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

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

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Abstract

A third bacteriological collaborative study was organized by the Community Reference Laboratory for *Salmonella.* All National Reference Laboratories for *Salmonella* (NRL) participated. The main objective of this study was to evaluate the results of the detection of different contamination levels (10 and 100 colony forming particles per capsule) of *Salmonella* Enteritidis and *Salmonella* Typhimurium in the presence of competitive micro-organisms among and within the NRLs.
As method an adapted ISO 6579 (proposed reference method) and, optionally, the laboratory's own routine method for the detection of *Salmonella* in chicken faeces were used. Significant differences were found between and within the laboratories. None of the laboratories succeeded to isolate *Salmonella* from all capsules containing 10 colony forming particles. The number of positives found strongly differed between laboratories.
With semi-solid media significantly more samples were found positive for *Salmonella*, especially for the samples containing *S.* Enteritidis compared to the reference method.

Contents

		page
Samenvatting		4
Summary		5
1	Introduction	6
2	Participants	7
3	Materials and methods	
3.1	Preparation of reference materials and faeces samples	9
3.1.1	Reference materials containing *Salmonella* Typhimurium and *Salmonella* Enteritidis	9
3.1.2	Samples containing competitive micro-organisms	9
3.2	Collaborative study	10
3.3	Statistical analysis of the data	11
4	Results	
4.1	Preparation of reference materials and faeces samples	12
4.1.1	Stability test and homogeneity test of reference materials	12
4.1.2	Testing faeces samples with antibiotic	13
4.2	Collaborative study	13
4.2.1	Control samples (n=18)	14
4.2.2	Reference method (n=42)	14
4.2.3	Own bacteriological method	18
5	Discussion	19
6	Conclusion	21
References		22
Appendix 1	Mailing list	23
Appendix 2	Protocol bacteriological collaborative study III	24
Appendix 3	SOP bacteriological collaborative study III	28
Appendix 4	Test Report bacteriological collaborative study III	35
Appendix 5	Data about media used	54

Samenvatting

Het Communautair Referentie Laboratorium voor *Salmonella* heeft een derde bacteriologisch ringonderzoek georganiseerd betreffende de detectie van *Salmonella*. De deelnemers waren de Nationale Referentie Laboratoria (NRLs) voor *Salmonella* uit de lidstaten van de Europese Unie.
Het belangrijkste doel van dit ringonderzoek was het vergelijken tussen en binnen de NRLs van de behaalde resultaten betreffende de detectie van verschillende besmettingsniveaus van *Salmonella* in de aanwezigheid van stoorflora.
Zestig individueel genummerde capsules werden door elk van de 15 laboratoria onderzocht op de aanwezigheid van *Salmonella*. Hiervan moesten 49 capsules onderzocht worden in combinatie met één gram kippenfaeces. De overige 11 capsules, waaraan geen faeces moest worden toegevoegd, waren controles. De 49 capsules waren onderverdeeld in 14 capsules met 10 kolonie vormende eenheden (kve) *Salmonella* Typhimurium (STM), 21 capsules met 100 kve STM en 14 capsules met ongeveer 100 kve *Salmonella* Enteritidis (SE). Aan zeven van de 21 capsules met 100 kve STM moest faeces worden toegevoegd dat een antibioticum bevatte.
De capsules werden getest met een van de ISO 6579 afgeleide methode waarbij alleen RV als selectief ophopingsmedium werd gebruikt en daarnaast eventueel de eigen methode van het laboratorium voor de detectie van *Salmonella* in kippenfaeces.
Drie laboratoria isoleerden *Salmonella* uit de capsules waaraan faeces met het antibioticum moest worden toegevoegd.
Drie laboratoria slaagden er niet in om *S.* Panama uit de controle capsules te isoleren. Eén van deze laboratoria slaagde er ook niet in om van drie van de vier controle-capsules, die 10 kolonie vormende eenheden (kve) STM bevatten, *Salmonella* te isoleren.
Er werden significante verschillen tussen de laboratoria gevonden. Acht laboratoria hadden significant meer positieve resultaten dan de andere laboratoria.
Geen van alle laboratoria isoleerde *Salmonella* uit alle 14 capsules die 10 kve STM bevatten. Het aantal positieven varieerde tussen de 0 en 13. Vijf laboratoria vonden significant minder negatieve capsules dan de overige laboratoria.
Vijf laboratoria isoleerden *Salmonella* uit alle 14 capsules met 100 kve STM. Drie laboratoria scoorden significant minder betreffende de isolatie van STM uit capsules die 100 kve bevatten. Ook voor de isolatie van SE werden verschillen gevonden tussen de laboratoria. Vijf laboratoria scoorden significant beter, 2 laboratoria isoleerden SE uit alle 14 capsules en 3 laboratoria isoleerden geen enkele *Salmonella* uit deze capsules.
Het gebruik van semi-solid media gaf significant betere resultaten dan de van de ISO afgeleide methode, in het bijzonder voor *S.* Enteritidis.

Summary

The Community Reference Laboratory for *Salmonella* organized a third bacteriological collaborative study into the methods for the detection of *Salmonella*. Participants were the National Reference Laboratories (NRLs) for *Salmonella* of the Member States of the European Union.
The main objective of this study was to evaluate the results among and within the NRLs of the detection of different contamination levels of *Salmonella* in the presence of competitive microorganisms.
Sixty individually numbered capsules were examined by 15 laboratories for the presence of *Salmonella*. Forty-nine capsules had to be examined in combination with one gram of chicken faeces. These 49 capsules were subdivided into 14 capsules containing 10 colony forming particles (cfp) *Salmonella* Typhimurium (STM), 21 capsules containing 100 cfp STM and 14 capsules containing 100 cfp *Salmonella* Enteritidis (SE). Faeces with an antibiotic had to be added to seven of the 21 capsules containing 100 cfp STM. The remaining 11 capsules were controls to which no faeces had to be added.
The capsules were tested using an adapted ISO 6579 method using RV as only selective enrichment broth and, optionally, the method routinely used by a laboratory for the detection of *Salmonella* in chicken faeces.
Three laboratories isolated *Salmonella* from the capsules to which the faeces containing an antibiotic was added.
Three laboratories did not succeed to isolate *S.* Panama from the control capsules. One of those laboratories did not succeed to isolate *Salmonella* from three of the four control capsules containing 10 cfp STM also.
Significant differences were found between laboratories. Eight laboratories found significantly more positive results compared to the other laboratories.
None of the laboratories isolated *Salmonella* from all 14 capsules containing 10 cfp STM. The number of positive capsules varied from 0 to 13. Five laboratories found significantly less positives compared to the other laboratories.
Five laboratories isolated *Salmonella* from all 14 capsules containing 100 cfp STM. Using the capsules containing 100 cfp STM three laboratories found significantly less positives.
Also differences were found between laboratories for the capsules containing SE. Five laboratories scored significantly better, 2 isolated SE from all 14 capsules and 3 laboratories isolated no *Salmonella* at all from those 14 capsules.
The use of semi-solid media gave significantly more positives compared to the reference method, especially for the samples containing *S.* Enteritidis.

