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EU Interlaboratory comparison study VIII (2004) on bacteriological detection of *Salmonella* spp.

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Abstract

EU Interlaboratory comparison study VIII (2004) on bacteriological detection of *Salmonella* spp.

In 2004 the eighth interlaboratory comparison study on bacteriological detection of *Salmonella* spp. was organized by the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*, Bilthoven, the Netherlands). National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*) of the EU Member States (26), the NRLs of Norway and of Romania 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* Enteritidis) were tested. 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 4 h and 18 h incubation of the samples in the pre-enrichment broth Buffered Peptone Water (BPW), followed by selective enrichment on Modified Semi-solid Rappaport Vassiliadis (MSRV) and plating-out on Xylose Lysine Deoxycholate agar (XLD).
Significant more positive isolations were obtained from the artificially contaminated samples (negative chicken faeces, artificially contaminated with reference materials) after 18 h of incubation in BPW. The accuracy rates for the artificially contaminated samples were 49% and 77% after respectively 4 and 18 h of incubation in BPW. The overall results for the naturally contaminated samples revealed significant more positive results after 4 h of incubation. The accuracy rates for these samples were respectively 81% and 56 % after 4 and 18 h of incubation in BPW.

Keywords: CRL-*Salmonella, Salmonella*, interlaboratory comparison, reference materials, detection methods.

Rapport in het kort

EU Ringonderzoek VIII (2004) voor bacteriologische detectie van *Salmonella* spp.

In 2004 werd door het Communautair Referentie Laboratorium voor *Salmonella* (CRL-*Salmonella*, Bilthoven, Nederland) het achtste bacteriologische ringonderzoek georganiseerd. Deelnemers aan de studie waren de Nationale Referentie Laboratoria voor *Salmonella* (NRL's-*Salmonella*) van de EU lidstaten (26), van Noorwegen en van Roemenië. Referentiematerialen in combinatie met of zonder de aanwezigheid van kippenfeces, evenals natuurlijk besmette feces (bevattende *Salmonella* Enteritidis) werden getest. De referentiematerialen bestonden uit gelatine capsules met verschillende besmettingsniveaus van *Salmonella* Typhimurium (STM), *Salmonella* Enteritidis (SE) en *Salmonella* Panama (SPan). Bovendien werd naast de uitvoering van de testen door de laboratoria een vergelijking gemaakt tussen 4 en 18 uur voorophoping van de monsters in gebufferd Pepton Water (BPW), gevolgd door selectieve ophoping op Modified Semi-solid Rappaport Vassiliadis en uitplating op Xylose Lysine Deoxycholate agar.
Significant meer positieve isolaties werden gevonden met de kunstmatig besmette monsters (negatieve kippenfeces, kunstmatig besmet met referentiematerialen) na 18 uur incubatie in BPW. De waardes voor nauwkeurigheid ("accuracy rates") van de kunstmatig besmette monsters waren 49% en 77% na respectievelijk 4 en 18 uur incubatie in BPW. De totale resultaten van de natuurlijk besmette monsters lieten significant meer positieve isolaties zien na 4 uur incubatie. De waardes voor nauwkeurigheid ("accuracy rates") voor deze monsters waren respectievelijk 81% en 56% na 4 en 18 uur incubatie in BPW.

Trefwoorden: CRL-*Salmonella, Salmonella*, ringonderzoek, referentie materialen, detectie methoden.

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Summary

In fall 2004 the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*) organized the eighth interlaboratory comparison study on bacteriological detection of *Salmonella*. Participants were the twenty-six National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*) of the EU Member States, the NRL from Norway and the NRL from Romania.
The main objective of the eighth interlaboratory comparison 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. In addition to the performance testing of the laboratories, a comparison was made between 4 h and 18 h incubation of the samples in the pre-enrichment broth Buffered Peptone Water (BPW), followed by selective enrichment on Modified Semi-solid Rappaport Vassiliadis (MSRV) and plating-out on Xylose Lysine Deoxycholate agar (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 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: 7 capsules with *ca* 10 colony forming particles (cfp) of *Salmonella* Typhimurium (STM10), 4 capsules with *ca* 100 cfp *S.* Typhimurium (STM100), 7 capsules with *ca* 100 cfp *S.* Enteritidis (SE100), 4 capsules with *ca* 500 cfp *S.* Enteritidis (SE500) and 3 blank capsules. The other 10 capsules, to which no faeces had to be added, were control samples, existing of 3 capsules with *ca* 10 cfp *S.* Typhimurium, 2 capsules with *ca* 100 cfp *S.* Enteritidis, 1 capsule with *ca* 500 cfp *S.* Enteritidis, 2 capsules with *ca* 5 cfp *S.* Panama and 2 blank capsules. Beside the reference materials, also 20 chicken faeces samples (10 g each) naturally contaminated with *Salmonella* Enteritidis were examined.
One laboratory did not test the prescribed medium combinations, which made comparison of their results with the other NRLs impossible. Five laboratories scored systematically below the average results of all laboratories for the artificially contaminated samples for both medium combinations. Four laboratories scored systematically below the average results for the naturally contaminated samples.
Significant more positive isolations were obtained from the artificially contaminated samples (negative chicken faeces, artificially contaminated with reference materials) after 18 h of incubation in BPW. The accuracy rates for the artificially contaminated samples were 49% and 77% after respectively 4 and 18 h of incubation in BPW. The overall results for the naturally contaminated samples revealed significant more positive results after 4 h of incubation. The accuracy rates for these samples were respectively 81% and 56 % after 4 and 18 h of incubation in BPW.

List of abbreviations

BGA	Brilliant Green Agar
BPLS and BPLSA	Brilliant Green Phenol-Red Lactose Sucrose agar
BPW	Buffered Peptone Water
cfp	colony forming particles
CRL	Community Reference Laboratory
dPCA	Double concentrated Plate Count Agar
dVRBG	Double concentrated Violet Red Bile Glucose agar
hcmp	Highly Contaminated Milk Powder
ISO	International Standardisation Organisation
LDC	Lysine Decarboxylase
MK	Mueller Kauffmann
MKTTn	Mueller Kauffmann Tetrathionate novobiocin broth
MLCB	Mannitol Lysine Crystal violet Brilliant green agar
MSRV	Modified Semi-solid Rappaport Vassiliadis
NRL	National Reference Laboratory
PCR	Polymerase Chain Reaction
RIVM	Rijks Instituut voor Volksgezondheid en het Milieu (National Institute for Public Health and the Environment.
RM	Reference Material
RV	Rappaport Vassiliadis
RVS	Rappaport Vassiliadis Soya broth
SC	Sub Committee
SE	*Salmonella* Enteritidis
SOP	Standard Operation Procedure
SPan	*Salmonella* Panama
STM	*Salmonella* Typhimurium
TC	Technical Committee
TSI	Triple Sugar Iron agar
UA	Urea Agar
XLD	Xylose Lysine Deoxycholate agar
XLT4	Xylose Lysine Tergitol 4 agar

1. Introduction

In pursuance of the Directive 2003/99/EC, which replaced the Council Directive 92/117/EEC, the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*) organizes bacteriological interlaboratory comparison studies with the objective that the examination of samples in the EU Member States is carried out uniformly and that comparable results should be obtained by all National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*).
Earlier studies (see Annex 1) have shown significantly better results when using Modified Semi-solid Rappaport-Vassiliadis with novobiocin (MSRV) compared to the use of Rappaport-Vassiliadis broth (RV) as selective enrichment. Since the fourth study, all laboratories used the selective enrichment medium MSRV, in addition to RV or RVS (Rappaport-Vassiliadis Soya broth). In 2002 a new version of ISO 6579 was published. In this ISO the selective broths Mueller Kaufmann Tetrathionate with novobiocin (MKTTn) and RVS are prescribed. Furthermore, this ISO prescribes Xylose Lysine Deoxycholate (XLD) as the plating out agar. In the studies of 2002 and 2003 these media were also prescribed to analyse the samples.
Also the 2002 version of ISO 6579 is mainly intended for the detection of *Salmonella* spp. in food and feeding stuff and is less appropriate for the detection of *Salmonella* spp. in animal faeces. It was therefore requested at ISO/TC34/SC9 (Subcommittee dealing with microbiology under Technical Committee Food and Feeding stuff) to standardise the detection of *Salmonella* spp. in animal faeces. A draft proposal including MSRV as selective enrichment was sent to the secretariat of ISO/TC34/SC9 in 2004. It was proposed to prepare a new annex to ISO 6579 (Annex D) which would describe the procedure of *Salmonella* spp. in animal faeces.
In the present study of 2004, the media MSRV and XLD as mentioned in this draft Annex D of ISO 6579 are prescribed. In a report of Heuvelman and in ‘t Veld (1998) it was described that a shorter incubation time of chicken faeces in BPW (4-7 h) would reveal more positive results. As different experiments at CRL-*Salmonella* and also earlier interlaboratory comparison studies did not always show the expected number of positive results, it was decided to try in this interlaboratory comparison study a short incubation time of BPW (4 h) beside the “normal” incubation time (18 h).
Ten control samples containing different reference materials had to be tested without the addition of chicken faeces. These reference materials consisted of 3 capsules with *ca* 10 cfp *Salmonella* Typhimurium (STM10), 2 capsules with *ca* 100 cfp *Salmonella* Enteritidis (SE100), 1 capsule with *ca* 500 cfp *Salmonella* Enteritidis (SE500), 2 capsules with *ca* 5 cfp *Salmonella* Panama (SPan5) and 2 blank capsules. Blank capsules were also tested without the addition of chicken faeces. Twenty-five samples of *Salmonella* negative chicken faeces spiked with four different reference materials had to be examined including blank capsules. The four different reference materials consisted of two levels of *Salmonella* Typhimurium

(STM10 and STM100) and two levels of *Salmonella* Enteritidis (SE100 and SE500).
Furthermore, 20 naturally contaminated samples of chicken faeces containing *Salmonella* Enteritidis were also examined by using the same medium combinations [BPW (4 h) / MSRV / XLD and BPW (18 h) / MSRV / XLD].

2. Participants

Country	City	Institute
Austria	Graz	Institut für Medizinische Mikrobiologie und Hygiene, Nationale Referenzzentrale für Salmonellen
Belgium	Brussels	Veterinary and Agrochemical Research Center (VAR)
Cyprus	Nicosia	Cyprus Veterinairy Services, Laboratory for the Control of Foods of Animal Origin (LCFAO)
Czech Republic	Prague	State Veterinary Institute
Denmark	Copenhagen	Danish Veterinary Laboratory
Estonia	Tartu	Estonian Veterinary and Food Laboratory, Diagnostic Department
Finland	Kuopio	National Veterinary and Food Research Institute, Kuopio Department
France	Ploufragan	Agence Française de Sécurité Sanitaire des Aliments (AFSSA) Laboratoire d'Etudes et de Recherches Avicoles et Porcines (LERAP)
Germany	Berlin	Federal Institute for Risk Assessment (BFR) National Salmonella Reference Laboratory
Greece	Halkis	Veterinary Laboratory of Halkis
Hungary	Budapest	National Food Investigation Institute
Ireland	Dublin	Department of Agriculture and Food Central Veterinary Research Laboratory
Italy	Venice	Istituto Zooprofilattico Sperimentale delle Venezie, Centro Nazionale di Referenza per le Salmonellosi
Latvia	Riga	State Veterinary Medicine Diagnostic Centre
Lithuania	Vilnius	National Veterinary Laboratory
Luxembourg	Luxembourg	Laboratoire de Médecine Vétérinaire de l'Etat , Animal Zoonosis
Malta	Marsa	Food and Veterinary Regulatory Division, Ministry of Rural Affairs and the Environment
The Netherlands	Bilthoven	Rijksinstituut voor Volksgezondheid en Milieu (RIVM)
Norway	Oslo	National Veterinary Institute, Section of Bacteriology
Poland	Pulawy	National Veterinary Research Institute Department of Microbiology

Country	City	Institute
Portugal	Lisbon	Laboratório Nacional de Investigaçã Veterinária
Romania	Bucharest	Institutul de diagnostic si Sanatate Animala
Slovak Republic	Bratislava	State Veterinary and Food Institute
Slovenia	Ljubljana	National Veterinary Institute, Veterinary Faculty
Spain	Madrid	Laboratorio de Sanidad Y Produccion Animal de Algete
Sweden	Uppsala	National Veterinary Institute, Department of Bacteriology
United Kingdom	Addlestone	Veterinary Laboratories Agency , Department of Bacterial Diseases, New Haw
United Kingdom	Belfast	Department of Agriculture for Northern Ireland, Veterinary Sciences Division, Bacteriology Department,

3. Materials and Methods

3.1 Reference materials

Five batches of reference materials were prepared. For this purpose milk, artificially contaminated with a *Salmonella* strain was spray-dried (In ‘t Veld *et al.*, 1996). The obtained highly contaminated milk powder (hcmp) was mixed with sterile (γ-irradiated) milk powder (Carnation, Nestlé, the Netherlands) to obtain the desired contamination level. The mixed powder was filled in gelatin capsules resulting in the final reference materials (RMs).
The target levels of the five batches of RMs were:

- 5 colony forming particles (cfp) per capsule for *Salmonella* Panama (SPan5);
- 10 and 100 colony forming particles (cfp) per capsule for *Salmonella* Typhimurium (STM10 and STM100);
- 100 and 500 colony forming particles (cfp) per capsule for *Salmonella* Enteritidis (SE100 and SE500).

Before filling the mixed powders into gelatin capsules, test batches capsules were prepared of each mixture to determine the mean number of cfp per capsule and the homogeneity of the mixture. The remaining mixed powders were stored at –20 ^{o}C. If the test batch fulfilled the pre-set criteria for contamination level and homogeneity, the relevant mixed powders were filled into gelatin capsules and stored at -20 ^{o}C. For the preparation of the STM10 and STM100 capsules the remaining of the mixed powder of the study of 2003 were used.
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 should be $T_2/(I-1) \leq 2$, where T_2 is a measure for the variation between capsules of one batch (see formula in Annex 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 ± 1) ^{o}C for (45 ± 5) minutes;
- repair of *Salmonella* by the addition of 5 ml molten double concentrated plate count agar (dPCA) to the reconstituted capsule solution, and after solidification incubation at (37 ± 1) ^{o}C for (4 ± ½) h;
- after incubation, 10 ml of molten double concentrated Violet Red Bile Glucose agar (dVRBG) was added as an overlayer and after solidification the plates were incubated for (20 ± 2) h at (37 ± 1) ^{o}C.

3.2 Faecal samples

3.2.1 General

Chicken faeces was obtained from poultry laying flocks. The faeces were tested for the presence or absence of *Salmonella* spp. For this purpose 10 portions of 10 g were each added to 90 ml BPW. After pre-enrichment at 37 °C for 16-18 h, selective enrichment was carried out on MSRV. Next, the cultures were plated-out on BGA and confirmed biochemically and serologically.
The suspected colonies of the positive faeces were isolated on TSI agar and sent for serotyping to the Diagnostic Laboratory for Infectious Diseases and Perinatal Screening (LIS/RIVM). 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 faecal samples (*Salmonella* negative as well as *Salmonella* positive faeces) were mixed and homogenized with sterilised glycerol/peptone solution (mixing ratio 1:1). One liter of this solution consisted of 300 ml glycerol, 7 gram of peptone and 700 ml distilled water. After mixing all faeces samples with the peptone/glycerol solution, they were again analysed for the presence or absence of *Salmonella* and were stored until sending the samples to the National Reference Laboratories for *Salmonella* (-20 ± 5 °C).

3.2.2 MPN of *Salmonella* in naturally contaminated faeces

To semi-quantify the number of Salmonellae in the *Salmonella* positive faeces, a Most Probable Number (MPN) method was used. For this purpose, ten gram of faeces was added to 90 ml of buffered peptone water (BPW) in a plastic bag and mixed by using a Stomacher (60 seconds for each sample). Next tenfold dilutions were prepared in BPW until a concentration of 0.01 mg faeces per 100 ml BPW. This procedure was repeated five times. The BPW jars with concentrations of 1000 mg till 0.01 mg faeces (per 100 ml BPW) were incubated and handled according to the same standard operating procedure as all other samples in this study with medium combination MSRV/BGA. After completion of the test the MPN was calculated using a complementary log-log link in SAS.Proc logistic (SAS Institute Inc, 2004).

3.2.3 Total bacterial count in faeces

For the naturally contaminated faeces with *Salmonella* as well as the negative faeces without *Salmonella* the total number of aerobic bacteria was investigated. The procedure of ISO 4833 (Anonymous, 2003) was used for this purpose. Portions of 10 gram chicken faeces were homogenized into 90 ml peptone saline solution in a plastic bag. The content was mixed by using a stomacher (60 sec). Next tenfold dilutions were prepared in peptone saline solution. Two times one ml of each dilution was brought into 2 empty Petri-dishes (diameter 9 cm). To these two dishes 25 ml of molten Plate Count Agar (PCA) was added. These plates were incubated at (30 ± 1) ^{o}C for (72 ± 3) h for the enumeration of the total number of aerobic bacteria.

3.2.4 Stability test

To test the possible influence of transport times and temperatures on the number of *Salmonella* spp. and on the background flora in the positive faeces samples a stability study was carried out. For this purpose portions of faeces samples mixed with peptone/glycerol solution were stored at -20 ^{o}C, +5 ^{o}C and +20 ^{o}C. The number of *Salmonella* and the total aerobic count were determined on days 0, 2, 7 and 14 according to the procedures as mentioned under 3.2.2 and 3.2.3.

3.3 Design of the interlaboratory comparison study

3.3.1 Samples

Two weeks before the study the reference materials (35 individually numbered capsules) and 300 grams of negative faeces and 250 grams of positive faeces for *Salmonella* were mailed (with cooling devices) as diagnostic specimens by courier service to the participants. After arrival at the laboratory the capsules and faecal samples had to be stored at –20 ^{o}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 (Annex 7) and Standard Operation Procedure (Annex 8). The testreport which was used during the study can be found at the CRL-*Salmonella* website: http://www.rivm.nl/crlsalmonella/collabstudies/detection.html.
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 these artificially contaminated samples, also 20 samples (numbered N1 – N20) of 10 grams each of naturally contaminated faeces samples (with *Salmonella* Enteritidis) were analysed. The types and the number of capsules and faeces samples to be tested are shown in Table 1.

Table 1 Overview of the types and the number of the capsules to be tested per laboratory in the interlaboratory comparison study

Capsules	Control capsules (n = 10) No faeces added	Test samples (n=25) with 10 g *Salmonella*-negative faeces	Test samples (n=20) with 10 g *Salmonella*-positive faeces
S. Panama 5	2	---	---
S. Enteritidis 100	2	7	---
S. Enteritidis 500	1	4	---
S. Typhimurium 10	3	7	---
S. Typhimurium 100	---	4	---
Blank	2	3	---
No capsules	---	---	20

3.3.2 Methods

During the workshop meeting at 13 and 14 May 2004 in Bilthoven (the Netherlands) it was decided that this interlaboratory comparison study would in principle have the same set-up as study IV, V and VI. Differences were the number of media to be used (less than in former studies) and two incubation times of the pre-enrichment broth BPW (4 h and 18 h).
The following media were prescribed in this study VIII (see also Standard Operation Procedure in Annex 8):

Pre-enrichment in:
- Buffered Peptone Water (BPW): (beside incubation of 18 h also incubation of 4 h)

Selective enrichment on/in:
- Modified semi-solid Rappaport Vassiliadis medium (MSRV)
- Own selective enrichment medium (not compulsory)

Plating-out on:
- Xylose lysine desoxycholate agar (XLD)
- Second plating-out medium for choice (obligatory!)
- Own plating-out medium (not compulsory)

Biochemical confirmation:
- Urea, Triple Sugar Iron agar (TSI) and Lysine Decarboxylase (LDC)

Beside the prescribed methods the NRLs were also allowed to use their own methods. This could be different medium combinations and/or investigation of the samples with a Polymerase Chain Reaction based method.

3.3.3 Temperature recording during shipment

For the control of exposure to abusive temperatures during shipment and storage, so called micro temperature loggers were used to record the temperature during transport. These loggers are tiny sealed units in a 16 mm diameter and 6 mm deep stainless steel case. Each package contained one logger. The loggers were programmed by the CRL-*Salmonella* to measure the temperature every hour. Each NRL had to return the temperature recorder immediately after receipt of the parcel to the CRL. At the CRL-*Salmonella* the loggers were read via the computer and all data from the start of the shipment until the arrival at the National Reference Laboratories were transferred to an Excel graphic which shows all recorded temperatures.

Before sending the materials to the NRLs the temperature variation in the package was checked, as well as the time period before the content of the package would become equal to room temperature. This test package was packed in the same way as the packages to be sent to the NRLs.
Three biopacks and six cooling devices were placed in one large shipping box according to the drawing below (Figure 1). In each of the three biopacks, one temperature recorder was enclosed. One temperature recorder was attached to the outside on top of the large package. The cooling devices are drawn as grey cells. For biopack number three one cooling device was put underneath the pack and the other on top of it.

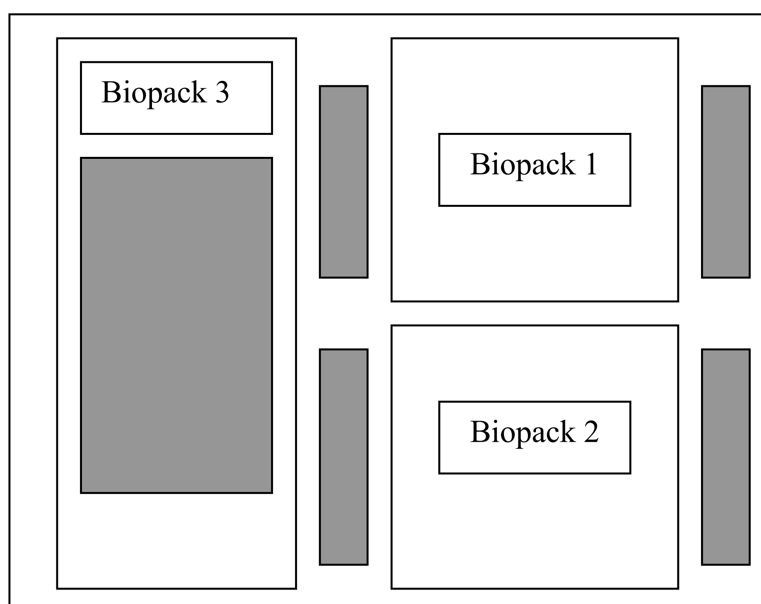

Figure 1 Lay-out of the experiment to control the temperature at storage

3.4 Accreditation/certification

Sixteen laboratories mentioned to be accredited for their quality system according to EN-ISO/IEC 17025 (labcodes 1, 2, 4, 6, 8, 9, 10, 12, 17, 19, 21, 23, 24, 26, 27 and 28). Four NRLs (labcodes 5, 16, 22 and 25) were accredited according to various quality systems like Cofrac and DAR. Two laboratories (labcodes 3 and 18) mentioned that they were not accredited nor certified to any system and mentioned no planning to do so in the near future. Six laboratories (labcodes 7, 11, 13, 14, 15 and 20) are planning to be accredited or certified in the near future.

3.5 Statistical analysis of the data

To be able to investigate the results of the participating laboratories the specificity, sensitivity and accuracy rates were calculated for the control samples, the artificially contaminated samples with faeces (negative for *Salmonella* spp.) as well as for the naturally contaminated samples.

The specificity, sensitivity and accuracy were calculated according to the following formulae:

$$\text{Specificity rate:} \quad \frac{\text{Number of negative results}}{\text{Total number of (expected) negative samples}} \times 100\ \%$$

$$\text{Sensitivity rate:} \quad \frac{\text{Number of positive results}}{\text{Total number of (expected) positive samples}} \times 100\ \%$$

$$\text{Accuracy rate:} \quad \frac{\text{Number of correct results (positive and negative)}}{\text{Total number of samples (positive and negative)}} \times 100\ \%$$

Further more a so-called mixed logistic model was used to analyse the differences between treatments (4h and 18h incubation of BPW) and laboratories.

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 2. All batches met the pre-set criteria as stated under 3.1. The enumerated minimum and maximum levels within each batch of capsules are also given in the table. For the preparation of the STM 10, STM 100 and the SPan 5 capsules the remaining of the mixed powders of the study of 2003 were used. Therefore no information of the test batches was available (see also Table 2). The final batches were tested twice; firstly immediately after preparing the batch and secondly at the time of the interlaboratory comparison study.

