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A collaborative study on the detection of *Salmonella* amongst the National Reference Laboratories for *Salmonella*

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ABSTRACT

A bacteriological collaborative study in which the National Reference Laboratories (NRLs) for *Salmonella* participated was organized by the Community Reference Laboratory (CRL) for *Salmonella*. The study aimed to compare the results of the proposed European reference method (ISO 6579 method) and routine methods for the detection of *Salmonella* among and within the participating laboratories.

Because of incorrect examination of the test samples the results of three laboratories could not be evaluated. One laboratory found significantly less positive results in comparison with other laboratories using the reference method. These three laboratories carried out the study again. In this additional study their results did not deviate anymore.

No significant difference was found between the routine (performed by eight laboratories) and the reference method for the individual laboratories.

SUMMARY

A bacteriological collaborative study was organized by the Community Reference Laboratory (CRL) for *Salmonella*. In this study the National Reference Laboratories (NRLs) for *Salmonella* of the Member States of the European Union (EU) participated.
The aim of this study was to compare the results of the proposed reference method for the detection of *Salmonella* (ISO 6579 method) among and within laboratories and the results of the reference and routine method within a laboratory.
Seventeen laboratories examined 30 individually numbered capsules of which 26 contained circa 5 colony forming particles (cfp) per capsule of S Typhimurium in total and 4 were negative control samples. The number of these capsules was unknown to the participants. Besides the reference method the laboratories were free to perform the routine method (their own method) in their laboratory.
Because one laboratory did not use the reference method and two laboratories reported positive *Salmonella* isolations from negative control samples, the results of these laboratories were excluded from statistical analysis.
Using the reference method, one laboratory found significantly more capsules negative in comparison with other laboratories. Among the remaining 13 laboratories no significant difference was found in the number of positive *Salmonella* isolations. Eight laboratories performed their own method and no significant difference was found between their own and the reference method for the individual laboratories.
Three laboratories with deviating results carried out the study once again. In that additional study no deviating results were found anymore.

SAMENVATTING

Het Communautair Referentie Laboratorium voor *Salmonella* heeft een bacteriologisch ringonderzoek georganiseerd met deelname van alle Nationale Referentie Laboratoria uit de lidstaten van de Europese Unie.
Doel van het ringonderzoek was om de resultaten te vergelijken van de voorgestelde referentiemethode voor de detectie van *Salmonella* (ISO 6579 methode) tussen en binnen de laboratoria en om de resultaten van de referentie en eigen methode binnen een laboratorium te vergelijken.
Zeventien laboratoria onderzochten in totaal 30 individueel genummerde capsules, die ongeveer 5 kolonie vormende eenheden S.Typhimurium per capsule bevatten. Vier capsules waren negatieve controles. De deelnemers wisten niet hoeveel negatieve controles er waren. Naast de referentiemethode konden de laboratoria de methode uitvoeren die ze normaal gebruiken (eigen methode).
De resultaten van 3 deelnemers werden niet gebruikt voor de statistische analyse; één laboratorium gebruikte de referentiemethode niet en 2 laboratoria isoleerden *Salmonella* uit negatieve controles.
Bij het uitvoeren van de referentiemethode vond één laboratorium in vergelijking met de andere laboratoria significant meer capsules negatief. Tussen de overige 13 laboratoria werd geen significant verschil gevonden in het aantal voor *Salmonella* positieve monsters. Acht laboratoria voerden daarnaast hun eigen methode uit, waarbij geen significant verschil gevonden werd tussen de eigen en de referentiemethode die binnen de afzonderlijke laboratoria gebruikt werden.
Drie laboratoria met afwijkende resultaten voerden de studie nogmaals uit. In deze aanvullende studie werden geen afwijkende resultaten meer gevonden.

1. INTRODUCTION

The Council Directive 92/117/EEC concerns measures for protection against specific zoonoses and specified zoonotic agents in animals and products of animal origin in order to prevent outbreaks of food borne infections and intoxications.

The Directive lays down rules for the collection of information on zoonoses and zoonotic agents and the relevant measures to be taken in the Member States.
As far as *Salmonella* is concerned the Commission has to be informed about:

- the occurrence of *Salmonella* in animals, products of animal origin and animal feeding stuffs,
- the occurrence of clinical cases of salmonellosis in humans and animals.

Furthermore the Directive predescribes intervention measures to reduce the contamination in poultry breeding flocks.

The Directive provides rules for sampling and examination of samples. A proposed bacteriological reference method for *Salmonella* is the ISO 6579 method.
It is a task of the Community Reference Laboratory (CRL) for *Salmonella* to organize collaborative studies. These studies aim that the examination of samples in Member States is carried out uniformly and that comparable results will be obtained.

This report describes the first bacteriological collaborative study organized by the CRL for *Salmonella*, in which all National Reference Laboratories (NRLs) for *Salmonella* of the Member States participated. Thirty vials of which twenty six containing low numbers of sublethally injured S.Typhimurium, were examined.

