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EU Collaborative study VI (2002) on bacteriological detection of *Salmonella* spp.

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Abstract

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

A sixth bacteriological collaborative study was organised by the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*, Bilthoven, The Netherlands) in 2002. Seventeen National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*) participated in the study.
Reference materials in combination with or without the presence of chicken faeces, as well as naturally contaminated faecal samples (containing *Salmonella* Infantis) were tested by all laboratories. The reference materials existed of gelatin capsules containing *Salmonella* Typhimurium (STM), *Salmonella* Enteritidis (SE) or *Salmonella* Panama (SPan) at different contamination levels.
In addition to the performance testing of the laboratories a comparison was made between the media described in the amended ISO 6579: 2002 [including Rappaport Vassiliadis Soya broth (RVS), Mueller Kauffmann Tetrathionate-novobiocin broth (MKTTn) and Xylose Lysine Deoxycholate agar (XLD)] and the alternative media Modified Semi-solid Rappaport Vassiliadis (MSRV) and Brilliant Green Agar (BGA).
Significantly more positive isolations were obtained from capsules containing a high level of STM than, in declining order, from capsules with a high level of SE, from capsules containing a low level of STM and from capsules with a low level of SE.
The overall results of all different capsules as well as the results of the naturally contaminated samples revealed better results for MSRV (with BGA and XLD as plating-out media) in comparison with the ISO 6579: 2002 method.

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Samenvatting

Het Communautair Referentie Laboratorium voor *Salmonella* (CRL-*Salmonella*) organiseerde in 2002 het zesde bacteriologisch ringonderzoek voor de detectie van *Salmonella*. De deelnemers waren de Nationale Referentie Laboratoria voor *Salmonella* (NRL's-*Salmonella*) in de lidstaten van de Europese Unie en het NRL in Noorwegen.
Het doel van dit zesde ringonderzoek was het vergelijken van de test tussen en binnen de NRLs bij gebruik van verschillende besmettingsniveaus en serotypen van *Salmonella* in aan- en afwezigheid van stoorflora (kippenfaeces). Daarnaast werden de resultaten, verkregen met Modified Semi-solid Rappaport Vassiliadis (MSRV) als selectief verrijkingsmedium, vergeleken met Mueller-Kauffmann Tetrathionate-novobiocin (MKTTn) en Rappaport-Vassiliadis Soya broth (RVS) zoals vermeld in ISO 6579: 2002. Als selectieve isolatie media werden Brilliant Groen agar (BGA, ISO 6579: 1993) en Xylose Lysine Deoxycholate agar (XLD) gebruikt. Hierdoor ontstonden 6 combinaties van media die onderling met elkaar zijn vergeleken, namelijk RVS/BGA, RVS/XLD, MKTTn/BGA, MKTTn/XLD, MSRV/BGA en MSRV/XLD. Eventueel kon een laboratorium ook andere, eigen media voor de detectie van *Salmonella* gebruiken naast de voorgeschreven media.
Vijfendertig individueel genummerde capsules en 20 natuurlijk besmette monsters werden door alle deelnemers onderzocht op de aan- of afwezigheid van *Salmonella*. Vijfentwintig van deze capsules moesten onderzocht worden in combinatie met 10 gram *Salmonella*-negatieve kippenfaeces. Deze 25 capsules waren onderverdeeld in de volgende groepen:
5 capsules met 10 kolonie vormende deeltjes (kvd) *Salmonella* Typhimurium (STM10),
5 capsules met 100 kvd *S.* Typhimurium (STM100), 5 capsules met 100 kvd *S.* Enteritidis (SE100), 5 capsules met 500 kvd *S.* Enteritidis (SE500) en 5 blanco capsules. De overige
10 capsules, waaraan geen faeces mocht worden toegevoegd, werden beschouwd als controle-capsules.

Summary

The Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*) organised the sixth collaborative study on bacteriological detection of *Salmonella*. Participants were the National Reference Laboratories for Salmonella (NRLs-*Salmonella*) of the EU Member States and the NRL from Norway.
The main objective of the sixth collaborative study was to make a comparison of the results obtained with the different levels of contamination and different serotypes of *Salmonella* in the presence or absence of competitive micro-organisms between and within the NRLs. Furthermore, the results obtained with Modified Semi-solid Rappaport Vassiliadis (MSRV) as the selective enrichment medium were compared with Mueller-Kauffmann Tetrathionate-novobiocin (MKTTn) and Rappaport-Vassiliadis Soya Broth (RVS) as mentioned in the ISO 6579: 2002. As selective isolation media Brilliant Green Agar (BGA, ISO 6579: 1993) and Xylose Lysine Deoxycholate agar (XLD) were used. Due to this, 6 combinations of media were created that were compared mutually, namely RVS/BGA, RVS/XLD, MKTTn/BGA, MKTTn/XLD, MSRV/BGA and MSRV/XLD. Optionally, a laboratory could also use other, own media for the detection of *Salmonella* in addition to the prescribed media.
Thirty five individually numbered capsules and 20 naturally contaminated samples had to be tested by the participants for the presence or absence of *Salmonella*. Twenty five of the capsules had to be examined in combination with 10 gram of Salmonella-negative chicken faeces. The 25 capsules were divided over the following groups: 5 capsules with 10 colony forming particles (cfp) of *Salmonella* Typhimurium (STM10), 5 capsules with 100 cfp *S*. Typhimurium (STM100), 5 capsules with 100 cfp *S*. Enteritidis (SE100), 5 capsules with 500 cfp *S*. Enteritidis (SE500) and 5 blank capsules. The other 10 capsules, to which no faeces had to be added, were control samples.

List of abbreviations

BGA	Brilliant Green Agar
BPLS	Brilliant-green Phenol-red Lactose Sucrose agar
BPW	Buffered Peptone Water
cfp	colony forming particles
CRL	Community Reference Laboratory
DIASALM	Diagnostic Semi-solid *Salmonella* medium
dPCA	Double concentrated Plate Count Agar
dVRBG	Double concentrated Violet Red Bile Glucose agar
HCMP	Highly Contaminated Milk Powder
ISO	International Organization for Standardization
kvd	kolonie vormende deeltjes
LDC	Lysine Decarboxylase
LIS	Diagnostic Laboratory for Infectious Diseases and Perinatal Screening
MK	Mueller Kauffmann
MKTTn	Mueller Kauffmann Tetrathionate novobiocin broth
MLCV	Mannitol Lysine Crystal Violet brilliant green agar
MSRV	Modified Semi-solid Rappaport Vassiliadis
NRL	National Reference Laboratory
PCR	Polymerase Chain Reaction
RM	Reference Material
RT	Room Temperature
RV	Rappaport Vassiliadis
RVS	Rappaport Vassiliadis Soya broth
SC	Selenite/Cystine broth
SE	*Salmonella* Enteritidis
SOP	Standard Operation Procedure
SPan	*Salmonella* Panama
STM	*Salmonella* Typhimurium
TBG	Tetrathionate Brilliant-Green Bile Enrichment Broth
TSI	Triple Sugar Iron agar
XLD	Xylose Lysine Deoxycholate agar
XLT4	Xylose Lysine Tergitol 4 agar

1. Introduction

In pursuance of the Council Directive 92/117/EEC the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*) organises bacteriological collaborative studies with the objective that the examination of samples in the EU Member States is carried out uniformly and that comparable results will be obtained by all National Reference Laboratories (NRLs).
Earlier studies (see Table 1.) have shown a significantly reduced number of positive isolations using Selenite/Cystine broth (SC) and significantly better results using Modified Semi-solid Rappaport-Vassiliadis (MSRV) compared to the use of Rappaport-Vassiliadis broth (RV) as selective enrichment medium. In the fourth study, all laboratories used the selective enrichment medium MSRV, in addition to RV (Rappaport Vassiliadis) or RVS. The design of the fifth study was identical to that of the fourth study to enable comparison of results over the years.
Moreover, the composition of the sixth study, described in this report, was identical to that in studies number IV and V. In addition to earlier studies, Mueller Kaufmann Tetrathionate with novobiocin (MKTTn) was used as a prescribed selective enrichment medium together with Rappaport-Vassiliadis Soya broth (RVS) and MSRV. For plating-out (isolation) media Brilliant Green agar (BGA) and Xylose Lysine Deoxycholate agar (XLD) were used.
All these combinations were chosen to enable comparison of results by using the newly published ISO 6579: 2002 (RVS/MKTTn and XLD) with those obtained by using earlier medium combinations.
Four different types of spiked capsules had to be examined, including 2 levels of *Salmonella* Typhimurium (STM10 and STM100) and 2 levels of *Salmonella* Enteritidis (SE100 and SE500). Furthermore, 20 naturally contaminated samples of chicken faeces containing *Salmonella* Infantis were examined by using the same six medium combinations as mentioned above.

Table 1. History of collaborative bacteriological studies

Study	Year	Number of samples	Capsules	Actual number of cfp/capsule	Salmonella negative faeces added	Selective enrichment medium	Plating-out medium	Reference
I	1995	26 4	STM5 Blank	6 0	No No	RV and SC	BGA and own	N.Voogt et al., 1996 (report 284500003)
II	1996	15 15 2 1 1	STM100 STM1000 SPan5 STM100 Blank	116 930 5 116 0	1 gram 1 gram No No No	RV, SC and own	BGA and own	N.Voogt et al., 1997 (report 284500007)
III	1998	14 14 7 14 4 2 5	STM10 STM100 STM100 SE100 STM10 SPan5 Blank	11 94 94 95 11 5 0	1 gram 1 gram 1 gram* 1 gram No No No	RV and own	BGA and own	M.Raes et al., 1998 (report 284500011)
IV	1999	5 5 5 5 5 3 3 2 2	STM10 STM100 SE100 SE500 Blank STM10 SE100 SPan5 Blank	4 210 60 220 0 5 60 5 0	10 gram 10 gram 10 gram 10 gram 10 gram No No No No	RV or RVS, MSRV and own	BGA and own	M. Raes et al., 2000 (report 284500014)
V	2000	5 5 5 5 5 3 3 2 2 20	STM10 STM100 SE100 SE500 Blank STM10 SE100 SPan5 Blank None	4 47 63 450 0 4 47 5 0 -	10 gram 10 gram 10 gram 10 gram 10 gram No No No No 25 gram**	RV or RVS, MSRV and own	BGA and XLD	M.Raes et al., 2001 (report 284500018)
VI	2002	5 5 5 5 5 3 3 2 2 20	STM10 STM100 SE100 SE500 Blank STM10 SE100 SPan5 Blank None	11 139 92 389 0 11 92 5 0 -	10 gram 10 gram 10 gram 10 gram 10 gram No No No No 25 gram**	RVS, MSRV, MKTTn and own	BGA, XLD and own	This report

* = with antibiotics

** = Naturally contaminated chicken faeces with *Salmonella*

2. Participants

Country	Institute/City
Austria	Bundesstaatliche bakteriologisch-serologischeUntersuchungsanstalt Graz
Belgium	Veterinary and Agrochemical Research Center (VAR) Brussels
Denmark	Danish Veterinary Laboratory Copenhagen
Finland	National Veterinary and Food Research Institute, Kuopio Department Kuopio
France	Agence Française de Sécurité Sanitaire des Aliments (AFSSA) Laboratoire d'Etudes et de Recherches Avicoles et Porcines (LERAP) Ploufragan
Germany	Federal Institute for Risk Assessment (BFR) National Salmonella Reference Laboratory Berlin
Greece	Veterinary Laboratory of Halkis Halkis
Ireland	Department of Agriculture and Food Central Veterinary Research Laboratory Dublin
Italy	Istituto Zooprofilattico Sperimentale delle Venezie Centro Nazionale di Referenza per le Salmonellosi Legnaro

Country	Institute/City
Luxembourg	Laboratoire de Médecine Vétérinaire de l'Etat , Animal Zoonosis Luxembourg
The Netherlands	Rijksinstituut voor Volksgezondheid en Milieu (RIVM) Bilthoven
Norway	National Veterinary Institute, Section of Bacteriology Oslo
Portugal	Laboratório Nacional de Investigaçã Veterinária Lisboa
Spain	Laboratorio de Sanidad Y Produccion Animal de Algete Madrid
Sweden	National Veterinary Institute, Department of Bacteriology Uppsala
United Kingdom	Veterinary Laboratories Agency Department of Bacterial Diseases New Haw, Addlestone
United Kingdom	Department of Agriculture for Northern Ireland Veterinary Sciences Division, Bacteriology Department Belfast

3. Materials and Methods

3.1 Reference materials (capsules) containing various levels of *Salmonella*

The Highly Contaminated Milk Powder (HCMP) containing *Salmonella* Enteritidis (SE) and *Salmonella* Typhimurium (STM) of which the low contaminated powders were mixed as described by Voogt et al. (2002), was obtained by spray-drying artificially contaminated milk as described by In 't Veld et al. (1996).

To obtain the target levels, the HCMP was diluted in steps (ratio 1:1 g/g) by mixing it with sterile (γ-irradiated) milk powder (Carnation, Nestlé, The Netherlands) using a mortar and a pestle. The target levels were 10 and 100 colony forming particles (cfp) per capsule for *Salmonella* Typhimurium (STM10 and STM100) and 100 and 500 for *Salmonella* Enteritidis (SE100 and SE500).

From each mixture test batches of 60 capsules were prepared to determine the mean number of cfp per capsule and the homogeneity of the test batch. The remaining mixtures were stored at –20°C. If the test batch fulfilled the pre-set criteria for contamination level and homogeneity, the capsules needed for the ring trial were prepared from the remaining mixtures and stored at -20°C.

The pre-set criteria were:

- mean contamination levels should lie between target level minus 30% and target level plus 50% (e.g. between 70 and 150 cfp if the target level is 100 cfp);
- for the homogeneity within one batch of capsules the maximum demand for the variation between capsules was $T_2/(I-1) \leq 2$, where T_2 is a measure for the variation between capsules of one batch (see formula in Appendix 2) and I is the number of capsules.

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

- reconstitution of each capsule in 5 ml peptone saline solution in a petri dish at 38.5°C for 45 minutes;
- repair of *Salmonella* by the addition of 5 ml double concentrated plate count agar (dPCA) to the reconstituted capsule solution, incubation at 37°C for 4 hours;
- after incubation 10 ml of double concentrated Violet Red Bile Glucose agar (dVRBG) was added as an overlayer and the plates were incubated for 20 hours at 37°C.

Capsules containing *Salmonella* Panama (5 cfp per capsule) were obtained from the Foundation for the Advancement of Public Health and Environmental Protection (SVM, Bilthoven, The Netherlands). These capsules were prepared according to the same protocol as mentioned above.