Table 2 Level of contamination and homogeneity of SE, SPan and STM capsules

	SE 100	SE 500	SPan 5	STM 10	STM 100
Test batch					
Date testing capsules	27-09-04	26-08-04			
Number of capsules tested	22	44			
Mean cfp per capsule	83	711			
Min-max cfp per capsule	58–102	470–1150			
T_2 / (I-1)	0.97	1.71			
Final batch; Test 1					
Date testing capsules	01-10-04	01-10-04	17-06-04	10-08-04	10-08-04
Number of capsules tested	25	25	46	50	50
Mean cfp per capsule	62	418	9	13	113
Min-max cfp per capsule	40 – 82	290-540	5-15	7-22	97-136
T_2 / (I-1)	1.09	0.79	0.97	0.66	0.89
Final batch; Test 2					
Date testing capsules	11-11-04	12-11-04	16-11-04	11-11-04	11-11-04
Number of capsules tested	23	25	25	23	19
Mean cfp per capsule	74	434	7	11	81
Min-max cfp per capsule	46 – 108	280-620	3-13	7-22	52-120
T_2 / (I-1)	1.92	1.85	0.56	1.32	1.54

cfp = colony forming particles;
min-max = enumerated minimumand maximum cfp;
formula T_2 see Annex 2; I is number of capsules;
Demand for homogeneity T_2 /(I-1) ≤ 2

4.2 Faecal samples

At the 29th of September 2004 the faeces samples were received at CRL-*Salmonella*. Before mixing the positive faeces with peptone/glycerol the number of *Salmonella* was determined by performing the MPN procedure (see 3.2.2) on 04-10-2004. The MPN result of this positive faeces was 2.4 x 10^3 cfp per gram (95% confidence interval: 0.8 – 5.5 x 10^3 cfp per gram). On 07-10-2004 the positive faeces was mixed with peptone/glycerol and stored at -20 °C. On 12-10-2004 the number of *Salmonella* was determined in the thawed samples of the mixed faeces using the MPN method. The MPN result was 8.7 x 10^3 cfp per gram (95% confidence interval: 2.2 – 20.3 x 10^3 cfp per gram).

In Table 3 the total number of aerobic bacteria is shown of the positive and the negative chicken faeces samples, mixed as well as not-mixed with peptone/glycerol.

Table 3 Number of aerobic bacteria per gram of naturally contaminated faeces with Salmonella and faeces without Salmonella

	Faeces not mixed with pepton/glycerol	**Faeces 1:1 mixed with pepton/glycerol**
Naturally contaminated chicken faeces with *Salmonella*	6.1 x 10^9 cfp/gram (determined on 04-10-2004)	3.4 x 10^9 cfp/gram (determined on 12-10-2004)
Negative chicken faeces without *Salmonella*	5.3 x 10^8 cfp/gram (determined on 04-10-2004)	3.0 x 10^8 cfp/gram (determined on 26-10-2004)

The total aerobic count of the mixed naturally contaminated chicken faeces with *Salmonella* Enteritidis determined at days 0, 2, 7 and 14 showed stable results at all storage temperatures (see Figure 2). The number of *Salmonella* Enteritidis bacteria was stable when the mixed faeces was stored at -20 °C. When stored at +5 °C the number of SE slowly decreased during the 14 days of storage. When the mixed positive chicken faeces was stored at +20 °C the *Salmonella* bacteria disappeared within two days of storage. The separate numbers of both *Salmonella* Enteritidis and total bacterial count are reported in Veenman *et al.* (under preparation).

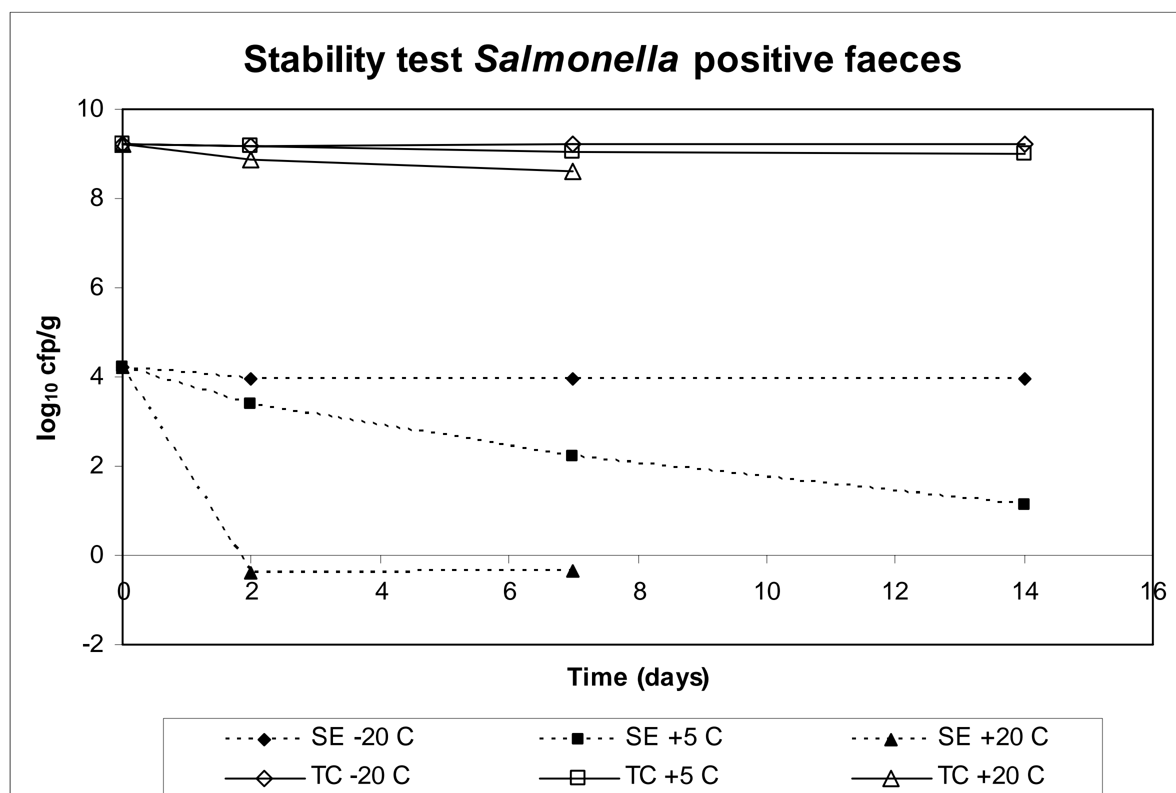

Figure 2 *Number of Salmonella Enteritidis (SE) bacteria and total bacterial count (TC) in Salmonella positive chicken faeces mixed with peptone/glycerol (30 % v/v) and stored at -20 °C, +5 °C and +20 °C*

4.3 Technical data interlaboratory comparison study

4.3.1 Pre-warming time and temperature of BPW

Before adding the capsules and/or faeces to the BPW, all jars had to be pre-warmed at (37 ± 1) °C overnight. All laboratories except two met the criteria as set in the standard operation procedure. The NRL with labcode 1 reported a pre-warming time of 5 h and 40 m. and the NRL with labcode 21 a pre-warming time of 2 hrs.

4.3.2 Incubation time and temperature for dissolving the capsules

Before adding the chicken faeces to the pre-enrichment medium (BPW), the capsules had to be dissolved in the BPW at (37 ± 1) °C for 45 minutes. Twenty-two laboratories dissolved the capsules in exactly forty-five minutes. One laboratory (labcode 15) reported a dissolving time of 35 minutes. Five laboratories used a dissolving time of more than 45 minutes, i.e. 47, 50,

55, 90 minutes, and 24 hrs and 50 minutes by respectively laboratories 24, 27, 4, 18 and 19. One laboratory (labcode 2) started the dissolving with an incubator temperature of 34.6 °C and a final temperature of 34.1 °C. The NRL with labcode 13 reported a final temperature of the incubator of 34 °C and laboratory 22 of 34.8 °C.

4.3.3 Incubation time and temperature of pre-enrichment

In this study two incubation times of the pre-enrichment medium BPW were compared being (4 ± ½) h and (18 ± 2) h. The last incubation time is according to ISO 6579: 2002. All laboratories except one laboratory (labcode 18) incubated the BPW for the prescribed short incubation time (4 ± ½) h (see Table 4). Laboratory 18 incubated the samples for 3 h and 10 m. Nine laboratories (labcodes 4, 8, 12, 15, 19, 21, 23, 24 and 25) exceeded the long incubation time (more than 20 h).
The prescribed temperature for the incubation of BPW is (37 ± 1) °C. All laboratories except four (labcode 2, 3, 13 and 20) incubated the BPW at the prescribed temperature (see Table 4).

Table 4 Incubation times and temperatures of pre-enrichment medium BPW

Labcode	Incubation time (h:min)	Incubation temperature in °C (min-max)	Incubation time (h:min)	Incubation temperature in °C (min-max)
Protocol	3:30 – 4:30	36-38	16 – 20	36-38
1	4:00	36.8 – 37.1	18:55	37.1 – 37.2
2	3:50	34.1 – 35.2	19:00	35.1 – 37.1
3	3:50	35.7 – 37.0	19:55	36.4 – 36.6
4	4:00	37	20:30	37
5	4:00	36.7 – 37.0	18:00	36.7 – 37.0
6	4:00	36.6 – 36.7	19:37	36.6 – 36.7
7	3:55	37	19:25	37
8	4:00	37.2 – 37.1	20:15	37.1 – 37.2
9	3:55	36.6 – 37.1	20:00	36.6 – 37.0
10	3:50	36.5 – 36.7	18:55	36.5 – 37.0
11	3:58	36	19:45	36
12	4:00	37	22:00	37
13	4:00	34 – 37	16:55	37
14	3:50	36.8 – 37.2	19:30	36.5 – 36.7
15	4:00	37	23:11	37
16	4:00	36.0 – 36.2	19:30	36.0 – 36.8
17	4:00	36.6 – 36.8	19:25	36.8
18	3:10	36 – 37	19:40	36.5 – 37.0
19	4:05	36.7 – 37.5	24:35	36.3 – 37.6
20	4:00	35 - 37	18:30	35 - 37
21	4:20	36.8	23:10	36.8
22	4:30	36.1 – 36.3	19:40	36.1 – 36.2
23	4:00	37	20:30	37
24	4:30	36.7 – 37.3	21:10	36.7 – 37.4
25	4:00	36.7 – 36.8	21:00	37.0 – 37.1
26	4:00	37.0 – 37.1	19:05	37.0
27	4:05	37.5	19:30	37.5 – 38.0
28	4:00	37	19:45	37

Times and temperatures deviating from the prescribed ones are indicated as gray cells.

4.3.4 Composition of selective enrichment medium MSRV

The prescribed composition of the MSRV was according to the draft Annex D of ISO 6579 (see Annex 9). All laboratories except for the laboratory with labcode 19 reported the correct composition (see Table 4.4 in Annex 4). However, according to draft Annex D the concentration of novobiocin in MSRV should be of 0.01 g/L. Six laboratories (labcodes 3, 4, 10, 16, 19 and 28) used a concentration of 0.02 g/L novobiocin in their selective enrichment medium MSRV. The NRL with labcode 21 did not add novobiocin to their MSRV.

4.3.5 Incubation times and temperatures of selective enrichment

The incubation time and temperature for MSRV according to draft Annex D of ISO 6579 should be between 21 – 27 h and (41.5 ± 1) ^{o}C, respectively. If plates were negative they should be incubated for another 21-27 h. Eight laboratories (labcodes 7, 11, 15, 17, 18, 19, 21 and 22) used a total incubation time outside the prescribed range of 42 – 54 h. Most laboratories complied with the required incubation time (see Table 5). All NRLs except six (labcodes 3, 4, 10, 15, 17 and 21) met the prescribed temperature of (41.5 ± 1) ^{o}C. Two laboratories (labcodes 4 and 21) incubated the MSRV at a temperature of around 37 ^{o}C instead of 41.5 ^{o}C.

Table 5 Incubation times and temperatures of selective enrichment medium MSRV after 4 and 18 h of incubation in BPW

	After 4 h incubation in BPW		After 18 h incubation in BPW	
Labcode	**Incubation time in h:min**	**Incubation temperature in °C (min-max)**	**Incubation time in h:min**	**Incubation temperature in °C (min-max)**
SOP	2 x (24 ± 3) h	40.5 – 42.5	2 x (24 ± 3) h	40.5 – 42.5
1	45:00	41.1 – 41.6	48:42	40.9 – 41.4
2	42:55	41.5 – 41.8	47:35	41.6 – 41.9
3	43:15	41.8 – 42.9	44:37	42.4
4	47:20	37	48:00	37
5	47:30	41.0 – 41.5	47:00	41.2 – 41.5
6	42:15	40.9 – 41.3	46:15	41.1 – 41.2
7	39:35	41.4 – 41.5	45:15	41.5
8	45:05	41.1 – 41.8	46:45	41.1 – 41.3
9	45:00	41.5 – 42.0	48:00	42.0
10	44:35	40.2 – 41.1	45:50	40.8 – 41.1
11	41:15	41.3 – 41.6	45:45	41.3 – 41.4
12	46:40	41.5	47:00	41.5
13	45:37	41.1 – 41.5	50:10	41.5
14	45:20	41.5 – 41.7	46:20	41.6 – 41.7
15	40:07	43	46:56	43
16	44:20	41.6 – 42.0	49:35	41.6 – 41.8
17	40:20	37.0 – 41.5	50:15	41.4 – 41.5
18	37:00	40.9 – 42.5	46:00	41
19	44:35	41.4 – 42.4	41:45	40.9
20	44:00	41 – 42	46:30	41 – 42
21	39:50	36.8 – 37.1	46:00	36. 8
22	41:40	40.7 – 41.1	41:20	40.6 – 41.6
23	43:00	41.5	47:00	41.5
24	43:35	41.7 – 42.0	45:00	42.0
25	44:55	41.1 – 42.0	44:25	41.6 – 41.8
26	43:10	41.5	47:15	41.5
27	44:30	42.0	48:45	42.0 – 42.4
28	46:25	41.5	45:45	41.5

Incubation times and temperatures according to SOP. Times and temperatures deviating from the prescribed ones are indicated as gray cells.

4.4 Control samples

General

All laboratories except one (labcode 19) tested the control samples (n = 10) with the requested two combinations of media, i.e. BPW(4hrs)/MSRV/XLD and BPW(18 hrs)/MSRV/XLD. Laboratory 19 used slightly different media combinations and their results are therefore not presented in the next tables. The laboratories with labcodes 3, 4, 10, 16 and 28 reported that they used 0.02 g/L novobiocin in the MRSV instead of 0.01 g/L. The laboratory with labcode 21 did not use novobiocin in their MSRV medium.
None of the laboratories isolated *Salmonella* from the procedure control (C11: no capsule/no faeces) and one laboratory (labcode 21) isolated *Salmonella* from the faeces control (C12: no capsule/negative faeces) with medium combinations BPW(4hrs)/MSRV/XLD and BPW(18hrs)/MSRV/XLD.

Blank capsules (n=2) without addition of faeces

The blank capsules contained only sterile milk powder. For the analyses no faeces was added. All twenty-seven participating laboratories did not isolate bacteria from these blank capsules (see Table 6).

Table 6 Number of positive isolations per laboratory for blank capsules (n=2) without addition of faeces

	Laboratory codes													
Medium combination	**1**	**2**	**3 #**	**4 #**	**5**	**6**	**7**	**8**	**9**	**10 #**	**11**	**12**	**13**	**14**
BPW 4 hrs/ MSRV/XLD	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BPW 18 hrs/ MSRV/XLD	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Laboratory codes													
Medium combination	**15**	**16 #**	**17**	**18**	**19**	**20**	**21 ***	**22**	**23**	**24**	**25**	**26**	**27**	**28 #**
BPW 4 hrs/ MSRV/XLD	0	0	0	0	Nt	0	0	0	0	0	0	0	0	0
BPW 18 hrs/ MSRV/XLD	0	0	0	0	Nt	0	0	0	0	0	0	0	0	0

*# 0.02 g/L novobiocin in MSRV instead of 0.01 g/L; * no novobiocin in MSRV; Nt = Not tested*

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

Fifteen laboratories (labcodes 1, 2, 3, 4, 6, 9, 13, 16, 17, 18, 21, 23, 24, 25, and 26) failed to isolate *Salmonella* from the capsules containing *S*. Panama at a level of *ca* 5 cfp/capsule with the medium combination BPW(4 hrs)/MSRV/XLD. Three laboratories (labcodes 4, 16 and 26) isolated no *Salmonella* or only from one of the two capsules (see Table 7).
Two laboratories (labcode 4 and 26) isolated *Salmonella* from only one of two *S*. Panama capsules with medium combination BPW(18 hrs)/MSRV/XLD and one laboratory (labcode 16) was not able to isolate *Salmonella* from any of the *S*. Panama capsules.

Table 7 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**
BPW 4 hrs/ MSRV/XLD	0	0	0	0	2	0	1	1	0	2	2	1	0	1
BPW 18 hrs/ MSRV/XLD	2	2	2	1	2	2	2	2	2	2	2	2	2	2

	Laboratory codes													
Medium combination	**15**	**16 #**	**17**	**18**	**19**	**20**	**21 ***	**22**	**23**	**24**	**25**	**26**	**27**	**28 #**
BPW 4 hrs/ MSRV/XLD	1	0	0	0	Nt	2	0	1	0	0	0	0	2	1
BPW 18 hrs/ MSRV/XLD	2	0	2	2	Nt	2	2	2	2	2	2	1	2	2

*# 0.02 g/L novobiocin in MSRV instead of 0.01 g/L; * no novobiocin in MSRV; Nt = Not tested*

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

Six laboratories (labcodes 5, 10, 11, 14, 20 and 27) were able to isolate *Salmonella* from all three capsules containing *Salmonella* Typhimurium at a mean level of *ca* 10 cfp/capsule with medium combination BPW(4 hrs)/MSRV/XLD (see Table 8). Thirteen laboratories were not able to isolate *Salmonella* from any of the three *S*. Typhimurium capsules with medium combination BPW(4 hrs)/MSRV/XLD. All laboratories except the NRL with labcode 16 isolated the maximum of three capsules positive with medium combination BPW(18 hrs)/MSRV/XLD. Laboratory 16 reported only one capsule positive of the three capsules.

Table 8 Number of positive isolations 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**
BPW 4 hrs/ MSRV/XLD	1	2	0	0	3	0	2	0	0	3	3	0	0	3
BPW 18 hrs/ MSRV/XLD	3	3	3	3	3	3	3	3	3	3	3	3	3	3

	Laboratory codes													
Medium combination	**15**	**16 #**	**17**	**18**	**19**	**20**	**21 ***	**22**	**23**	**24**	**25**	**26**	**27**	**28 #**
BPW 4 hrs/ MSRV/XLD	0	0	0	1	Nt	3	2	2	0	0	0	2	3	1
BPW 18 hrs/ MSRV/XLD	3	1	3	3	Nt	3	3	3	3	3	3	3	3	3

*# 0.02 g/L novobiocin in MSRV instead of 0.01 g/L; * no novobiocin in MSRV; Nt = Not tested*

Salmonella Enteritidis 100 capsules (n=2) without addition of faeces

Only three laboratories (labcode 5, 14 and 26) reported the maximum number of two positive isolations from the capsules containing *S*. Enteritidis at a mean level of *ca* 100 cfp/capsule with medium combination BPW(4 hrs)/MSRV/XLD (see Table 9). Fourteen laboratories (labcodes 2, 3, 4, 6, 8, 9, 11, 13, 15, 17, 18, 20, 23 and 28) were not able to isolate Salmonella from the two SE capsules with the above mentioned medium combination. All laboratories except the NRL with labcode 16 isolated *Salmonella* in all two capsules with BPW(18 hrs)/MSRV/XLD. Laboratory 16 reported one capsule positive with this medium combination.

Table 9 Number of positive isolations per laboratory for SE 100 (n=2) without addition of faeces

	Laboratory codes													
Medium combination	**1**	**2**	**3 #**	**4 #**	**5**	**6**	**7**	**8**	**9**	**10 #**	**11**	**12**	**13**	**14**
BPW 4 hrs/ MSRV/XLD	1	0	0	0	2	0	1	0	0	1	0	1	0	2
BPW 18 hrs/ MSRV/XLD	2	2	2	2	2	2	2	2	2	2	2	2	2	2

	Laboratory codes													
Medium combination	**15**	**16 #**	**17**	**18**	**19**	**20**	**21 ***	**22**	**23**	**24**	**25**	**26**	**27**	**28 #**
BPW 4 hrs/ MSRV/XLD	0	1	0	0	Nt	0	1	1	0	1	1	2	1	0
BPW 18 hrs/ MSRV/XLD	2	1	2	2	Nt	0	2	2	2	2	2	2	2	2

*# 0.02 g/L novobiocin in MSRV instead of 0.01 g/L; * no novobiocin in MSRV; Nt = Not tested*

Salmonella Enteritidis 500 capsules (n=1) without addition of faeces

Twenty-one laboratories (labcodes 1, 2, 4, 5, 6, 7, 8, 10, 11, 12, 14, 15, 18, 20, 21, 22, 23, 25, 26, 27 and 28) isolated *Salmonella* from the capsule containing *S*. Enteritidis at a mean level of *ca* 500 cfp/capsule with both medium combinations (see Table 10). The NRLs with labcodes 3, 9, 13 17 and 24 only isolated *Salmonella* with medium combination BPW(18 hrs)/MSRV/XLD.

The NRL with labcode 16 only isolated *Salmonella* with the medium combination BPW(4 hrs)/MSRV/XLD.

Table 10 Number of positive isolations per laboratory for SE 500 (n=1) without addition of faeces

	Laboratory codes													
Medium combination	**1**	**2**	**3 #**	**4 #**	**5**	**6**	**7**	**8**	**9**	**10 #**	**11**	**12**	**13**	**14**
BPW 4 hrs/ MSRV/XLD	1	1	0	1	1	1	1	1	0	1	1	1	0	1
BPW 18 hrs/ MSRV/XLD	1	1	1	1	1	1	1	1	1	1	1	1	1	1

	Laboratory codes													
Medium combination	**15**	**16 #**	**17**	**18**	**19**	**20**	**21 ***	**22**	**23**	**24**	**25**	**26**	**27**	**28 #**
BPW 4 hrs/ MSRV/XLD	1	1	0	1	Nt	1	1	1	1	0	1	1	1	1
BPW 18 hrs/ MSRV/XLD	1	0	1	1	Nt	1	1	1	1	1	1	1	1	1

*# 0.02 g/L novobiocin in MSRV instead of 0.01 g/L; * no novobiocin in MSRV; Nt = Not tested*

In Table 11 the specificity, sensitivity and accuracy for the control capsules without the addition of faeces are shown. The results of the NRLs with labcodes 16, 19 and 28 were not used for the calculation, because:

- laboratory 16 stored the RMs and faeces at +5 °C instead of -20 °C;
- laboratory 19 did not use the prescribed medium combination;
- laboratory 28 stored the RMs and faeces at room temperature instead of -20 °C.

For medium combinations MSRV/XLD after 4 h of incubation of BPW as well as after 18 h of incubation of BPW the specificity was in both cases 100 %. The sensitivity for SPan 5 capsules, STM 10 capsules, SE 100 capsules and SE 500 capsules after 4 h of incubation in BPW was respectively 32%, 40%, 30% and 80 %. The sensitivity after 18 h of incubation in BPW was for all four kinds of samples more than 95 % (see Table 11). The sensitivity for all capsules containing *Salmonella* was for the medium combination MSRV/XLD after 4 h of incubation of the BPW 40%, but after 18 h of incubation in BPW 95 %. The accuracy rate for all capsules (blank and capsules containing *Salmonella*) was 52 % after 4 h of incubation of BPW and 99 % after 18 h of incubation of BPW.