2. PARTICIPANTS

2.1. National Reference Laboratories

Austria	Bundesstaatliche bakteriologisch-serologische Untersuchungsanstalt Graz Graz
Belgium	Institut National de Recherches Veterinaires Bruxelles
Denmark	Danish Veterinary Laboratory Copenhagen
Finland	National Veterinary and Food Research Institute Department of Food Microbiology Helsinki
France	Centre National d'Etudes Vétérinaires et Alimentaires Laboratoire central de recherches avicole et porcine Ploufragan
Germany	Bundesinstitut für gesuntheitlichen Verbraucherschutz und Veterinär-medizin Berlin
Greece	Centre of Athens Veterinary Institutions Microbiology Department Athens
Ireland	Department of Agriculture Food and Forestry Veterinary Research Laboratory Dublin
Italy	Istituto Zooprofilattico Sperimentale delle Venezie Vicenza

Luxembourg	Laboratoire de Médecine vétérinaire de l'Etat Luxembourg
The Netherlands	National Institute of Public Health and the Environment Bilthoven
Portugal	Laboratorio Nacional de Veterindria Lisboa
Spain	Laboratorio de Sanidad y Producción Animal de Algete Madrid
Sweden	National Veterinary Institute Laboratory of Bacteriology Uppsala
United Kingdom	Central Veterinary Laboratory Bacteriological Department Surrey
Northern Ireland	Veterinary Sciences Division Bacteriology Department Belfast

2.2. Other laboratories

Denmark	Danish Veterinary Laboratory Aarhus

3. MATERIALS AND METHODS

3.1. Preparation of the batch reference materials of S.Typhimurium

The reference materials were prepared from a highly contaminated milk powder (HCMP) containing S. Typhimurium. This powder was obtained by spray-drying artificially contaminated milk as described earlier by in 't Veld et al (1).
From the HCMP 0.03 gram was diluted in steps by mixing with uncontaminated milkpowder using a mortar and pestle to circa 500 gram. The target contamination level of the mixed powder was circa 5 colony forming particles (cfp's) per capsule. Two times 50 capsules were filled with the mixed powder (0.34 g per capsule) and used to determine the number of cfp's of *Salmonella* per capsule (called *z*) and the homogeneity of the batch (named 1-5/250795/3). The determination of *z* and the homogeneity of the batch was carried out as described by in 't Veld et al (1).
For the collaborative study and testing the stability at storage temperature of -20 °C 960 capsules were filled. The stability test started 5 weeks before the collaborative study, each time of analysis 50 capsules were used to enumerate the number of cfp of *Salmonella*.

3.2. Collaborative study

The reference materials were mailed 3 weeks before the study by cargo freight to limit the time between mailing and arrival. Cooling devices were enclosed with the reference materials to keep the temperature low during transport. The capsules were stored at -20 °C upon receipt until the start of the study. In the protocol (annex 1) all details about the mailing and storing are mentioned.
The Standard Operating Procedure (SOP, annex 2) describes the procedure used for the collaborative study. As reference method, the ISO 6579 method, for the detection of *Salmonella* (2) was used. Besides the reference method the participants were asked to perform the method routinely used in their laboratory (= their own method).
All laboratories received 30 vials, each containing one capsule, to be tested for the presence of S. Typhimurium. From the 30 capsules 4 were negative control samples, the number of these capsules was unknown to the participants. The results of the study and operational details were reported in a test report (annex 3) to the CRL.
The protocol, SOP and test report were mailed to the participants 4 weeks before the collaborative study.

3.2.1. Statistical analysis of data

The distribution of the cfp over the capsules and the probability of finding negative capsules in the number of samples used for the collaborative study were calculated before the results of the participants were analyzed.
The CRL evaluated the results based on the number of positive and negative isolations reported by the laboratories. For significant differences ($\alpha = 0.05$) the Fisher's exact test (using the mid p-value) was used (3) to test among laboratories using the reference method, between the two selective enrichment media of the reference method within a laboratory and between the reference and routine method within a laboratory. The Pearson residual (3) was used to identify laboratories with a deviating proportion of negative isolations in comparison with the other laboratories.

4. RESULTS

4.1. Stability and homogeneity test of reference material

The contamination level of batch 1-5/250795/3 was determined twice before the capsules were filled for the stability test and the collaborative study. The mean *z* was 6.42 and 5.84. Corresponding values for the homogeneity of the batch, expressed as the measure of dispersion $T_2/(I-1)$, were 1.23 and 0.99 respectively (T_2 is the Cochran's dispersion; measure of spread between capsules and I is the number of capsules (3)). In case of an ideal homogeneous distribution (Poisson distribution) a value of $T_2/(I-1)$ of 1 is expected. Based on these results it was decided to use the batch for the stability test and the collaborative study.

The results of the stability and homogeneity tests of the batch are shown in Table 1. Based on the T_2 values shown in Table 1 a mean value for the homogeneity of the batch (called T_{hom}/N) was calculated; this value was 1.05. In case of a Poisson distribution, T_2 follows a χ^2-distribution with (I-1) degrees of freedom. The value 1.05 was not significant different from the χ^2-distribution with 364 df and $\alpha = 0.05$, so the variation in *z* between the capsules followed a Poisson distribution.

Table 1: *Results of the stability and homogeneity tests of batch 1-5/250795/3, stored at -20 °C.*

Storage time (weeks)	mean *z*	number of capsules (I)	T_2	$T_2/(I-1)$
0	5.27	48	48.09	1.02
1	4.98	48	66.07	1.41
2	4.68	47	53.45	1.16
3	3.96	46	58.11	1.29
4	4.91	46	33.72	0.75
5	4.61	44	43.01	1.00
6	5.43	47	43.77	0.95
8	3.89	46	36.61	0.81

4.2. Media applied and performance of the method

The ISO 6579 method (2) is based on 4 stages, namely pre-enrichment, selective enrichment, isolation on selective solid media and confirmation of typical colonies as *Salmonella*. The media are predescribed in the ISO 6579 document.