1 Introduction

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

In the first collaborative study (October 1995) *Salmonella* had to be isolated from capsules containing high levels of *Salmonella* Typhimurium (STM) only. No significant difference was found between the performance of the proposed reference method (International Standard Organization (ISO) 6579 (1)) and routine methods both between and within the National Reference Laboratories (NRLs) for *Salmonella.*
The second bacteriological collaborative study was organized in October 1996 (2). In this second study *Salmonella* had to be isolated from capsules in combination with competitive flora in the form of chicken faeces. In this study only 8 of the 17 laboratories isolated *Salmonella* from all 30 positive capsules using the full ISO 6579 method. A significantly reduced number of positive isolations was found using Selenite/Cystine broth (SC) compared to Rappaport-Vassiliadis broth (RV) as selective enrichment medium. Therefore it was decided that SC will not be used anymore in future collaborative studies. Further separate research whether SC is suitable to detect *Salmonella* in the presence of competitive flora is necessary.
In the third study, described in this report, *Salmonella* had to be isolated from capsules in combination with chicken faeces again. Besides capsules containing different levels of STM, capsules containing *Salmonella* Enteritidis (SE) were included.

2 Participants

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

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

3 Materials and methods

3.1 Preparation of reference materials and faeces samples

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

The reference materials (RMs) were prepared from milk powder containing 1000 cfp STM per capsule and from highly contaminated milk powder (HCMP) containing SE. The powder containing STM was obtained as described earlier by Voogt et al (2). The powder containing SE was obtained by spray-drying artificially contaminated milk as described earlier by In 't Veld et al (3).
To obtain the target levels, 28 gram from the milk powder containing 1000 cfp STM per capsule was diluted in steps (mixing ratio 1:1 g/g) by mixing with uncontaminated milk powder (Carnation-koffiemelkpoeder Nestlé, The Netherlands) using a mortar and pestle to a total of 280 gram. From this milk powder 15 gram was diluted further with uncontaminated milk powder to a total of 150 gram. The target contamination level of the mixed powders was about 10 and 100 cfp per capsule for STM, respectively. The stability of the STM capsules was tested earlier and described by Voogt et al (2).
From the HCMP containing SE, 3 gram was diluted as described for STM with uncontaminated milk powder to a total of 240 gram. The target contamination level of the mixed powder was between 100 and 500 cfp per capsule.
Sixty capsules were filled with the mixed powder containing STM and also 60 with SE (0.34 g per capsule). These capsules were used to determine the number of cfp per capsule and the homogeneity of the batch. The capsules were stored at -20°C.
For use in the collaborative study and for testing the stability at storage temperature, 360 capsules containing 10 cfp STM, 840 capsules containing 100 cfp STM and 300 capsules containing 100 cfp SE were filled.
The stability of the SE capsules was determined over a period of 7 weeks. Each week 10 capsules were dissolved according to standard procedures of the RIVM, Bilthoven (The Netherlands) (4). The contamination level was determined by pouring plates with 5 ml plate count agar (PCA) and 10 ml violet red bile glucose agar (VRBG). Four hours after addition of PCA, VRBG was poured as an overlayer. The plates were incubated at 37°C overnight.

3.1.2 Samples containing competitive micro-organisms

Two and a half kilogram of faeces of a poultry laying flock, which was found negative for *Salmonella* in the Dutch Monitoring program, was used to prepare the samples containing competitive micro-organisms. Five portions of 25 g chicken faeces were tested for presence/absence of *Salmonella* according to routine method of the RIVM, Bilthoven (The Netherlands) (5). The faeces was mixed and homogenised before freezing with glycerol/ peptone solution (mixing ratio 1:1 (g/l)) and stored in portions of 10 grams at -20°C in order to stabi-

lise the micro-organisms. The glycerol/peptone solution consisted of 945 g glycerol and 17.5 g pepton dissolved in 1750 ml distilled water.
In this collaborative study a check was included to test whether all participants added the faeces to the capsules. This check consisted of faeces supplemented with an antibiotic for which *Salmonella* is sensitive.
Before use in the study a suitable concentration of the antibiotic was determined. Two concentrations of the antibiotic were tested. The faeces with antibiotic in combination with capsules containing ±100 cfp STM, was tested over a time period of 6 weeks. Both concentrations of antibiotic were tested in eight samples each week.

3.2 Collaborative study

Two weeks before the study the RMs (in total 60 individually numbered capsules) and 70 grams of chicken faeces (in total 7 individually numbered portions of 10 g) were mailed with cooling devices by cargo freight to the 15 participants. The content of the 60 capsules and the presence of faeces containing the growth inhibitor was unknown to the participants. 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.
In total 60 samples, consisting of faeces and/or capsules, were tested as presented in Table 1.

Table 1: *Overview of the number of samples tested and their composition.*

Number of samples tested	Capsule containing	Faeces added
14	10 cfp STM	Yes
14	100 cfp STM	Yes
7	100 cfp STM	Yes, containing antibiotic
14	100 cfp SE	Yes
4	10 cfp STM	No
2	5 cfp *S.* Panama	No
5	Blank capsule	No

Forty-nine capsules had to be tested in combination with chicken faeces. The portion of faeces containing the antibiotic was portion 4 and had to be tested in combination with capsules containing 100 cfp STM (capsule numbers 22 to 28). The eleven samples consisting of only capsules were control samples. Four of these 11 capsules contained 10 cfp STM, two capsules contained circa 5 cfp *S.* Panama and 5 were blank capsules. Additionally a procedure control (C12) and a faeces control (C13) had to be included (Table 2).

Table 2: *Overview of the control capsules tested and their composition.*

Control capsule	Capsule containing
C1	5 cfp *S.* Panama
C2	10 cfp STM
C3	Blank capsule
C4	5 cfp *S.* Panama
C5	Blank capsule
C6	10 cfp STM
C7	Blank capsule
C8	10 cfp STM
C9	Blank capsule
C10	10 cfp STM
C11	Blank capsule
C12	no capsule
C13	1 gram of faeces

The capsules had to be tested as described in the SOP and the results and operational details had to be reported to the CRL as proposed in the test report.

3.3 Statistical analysis of the data

The values for the homogeneity of the RMs were expressed as $T_2/(I-1)$ (measure of dispersion). T_2 is the Cochran's dispersion test (measure of spread between capsules) and I is the number of capsules. In case of a true homogeneous distribution (Poisson distribution) a value for $T_2/(I-1)$ of 1 is expected (6). The following SAS program options were used for analysis of the data:

Proc glm: Duncan's multiple range test was used to identify sets of laboratories which can be considered homogeneous, i.e. which are not mutually statistically different from each other, and are statistically significantly different from laboratories not belonging to that set (7).