Table 11 Specificity, sensitivity and accuracy for all participating laboratories (n = 25) with all control capsules and all medium combinations without addition of faeces*

		4 h BPW	18 h BPW
Capsules		**MSRV/XLD**	**MSRV/XLD**
Blank (n = 2 per lab)	Number of samples	50	50
	Negative samples	50	50
	Specificity in %	**100**	**100**
SPan 5 (n = 2 per lab)	Number of samples	50	50
	Positive samples	16	48
	Sensitivity in %	**32**	**96**
STM 10 (n = 3 per lab)	Number of samples	75	75
	Positive samples	30	75
	Sensitivity in %	**40**	**100**
SE 100 (n = 2 per lab)	Number of samples	50	50
	Positive samples	15	49
	Sensitivity in %	**30**	**98**
SE 500 (n = 1 per lab)	Number of samples	25	25
	Positive samples	20	25
	Sensitivity in %	**80**	**100**
All capsules with *Salmonella*	Number of samples	200	200
	Positive samples	81	197
	Sensitivity in %	**41**	**99**
All capsules	Number of samples	250	250
	Correct samples	131	247
	Accuracy in %	**52**	**99**

** Results of lab 16, 19 and 28 were not used for the calculations*

4.5 Results faeces samples artificially contaminated with *Salmonella* spp.

4.5.1 Results per type of capsule and per laboratory

General

All laboratories except one (labcode 19) tested the artificially contaminated samples (n = 25) with the requested two combinations of media, i.e. BPW(4hrs)/MSRV/XLD and BPW(18 hrs)/MSRV/XLD (See 4.4 *general*). The laboratories with labcodes 3, 4, 10, 16 and 28 reported that they used 0.02 g/L novobiocin in the MRSV instead of 0.01 g/L. The laboratory with labcode 21 did not use novobiocin in their MSRV medium.

Blank capsules with negative faeces

The NRL with labcode 12 isolated *Salmonella* spp. from 2 of 3 blank capsules with both medium combinations (see Table 12). Laboratory 8 reported the isolation of *Salmonella* spp. of all three capsules, but only with medium combination MSRV/XLD after 18 h of incubation of the BPW.

Table 12 Number of isolations per laboratory for blank capsules (n=3) 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**
BPW 4 hrs/ MSRV/XLD	0	0	0	0	0	0	0	0	0	0	0	2	0	0
BPW 18 hrs/ MSRV/XLD	0	0	0	0	0	0	0	3	0	0	0	2	0	0

	Laboratory codes													
Medium combination	**15**	**16 #**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28 #**
BPW 4 hrs/ MSRV/XLD	0	0	0	0	Nt	0	0	0	0	0	0	0	0	0
BPW 18 hrs/ MSRV/XLD	0	0	0	0	Nt	0	0	0	0	0	0	0	0	0

*# 0.02 g/L novobiocin in MSRV instead of 0.01 g/L; * no novobiocin in MSRV; Nt = Not tested*

S. *Typhimurium 10 capsules (STM10) with negative faeces*
In Table 13 the results are summarized of the *Salmonella*-negative faeces samples artificially contaminated with capsules containing STM10. Six laboratories (labcode 4, 8, 13, 21, 23 and 25) did not isolate *Salmonella* from medium combination BPW(4 hrs)/MSRV/XLD. The maximum number of positive isolations (7) for this medium combination was only found by laboratory 20. For medium combination MSRV/XLD after 18 h of incubation of BPW the maximum number of positives were found by laboratories 2, 4, 6, 8, 10, 11, 12, 13, 17, 20, 22, 25, 26, 27. No isolation of *Salmonella* spp. for this combination was reported by NRLs with labcodes 1, 18 and 28.

Table 13 Number of positive isolations per laboratory for STM 10 (n=7) 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**
BPW 4 hrs/ MSRV/XLD	6	1	1	0	4	2	1	0	1	4	5	6	0	1
BPW 18 hrs/ MSRV/XLD	0	7	5	7	5	7	6	7	6	7	7	7	7	6

	Laboratory codes													
Medium combination	**15**	**16 #**	**17**	**18**	**19**	**20**	**21 ***	**22**	**23**	**24**	**25**	**26**	**27**	**28 #**
BPW 4 hrs/ MSRV/XLD	2	1	1	3	Nt	7	0	6	0	4	0	5	5	4
BPW 18 hrs/ MSRV/XLD	1	1	7	0	Nt	7	2	7	6	6	7	7	7	0

*# 0.02 g/L novobiocin in MSRV instead of 0.01 g/L; * no novobiocin in MSRV; Nt = Not tested*

S. *Typhimurium 100 (STM100) with negative faeces*
Considerably more positive isolations were found with the STM100 than with the STM10 capsules, in combination with *Salmonella*-negative faeces (see Table 14). Laboratories 6, 10, 11, 12, 14, 17, 20, 22, 24, 26 and 27 found all capsules positive for both medium combinations. Laboratories 4, 23 and 25 were not able to isolate *Salmonella* from medium combination BPW(4 hrs)/MSRV/XLD and laboratories 1, 16 and 28 not from medium combination BPW(18 hrs)/MSRV/XLD.

Table 14 Number of positive isolations per laboratory for STM 100 (n=4) with the addition of 10 g Salmonella negative chicken faeces

Medium combination	1	2	3 #	4 #	5	6	7	8	9	10 #	11	12	13	14
BPW 4 hrs/ MSRV/XLD	4	3	2	0	4	4	3	1	1	4	4	4	1	4
BPW 18 hrs/ MSRV/XLD	0	4	4	3	2	4	2	4	4	4	4	4	4	4

Medium combination	15	16 #	17	18	19	20	21 *	22	23	24	25	26	27	28 #
BPW 4 hrs/ MSRV/XLD	1	1	4	4	Nt	4	2	4	0	4	0	4	4	4
BPW 18 hrs/ MSRV/XLD	1	0	4	1	Nt	4	1	4	3	4	4	4	4	0

*# 0.02 g/L novobiocin in MSRV instead of 0.01 g/L; * no novobiocin in MSRV; Nt = Not tested*

S. *Enteritidis 100 (SE100) with negative faeces*

Laboratories with labcodes 1, 2, 15 and 16 were not able to isolate *Salmonella* from the SE100 capsules with any of the medium combinations (see Table 15). Furthermore, laboratories 3, 6, 13, 21, 23 and 27 reported no positive isolations with medium combination BPW(4h)/MSRV/XLD and laboratories 18 and 28 with medium combination BPW(18 h)/MSRV/XLD. Only laboratories 8, 9, 11, 20, 25 and 27 reported the maximum number positive isolation (7) with either of the medium combinations.

Table 15 Number of positive isolations per laboratory for SE 100 (n=7) with the addition of 10 g Salmonella negative chicken faeces

Medium combination	1	2	3 #	4 #	5	6	7	8	9	10 #	11	12	13	14
BPW 4 hrs/ MSRV/XLD	0	0	0	1	3	0	2	1	2	3	3	4	0	1
BPW 18 hrs/ MSRV/XLD	0	0	4	5	2	3	3	7	7	6	7	6	6	5

Medium combination	15	16 #	17	18	19	20	21 *	22	23	24	25	26	27	28 #
BPW 4 hrs/ MSRV/XLD	0	0	3	1	Nt	7	0	4	0	1	1	2	0	5
BPW 18 hrs/ MSRV/XLD	0	0	2	0	Nt	5	5	6	4	6	7	5	7	0

*# 0.02 g/L novobiocin in MSRV instead of 0.01 g/L; * no novobiocin in MSRV; Nt = Not tested*

S. Enteritidis 500 (SE500) with negative faeces

The maximum number of positives for capsules SE 500 and both medium combinations was only obtained by laboratories 10, 12, 22, 24 and 26 (see Table 16). No *Salmonella* could be isolated from medium combination MSRV/XLD after 4 h of incubation in BPW by laboratories 13 and 25 and from medium combination MSRV/XLD after 18 h of incubation in BPW by laboratories 1, 15, 16, 18 and 28.

Table 16 Number of positive isolations per laboratory for SE 500 (n=4) 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**
BPW 4 hrs/ MSRV/XLD	2	1	2	2	4	2	2	2	1	4	3	4	0	2
BPW 18 hrs/ MSRV/XLD	0	3	4	3	3	2	4	4	4	4	4	4	4	4

	Laboratory codes													
Medium combination	**15**	**16 #**	**17**	**18**	**19**	**20**	**21 ***	**22**	**23**	**24**	**25**	**26**	**27**	**28 #**
BPW 4 hrs/ MSRV/XLD	2	1	2	2	Nt	4	1	4	3	4	0	4	2	4
BPW 18 hrs/ MSRV/XLD	0	0	2	0	Nt	3	3	4	4	4	4	4	4	0

*# 0.02 g/L novobiocin in MSRV instead of 0.01 g/L; * no novobiocin in MSRV; Nt = Not tested*

In Figure 3 all positive isolations for all capsules containing *Salmonella* and medium combination BPW(4h)/MSRV/XLD per laboratory are given.

In Figure 4 all positive isolations for all capsules containing *Salmonella* and medium combination BPW(18h)/MSRV/XLD per laboratory are given.

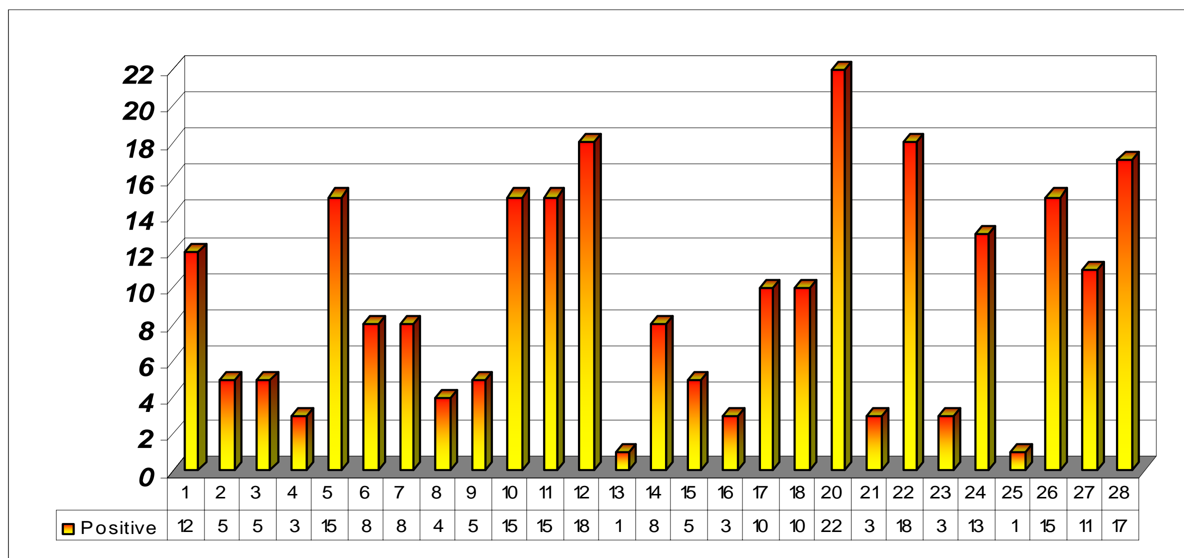

Figure 3 Number of positive isolations per laboratory (labcodes 1-18 and 20-28) for all capsules (n=22) for medium combination MSRV/XLD ***after 4 h*** *incubation of BPW with the addition of 10 g Salmonella negative chicken faeces*

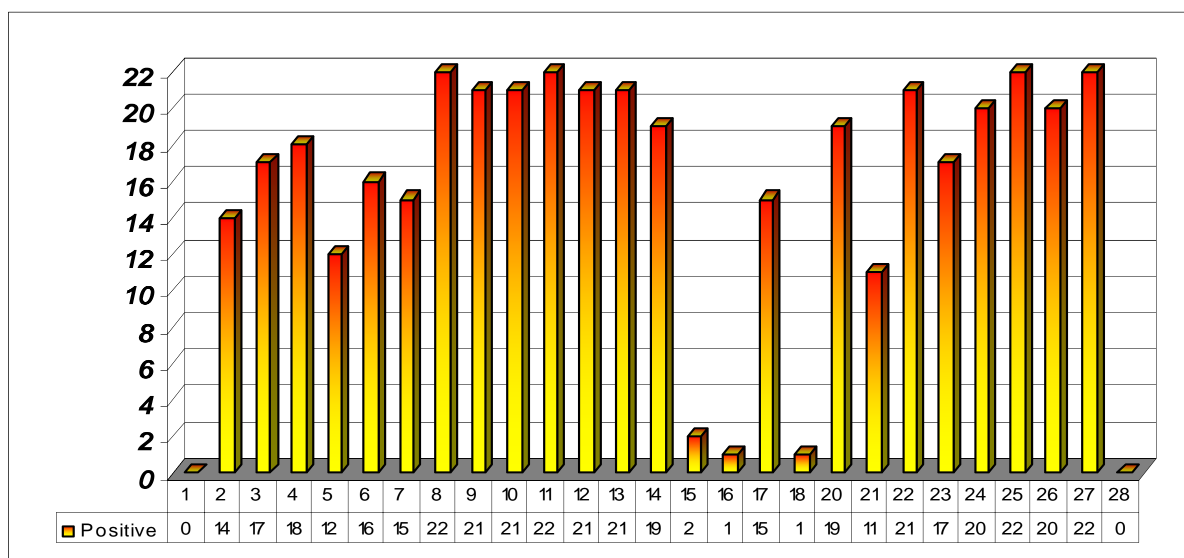

Figure 4 Number of positive isolations per laboratory (labcodes 1-18 and 20-28) for all capsules (n=22) for medium combination MSRV/XLD ***after 18 h*** *incubation of BPW with the addition of 10 g Salmonella negative chicken faeces*

4.5.2 Specificity, sensitivity and accuracy rates of artificially contaminated samples

The specificity, sensitivity and accuracy rates per medium combination for all types of capsules with the addition of *Salmonella*-negative faeces are shown in Table 17. The results of laboratories with labcodes 16, 19 and 28 were not used for the calculations (see 4.4).
The specificity rate for medium combination MSRV/XLD after 4 h of incubation of BPW was 97 %, and after 18 h of incubation 93 %. For the medium combination MSRV/XLD after 4 h of incubation of BPW the sensitivity in declining order of the capsules containing *Salmonella* was 22, 37, 59 and 70 % for respectively SE 100, STM 10, SE 500 and STM 100 capsules. After 18 h of incubation of BPW the sensitivity was more than 70 % except for the SE 100 capsules. The sensitivity rate for all capsules containing *Salmonella* was 42 % after 4 h of incubation of BPW and 74 % after 18 h of incubation of BPW. The accuracy rate was 49 % and 77 % for all capsules after 4 h and 18 h of incubation of BPW, respectively.

Table 17 Specificity, sensitivity and accuracy rates for all participating laboratories (n = 25) with all capsules and all medium combinations with addition of 10 g Salmonella negative chicken faeces*

Capsules		4 h BPW MSRV/XLD	18 h BPW MSRV/XLD
Blank (n = 3 per lab)	Number of samples	75	75
	Negative samples	73	70
	Specificity in %	**97**	**93**
STM 10 (n = 7 per lab)	Number of samples	175	175
	Positive samples	65	141
	Sensitivity in %	**37**	**81**
STM 100 (n = 4 per lab)	Number of samples	100	100
	Positive samples	70	81
	Sensitivity in %	**70**	**81**
SE 100 (n = 7 per lab)	Number of samples	175	175
	Positive samples	39	108
	Sensitivity in %	**22**	**62**
SE 500 (n = 4 per lab)	Number of samples	100	100
	Positive samples	59	79
	Sensitivity in %	**59**	**79**
All capsules with *Salmonella*	Number of samples	550	550
	Positive samples	233	409
	Sensitivity in %	**42**	**74**
All capsules	Number of samples	625	625
	Correct samples	306	479
	Accuracy in %	**49**	**77**

** Results of lab 16, 19 and 28 were not used for the calculations*

4.5.3 Results of other medium combinations

All participating laboratories (28) also tested the artificially contaminated samples with their own medium combination(s). In Table 18 the results obtained with the prescribed medium combination BPW(4h)/MSRV/XLD giving the highest number of positive results are compared with the results of their best own medium (also after 4h of incubation of BPW) being the own medium which gives the highest number of positive results (see also Annex 3). Only those results are shown in Table 18 that were different from each other. Five laboratories (labcode 5, 7, 9, 24 and 26) reported more positive isolations with the medium combination BPW(4h)/MSRV/XLD than with their own best medium combination. The NRL with labcode 12 found more positives with their own best medium combination after 4 h of incubation in BPW. No comparison was possible for the results of laboratory with labcode 19. This laboratory did not test the prescribed medium combination.

Table 18 Comparison of results between BPW(4 h)/MSRV/XLD and best own medium combination (4h incubation of BPW) for artificially contaminated samples

Labcode	Medium	STM10 (n=7)	STM100 (n=4)	SE100 (n=7)	SE500 (n=4)	All capsules (n=22)
5	MSRV/XLD	4	4	3	4	15
	Own best	4	4	2	4	14
7	MSRV/XLD	1	3	2	2	8
	Own best	0	3	1	2	6
9	MSRV/XLD	1	1	2	1	5
	Own best	0	0	3	1	3
12	MSRV/XLD	6	4	4	4	18
	Own best	6	4	5	4	19
19	MSRV/XLD	Nt	Nt	Nt	Nt	Nt
	Own best	6	4	4	4	18
24	MSRV/XLD	4	4	1	4	13
	Own best	4	3	1	4	12
26	MSRV/XLD	5	4	2	4	15
	Own best	4	4	2	4	14

Nt = Not tested

In Table 19 the results obtained with medium combination BPW(18h)/MSRV/XLD, giving the highest number of positive results are also compared with the results of the best own medium of the NRLs (after 18h of incubation of BPW) being the own medium which gives the highest number of positive results (see also Annex 3). Only those results are shown in Table 19 that were different from each other. Two laboratories (labcode 12 and 23) reported more positive isolations with their own best medium combination after 18h of incubation of BPW than with the medium combination BPW(18h)/MSRV/XLD.

Table 19 Comparison of results between BPW(18 h)/MSRV/XLD and best own medium combination for artificially contaminated samples

Labcode	Medium	STM10 (n=7)	STM100 (n=4)	SE100 (n=7)	SE500 (n=4)	All capsules (n=22)
12	MSRV/XLD	7	4	6	4	21
	Own best	7	4	7	4	22
19	MSRV/XLD	Nt	Nt	Nt	Nt	Nt
	Own best	2	1	2	0	5
23	MSRV/XLD	6	3	4	4	17
	Own best	6	4	5	4	19

Nt = Not tested

4.5.4 Comparison between laboratories

To be able to compare the positive isolations with the two medium combinations (BPW(4h)/MSRV/XLD and BPW(18h)/MSRV/XLD) separately and both medium combinations together the differences between NRLs were calculated in relation to the average results for all NRLs (see Figure 5). Fourteen laboratories (labcodes 2, 3, 4, 6, 7, 8, 9, 13, 14, 15, 16, 21, 23 and 25) scored below the average number of positive isolations (average = 9.4; n = 22) from all laboratories with the medium combination BPW(4h)/MSRV/XLD and of 13 laboratories the results were above the average. For the medium combination BPW(18h)/MSRV/XLD ten laboratories (labcodes 1, 2, 5, 7, 15, 16 17, 18 21 and 28) scored below the average of all laboratories (average = 15.2; n = 22) and of 17 laboratories the results were above the average results. For both medium combinations together fourteen laboratories (labcodes 1, 2, 3, 4, 6, 7, 13, 15, 16, 18, 21, 23, 25 and 28) scored below the average of all laboratories (average = 24.6; n = 44) and 13 laboratories above the average of all laboratories.

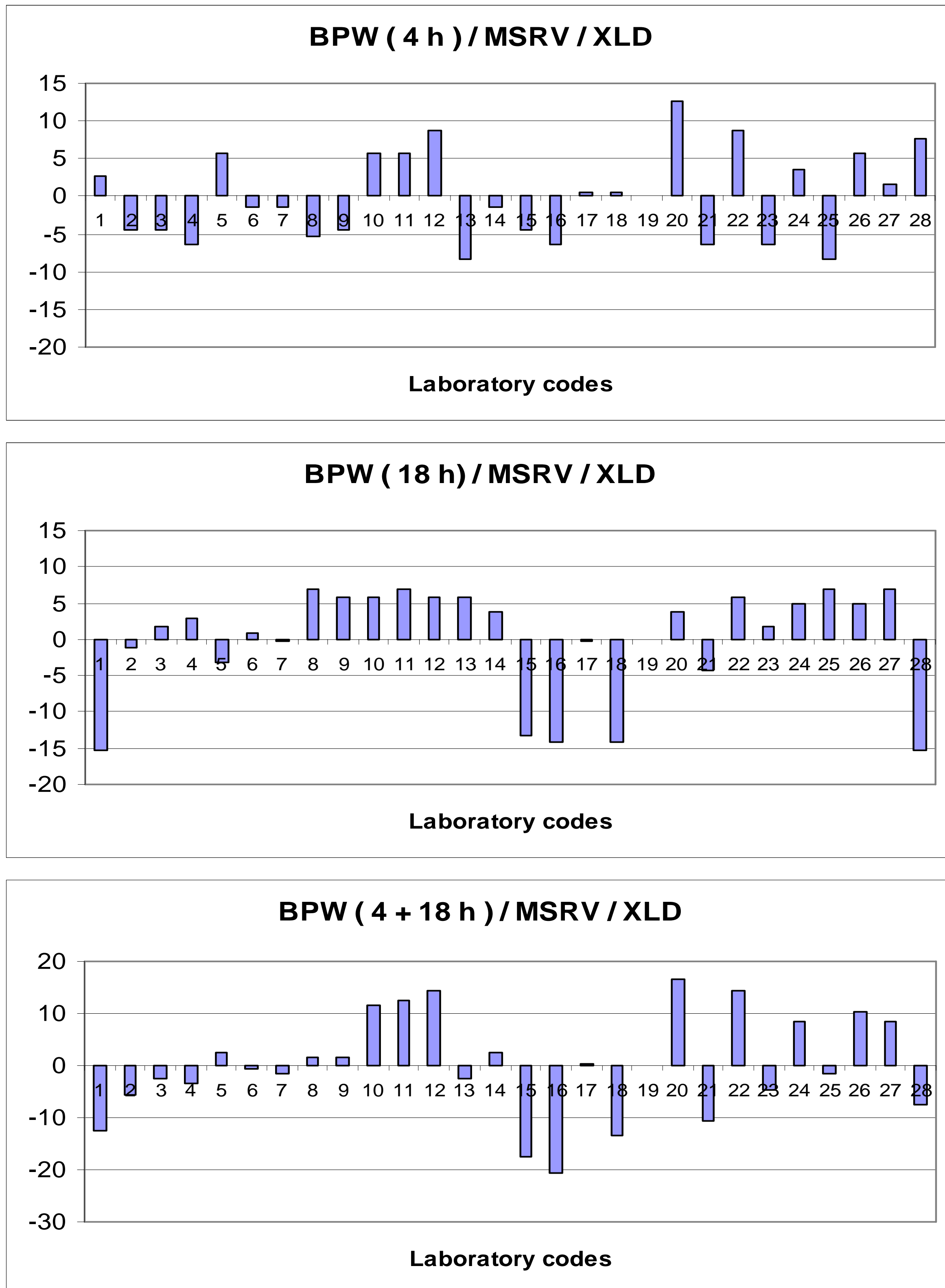

Figure 5 *Results obtained with two medium combinations per laboratory compared to the average results of all laboratories (y-axis: arithmetical variation values) for the artificially contaminated samples*

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

The results in Table 20 and Figures 6 and 7 shows that two laboratories (labcodes 5 and 22) were able to recover *Salmonella* from all faeces samples with the use of medium combination MSRV/XLD after incubation of BPW of 4 h as well as after 18 h. Furthermore laboratories 1, 2, 7, 11, 13, 14, 17, 20, 21, 25, 26 and 27 scored the maximum number of positives of all samples only with medium combination MSRV/XLD after 4 h of incubation of BPW. Laboratories 5, 6, 8 and 22 scored the maximum number of positives with combinations MSRV/XLD after 18 h of incubation of BPW. Laboratory 19 did not test these samples with MSRV/XLD.

