sal [b] = number of colonies confirmed as *Salmonella*

Table 2 (continued): Results of isolation using MSRV (dish numbers C1-C12)

	MSRV 24 hour						MSRV 48 hour					
	BGA		XLD		third medium		BGA		XLD		third medium	
no.	col [a]	Sal [b]	col	Sal	Col	Sal	col	Sal	col	Sal	col	Sal
C1												
C2												
C3												
C4												
C5												
C6												
C7												
C8												
C9												
C10												
C11												
C12												

col [a] = number of colonies used for confirmation
Sal [b] = number of colonies confirmed as *Salmonella*

Table 3: Results of isolation using own enrichment (dish numbers 1-25)

	Own enrichment 24 hour						Own enrichment 48 hour					
	BGA		XLD		third medium		BGA		XLD		third medium	
no.	col [a]	Sal [b]	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												

col [a] = number of colonies used for confirmation
Sal [b] = number of colonies confirmed as *Salmonella*

Table 3 (continued): Results of isolation using own enrichment (dish numbers N1-N20)

	Own enrichment 24 hour						Own enrichment 48 hour					
	BGA		XLD		third medium		BGA		XLD		third medium	
no.	col [a]	Sal [b]	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												

col [a] = number of colonies used for confirmation
Sal [b] = number of colonies confirmed as *Salmonella*

Table 3 (continued): Results of isolation using own enrichment (dish numbers C1-C12)

	Own enrichment 24 hour						Own enrichment 48 hour					
	BGA		XLD		third medium		BGA		XLD		third medium	
no.	col [a]	Sal [b]	col	Sal	Col	Sal	col	Sal	col	Sal	col	Sal
C1												
C2												
C3												
C4												
C5												
C6												
C7												
C8												
C9												
C10												
C11												
C12												

col [a] = number of colonies used for confirmation
Sal [b] = number of colonies confirmed as *Salmonella*

Table 4: Results of detection using PCR (dish numbers 1-25)

	PCR + or -				
no.		no.		no.	
1		N1		C1	
2		N2		C2	
3		N3		C3	
4		N4		C4	
5		N5		C5	
6		N6		C6	
7		N7		C7	
8		N8		C8	
9		N9		C9	
10		N10		C10	
11		N11		C11	
12		N12		C12	
13		N13			
14		N14			
15		N15			
16		N16			
17		N17			
18		N18			
19		N19			
20		N20			
21					
22					
23					
24					
25					

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

Date: ..... - ..... - 2000

Name of technician/technologist carrying out the fifth 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	Batch
1	Own preparation		
2	Merck	7228	vk457628
3	Biomerieux	42043	7,45E+08
4	Merck	7228	v321628912
5	Own preparation		
6	Merck		v253028844
7	Oxoid	CM509	226620
8	Oxoid	CM509	229810
9	Oxoid	CM509	214945
10	Difco	1810-17	14014xd
11	Merck	7228	vk477528008
12	Lab-M	lab46	29207
13	AES	AEB140302	932734
14	Oxoid	CM509	234167
15	Merck	7228	8E+08
16	Oxoid	CM509	6785

Table 2: Manufacturer of RV from participants

Labcode	Manufacturer RV	Code	Batch
1	Oxoid	CM669	
3	Biomerieux	42073	744410601
4	Oxoid	CM669	215647
8	Oxoid	CM669	232724
9	Own preparation	-	
13	Oxoid	CM669	227490
14	Oxoid	CM669	232724
16	own preparation	-	

Table 3: Manufacturer of RVS from participants

Labcode	Manufacturer RVS	Code	Batch
2	Oxoid	CM866	233675
5	Oxoid	CM866	229815
6	Merck	7700	v915500721
7	Oxoid	CM866	233675
9	Oxoid	CM866	233675
10	LabM	lab86	41882
11	Merck	7700	vk493500012
12	Oxoid	CM866	30512
13	LabM	lab86	222581

Table 4: Manufacturer of MSRV from participants

Labcode	Manufacturer MSRV	Code	Batch
1	Difco	141883	141883
2	Oxoid	CM910	233058
3	Oxoid	CM910	b234473
4	Merck	9878	v179978817
5	Difco	1868-17	94376jb
6	Oxoid	CM910	234473
7	Lab M	lab150	43427
8	Lab M	lab150	30121
9	Oxoid	CM910	234473
10	Lab M	lab150	44642
11	Merck	9878	v179978817
12	Lab M	lab150	46758
13	Biokar	BK134	gc570
14	Oxoid	CM910	231292
15	Oxoid	CM910	217730
16	Oxoid	CM910	6,806E+09

Table 5: Manufacturer of optional further selective enrichments from participants

Labcode	Name medium	Manufacturer	Code	Batch
1	DIASALM	LabM	lab537	43435
2	MSRV+ [1)]	Oxoid	CM910	233058
2	SC	Merck	7709	v394709
4	Rappaport broth	Biomerieux	42091	7,44E+08
6	TBG	Merck		v173878816
7	SC	Oxoid	tv50051	8568
8	DIASALM	Merck	9803	vk460303
10	SC	Oxoid	CM699	60187
13	MK	AES	aeb140702	932925
14	DIASALM	LabM	lab537	47628
16	RVS	Oxoid	CM866	6,72E+09

1) additions: Bromcresolpurpur, saccharose and novobiocine

Table 6: Manufacturer of BGA from participants

Labcode	Manufacturer BGA	Code	Batch
1	Oxoid	CM329	
2	Oxoid	CM329	234181
3	Oxoid	CM329	
4	Difco	0285-17-7	126327jb
5	Merck	7237	v368137
6	Oxoid	CM329	215873
7	Oxoid	CM329	218040
8	Oxoid	CM329	228150/232919
9	Difco	1880	142428xa
10	LabM	lab34	28454
11	Merck	747	v273947850
12	LabM	lab34	44107
13	AES	aeb151492	911630
14	Oxoid	CM329	228750
15	Merck	747	v212347828
16	Acumedia	7117	687000906

Table 7: Manufacturer of XLD from participants

Labcode	Manufacturer XLD	Code	Batch
1	Oxoid	CM469	202376
2	Oxoid	CM469	230180
3	Biomerieux	43563	745783401
4	Biomerieux	43563	746184701
5	Difco	278850	130kyr
6	Merck		v248887843
7	Difco	278850	c3dkgn
8	Oxoid	CM469	233269/208108
9	Difco	788	c3dkgn
10	LabM	lab32	47064
11	Merck	5278	v424687945
12	LabM	lab32	28065
13	AES	aeb153402	924924
14	Oxoid	CM469	234760
15	Merck	5278	v343487918
16	LabM	lab32	679000905

Table 8: Manufacturer of own isolation medium from participants

Labcode	Name medium	Manufacturer	Code	Batch
3	SMID	Biomerieux	43291	744961901
3	SMID	Biomerieux	43291	745782801
5	XLT-4	Merck	3919	vk495819013
6	Rambach	Merck		13078
7	XLT-4	Difco		140407
9	BHIagar	Difco	44	c3dxnaxa
10	MLCVBGA	LabM	lab116	26988
11	Rambach	Merck	7500	29079
12	Onoz	Merck	5034	v430634
13	Rambach	Merck	7500	28059
13	XLT-4	Difco	0234-17	140407
14	Rambach	Merck	7500	28059
15	Gassner	Merck	1282	v416782942
16	XLD+novo	LabM	lab325	686000906