Proc freq: The McNemar's (8) test was used to identify differences between the reference method and other methods for laboratories using non-reference methods.

4 Results

4.1 Preparation of reference materials and faeces samples

4.1.1 Stability test and homogeneity test of reference materials

The stability of the contamination level of the SE capsules was determined over a period of 7 weeks (Table 3). The initial level of contamination was about 200 cfp per capsule. The level of contamination decreased over 7 weeks when stored at -20°C (Figure 1). The values for the homogeneity of the batch are also shown in Table 3. The mean value for $T_2/(I-1)$ was 1.9.
The average number of *Salmonella* per capsule for the capsules containing ± 10 cfp STM was 10.7 with an $T_2/(I-1)$ value of 0.90. For the capsules containing ± 100 cfp STM the average was 93.9 cfp with an $T_2/(I-1)$ value of 1.47. Stability was not tested for the STM capsules in this study as they have been proven to be stable in an earlier study described by Voogt et al (2).

Table 3: *Results of the stability and homogeneity tests of capsules containing SE.*

no. of days in freezer	Average no. of cfp per capsule	$T_2/(I-1)$
1	212	1.12
14	219	2.56
20	153	1.66
28	112	1.52
35	163	1.81
43	188	2.81
50	95	1.82

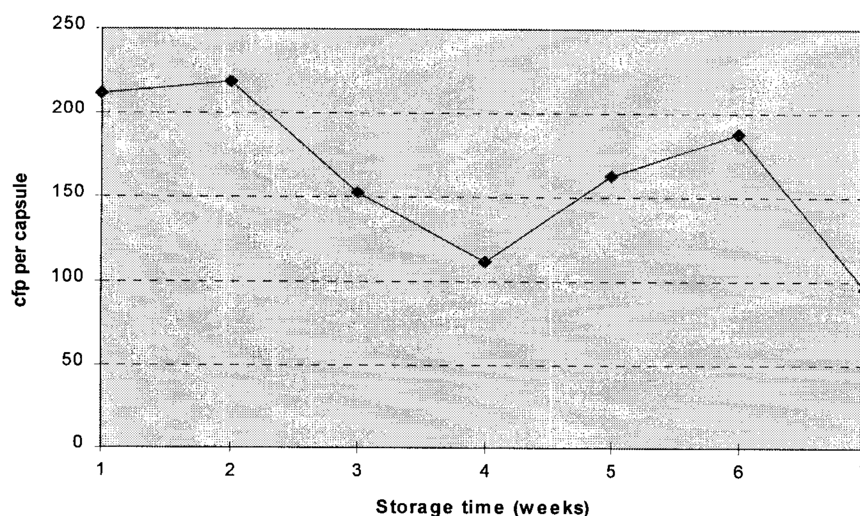

Figure 1: *Level of contamination of capsules containing SE stored at -20°C over 7 weeks.*

4.1.2 Testing faeces samples with antibiotic

Table 4 shows the results of the stability test of the faeces containing antibiotic stored at -20°C. Using the lower concentration of antibiotic *Salmonella* was isolated from the capsules after 3, 4, 5 and 6 weeks of storage at -20°C. In total 15 positive isolations were found from the 56 capsules. Using the higher concentration *Salmonella* was isolated only twice from the 56 capsules (after 2 and 4 weeks of storage).

Table 4: *Detection of Salmonella from capsules containing 100 cfp STM in the presence of faeces containing antibiotic.*

no. of weeks at -20°C	Lower antibiotic concentration	Higher antibiotic concentration
0	0 / 8*	0 / 8
1	0 / 8	0 / 8
2	3 / 8	1 / 8
3	4 / 8	0 / 8
4	7 / 8	1 / 8
5	1 / 8	0 / 8
6	0 / 8	0 / 8

* : Number of positive isolations / number of tests

4.2 Collaborative study

The media needed to conduct the study were predescribed in the SOP. Data about the media used and manufacturers are presented in appendix 5 (Table 12 to 14). The labcodes for the NRLs used in this third study were the same as those in the first and second study.

Pre-enrichment

Before adding one gram of 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 predescribed time, except for laboratory 2 (60 min), laboratory 7 (39 min) and laboratory 8 (45 min).

The predescribed time for the pre-enrichment was 16 - 20 hours. Except for four laboratories all laboratories incubated between 16 and 20 hours. The laboratories 2, 3, 6 and 8 incubated for 20 h 57 min, 20 h 50 min, 21 h 30 min and 25 h, respectively.

Selective enrichment

The predescribed incubation time for RV is 24 ± 1 hours and subsequently a further 24 ± 1 hours. Ten of the 15 laboratories incubated the first period for 24 ± 1 hours. Three laboratories (codes 1, 2 and 6) incubated shorter and two laboratories (codes 3 and 5) incubated longer.

Twelve laboratories incubated RV 48 ± 2 hours. Laboratories 1, 2 and 6 incubated for 51, 45 and 44 hours, respectively.

Isolation

The isolation media, brilliant green agar (BGA) and a medium of own choice, had to be incubated for 18 - 24 hours. Except for laboratory 1 (27 hours) and 5 (16 h 30 min) all laboratories incubated the media for the predescribed time.

4.2.1 Control samples (n=18)

In total 18 control samples were examined per laboratory. Seven faeces samples containing the antibiotic in combination with capsules containing 100 cfp STM and 11 capsules without faeces.

Capsules tested without faeces

Only laboratory 12 isolated *Salmonella* from the procedure control (C12) to which no faeces and capsule was added using the reference method on both isolation media. Laboratory 1 found all blank capsules (C3, C5, C7, C9 and C11) positive using Muller-Kauffmann Tetrathionate broth (MK-TT) for selective enrichment and hektoen agar for isolation. None of the other participants isolated *Salmonella* from the procedure control, negative control (C13, only faeces added) or blank capsules.

Laboratory 6 and 8 could not detect *Salmonella* from the capsules containing 5 cfp *S.* Panama (C1 and C4) using both the reference method and their own method. Laboratory 3 could not detect *S.* Panama using one of their two own methods using SC for selective enrichment. The alternative selective enrichments used by laboratories 3, 6 and 8 are shown in Table 8. Laboratory 6 could not detect 10 cfp STM from samples C2, C6 and C8 using SC for selective enrichment and BGA for isolation.

Faeces containing antibiotic

Three laboratories (1, 6 and 13) isolated *Salmonella* from one or more of the capsules to which faeces containing antibiotic was added. Laboratory 1 isolated *Salmonella* from sample 27 with all isolation media used. Laboratory 6 isolated *Salmonella* from sample 27 only with the use of the reference method. Laboratory 13 isolated *Salmonella* from samples 24 to 28 with the reference method. Using their own isolation medium all five samples were found positive. Using BGA for isolation they found one less positive. No *Salmonella* was isolated from sample 22 to 28 with the use of MSRV by this laboratory.