Table 20 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**
BPW 4 hrs/ MSRV/XLD	20	20	2	19	20	15	20	12	11	19	20	9	20	20
BPW 18 hrs/ MSRV/XLD	10	18	7	0	20	20	1	20	19	15	17	15	6	15

	Laboratory codes													
Medium combination	**15**	**16 #**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28 #**
BPW 4 hrs/ MSRV/XLD	9	18	20	2	Nt	20	20	20	10	15	20	20	20	0
BPW 18 hrs/ MSRV/XLD	1	1	0	0	Nt	0	19	20	19	14	9	8	7	0

 # 0.02 g/L novobiocin in MSRV instead of 0.01 g/L; Nt = Not tested

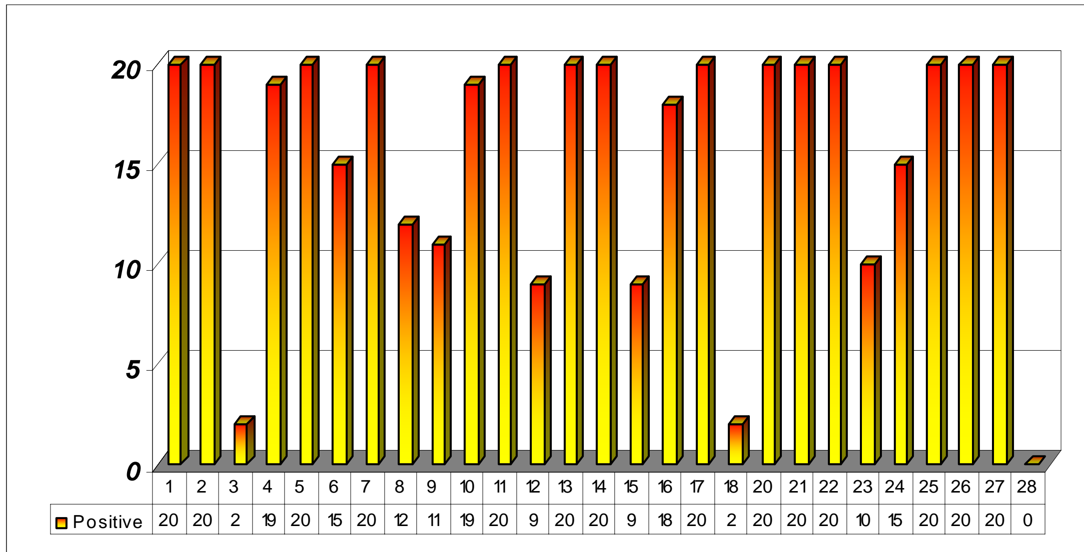

Figure 6 *Number of positive isolations (max. 20) per laboratory (labcodes 1-18 and 20-28) for medium combination MSRV/XLD* ***after 4 h*** *incubation of BPW when analyzing 10 g* Salmonella *positive faeces*

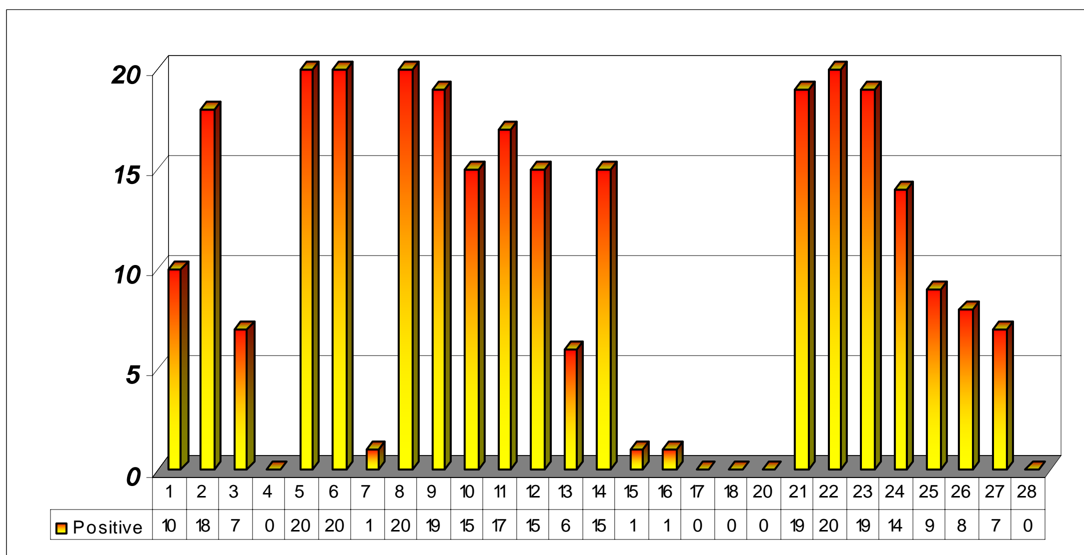

Figure 7 *Number of positive isolations (max. 20) per laboratory (labcodes 1-18 and 20-28) for medium combination MSRV/XLD* ***after 18 h*** *incubation of BPW when analyzing 10 g* Salmonella *positive faeces*

The sensitivity rates for 25 participating laboratories for the naturally contaminated samples per medium combination are given in Table 21. The isolation of *Salmonella* from the naturally contaminated samples showed more positive isolations with MSRV/XLD after 4 h of incubation of BPW than with MSRV/XLD after 18 h of incubation of BPW, resulting in a sensitivity of 81 % and 56 %, respectively.

Table 21 Sensitivity rates of all participating laboratories (n = 25) and two medium combinations for the naturally contaminated faeces with Salmonella (n=20)*

		4 h BPW	18 h BPW
Capsules		**MSRV/XLD**	**MSRV/XLD**
None	Number of samples	500	500
	Positive samples	403	280
	Sensitivity[1] in %	**81**	**56**

** Results of laboratories 16, 19 and 28 were not used for the calculations;*
[1] No negative samples were tested; therefore the accuracy rate is equal to the sensitivity rate

To be able to compare the positive isolations with the two medium combinations (BPW(4h)/MSRV/XLD and BPW(18h)/MSRV/XLD) separately and both medium combinations together the differences between NRLs were calculated in relation to the average results for all NRLs (see Figure 8). The results of laboratories 3, 6, 8, 9, 12, 15, 18, 23, 24 and 28 for the medium combination BPW(4h)/MSRV/ XLD were below the average number (average = 15.6; n = 20) of positive isolations from all laboratories and 17 laboratories scored above the average. For the medium combination BPW(18h)/MSRV/XLD fourteen laboratories (labcodes 1, 3, 4, 7, 13, 15, 16, 17, 18, 20, 25, 26, 27 and 28) scored below the average (average = 10.4; n = 20) of all laboratories and 13 laboratories scored above average. For both medium combinations together only ten laboratories (labcodes 3, 4, 7, 12, 15, 16, 17, 18, 20 and 28) scored below average (average = 26.0; n = 40) of all laboratories while 16 laboratories scored above average of all laboratories.

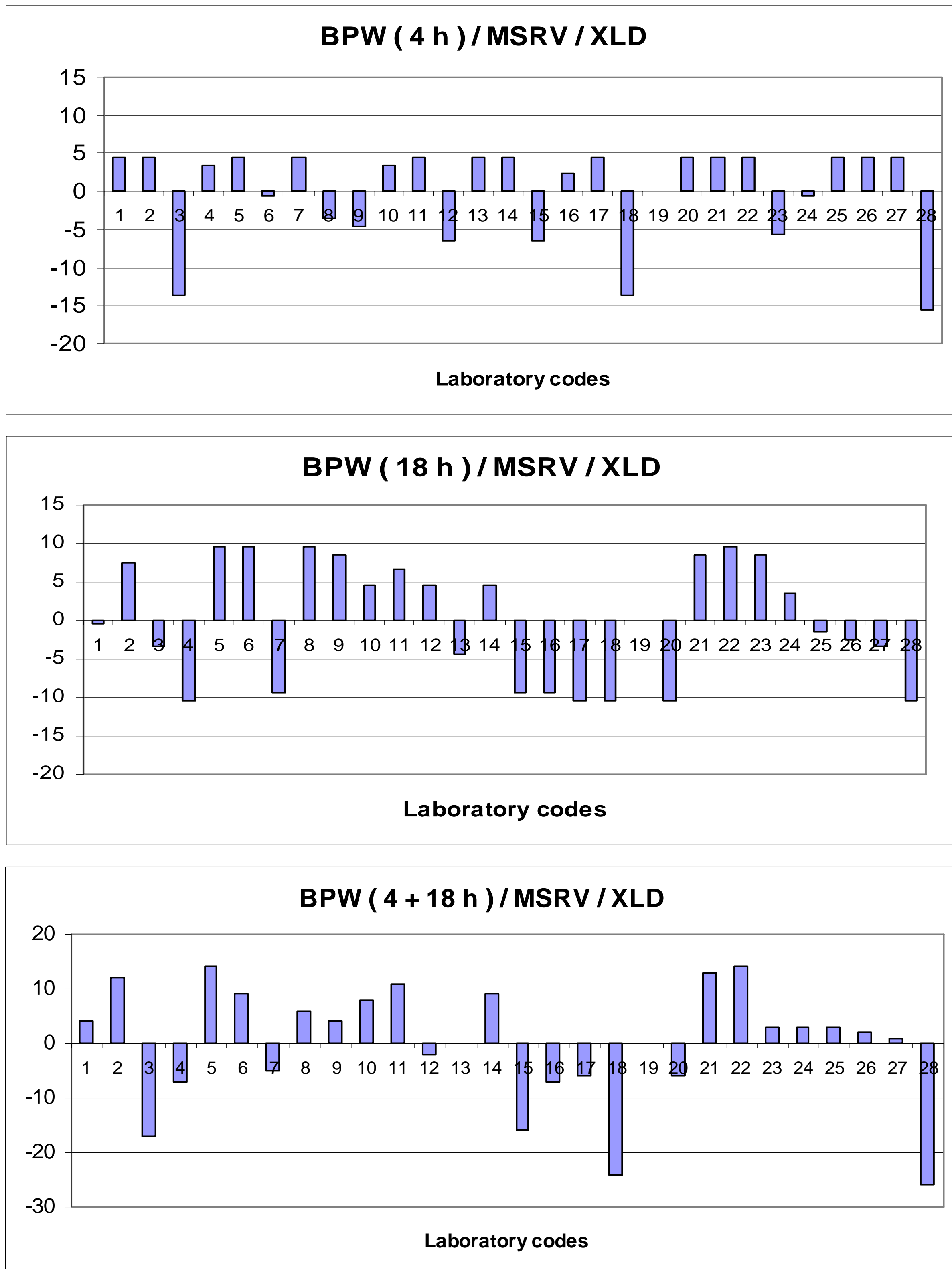

Figure 8 *Results obtained with two medium combinations per laboratory compared to the average results of all laboratories (y-axis: arithmetical variation values) for the naturally contaminated samples*

4.7 PCR

Four laboratories (labcodes 3, 6, 17 and 27) applied the PCR and one of these four laboratories (labcode 17) also applied an ELISA test as their additional detection technique.

Table 22 Details on the Polymerase Chain Reaction method, used as own method during the interlaboratory comparison study by four laboratories

Labcode	Volume of BPW (µl)	Volume of DNA sample (µl)	Volume DNA added to PCR (µl)
3	1000	150	2
6	1000	150	1.5
17	Not reported	Not reported	Not reported
27	2000	200	5

Two laboratories (labcodes 3 and 17) used a PCR procedure which was commercially available. Laboratory with labcode 3 only carried out PCR on the artificially contaminated samples. Lab 6 and 27 performed the PCR only on the control samples without the addition of chicken faeces.
The NRL with labcode 17 tested the artificially and naturally contaminated samples with three different PCR tests beside an ELISA test. This laboratory also performed a PCR directly after the incubation on MSRV.
The PCR results of all laboratories are shown in the tables 5.1 - 5.5 in Annex 5.
The PCR on the control samples revealed excellent results in relation to the bacteriological methods.
For the naturally contaminated samples laboratory 6 tested all samples positive in the PCR as well as with the culture methods after 18 h of incubation of BPW. Laboratory 27 tested all samples negative in the PCR and seven samples positive with the culture methods. The NRL with labcode 17 tested the naturally contaminated samples in three different PCR tests. Comparable numbers of positives were found with the iQ-check from Biorad (12 out of 20) and with the IMS-PCR from Selekta (11 out of 20). No samples were tested positive neither with another PCR from Selekta nor with the Bioline ELISA from the same manufacturer. The confirmation PCR tested all samples positive on MSRV after 4 h of incubation of BPW.
The number of positive results of laboratory 27 for the artificially contaminated samples was the same as the number of positive results found with the culture methods. The NRLs with labcodes 3 and 6 tested 22 samples positive with their PCR and respectively 17 and 16 with the culture methods. However, laboratory 3 tested also one blank capsule positive with the PCR. For the artificially contaminated samples the ELISA test results of laboratory 17 were comparable to the culture methods. Furthermore, this laboratory tested more samples positive with the three different PCRs than with the culture methods.

4.8 Transport of samples

The temperature recorders were returned immediately after receipt to CRL-*Salmonella* by twenty-six NRLs. One laboratory received the package but did not return the temperature recorder. Print-outs of the temperature recorders can be found in Annex 6. The majority of the laboratories received the materials within 1 to 2 days. Only for 5 laboratories the transport time was longer. Laboratories 15, 21 and 23 received the materials within three days and laboratory 12 within four days. The transport time for laboratory 28 was 18 days due to problems with the customs. The average number of transport time was 1.6 days (without taking the transport time of laboratory 28 into account). The temperature of the content of the parcel was below 5 ^{o}C in most cases, except for laboratories 5, 12 and 21. The maximum transport temperature for the parcels of these three laboratories was between 5 and 10 ^{o}C. The temperature of the parcel for laboratory 28 was approximately 20 ^{o}C for more than ten days.

The results of the experiments of CRL-Salmonella revealed that when the transportbox was taken out of the freezer (-20 ^{o}C) and placed at +5 ^{o}C the temperature within the box remained at approximately 0 ^{o}C for at least 140 h (Figure 9).
When the transport box was taken out of the freezer (-20 ^{o}C) and placed at room temperature (ca +20 ^{o}C), the temperature within the box remained between 0 and 10 ^{o}C for approximately 48 h (see Figure 10).

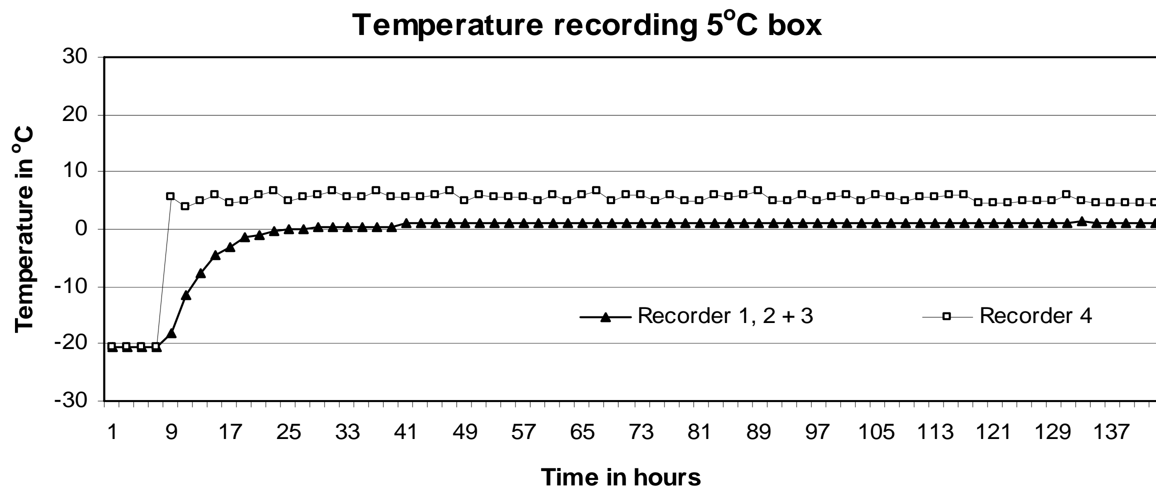

Figure 9 *Temperature recording of a transport box when stored at +5 °C after freezing at -20 °C. Recorders 1, 2 and 3 were placed inside the bottles in the box. Recorder 4 was placed at the outside of the box.*

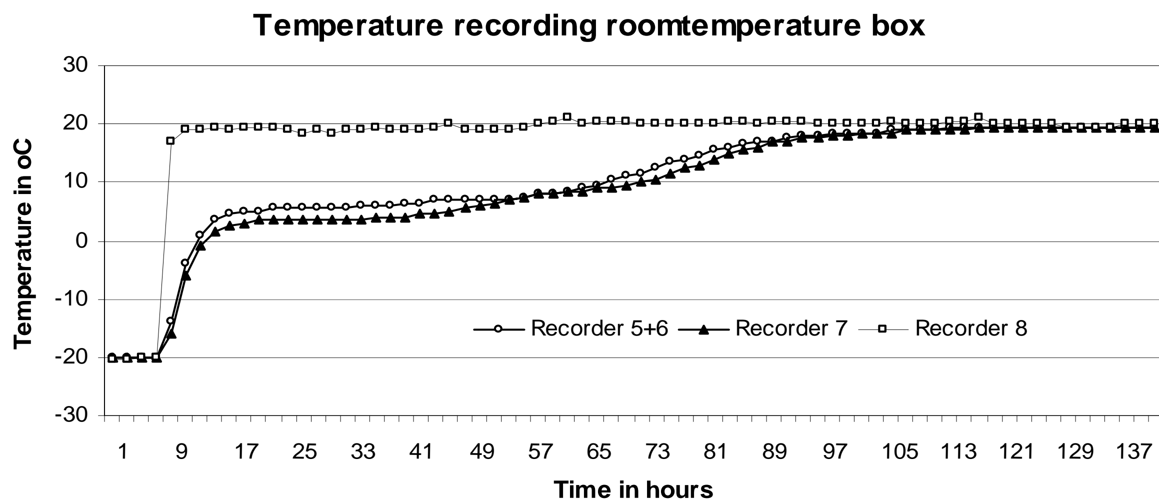

Figure 10 *Temperature recording of a transport box when stored at room temperature after freezing at -20 °C; Recorders 5, 6 and 7 were placed inside the bottles in the box and recorder 8 was placed at the outside of the box.*

5. Discussion

Transport of the samples

For this study the samples (faeces as well as capsules) were shipped by courier service from door-to-door as diagnostic specimens, resulting in short transport times, so that the temperature in the parcels remained low. The package of the NRL with labcode 28 was delayed at the border of that particular country for more than 2 weeks due to clearing problems through customs. The temperature of the contents of their package was around 20 ^{o}C for more than 10 days. This fact is a plausible explanation for the low number of *Salmonella* isolations.

Faecal samples

Additional to earlier studies the MPN of *Salmonella* in naturally contaminated faeces was determined as well as the total bacterial count in both positive and negative chicken faeces samples. By determining the MPN, an indication of the number of *Salmonella* spp. in the naturally contaminated faeces is obtained. The total bacterial count was determined to build up history about the possible effect of the total number of competitive bacteria in the chicken faeces on the detection of *Salmonella*.

The results of a stability study carried out by CRL-*Salmonella* on the naturally contaminated faeces (with *Salmonella* Enteritidis) mixed with peptone/glycerol, revealed that after a storage time of 14 days at three different temperatures (-20 ^{o}C, +5 ^{o}C and +20 ^{o}C) the total aerobic bacterial count almost remained the same. However, the number of *Salmonella* Enteritidis bacteria decreased rapidly after two days of storage at +20 ^{o}C. The number of SE bacteria decreased slowly but steadily at a storage temperature of +5 ^{o}C. When the (mixed) faeces was stored at -20 ^{o}C the number of SE remained stable for the whole measurement period (14 days).

Control samples

The majority of the NRLs found good results with the control samples (without faeces added). However, an incubation time of 4h of the pre-enrichment broth (BPW) seems to be too short for sufficient growth of *Salmonella,* resulting in less positive isolations.

One laboratory (labcode 21) found *Salmonella* in the negative faeces control, due to a possible cross-contamination or mislabeling of the BPW jars. Many laboratories failed to isolate the maximum number of isolations after 4 h of incubation of BPW. After 18 h of incubation of BPW two laboratories (labcodes 4 and 26) found only one SPan 5 control sample positive (out of 2), and one laboratory (labcode 16) could not isolate *Salmonella* from either of the two SPan 5 control samples. This might have been caused by the fact that the package of this latter laboratory was stored at +5 ^{o}C instead of -20 ^{o}C.

Statistically it is possible that at low contamination levels (< 10 cfp/capsule) occasionally a negative capsule can be found. This may be the explanation for the negative results found

with SPan5. Statistically it is also possible that occasionally a STM10 capsule is found negative, but the changes are very small to find 2 or 3 STM10 capsules negative at the same time.

Media

Each laboratory was asked to test the samples with the selective enrichment medium MSRV and the plating out medium XLD after 4 h and 18 h pre-enrichment in BPW. All NRLs except one (labcode 19) tested the samples with the prescribed medium combinations.
The prescribed range of the pH of the BPW according to ISO 6579: 2002 (E) is between 6.8 and 7.2. Most laboratories met this criterion. Eleven laboratories used MSRV with a pH higher (at the day of use) than the prescribed pH range of 5.0 – 5.4 varying from 5.44 to 5.74. A higher pH can stimulate the growth of background bacteria and might influence the results. In this study no unambiguous effect of the pH on the positive isolations was found.

Incubation time and temperature of the media

Two laboratories (labcodes 4 and 21) incubated the MSRV at a temperature of around 37 ^{o}C instead of at the prescribed (41.5 ± 1.0) ^{o}C. This may result in less selectivity of MSRV and easier outgrowth (overgrowth) of disturbing background flora. The effect of this incubation temperature on the results of the study was not clear. Laboratory 18 reported a total incubation time for the MSRV of 37 h (instead of the prescribed 42-54 h). It is not clear whether this short incubation time resulted in the low number of positive isolations of this laboratory, but it could have been one of the causes.

PCR

With the PCR method all blank control samples were tested correctly except for one laboratory (labcode 3). No direct explanation could be found. One laboratory (labcode 27) tested more samples positive with the culture methods than with the PCR for the naturally contaminated samples. For the artificially contaminated two laboratories (labcodes 3 and 6) found more positive results with the PCR than with the culture methods.

Performance of the laboratories

One laboratory (labcode 19) did not test the prescribed medium combination which made comparison of their results impossible. Fourteen laboratories (labcodes 2, 3, 4, 6, 7, 8, 9, 13, 14, 15, 16, 21, 23 and 25) scored below the average results of all laboratories for the artificially contaminated samples after 4 h of incubation of the BPW and ten laboratories (labcodes 1, 2, 5, 7, 15, 16, 17, 18, 21 and 28) scored below the average after 18 h of incubation of the BPW. Only five of the laboratories (labcodes 2, 7, 15, 16 and 21) scored below the average for both incubation times. Ten laboratories (labcodes 3, 6, 8, 9, 12, 15, 18, 23, 24 and 28) scored below the average results of all laboratories for the naturally contaminated samples after 4 h of incubation of the BPW and fourteen laboratories (labcodes 1, 3, 4, 7, 13, 15, 16, 17, 18, 20 25, 26, 27 and 28) after 18 h of incubation of the BPW. Four laboratories (labcodes 3, 15, 18 and 28) scored below the average for both incubation times.

Only one laboratory (labcode 15) scored below the average for the artificially as well as for the naturally contaminated samples.

For the calculation of the specificity rates, sensitivity rates and the accuracy rates, the results of laboratories 16, 19 and 28 were not used because of technical deviations.

- laboratory 16 stored the RMs and faeces at +5 ^{o}C instead of -20 ^{o}C;
- laboratory 19 did not use the prescribed medium combination;
- laboratory 28 stored the RMs and faeces at room temperature instead of -20 ^{o}C.

The accuracy rates of the control capsules (without addition of faeces) were obviously higher after 18h of incubation of BPW (99%) than after 4h of incubation of BPW (52%). The same is applicable to the artificially contaminated samples (capsules and faeces). For these samples the accuracy rate after 4h incubation of BPW was only 49% whereas after 18h of incubation of the BPW this was 77%. Opposite results were found with the naturally contaminated samples. For these samples the accuracy rate (here the same as the sensitivity rate) after 4h of incubation of BPW was 81%, whereas after 18h of incubation of BPW it was only 56%. Explanations for the somewhat low accuracy rates and for the opposite results between the artificially contaminated samples and the naturally contaminated samples were sought in the influence of glycerol on the growth of *Salmonella*. Chun *et al.* (1972) described an inhibitory effect of glycerol on the growth of *Salmonella*. Several experiments performed by the CRL-Salmonella on the effect of different concentrations of glycerol in chicken faeces on the growth of *Salmonella* also revealed an inhibitory effect of glycerol (Veenman *et al.* in preparation).

Future studies

In the following interlaboratory comparison study (IX, 2005) the faeces will no longer be mixed with a peptone/glycerol solution because of the possible inhibitory effect of glycerol on the growth of *Salmonella*. To make comparison with the present study possible both incubation times of BPW will again be included in the next study as well.