3.2 Faecal samples

Chicken faeces was obtained from poultry laying flocks which were examined in the context of a Dutch national monitoring programme. One portion of faeces from a flock tested positive in this programme according to the routine method as used by the National Reference Laboratory for *Salmonella* at RIVM, Bilthoven, The Netherlands (RV and MSRV as selective enrichment media and BGA as the selective isolation medium). The suspected colonies were incubated on TSI agar and were sent for serotyping to the Diagnostic Laboratory for Infectious Diseases and Perinatal Screening (RIVM). All *Salmonella* cultures were typed as *Salmonella* Infantis. The faeces, bacteriologically positive for *Salmonella,* was used to prepare the naturally contaminated samples.
From another poultry laying flock, which was found negative for *Salmonella*, faeces was used to prepare the samples containing non-*Salmonella* competitive micro-organisms.
All faeces samples (*Salmonella* negative as well as *Salmonella* positive faeces) were mixed and homogenised with sterilised glycerol/peptone solution (mixing ratio 1:1). One litre of this solution consisted of 300 ml glycerol, 7 gram of peptone and 700 ml distilled water. After mixing all faeces samples with the glycerol peptone solution, they were again analysed for the presence of *Salmonella* and were frozen at a temperature of – (20±2) ^{o}C untill sending the samples to the National Reference Laboratories for *Salmonella*.

3.3 Design of the collaborative study

Two weeks before the study the reference materials (45 individually numbered capsules) and 300 grams of negative faeces and 550 grams of positive faeces for Salmonella were mailed with cooling devices by road transport to the participants. After arrival at the laboratory the capsules and faeces samples had to be stored at –20°C until the start of the study.

Details about mailing and handling of the samples and reporting of test results can be found in the Protocol (Appendix 6), Standard Operation Procedure (Appendix 7) and Test Report (Appendix 8).

Ten control capsules had to be tested without faeces. Twenty-five capsules (numbered 1-25) were tested in combination with 10 grams of chicken faeces each (negative for *Salmonella*). Beside the artificially contaminated samples also 20 samples (numbered N1-N20) of 25 grams each of naturally contaminated faeces samples (with *S.* Infantis) were analysed. The contents of the capsules and faeces samples are shown in table 2.

Table 2. Overview of the content and number of the capsules

Capsules	Control capsules (n = 10) No faeces added	Test samples (n=25) with 10 g *Salmonella*- negative faeces	Test samples (n=20) with 25 g *Salmonella*- positive faeces
S. Panama 5	2	---	---
S. Enteritidis 100	3	5	---
S. Enteritidis 500	---	5	---
S. Typhimurium 10	3	5	---
S. Typhimurium 100	---	5	---
Blank	2	5	---
No capsules		---	20

3.4 Media

During the workshop meeting at 28 May 2002 in Ploufragan (France) it was decided that this collaborative study would in principle have the same set-up as study IV and V (Korver et al., 2002). Small changes were introduced in this study due to the newly accepted ISO 6579: 2002. The following media were used in study VI (see also Standard Operation Procedure in Appendix 7):

Pre-enrichment in:

- Buffered Peptone Water: BPW (same as study V)

Selective enrichment in:

- Rappaport-Vassiliadis medium with soya = RVS (was RV in study V)
- Mueller-Kauffmann Tetrathionate-novobiocin broth = MKTTn (extra to study V)
- Modified semi-solid Rappaport Vassiliadis medium = MSRV (same as study V)

Plating-out on:

- Brilliant Green agar = BGA (same as study V)
- Xylose lysine deoxycholate agar = XLD (same as study V)

Biochemical confirmation:

- Urea, Triple Sugar Iron agar (TSI) and Lysine Decarboxylase (LDC) (same as study V)

3.5 PCR detection

In addition to the earlier mentioned prescribed methods some laboratories also used a Polymerase Chain Reaction based method to investigate the samples. Five laboratories (see Table 3) applied this detection technique (labs 1, 5, 10, 11 and 13).

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

Labcode	Volume of BPW (ml)	Volume of DNA extract (ml)	Volume used in PCR reaction (µl)	Actual volume of BPW tested in PCR (µl)
1	0.5	0.1	5	25
5	1	0.05	5	100
10	1	0.15	5	33
11	1	0.01	10	1000
13	0.25	0.05	2.5	12.5

3.6 Time-temperature monitoring during shipment

To cool the content of the packages three cooling devices per package were included and for the control of exposure to abusive temperatures during shipping and storage so called WarmMark™ Time-Temperature Tags (IntroTech, Inc) were used. These tags are designed for monitoring of temperatures for extended periods of time. One of the basic components is a blotter paper pad saturated with a red-dyed chemical specially formulated to melt at the tag's response temperature. Two tags (one for the response temperature of 10°C and another

Figure 1. Example of Time-Temperature tags

for 20°C) were sticked to the inside lid of the package. The temperature devices react and show a change in color when the temperature is more than 2°C above the indicated temperature of the tag. Below this temperature there will be no change of colour. Colouring of the brief part of the tag occurs within several hours and prolonged colouring within several days of exposure.

3.7 Accreditation/certification

Ten laboratories mentioned to be accredited for their quality system according to EN 45001 (2 labs) or ISO 17025 (8 labs). Four laboratories are planning to be accredited or certified in the near future and one laboratory did not answer this question. Two labs mentioned to be certified according to ISO 9001.

3.8 Statistical analysis of the data

The results of the collaborative study were statistically analysed in order to compare the results of the participating laboratories, the different types of samples and the various methods (selective enrichment and plating-out/isolation media).
Results were analysed using SAS v 8.2. In order to detect differences among media, labs and capsules, logistic regression (using PROC GENMOD) was used. Correlation between observations within labs was taken into account by using Generalized Estimating Equations (GEE). Contrasts (as p-values) were used to test for formal differences between specific subsets of media.
To identify laboratories that differed in performance compared to all participating laboratories, the arithmetical mean value per category was compared to the results obtained by every separate NRL.

4. Results

4.1 Reference materials

The level of contamination and the homogeneity of the test batches as well as of the final batches of capsules are presented in Table 4. All batches met the pre-set criteria as stated under 3.1. The enumerated minimum and maximum levels within each batch of capsules are given between brackets.

Table 4. Level of contamination and homogeneity of SE and STM capsules

	Test batch (n=10)		Final batch (n=25)	
	Mean cfp per capsule (min.+max. cfp)	Homogeneity (T_2 / (I-1)	Mean cfp per capsule (min.+max. cfp)	Homogeneity (T_2 / (I-1)
STM 10	10 (6-14)	0.49	11 (5-16)	0.75
STM 100	150 (126-170)	0.92	139 (114-170)	0.90
SE 100	111 (90-132)	0.73	92 (72-122)	1.28
SE 500	487 (430-550)	0.40	389 (290-480)	0.77

cfp = colony forming particles min. = enumerated minimum cfp max. = enumerated maximum cfp

formula T_2 see Appendix 2; I is number of capsules

4.2 Technical data collaborative study for artificially and naturally contaminated samples

4.2.1 Dissolving time and temperature of capsules

Before adding the chicken faeces to the pre-enrichment medium (BPW), the capsules had to be dissolved in the BPW at 37°C for 30 minutes. For this purpose the BPW had to be pre-warmed at 37°C. The prescribed dissolving time of the capsules in buffered peptone water

(BPW) according to the standard operation procedure is 30 minutes. Twelve NRLs (labcodes 1, 2, 4, 5, 6, 7, 8, 9, 11, 12, 13 and 17) dissolved the capsules in exactly thirty minutes. Five other laboratories used a dissolving time of more than 30 minutes, i.e. 32, 33, 39, 60 and 90 minutes by respectively laboratories 10, 3, 16, 14 and 15.

Laboratories 8, 10, 13, 14, 16 and 17 did not preheat the BPW to 37°C as prescribed, but left the BPW come to roomtemperature before adding the capsules. Two laboratories (labcode 2 and 5) did not mention the temperature for pre-heating BPW.

4.2.2 Incubation time and temperature of pre-enrichment

According to the standard operation procedure and the ISO 6579:2002 the incubation time for the pre-enrichment is between 16 and 20 hrs. Except for four laboratories all laboratories incubated the BPW for the prescribed time. Laboratories 3, 8, 11 and 15 incubated this pre-enrichment medium longer than the prescribed time (see Table 5). The prescribed temperature for the incubation of BPW is (37 ± 1) °C. All laboratories except two (labcode 14 and 16) incubated the BPW at the prescribed temperature.

Table 5. Incubation time and temperatures of pre-enrichment medium BPW

Labcode	Incubation time (h)	Incubation temperature in °C (minimum-maximum)
Prescribed*	16 – 20	37 ± 1
1	20.00	37 – 37
2	20.00	37 – 37
3	21.15	37.3 – 37.3
4	16.55	36.7 – 36.8
5	18.02	37.0 – 37.6
6	17.35	37 – 37
7	19.55	37 – 37
8	20.30	36 – 37
9	19.20	36.5 – 37.2
10	17.12	36.2 – 36.3
11	20.26	36.6 – 36.7
12	17.45	37.1 – 37.6
13	17.45	36.8 – 36.9
14	18.00	32.5 – 37
15	22.40	36 – 36
16	18.01	33.5 – 37.7
17	19.48	37.0 – 37.1

**Incubation time and temperature according to ISO 6579 (2002): 16-20 hours at (37 ± 1) °C.*

4.2.3 Incubation time and temperature of selective enrichment media

RVS

The incubation temperature for RVS as mentioned in the standard operation procedure should be between 40.5°C and 42.5°C. Twelve laboratories incubated their RVS medium between these temperatures (see table 6). Of five laboratories (labcodes 3, 11, 12, 13 and 15) the incubation temperatures varied between 39°C and 43°C. Fourteen NRLs incubated the RVS medium for the prescribed time of 21 to 27 hours. The incubation time for NRLs 7, 12 and 15 was between 17 and 21 hrs.

Table 6. Incubation times and temperatures of selective enrichment media

	RVS		MKTTn		MSRV	
Labcode	Time (h)	Temp. (°C)	Time (h)	Temp. (°C)	Time (h)	Temp.(°C)
Prescribed*	24 ± 3	41.5 ±1	24 ± 3	37 ± 1	24 ± 3	41.5±1
1	21.00	41.5-42	21.00	36.5-37	21.00	41.5
2	23.00	41.3-42	23.00	36.5-37	23.00	41.3-42
3	21.10-22.55	42.0-43.0	20.25-20.56	36.8-37.3	21.10-23.05	42.0-43.0
4	23.25-24.00	41.5	23.05-23.55	36.8-36.9	23.20-24.00	41.1-41.5
5	21.10-24.17	41.2-42.6	22.45-23.56	36.7-37.4	21.35-24,53	41.6-42.4
6	21.55-23.10	41.6	21.00-24.04	37	21.50-23.56	41.6-41.7
7	20.45-23.18	42	20.45-23.20	37	20.45-23.00	42
8	---	---	---	---	22.30	42-42.2
9	21.58-24.15	41.5-41.6	22.33-24.20	37-37.5	23.55-23.58	37-41.5
10	21.25-22.45	41.4-42.1	20.45-22.45	35.9-36.5	18.50-21.20	41.4-41.5
11	22.55-23.05	41.1-41.5	22.40-22.41	37.4	23.40-26.15	41.8-42.0
12	17.10-23.45	39.1-42.8	18.30-24.30	41.2-42.8	19.00-24.00	40.1-41.9
13	21.30-22.05	42.3-42.8	21.30-22.05	36.4-37.3	21.25-22.05	42.2-42.8
14	21.30-22.00	41-42	21.30-22.00	35-37	20.30-22.00	41-42
15	19.15-21.50	40-41	19.45-20.50	34-37	19.15-20.30	40-41
16	22.01-22.52	41.3-41.5	22.01-22.52	41.3-41.5	22.01-22.52	41.3-41.5
17	21.46-26.54	41.5-41.6	21.47-27.13	36.5-37.0	19.55-24.15	41.5

** Incubation times and temperatures according to SOP, only minimum and maximum times and temperatures are mentioned in this table (see Appendix 7)*

MKTTn

The prescribed time and temperature for incubation of MKTTn are 21-27 hrs at 36 – 38°C, respectively. Laboratories with labcodes 3, 7, 10, 12, 15 and 17 incubated their MKTTn medium plates for a period of 18 - 28 hrs. All other NRLs fulfilled the prescribed incubation time. Three laboratories (labcodes 10, 13 and 14) incubated their plates at a temperature between 34°C and 36.5°C. Laboratories with labcodes 12 and 16 used a temperature of 41.2 – 42.8°C. All other NRLs used the correct incubation time and temperature.

MSRV

The incubation time and temperature for MSRV according to the SOP should be between 21-27 hrs and (41.5±1) °C, respectively. Six laboratories (7, 10, 12, 14, 15 and 17) used an incubation time between 18 and 21 hrs. All other laboratories complied with the required incubation time.

All NRLs except three (labcode 12, 13 and 15) met the prescribed temperature of (41.5±1) °C. These three laboratories incubated the MSRV plates between 40°C and 43°C. Laboratory with labcode 9 had a final temperature of 37°C.

4.2.4 Composition of MKTTn

Some NRLs prepared their own MKTTn medium according to the formula as given in the ISO 6579:2002. At the time of the collaborative study the MKTTn medium with the composition of the ISO was not yet available as dehydrated medium. Most laboratories used MKTTn from various sources with different formula. The manufacturer and the final pH of the MKTTn–media used in this study are shown in table 7. Information concerning the composition of the MKTTn is given in Appendix 4. Laboratory 8 did not test the samples with this medium.

Table 7. Manufacturer and final pH of MKTTn medium

Manufacturer	Laboratory code	Final pH (temp.°C)
Home made	1	7.0 (21)
Home made according to ISO	11	8.2 (RT)
	16	8.22 (RT)
Biokar	12	7.8 (22)
	14	7.3 (25)
Biolife	2	?
Biorad	3	?
	13	8 (25)
Difco	4	8.14 (25)
Oxoid	5	8.0 (RT)
	6	8.0 (?)
	10	8.01 (25)
	15	7.92 (29)
	17	?
Merck	9	7.4 (22)
Scharlau	7	8.3 (25)

RT = Room Temperature; pH according to ISO 6579 (2002): 8.2 ± 0.2 at 25 °C (outside this range in red)

4.3 Control samples

Control samples

All laboratories except one (labcode 8) tested the control samples (n = 10) with the requested six combinations of media, i.e. RVS, MKTTn and MSRV as the selective enrichment medium and BGA and XLD as the isolation/plating out media. The laboratory with labcode 8 only tested the combination MSRV with XLD.

None of the laboratories isolated *Salmonella* from the procedure (C11: no capsule/no faeces) or faeces control (C12: no capsule/negative faeces).