In the Tables 2 - 4 the data of the media used by the participants and the incubation times of the various stages are presented. The results of laboratory 2 are not shown. Due to a reorganization they only performed their own method and reported only the number of capsules found positive or negative for *Salmonella*. Laboratory 15 divided the 30 capsules in two times 15 and examined these on two successive weeks.

The incubation time of the pre-enrichment step according to the reference method is 16-20 h. This time varied between 18 h 05 min and 24 h 50 min (Table 2).

For the enrichment step the predescribed incubation time is 24 h and a further 24 h. Using Rappaport Vassiliadis (RV) the laboratories, except the laboratories 15 and 16, reported an incubation period of 24 h ± 1 h. The incubation time varied between 20 h 45 min and 25 h (Table 3a). After 48 hours the laboratories, except the numbers 9, 12 and 15, reported an incubation time of 48 h ± 2 h. The incubation time varied between 44 h 50 min and 51 h 45 min. With the use of Selenite/cystine (SC) as enrichment medium, the laboratories, except the laboratories 12, 15 and 16, reported an incubation period of 24 h ± 1 h. The incubation time varied between 20 h 45 min and 25 h and 35 min (Table 3b). After 48 hours the laboratories, except the numbers 12 and 15, reported an incubation time of 48 h ± 2 h. The incubation time varied between 44 h 50 min and 51 h 45 min.

The predescribed incubation time for the isolation on a selective medium is 18-24 h. This time varied between 19 h 05 min and 24 h 10 min for the BGA (Table 4a) and between 19 h 05 min and 25 h 15 min for the second isolation medium (Table 4b).

Table 2: *Manufacturers and incubation times of pre-enrichment medium used by the participating laboratories*

Buffered Peptone water			
labcode	manufacturer	(code number)	incubation time
1	own preparation		20 h 00 min
3	Merck	(1 07228 001)	20 h 26 min
4	Difco	(69524 JA)	20 h 00 min
5	LAB M	(LAB46)	19 h 35 min
6	AES	(AEB 140 30 2)	18 h 05 min
7	Difco	(0118-01-8)	20 h 00 min
8	Oxoid	(CM 509)	19 h 45 min
9	LAB M	(LAB46)	21 h 55 min
10	Oxoid	(CM 509)	19 h 05 min
11	BioMerieux	(42 043)	22 h 50 min
12	SVM	(E4900z)	19 h 35 min
13	Difco	(1810-17-9)	20 h 00 min
14	Difco	(0118-01-8 (a.o.))	18 h 20 min
15	Difco	(301186)	20 h 33 min[1] 24 h 50 min[2]
16	Oxoid	(CM 509)	19 h 00 min
17	Oxoid	(CM 509)	19 h 00 min

[1] = for capsules 1 - 15

[2] = for capsules 16 - 30

Table 3a: *Manufacturers and incubation times of the RV selective enrichment medium used by the participating laboratories*

labcode	manufacturer (code number)	incubation time (first period (total))
1	Oxoid (CM 669)	24h15min (47h15min)
3	Oxoid (CM 669)	24h00min (47h55min)
4	Oxoid (CM 669)	24h15min (48h30min)
5	LAB M (LAB86)	23h30min (47h15min)
6	Merck (7700)	24h00min (49h00min)
7	Oxoid (CM 669)	24h00min (48h00min)
8	Oxoid (CM 669)	24h00min (48h00min)
9	LAB M (LAB86)	23h45min (45h05min)
10	Oxoid (CM 669)	24h00min (48h00min)
11	BioMerieux (42 003)	23h45min (47h45min)
12	SVM (E5125z)	25h00min (51h45min)
13	Difco (1858-17)	24h00min (49h00min)
14	own preparation	24h20min (48h05min)
15	Oxoid (321465)	24h40min (48h30min)[1] 20h45min (44h50min)[2]
16	Oxoid (CM 669)	22h35min (46h05min)
17	Oxoid (CM 669)	24h10min (49h15min)

[1] = for capsules 1 - 15

[2] = for capsules 16 - 30

Table 3b: *Manufacturers and incubation times of the Selenite selective enrichment medium used by the participating laboratories*

labcode	manufacturer	(code number)	incubation time (first period (total))
1	Oxoid	(CM 699)	23h45min (46h58min)
3	Merck	(1 07709)	24h00min (48h00min)
4	Oxoid	(CM 699)	24h00min (48h00min)
5	LAB M	(LAB55A+44B)	23h30min (47h15min)
6	AES	(AES 1412 52)	23h00min (49h00min)
7	Merck	(6340+2837)	24h00min (48h00min)
8	Oxoid	(CM 699)	24h00min (48h00min)
9	LAB M	(LAB55A +Oxoid L121)	23h25min (47h30min)
10	Oxoid	(CM699+L121)	24h00min (48h00min)
11	Sanofi/Diagn	(78752)	24h15min (47h45min)
12	Oxoid	(CM699+L121)	25h35min (51h45min)
13	Difco	(0687-17-1)	24h00min (49h00min)
14	own preparation		23h30min (47h15min)
15	Difco	(311085)	24h25min (48h15min)[1] 20h45min (44h50min)[2]
16	Oxoid	(CM 699)	22h40min (46h50min)
17	Oxoid	(CM 699)	23h27min (47h47min)