4.2.2 Reference method (n=42)

The number of positive isolations and the serotypes identified from all 42 capsules are presented in Table 5 per laboratory and per level of contamination. For all laboratories finding not all samples positive or negative, the 95% confidence intervals for the percentage of positive isolations were determined (Table 6). This interval can be used to compare the results

between laboratories. When the intervals do not overlap the percentage of positive isolations of those laboratories are significantly different from each other.
The total number of positive isolations varied from 0 to 39. Based on SAS (proc glm, Duncan's multiple range test) significant differences ($p<0.05$) were found between the laboratories over all 42 capsules examined. Laboratory 2 could not isolate *Salmonella* from any of the capsules. Four laboratories (labcodes 2, 6, 9 and 11) found significantly less positive isolations ($p<0.05$, Duncan's multiple range test) compared with the remaining 11 laboratories. None of the laboratories isolated *Salmonella* from all capsules containing 10 cfp STM (STM10). The number of capsules which were found positive for this level of contamination varied from 0 to 13. Laboratories 2, 3, 6, 9, 11 and 16 found significantly less positives ($p<0.05$, Duncan's multiple range test) in comparison with the other participants. *Salmonella* was isolated 0 to 14 times, from the capsules containing 100 cfp STM (STM 100) and SE. Laboratories 1, 4, 5, 7, 10 and 13 found all capsules containing 100 cfp STM positive. For the capsules containing 100 cfp STM, laboratories 2 and 11 found significantly less positives capsules ($p<0.05$, Duncan's multiple range test) compared to the other participants. Laboratories 1, 2, 3, 6, 8, 9, 11 and 16 found significantly less positives ($p<0.05$, Duncan's multiple range test) with the isolation of *Salmonella* from the capsules containing 100 cfp SE compared to the other participants. Laboratories 4, 10 and 13 isolated SE from all 14 capsules and laboratories 2, 6, and 16 did not isolate *Salmonella* from those capsules at all.

Table 5: *Number of positive isolations with the use of the reference method.*

Labcode	Total (n=42)	STM10 (n=14)	STM100 (n=14)	SE (n=14)
1	27	11	14	2
2	0	0	0	0
3	16	3	8	5
4	39	11	14	14
5	33	13	14	6
6	11	1	10	0
7	38	12	14	12
8	20	7	9	4
9	9	1	7	1
10	38	10	14	14
11	7	1	5	1
12	34	9	12	13
13	39	11	14	14
15	27	9	12	6
16	15	3	12	0

Second isolation media

In total 14 laboratories used an own isolation media besides BGA. The results are shown in Table 7. Significant differences were found with respect to laboratory 3 and 12. Laboratory 3 isolated *Salmonella* from 15 capsules using BGA and from 5 capsules using Rambach agar. Laboratory 12 isolated *Salmonella* from 34 capsules using SMID and from 26 capsules using BGA.

Table 6: *95% confidence intervals for the percentage of positive Salmonella isolations in relation to the level of contamination of the capsules.*

Labcode	no. of positive capsules	percentage positive	95% confidence intervals
STM 10 (n=14)			
1	11	79	54-95
3	3	21	5-46
4	11	79	54-95
5	13	93	74-100
6	1	7	0-26
7	12	86	63-99
8	7	50	25-75
9	1	7	0-26
10	10	71	45-91
11	1	7	0-26
12	9	64	38-87
13	11	79	54-95
15	9	64	38-87
16	3	21	5-46

Table 6 Continued

Labcode	no. of positive capsules	percentage positive	95% confidence intervals
STM 100 (n=14)			
3	8	57	31-81
6	10	71	45-91
8	9	64	38-87
9	7	50	25-75
11	5	36	13-62
12	12	86	63-99
15	12	86	63-99
16	12	86	63-99

Table 6 Continued

Labcode	no. of positive capsules	percentage positive	95% confidence intervals
SE (n=14)			
1	2	14	1-37
3	5	36	13-62
5	6	43	19-69
7	12	86	63-99
8	4	29	9-54
9	1	7	0-26
11	1	7	0-26
12	13	93	74-100
15	6	43	19-69

Table 7: *Number of positive isolations using second isolation media compared with BGA.*

Labcode	BGA (n=42)			XLD (n=42)			Gassner (n=42)			Rambach (n=42)			Önöz (n=42)		
	10[1]	100[2]	SE[3]	10	100	SE	10	100	SE	10	100	SE	10	100	SE
1	7	14	2	12	13	2									
2	0	0	0				0	0	0						
3	3	8	5							1	2	2			
4	12	14	14	12	14	14							12	14	14
6	1	10	0				1	9	0						
7	12	14	12							12	14	12			
13	11	14	14	11	14	14									
15	8	12	6							8	12	6			
16	3	12	0							3	12	0			

Table 7 Continued

Labcode	BGA (n=42)			Compass (n=42)			MLCV BGA (n=42)			XLT 4 (n=42)			SMID (n=42)		
	10[1]	100[2]	SE[3]	10	100	SE	10	100	SE	10	100	SE	10	100	SE
5	13	14	6	12	13	6									
8	7	9	1				7	9	2						
9	1	7	1							1	7	0			
10	11	14	9										9	13	13
12	5	10	11										9	12	13

1= capsules containing 10 cfp STM (n=14)
2= capsules containing 100 cfp STM (n=14)
3= capsules containing 100 - 500 cfp SE (n=14)

4.2.3 Own bacteriological method

Own selective enrichment

Nine laboratories used also an own selective enrichment besides RV (Table 8). Five of those laboratories used a semi-solid medium. Based on SAS (proc freq, McNemar's test), for 4 of those laboratories (3, 5, 15 and 16) significant differences ($p<0.05$) in the total number of positive isolations obtained were found between the reference method and their own method indicating that with the semi-solid media (MSRV and DIASALM) significantly more positives were obtained. Also for the capsules containing SE significant differences ($p<0.05$) between the reference method and their own method were found for those laboratories indicating that *S.* Enteritidis was isolated more frequently with the semi-solid media.
For the capsules containing 10 and 100 cfp STM significant differences were found for laboratory 3 only.