Blank capsules (n = 2) without addition of faeces

The blank capsules only contained sterile milk powder. For the analyses no faeces was added. Sixteen laboratories did not isolate bacteria from these blank capsules. One laboratory (labcode 3) reported the isolation of *Salmonella* in one blank capsule with the combinations RVS/BGA, RVS/XLD and MKTTn/XLD. The laboratory with labcode 8 only tested the combination MSRV with XLD and did not isolate *Salmonella* from the blank capsules.

Salmonella *Panama 5 capsules (n = 2) without addition of faeces*

Four laboratories (labcodes 1, 3, 8 and 11) failed to isolate *Salmonella* from one or both capsules containing *S.* Panama at a level of 5 cfp/capsule with some of the medium combinations (see Table 8).

Table 8. Number of positive isolations per laboratory for SPan 5 (n=2) without addition of faeces

	Laboratory codes																
Medium combination	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
RVS/BGA	0	2	2	2	2	2	2	--	2	2	2	2	2	2	2	2	2
RVS/XLD	2	2	0	2	2	2	2	--	2	2	2	2	2	2	2	2	2
MKTTn/BGA	0	2	1	2	2	2	2	--	2	2	2	2	2	2	2	2	2
MKTTn/XLD	2	2	1	2	2	2	2	--	2	2	1	2	2	2	2	2	2
MSRV/BGA	2	2	2	2	2	2	2	--	2	2	2	2	2	2	2	2	2
MSRV/XLD	2	2	0	2	2	2	2	1	2	2	2	2	2	2	2	2	2

-- = not tested Yellow cells = Unexpected results

Salmonella *Typhimurium 10 capsules (n = 3) without addition of faeces*

All laboratories except for NRL with labcode 3 isolated *Salmonella* from all capsules containing *Salmonella* Typhimurium at a mean level of 10 cfp/capsule with all medium combinations (see Table 9). Laboratory 3 isolated *Salmonella* in two out of three capsules with RVS/BGA and MSRV/BGA, in one capsule with RVS/XLD and MSRV/XLD and in none with MKTTn/BGA.

Table 9. Number of positive control samples per laboratory for STM 10 (n=3) without addition of faeces

	Laboratory codes																
Medium combination	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
RVS/BGA	3	3	2	3	3	3	3	--	3	3	3	3	3	3	3	3	3
RVS/XLD	3	3	1	3	3	3	3	--	3	3	3	3	3	3	3	3	3
MKTTn/BGA	3	3	0	3	3	3	3	--	3	3	3	3	3	3	3	3	3
MKTTn/XLD	3	3	3	3	3	3	3	--	3	3	3	3	3	3	3	3	3
MSRV/BGA	3	3	2	3	3	3	3	--	3	3	3	3	3	3	3	3	3
MSRV/XLD	3	3	1	3	3	3	3	3	3	3	3	3	3	3	3	3	3

-- = not tested Yellow cells = Unexpected results

Salmonella *Enteritidis 100 capsules (n = 3) without addition of faeces.*

All laboratories except for NRL with labcode 3 isolated *Salmonella* from all capsules containing SE at a mean level of 100 cfp/capsule with all medium combinations (see Table 10). Laboratory 3 isolated Salmonella in two out of three capsules with RVS/XLD and MKTTn/XLD, in one capsule with RVS/BGA and MKTTn/BGA and in none with MSRV/XLD.

Table 10. Number of positive control samples per laboratory for SE 100 (n=3) without addition of faeces

	Laboratory codes																
Medium combination	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
RVS/BGA	3	3	1	3	3	3	3	--	3	3	3	3	3	3	3	3	3
RVS/XLD	3	3	2	3	3	3	3	--	3	3	3	3	3	3	3	3	3
MKTTn/BGA	3	3	1	3	3	3	3	--	3	3	3	3	3	3	3	3	3
MKTTn/XLD	3	3	2	3	3	3	3	--	3	3	3	3	3	3	3	3	3
MSRV/BGA	3	3	3	3	3	3	3	--	3	3	3	3	3	3	3	3	3
MSRV/XLD	3	3	0	3	3	3	3	3	3	3	3	3	3	3	3	3	3

-- = not tested Yellow cells = Unexpected results

4.4 Results *Salmonella* capsules with addition of *Salmonella* negative chicken faeces

4.4.1 Results per type of capsule and per laboratory

STM 10

The results obtained with the different medium combinations for STM10 with the addition of 10 grams of *Salmonella* negative faeces are shown in Table 11. Five laboratories (labcode 2, 8, 11, 12 and 16) did not isolate *Salmonella* from all medium combinations. The maximum number of isolations was only obtained by laboratories 4, 6, 9, 10 and 14 for medium combinations MSRV/BGA and MSRV/XLD. Furthermore laboratory 9 also found the maximum number of positive isolations for RVS/BGA and RVS/XLD and laboratories 14 and 17 for MKTTn/XLD.

Table 11. Number of positive isolations per laboratory for STM 10 (n=5) with the addition of 10 g Salmonella negative chicken faeces

	Laboratory codes																
Medium combination	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
RVS/BGA	3	0	0	0	3	0	0	--	5	2	0	0	1	2	3	0	0
RVS/XLD	1	0	0	0	3	0	1	--	5	2	0	0	0	3	3	0	0
MKTTn/BGA	0	0	0	0	3	1	0	--	3	1	0	0	1	2	4	0	1
MKTTn/XLD	0	0	1	3	3	0	1	--	3	1	0	0	1	5	3	0	5
MSRV/BGA	1	0	0	5	3	5	0	--	5	5	0	0	0	5	1	0	0
MSRV/XLD	0	0	0	5	3	5	2	0	5	5	0	0	0	5	1	0	0
All combinations (n=30)	**5**	**0**	**1**	**13**	**18**	**11**	**4**	**0**	**26**	**16**	**0**	**0**	**3**	**22**	**15**	**0**	**6**

STM 100

Considerably more positive isolations were found with the STM100 than with the STM10 capsules (see Table 12). Laboratories 5 and 10 found all capsules positive for all medium combinations. Most laboratories found more positive isolations with MSRV except for

laboratories 13 and 17. Laboratory 3 was not able to isolate *Salmonella* from any medium combination.

Table 12. Number of positive isolations per laboratory for STM 100 (n=5) with the addition of 10 g Salmonella negative chicken faeces

	Laboratory codes																
Medium combination	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
RVS/BGA	2	1	0	1	5	3	0	--	4	5	0	2	5	4	2	0	5
RVS/XLD	5	1	0	1	5	2	3	--	4	5	0	2	3	5	2	0	5
MKTTn/BGA	0	1	0	0	5	1	1	--	4	5	0	3	5	5	2	0	3
MKTTn/XLD	0	0	0	5	5	1	0	--	1	5	0	3	5	4	2	0	5
MSRV/BGA	4	0	0	5	5	5	0	--	4	5	1	3	1	5	2	3	0
MSRV/XLD	4	0	0	5	5	5	3	1	4	5	1	3	4	5	2	3	3
All combinations (n=30)	**15**	**3**	**0**	**17**	**30**	**17**	**7**	**1**	**21**	**30**	**2**	**16**	**23**	**28**	**12**	**6**	**21**

SE 100

Laboratories 2, 8, 11, 15 and 16 were not able to isolate *Salmonella* from the SE100 capsules with any of the medium combinations (see Table 13). Only laboratories 4 and 5 isolated the maximum number of five isolations per medium combination using MSRV.

Table 13. Number of positive isolations per laboratory for SE 100 (n=5) with the addition of 10 g Salmonella negative chicken faeces

	Laboratory codes																
Medium combination	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
RVS/BGA	0	0	0	0	4	1	0	--	4	0	0	0	1	0	0	0	1
RVS/XLD	0	0	0	0	4	2	0	--	4	0	0	0	0	1	0	0	0
MKTTn/BGA	0	0	0	1	4	0	0	--	3	1	0	1	1	3	0	0	0
MKTTn/XLD	0	0	1	1	4	2	1	--	4	1	0	1	1	3	0	0	2
MSRV/BGA	1	0	0	5	5	4	0	--	4	2	0	0	0	2	0	0	0
MSRV/XLD	0	0	0	5	5	4	2	0	4	2	0	0	1	3	0	0	1
All combinations (n=30)	**1**	**0**	**1**	**12**	**26**	**13**	**3**	**0**	**23**	**6**	**0**	**2**	**4**	**12**	**0**	**0**	**4**

SE 500

The maximum number of positives for capsules SE500 and all medium combinations was only obtained by laboratory 5 (see Table 14). No *Salmonella* could be isolated from any medium combination by NRLs 2, 3 and 11.

Table 14. *Number of positive isolations per laboratory for SE 500 (n=5) with the addition of 10 g Salmonella negative chicken faeces*

	Laboratory codes																
Medium combination	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
RVS/BGA	3	0	0	0	5	0	1	--	4	1	0	1	2	1	1	0	2
RVS/XLD	1	0	0	0	5	2	0	--	4	2	0	2	1	1	1	0	1
MKTTn/BGA	0	0	0	2	5	1	0	--	4	5	0	3	2	4	3	1	1
MKTTn/XLD	0	0	0	5	5	1	0	--	4	5	0	3	3	4	4	2	3
MSRV/BGA	4	0	0	5	5	5	2	--	4	5	0	2	2	5	0	1	1
MSRV/XLD	3	0	0	5	5	5	2	1	4	5	0	2	1	5	3	1	2
All combinations (n=30)	**11**	**0**	**0**	**17**	**30**	**14**	**5**	**1**	**24**	**23**	**0**	**13**	**11**	**20**	**12**	**5**	**10**

In Figure 2 all positive isolations for all capsules and all medium combinations per laboratory are given.

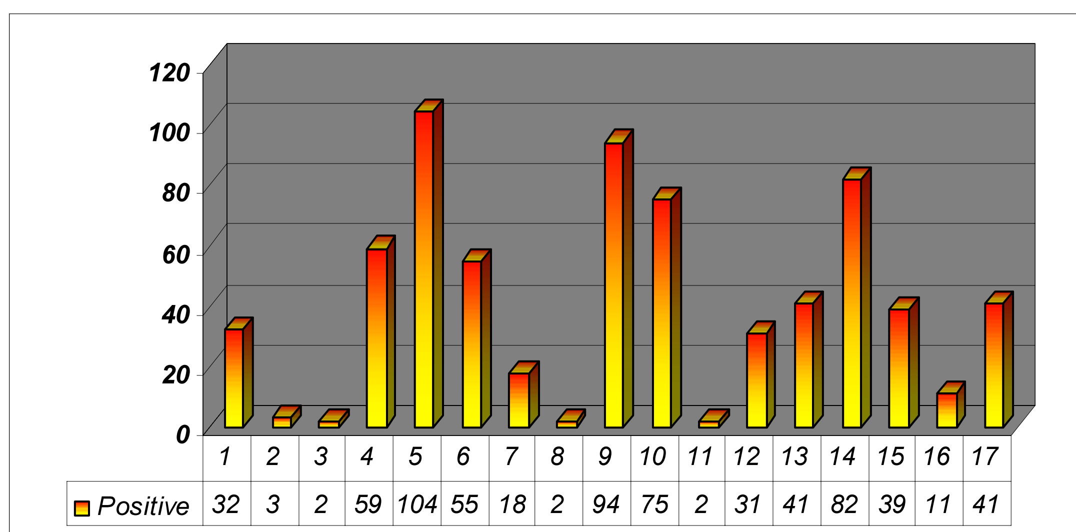

Figure 2. *Number of positive isolations (max. 120) per laboratory (1-17) for all capsules (n=20) and all medium combinations (n=6) with the addition of 10 g Salmonella negative chicken faeces*

4.4.2 Results per medium combination tested by all laboratories

The cumulative results per combination of selective enrichment and isolation medium for all kind of capsules with the addition of faeces are shown in Table 15.

For the STM10 and STM100 capsules, the results obtained with RVS and MKTTn are not significantly different from those with MSRV (see also Table 16).

For the SE100 and SE500 capsules significantly more positive isolations were obtained with MSRV compared with RVS. Also MKTTn showed significantly more positive isolations than RVS. The results of MKTTn versus MSRV are not significantly different from each other. The isolation of *Salmonella* for all capsules tested only revealed significantly more positive isolations for MSRV when compared with RVS.

The comparison between BGA and XLD revealed that for XLD significantly more positive isolations were obtained. This was also the case for the SE capsules but not for the STM capsules.

Table 15. Number and percentages positive isolations for all participating laboratories (n = 17) with all capsules and all medium combinations

		RVS		MKTTn		MSRV	
Capsules		BGA*	XLD*	BGA*	XLD*	BGA*	XLD**
STM 10	Positives	19	17	16	26	30	31
(n=5)	%	24	21	20	33	38	36
STM 100	Positives	39	43	35	36	43	53
(n=5)	%	49	54	44	45	54	62
SE 100	Positives	11	11	14	21	23	27
(n=5)	%	14	14	18	26	29	32
SE 500	Positives	21	20	31	39	41	44
(n=5)	%	26	25	39	49	51	52
All	Positives	90	91	96	122	137	155
(n=20)	%	28	28	30	38	43	46

* 16 participating laboratories ** 17 participating laboratories

Table 16. Comparison of media as p-values for capsules with the addition of negative faeces

Media	SE 100	SE 500	STM 10	STM 100
MSRV vs RVS	0.0147	0.0020	0.1151	0.3709
MKTTn vs MSRV	0.1185	0.2832	0.2207	0.1236
MKTTn vs RVS	0.0976	0.0166	0.5708	0.2381
BGA vs XLD	0.0400	0.2444	0.2638	0.1528

Media	All capsules	SE capsules	STM capsules
MSRV vs RVS	0.0210	0.0042	0.1522
MKTTn vs MSRV	0.1125	0.1482	0.1277
MKTTn vs RVS	0.2049	0.0233	0.7381
BGA vs XLD	0.0278	0.0443	0.1440

p-values <0.05 are shown in red

4.4.3 Results of other medium combinations

Eleven laboratories also tested the capsules with their own medium combination(s). In Table 17 the results obtained with medium combination MSRV/XLD are compared with the results of their best own medium (see also Appendix 3).

Table 1.7 Comparison of results between MSRV/XLD and best own medium combination

Labcode	Medium	STM10	STM100	SE100	SE500	All capsules
1	MSRV/XLD	0	4	0	3	7
	Own best	0	3	0	1	4
6	MSRV/XLD	5	5	4	5	19
	Own best	0	5	0	2	7
7	MSRV/XLD	2	3	2	2	9
	Own best	2	4	2	2	10
8	MSRV/XLD	0	1	0	1	2
	Own best	0	1	0	1	2
9	MSRV/XLD	5	4	4	4	17
	Own best	5	4	4	4	17
12	MSRV/XLD	0	3	0	2	5
	Own best	0	5	1	3	9
13	MSRV/XLD	0	4	1	1	6
	Own best	1	5	1	2	9
14	MSRV/XLD	5	5	3	5	18
	Own best	3	5	2	4	14
15	MSRV/XLD	1	2	0	3	6
	Own best	3	2	0	3	8
16	MSRV/XLD	0	3	0	1	4
	Own best	0	0	0	0	0
17	MSRV/XLD	0	3	1	2	6
	Own best	3	5	1	3	12

Four laboratories (labcodes 1, 6, 14 and 16) found more positive isolations with MSRV/XLD than with their best own medium. Laboratories (labcodes 7, 12, 13, 15 and 17) scored less positive isolations with MSRV/XLD than with their best own medium.