[1] = for capsules 1 - 15

[2] = for capsules 16 -30

Table 4a: *Manufacturers and incubation times of the BGA isolation medium used by the participating laboratories*

labcode	manufacturer	(code number)	incubation time
1	Oxoid	(CM 263)	23 h 43 min
3	Oxoid	(CM 329)	19 h 05 min
4	Oxoid	(CM 329)	20 h 00 min
5	LAB M	(LAB34)	22 h 30 min
6	AES	(AEB 151492)	22 h 30 min
7	Merck	(10747)	24 h 00 min
8	Merck	(10747)	23 h 15 min
9	LAB M	(LAB34)	22 h 15 min
10	Oxoid	(CM 329)	23 h 00 min
11	Difco	(0285-01-5)	24 h 10 min
12	SVM	(E1800y)	22 h 35 min
13	Oxoid	(CM 329)	24 h 00 min
14	own preparation		23 h 15 min
15	Oxoid	(341100)	20 h 10 min[1] 22 h 55 min[2]
16	Oxoid	(CM 329)	22 h 00 min
17	Oxoid	(CM 263)	22 h 15 min

[1] = for capsules 1 - 15

[2] = for capsules 16 - 30

Table 4b: *Manufacturers and incubation times of the second isolation medium used by the participating laboratories*

labcode	medium	manufacturer	(code number)	incubation time
1	Hektoen	BBL	(12211)	23 h 43 min
3	XLT4	Difco suppl: Difco	(0234-17-9) (0353-72-6)	19 h 05 min
4	XLD	Oxoid	(CM 469)	20 h 00 min
5	ÖNÖZ XLD	Merck LAB M	(15034) (LAB32)	22 h 30 min 22 h 30 min
6	Rambach XLT4	Merck Difco	(7500/000 2) (0234-17-9)	22 h 30 min 25 h 15 min
7	Gassner	Merck	(1282)	24 h 00 min
8	SS agar	Oxoid	(CM 99)	22 h 45 min
9	MLCB	LAB M	(LAB116)	24 h 05 min
10	XLT4		different	23 h 00 min
11	Rambach	Merck	(7500)	23 h 35 min
12	Bismuth	Oxoid	(CM 201)	22 h 35 min
13	MacConkey	Difco	(0075-01-9)	24 h 00 min
14	MacConkey BGA	Difco Difco	(0075-17-1) (0285-17-7)	23 h 30 min 23 h 30 min
15	XLD	BBL	(342520)	20 h 10 min[1] 22 h 55 min[2]
16	Rambach	Merck	(7500/000 2)	22 h 00 min
17	DCA	Oxoid	(CM 35)	22 h 25 min

[1] = for capsules 1 - 15

[2] = for capsules 16 - 30

4.3. Reference method

The ISO 6579 method (2) describes as selective enrichment medium both RV and SC. In Table 5 the results of the capsules are presented, excluding the negative control samples.

Two laboratories reported positive *Salmonella* isolations from negative control samples. Laboratory 11 reported 3 of the 4 negative control samples with both RV and SC as positive after 48 hours incubation, laboratory 15 reported one of the four as positive using SC after 24 and 48 hours incubation.

Table 6 presents the number of positive isolations for the selective enrichment media separately. Except the laboratories 1 and 16 the laboratories found no difference in the number of capsules positive for *Salmonella* using either RV or SC medium. Laboratory 1 reported 6 positive isolations less with the use of the RV medium, while laboratory 16 found 1 positive isolation more. For the remaining laboratories the same capsule numbers were found positive for *Salmonella* using the RV or the SC medium.

For the isolation on a selective medium BGA is described as first medium. The choice of the second medium is free. The medium used by the participants is shown in Table 4b. There was no difference in results between the both selective media within a laboratory, except laboratory 1. They reported for 4 capsules a positive isolation on BGA and a negative isolation on Hektoen. The difference was only reported with the use of RV as selective enrichment medium.

Table 5: *Results of the full ISO 6579 method*

labcode	**positive** isolations	**negative** isolations
1	26	0
3	25	1
4	26	0
5	26	0
6	21	5
7	26	0
8	24	2
9	26	0
10	24	2
11	26	0
12	25	1
13	26	0
14	26	0
15	26	0
16	25	1
17	25	1
Total	403	13

Table 6: *Number of positive isolations with the use of RV and SC separately*

labcode	RV	SC
1	19	26
3	25	25
4	26	26
5	26	26
6	21	21
7	26	26
8	24	24
9	26	26
10	24	24
11	26	26
12	25	25
13	26	26
14	26	26
15	26	26
16	25	24
17	25	25
Total	396	402

4.4. Own bacteriological method

Only nine laboratories applied their own method. For 8 laboratories the difference between the reference method and their own method was the use of another medium as selective enrichment medium. Table 7 presents the number of positive isolations and the types of enrichment medium used by these laboratories. In total 5 different enrichment media were used; three laboratories (numbers 11, 14 and 16) reported a difference between the methods in the number of positive isolations.
Besides one laboratory (labcode 9) tested the 30 capsules on Malthus 2000 system. The results (26 positive isolations) were identical to the results of the reference method.