Table 8: *Number of positive isolations using own selective enrichment compared with reference method (RV).*

Labcode	RV (n=42)			MK-TT (n=42)			MSRV (n=42)			SC (n=42)			RVS (n=42)			DIASSALM (n=42)		
	10[1]	100[2]	SE[3]	10	100	SE	10	100	SE	10	100	SE	10	100	SE	10	100	SE
1	11	14	2	10	14	2												
2	0	0	0	1	0	2												
3	3	8	5				9	14	10	0	0	0						
4	11	14	14										12	14	14			
5	13	14	6				13	14	13									
6	1	10	0							0	9	0						
13	11	14	14				9	13	14									
15	9	12	6													12	14	13
16	3	12	0				7	14	7									

1= capsules containing 10 cfp STM (n=14)
2= capsules containing 100 cfp STM (n=14)
3= capsules containing 100 - 500 cfp SE (n=14)

5 Discussion

In this study *Salmonella* had to be isolated from capsules in the presence of competitive micro-organisms in the form of chicken faeces. Two different contamination levels of STM and two serotypes (STM and SE) were used. For the batch of capsules containing *ca* 100 cfp SE, the (mean) value for homogeneity, expressed as $T_2/(I-1)$, was 1.9. 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 (6). For the capsules containing ± 10 and ± 100 cfp STM the value for homogeneity, expressed as $T_2/(I-1)$, was 0.90 and 1.47, respectively, with an average of 10.7 and 93.9 cfp per capsule. The level of contamination of the capsules containing SE decreased over the storage period of 7 weeks. An estimate (based on linear regression) of the level of contamination at the time of the collaborative study (one week after the end of the storage test) is *ca* 100 cfp. As the stability of the capsules containing SE is not optimal more research is needed to improve stability. As all laboratories carried out the collaborative study at the same time it was decided to use the capsules in the collaborative study.

A control was included to check whether all participants added the faeces to the capsules. Therefore an antibiotic was added to one portion of the faeces. Prior investigations were done to determine the level of antibiotic to be used. *Salmonella* was isolated 15 out of 56 times using the lower concentration of antibiotic and only isolated twice using the higher concentration. Therefore, the highest concentration was used for the faeces control.
Three laboratories isolated *Salmonella* from the capsules 22 to 28 to which faeces containing antibiotic was added. Two laboratories isolated *Salmonella* once. The other laboratory isolated *Salmonella* from 5 capsules using the reference method only. With the use of the own method (MSRV) they did not isolated *Salmonella* from the capsules to which faeces containing antibiotic was added. An explanation for finding *Salmonella* in the samples tested in combination with the faeces containing antibiotic can be that the laboratory did not add or added less than 1 gram of faeces to the BPW. If so, it is expected that both the reference and own method give false positive results. However, the false positive results were only obtained using the reference method and not with the own method although both showed a comparable performance. So, no clear explanation could be found.

One laboratory made an error with the procedure control. *Salmonella* was isolated from this control using both the reference and own method. This is likely due to cross contamination or switching of samples. Also one laboratory found all blank capsules positive with the use of an alternative method.
Using the reference method two laboratories (codes 6 and 8) could not detect *S.* Panama. Using an alternative method 3 laboratories (codes 3, 6 and 8) were not able to isolate *S.* Panama from the capsules. The chance for a *S.* Panama capsule to be blank (containing no organisms

at all) is very low ($p<0.2\%$). Laboratory 6 could not detect 10 cfp STM from 3 out of 4 control capsules with the alternative method. It is possible the laboratories are not able to detect low numbers of *Salmonella*. It might also be caused by adding the capsule to BPW which is not at room temperature.

Using the reference method one laboratory found all capsules negative. However, they found all control capsules as expected. It could mean that this laboratory is not able to detect *Salmonella* in combination with chicken faeces. None of the laboratories found all capsules containing 10 cfp STM in the presence of chicken faeces positive. The number of positive isolations varied from 0 to 13. It seems that this level of contamination is critical for the detection of *Salmonella* as this may be close to the limit of detection. As the number of positive isolations varied from lab to lab, the detection limit seems to differ from lab to lab.
Salmonella was isolated 0 to 14 times from the capsules containing 100 cfp STM. The number of positives obtained using SE (containing *ca* 100 cfp per capsule) varied, as for the 100 cfp STM, between 0 and 14. However on average less positives were found using SE. The average number of positives was comparable to the number of positives obtained using 10 cfp STM. Therefore, it seems that the limit of detection of SE is higher than for STM.
A relation between the manufacturers of the media and the number of negative isolations could not be observed.

Nine laboratories used their own selective enrichment method besides the reference method. In total five laboratories used a semi-solid medium for their own method. For these laboratories significantly more *Salmonella* isolations were obtained using their own method. Four of these five laboratories found significant differences between the reference and own methods. Three laboratories scored significantly more positives using MSRV and 1 laboratory using DIASSALM. The use of semi-solid media for the detection of *Salmonella* from chicken faeces seems to be a good alternative for RV as described in the ISO method.

In this study capsules containing SE and two levels of STM were used. Further studies are needed to evaluate the difference in the ability to detect a certain level of STM or SE and to evaluate the performance of the semisolid media.

6 Conclusion

Compared to collaborative study I and II differences in detection of *Salmonella* between and within laboratories were considerably larger.
The semi-solid media seem to be more suitable for isolation of *Salmonella* from chicken faeces, and especially for detection of *S.* Enteritidis, in comparison to the reference method.
The high levels of STM (100 cfp) are easier to isolate than the low levels of STM (10 cfp).
The lower level of STM (10 cfp/capsule) and the level of SE (*ca* 100 cfp/capsule) examined in combination with chicken faeces showed to be, on average, (close to) the detection limit of the reference method, although a large variation between laboratories was found.

References

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Appendix 1 Mailing list

01	European Commission	A. Checchi Lang
02	European Commission	B. Hogben
03	European Commission	V. Niemi
04	Veterinary Public Health Inspector	drs. H. Verburg
05-20	Participants of the study (National Reference Laboratories)	
21	Board of Directors RIVM	dr. G. Elzinga
22	Director Sector Public Health Research	prof. dr. ir. D. Kromhout
23	Head of Microbiological Laboratory for Health Protection and Director CRL *Salmonella*	dr. ir. A.M. Henken
24-26	Project Workers	
27-30	Authors	
31	Dutch National Library for Publications and Bibliography	
32	SBD/Information and Public Relations	
33	Registration agency for Scientific Reports	
33	Library RIVM	
35-50	Sales department ot RIVM Reports	
51-61	Spare copies	

Appendix 2 Protocol

BACTERIOLOGICAL COLLABORATIVE STUDY III
ORGANIZED BY CRL SALMONELLA

PROTOCOL:

Introduction:
The Community Reference Laboratory (CRL) Salmonella organizes a third bacteriological collaborative study on the methods for the detection of *Salmonella* amongst the National Reference Laboratories (NRLs). In the first collaborative study (October 1995) no significant difference was found between the routine method (performed by 8 of the 17 laboratories) and the reference method for the individual laboratories. In the second study (October 1996) only 8 of the 17 laboratories isolated *Salmonella* from all 30 positive capsules using the full International Standard Organization (ISO) 6579 method. The number of positive isolations was on average significantly lower with selenite/cystine compared to Rappaport-Vassiliadis as selective enrichment medium. In this third study the ability of the laboratories to detect different contamination levels of *Salmonella* in the presence of competitive organisms will be tested. The NRLs have to use the principles of the adapted method of the ISO 6579 for the detection of *Salmonella* (excluding selenite/cystine). The adapted method is described in the Standard Operating Procedure (SOP) enclosed. The NRLs may optionally use their own method (= the routine method).
Reference Materials (RMs) produced by the CRL and poultry faeces will be used for testing the performance of this study. The RMs consist of gelatin capsules containing sublethally injured Salmonella Typhimurium or Salmonella Enteritidis.
Each laboratory will examine 49 faeces samples in combination with a capsule containing Salmonella Typhimurium or Salmonella Enteritidis and 13 control samples. The results will be evaluated by the CRL.