4.5 Comparison between laboratories

To be able to compare the positive isolations with the six medium combinations separately and all medium combinations together the differences between NRLs were calculated in relation to the average results for all laboratories (see Figure 3).
Laboratories with labcodes 5 and 9 obtained significantly better results for all medium combinations. This was partly caused by the high number of positive isolations when RVS was used. Laboratories 10 and 14 also tested more samples positive in relation to the average of all laboratories for medium combinations separately and all medium combinations together.
The NRLs with labcodes 2, 3, 7, 11 and 16 scored with all medium combinations below the average of all laboratories. Differences between enrichment media found in laboratories 4 and 6 revealed that in these laboratories significantly more positive isolations of *Salmonella* were obtained with MRSV than with MKTTn or RVS.

Figure 3. Results obtained with various medium combinations per laboratory compared to the average results of all laboratories (y-axis:arithmetical variation values)

4.6 Results of faeces samples naturally contaminated with *Salmonella*.

The results in Table 18 and Figure 4 showed that only one laboratory (labcode 10) was able to recover *Salmonella* from all faeces samples with the use of all medium combinations. Laboratories 4 and 6 only scored the maximum of all samples with medium combinations MSRV/BGA and MSRV/XLD. Laboratory 8 analysed the samples only with MSRV/XLD, and obtained the maximum positive score with that medium combination.

Table 18. Number of positive isolations per medium combination and per laboratory for naturally contaminated samples (n=20)

	Laboratory codes																
Medium combination	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
RVS/BGA	**7**	**20**	**0**	**15**	**20**	**3**	**0**	**--**	**19**	**20**	**8**	**15**	**7**	**12**	**19**	**17**	**18**
RVS/XLD	**6**	**20**	**20**	**15**	**20**	**7**	**6**	**--**	**19**	**20**	**9**	**20**	**2**	**11**	**19**	**12**	**19**
MKTTn/BGA	**2**	**19**	**0**	**1**	**17**	**2**	**2**	**--**	**4**	**20**	**10**	**20**	**9**	**18**	**20**	**20**	**7**
MKTTn/XLD	**0**	**20**	**6**	**18**	**20**	**0**	**3**	**--**	**11**	**20**	**18**	**20**	**7**	**18**	**19**	**20**	**15**
MSRV/BGA	**5**	**4**	**1**	**20**	**19**	**20**	**0**	**--**	**19**	**20**	**14**	**20**	**9**	**19**	**19**	**13**	**8**
MSRV/XLD	**1**	**20**	**17**	**20**	**19**	**20**	**5**	**20**	**19**	**20**	**14**	**20**	**6**	**19**	**19**	**16**	**17**

Table 19. Overall results of all participating laboratories and all medium combinations (n=6) for the naturally contaminated faeces with Salmonella (n=20)

		RVS		MKTTn		MSRV	
Capsules		BGA*	XLD*	BGA*	XLD*	BGA*	XLD**
None	Positives	200	225	171	215	210	272
	%	63	70	53	67	66	85

* 16 participating laboratories ** 17 participating laboratories

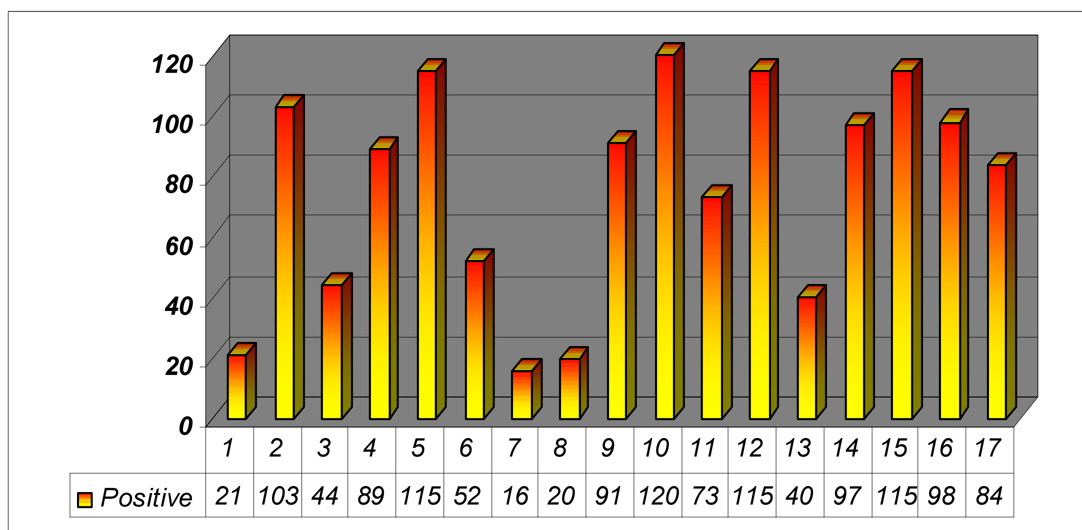

Figure 4. Number of positive isolations (max.120) per laboratory (1-17) for all medium combinations (n=6) when analysing 25 g Salmonella positive faeces

The cumulative results of all laboratories for the naturally contaminated samples per medium combination are given in Table 19. The isolation of *Salmonella* from the naturally contaminated samples showed somewhat more positive isolations when RVS and MSRV were used as the selective enrichment media compared to MKTTn, although these differences were not significantly better (see Table 20). The comparison between BGA and XLD revealed that for XLD significantly more positive isolations were obtained.

Table 20. Comparison of media as p-values for naturally contaminated samples

Media	p-values
MSRV vs RVS	0.3320
MKTTn vs MSRV	0.1134
MKTTn vs RVS	0.3492
BGA vs XLD	0.0249

p-values <0.05 are shown in red

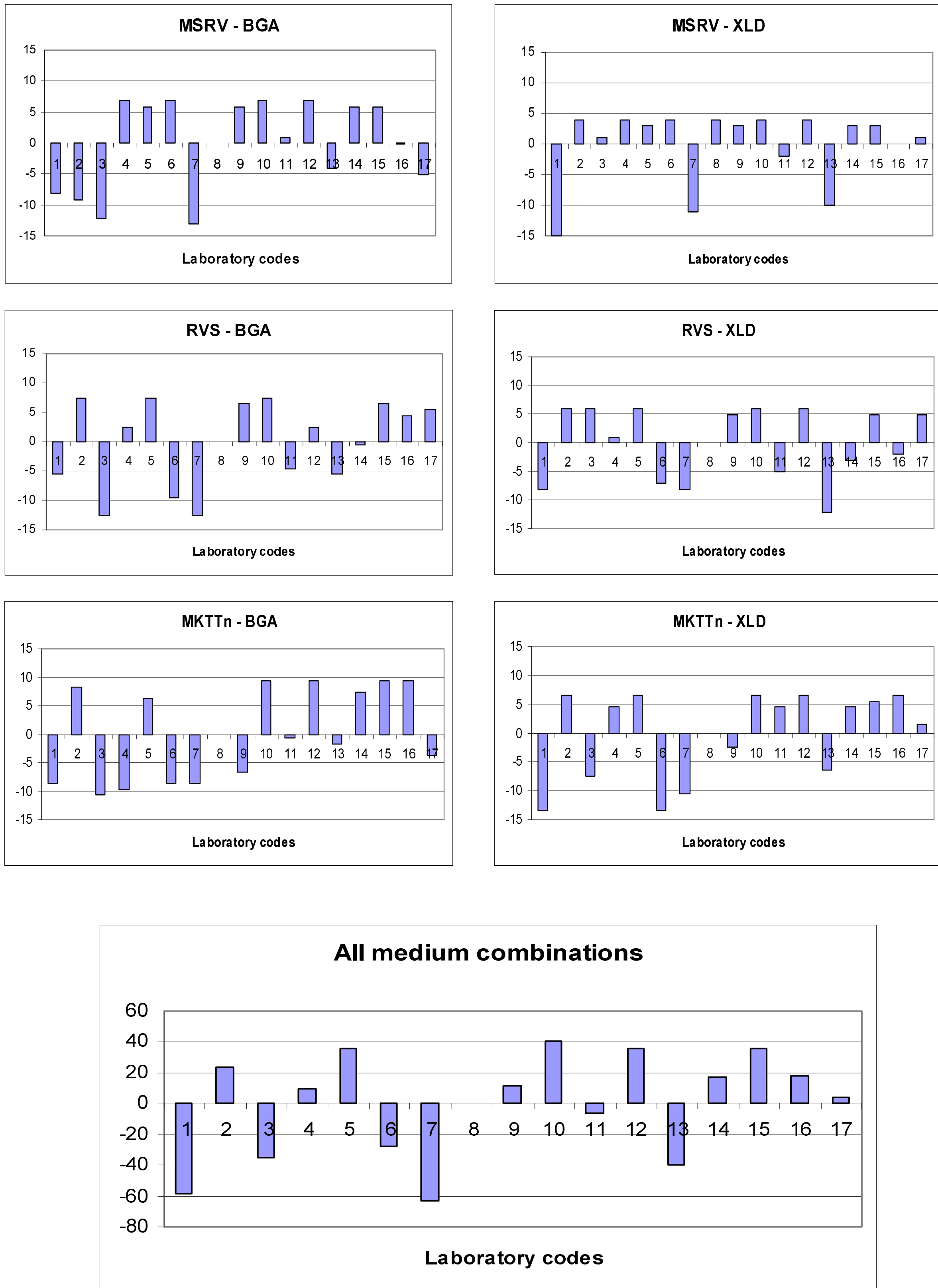

Figure 5. Results obtained with various medium combinations for the naturally contaminated samples per laboratory compared to the average result of all laboratories (y-axis:arithmetical variation values)

To be able to compare the positive isolations with the six medium combinations separately and all medium combinations together the differences between NRLs were calculated in relation to the average results for all NRLs (see Figure 5).
For all medium combinations laboratories 5, 10, 12 and 15 found considerably more samples positive than the other laboratories. Laboratories 1 and 7 and to a lesser extent 3, 6 and 13 obtained less positive results in relation to the average of all laboratories. Laboratory 2 found more samples positive with MSRV/XLD than with MSRV/BGA. The NRL with labcode 6 got better results when MSRV was used as the selective enrichment medium

4.7 PCR

Five NRLs also carried out PCR on all artificially and naturally contaminated samples. The results of these five laboratories are shown in the tables 5.1, 5.2 and 5.3 in Appendix 5.
The PCR on the control samples revealed excellent results in relation to the bacteriology. For the naturally contaminated samples only one laboratory (labcode 1) tested more samples positive with the PCR than with the bacteriological investigations, i.e. 13 PCR positives to 7 bacteriology positives. Laboratory 10 tested all these samples negative and laboratory 5 did not test all samples. Laboratory 11 faced problems with inhibition of the PCR reaction. This laboratory used the highest actual volume of BPW in the PCR in relation to the other laboratories.
For the artificially contaminated samples the PCR results of laboratories 1 and 5 were comparable to the bacteriological results. Laboratory 10 found less positives with their PCR than with the bacteriological detection. Laboratory 11 faced problems with inhibition of their PCR reaction and laboratory 13 tested all samples negative. Laboratories 1 and 5 tested respectively one and two blank capsules positive.

4.8 Transport

As all samples (capsules as well as faeces) contained risk class 2 micro-organisms, they had to be packed and transported as dangerous goods. Due to the amount of the materials (ca. 1kg capsules and chicken faeces) it was transported by road instead of by air, meaning more days en route. Figure 6 shows the days between sending by CRL-*Salmonella* and arrival of the parcels at the lab of destination.

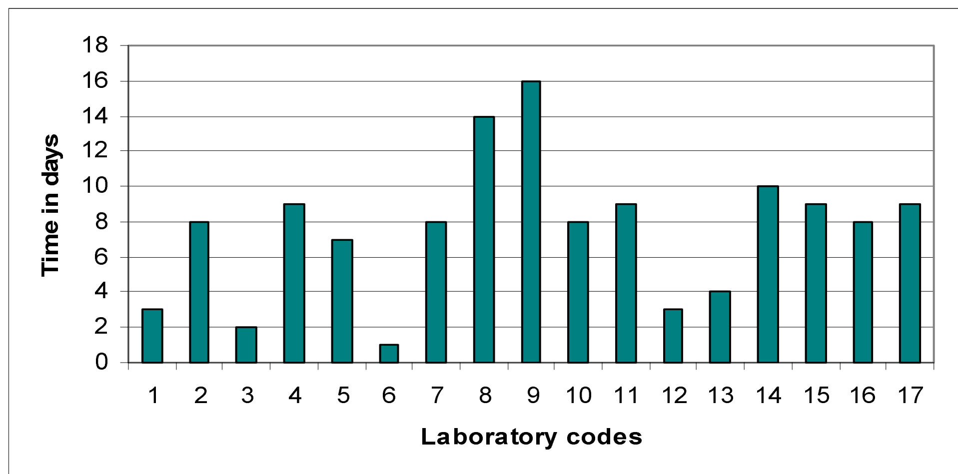

Figure 6. Days of transport of the packages

According to the protocol, sent to all NRLs one month before transporting the parcels, all samples had to be stored at (- 20 ± 5) ^{o}C after arrival at the laboratory. Eleven laboratories stored the samples according to this protocol. NRLs with laboratory codes 2, 5, 7, 10, 15 and 16 immediately tested the samples after arrival.

Information on the colouring of the different compartments of the temperature recorders is shown table 21. The NRLs with labcode 2, 3 and 15 did not record their results. The yellow and brown cells in the table indicate the colouring of the different compartments of the tags.

Table 21. Reading of the colouring of the compartments of the temperature tags.

Lab codes	Temp. tags	Brief			Moderate			Prolonged			Appr.max. temperature
		Colour	Partly	White	Colour	Partly	White	Colour	Partly	White	
1, 6	10 °C										< 10 °C
	20 °C										
8, 12, 13	10 °C										12 ± 2 °C
	20 °C										
10	10 °C										14 ± 2 °C
	20 °C										
4, 5, 7, 11, 14, 16, 17	10 °C										16 ± 2 °C
	20 °C										
9	10 °C										> 20 °C
	20 °C										

The temperature of two packages remained below 10°C (labcodes 1 and 6). The colouring of the compartments on the tags of three laboratories (labcodes 8, 12 and 13) indicated that their package did not exceed the temperature of approximately (12 ± 2) °C and of laboratory 10 with a temperature of appr. (14 ± 2) °C. The maximum temperature of the contents of the packages from seven NRLs (labcodes 4, 5, 7, 11, 14, 16 and 17) was approximately (16 ± 2) °C. The content of the package of laboratory 9 has probably been at temperatures above 20°C.