Table 7: *Number of positive isolations using own bacteriological method in relation to the results of the reference method and the types of enrichment medium used*

labcode	reference	medium 1	medium 2	medium 3	medium 4	medium 5
1	26	26				
3	25		25		25*	
6	21	21	21			
10	24			24		24#
11	25	13				
13	26					26
14	26	24				
16	25			25	24	

medium 1 = Muller/Kauffmann
medium 2 = Modified Semi-solid Rappaport Vassiliadis
medium 3 = Rappaport Vassiliadis Soya Peptone Broth
medium 4 = Selenite medium
medium 5 = Tetrathionate broth
* = incubation temperature: 41.5°C
# = base Hajna

4.5. Statistical analysis of data

For the statistical analysis the results of the laboratories 11 and 15 were rejected, because of errors made with the negative control samples.

Based on the distribution of cfp over the capsules at a low level of contamination there is a change a capsule will not contain *Salmonella*. The probability (P_x) of finding x negative capsules can be calculated (4) based on the assumption of a Poisson distribution of cfp over the capsules. In Table 8 the P_x of finding x negative capsules out of 26 capsules examined (= total number of capsules examined minus negative control samples) are shown. The overall mean z (= 4.72) of batch 1-5/250795/3 (Table 1) was used for the calculation. Also the expected and observed number of laboratories finding x numbers of negative isolations are shown.

Table 8: *Probability (P_x) of finding x negative capsules out of 26 capsules examined in relation to the expected and observed number of laboratories*

number of negative capsules (x)	P_x	Expected number of laboratories	Observed number of laboratories
0	0.79	11	7
1	0.18	2	4
2	0.02	1	2
3	0.001	0	0

Differences in the number of positive isolations among the laboratories using the reference method (Table 5) were tested by means of the Fisher's exact test (3). A significant difference was found ($p = 0.02$; a p-value of less than 0.05 is regarded as significant) among the laboratories. The Pearson residual (3) was calculated per laboratory to identify laboratories with a significant deviating number of negative isolations. The results of laboratory 6 were identified as an outlier and were excluded. The data were analyzed again. Using the Fisher's exact test no significant difference ($p = 0.46$) was found among the laboratories.

The difference in the number of positive isolations using the RV or SC medium reported by laboratory 1 and 16 (Table 6) were tested by means of the Fisher's exact (3). Laboratory 1 showed a highly significant difference between RV and SC ($p = 0.005$), while the difference found by laboratory 16 was not significant ($p = 0.62$).

The reference method compared with their own method (Table 7) showed differences in the laboratories 11, 14 and 16. The results of laboratory 11 were not statistically analyzed, because of errors made with the negative control samples. No significant difference was found, when the results of laboratory 14 and 16 were analyzed using the Fisher's exact test (laboratory 14: $p = 0.24$ and laboratory 16: $p = 0.62$).

5. DISCUSSION

A stability test with the batch reference materials was carried out to demonstrate possible changes in the number of cfp's of *Salmonella* per capsule. During two months storing at - 20 °C the mean number of cfp's of S.Typhimurium in batch 1-5/250795/3 did not show a decrease, although no test was used to confirm this statistically.

Two laboratories did not carry out the collaborative study according to the SOP. Laboratory 2 only used their own method and reported the results without filling in the test report. Laboratory 15 examined 15 capsules in two successive weeks. In a collaborative study all participants have to follow the predescribed procedure for a reliable evaluation.

The ISO 6579 method predescribes the media for the performance of the method. There are different manufacturers who produce these media. In this study the Buffered Peptone Water was produced by 7 different manufacturers, whereas 6 manufacturers provided the laboratories with Rappaport Vassiliadis and also 6 manufacturers with Selenite. The first isolation medium BGA was produced by 6 different manufacturers. The choice of the second isolation medium was optional, the participants used 11 different media.
By most of the laboratories the media for pre-enrichment and selective isolation were incubated within the predescribed time. The variation in the incubation periods of the selective enrichment media mostly varied between 24 h ± 1 h and 48 h ± 2 h.

Two participants (laboratories 11 and 15) were rejected before statistical analysis was carried out, because of errors made with the negative control samples. The occurrence of false positive results indicates that cross contamination and/or switching of samples took place. The laboratories should investigate the cause of the error.

The number of laboratories which found all of the intended capsules positive was less as expected on the basis of the enumeration results (Table 8). Using the reference method laboratory 6 found significantly more capsules negative in comparison with the other laboratories. This laboratory should investigate the cause of this difference. No significant difference was found among the remaining laboratories in the number of positive isolations.

One laboratory found a significant difference between the use of RV and SC; RV presented more negative capsules in comparison with Selenite. No deviations in the test report were found to explain this difference.

Nine laboratories reported the results of their own method besides the reference method. The difference in the use of the reference method or their own method for the examination of the capsules was not significant.

6. CONCLUSION

The objective of this study was to test the performance of the reference method among and within the laboratories. Furthermore the routine method for the detection of *Salmonella* used by several laboratories was evaluated. The reference materials used were convenient for testing the performance of the reference and the routine method in different laboratories.

The conclusions of this study are:
- two laboratories found false positive isolations. These laboratories should investigate whether there was cross contamination or switching of the samples;
- using the reference method one laboratory reported a significantly higher number of negative isolations in comparison with the other laboratories. Among the remaining laboratories no significant difference was found in the performance of the reference method;
- one laboratory reported a significantly lower number of positive isolations using RV in comparison with the use of SC. The other laboratories found no significant difference between the two selective enrichment media;
- no significant difference was found between the reference method and the routine method of eight different laboratories.