Objective:
The main objective of the third 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.

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:

- 49 numbered vials; each containing one Salmonella Typhimurium or Salmonella Enteritidis capsule;
- 11 control vials; each containing one capsule with or without *Salmonella*;
- 7 portions of 10 gr. frozen poultry faeces.

The performance of the study will be in week 17 (starting on 20 april 1998). 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 reference and routine method in the collaborative study will not be supplied by the CRL.

Time table of bacteriological collaborative study III

The performance of the study must be in **week 17 (starting on 20 april 1998)** or one week earlier or later.

30 March - 03 April Mailing the protocol, SOP and test report to the NRLs.

08 April Mailing the parcel to the NRLs.
CRL will mail the parcel by cargo freight from the Dutch airport (Schiphol) to the airport of destination.
The participants have to collect the parcel at the airport. Please collect the parcel directly after arrival at the airport. For this you need the airway bill number. This number and other necessary information will be indicated in a fax in the week before mailing.

The transport costs from the airport of destination to the laboratory can't be paid by the CRL, so this will be at the expense of the NRL.

The parcel will be mailed with cooling devices to keep the temperature low during transport and storage at the airport. A cold chain monitor is included in the parcel to check the temperature during shipment. For collecting the parcel at the airport take your own cooling box with cooling devices or ice with you. Open the parcel at the airport and check the contents for damage. Put the contents of the parcel into your own cooling box. Check the cold chain monitor and *note on test report (a copy of the concerning page is enclosed in the parcel) the date, time, the colour of the different compartments and whether the complete compartment has become blue.* Place the cold chain monitor in the cooling box with the reference materials. Immediately after arrival at the laboratory **store the materials at -20 °C**. Check the cold chain monitor again and *note on test report date, time, the colour of the different compartments and whether the complete compartment has become blue.*

For a small number of laboratories the CRL will mail the parcel by courier service. The parcel will be mailed with cooling devices to keep the temperature low during transport. A cold chain monitor is included in the parcel to check the temperature during shipment.
Check the cold chain monitor upon arrival as described above.
Immediately after arrival at the laboratory **store the materials at -20 °C.**

If you did not receive the parcel before or at 10 April, do contact the CRL immediately.

14 - 17 April — 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)

20 - 24 April — 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.

06 - 08 May — Completion of the test report and faxing it to the CRL. The original test report will be sent to CRL.

18 - 22 May — Checking the results by the National Reference Laboratories.

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

Nelly Voogt / Maurice Raes (research assistants CRL)
P.O. Box 1
3720 BA Bilthoven
The Netherlands
tel. number: ..-31-30-2743927 / ..-31-30-2744263
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Appendix 3 Standard Operating Procedure

BACTERIOLOGICAL COLLABORATIVE STUDY III SOP/CRL/04
ORGANIZED BY CRL SALMONELLA Version no.1
170298

STANDARD OPERATING PROCEDURE (SOP):

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

1. Scope and field of application

This standard operating procedure (SOP) describes the procedure for the detection of *Salmonella* in the presence of competitive micro-organisms. For this purpose Reference Materials (RMs) containing sublethally injured Salmonella Typhimurium or Salmonella Enteritidis as prepared by the Community Reference Laboratory (CRL) Salmonella and poultry faeces are used. Its application is limited to the bacteriological collaborative study for *Salmonella* described is this sop.

2. References

International Organization of Standardization.
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 gelatin 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.

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)
5.3. Solid selective media
5.3.1. Phenol red/brilliant green agar (Annex B.4)
5.3.2. Second medium (ISO 6579; 4.3)
5.4. Confirmation media
Selection of colonies for confirmation (optionally)
5.4.1. Nutrient agar (Annex B.5)
Biochemical confirmation
5.4.2. Triple sugar/iron agar (TSI agar) (Annex B.6)
5.4.3. Urea agar (Annex B.7)
5.4.4. l-Lysine decarboxylation medium (Annex B.8)

6. Apparatus and glassware

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

6.1. Apparatus

6.1.1. Oven (for dry sterilization) or autoclave (for wet sterilization);
6.1.2. Incubator, capable of operating at 37 °C ± 1 °C;
6.1.3. Water bath, capable of operating at 42 °C ± 0.1 °C or incubator, capable of operating at 42 °C ± 0.5 °C;
6.1.4. Water bath, capable of operating at 37 °C ± 0.1 °C;
6.1.5. Loops;
6.1.6. pH-meter; having an accuracy of calibration of ± 0.1 pH unit at 25 °C.

6.2. Glassware

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

6.2.1. Culture bottles or jars, capacity ≥ 250 ml;
6.2.2. Culture tubes; 8 mm in diameter and 160 mm in length;
6.2.3. Micro-pipettes; nominal capacity 0,1 ml.
6.2.4. Petri dishes; small size (diameter 90 mm to 100 mm) and/or large size (diameter 140 mm).

7. Procedure

Thaw the 7 portions of 10 gr. frozen faeces **overnight** at 5 °C. Take the frozen faeces out of the freezer at the end of the working day.

7.1. Pre-enrichment

Allow the BPW to equilibrate to room temperature, if it was stored at a lower temperature. Record on test report (page 3) the requested data of the BPW. Take the numbered vials with the *Salmonella* capsules and the control capsules out of the freezer one hour before they are added to the pre-enrichment broth BPW, to allow them to equilibrate to room temperature. Label 49 jars containing 225 ml of BPW from 1 to 49. Also label 13 BPW from C1 to C13 (control capsules). One jar is a procedure control (= C12) to which no capsule/faeces is added and one jar is a negative control to which only 1 gr. faeces is added (= C13). These control jars should be handled in the same way as the other jars from then on.
After equilibration add to 60 labelled jars a gelatin capsule from the vial with the corresponding label number. Do <u>not</u> open the gelatin capsule and/or do not shake the BPW to dissolve the capsule more rapidly. Place the jars in the 37 °C incubator for **30 minutes** for dissolving

of the capsules. Note the temperature and time at the start and at the end of this period on test report (page 3).After 30 minutes add the thawed faeces to the jars according to the following scheme, except to the procedure and 11 control jars;

- **add 1 gr. faeces from portion 1 to jars labeled 1-7,**
- **add 1 gr. faeces from portion 2 to jars labeled 8-14,**
- **add 1 gr. faeces from portion 3 to jars labeled 15-21,**
- **add 1 gr. faeces from portion 4 to jars labeled 22-28,**
- **add 1 gr. faeces from portion 5 to jars labeled 29-35,**
- **add 1 gr. faeces from portion 6 to jars labeled 36-42 and to C13,**
- **add 1 gr. faeces from portion 7 to jars labeled 43-49,**
- **add no faeces to jars labeled C1 - C12.**

Do not shake the jars after adding the faeces.
Place all jars in the 37 °C incubator for 16 h to 20 h. Note the temperature and time at the start and at the end of the incubation period and other requested data on test report (page 3).