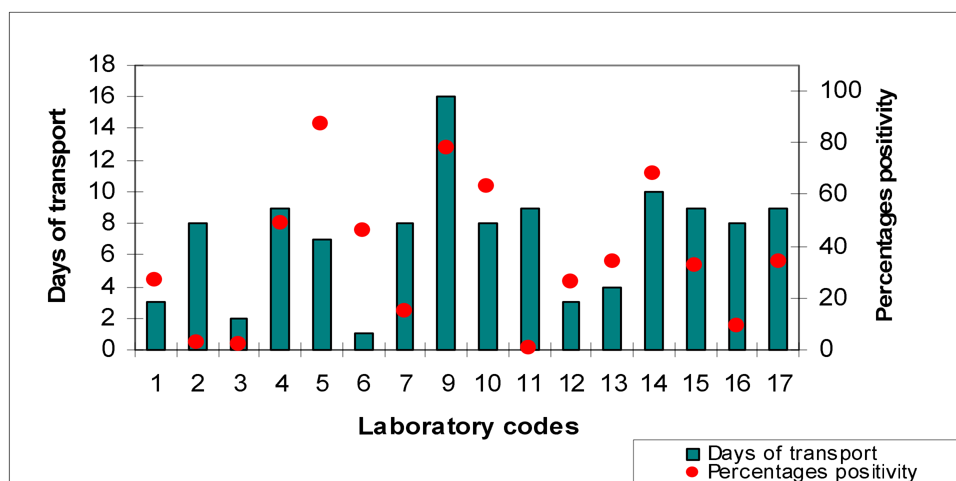

Figure 7. Relationship between days of transport and results of the faeces samples artificially contaminated with Salmonella for all medium combinations

Long transport time at elevated temperatures could have had a negative effect on the capsules and/or the faeces samples. Figures 7 and 8 show the days of transport in relation to the percentages positivity for the artificially and naturally contaminated samples, respectively. When a comparison is made for the artificially contaminated samples among laboratories 2, 4, 7, 10 and 11 (8 and 9 days of transport time), Figure 7 shows percentages positivity of 3, 49, 15, 63 and 1, respectively. For the naturally contaminated samples the days of transport seem to be of even lesser importance than for the artificially contaminated samples (see Figure 8).

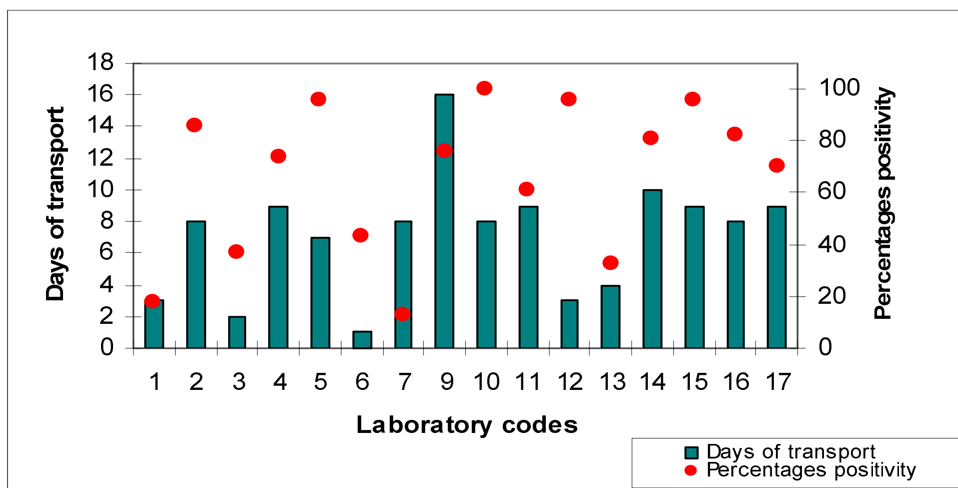

Figure 8. *Relationship between days of transport and results of faeces samples naturally contaminated with Salmonella for all medium combinations*

5. Discussion

Pre-enrichment medium (BPW)

The prescribed pre-heating temperature of BPW is 37°C. This temperature was used by most of the laboratories. Incubating the BPW for the prescribed time of 16-20 hours reveals a higher percentage of positive isolations compared to incubating > 20 hours. Laboratories 3, 8 and 11 used an incubation time of the BPW of more than 20 hours. These latter laboratories tested less positive samples in relation to the other laboratories. The pH for the BPW as prescribed by ISO 6579: 2002 is 7.0 ± 0.2. Three laboratories used a pH for the pre-enrichment of more than 7.2, but this did not seem to influence their results.

Selective enrichment medium for artificially contaminated samples

The newly accepted ISO 6579: 2002 (E) prescribes the use of RVS and MKTTn as selective enrichment media. In this study each laboratory was asked to test these media together with MSRV as the selective enrichment. MSRV was tested and compared starting from study IV on. The medium MKTTn was not used in earlier collaborative studies. The composition of the MKTTn from all manufacturers differed from the composition as mentioned in the ISO. Some laboratories had problems in isolating *Salmonella* from artificially contaminated samples using MKTTn but no reason could be found for these results. These laboratories prepared either their MKTTn in accordance with the ISO-prescription or according to the different formulae from various MKTTn suppliers. A probable explanation for the poor results on MKTTn could have been the pH of the MKTTn medium. The pH value of MKTTn differed largely between laboratories (7.0 – 8.2).

Instead of an incubation temperature for MKTTN of 37°C two laboratories incubated their medium plates with MKTTn at 41.5°C, but this did not seem to influence their results.

The contrast results in p-values for all capsules indicate that MSRV is a considerably better selective enrichment medium than RVS for the artificially contaminated samples. Although more positive isolations were found with MSRV in relation to MKTTn, this difference was not significant.

The results of the STM10 and STM100 capsules was not obviously influenced by the choice of the selective enrichment medium. However, for the SE capsules less positive results were seen with RVS in relation to both other enrichment media. The differences between MKTTn and MSRV were not significant for the average result of all capsules.

Selective enrichment medium for naturally contaminated samples

The number of positive isolations for all laboratories together showed that, independent of the plating-out media, MSRV was responsible for more positive isolations of *Salmonella* than MKTTn and RVS, while RVS also showed more isolations than MKTTn. However, the differences between the three selective enrichment media were not significant.

Plating-out (isolation) medium

For the artificially contaminated samples the choice of a certain plating-out medium (BGA or XLD) seemed to influence the results significantly except for the STM capsules. In general, more *Salmonella* could be isolated when XLD was used.
The differences were even more obvious for the naturally contaminated samples. Here the use of XLD showed significantly more positive results than the use of BGA as the isolation medium.

Transport

In earlier studies the capsules were packed as dangerous goods and the faeces samples not. It was decided by CRL-*Salmonella* that all samples should be sent under dangerous goods management, meaning that the amount of infectious samples exceeded the maximum amount of material allowed to be sent per package (50 grams). Due to the amount of materials the packages were transported by road, resulting in considerably longer transport time than in earlier studies. Long transport time at elevated temperatures could affect the results. However, in this study no significant relation was found between transport time and “poor”or “good” results.

PCR

The use of a PCR detection method showed similar or poorer results as the bacteriological detection method. No positive PCR results were seen for the laboratory that used the smallest actual volume of BPW (volume of DNA extract used in comparison with the volume of BPW). One laboratory tested some of the blank capsules positive. The laboratory with the highest actual volume of BPW had problems with an inhibition of the PCR reaction. All control samples were tested correctly meaning that negative results in the PCR reaction are probably caused by the presence of faeces.

Action items

A critical point in the analyses of the artificially contaminated samples is the complete dissolving of the capsules in BPW. CRL-*Salmonella* will investigate whether it is necessary to adjust the Standard Operation Procedure at this point.

Comparison of results obtained with the selective enrichment medium MKTTn is hampered by the varying compositions of this medium used by the different laboratories. Only three laboratories used MKTTn in the same composition as described in ISO 6579:2002. Special attention will be paid to this item in future collaborative studies.

Laboratories with poor results will be contacted individually by CRL-*Salmonella* to discuss how these laboratories can be offered assistance to improve their results. In the present study one laboratory received additional capsules and faeces to perform additional tests for searching the reason of their previous poor performance.

6. Conclusions

- It was easier to isolate *Salmonella* from capsules with a high level of STM (139 cfp/capsule) than, in declining order, from capsules with a high level of SE 500 (389 cfu/capsule), from capsules containing a low level of STM (11 cfp/capsule) and from capsules with a low level of SE (92 cfp/capsule).

- The use of MSRV as selective enrichment medium yielded significantly more positive *Salmonella* isolations in comparison with the use of RVS. MSRV also showed more positive *Salmonella* isolations than MKTTn, but the difference was not significant. The differences between the results of the three selective enrichment media for the examination of the naturally contaminated samples were not significant.

- Significantly more positive isolations of *Salmonella* were obtained by using XLD as the plating-out/isolation medium in comparison with the use of BGA, except for the STM capsules

- More positive results were obtained from the naturally contaminated samples in comparison with the artifically contaminated samples.

- Some laboratories found few positive *Salmonella* isolations in all samples. Explanations could not always be found. Further discussion on these poor results will be necessary.

Acknowledgments

We are grateful to all the contact persons and laboratory technicians from the National Reference Laboratories for *Salmonella* in the Member States of the European Union and Norway.
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Appendix 1 Mailing list

01	European Commission, Director of Directorate D	P. Testori-Coggi
02	European Commission, Head of Unit D 2	E. Poudelet
03	European Commission	J.C. Cavitte
04	European Commission	P. Mäkelä
05	President of the Council of Health, The Netherlands	prof. dr. J.A. Knottnerus
06	Veterinary Public Health Inspector	drs. H. Verburg
07	Board of Directors	H.A.P.M. Pont
08	Director Sector VCV	prof. dr. ir. D. Kromhout
09	Head of Microbiological Laboratory for Health Protection and Director CRL-*Salmonella*	dr. ir. A.M.Henken
10	Head of Laboratory for Analytical Residue Research	prof. dr. W.R. Stephany
11-43	Participants of the study	
44-48	Authors	
49	Dutch National Library for Publications and Bibliography	
50	SBC/Communication	
51	Registration agency for Scientific Reports	
52	Library RIVM	
53-60	Sales department of RIVM Reports	
61-80	Spare copies	

Appendix 2 Calculation of T_2

Calculation of T_2 in homogeneity studies

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

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

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

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

Appendix 3 Results by laboratory, sample and medium combination

Table 3.1 Number of positive isolations per laboratory with own medium combinations for all control samples

	Medium combination		Capsules			
Labcode	Selective enrichment	Isolation medium	Blank (n=2)	S.Pan 5 (n=2)	STM 10 (n=3)	SE 100 (n=3)
1	RVS	Gassner	0	1	3	3
	MKTTn	Gassner	0	1	3	3
	MSRV	Gassner	0	2	3	3
6	RV	BGA	0	2	3	3
	RV	XLD	0	2	3	3
7	RVS	XLT4	0	2	3	3
	MKTTn	XLT4	0	2	3	3
	MSRV	XLT4	0	2	3	3
8	MSRV	Rambach	0	1	0	3
	TBG	XLD	0	1	0	3
	TBG	Rambach	0	1	0	3
9	RVS	Rambach	0	2	3	3
	MKTTn	Rambach	0	2	3	3
	MSRV	Rambach	0	2	3	3
12	MKTTn	XLT4	0	2	3	3
	MSRV	Rambach	0	2	3	3
	MK	XLT4	0	2	3	3
13	RVS	SMID	0	2	3	3
	MKTTn	SMID	0	2	3	3
	MSRV	SMID	0	2	3	3
14	Rappaport	BGA	0	2	3	3
	Rappaport	SMID	0	2	3	3
15	RVS	MLCV	0	2	3	3
	MKTTn	MLCV	0	2	3	3
	MSRV	MLCV	0	2	3	3
16	BPLS	BGA	0	2	3	3
17	Diasalm	BGA	0	2	3	3
	Diasalm	XLD	0	2	3	3

** Unexpected results are indicated in red*

Table 3.2 Number of positive isolations per laboratory for STM 10 (n=5) with the addition of Salmonella negative faeces

Medium combination	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
RVS/BGA	3	0	0	0	3	0	0	--	5	2	0	0	1	2	3	0	0
RVS/XLD	1	0	0	0	3	0	0	--	5	2	0	0	0	3	3	0	0
MKTTn/BGA	0	0	0	0	3	1	0	--	3	1	0	0	1	2	4	0	1
MKTTn/XLD	0	0	1	3	3	0	1	--	3	1	0	0	1	5	3	0	5
MSRV/BGA	1	0	0	5	3	5	0	--	5	5	0	0	0	5	1	0	0
MSRV/XLD	0	0	0	5	3	5	2	0	5	5	0	0	0	5	1	0	0
RV/BGA						0											
RV/XLD						0											
RVS/Gassner	0																
RVS/XLT4							0										
RVS/Rambach									5								
RVS/SMID													0				
RVS/MLCV															3		
MKTTn/Gassner	0																
MKTTn/XLT4							1					0					
MKTTn/Rambach									3								
MKTTn/SMID													1				
MKTTn/MLCV															2		
MSRV/Gassner	0																
MSRV/XLT4							2										
MSRV/Rambach								0	5			0					
MSRV/SMID													1				
MSRV/MCLV															1		
MK/XLT4												0					
TBG/XLD								0									
TBG/Rambach								0									
Rappaport/SMID														3			
BPLS/BGA																0	
Diasalm/BGA																	3
Diasalm/XLD																	3

Table 3.3 Number of positive isolations per laboratory for STM 100 (n=5) with the addition of Salmonella negative faeces

Medium combination	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
RVS/BGA	2	1	0	1	5	3	0	--	4	5	0	2	5	4	2	0	5
RVS/XLD	5	1	0	1	5	2	3	--	4	5	0	2	3	5	2	0	5
MKTTn/BGA	0	1	0	0	5	1	1	--	4	5	0	3	5	5	2	0	3
MKTTn/XLD	0	0	0	5	5	1	0	--	1	5	0	3	5	4	2	0	5
MSRV/BGA	4	0	0	5	5	5	0	--	4	5	1	3	1	5	2	3	0
MSRV/XLD	4	0	0	5	5	5	3	1	4	5	1	3	4	5	2	3	3
RV/BGA						5											
RV/XLD						5											
RVS/Gassner	0																
RVS/XLT4							3										
RVS/Rambach									4								
RVS/SMID													3				
RVS/MLCV															2		
MKTTn/Gassner	0																
MKTTn/XLT4							1					3					
MKTTn/Rambach									3								
MKTTn/SMID													5				
MKTTn/MLCV															2		
MSRV/Gassner	3																
MSRV/XLT4							4										
MSRV/Rambach								1	4			3					
MSRV/SMID													4				
MSRV/MCLV															1		
MK/XLT4												5					
TBG/XLD								0									
TBG/Rambach								0									
Rappaport/SMID														5			
BPLS/BGA																0	
Diasalm/BGA																	5
Diasalm/XLD																	5