The four laboratories with deviating results were asked to carry out this study once again. The CRL for *Salmonella* contacted these participants about the procedure. The results are reported in annex 4.

LITERATURE

1. The certification of a reference material for the evaluation of the ISO method for the detection of *Salmonella*
P.H. in 't Veld, N.G.W.M. van Strijp-Lockefeer, A.H. Havelaar, E.A. Maier
accepted for publication in Journal of Applied Bacteriology, 1996.

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

3. Statistical analysis of certification trials for microbiological reference materials
S.H. Heisterkamp, J.A. Hoekstra, N.G.W.M. van Strijp-Lockefeer, A.H. Havelaar, K.A. Mooijman, P.H. in 't Veld, S.H.W. Notermans, E.A. Maier, B. Griepink
Commission of the European Communities; Community Bureau of Reference, Brussel (1993) EUR 15008 EN

4. Practical Statistics for Experimental Biologists
A.C Wardlaw, september 1993 (page 103-104)

ANNEX 1

BACTERIOLOGICAL COMPARATIVE STUDY ORGANIZED BY CRL SALMONELLA

PROTOCOL:

Introduction:

The Community Reference Laboratory (CRL) Salmonella is organizing a bacteriological comparative study on the methods for the detection of *Salmonella* amongst the National Reference Laboratories (NRLs). All NRLs have to use the principles of the standard method of the International Standard Organization (ISO 6579) for the detection of *Salmonella*. In this study the ISO 6579 method will be used as reference method. Besides the reference method the NRLs will use their own method, which is used in the framework of the directive for screening samples.

Reference materials produced by the CRL will be used for testing the performance in this study. These materials consist of gelatin capsules containing a low number of sublethally injured S.Typhimurium. Each laboratory will examine 30 capsules. The results of all laboratories will be evaluated by the CRL.

Objective:

The main objective of this first bacteriological study is the comparison of the results among and within the laboratories.

Outline of the comparative study:

Each NRL will receive a parcel containing:

30 numbered vials (numbered 1 to 30); each containing one *Salmonella* capsule to be tested for presence/absence of *Salmonella*.

The examination of the capsules will be in week 40 (starting at 02 October 1995). 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 comparative study will not be supplied by the CRL.

Time table of the bacteriological comparative study

The examination of the capsules must be performed in week 40 (starting at 02 October) or one week earlier or later.

04-08 September	Mailing the protocol, procedure and test report to the NRLs.
11-15 September	Mailing the 30 vials containing the *Salmonella* capsules to the NRLs. CRL will mail the parcel by cargo freight from the Dutch airport (Schiphol) to the airport of destination. The reference materials are packed 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. The participants have to collect the parcel at the airport. All necessary information will be indicated in a fax. 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 all vials with reference materials 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 reference 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 reference materials are packed 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 reference materials at -20°C.**

	If you did not receive the parcel before or at <u>15 September</u>, do contact the CRL immediately.
25-29 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)
02-06 October	Starting with examining the capsules. **Note:** Each laboratory is free te determine when they want to examine the capsules as long as it will be done in the scheduled weeks.
16 October	Completion of the test report and faxing it to the CRL. The original test report will be sent by express mail to CRL.
30 October	Check of the results by the National Reference Laboratories.

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

Nelly Voogt
(research assistant CRL)
P.O. Box 1
3720 BA Bilthoven
The Netherlands
tel. number: ..-31-30-2742093
fax. number: ..-31-30-2744434

ANNEX 2

BACTERIOLOGICAL COMPARATIVE STUDY ORGANIZED BY CRL SALMONELLA

SOP/CRL/01
page 1 of 8
Version no.1
230895

STANDARD OPERATING PROCEDURE (SOP):

Detection of *Salmonella* in reference materials.

1. Scope and field of application

This standard operating procedure (SOP) describes the procedure for the detection of *Salmonella* in reference materials. For this purpose reference materials are used as prepared by the Community Reference Laboratory Salmonella (CRL *Salmonella*), Bilthoven, The Netherlands. It application is limited to bacteriological comparative studies for *Salmonella*.

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*: microorganism 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*: Determination of the presence or absence of *Salmonella* in reference materials, when the test is carried out in accordance with this SOP.

3.3 *Reference material*: A gelatin capsule containing a quantified amount of artificially contaminated spray dried milk.

4. Principle

The detection of *Salmonella* in reference materials 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. If you don't have a copy of this method please contact the CRL *Salmonella.*

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. Selenite/cystine medium (Annex B.3)

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

5.4.1. Nutrient agar (Annex B.5)

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)

5.4.5. Reagent for detection of β-galactosidase (Annex B.9)

5.4.6. Reagents for Voges Proskauer (VP) (Annex B.10)
- **5.4.6.1.** VP medium
- **5.4.6.2.** Creatine solution
- **5.4.6.3.** 1-Naphthol, ethanolic solution
- **5.4.6.4.** Potassium hydroxide solution

5.4.7. Reagents for indole reaction (Annex B.11)
- **5.4.7.1.** Tryptone/trypthophan medium
- **5.4.7.2.** Kovacs reagent

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.** Graduated pipets; nominal capacities 10 ml and 1 ml, graduated respectively in 0.5 ml and 0.1 ml divisions;
- **6.2.4.** Petri dishes; small size (diameter 90 mm to 100 mm) and/or large size (diameter 140 mm).