7.2. Selective enrichment

Allow the selective enrichment broths to equilibrate to room temperature, if they were stored at a lower temperature. Record on test report (page 4-5) the requested data of the selective enrichment broths. Label 49 jars/tubes of each selective enrichment broth from 1 to 49. Also label 13 selective enrichment broths from C1 to C13 (control jars).

After equilibration:
- transfer 0.1 ml of homogenised BPW culture to a tube containing 10 ml RV medium with the corresponding label number. Incubate at 42 °C for 24 h and a further 24 h;
- inoculate the routinely used selective medium/media (other than the one mentioned above), with the corresponding label number, from the homogenised BPW culture (note the inocula-tion volume of BPW used and the volume of the selective medium/media on test report). In-cubate at the temperature routinely used.
Place the jars/tubes in the appropiate 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 4-5).

7.3. Isolation on media (first and second isolation)

Note: In the case that you do not have large dishes at your disposal use two small dishes, one after the other, using the same loop.
Record on test report (page 6-8) the requested data of the isolation media used. Label 49 large petri dishes of the isolation media from 1 to 49 and label 13 large petri dishes from C1 to C13

(these petri dishes are inoculated with the control selective enrichment broths). (see also *note* at the beginning of section 7.3)

7.3.1. First isolation

Inoculation:

Inoculate, by means of a loop, from all selective enrichment cultures the surface of a large size petri dish with the corresponding label number (see also *note* at the beginning of section 7.3) containing:

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

Incubation:

ad 1/2. Place the petri dishes with the bottom up in the incubator set at 37 °C (note the temperature and time at the start and at the end of the incubation and other requested data on test report, page 6-7).

ad 3. Incubate the medium/media at the temperature routinely used (note temperature and time and other requested data on test report, page 8).

After incubation for 20 h to 24 h, examine the petri dishes for the presence of typical colonies of *Salmonella*. Typical colonies of *Salmonella* grown on phenol red/brilliant green agar cause to change the colour of the medium from pink to red.

If growth is slight or no typical colonies of *Salmonella* are present, re-incubate at 37 °C for 18 h to 24 h. Re-examine for the presence of typical colonies of *Salmonella*.

7.3.2. Second isolation

After a total incubation time of 48 h of the selective enrichment media, repeat the procedure described above (**7.3.1. First isolation**).

7.4. 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 biochemically 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 9) 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 9 (first isolation)

and 10 (second isolation) on test report (page 11-16). The results of the control capsules must be reported in Table 11 (page 17-18).

7.4.1. 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 10) the requested data of the media.

7.4.1.1. TSI agar

7.4.1.2. Urea agar

7.4.1.3. l-Lysine decarboxylation medium

7.4.2. Interpretation of the biochemical tests

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

7.4.2.1. TSI agar:

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

Slant: red or unchanged

7.4.2.2. Urea agar: coloured yellow

7.4.2.3. l-Lysine decarboxylation medium: coloured purple

8. 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 1 gr faeces to BPW 16-20 h at 37 °C
day 2	**Selective enrichment**	0.1 ml BPW culture in 10 ml RV 24 h at 42 °C
day 3	**First isolation**	inoculate from RV: a. phenol red/brilliant green agar b. other selective medi(um)(a) incubate both 20 -24 h at the specified temperature
	Continue sel. enrichment	incubate RV medium (see day 2) another 24 h at 42 °C
day 4	**Second isolation**	inoculate from RV (see day 3) a. phenol red/brilliant green agar b. other selective medi(um)(a) incubate both 20 -24 h at the specified temperature
	Biochemical confirmation	inoculate the media from first isolation media (day 3) for biochemical identification and incubate 20 -24 h at the specified temperature
day 5	**Biochemical confirmation**	inoculate the media from first isolation media (day 4) for biochemical identification and incubate 20 -24 h at the specified temperature

Appendix 4 Test Report

BACTERIOLOGICAL COLLABORATIVE STUDY III
ORGANIZED BY CRL SALMONELLA

TEST REPORT
OF THE THIRD BACTERIOLOGICAL COLLABORATIVE STUDY

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

Laboratory code :
Laboratory name : ....

Date of receipt of the parcel : .... - .... - 1998
Starting date for detection : .... - .... - 1998

Shipment

Cold chain monitor:

1. Check at airport:

date :............. - 1998
time :............. h .... min

Parcel damaged ☐ YES
☐ NO

Colour of compartment

A	☐ completely coloured		☐ partly coloured
	☐ white	☐ light blue	☐ dark blue
B	☐ completely coloured		☐ partly coloured
	☐ white	☐ light blue	☐ dark blue
C	☐ completely coloured		☐ partly coloured
	☐ white	☐ light blue	☐ dark blue
D	☐ completely coloured		☐ partly coloured
	☐ white	☐ light blue	☐ dark blue

2. Check at laboratory:

date :............. - 1998
time :............. h .... min

Parcel damaged ☐ YES
☐ NO

Colour of compartment

A	☐ completely coloured		☐ partly coloured
	☐ white	☐ light blue	☐ dark blue
B	☐ completely coloured		☐ partly coloured
	☐ white	☐ light blue	☐ dark blue
C	☐ completely coloured		☐ partly coloured
	☐ white	☐ light blue	☐ dark blue
D	☐ completely coloured		☐ partly coloured
	☐ white	☐ light blue	☐ dark blue

Pre-enrichment

Manufacturer of the BPW:

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

Incubation time and temperature for dissolving the capsules

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

Incubation time and temperature for pre-enrichment

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

Type of incubator: ☐ vented incubator
☐ nonvented incubator

Thermometer used for reading the incubation temperature:

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

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

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

Selective enrichment

1. Rappaport Vassiliadis medium

Manufacturer of the RV medium:

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

Incubation time and temperature for selective enrichment:

- at the start : time: .... h .... min
  : temperature incubator: .... °C
- at the end first 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. Selective medium, routinely used in your laboratory

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

Medium : ....

Manufacturer of the medium:

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

Volume of the medium per jar/tube : .... ml

Inoculation volume of BPW : .... ml

Incubation temperature : .... °C

Incubation time and temperature for selective enrichment:

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

First and second isolation

1. Phenol red/brilliant green agar

Manufacturer of the phenol red/brilliant green agar:

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

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

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

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

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

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

Type of incubator: □ vented incubator
□ nonvented incubator

Thermometer used for reading the incubation temperature:

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

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

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

2. Isolation medium of your own choice

Medium : ....