Table 3.4 Number of positive isolations per laboratory for SE 100 (n=5) with the addition of Salmonella negative faeces

Medium combination	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
RVS/BGA	0	0	0	0	4	1	0	--	4	0	0	0	1	0	0	0	1
RVS/XLD	0	0	0	0	4	2	0	--	4	0	0	0	0	1	0	0	0
MKTTn/BGA	0	0	0	1	4	0	0	--	3	1	0	1	1	3	0	0	0
MKTTn/XLD	0	0	1	1	4	2	1	--	4	1	0	1	1	3	0	0	2
MSRV/BGA	1	0	0	5	5	4	0	--	4	2	0	0	0	2	0	0	0
MSRV/XLD	0	0	0	5	5	4	2	0	4	2	0	0	1	3	0	0	1
RV/BGA						0											
RV/XLD						0											
RVS/Gassner	0																
RVS/XLT4							1										
RVS/Rambach									4								
RVS/SMID													0				
RVS/MLCV															0		
MKTTn/Gassner	0																
MKTTn/XLT4							2					1					
MKTTn/Rambach									3								
MKTTn/SMID													1				
MKTTn/MLCV															0		
MSRV/Gassner	0																
MSRV/XLT4							2										
MSRV/Rambach								0	4			0					
MSRV/SMID													1				
MSRV/MCLV															0		
MK/XLT4												0					
TBG/XLD								0									
TBG/Rambach								0									
Rappaport/SMID														2			
BPLS/BGA																0	
Diasalm/BGA																	1
Diasalm/XLD																	1

Table 3.5 Number of positive isolations per laboratory for SE 500 (n=5) with the addition of Salmonella negative faeces

Medium combination	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
RVS/BGA	3	0	0	0	5	0	1	--	4	1	0	1	2	1	1	0	2
RVS/XLD	1	0	0	0	5	2	0	--	4	2	0	2	1	1	1	0	1
MKTTn/BGA	0	0	0	2	5	1	0	--	4	5	0	3	2	4	3	1	1
MKTTn/XLD	0	0	0	5	5	1	0	--	4	5	0	3	3	4	4	2	3
MSRV/BGA	4	0	0	5	5	5	2	--	4	5	0	2	2	5	0	1	1
MSRV/XLD	3	0	0	5	5	5	2	1	4	5	0	2	1	5	3	1	2
RV/BGA						2											
RV/XLD						2											
RVS/Gassner	1																
RVS/XLT4							1										
RVS/Rambach									4								
RVS/SMID													0				
RVS/MLCV															1		
MKTTn/Gassner	0																
MKTTn/XLT4							0					3					
MKTTn/Rambach									4								
MKTTn/SMID													1				
MKTTn/MLCV															3		
MSRV/Gassner	0																
MSRV/XLT4							2										
MSRV/Rambach								1	4			2					
MSRV/SMID													2				
MSRV/MCLV															2		
MK/XLT4												3					
TBG/XLD								1									
TBG/Rambach								1									
Rappaport/SMID														4			
BPLS/BGA																0	
Diasalm/BGA																	3
Diasalm/XLD																	3

Appendix 4 Information on the media used

Table 4.1 Manufacturer of BPW

Labcode	Name	Code	Batch	pH
ISO 6579	--	--	--	7.0 ± 0.2
1	Merck	1.07228	VK5387824	7.2
2	Biolife	5112793	32H030	---
3	Oxoid	BO 01445	827682	7.2
4	Merck	107228	VM 870428214	7.17
5	Merck	107228	VM 909928	7.2
6	SVM	E 4900	2204128	-----
7	Merck	1.07228	VM870228	7.2
8	Merck	1.07228	VN 845128	7.3
9	Merck	1.07228	VM 885868/218	7.15
10	Lab M	Lab 46	1) 054833 2) 060698	1) 7.21 2) 7.31
11	Oxoid	CM 509	250695	7.1
12	AES Lab	AEB 140302	122638	7.1
13	Biomerieux	42043	768924201	7.0
14	Merck	1.07228	VM 870228	7.2
15	Difco	218105	1163007	7.39
16	Oxoid	CM 509	276 505	7.1
17	Oxoid	CM 509	B 278574	7.2

Table 4.2 Manufacturer of MSRV medium

Labcode	Name	Code number	Batch number	pH
SOP	--	--	--	5.0 ± 0.2
1	Merck	1.09878	VL 811178 202	5.6
2	Oxoid	CM 910	982/D	5.2
3	Becton Dickinson	257234	2261659	--
4	Oxoid	CM 910	271841	5.42
5	Oxoid	CM 910	B 284530	5.3
6	Becton Dickinson	218681	2017008	--
7	Becton Dickinson	218681	1108002	5.1
8	Oxoid	CM 911	261170	5.2
9	Oxoid	CM 910	281488	5.3
10	Lab M	Lab 150	048468	5.40
11	Oxoid SVA	CM 910 341927	B210783 7104	5.3
12	AES	AEB 140672	116352	5.5
13	Oxoid	CM 910	278000	5.4
14	Merck	1.09878	V439078 948	5.4
15	Oxoid	CM 910	B 282661	5.48
16	Oxoid	CM 910	281488	5.41
17	Oxoid	CM 910	B284530	5.4

Table 4.3 Manufacturers of Rappaport Vassiliadis medium with soya broth (RVS)

Labcode	Name	Code	Batch	pH
ISO 6579	--	--	--	5.2 ± 0.2
1	Merck	--	--	5.2
2	Biolife	521980	32M096	--
3	Oxoid	BO 0499E	833463	--
4	Oxoid	CM 866	B 270616	5.17
5	Oxoid	CM 866	B 267 957	5.2
6	Oxoid	CM 866	239145	--
7	Oxoid	CM 866	B 263 943	5.3
9	Merck	1.07700	VK 493500	5.25
10	Oxoid	CM 866	263943	5.22
11	Oxoid	CM 866	276557	5.3
12	Oxoid	CM 866	B 262 584	5.4
13	Oxoid	TV 5036 F	14917	5.2
14	Biorad	55777	2E0017	--
15	Lab M	LAB 86	045327	5.01
16	Oxoid	CM 866	12A 007	5.38
17	Oxoid	CM 866	B 259255	5.2

Table 4.4 Composition of RVS selective enrichment mediumin g/l

Labcode	Soya peptone	NaCl	Potassium dihydrogen phosphate	Dipotassium hydrogen phosphate	Mg chloride anhydrous	Mg chloride. 6H20	Malachite green
ISO 6579	4.5	7.2	1.44	0.2	13,4	--	0.036
1	--	--	--	--	--	--	--
2	--	--	--	--	--	--	--
3	4.5	7.2	1.4	0.2	13,4	--	0.036
4	4.5	7.2	1.26	0.18	13,58	--	0.036
5	4.5	7.2	1.26	0.18	13,58	--	0.036
6	4.5	7.2	1.26	0.18	13,58	--	0.036
7	4.5	7.2	1.26	0.18	13,58	--	0.036
8	---	---	---	---	---	---	---
9	4.5	8.0	0.6	0.4	--	29.0	0.036
10	4.5	7.2	1.26	0.18	13,58	--	0.036
11	4.5	7.2	1.26	0.18	13,58	--	0.036
12	4.5	7.2	1.26	0.18	13,58	--	0.036
13	4.5	7.2	1.26	0.18	13,58	--	0.036
14	4.5	7.2	1.26	0.18	28,60	---	0.036
15	4.5	7.2	1.26	0.18	13,58	--	0.033
16	4.5	7.2	1.26	0.18	13,58	--	0.036
17	4.5	7.2	1.26	0.18	13,58	--	0.036

Table 4.5 Manufacturer of MKTTn medium

Labcode	Name	Code	Batch	pH
ISO 6579	--	--	--	8.2 ± 0.2
1	Home made	--	--	7.0
2	Biolife	521743	32H-036	--
3	Biorad	355 6140	F3194	--
4	Difco	218531	1283004	8.14
5	Oxoid	CM 343	B 247547	8.0
6	Oxoid	CM 343	236176	--
7	Scharlau	02-335	10008	8.3
8	--	--	--	--
9	Merck	1.10863	VK 582963	7.4
10	Oxoid	CM 343	264568	8.01
11	Home made	--	7172	8.2
12	Biokar	BK 135 HA	OM 922	7.8
13	Biorad	35561440	I 3194	8
14	Biokar	BK 135HA	1G412	7.3
15	Oxoid	CM 343	B 239428	7.92
16	Home made	--	--	8.22
17	Oxoid	CM 343	B 239428	--

Table 4.6 Composition of MKTTn mediumin g/l

Labcode	Meat extract	Casein	NaCl	$CaCO_3$	$Na_2S_2O_3.5H_20$	Ox bile	Brilliant green
ISO 6579	4.3	8.6	2.6	38.7	47.8	4.78	9.6 mg
1	--	--	--	--	--	--	--
2	--	--	--	--	--	--	--
3	2.3	7	2.3	25	40.7	4.75	9.5 mg
4	5.0	10.0	3.0	45.0	38.1	4.7	9.5 mg
5	7.0	2.3	2.23	25.0	40.7	4.75	9.6 mg
6	7.0	2.3	2.3	25.0	40.7	4.75	10 mg
7	4.5	9.0	2.7	40.5	50.0	5.0	10 mg
8	--	--	--	--	--	--	--
9	0.9	4.5	4.5	25.0	40.7	4.75	10 mg
10	7.0	2.3	2.3	25.0	40.7	4.75	--
11	4.3	8.6	2.6	38.7	47.8	4.78	9.6 mg
12	4.23	8.45	2.54	38.04	30.27	4.75	9.5 mg
13	2.3	7	2.3	25.0	40.7	4.75	9.5 mg
14	4.23	8.45	2.54	38.04	30.27	4.75	9.5 mg
15	?	?	2.3	25	40.7	4.75	?
16	4.35	8.63	2.63	38.7	47.81	4.8	9.6 mg
17	7.0	2.3	2.3	25.0	40.7	4.75	10 mg

Table 4.7 Manufacturer of own selective medium

Labcode	Name	Code number	pH of broth	Volume per jar/tube	Inoculation volume of BPW	Incubat. Temp.
6	RV	SVM E5125z	--	10 ml	0.1 ml	42
8	TBG	Merck 1.05178	7.2	50	1.5 ml	First period 42 Second period 37
12	AES	AEB 140702	8.2	20 ml	2 ml	42
14	Rappaport broth	Biomerieux 42091	--	9 ml	1 ml	37
17	DIASSALM	Lab M Lab 537	5.5	20 ml	0.1 ml	41.5

Table 4.8 Manufacturer of BGA

Labcode	Name	Code	pH	Volume	Inoculation volume of BPW	Incubation temp.
1	Merck	1.07237	6.9	16 ml	--	37
2	DID	0285-30	--	--	--	37
3	Oxoid	15574	--	--	--	--
4	Oxoid	CM 329	--	17 ml	--	37
5	Oxoid	CM 329	6.8	--	--	--
6	SVM	E1800y	--	--	0.1 ml	37
7	Merck	1.07237	6.9	--	--	--
8	--	--	--	--	--	--
9	Lab M	Lab 37	6.9	--	--	--
10	Oxoid	CM 263	6.86 and 6.79	20 ml	0.01 ml	37
11	Oxoid	CM 329	7.0	20 ml	0.01 ml	37
12	AES	AEB 151492	7.0	17 ml	0.01 ml	37
13	Biomerieux	43588	7.0	--	--	35-37
14	BPLS agar from Merck	1.10747	6.9	15-20 ml	--	37
15	Lab M	Lab 34	6.85	--	--	37
16	Merck	1.10747	6.94	20 ml	1 loopfull	37
17	Oxoid	CM 329	6.9	20 ml	--	37

Table 4.9 Manufacturer of XLD

Labcode	Name	Code number	pH of broth	Size Petri dishes
1	Merck	1.05287	7.4	90 mm
2	Biolife	542206	--	--
3	Oxoid	--	--	90 mm
4	Oxoid	CM 469	7.5	90 mm
5	Oxoid	CM 469	7.4	90 mm
6	Oxoid	CM 469	--	90 mm
7	Difco	278850	7.4	140 mm
8	Merck	1.05287	7.4	90 mm
9	Merck	1.05287	7.4	90 mm
10	Lab M	Lab 32	7.40	90 mm
11	Lab M	Lab 32	7.3	90 mm
12	AES	AEB 153402	7.4	90 mm
13	Biomerieux	43563	7.4	90 mm
14	Biokar	BK058HA	7.4	90 mm
15	Oxoid	CM 469	7.29	--
16	Difco	0788	7.46	90 mm
17	Oxoid	CM 469	7.4	90 mm

Table 4.10 Manufacturer of own isolation medium

Labcode	Name Medium	Incub. temp.	Manufacturer medium	Code	pH	Size Petri dishes
1	Gassner-Agar	37	Merck	1.01282	7.2	90 mm
7	XLT4	37	Difco	223420	7.4	140 mm
8	Rambach	37	Merck	15999	7.3	90 mm
9	Rambach	37	Merck	1.0750	7.3	90 mm
12	Rambach	37	Merck	1.0750	7.3	90 mm
	XLT4	37	Biokar	BK 156 HA	7.2	90 mm
13	SMID	37	Biomerieux	43291	7.6	90 mm
14	SMID	37	Biomerieux	43291	--	90 mm
15	Mannitol Lysine Crystal Violet BGA	37	Oxoid	CM 785	6.9	--
16	BGA/ BPLS	37	Merck	1.10747	6.94	90 mm

Appendix 5 Results of PCR detection

Table 5.1 PCR results of artificially contaminated samples in comparison with bacteriological results