7. Procedure

7.1 Pre-enrichment

Take the numbered vials with the *Salmonella* capsules out of the freezer one hour before they are added to the pre-enrichment broth BPw, to let them equilibrate to room temperature. Let the BPw equilibrate to room temperature, if it was stored at a lower temperature. Record on test report (page 3) the requested data of the BPw. Label 30 jars containing 225 ml of BPw from 1 to 30. Also use 1 jar as a procedure control (no capsule is added but this jar is handled further as the other jars). After equilibration add to each of the 30 labelled jars a gelatin capsule from the vial with the corresponding label number. Do **not** open the gelatin capsule and/or do not shake the BPw to dissolve the capsule more rapidly. 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

Let the selective enrichment broths equilibrate to room temperature, if they were stored at a lower temperature. Record on test report (page 4-6) the requested data of the selective enrichment broths. Label 30 jars/tubes of each selective enrichment broth from 1 to 30. Also use 1 jar/tube for each selective enrichment broth as a procedure control (this jar/tube is inoculated with the procedure control BPw). After equilibration:

- transfer 0.1 ml 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;
- transfer 10 ml BPw culture to a bottle containing 100 ml selenite/cystine medium with the corresponding label number. Incubate at 37 °C for 24 h and a further 24 h;
- inoculate the normally used selective medium/media (other than those mentioned above), with the corresponding label number, from the 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 appropiate incubators 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).

7.3 Isolation on media (first and second isolation)

Record on test report (page 7-9) the requested data of the isolation media used. Label 30 petri dishes of the isolation media from 1 to 30. Also use 1 petri dish as a procedure control (this petri dish is inoculated with the procedure control selective enrichment broth).

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 end 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 normally used in your laboratory (using a new loop). Only if media are used other than those mentioned above.

Incubation:

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

ad 3 Incubate the medium/media at the temperature routinely used (note temperature and time and other requested data on test report, page 9).

After incubation for 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).

Note: In absence of large dishes use two small dishes, one after the other, using the same loop.

7.4 Confirmation of colonies from first and/or second isolation

For confirmation take from each petri dish of each selective medium at least 2 colonies considered to be typical or suspect (use only pure cultures).
If on a dish there is only one typical or suspect colony, take this for confirmation.
Streak the selected colonies onto the surface of nutrient agar plates with the corresponding label numbers, in a manner which allow to develop well isolated colonies. Record on test report (page 10) the requested data of the nutrient agar.
Incubate the inoculated plated at 37 °C for 18 h to 24 h.
The colonies can be confirmed biochemically and/or serologically. If only a serological confirmation is carried out, auto- or non agglutinating colonies must also be confirmed biochemically. Report the number of colonies tested and the number of colonies confirmed as *Salmonella* for each dish in table 1 (first isolation) and 2 (second isolation) on test report (page 12-15).

7.4.1 Biochemical confirmation

By means of a loop, inoculate the media specified below with each of the cultures obtained from the colonies selected as described above. Record on test report (page 11) 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
7.4.1.4. detection of β-galactosidase
7.4.1.5. medium for Voges Proskauer (VP)
7.4.1.6. medium for indole reaction

7.4.2. Interpretation of the biochemical tests

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

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 specificied 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 full ISO 6579 method

day 1	**Pre-enrichment**	1 capsule to 225 ml BPw 16 - 20 h at 37 °C
day 2	**Selective enrichment**	a. 0.1 ml BPw culture in 10 ml RV 24 h at 42 °C and b. 10 ml BPw culture in 100 ml selenite/cystine 24 h at 37 °C
day 3	**First isolation**	inoculate from RV and selenite/cystine: a. phenol red/brilliant green agar b. own medium incubate both 20 -24 h at 37 °C
	Continue sel. enrichment	incubate RV and selenite/cystine medium (see day 2) another 24 h at resp. 37 °C and 42 °C
day 4	**Second isolation**	inoculate from RV and selenite/cystine (see day 3) a. phenol red/brilliant green agar b. own medium incubate both 20 -24 h at 37 °C
	Confirmation	streak at least **2** characteristic colonies (for each plate) from <u>first</u> isolation on nutrient agar plates 18 - 24 h at 37 °C
day 5	**Confirmation**	streak at least **2** characteristic colonies (for each plate) from <u>second</u> isolation on nutrient agar plates 18-24 h at 37 ° C
	Biochemical confirmation	inoculate the specific media for from nutrient agar (day 4) biochemical identification and incubate

day 6	**Biochemical confirmation**	inoculate the specific media for from nutrient agar (day 5) biochemical identification and incubate

ANNEX 3

BACTERIOLOGICAL COMPARATIVE STUDY
ORGANIZED BY CRL SALMONELLA

page 1 of 16

TEST REPORT
OF THE FIRST BACTERIOLOGICAL COMPARATIVE STUDY

Detection of *Salmonella* in reference materials.

Laboratory code	: .....
Laboratory name	: .....
Date of receipt of the parcel	: ..... - ..... - *1995*
Starting date for detection	: ..... - ..... - *1995*

Shipment

Cold chain monitor:

1. Check at airport:

date :..... - 1995
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 :..... - 1995
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 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 recorder 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 medium</u>

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

Thermometer used for reading the incubation temperature:

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

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

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

2. Selenite/cystine medium

Manufacturer of the selenite/cystine 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

Thermometer used for reading the incubation temperature:

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

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

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

3. <u>Selective medium, normally 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

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

Thermometer used for reading the incubation temperature:

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

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

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

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

First and second isolation

1. <u>Phenol red/brilliant green agar</u>

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 recorder automatically, please provide printout of the temperatures)

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

2. Isolation medium of your own choice

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 recorder automatically, please provide printout of the temperatures)

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

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

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

Medium : .....