6. Conclusions

- Significant more positive isolations were obtained from the artificially contaminated samples (negative chicken faeces, artificially contaminated with reference materials) after 18h of incubation in the pre-enrichment broth BPW, when compared with 4h of incubation.
- The accuracy rates for the artificially contaminated samples were 49% and 77% after respectively 4h and 18h of incubation in BPW.
- For the naturally contaminated samples, significant more positive isolations were found after 4h of incubation in the pre-enrichment broth BPW, when compared with 18h of incubation.
- The accuracy rates for the naturally contaminated samples were respectively 81% and 56% after 4h and after 18h of incubation in BPW.
- After 4h of incubation of BPW it was easier to isolate *Salmonella* from capsules with a high level of *Salmonella* Typhimurium (STM100), combined with (*Salmonella* negative) faeces than, in declining order, from capsules with a high level of *Salmonella* Enteritidis (SE500) or a low level of *Salmonella* Typhimurium STM10), and from capsules with a low level of *Salmonella* Enteritidis (SE100).
- After 18h of incubation of BPW it was easier to isolate *Salmonella* from capsules with both levels of *Salmonella* Typhimurium (STM10 and STM100), combined with (*Salmonella* negative) faeces than, in declining order, from capsules with a high level of *Salmonella* Enteritidis (SE500) and from capsules with a low level of *Salmonella* Enteritidis (SE100).
- Sending the materials as diagnostic specimens by door-to-door courier service shortened the transport time to an average of *ca* 1.5 days. This is a large improvement when compared with earlier studies when the materials were sent as dangerous goods.
- The temperature recorders in the parcels gave important information about the temperature during transport.
- Stability studies of the naturally contaminated faeces revealed a negative effect of elevated storage temperatures on the detection of *Salmonella*.
- The presence of glycerol in the chicken faeces may negatively have affected the growth of *Salmonella*.

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References

Anonymous, ISO 6579, 1993 (E). Microbiology – General guidance on methods for the detection of *Salmonella*. International Organisation for Standardisation, Geneve, Switzerland.

Anonymous, ISO 6579, 2002 (E). Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. International Organisation for Standardisation, Geneve, Switzerland.

Anonymus, ISO 4833, 2003. Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of microorganisms – Colony-count technique at 30 degrees C International Organisation for Standardisation, Geneve, Switzerland.

Chun D,Yong Seol S, Tak R and Kyu Park C, 1972. Inhibitory effect of glycerol on *Vibrio parahaemolyticu*s and *Salmonella*. Applied Microbiology, 24 (5) 675-678.

Heisterkamp SH, Hoekstra JA, van Strijp-Lockefeer NGWM, Havelaar A, Mooijman KA, In ‘t Veld PH, Notermans SHW, 1993. Statistical analysis of certification trials for microbiological reference materials. Commission of European Communities, Community Bureau of Reference, Brussels, Luxembourg. EUR Report; EUR 15008 EN.

Heuvelman CJ, and In ’t Veld PH, 1998. Comparison of direct selective enrichment with pre-enrichment in BPW on the detection of *Salmonella* in chicken faeces. National Institute for Public Health and the Environment, Bilthoven, the Netherlands RIVM. report 284500009.

In ‘t Veld PH, Strijp-Lockefeer van NGWM, Havelaar AH, Maier EA, 1996. The certification of a reference material for the evaluation of the ISO method for the detection of *Salmonella*. J.Appl.Bacteriol; 80: 496-504

Korver H, Mooijman KA, Nagelkerke NJD, van de Giessen AW, Henken AM, 2003. EU Collaborative study VI (2002) on bacteriological detection of Salmonella spp. National Institute for Public Health and the Environment, Bilthoven, the Netherlands. RIVM report 330300001.

Raes M, Voogt N, In ’t Veld PH, Nagelkerke N, Henken AM, 1998. Bacteriological detection of *Salmonella* in the presence of competitive micro-organisms. Bacteriological collaborative study III amongst the National Reference Laboratories for *Salmonella.* National Institute for Public Health and the Environment, Bilthoven, the Netherlands. RIVM report 284500011.

Raes M, Nagelkerke N, Henken AM, 2000. Bacteriological detection of *Salmonella* in the presence of competitive micro-organisms. Bacteriological collaborative study IV amongst the National Reference Laboratories for *Salmonella*, the use of MSRV as selective enrichment. National Institute for Public Health and the Environment, Bilthoven, the Netherlands. RIVM report 284500014.

Raes M, Nagelkerke N, Henken AM, 2001. Bacteriological detection of *Salmonella* in the presence of competitive micro-organisms. Bacteriological collaborative study V amongst the National Reference Laboratories for *Salmonella.* National Institute for Public Health and the Environment, Bilthoven, the Netherlands. RIVM report 284500018.

SAS Institute inc., Gary, NC, USA, 2004

Schulten SM, In 't Veld PH, Ghameshlou Z, Schimmel H, Linsinger T, 2000. The certification of the number of colony forming particles of *Salmonella* Typhimurium and number fraction of negative capsules from artificially contaminated milk powder. Commission of European Communities, Community Bureau of Reference, Brussels, Luxembourg. CRM 507R, EUR 19622 EN.

Veenman C, Korver H, Mooijman KA, 2005. Research activities CRL-*Salmonella.* National Institute for Public Health and the Environment, Bilthoven, the Netherlands. RIVM report 330300010 in preparation.

Voogt N, In 't Veld PH, Notermans SHW, Henken AM, 1996. A collaborative study amongst the National Reference Laboratories for *Salmonella.* National Institute for Public Health and the Environment, Bilthoven, the Netherlands. RIVM report 284500003.

Voogt N, In 't Veld PH, Nagelkerke N, Henken AM, 1997. Bacteriological detection of *Salmonella* in the presence of competitive micro-organisms. A collaborative study amongst the National Reference Laboratories for *Salmonella.* National Institute for Public Health and the Environment, Bilthoven, the Netherlands. RIVM report 284500007.

Voogt N, In 't Veld PH, Nagelkerke N, Henken AM, 2002. Differences Between Reference Laboratories of the European Community in Their Ability to Detect Salmonella species, Eur.J.Microbiol.Inf.Dis.(21): 449-454

Annex 1. History of bacteriological studies

Table 1.1 History of bacteriological studies (1)

Study	Year	Number of samples	Capsules	Actual number of cfp/capsule	Salmonella negative faeces[1] 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 63 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	Korver et al., 2002 (report 330300001)

** = with antibiotics ; ** = Naturally contaminated chicken faeces with Salmonella*

[1]Faeces mixed (1:1) with a solution of peptone/glycerol. Final concentration glycerol in the faeces mixture was 15% (v/v)

Table 1.2 History of bacteriological studies (2)

Study	Year	Number of samples	Capsules	Actual number of cfp/capsule	Salmonella negative faeces[1] added	Selective enrichment medium	Plating-out medium	Reference
VII	2003	5	STM10	12	10 gram	RVS, MSRV, MKTTn and own	BGA, XLD and own	Korver et al., 2003 (report 330300004)
		5	STM100	96	10 gram			
		5	SE100	127	10 gram			
		5	SE500	595	10 gram			
		5	Blank	0	10 gram			
		3	STM10	12	No			
		3	SE100	127	No			
		2	SPan5	9	No			
		2	Blank	0	No			
		20	None	-	10 gram**			
VIII	2004	7	STM10	13	10 gram	MSRV and own	XLD and own	This report
		4	STM100	81	10 gram			
		7	SE100	74	10 gram			
		4	SE500	434	10 gram			
		3	Blank	0	10 gram			
		3	STM10	13	No			
		2	SE100	74	No			
		1	SE500	434	No			
		2	SPan5	7	No			
		2	Blank	0	No			
		20	None	-	10 gram**			

*** = Naturally contaminated chicken faeces with Salmonella*

[1]Faeces mixed (1:1) with a solution of peptone/glycerol. Final concentration glycerol in the faeces mixture was 15% (v/v)

Annex 2. Calculation of T_2

The variation between capsules of one batch of reference materials 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 (*i*)
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 and thus 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). An acceptable variation for a batch of capsules will be $T_2/(I-1) \leq 2$.

Annex 3. Results per laboratory, sample and medium combination

Table 3.1 Number of positive isolations per laboratory with own medium combinations for all control samples ***after 4 h*** *of incubation of BPW*

	Medium combination		Capsules				
Labcode	Selective enrichment	Isolation medium	Blank (n=2)	SPan 5 (n=2)	STM 10 (n=3)	SE 100 (n=2)	SE 500 (n=1)
1	MSRV	BGA	0	0	1	1	1
2	MSRV	BPLS	0	0	2	0	1
3	MSRV	SMID	0	0	0	0	0
4	MSRV	BGA	0	0	0	0	1
5	MSRV	BGA	0	2	3	2	1
6	MSRV	Onoz	0	0	0	0	1
7	MSRV	BGA	0	1	2	1	1
8	MSRV	BGA	0	1	0	0	1
9	MSRV	BGA	0	0	0	0	0
10	MSRV	BGA	0	2	3	1	1
11	MSRV	BPLS	0	2	3	0	1
12	MSRV	BGA	0	1	0	1	1
13	MSRV	XLT4	0	0	0	0	0
14	MSRV	Rambach	0	1	3	2	1
15	MSRV	XLT4	0	0	0	0	1
16	MSRV	BGA	0	0	0	1	1
17	MSRV	BGA	0	0	0	0	0
18	MSRV	BGA	0	0	1	0	1
	MSRV	MLCB	0	0	1	0	1
19	MSRV	XLT4	0	0	0	0	1
	MSRV	BGA	0	0	0	0	1
20	MSRV	SMID	0	2	3	0	1
21	MSRV	Rambach	0	0	2	1	1
22	MSRV	Rambach	0	1	2	1	1
23	MSRV	BGA	0	0	0	0	1
24	MSRV	BxLH	0	0	0	1	0
25	MSRV	Rambach	0	0	0	1	1
26	MSRV	BGA	0	0	2	2	1
	RVS	XLD	0	1	1	0	1
	RVS	BGA	0	1	1	0	1
27	MSRV	BPLSA	0	2	3	1	1
28	MSRV	McConkey	0	1	1	0	1
	SC	XLD	0	1	0	0	1
	SC	McConkey	0	1	0	0	1

*Table 3.2 Number of positive isolations per laboratory with own medium combinations for all control samples **after 18 h** of incubation of BPW*

	Medium combination		Capsules				
Labcode	Selective enrichment	Isolation medium	Blank (n=2)	SPan 5 (n=2)	STM 10 (n=3)	SE 100 (n=2)	SE 500 (n=1)
1	MSRV	BGA	0	2	3	2	1
2	MSRV	BPLS	0	2	3	2	1
3	MSRV	SMID	0	2	3	2	1
4	MSRV	Rambach	0	1	3	2	1
5	MSRV	BGA	0	2	3	2	1
6	MSRV	Onoz	0	2	3	2	1
7	MSRV	BGA	0	2	3	2	1
8	MSRV	BGA	0	2	3	2	1
	RV	XLD	0	2	3	2	1
	RV	BGA	0	2	3	2	1
9	MSRV	BGA	0	2	3	2	1
10	MSRV	BGA	0	2	3	2	1
	RV	XLD	0	2	3	2	1
	RV	BGA	0	2	3	2	1
11	MSRV	BPLS	0	2	3	2	1
12	MSRV	BGA	0	2	3	2	1
	RVS	XLD	0	2	3	2	1
	RVS	BGA	0	2	3	2	1
13	MSRV	XLT4	0	2	3	2	1
14	MSRV	Rambach	0	2	3	2	1
15	MSRV	XLT4	0	2	3	2	1
16	MSRV	BGA	0	0	1	1	0
17	MSRV	BGA	0	2	3	2	1
	RVS	XLD	0	2	3	2	1
	RVS	BGA	0	2	3	2	1
18	MSRV	BGA	0	2	3	2	1
	MSRV	MLCB	0	2	3	2	1
	MKTTn	XLD	0	2	3	2	1
	MKTTn	BGA	0	2	3	2	1
	MKTTn	MLCB	0	2	3	2	1
19	MSRV	XLT4	0	0	2	1	0
	MSRV	BGA	0	2	1	1	1
20	MSRV	SMID	0	2	3	0	1
21	MSRV	Rambach	0	2	3	2	1
22	MSRV	Rambach	0	2	3	2	1
	MK	XLD	0	2	3	2	1
	MK	Rambach	0	2	3	2	1
	MK	XLT4	0	2	3	2	1
23	MSRV	BGA	0	2	3	2	1
	RV	XLD	0	2	3	2	1
	RV	BGA	0	2	3	2	1
	MKTTn	XLD	0	2	3	2	1
	MKTTn	BGA	0	2	3	2	1
24	MSRV	BxLH	0	2	3	2	1
25	MSRV	Rambach	0	2	3	2	1
26	MSRV	BGA	0	1	3	2	1
	RVS	XLD	0	1	3	2	1
	RVS	BGA	0	1	3	2	1
27	MSRV	BPLSA	0	2	3	2	1
28	MSRV	McConkey	0	2	3	2	1
	SC	XLD	0	2	3	2	1
	SC	McConkey	0	2	3	2	1

Table 3.3 *Number of positive isolations per laboratory with own medium combinations for STM 10 (n=7) with the addition of* Salmonella *negative faeces* ***after 4 h*** *of incubation of BPW*

	Laboratory code													
Medium combination	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MSRV/BGA	6			0	4		0	0	0	4		6		
MSRV/BPLS		1									5			
MSRV/SMID			1											
MSRV/Onoz						2								
MSRV/XLT4													0	
MSRV/Rambach														1

	Laboratory code													
Medium combination	15	16	17	18	19	20	21	22	23	24	25	26	27	28
MSRV/XLT4	2				6									
MSRV/BGA		1	1	3	6				0			4		
MSRV/MLCB				3										
MSRV/SMID						7								
MSRV/Rambach							0	6			0			
MSRV/BxLH										4				
MSRV/BPLSA													5	
MSRV/McConkey														4
MKTTn/XLD				1										
MKTTn/BGA				0										
MKTTn/MLCB				0										
RVS/XLD												4		
RVS/BGA												4		
SC/XLD														4
SC/McConkey														4

Table 3.4 Number of positive isolations per laboratory with own medium combinations for STM 10 (n=7) with the addition of Salmonella *negative faeces* ***after 18 h*** *of incubation of BPW*

	Laboratory code													
Medium combination	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MSRV/BGA	0				5		6	7	6	7		7		
MSRV/BPLS		7									7			
MSRV/SMID			5											
MSRV/Rambach				7										6
MSRV/Onoz						7								
MSRV/XLT4													7	
RV/XLD								7		6				
RV/BGA								7		6				
RVS/XLD												7		
RVS/BGA												7		

	Laboratory code													
Medium combination	15	16	17	18	19	20	21	22	23	24	25	26	27	28
MSRV/XLT4	1				2									
MSRV/BGA		1	7	0	2				6			7		
MSRV/MLCB				0										
MSRV/SMID						7								
MSRV/Rambach							2	7			7			
MSRV/BxLH										6				
MSRV/BPLSA													7	
MSRV/McConkey														0
MKTTn/XLD				0					6					
MKTTn/BGA				0					6					
MKTTn/MLCB				0										
RVS/XLD			2									6		
RVS/BGA			2									6		
SC/XLD														0
SC/McConkey														0
MK/XLD								6						
MK/Rambach								6						
MK/XLT4								6						
RV/XLD									6					
RV/BGA									6					

Table 3.5 Number of positive isolations per laboratory with own medium combinations for STM 100 (n=4) with the addition of Salmonella *negative faeces* ***after 4 h*** *of incubation of BPW*

	Laboratory code													
Medium combination	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MSRV/BGA	4			0	4		3	1	0	4		4		
MSRV/BPLS		3									4			
MSRV/SMID			2											
MSRV/Onoz						4								
MSRV/XLT4													1	
MSRV/Rambach														4

	Laboratory code													
Medium combination	15	16	17	18	19	20	21	22	23	24	25	26	27	28
MSRV/XLT4	1				4									
MSRV/BGA		1	4	4	4				0			4		
MSRV/MLCB				4										
MSRV/SMID						4								
MSRV/Rambach							2	4			0			
MSRV/BxLH										3				
MSRV/BPLSA													4	
MSRV/McConkey														4
MKTTn/XLD				0										
MKTTn/BGA				0										
MKTTn/MLCB				0										
RVS/XLD												4		
RVS/BGA												4		
SC/XLD														4
SC/McConkey														4

Table 3.6 *Number of positive isolations per laboratory with own medium combinations for STM 100 (n=4) with the addition of* Salmonella *negative faeces* ***after 18 h*** *of incubation of BPW*

	Laboratory code													
Medium combination	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MSRV/BGA	0				2		2	4	4	4		4		
MSRV/BPLS		4									4			
MSRV/SMID			4											
MSRV/Rambach				3										4
MSRV/Onoz						4								
MSRV/XLT4													4	
RV/XLD								4		3				
RV/BGA								4		3				
RVS/XLD												4		
RVS/BGA												4		

	Laboratory code													
Medium combination	15	16	17	18	19	20	21	22	23	24	25	26	27	28
MSRV/XLT4	1				1									
MSRV/BGA		0	4	1	1				3			4		
MSRV/MLCB				1										
MSRV/SMID						4								
MSRV/Rambach							1	4			4			
MSRV/BxLH										4				
MSRV/BPLSA													4	
MSRV/McConkey														0
MKTTn/XLD				1					4					
MKTTn/BGA				1					4					
MKTTn/MLCB				1										
RVS/XLD			3									4		
RVS/BGA			3									4		
SC/XLD														0
SC/McConkey														0
MK/XLD								4						
MK/Rambach								3						
MK/XLT4								3						
RV/XLD									4					
RV/BGA									4					

Table 3.7 Number of positive isolations per laboratory with own medium combinations for SE 100 (n=7) with the addition of Salmonella *negative faeces* ***after 4 h*** *of incubation of BPW*

	Laboratory code													
Medium combination	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MSRV/BGA	0			1	2		1	1	3	3		5		
MSRV/BPLS		0									3			
MSRV/SMID			0											
MSRV/Onoz						0								
MSRV/XLT4													0	
MSRV/Rambach														1

	Laboratory code													
Medium combination	15	16	17	18	19	20	21	22	23	24	25	26	27	28
MSRV/XLT4	0				4									
MSRV/BGA		0	3	1	4				0			2		
MSRV/MLCB				1										
MSRV/SMID						7								
MSRV/Rambach							0	4			1			
MSRV/BxLH										1				
MSRV/BPLSA													0	
MSRV/McConkey														5
MKTTn/XLD				0										
MKTTn/BGA				0										
MKTTn/MLCB				0										
RVS/XLD												2		
RVS/BGA												1		
SC/XLD														4
SC/McConkey														4

Table 3.8 Number of positive isolations per laboratory with own medium combinations for SE 100 (n=7) with the addition of Salmonella *negative faeces* ***after 18 h*** *of incubation of BPW*

	Laboratory code													
Medium combination	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MSRV/BGA	0				2		3	6	7	6		6		
MSRV/BPLS		0									7			
MSRV/SMID			4											
MSRV/Rambach				5										5
MSRV/Onoz						3								
MSRV/XLT4													6	
RV/XLD								7		3				
RV/BGA								6		3				
RVS/XLD												7		
RVS/BGA												6		

	Laboratory code													
Medium combination	15	16	17	18	19	20	21	22	23	24	25	26	27	28
MSRV/XLT4	0				0									
MSRV/BGA		0	2	0	2				4			5		
MSRV/MLCB				0										
MSRV/SMID						5								
MSRV/Rambach							5	6			7			
MSRV/BxLH										6				
MSRV/BPLSA													7	
MSRV/McConkey														0
MKTTn/XLD				0					5					
MKTTn/BGA				0					5					
MKTTn/MLCB				0										
RVS/XLD			0									1		
RVS/BGA			0									4		
SC/XLD														0
SC/McConkey														0
MK/XLD								5						
MK/Rambach								5						
MK/XLT4								5						
RV/XLD									4					
RV/BGA									4					

Table 3.9 Number of positive isolations per laboratory with own medium combinations for SE 500 (n=4) with the addition of Salmonella *negative faeces* ***after 4 h*** *of incubation of BPW*

	Laboratory code													
Medium combination	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MSRV/BGA	2			2	4		2	2	1	4		4		
MSRV/BPLS		1									3			
MSRV/SMID			2											
MSRV/Onoz						2								
MSRV/XLT4													0	
MSRV/Rambach														2

	Laboratory code													
Medium combination	15	16	17	18	19	20	21	22	23	24	25	26	27	28
MSRV/XLT4	2				4									
MSRV/BGA		1	2	2	4				3			4		
MSRV/MLCB				2										
MSRV/SMID						4								
MSRV/Rambach							1	4			0			
MSRV/BxLH										4				
MSRV/BPLSA													2	
MSRV/McConkey														4
MKTTn/XLD				1										
MKTTn/BGA				1										
MKTTn/MLCB				1										
RVS/XLD												4		
RVS/BGA												4		
SC/XLD														3
SC/McConkey														3

Table 3.10 Number of positive isolations per laboratory with own medium combinations for SE 500 (n=4) with the addition of Salmonella *negative faeces* ***after 18 h*** *of incubation of BPW*

	Laboratory code													
Medium combination	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MSRV/BGA	0				3		4	4	4	4		4		
MSRV/BPLS		3									4			
MSRV/SMID			4											
MSRV/Rambach				3										4
MSRV/Onoz						2								
MSRV/XLT4													4	
RV/XLD								4		1				
RV/BGA								4		1				
RVS/XLD												4		
RVS/BGA												4		

	Laboratory code													
Medium combination	15	16	17	18	19	20	21	22	23	24	25	26	27	28
MSRV/XLT4	0				0									
MSRV/BGA		0	2	0	0				4			4		
MSRV/MLCB				0										
MSRV/SMID						3								
MSRV/Rambach							3	4			4			
MSRV/BxLH										4				
MSRV/BPLSA													4	
MSRV/McConkey														0
MKTTn/XLD				0					3					
MKTTn/BGA				0					3					
MKTTn/MLCB				0										
RVS/XLD			0									2		
RVS/BGA			0									2		
SC/XLD														0
SC/McConkey														0
MK/XLD								3						
MK/Rambach								3						
MK/XLT4								3						
RV/XLD									1					
RV/BGA									2					

Table 3.11 Number of positive isolations per laboratory with own medium combinations for blank capsules (n=3) with the addition of Salmonella *negative faeces* ***after 4 h*** *of incubation of BPW*

	Laboratory code													
Medium combination	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MSRV/BGA	0			0	0		0	0	0	0		1		
MSRV/BPLS		0									0			
MSRV/SMID			0											
MSRV/Onoz						0								
MSRV/XLT4													0	
MSRV/Rambach														0

	Laboratory code													
Medium combination	15	16	17	18	19	20	21	22	23	24	25	26	27	28
MSRV/XLT4	0				0									
MSRV/BGA		0	0	0	0				0			0		
MSRV/MLCB				0										
MSRV/SMID						0								
MSRV/Rambach							0	0			0			
MSRV/BxLH										0				
MSRV/BPLSA													0	
MSRV/McConkey														0
MKTTn/XLD				0										
MKTTn/BGA				0										
MKTTn/MLCB				0										
RVS/XLD												0		
RVS/BGA												0		
SC/XLD														0
SC/McConkey														0

Gray cells = unexpected results

Table 3.12 Number of positive isolations per laboratory with own medium combinations for blank capsules (n=4) with the addition of Salmonella *negative faeces* ***after 18 h*** *of incubation of BPW*

	Laboratory code													
Medium combination	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MSRV/BGA	0				0		0	0	0	0		1		
MSRV/BPLS		0									0			
MSRV/SMID			0											
MSRV/Rambach				0										0
MSRV/Onoz						0								
MSRV/XLT4													0	
RV/XLD								1		0				
RV/BGA								1		0				
RVS/XLD												2		
RVS/BGA												1		

	Laboratory code													
Medium combination	15	16	17	18	19	20	21	22	23	24	25	26	27	28
MSRV/XLT4	0				0									
MSRV/BGA		0	0	0	0				0			0		
MSRV/MLCB				0										
MSRV/SMID						0								
MSRV/Rambach							0	0			0			
MSRV/BxLH										0				
MSRV/BPLSA													0	
MSRV/McConkey														0
MKTTn/XLD				0					0					
MKTTn/BGA				0					0					
MKTTn/MLCB				0										
RVS/XLD			0									0		
RVS/BGA			0									0		
SC/XLD														0
SC/McConkey														0
MK/XLD								0						
MK/Rambach								0						
MK/XLT4								0						
RV/XLD									0					
RV/BGA									0					

Gray cells = unexpected results

Table 3.13 Number of positive isolations per laboratory with own medium combinations for naturally contaminated samples (n=20) ***after 4 h*** *of incubation of BPW*