	Lab 1		Lab 5		Lab 10		Lab 11		Lab 13	
Number	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR
1	+	+	+	+	+	-	-	I	-	-
2	+	+	-	-	+	-	-	I	-	-
3	+	+	+	+	+	-	-	I	+	-
4	+	+	+	+	+	-	-	I	+	-
5	+	+	+	+	+	+	-	I	+	-
6	+	+	+	+	+	-	-	I	+	-
7	+	+	+	+	+	+	-	I	+	-
8*	-	+	+	-	-	-	-	I	-	-
9*	-	-	+	+	-	-	-	I	-	-
10	-	-	+	+	+	-	-	I	-	-
11	+	+	+	+	+	-	-	I	-	-
12	-	+	+	+	-	-	-	I	-	-
13	-	-	-	+	+	-	-	I	-	-
14	-	-	+	+	-	-	-	I	-	-
15	+	+	+	+	+	-	-	I	+	-
16*	-	-	+	+	-	-	-	I	+	-
17	-	+	+	+	+	-	-	I	+	-
18	+	-	+	+	+	-	-	I	-	-
19	+	+	+	+	+	+	+	I	+	-
20	-	-	+	+	+	-	-	I	-	-
21*	-	-	-	nt	-	-	-	I	-	-
22*	-	-	-	nt	-	-	-	I	-	-
23	-	-	+	nt	-	-	-	I	-	-
24	+	+	+	nt	+	+	-	I	+	-
25	+	+	+	nt	+	+	-	I	+	-

I = Inhibition of PCR reaction * = Blank capsules BAC = Bacteriology nt=not tested

Table 5.2 PCR results of naturally contaminated samples in comparison with bacteriological results

	Lab 1		Lab 5		Lab 10		Lab 11		Lab 13	
	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR
N1	-	-	+	+	+	-	+	I	+	-
N2	-	+	+	nt	+	-	+	I	+	-
N3	-	+	+	nt	+	-	+	I	-	+
N4	-	+	+	nt	+	-	+	I	-	-
N5	-	+	+	+	+	-	+	I	+	+
N6	-	-	+	nt	+	-	+	I	+	-
N7	-	+	+	nt	+	-	+	I	-	-
N8	-	+	+	nt	+	-	-	I	+	+
N9	+	+	+	nt	+	-	+	I	-	-
N10	-	+	+	+	+	-	+	I	-	-
N11	-	-	+	nt	+	-	+	I	-	-
N12	-	-	+	nt	+	-	-	I	+	-
N13	+	+	+	nt	+	-	+	I	+	-
N14	+	-	+	nt	+	-	+	I	-	-
N15	+	+	+	+	+	-	+	I	+	-
N16	-	-	+	nt	+	-	+	I	-	-
N17	-	-	+	nt	+	-	+	I	-	-
N18	+	+	+	nt	+	-	+	I	-	-
N19	+	+	+	nt	+	-	+	I	+	-
N20	+	+	+	+	+	-	+	I	-	-

I = Inhibition of PCR reaction BAC = Bacteriology nt=not tested

Table 5.3 PCR results of control samples in comparison with bacteriological results

	Lab 1		Lab 5		Lab 10		Lab 11		Lab 13	
	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR
C1	+	+	+	nt	+	+	+	+	+	+
C2	+	+	+	nt	+	+	+	+	+	+
C3	+	+	+	nt	+	+	+	+	+	+
C4	+	+	+	nt	+	+	+	+	+	+
C5	-	-	-	nt	-	-	-	-	-	-
C6	-	-	-	nt	-	-	-	-	-	-
C7	+	+	+	nt	+	+	+	+	+	+
C8	+	+	+	nt	+	+	+	+	+	+
C9	+	+	+	nt	+	+	+	+	+	+
C10	+	+	+	nt	+	+	+	+	+	+
C11	-	-	-	nt	-	-	-	-	-	-
C12	-	-	-	-	-	-	-	-	-	-

BAC = Bacteriology nt=not tested

Appendix 6 Protocol

BACTERIOLOGICAL COLLABORATIVE STUDY VI (2002)
ORGANISED BY CRL *SALMONELLA*

PROTOCOL

Introduction

The Community Reference Laboratory (CRL) *Salmonella* organises the sixth bacteriological collaborative study on the methods for the detection of *Salmonella* amongst the National Reference Laboratories (NRLs). In the first and second collaborative study (October 1995 and October 1996 respectively) no significant difference was found between the routine method and the reference method for the individual laboratories. The number of positive isolations was on average significantly lower with selenite/cystine compared to Rappaport-Vassiliadis (RV) as selective enrichment medium. In the third study the ability of the laboratories to detect different contamination levels of *Salmonella* in the presence of competitive organisms was tested. Some laboratories used a semi solid medium for selective enrichment. These media seemed to be superior to RV as selective enrichment, especially for the detection of *Salmonella* Enteritidis (SE). Therefore, in the fourth collaborative study all laboratories used Modified Semi solid Rappaport Vassiliadis (MSRV) as selective enrichment next to RV(S). In this study significantly better results were revealed with MSRV compared to RV. Results obtained with RVS were not significantly different from the results obtained with MSRV.
The revised ISO 6579 will prescribe Muller Kauffmann Tetra Thionate + novobiocin (MKTTn) and Rappaport-Vassiliadis Soy (RVS) instead of RV. BGA as well as XLD will be used in this study as an isolation medium (see Workshop 2000). Laboratories that are interested can perform PCR on the samples.

For the performance of this study Reference Materials (RMs) produced by the CRL and poultry faeces will be used. The RMs consist of gelatin capsules containing sublethally injured *Salmonella* Typhimurium (STM) or SE.
Each laboratory will examine 25 faeces samples (10 g each) in combination with a capsule containing STM or SE and 10 control samples (no faeces added to the capsule). Next to the capsules, 20 faeces samples (25 g each) which are naturally contaminated with *Salmonella* will be examined (no addition of capsules). The results will be evaluated by the CRL.

Objectives

The main objective of the sixth 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 newly adapted ISO 6579 method and optionally the routine method of a laboratory.

Each participant will receive a parcel containing:

- 25 numbered vials; each containing one *Salmonella* Typhimurium or one *Salmonella* Enteritidis capsule;
- 10 control vials; each containing one capsule with or without *Salmonella*;
- 300 g of frozen poultry faeces (free from *Salmonella*).
- 550 g of naturally contaminated (with *Salmonella*) frozen faeces.

The performance of the study will be in week 46 (starting on 11 November 2002). All data will be reported in the test report and send to the CRL-*Salmonella* and will be used for (statistical) analysis.

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

Time table of bacteriological collaborative study VI (2002)

Week	Date	Topic
40	30 September - 4 October	Mailing of the protocol, standard operation procedure and test report to the NRLs
44	28 October - 1 November	In this week the airway bill number and other important information will be (e-)mailed to your institute.
45	4 - 8 November	Mailing of the parcels to the NRLs. The participants have to collect the parcel at the airport. The parcel will be mailed with cooling devices to keep the temperature low during transport and storage at the airport. Two cold chain monitors (for 10°C and 20°C) are included in the parcel (at the backside of the lid) 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 reference materials with the cold chain monitors in the cooling box. Immediately after arrival at the laboratory **store all materials at -20°C ± 5°C**. Before placing the materials in the freezer check the cold chain monitor again and *note in test report date, time, the colour of the different compartments and whether the complete compartment has become coloured.* If you did not receive the parcel before or at 8 November, do contact the CRL immediately. 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)
46	11 - 15 November	Performance of the study
48	25 - 29 November	Completion of the test report and faxing or e-mailing it to the CRL. The original test report will be sent to CRL.
50	9 - 13 December	Checking the results by the National Reference Laboratories.

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

Hans Korver or Edda van Raamsdonk (research assistants CRL)
P.O. Box 1
3720 BA Bilthoven
The Netherlands
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Appendix 7 Standard Operation Procedure

BACTERIOLOGICAL COLLABORATIVE STUDY VI (2002)
ORGANISED BY CRL *SALMONELLA*

STANDARD OPERATING PROCEDURE FOR THE 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 for *Salmonella* (CRL). Furthermore poultry faeces is used. The application of this SOP is limited to the bacteriological collaborative study for *Salmonella* described in this SOP.

2 References

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

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:

- *Salmonella*: micro-organisms which form typical colonies on isolation media for *Salmonella* and which display the serological and/or biochemical reactions described when tests are carried out in accordance with this SOP.

- *Detection of Salmonella*: detection of *Salmonella* from reference materials in the presence of competitive organisms, when the test is carried out in accordance with this SOP.

- *Reference Material*: a gelatine capsule containing a quantified amount 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 6579:2002(E).

5.1 Non selective pre-enrichment medium

- Buffered Peptone water (Annex B.1)

5.2 Selective enrichment medium

- Rappaport Vassiliadis medium with soya (RVS broth) (Annex B.2)
- Muller Kauffmann tetrathionate-novobiocin broth (MKTTn) (Annex B.3)
- Modified Semi solid Rappaport Vassiliadis (Newsletter, Vol.5, No.2, June 1999)
  This medium must be boiled to dissolve (instructions manufacturer). After boiling the medium must be transparent blue. After cooling down to 50°C the supplement or the novobiocine has to be added.
  The final concentration of the novobiocine in the medium should be 0.01 g/l. Plates should be poured with a volume of 15 to 20 ml.
- Selective enrichment medium routinely used in your laboratory

5.3 Solid selective media for first and second isolation

- Phenol red/brilliant green agar (Annex B.4, ISO 6579,1993)
  The medium must be boiled to dissolve (instructions manufacturer). After boiling the medium must be transparent red. Plates should be poured with a volume of 30-40 ml (140 mm-plates).
- Xylose-Lysine-Desoxycholate (Annex B.4)
  This medium must be boiled to dissolve (instructions manufacturer). After boiling the medium must be transparent red. Plates should be poured with a volume of 30-40 ml (140 mm-plates).
- Third medium (optionally) (Paragraph 4.4)

5.4 Confirmation media

Biochemical confirmation

- Triple sugar/iron agar (TSI agar) (Annex B.6)
- Urea agar (Annex B.7)
- l-Lysine decarboxylation medium (Annex B.8)
- Nutrient agar (optionally) (Annex B.5)

6 Apparatus and glassware

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

Apparatus

- Oven (for dry sterilisation) or autoclave (for wet sterilisation);
- Incubator, capable of operating at 37°C ± 1°C;
- Water bath, capable of operating at 41,5°C ± 1°C or incubator, capable of operating at 41,5°C ± 1°C;
- Water bath, capable of operating at 37°C ± 1°C;
- Loops;
- pH-meter; having an accuracy of calibration of ± 0.1 pH unit at 25°C.

Glassware

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

7 Procedure

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

7.1 Pre-enrichment

Allow the BPW to equilibrate to 37°C for better dissolving of the capsules.
Record in test report (page 3) the requested data of the BPW. Take the numbered vials with the *Salmonella* capsules and the control capsules out of the freezer one hour before they are added to the BPW, to allow them to equilibrate to room temperature. Label 25 jars containing 225 ml of BPW from 1 to 25. For the naturally contaminated samples number 20 jars of BPW from N1 to N20. Also label 12 jars of BPW from C1 to C12 (control capsules). One jar is a procedure control (= C11) to which no capsule or faeces is added and one jar is a negative faeces control to which only 10 gr. faeces is added (= C12). These control jars should further be handled in the same way as the other jars.

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

- **Add 10 grams of faeces from portion A to jars labelled 1-25 and C12,**
- **Add no faeces to jars labelled C1 - C11,**
- **Add 25 grams of faeces from portion B to jars labelled N1-N20.**

Do not shake the jars after adding the faeces.

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

If PCR is performed, fill in all requested data in the test report page 13. Results of PCR can be written in the test report Table 5 (page 26).

7.2 Selective enrichment

Allow the selective enrichment broths to equilibrate to room temperature, if they were stored at a lower temperature. Dry the surface of the MSRV plates in a Laminair Air Flow cabinet. Record (page 4-7) the requested data of the selective enrichment broths and MSRV plates in the test report. Label 25 jars/tubes/plates of each selective enrichment broth from 1 to 25. Also label 20 selective enrichment jars/tubes/plates from N1 to N20 and 12 jars/tubes/plates from C1 to C12. All selective media are incubated for 24 h and later on for another 24 h.

After equilibration:

- Transfer 0.1 ml of homogenised BPW culture to each tube containing 10 ml RVS medium. Incubate at 41.5°C ± 1°C for 24 h ± 3 h and later on another 24 h ± 3 h;
- Transfer 1 ml of homogenised BPW culture to each tube containing 10 ml MKTTn medium. Incubate at 37°C ± 1°C for 24 h ± 3 h and later on another 24 h ± 3 h;
- Inoculate the MSRV plates with three drops of BPW culture, with a total volume of 0.1 ml. Incubate **(not upside down)** at 41.5°C ± 1°C for 24 h ± 3 h and later on another 24 h ± 3 h;
- Inoculate the routinely used selective medium/media (other than those mentioned above), with the corresponding BPW culture (note the inoculation volume of BPW used and the volume of the selective medium/media on test report). Incubate at the temperature routinely used.

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

7.3 Isolation on media (first and second isolation)

Note: In the case that you do not have large dishes (140 mm) at your disposal use two small (90-100mm) dishes, one after the other, using the same loop.

Record in the test report (page 8-10) the requested data of the isolation media used. Label 25 large petri dishes of the isolation media from 1 to 25, label 20 large petri dishes from N1 to N20 and label 12 large petri dishes from C1 to C12.

First isolation after 24 h
Inoculation:
Inoculate, by means of a loop, from all selective enrichment cultures and from suspect MSRV plates, the surface of an isolation medium in a large size petri dish with the corresponding label number (see also *note* at the beginning of section 7.3). The following isolation media will be used:

1) Phenol red/brilliant green agar;
   Place the petri dishes with the bottom up in the incubator set at 37°C ± 1°C (record the temperature and time at the start and at the end of the incubation and other requested data in test report, page 8).

2) Xylose Lysine Desoxycholate agar
   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 9).

3) Optionally: selective isolation medium/media routinely used in your laboratory. Only if media used are different from those mentioned above.
   Incubate the medium/media at the temperature routinely used (record temperature and time and other requested data in test report, page 10).

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

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

7.4 Confirmation of colonies from first and 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). Store the plates at 5°C ± 3°C. Before biochemical confirmation, optionally, streak the typical colonies onto the surface of nutrient agar plates with the corresponding label numbers, in a manner which allows to develop well isolated colonies. Record on test report (page 11) the requested data of the nutrient agar. Incubate the inoculated plates at 37°C ± 1°C for 24 h ± 3h.

If the selected colony is not confirmed as *Salmonella*, test at maximum another 5 typical colonies. Report the number of colonies tested and the number of colonies confirmed as *Salmonella* for each dish in Table 1 (isolation using RVS), Table 2 (isolation using MKTTn), Table 3 (isolation using MSRV) and Table 4 (isolation using own enrichment) on test report page 14-25. For the results of detection of *Salmonella* using PCR fill in Table 4 on test report page 26.

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 in test report (page 11+12) the requested data of the media.

- TSI agar
- Urea agar
- l-Lysine decarboxylation medium
- Nutrient agar (optionally)

Interpretation of the biochemical tests
Salmonella generally show the reactions given in Table 1 of ISO 6579:2002(E) on page 9).