Manufacturer of this medium:

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

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

Incubation temperature : ..... °C

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

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

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

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

Type of incubator: vented incubator
nonvented incubator

Thermometer used for reading the incubation temperature:

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

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

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

Place of dishes in the incubator : .....

Confirmation

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 recorder automatically, please provide printout of the temperatures)

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

page 11 of 16

Biochemical confirmation

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 : .....

Manufacturer of β-galactosidase:
- name : .....
- code number : .....
- batch number : .....
- expire date : .....

Manufacturer of Voges Proskauer:
- name : .....
- code number : .....
- batch number : .....
- expire date : .....

Manufacturer of indole:
- name : .....
- code number : .....
- batch number : .....
- expire date : .....

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

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

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

Table 1 (continued): dish numbers 16-30

	RV[a]				SEL[b]				Own enrichment broth			
	BGA[c]		second[d] medium		BGA		second medium		first[e] medium		second medium	
no.	col[f]	Sal[g]	col	Sal	col	Sal	col	Sal	col	Sal	col	Sal
16												
17												
18												
19												
20												
21												
22												
23												
24												
25												
26												
27												
28												
29												
30												

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

Table 2: Results of confirmation tests of the **second isolation** (dish numbers 1-15)

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

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

Table 2 (continued): dish numbers 16-30

	RV[a]				SEL[b]				Own enrichment broth			
	BGA[c]		second[d] medium		BGA		second medium		first[e] medium		second medium	
no.	col[f]	Sal[g]	col	Sal	col	Sal	col	Sal	col	Sal	col	Sal
16												
17												
18												
19												
20												
21												
22												
23												
24												
25												
26												
27												
28												
29												
30												

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

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

Date: ..... - ..... - 1995

Name of technician/technologist carrying out the bacteriological comparative study:

.....

signature:.....

Name of person in charge: .....

signature:.....

ANNEX 4

ADDITIONAL BACTERIOLOGICAL COLLABORATIVE STUDY

INTRODUCTION

In the first study three laboratories reported deviating results in comparison with the majority of the participants. A fourth laboratory did not follow the protocol. These four laboratories were asked to carry out the study once again.

PERFORMANCE OF THE STUDY

The protocol of the additional study was identical to the first study. The protocol, SOP and test report were mailed to the participants three weeks before the start of the study.
The reference materials belonged to the same batch (1-5/250795/3) as those used in the first study. They were mailed to the participants one week before the performance. In this study each laboratory was free to determine when they wanted to examine the capsules as long as it was done in the scheduled period of four weeks.

RESULTS

Laboratory 2 was not able to perform the additional study according to the predescribed procedure and, therefore again, did not participate. Laboratory 6 reported that compartment A of the cold chain monitor was coloured dark blue during shipment.

1. Media applied and performance of the method

There was no difference in the media used in comparison with the first study. The incubation times of the pre-enrichment step were 18 h 30 min (laboratory 6), 25 h (laboratory 11) and 19 h 26 min (laboratory 15).
For both the RV and SC medium, the laboratories reported an incubation time of 24 ± 1 h, except laboratory 11 (incubation time of 21 h 50 min). The incubation time for the isolation on both BGA and own medium, varied between 20 h and 22 h 30 min.

2. Reference and own method

The results of the ISO 6579 method, excluding the negative control samples, are presented in Table 9. All laboratories reported the four blank samples as negative. There was no difference in the number of capsules positive for *Salmonella* using either the RV or SC medium.

Only laboratory 6 used its own method. As selective enrichment media Muller/Kauffmann was used. No difference was found between the number of positive isolations using the reference method or their own method. The same capsule numbers were found negative.

3. Statistical analysis of data

No separate statistical analysis was carried out with the results. The data showed only small differences between the methods used and the laboratories. The numbers were too low to allow for a formal statistical analysis.

Table 9: *Results of the full ISO 6579 method*

labcode	positive isolations	negative isolations
6	25	1
11	25	1
15	26	0

DISCUSSION

The discoloration of compartment A of the cold chain monitor reported by one laboratory had no influence on the results because the start of the detection was five days after arrival. A discoloration of compartment A means that the reference materials had been exposed to a temperature above 10 °C but below 34 °C and must be used within a given period.

In the first study the results of the laboratories 11 and 15 were not analysed due to errors made with the negative control (=blank) samples. In the additional study they detected the four blank samples correctly.

Laboratory 6 found significantly more capsules negative in comparison with the other laboratories in the first study. As a possible explanation, this laboratory reported the storage of the reference materials at 4 °C instead of -20 °C after arrival. In the additional study they detected one positive capsule as negative.

The laboratories found a small difference in the number of positive isolations. As mentioned in chapter 4.5 (page 23) the finding of 1 capsule per laboratory is acceptable. No difference was found between using the full ISO 6579 method or the RV and SC medium separately.

CONCLUSION

The objective of this additional collaborative study was to check up the results of the bacteriological detection method of the laboratories with deviating results in the first study.

The conclusion of this study is that three of the four participants found only a small difference in examination of the capsules for presence/absence of *Salmonella*. The deviating results reported in the first study were not found again in the additional study.