	Laboratory code													
Medium combination	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MSRV/BGA	20			19	11		20	9	6	8		4		
MSRV/BPLS		20									20			
MSRV/SMID			9											
MSRV/Onoz						16								
MSRV/XLT4													20	
MSRV/Rambach														20

	Laboratory code													
Medium combination	15	16	17	18	19	20	21	22	23	24	25	26	27	28
MSRV/XLT4	10				8									
MSRV/BGA		18	20	2	6				9			20		
MSRV/MLCB				2										
MSRV/SMID						20								
MSRV/Rambach							20	20			20			
MSRV/BxLH										12				
MSRV/BPLSA													20	
MSRV/McConkey														0
MKTTn/XLD				3										
MKTTn/BGA				14										
MKTTn/MLCB				9										
RVS/XLD												15		
RVS/BGA												20		
SC/XLD														0
SC/McConkey														0

Table 3.14 Number of positive isolations per laboratory with own medium combinations for naturally contaminated samples (n=20) ***after 18 h*** *of incubation of BPW*

	Laboratory code													
Medium combination	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MSRV/BGA	9				14		1	16	19	6		12		
MSRV/BPLS		18									17			
MSRV/SMID			6											
MSRV/Rambach				0										15
MSRV/Onoz						20								
MSRV/XLT4													6	
RV/XLD								18		10				
RV/BGA								19		11				
RVS/XLD												16		
RVS/BGA												16		

	Laboratory code													
Medium combination	15	16	17	18	19	20	21	22	23	24	25	26	27	28
MSRV/XLT4	1				6									
MSRV/BGA		1	0	1	5				11			7		
MSRV/MLCB				0										
MSRV/SMID						0								
MSRV/Rambach							19	20			9			
MSRV/BxLH										14				
MSRV/BPLSA													7	
MSRV/McConkey														0
MKTTn/XLD				3					7					
MKTTn/BGA				1					6					
MKTTn/MLCB				2										
RVS/XLD			1									1		
RVS/BGA			2									2		
SC/XLD														0
SC/McConkey														0
MK/XLD								20						
MK/Rambach								20						
MK/XLT4								20						
RV/XLD									12					
RV/BGA									9					

Annex 4. Information on the media used

Table 4.1 Manufacturer of BPW

Labcode	Kind of medium	Name	Code	Batch
1	Ready-to-use	Oxoid	CM 509	354805 and 340788
2	Dehydrated	Oxoid	CM 509	335025
3	Ready-to-use	Biomerieux	42042	786916901
4	Individual ingredients	---	---	---
5	Dehydrated	Hi-media	M 028	2C18307
6	Dehydrated	Oxoid	CM 509	325700
7	Ready-to-use	Biotrading	0045	430285360
8	Dehydrated	Oxoid	CM 1049	302081
9	Dehydrated	Oxoid	CM 509	308583
10	Dehydrated	Oxoid	CM 509	344588
11	Dehydrated	Merck	1.07228	VM 304428436
12	Dehydrated	BSL Global	KM 1016	B 00614
13	Dehydrated	Merck	1.07228	VM 214428410
14	Dehydrated	Merck	1.07228	VM 259028421
15	Dehydrated	Oxoid	CM 509	335025
16	Dehydrated	Oxoid	CM 509	350687
17	Dehydrated	Oxoid	CM 509	359915
18	Dehydrated	Oxoid	CM 509	305196
19	Ready-to-use	---	BO 0144 S	887521
20	Dehydrated	Merck	1.07228	VM 182128402
21	Dehydrated	---	V11259024	---
22	Dehydrated	AES Lab	AEB 140302	421 232
23	Dehydrated	Oxoid	CM 1049	323527
24	Dehydrated	Oxoid	CM 1049	335861
25	Dehydrated	Merck	1.07228	VM 232428420
26	Dehydrated	Oxoid	CM 509	335858
27	Dehydrated	Merck	1.07228	VK 538728024
28	Dehydrated	Oxoid	CM 509	346349

--- = not reported

Table 4.2 Composition (in g/L) and pH of BPW medium

Labcode	Peptone	Sodium chloride	Disodium phosphate	Potassium dihydrogen phosphate	pH after preparation	pH at day of use
ISO 6579	10.0	5.0	9.0	1.5	7.0 ± 0.2	7.0 ± 0.2
1	10.0	5.0	9.0	1.5	7.14	---
2	10.0	5.0	3.5	1.5	7.23	---
3	10.0	5.0	9.0	1.5	---	---
4	10.0	5.0	9.0	1.5	7.0	7.0
5	10.0	5.0	9.0	1.5	7.06	7.05
6	10.0	5.0	3.5	1.5	7.32	7.28
7	10.0	5.0	3.7	1.5	---	6.93
8	10.0	5.0	3.5	1.5	7.08	7.09
9	10.0	5.0	3.5	1.5	7.2	7.2
10	10.0	5.0	3.5	1.5	7.1	---
11	10.0	5.0	9.0	1.5	7	7
12	10.0	5.0	3.5	1.5	7.2	7.15
13	10.0	5.0	9.0	1.5	7.1	7.1
14	10.0	5.0	9.0	1.5	7.06	---
15	10.0	5.0	3.5	1.5	7.2	7.2
16	10.0	5.0	3.5	1.5	7.2	7.2
17	10.0	5.0	3.5	1.5	7.2	---
18	10.0	5.0	3.5	1.5	7.2	---
19	10.0	5.0	3.5	1.5	---	---
20	10.0	5.0	9.0	1.5	7.1	7.2
21	10.0	5.0	9.0	1.5	7.2	7.2
22	10.0	5.0	3.5	1.5	7.0	7.1
23	10.0	5.0	3.5	1.5	7.18	7.22
24	10.0	5.0	3.5	1.5	7.00	---
25	10.0	5.0	9.0	1.5	7.1	7.1
26	10.0	5.0	3.5	1.5	7.22	7.21
27	10.0	5.0	5.25	5.25	7.2	---
28	10.0	5.0	3.5	1.5	7.21	7.27

--- = not reported; gray cells = deviating from ISO 6579

Table 4.3 Manufacturer of MSRV

Labcode	Kind of medium	Name	Code	Batch
1	Ready-to-use	Oxoid	CM 910	325778
2	Dehydrated	Oxoid	CM 910	344726
3	Dehydrated	Oxoid	CM 910	359040
4	Dehydrated	Merck	1.09878	VM 963978239
5	Dehydrated	Oxoid	CM 910	359040
6	Dehydrated	Oxoid	CM 910	340224
7	Dehydrated	Difco	218681	4138799
8	Dehydrated	Oxoid	CM 910	335733
9	Dehydrated	Difco	218681	4138799
10	Dehydrated	Biolife	4019822	3L1701
11	Dehydrated	Biolife	4019822	4C4601
12	Dehydrated	Oxoid	CM 910	312950
13	Dehydrated	Difco	218681	2197382
14	Dehydrated	Difco	218681	4138799
15	Dehydrated	Oxoid	CM 910	328754
16	Dehydrated	Oxoid	CM 917	351810
17	Dehydrated	Oxoid	CM 910	351810
18	Dehydrated	Oxoid	CM 910	359040
19	Ready-to-use	Biorad	355-6139	---
20	Dehydrated	Merck	1.09878	VM 963978239
21	Dehydrated	Oxoid	CM 910	344726
22	Dehydrated	AES Lab	AEB 140672	328244
23	Dehydrated	Difco	218681	4208808
24	Dehydrated	Merck	1.09878	YM 963978239
25	Dehydrated	Biokar	4C757	4C757
26	Dehydrated	Merck	1.09878	VM 963978
27	Dehydrated	Oxoid	CM 910	302075
28	Dehydrated	Oxoid	CM 910	359040

--- = not reported

Table 4.4 Composition (in g/L) and pH of MSRV

Labcode	Tryp-tose	Casein hydro-lysate	NaCl	Potass. dihydrogen phosphate	$MgCl_2$ anhydrous	Malachite green oxalate	Agar	pH after preparation	pH at day of use
draft Annex D	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.2±0.2	5.2±0.2
1	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.4	---
2	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.56	---
3	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.4	5.4
4	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.7	5.7
5	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.37	5.35
6	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.44	5.37
7	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.23	5.36
8	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.4	5.3
9	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.2	5.2
10	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.2	---
11	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.26	5.29
12	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.4	5.3
13	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.1	---
14	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.45	---
15	4.59	4.59	7.34	1.47	10.93	0.037	2.7	---	5.5
16	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.4	5.4
17	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.5	---
18	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.5	---
19	Peptone 9.2	---	7.3	1.5	10.9	0.037	2.7	---	---
20	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.6	---
21	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.4	5.4
22	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.6	5.6
23	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.2	---
24	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.55	---
25	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.2	5.2
26	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.74	---
27	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.4	---
28	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.25	5.34

--- = not reported; gray cells = deviating from draft Annex D

Table 4.5 Manufacturer of own selective medium

Labcode	Kind of medium	Name Medium	Manu-facturer	Code number	Batch	pH after preparation	pH at day of use
8	Dehydrated	RV	Oxoid	CM 669	346717	5.2	5.3
10	Dehydrated	RVS	Lab M	Lab 86	069514	5.1	---
12	Dehydrated	RVS	Lab M	Lab 86	066736	5.2	5.09
17	Dehydrated	RVS	Oxoid	CM 866	343723	5.4	---
18	Dehydrated	MKTTn	Oxoid	CM 343	248782	8.0	---
22	Dehydrated	MK	AES Lab	AEB 140702	220435	8.3	8.2
23	Dehydrated	RV	Lab M	Lab 86	-73936	5.18	5.31
26	Dehydrated	RVS	Oxoid	CM 866	333034	5.4	5.4
28	Dehydrated	SC	Oxoid	CM 699	355993	7.12	7.05

--- = not reported

Table 4.6 Composition (in g/L) of own selective medium

Labcode	Name medium	Composition	
8	RV	Soya pepton	5.0
		Sodium chloride	8.0
		K dihydrogen phosphate	1.6
		Mg Chloride	40.0
		Malachite green	0.04
10	RVS	Soy peptone	4.5
		NaCl	7.2
		Pot.dihydrogen phosphate	1.26
		Dipotassium hydrogen phosphate	0.18
		Mg chloride anhydrous	13.58
		Malachite green	0.033
12	RVS	Soy peptone	4.5
		NaCl	7.2
		Pot.dihydrogen phosphate	1.26
		Dipotassium hydrogen phosphate	0.18
		Mg chloride anhydrous	13.58
		Malachite green	0.033
17	RVS	Soy peptone	4.5
		NaCl	7.2
		Pot.dihydrogen phosphate	1.26
		Dipotassium hydrogen phosphate	0.18
		Mg chloride	13.58
		Malachite green	0.036
18	MKTTn	Tryptone	7.0
		Soya peptone	2.3
		NaCl	2.3
		CaCO3	25.0
		Sodium thiosulphate	40.7
		Ox bile	4.75
22	MK	Tryptone	7.0
		Soya peptone	2.30
		NaCl	2.30
		CaCO3	25
		Sodium thiosulphate	40.7
		Bile de boeuf	4.75
23	RV	Soy peptone	4.5
		NaCl	7.2
		Pot.dihydrogen phosphate	1.2
		Dipotassium hydrogen phosphate	0.18
		Mg chloride anhydrous	13.58
		Malachite green	0.033
26	RVS	Soy peptone	4.5
		NaCl	7.2
		Pot.dihydrogen phosphate	1.26
		Dipotassium hydrogen phosphate	0.18
		Mg chloride anhydrous	13.58
		Malachite green	0.036
28	SC	Tryptone	5.0
		Lactose	4.0
		Disodium phosphate	10.0
		L-cystine	0.01

Table 4.7 Manufacturer and pH of XLD

Labcode	Kind of medium	Manu-facturer	Code number	Batch number	pH after preparation	pH at day of use
ISO 6579	--	--	--	--	7.4 ± 0.2	7.4 ± 0.2
1	Ready-to-use	Oxoid	CM 469	355657	7.5	---
2	Dehydrated	Oxoid	CM 469	281940 and 355657	7.44 and 7.45	---
3	Ready-to-use	Biomerieux	43563	789251101	---	---
4	Dehydrated	Merck	1.05287	V 343487/918	7.4	7.4
5	Dehydrated	Merck	1.05287	WM 12867331	7.21	7.21
6	Dehydrated	Lab M	Lab 32	073837	7.26	7.33
7	Ready-to-use	Oxoid	X04-004	314459	7.11	7.03
8	Dehydrated	Oxoid	CM 469	332765	7.35	7.35
9	Dehydrated	Oxoid	Lab 32	069663	7.3	7.3
10	Dehydrated	Oxoid	CM 469	---	7.3	---
11	Dehydrated	Merck	1.05287	VM 191987 404	7.34	7.47
12	Dehydrated	Oxoid	CM 469	305175	7.4	7.36
13	Ready-to-use	Pronadisa	930	3804	---	---
14	Dehydrated	Becton D.	211838	4103337	7.6	---
15	Dehydrated	Oxoid	CM 469	335144	---	7.4
16	Dehydrated	BBL/BD	211838	3211664	7.3	7.2
17	Dehydrated	Lab M	Lab 32	073365	7.3	---
18	Dehydrated	Mast	D 230D	149608	7.3	---
19	Not tested	---	---	---	---	---
20	Dehydrated	Biorad	69124	2F0118	7.4	---
21	Ready-to-use	---	---	---	---	---
22	Dehydrated	AES Lab	AEB 153402	416732	7.5	7.7
23	Dehydrated	BBL/Difco	211838	410337	7.54	---
24	Dehydrated	Oxoid	CM 469	332765	7.40	---
25	Dehydrated	Merck	1.05287	VM 191987 404	7.4	7.5
26	Dehydrated	Oxoid	CM 469	B: 355657	7.1	---
27	Dehydrated	Merck	1.05287	VM 252 887	7.2	---
28	Dehydrated	Biomerieux	51049	780897601	7.04	7.10

--- = not reported; grey cells = deviating from ISO 6579

Table 4.8 Composition of XLD in g/L (1)

Labcode	Xylose	L-lysine	Lactose	Sucrose	NaCl	Yeast extract
ISO 6579	3.75	5.0	7.5	7.5	5.0	3.0
1	3.75	5.0	7.5	7.5	5.0	3.0
2	3.75	5.0	7.5	7.5	5.0	3.0
3	3.5	5.0	7.5	---	5.0	3.0
4	3.75	5.0	7.5	7.5	5.0	3.0
5	3.75	5.0	7.5	---	5.0	3.0
6	3.75	5.0	7.5	7.5	5.0	3.0
7	3.75	5.0	7.5	7.5	5.0	3.0
8	3.75	5.0	7.5	7.5	5.0	3.0
9	3.75	5.0	7.5	7.5	5.0	3.0
10	3.75	5.0	7.5	7.5	5.0	3.0
11	3.75	5.0	7.5	7.5	5.0	3.0
12	3.75	5.0	7.5	7.5	5.0	3.0
13	3.0	5.0	7.5	---	5.0	3.0
14	3.5	5.0	7.5	7.5	5.0	3.0
15	3.75	5.0	7.5	7.5	5.0	3.0
16	3.5	5.0	7.5	7.5	5.0	3.0
17	3.75	5.0	7.5	7.5	5.0	3.0
18	3.75	5.0	7.5	7.5	5.0	2.0
19	---	---	---	---	---	---
20	3.5	5.0	7.5	7.5	5.0	3.0
21	---	---	---	---	---	---
22	3.5	5.0	7.5	---	5.0	3.0
23	3.5	5.0	7.5	7.5	5.0	3.0
24	3.75	5.0	7.5	7.5	5.0	3.0
25	3.75	5.0	7.5	7.5	5.0	3.0
26	3.75	5.0	7.5	7.5	5.0	3.0
27	3.75	5.0	7.5	---	5.0	3.0
28	3.5	5.0	7.5	7.5	5.0	3.0

--- = not reported; grey cells = deviating from ISO 6579

Table 4.9 Composition of XLD in g/L (2)

Labcode	Phenol red	Agar	Sodium desoxy-cholate	Sodium thio-sulphate	Ferric. amm. citrate	Saccharose
ISO 6579	0.08	9 – 18	1.0	6.8	0.8	---
1	0.08	12.5	1.0	6.8	0.8	---
2	0.08	12.5	1.0	6.8	0.8	---
3	0.08	13.5	2.5	6.8	0.8	7.5
4	0.08	14.5	1.0	6.8	0.8	7.5
5	0.08	14.5	1.0	6.8	0.8	7.5
6	0.08	13.0	1.0	6.8	0.8	---
7	0.08	12.5	1.0	6.8	0.8	---
8	0.08	12.0	1.0	6.8	0.8	---
9	0.08	13.0	1.0	6.8	0.8	---
10	0.08	12.5	1.0	6.8	0.8	---
11	0.08	14.5	1.0	6.8	0.8	7.5
12	0.08	12.5	1.0	6.8	0.8	---
13	0.08	13.5	2.5	6.8	0.8	7.5
14	0.08	13.5	2.5	6.8	0.8	---
15	0.08	12.5	1.0	6.8	0.8	---
16	0.08	13.5	2.5	6.8	0.8	---
17	0.08	13.0	1.0	6.8	0.8	7.5
18	0.072	---	1.0	4.34	0.8	---
19	---	---	---	---	---	---
20	0.08	13.5	2.5	6.8	0.8	7.5
21	---	---	---	---	---	---
22	0.08	13.5	2.5	6.8	0.8	7.5
23	0.08	13.5	2.5	6.8	0.8	---
24	0.08	12.5	1.0	6.8	0.8	---
25	0.08	14.5	1.0	6.8	0.8	7.5
26	0.08	12.5	1.0	6.8	0.8	---
27	0.08	14.5	1.0	6.8	0.8	7.5
28	0.08	13.5	2.5	6.8	0.8	---

--- = not reported; grey cells = deviating from ISO 6579

Table 4.10 Manufacturer of own isolation medium

Labcode	Name medium	Prescribed incubation temp.	Kind of medium	Manu-facturer	Code number	Batch number
1	BGA	37	Ready-to-use	Oxoid	CM 329	340651
2	BPLS	37 ± 1	Dehydrated	Merck	1.10747	VM 136947
3	SMID	37	Ready-to-use	---	43621	788882201
4	BGA	37	Individual ingredients	---	---	---
	Rambach	37	Dehydrated	Merck	1.07500	OC 468580
5	BGA	37	Dehydrated	Himedia	M 971	2/192
6	Onoz	37 ± 1	Dehydrated	Merck	15034	VM 224234
7	BGA	37	Ready-to-use	Biotrading/ Lab M	Lab 34	04243008
8	BGA	37	Dehydrated	Hi Media	M 971	ZK 024
9	BGA	37± 1	Dehydrated	Oxoid	CM 329	301648
10	BGA	37	Dehydrated	Biogenetics	BM 730	4L4203
11	BPLS	37	Dehydrated	Merck	1.10747	VM 136947 338
12	BGA	37	Dehydrated	Lab M	Lab 34	066000
13	XLT4	37 ± 1	Ready-to-use	Reactivos Diagnostico	PA 0003	34897
14	Rambach	41.5	Dehydrated	Merck	1.07500	OC 468580
15	XLT4	37	Dehydrated	---	7517A	0304-138
16	BGA	37	Dehydrated	Oxoid	CM 329	346531
17	BGA	37	Dehydrated	Oxoid	CM 329	321309
18	BGA	37	Dehydrated	Oxoid	CM 329	346531
	MLCB	37	Dehydrated	Oxoid	CM 783	257055
19	XLT4	37	Ready-to-use	Biorad	356 3654	4J0135
	BGA	37	Raedy-to-use	Oxoid	PO 5033A	993723
20	SMID	37	Ready-to-use	SMID2	43621	789370601
21	Rambach	37	Dehydrated	Merck	1.07500	OC353247
22	Rambach	37	Dehydrated	Merck	1.07500	OC360063
	XLT4	37	Dehydrated	Biokar	BK 156 HA	3L 154
23	BGA	37	Dehydrated	Difco	228530	4111373
24	BxLH	37.0	Individual ingredients	---	---	Not repored
25	Rambach	37	Dehydrated	Merck	1.075000	OC 468580
26	BGA	37.0	Dehydrated	Oxoid	CM 329	321309
27	BPLSA	37	Dehydrated	Merck	1.07237	VM 003 637 311
28	McConkey	37	Dehydrated	Oxoid	CM 115	344546

--- = not reported

Table 4.11 Manufacturer and pH of nutrient agar

Labcode	Kind of medium	Manu-facturer	Code number	Batch number	pH after preparation	pH at day of use
ISO 6579	---	---	---	---	7.0 ± 0.2	7.0 ± 0.2
1	Ready-to-use	Oxoid	CM 331	347411	7.24	---
2	Individual ingredients	Difco	244400	4026902	7.38	---
4	Individual ingredients	---	---	---	7.2	7.2
8	Dehydrated	Imuna	---	850204	7.2	7.2
9	Dehydrated	Biorad	64484	3L2109	7.2	7.2
12	Dehydrated	BSL Global	KM 1073	B 00626	7.3	7.2
13	Dehydrated	Difco	21300	2298497	6.8	---
15	Dehydrated	Oxoid	CM 3	288793	---	7.1
17	Individual ingredients	---	---	040628	7.2	---
18	Dehydrated	Oxoid	CM 7B	329515	7.2	---
23	Dehydrated	Oxoid	CM 1	237111	7.29	7.23
24	Dehydrated	Biomed	10204	40903	7.35	---

--- = not reported; grey cells = deviating from ISO 6579

Table 4.12 Manufacturer and pH of TSI

Labcode	Name manufacturer	Code number	Batch number	pH of medium
ISO 6579	---	---	---	7.4 ± 0.2
2	Difco	226540	0053002	7.17
4	Home-made	---	---	---
5	Biomark	B 1116	240502	7.41
6	Merck	3915	VM 010515	7.46
7	Individual ingredients	---	---	7.15
8	Oxoid	CM 277	B 209693	7.4
9	Oxoid	CM 277	281276	7.2
10	Lab M	Lab 59	062953	7.2
11	Merck	1.03915	VM 010515311	7.45
12	Lab M	Lab 53	013093	7.4
13	Merck	1.03915	VM 010515311	---
16	Oxoid	CM 277	330767	7.4
17	BBL	211749	3007215	7.3
18	Scharlau	01-1992	11/69	7.2
20	BD/Difco	226540	2303681	7.4
23	BBL/Difco	211749	3057743	7.28
24	Home-made	---	---	7.08
25	Biokar	1K756	---	7.3
28	Oxoid	CM 277	346722	7.6

--- = not reported; grey cells = deviating from ISO 6579

Table 4.13 Manufacturer and pH of ureum agar

Labcode	Name manufacturer	Code number	Batch number	pH of medium
ISO 6579	---	---	---	6.8 ± 0.2
2	Oxoid	CM 53	326371	6.67
4	Individual ingredients	---	---	6.8
5	Individual ingredients	---	---	6.91
6	Oxoid	CM 53	326371	6.80
7	Individual ingredients	---	---	6.69
8	Oxoid	CM 53	242418	6.7
9	Sifin	TN 1143	0710404	6.8
10	Oxoid	CM 53	269475	6.8
11	Merck	1.08492	VL 864392214	6.78
12	Merck	1.08492	VL 864392214	6.8
13	Pronadisa	4233	2640	---
15	Oxoid	CM 53	293716	---
16	Oxoid	CM 53	326371	6.7
17	Individual ingredients	---	---	6.8
20	BD/Difco	228320	0313008	6.8
22	AES Lab	AEB 111545	428210	---
23	BBL/Difco	211795	3051177	6.82
25	Individual ingredients	---	686	6.6
26	Mast	DM 138-1A	168139/15	6.85
28	INCDMI	623	03-03	6.8

--- = not reported

Table 4.14 Manufacturer and pH of LDC

Labcode	Name manufacturer	Code number	Batch number	pH of medium
ISO 6579	---	---	---	6.8 ± 0.2
2	Individual ingredients	---	---	6.88
4	Individual ingredients	---	---	6.8
5	Individual ingredients	---	---	6.85
6	Difco	287220	1288012	6.91
7	Individual ingredients	---	---	6.24
8	Individual ingredients	---	---	6.8
9	Sifin	TN 1154	2330903	6.8
10	Difco	211759	1341012	6.9
11	Individual ingredients	---	---	6.9
12	Fluka	62895	47883/1	6.5
13	BD	211759	3237358	6.7
16	Oxoid	CM 381	310679	6.7
17	BBL/Sigma	43211430	1000I1DJ	6.0
19	Oxoid	TV 5028 N	893 685	---
20	BD/Difco	211759	3210447	6.8
22	BD/Difco	211759	1341012	6.8
23	Oxoid	CM 308	1178101	6.20
25	Individual ingredients	---	685	6.8
28	INCDMI	622	04-01	6.8