- TSI agar:
  Butt: -yellow by fermentation of glucose;
  -black by formation of hydrogen sulfide; and
  -bubbles or cracks due to gas formation from glucose
  Slant: -red or unchanged
- Urea agar: red to rose pink and later to deep cerise
- l-Lysine decarboxylation medium: coloured purple

8 Test report

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

Schedule of the adapted ISO 6579: 2002(E) method

Day	Topic	Description
1	**Pre-enrichment**	1 capsule to 225 ml BPW Do not shake 30 min. at 37°C Add 10 or 25 gr. faeces to BPW Incubate 16-20 h at 37°C
2	**Selective enrichment**	0.1 ml BPW culture in 10 ml RVS 1 ml BPW culture in 10 ml MKTTn 0.1 ml BPW culture on MSRV plate Other selective enrichment medi(um)(a) Incubate 24 h at 41.5°C
3	**First isolation after 24 h**	Inoculate from RVS, MKTTn, suspect MSRV plates and other medi(um)(a) ➢ phenol red/brilliant green agar ➢ Xylose Lysine Desoxycholate agar ➢ other selective medi(um)(a) Incubate 24 h at the specified temperature
3	**Continue selective enrichment**	Incubate RVS and MSRV medium (see day 2) another 24 hours at 41.5°C
4	**Second isolation after 24 h**	Inoculate from RVS, MKTTn,suspect MSRV plates and Other medi(um)(a) ➢ phenol red/brilliant green agar ➢ Xylose Lysine Desoxycholate agar ➢ other selective medi(um)(a) Incubate another 24 h at the specified temperature
4	**Biochemical confirmation**	Inoculate the media from first isolation media (day 3) for biochemical identification and incubate 24 h at the specified temperature
5	**Biochemical confirmation**	inoculate the media from second isolation media (day 4) for biochemical identification and incubate 24 h at the specified temperature

Appendix 8 Test report

TEST REPORT OF THE SIXTH (2002)

BACTERIOLOGICAL COLLABORATIVE STUDY

ORGANISED BY CRL *SALMONELLA*

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

Laboratory code	
Laboratory name	
Address	
Country	
Date of collecting the parcel	………… - ……………………….. - 2002
Starting date testing	………… - ……………………….. - 2002

Is your laboratory accredited or certified for the determination of *Salmonella*. If yes, according to which system ? If no, are you planning to be accredited or certified in the near future ?	

SHIPMENT

Cold chain monitor:	
Check at airport:	date : ………….. - ………..………2002 time : ………….. h ………………… min
Parcels damaged	☐ YES ☐ NO
Colour of compartment 10°C Brief ☐ completely coloured ☐ partly coloured ☐ white Moderate ☐ completely coloured ☐ partly coloured ☐ white Prolonged ☐ completely coloured ☐ partly coloured ☐ white	Colour of compartment 20°C Brief ☐ completely coloured ☐ partly coloured ☐ white Moderate ☐ completely coloured ☐ partly coloured ☐ white Prolonged ☐ completely coloured ☐ partly coloured ☐ white
Check at laboratory	date : ………….. - ………..………2002 time : ………….. h ………………… min
Parcels damaged	☐ YES ☐ NO
Colour of compartment 10°C Brief ☐ completely coloured ☐ partly coloured ☐ white Moderate ☐ completely coloured ☐ partly coloured ☐ white Prolonged ☐ completely coloured ☐ partly coloured ☐ white	Colour of compartment 20°C Brief ☐ completely coloured ☐ partly coloured ☐ white Moderate ☐ completely coloured ☐ partly coloured ☐ white Prolonged ☐ completely coloured ☐ partly coloured ☐ white

PRE-ENRICHMENT – Buffered Peptone Water (BPW)

!!!!! Record temperatures and times outside the range as indicated in the SOP on page 27 !!!!!

Manufacturer of the BPW	
Name	
Code number	
Batch number	
Expire date	
pH of the BPW :……………………………..	Measured at …………………………… °C

Incubation time and temperature for dissolving the capsules	
At the start	time: …………... h ……………….……… min temperature incubator: ………………..…… °C temperature of BPW at the start: ☐ Room temperature ☐ 37°C
At the end	time: …………… h …………………… min temperature incubator: …………...…… °C

Incubation time and temperature for pre-enrichment	
At the start	time: ………….. h …………………..…… min temperature incubator: ……………..…… °C
At the end	time: ………….. h …………………..…… min temperature incubator: ……………..…… °C

SELECTIVE ENRICHMENT - Rappaport Vassiliadis Soya medium (RVS)

!!!!! Record temperatures and times outside the range as indicated in the SOP on page 27 !!!!!

Manufacturer of the RVS medium	
Name	
Code number	
Batch number	
Expire date	
pH of broth :……………………………..	Measured at ……………………………. °C

Specific data of composition of RVS medium. What is the concentration of the following compounds:	
Soya Peptone	
Sodium chloride	
Potassium dihydrogen phosphate	
Dipotassium hydrogen phosphate	
Magnesium chloride anhydrous	
Magnesium chloride.6H_2O	
Malachite green	

Incubation time and temperature for selective enrichment	
At the start of the first period	time: ………….. h ……………….….… min temperature incubator: ………………..…… °C
At the end of the first period	time: ………….. h ……………….….… min temperature incubator: ………………..…… °C
At the start of the second period	time: ………….. h ……………….….… min temperature incubator: ………………..…… °C
At the end of the second period	time: ………….. h ……………….….… min temperature incubator: ………………..…… °C

SELECTIVE ENRICHMENT -	**Muller Kauffmann Tetra Thionate + novobiocin (MKTTn)**

!!!!! Record temperatures and times outside the range as indicated in the SOP on page 27 !!!!!

Manufacturer of the MKTTn medium	
Name	
Code number	
Batch number	
Expire date	
pH of the broth :……………………………..	Measured at ……………………………. °C

Specific data of composition of MKTTn medium. What is the concentration of the following compounds:	
Enzymatic digest of meat extract	
Enzymatic digest of casein	
Sodium chloride (NaCl)	
Calcium carbonate ($CaCO_3$)	
Sodium thiosulfate pentahydrate ($Na_2S_2O_3.5H_2O$)	
Ox bile for bacteriological use	
Brilliant green	

Incubation time and temperature for selective enrichment	
At the start of the first period	time: ………….. h …………………..…… min temperature incubator: ………………..…… °C
At the end of the first period	time: ………….. h …………………..…… min temperature incubator: ………………..…… °C
At the start of the second period	time: ………….. h …………………..…… min temperature incubator: ………………..…… °C
At the end of the second period	time: ………….. h …………………..…… min temperature incubator: ………………..…… °C

SELECTIVE ENRICHMENT -	**Modified Semi solid Rappaport Vassiliadis medium (MSRV)**

!!!!! Record temperatures and times outside the range as indicated in the SOP on page 27 !!!!!

How much samples did you test in 2000 and 2001 using MSRV as selective enrichment	
2000:…………………………………………	2001:…………………………………………

Manufacturer of the MSRV medium	
Name	
Code number	
Batch number	
Expire date	
pH of the broth :……………………………..	Measured at ……………………………^{o}C

Incubation time and temperature for selective enrichment	
At the start of the first period	time: ………….. h ………………..…… min temperature incubator: ………………..…… °C
At the end of the first period	time: ………….. h ………………..…… min temperature incubator: ………………..…… °C
At the start of the second period	time: ………….. h ………………..…… min temperature incubator: ………………..…… °C
At the end of the second period	time: ………….. h ………………..…… min temperature incubator: ………………..…… °C

SELECTIVE ENRICHMENT - Selective medium, routinely used in your laboratory

!!!!! Record temperatures and times outside the range as indicated in the SOP on page 27 !!!!!

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 :……………………………..	Measured at …………………………….°C
Volume of the medium per jar/tube in ml	
Inoculation volume of BPW	
Prescribed incubation temperature in °C	

Incubation time and temperature for selective enrichment	
At the start of the first period	time: ………….. h ………………..…… min temperature incubator: ………………..…… °C
At the end of the first period	time: ………….. h ………………..…… min temperature incubator: ………………..…… °C
At the start of the second period	time: ………….. h ………………..…… min temperature incubator: ………………..…… °C
At the end of the second period	time: ………….. h ………………..…… min temperature incubator: ………………..…… °C

FIRST AND SECOND ISOLATION - Phenol red/brilliant green agar (BGA)

!!!!! Record temperatures and times outside the range as indicated in the SOP on page 27 !!!!!

Manufacturer of the phenol red/brilliant green agar	
Name	
Code number	
Batch number	
Expire date	
pH of the broth:…………………………….	Measured at ………………………………^{o}C
Volume of the medium per jar/tube in ml	
Inoculation volume of BPW	
Prescribed incubation temperature in ^{o}C	

Size of petri dishes			
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

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

!!!!! Record temperatures and times outside the range as indicated in the SOP on page 27 !!!!!

Manufacturer of the XLD medium	
Name	
Code number	
Batch number	
Expire date	
pH of the broth :……………………………..	Measured at …………………………….°C

Size of petri dishes			
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

FIRST AND SECOND ISOLATION - Isolation medium routinely used in your lab.

!!!!! Record temperatures and times outside the range as indicated in the SOP on page 27 !!!!!

If you use more selective media, please write these on an annex.	
Name of the medium	
Prescribed incubation temperature in °C	

Manufacturer of this medium	
Name	
Code number	
Batch number	
Expire date	
pH of the broth :……………………………..	Measured at …………………………….°C

Size of petri dishes			
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

CONFIRMATION – Nutrient agar

!!!!! Record temperatures and times outside the range as indicated in the SOP on page 27 !!!!!

Manufacturer of the nutrient agar	
Name	
Code number	
Batch number	
Expire date	
pH of the broth:……………………………..	Measured at……………………………….°C

Size of petri dishes			
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

BIOCHEMICAL CONFIRMATION

Manufacturer of the TSI agar	
Name	
Code number	
Batch number	
Expire date	
pH of the broth:……………………………..	Measured at………………………………^{o}C

Manufacturer of the urea agar	
Name	
Code number	
Batch number	
Expire date	
pH of the broth:……………………………..	Measured at………………………………^{o}C

Manufacturer of the l-Lysine decarboxylation medium	
Name	
Code number	
Batch number	
Expire date	
pH of the broth:……………………………...	Measured at………………………………^{o}C

Manufacturer of other confirmation tests - …………………………………………………	
Name	
Code number	
Batch number	
Expire date	
pH of the broth:……………………………...	Measured at………………………………^{o}C

DETECTION BY PCR

General questions	
Is the PCR used commercially available	□ Yes □ No
If yes, name of PCR, manufacturer and batch used in the study:	
Is the PCR validated	□ Yes □ No
How much samples did you test for *Salmonella* using this PCR in 2001 ?	
At what moment did you start with the extraction/detection?	before or after incubation of BPW
Volume of pre-enrichment used for extraction	
Volume of DNA-sample obtained from extraction	
Volume of DNA-sample added to PCR-mixture	

Composition of PCR-mixture		
Compound	Volume per sample	Manufacturer and batch of specific compound

Name of thermocycler	
Write down the cycles	
What kind of detection system is used	

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

	RV 24 hours						RV 48 hours					
	BGA		XLD		Third medium		BGA		XLD		Third medium	
no.	Col [a]	Sal [b]	Col	Sal	Col	Sal	Col	Sal	Col	Sal	Col	Sal
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												
13												
14												
15												
16												
17												
18												
19												
20												
21												
22												
23												
24												
25												

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

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

	RV 24 hours						RV 48 hours					
	BGA		XLD		third medium		BGA		XLD		third medium	
no.	Col [a]	Sal [b]	Col	Sal	Col	Sal	Col	Sal	Col	Sal	Col	Sal
N1												
N2												
N3												
N4												
N5												
N6												
N7												
N8												
N9												
N10												
N11												
N12												
N13												
N14												
N15												
N16												
N17												
N18												
N19												
N20												

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

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

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

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

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

	RV 24 hours						RV 48 hours					
	BGA		XLD		Third medium		BGA		XLD		Third medium	
no.	Col [a]	Sal [b]	Col	Sal	Col	Sal	Col	Sal	Col	Sal	Col	Sal
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												
13												
14												
15												
16												
17												
18												
19												
20												
21												
22												
23												
24												
25												

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

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

	RV 24 hours						RV 48 hours					
	BGA		XLD		third medium		BGA		XLD		third medium	
no.	Col [a]	Sal [b]	Col	Sal	Col	Sal	Col	Sal	Col	Sal	Col	Sal
N1												
N2												
N3												
N4												
N5												
N6												
N7												
N8												
N9												
N10												
N11												
N12												
N13												
N14												
N15												
N16												
N17												
N18												
N19												
N20												

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

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

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

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

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

	MSRV 24 hours						MSRV 48 hours					
	BGA		XLD		Third medium		BGA		XLD		Third medium	
no.	Col [a]	Sal [b]	Col	Sal	Col	Sal	Col	Sal	Col	Sal	Col	Sal
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												
13												
14												
15												
16												
17												
18												
19												
20												
21												
22												
23												
24												
25												

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

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

	MSRV 24 hours						MSRV 48 hours					
	BGA		XLD		Third medium		BGA		XLD		Third medium	
no.	Col [a]	Sal [b]	Col	Sal	Col	Sal	Col	Sal	Col	Sal	Col	Sal
N1												
N2												
N3												
N4												
N5												
N6												
N7												
N8												
N9												
N10												
N11												
N12												
N13												
N14												
N15												
N16												
N17												
N18												
N19												
N20												

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

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

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

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

Table 4: Results of isolation using own enrichment (dish numbers 1-25)

	Own enrichment 24 hours						Own enrichment 48 hours					
	BGA		XLD		Third medium		BGA		XLD		Third medium	
no.	Col [a]	Sal [b]	Col	Sal	Col	Sal	Col	Sal	Col	Sal	Col	Sal
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												
13												
14												
15												
16												
17												
18												
19												
20												
21												
22												
23												
24												
25												

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

Table 4 (continued): Results of isolation using own enrichment (dish numbers N1-N20)

	Own enrichment 24 hours						Own enrichment 48 hours					
	BGA		XLD		Third medium		BGA		XLD		Third medium	
no.	Col [a]	Sal [b]	Col	Sal	Col	Sal	Col	Sal	Col	Sal	Col	Sal
N1												
N2												
N3												
N4												
N5												
N6												
N7												
N8												
N9												
N10												
N11												
N12												
N13												
N14												
N15												
N16												
N17												
N18												
N19												
N20												

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

Table 4 (continued): Results of isolation using own enrichment (dish numbers C1-C12)

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

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

Table 5: Results of detection using PCR (dish numbers 1-25)

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

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

Name of person carrying out the sixth bacteriological collaborative study	
Date and signature	

Name of person in charge	
Date and signature	