Manufacturer of your own medium:

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

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

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

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

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

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

Type of incubator: □ vented incubator
□ nonvented incubator

Thermometer used for reading the incubation temperature:

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

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

Place of thermometer in the incubator:...

Place of jars in the incubator :...

3. Isolation medium routinely used in your laboratory

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

Medium : ....

Manufacturer of this medium:

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

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

Incubation temperature : .... °C

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

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

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

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

Type of incubator: ☐ vented incubator
☐ nonvented incubator

Thermometer used for reading the incubation temperature:

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

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

Place of thermometer in the incubator:..

Place of jars in the incubator :..

Confirmation

Optionally

Manufacturer of the nutrient agar:

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

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

Incubation time and temperature for confirmation:

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

Type of incubator: □ vented incubator
□ nonvented incubator

Thermometer used for reading the incubation temperature:

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

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

Place of thermometer in the incubator:..

Place of jars in the incubator :..

Biochemical confirmation

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

Manufacturer of TSI agar:

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

Manufacturer of urea agar:

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

Manufacturer of l-Lysine decarboxylation medium:

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

Table 9: Results of confirmation tests of the **first isolation** (dish numbers 1-15)

no.	RV [a]				Own enrichment broth			
	BGA [b]		second [c] medium		first [d] medium		second medium	
	col [e]	Sal [f]	col	Sal	col	Sal	col	Sal
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								
16								

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

Table 9 (continued): dish numbers 17-32

no.	RV [a]				Own enrichment broth			
	BGA [b]		second [c] medium		first [d] medium		second medium	
	col [e]	Sal [f]	col	Sal	col	Sal	col	Sal
17								
18								
19								
20								
21								
22								
23								
24								
25								
26								
27								
28								
29								
30								
31								
32								

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

Table 9 (continued): dish numbers 33-49

	RV [a]				Own enrichment broth			
	BGA [b]		second [c] medium		first [d] medium		second medium	
no.	col [e]	Sal [f]	col	Sal	col	Sal	col	Sal
33								
34								
35								
36								
37								
38								
39								
40								
41								
42								
43								
44								
45								
46								
47								
48								
49								

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

Table 10: Results of confirmation tests of the **second isolation** (dish numbers 1-16)

no.	RV [a]				Own enrichment broth			
	BGA [b]		second [c] medium		first [d] medium		second medium	
	col [e]	Sal [f]	col	Sal	col	Sal	col	Sal
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								
16								

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

Table 10 (continued): dish numbers 17-32

	RV [a]				Own enrichment broth			
	BGA [b]		second [c] medium		first [d] medium		second medium	
no.	col [e]	Sal [f]	col	Sal	col	Sal	col	Sal
17								
18								
19								
20								
21								
22								
23								
24								
25								
26								
27								
28								
29								
30								
31								
32								

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

Table 10 (continued): dish numbers 33-49

	RV [a]				Own enrichment broth			
	BGA [b]		second [c] medium		first [d] medium		second medium	
no.	col [e]	Sal [f]	col	Sal	col	Sal	col	Sal
33								
34								
35								
36								
37								
38								
39								
40								
41								
42								
43								
44								
45								
46								
47								
48								
49								

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

Table 11a: Results of the confirmation tests of the control capsules (**first isolation**)

no.	RV [a]				Own enrichment broth			
	BGA [b]		second [c] medium		first [d] medium		second medium	
	col [e]	Sal [f]	col	Sal	col	Sal	col	Sal
C 1								
C 2								
C 3								
C 4								
C 5								
C 6								
C 7								
C 8								
C 9								
C 10								
C 11								
C 12								
C 13								

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

Table 11b: Results of the confirmation tests of the control capsules (**second isolation**)

	RV [a]				Own enrichment broth			
	BGA [b]		second [c] medium		first [d] medium		second medium	
no.	col [e]	Sal [f]	col	Sal	col	Sal	col	Sal
C 1								
C 2								
C 3								
C 4								
C 5								
C 6								
C 7								
C 8								
C 9								
C 10								
C 11								
C 12								
C 13								

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

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

Date: .... - .... - 1998

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

....

signature:..

Name of person in charge:

....

signature:..

Appendix 5 Data about media used

Table 12: *Manufacturers of the pre-enrichment medium (BPW) used by the participating laboratories*

Labcode	manufacturer	code number
1	OXOID	CM 509
2	own preparation	
3	Merck	07228
4	LAB M	LAB 46
5	AES Laboratoires	AEB 140302
6	Merck	1.07228
7	Merck	1.07228
8	LAB M	LAB 46
9	OXOID	CM 509
10	Bio-Merieux	42043
11	SVM	E4900z
12	OXOID	CM 509
13	own preparation	
15	OXOID	CM 509
16	OXOID	CM 509

Table 13: *Manufacturers of the first (=RV) and second selective enrichment medium used by the participating laboratories*

Labcode	RV		second selective enrichment medium		
	manufacturer	code number	name	manufacturer	code number
1	OXOID	CM 669	MK-TT	OXOID	CM 343
2	Bio-Merieux	42003	MK-TT	OXOID	CM 343
3	OXOID	CM 866			
4	LAB M	LAB 86			
5	Merck	7700	MSRV	Biokar	BK 134
6	OXOID	CM 669	SC	own preparation	
7	Merck	1.07700			
8	LAB M	LAB 46			
9	OXOID	CM 669			
10	Bio-Merieux	42003			
11	SVM	E5125z			
12	Bio-Merieux	41214			
13	own preparation		MSRV	Difco	1868-17
15	OXOID	CM 669	DIASSALM	LAB M	LAB 537
16	OXOID	CM 669	MSRV	OXOID	CM 910

Table 14: *Manufacturers of the first (=BGA) and second selective isolation medium used by the participating laboratories*

Labcode	BGA		second selective isolation medium		
	manufacturer	code number	name	manufacturer	code number
1	OXOID	CM 329	XLD Hektoen	OXOID OXOID	CM 469 CM 419
2	OXOID	CM 329	Gassner	own production	
3	OXOID	CM 329	Rambach	Merck	7500/0000
4	LAB M	LAB 34	XLD Önöz	LAB M Merck	LAB 32 15034
5	AES laboratoire	AEB 151 492	Compass Rambach XLT4	Biokar Merck Difco	BKRD 002 1.07500/0002 0234-17
6	Merck	1.10747	Gassner	Merck	1282
7	Merck	1.10747	Rambach	Merck	1.07500/0002
8	LAB M	LAB 34	MLCV BGA	LAB M	LAB 116
9	OXOID	CM 329	XLT4	Difco	0234-17
10	Difco	0285-17-7	SMID	Bio Merieux	43291
11	SVM	E1800z			
12	Difco	0285-17-7	SMID	Bio Merieux	43291
13	Merck	7237	XLD	Difco	0788-17-9
15	Difco	0285-17-7	Rambach	Merck	107500 002
16	OXOID	CM 329	Rambach	Merck	1.07500