--- = not reported; grey cells = deviating from ISO 6579

Table 4.15 Manufacturer and pH of other confirmation media

Labcode	Name media or otherwise	Name manufacturer	Code number	Batch number	pH of medium
1	Confirmation by serotyping	---	---	---	---
4	ONPG	Home-made	---	---	7.2
10	SIM medium	Oxoid	CM 435	316085	7.1
13	Indoltest	Scharlau	064-TA0132	34786	7.34
14	Confirmation by serotyping	---	---	---	---
15	McConkey	Oxoid	CM 1	287518	7.0
16	SIM medium	Oxoid	CM 435	260110	7.3
18	API	Api	ZOE	787150501	---
19	HY Enterotest	Audit Diagnostic	TT-146	355	---
21	Confirmation by serotyping	---	---	---	---
22	Kligler agar	AES Lab	---	AEB 151252	7.4
23	Simmons citrate	Oxoid	CM 155	235670	7.04
24	ONPG	Home-made	---	---	7.39
25	Control medium for LDC	---	684	---	6.8
28	API	API	REF 20100	783353601	---

--- = not reported

Annex 5. Results of alternative methods

*Table 5.1 PCR and bacteriological culture method results of artificially contaminated samples of laboratories 3, 6 and 27 **after 18 h** of incubation in BPW*

	Labcode 3		Labcode 6		Labcode 27	
Number	BAC	PCR	BAC	PCR	BAC	PCR
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	-	+	-	+	+	+
Total	17	23	16	22	22	22

*BAC = bacteriological culture method; * = Blank capsules; + = positive; - = negative; grey cells = unexpected results*

Table 5.2 PCR, ELISA and bacteriological culture method results of artificially contaminated samples of laboratory 17

Number	MSRV 4 h	MSRV 4 h PCR con	MSRV 18 h	MSRV 18 h PCR con	Bioline ELISA Selekta	PCR Selekta	IMS-PCR Selekta	iQ-check Biorad
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	+	+	-		-	-	+	+
Total	10	10	15	15	17	21	22	22

*BAC = bacteriological culture method; * = Blank capsules; + = positive; - = negative; PCR con = PCR confirmation*

Table 5.3 PCR and bacteriological culture method results of naturally contaminated samples of laboratories 6 and 27 ***after 18 h*** *of incubation in BPW*

	Labcode 6		Labcode 27	
Number	BAC	PCR	BAC	PCR
N1	+	+	-	-
N2	+	+	+	-
N3	+	+	-	-
N4	+	+	-	-
N5	+	+	+	-
N6	+	+	-	-
N7	+	+	-	-
N8	+	+	-	-
N9	+	+	+	-
N10	+	+	+	-
N11	+	+	-	-
N12	+	+	-	-
N13	+	+	-	-
N14	+	+	-	-
N15	+	+	-	-
N16	+	+	+	-
N17	+	+	-	-
N18	+	+	+	-
N19	+	+	-	-
N20	+	+	+	-
Total	20	20	7	0

BAC = bacteriological culture method; + = positive; - = negative

Table 5.4 PCR, ELISA and bacteriological culture method results of naturally contaminated samples of laboratory 17

Number	MSRV 4 h	MSRV 4 h PCR con	MSRV 18 h	MSRV 18 h PCR con	Bioline ELISA Selekta	PCR Selekta	IMS-PCR Selekta	iQ-check Biorad
N1	+	+	-		-	-	+	+
N2	+	+	-		-	-	-	+
N3	+	+	-		-	-	-	+
N4	+	+	-		-	-	-	-
N5	+	+	-		-	-	-	+
N6	+	+	-		-	-	+	+
N7	+	+	-		-	-	+	+
N8	+	+	-		-	-	+	-
N9	+	+	-		-	-	+	-
N10	+	+	-		-	-	-	+
N11	+	+	-		-	-	+	+
N12	+	+	-		-	-	+	-
N13	+	+	-		-	-	+	-
N14	+	+	-		-	-	-	-
N15	+	+	-		-	-	+	+
N16	+	+	-		-	-	+	-
N17	+	+	-		-	-	-	+
N18	+	+	-		-	-	+	+
N19	+	+	-		-	-	-	-
N20	+	+	-		-	-	-	+
Total	20	20	0	0	0	0	11	12

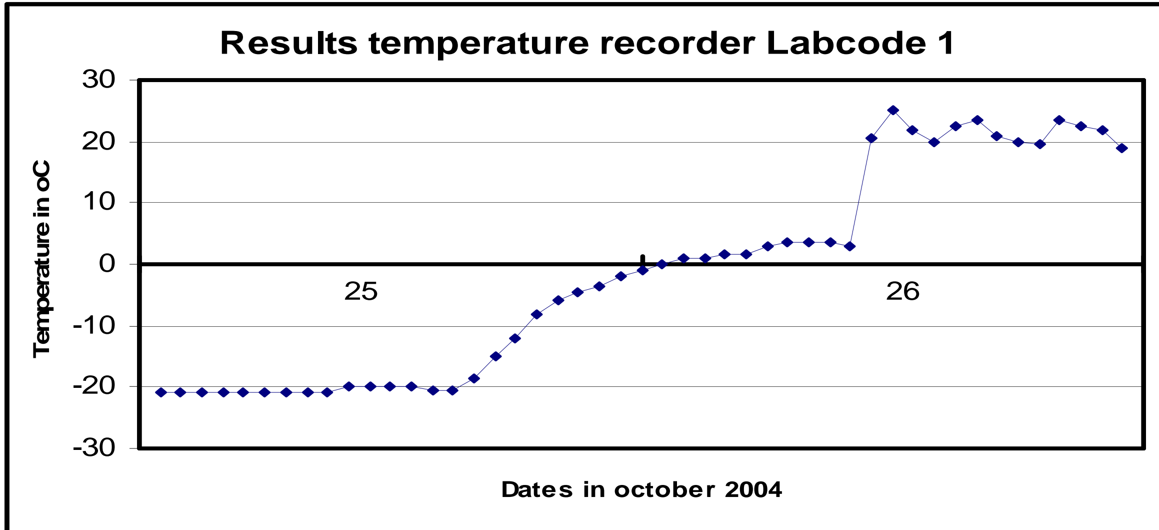
*BAC = bacteriological culture method; * = Blank capsules; + = positive; - = negative; PCR con = PCR confirmation*

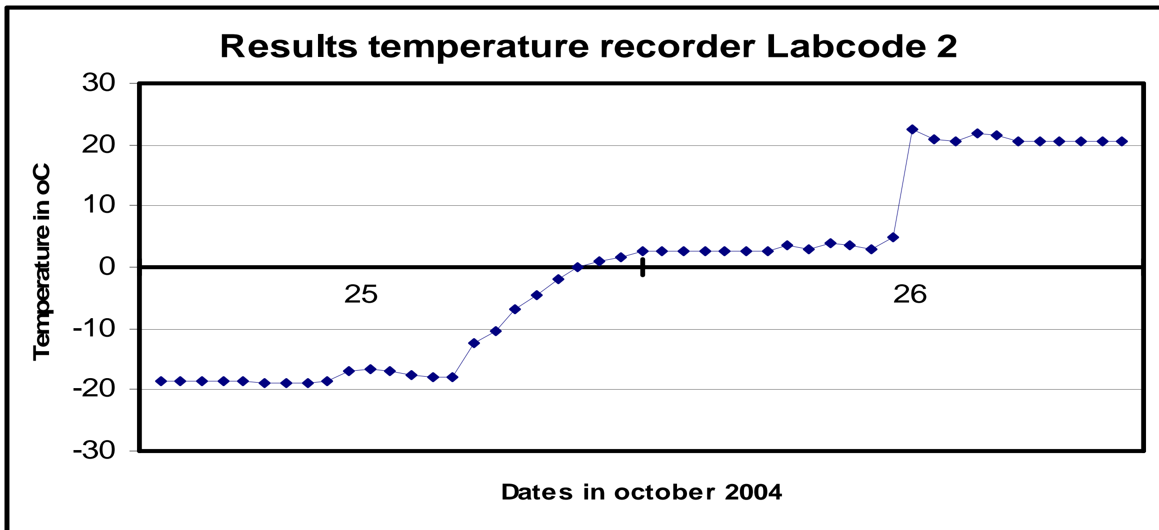
Table 5.5 PCR and bacteriological culture method results of control samples of laboratories 6 and 27 ***after 18 h*** *of incubation in BPW*

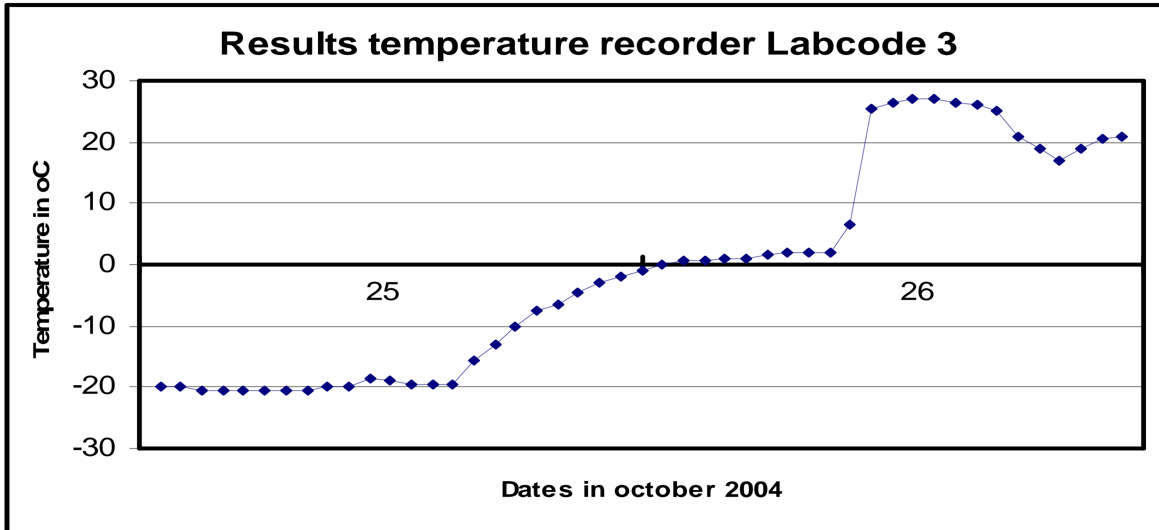
	Labcode 6		Labcode 27	
Number	BAC	PCR	BAC	PCR
C1	-	-	-	-
C2	+	+	+	+
C3	-	-	-	-
C4	+	+	+	+
C5	+	+	+	+
C6	+	+	+	+
C7	+	+	+	+
C8	+	+	+	+
C9	+	+	+	+
C10	+	+	+	+
C11	-	-	-	-
C12	-	-	-	-
Total	8	8	8	8

BAC = bacteriological culture method; + = positive; - = negative

Annex 6. Temperature recording



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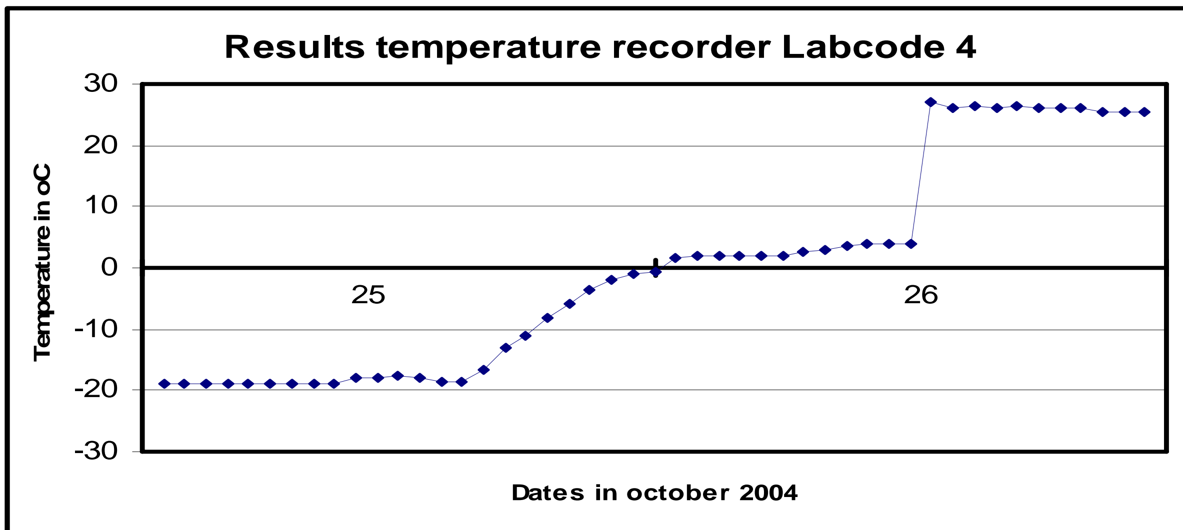
-30
25
26
Dates in october 2004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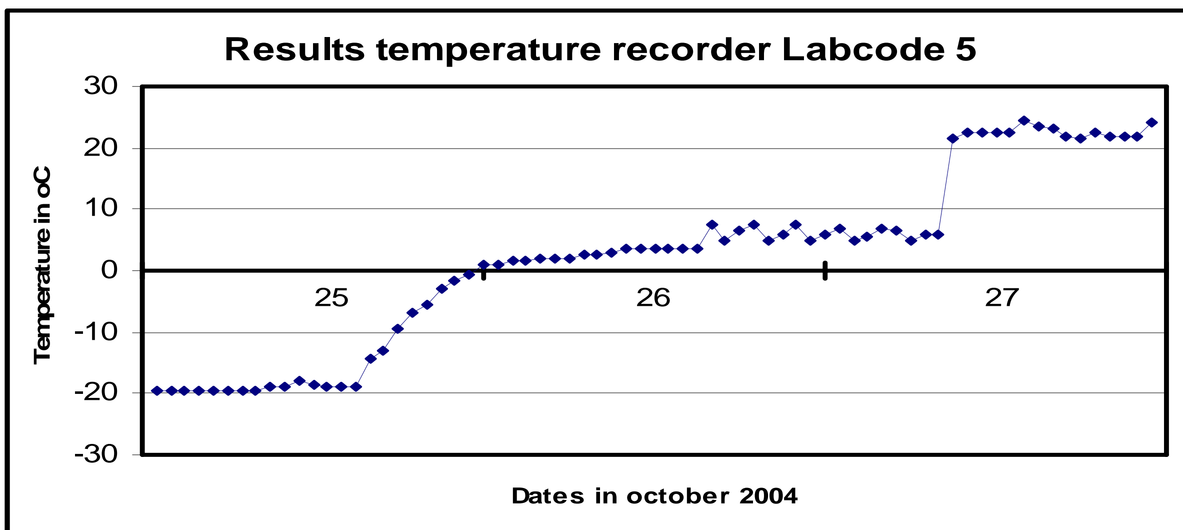
-30
25
26
Dates in october 2004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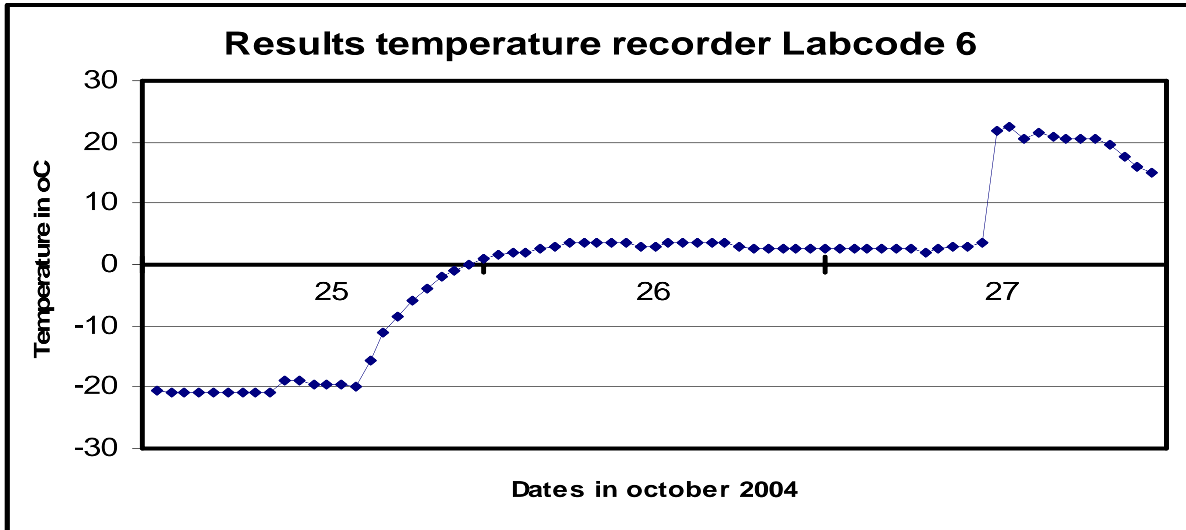
-10
-20
-30
25
26
27
Dates in october 2004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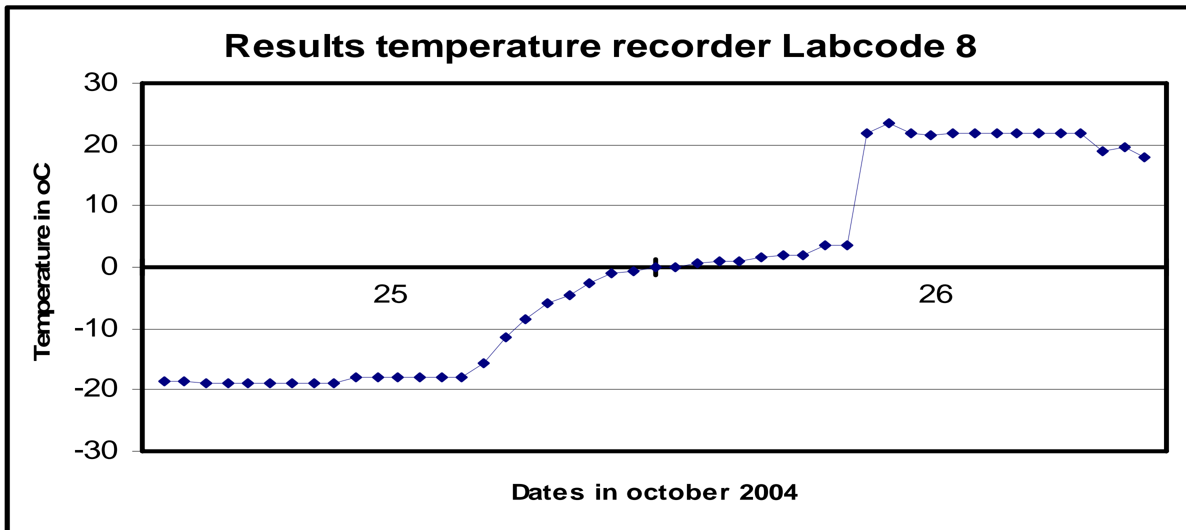
25
26
27
Dates in october 2004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-10
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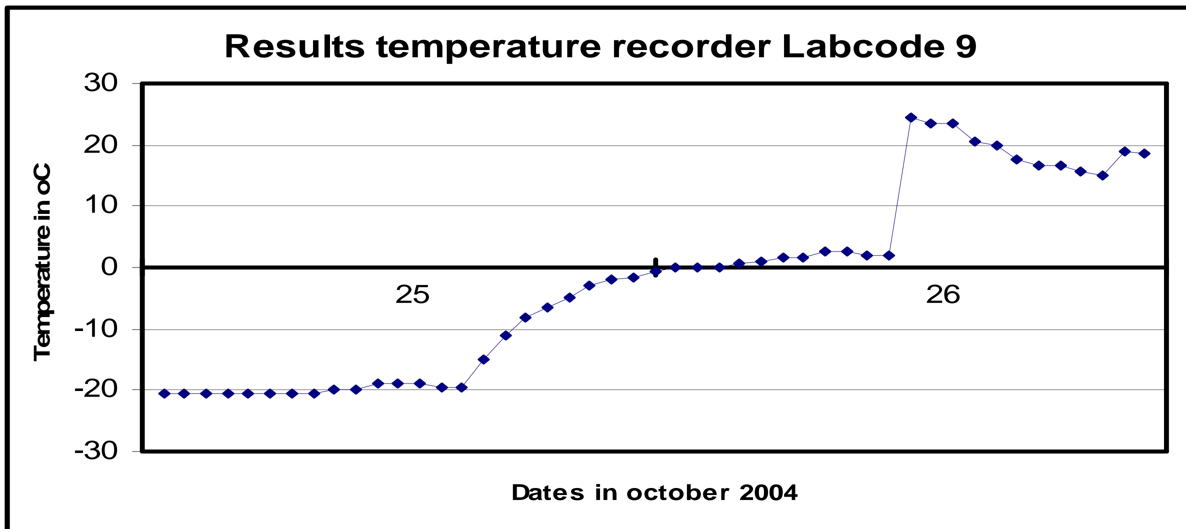
-30
25
26
Dates in october 2004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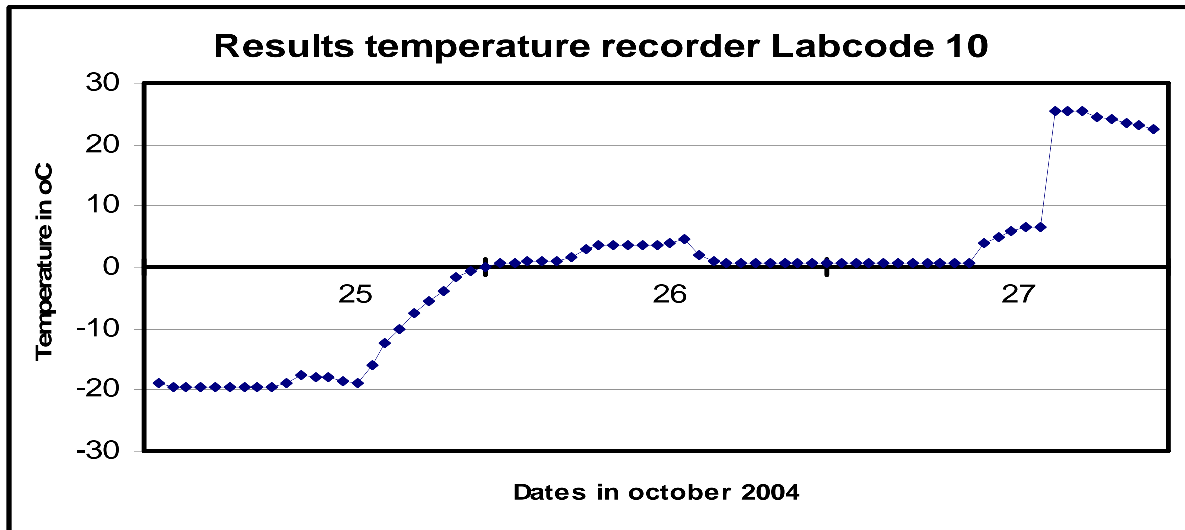
0
-10
-20
-30
25
26
Dates in october 2004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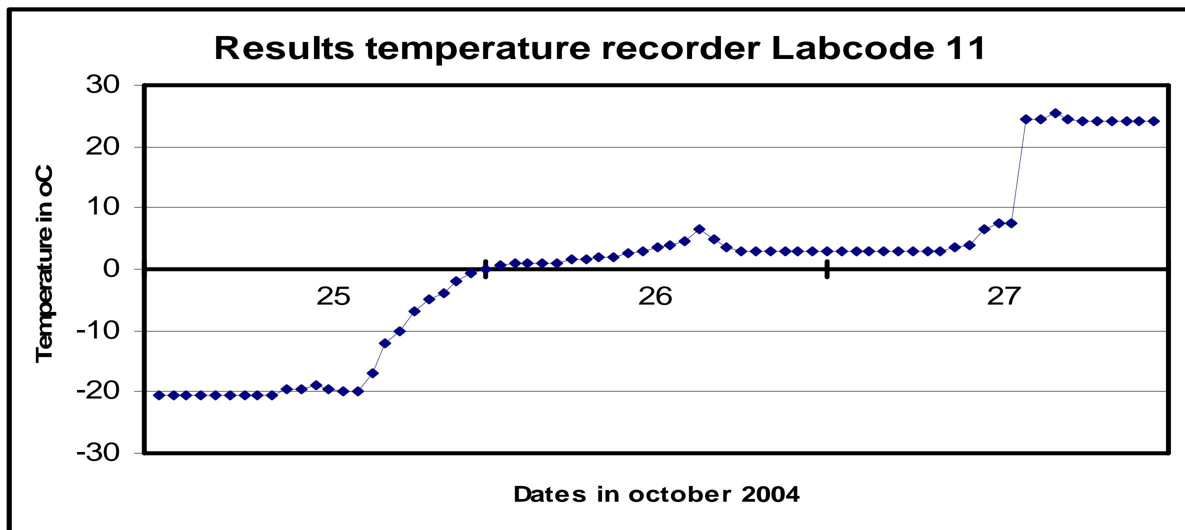
-20
-30
Temperature in oC
25
26
27
Dates in october 2004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Temperature in oC
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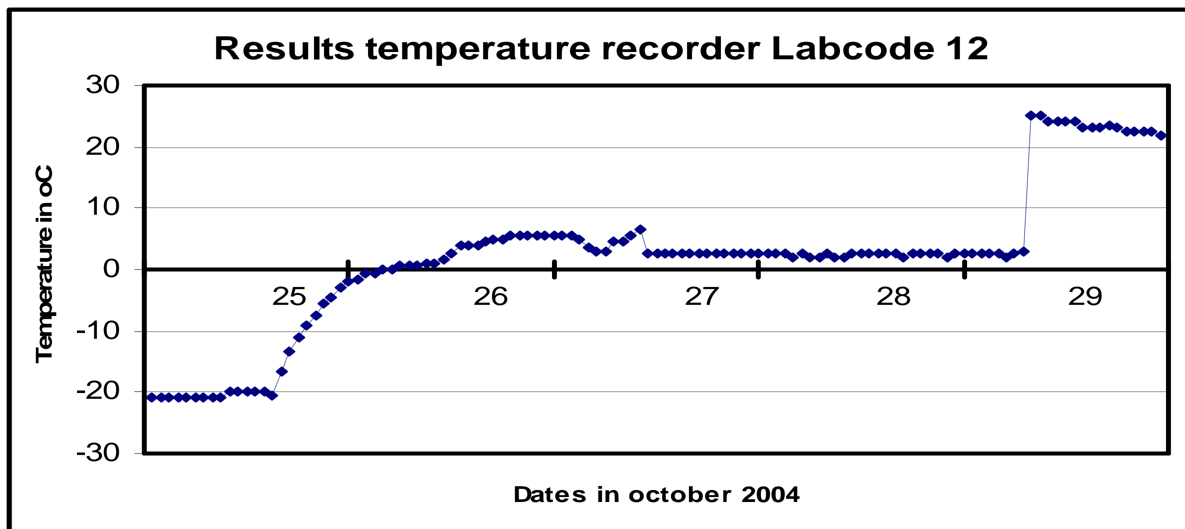
27
Dates in october 2004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Temperature in oC
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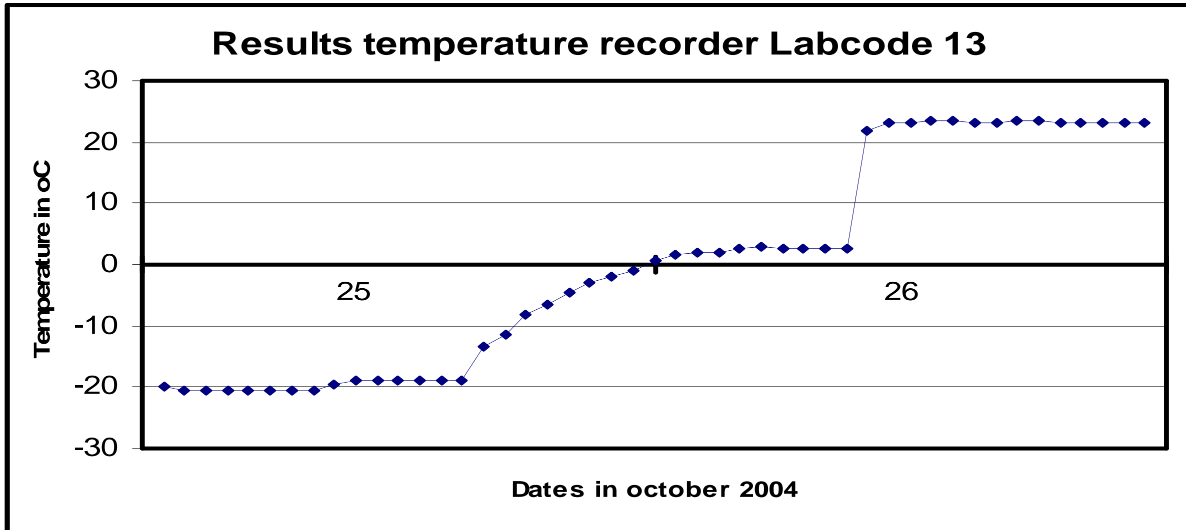
26
27
28
29
Dates in october 2004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25
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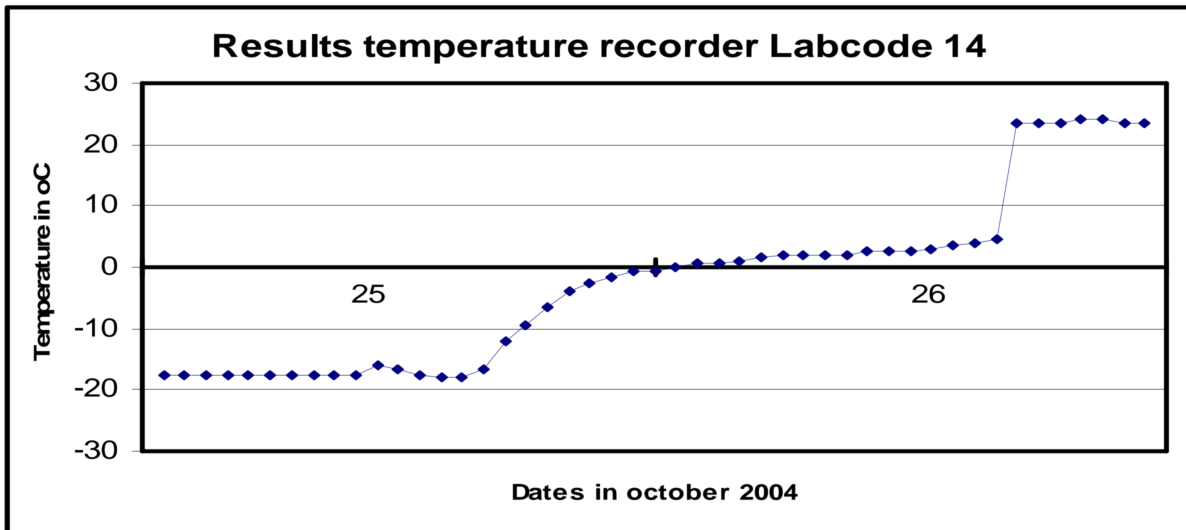
Dates in october 2004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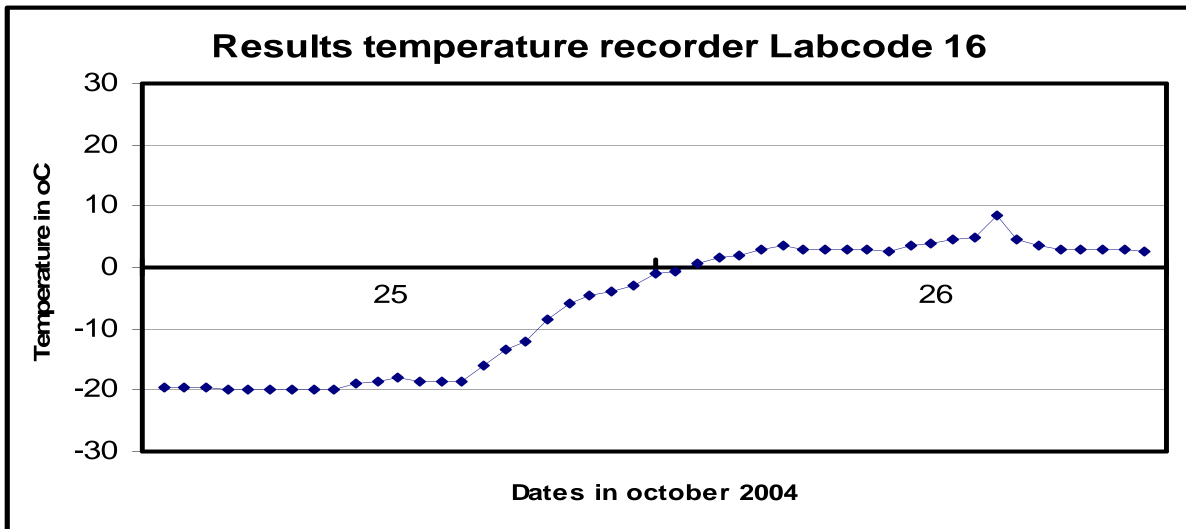
-10
-20
-30
25
26
Dates in october 2004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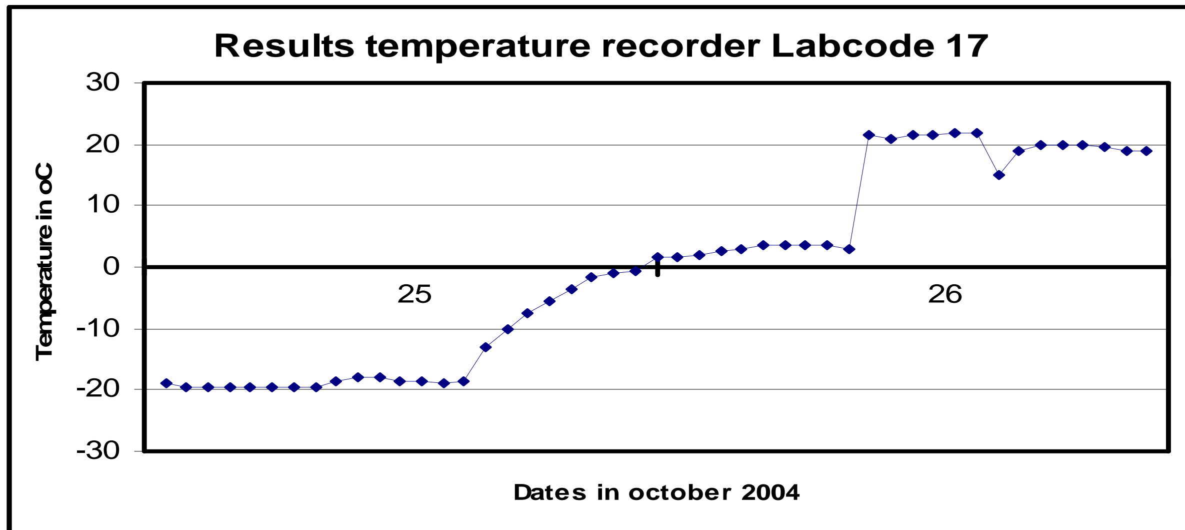
20
10
0
-10
-20
-30
25
26
Dates in october 2004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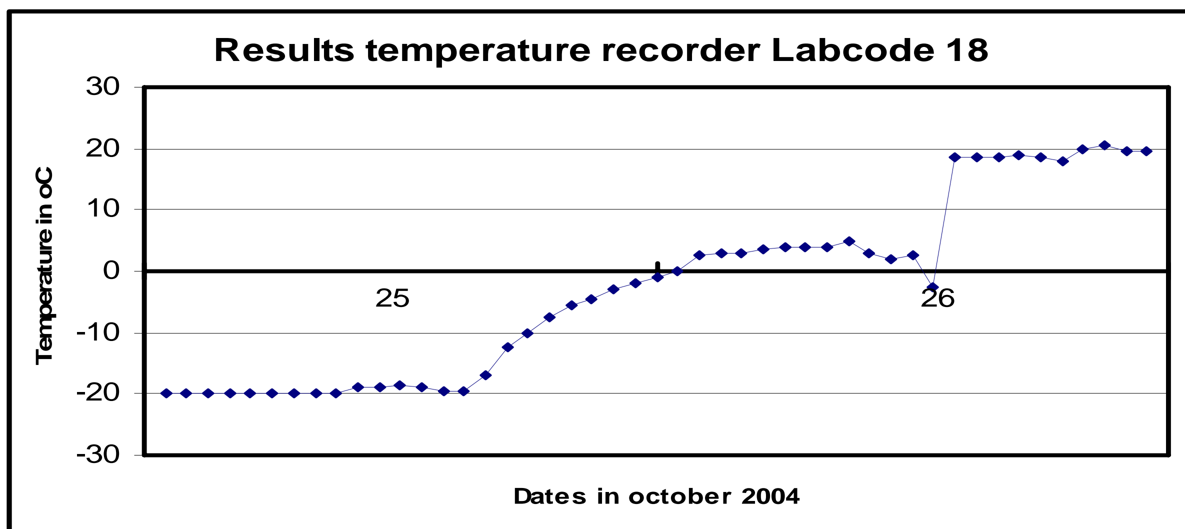
0
-10
-20
-30
25
26
Dates in october 2004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20
10
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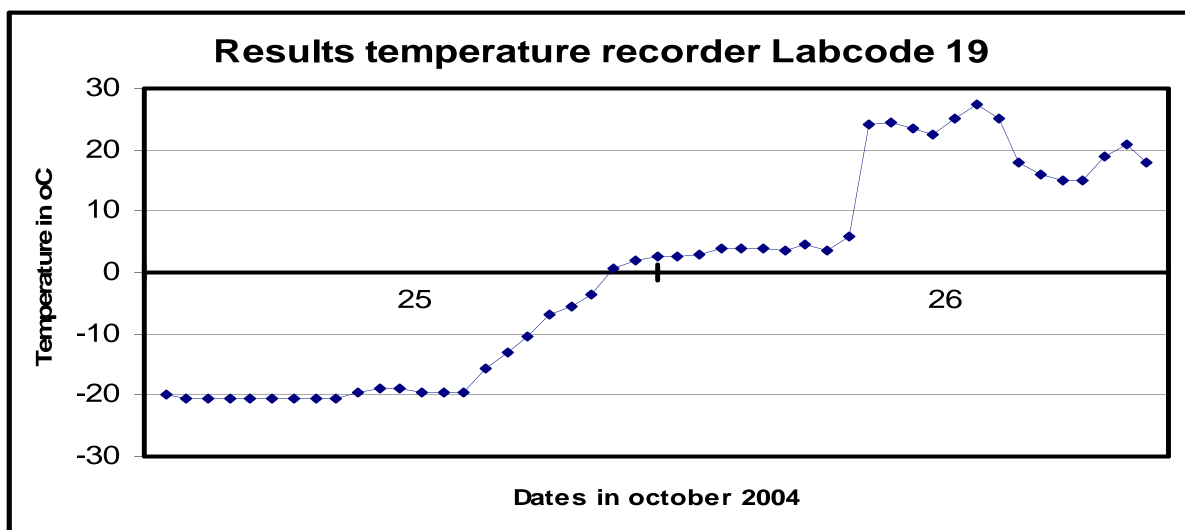
-30
25
26
Dates in october 2004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Temperature in oC
30
20
10
0
-10
-20
-30
25
26
Dates in october 2004

Results temperature recorder Labcode 20

Temperature in oC

30
20
10
0
-10
-20
-30

25
26

Dates in october 2004

Results temperature recorder Labcode 21

Temperature in oC

30
20
10
0
-10
-20
-30

25
26
27
28

Dates in october 2004

Results temperature recorder Labcode 22

Temperature in oC

30
20
10
0
-10
-20
-30

25
26
27

Dates in october 2004

Results temperature recorder Labcode 23

Temperature in oC

30
20
10
0
-10
-20
-30

25 26 27

Dates in october 2004

Results temperature recorder Labcode 24

Temperature in oC

30
20
10
0
-10
-20
-30

25 26 27 28

Dates in october 2004

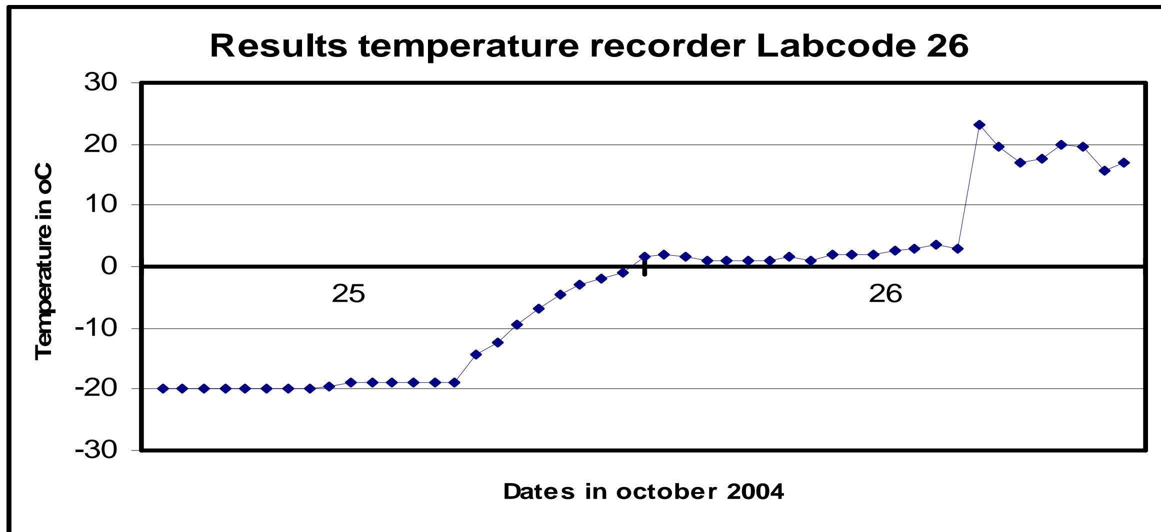
Results temperature recorder Labcode 25

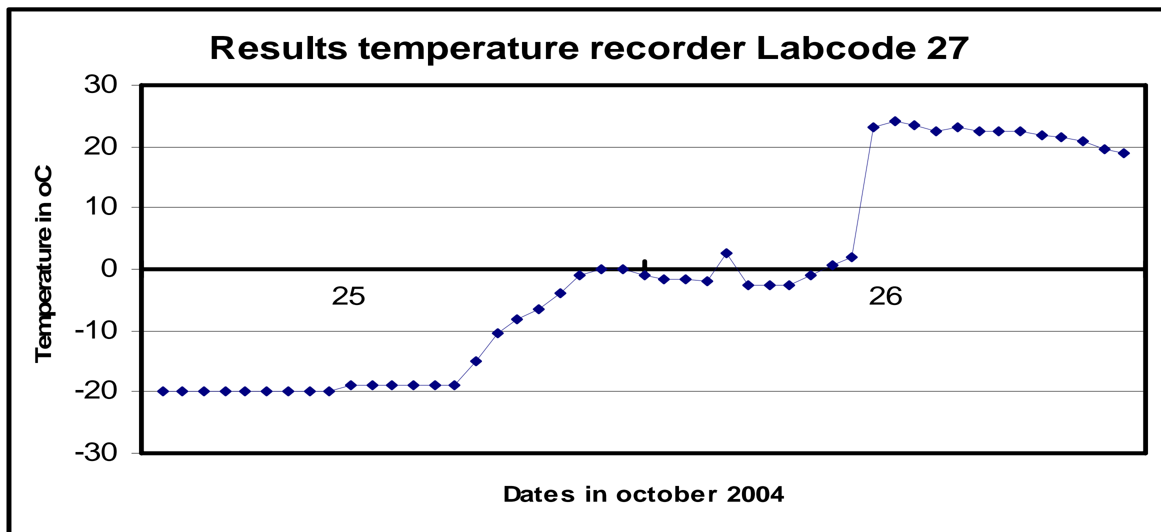
Temperature in oC

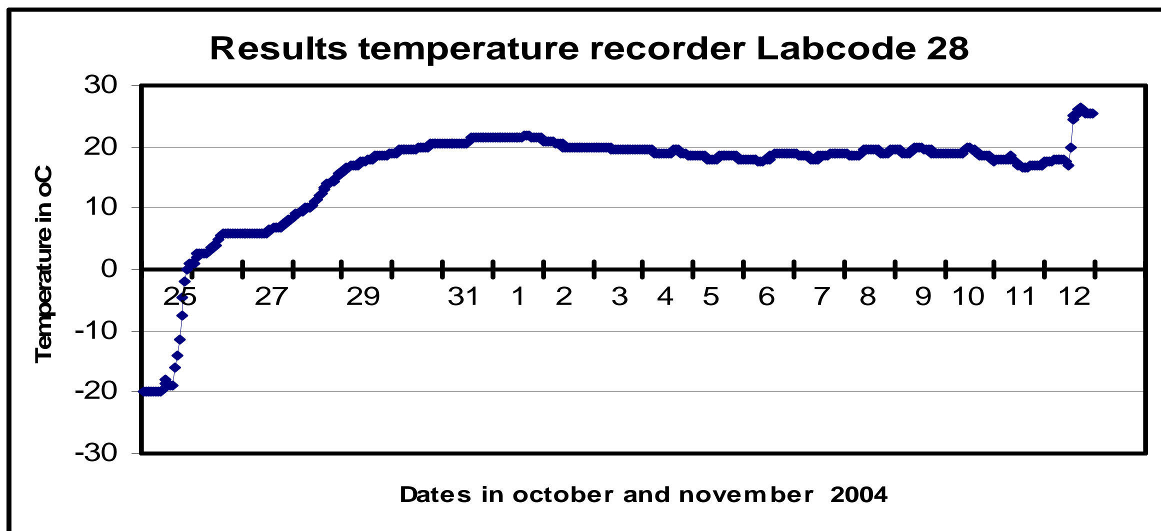
30
20
10
0
-10
-20
-30

25 26

Dates in october 2004


Results temperature recorder Labcode 26
Temperature in oC
30
20
10
0
-10
-20
-30
25
26
Dates in october 2004


Results temperature recorder Labcode 27
Temperature in oC
30
20
10
0
-10
-20
-30
25
26
Dates in october 2004


Results temperature recorder Labcode 28
Temperature in oC
30
20
10
0
-10
-20
-30
25
27
29
31
1
2
3
4
5
6
7
8
9
10
11
12
Dates in october and november 2004

Annex 7. Protocol

INTERLABORATORY COMPARISON STUDY VIII (2004) ON THE DETECTION OF *SALMONELLA* spp. organised by CRL-*Salmonella*

Introduction

This 8th interlaboratory comparison study on the detection of *Salmonella* spp. amongst the National Reference Laboratories (NRLs) in the EU, will be slightly different when compared to the 7th study.
At the workshop of May 2004 it was discussed to simplify the 8th study by decreasing the number of methods and it was discussed whether other samples than faecal samples, like dust, could be introduced. For the latter it was unfortunately not possible to obtain and test dust samples at the CRL-*Salmonella* before the study. Therefore dust will not be introduced in this study. Concerning the methods it was agreed to prescribe only the method which will be introduced in the new (draft) Annex D of ISO 6579. The method in this annex is especially intended for the detection of *Salmonella* spp. in animal faeces and samples of the primary production stage (like dust).
The procedure in this Annex D is based on ISO 6579, with the only difference that both selective broths of ISO 6579 are replaced by one semi-solid agar: modified semi-solid Rappaport Vassiliadis (MSRV), to be incubated for in total 48 h. By prescribing the method of the new annex the study of 2004 is therefore ‘simplified’ when compared to the study of 2003. However, newly introduced will be different incubation times of the pre-enrichment (Buffered Peptone Water). Recent studies of the CRL where naturally and artificially contaminated chicken faeces were incubated in BPW for 4 h and 18 h revealed (much) more positive results with the shortly incubated BPW. Both incubation times of BPW will therefore be introduced in this 2004 study [(4 ± ½) h and (18 ± 2) h].
Another difference from the study of 2003 will be that the laboratories will not be informed in advance on the number per type of capsules. This may be better for the randomisation of the study.
Finally, similar to former studies, laboratories who are interested can also perform PCR on the samples and/or use additional methods (routinely) used in their laboratories.

For the samples again reference materials (RMs) and poultry faeces will be used. The RMs consists of gelatin capsules containing sublethally injured *Salmonella* Typhimurium (STM), *Salmonella* Enteritidis (SE) or *Salmonella* Panama (SPan) at different contamination levels. Each laboratory will again examine 25 faeces samples (**10 g each** and negative for *Salmonella* spp.) in combination with a capsule containing STM or SE and 10 control samples (no faeces added to the capsule). Next to the capsules, again 20 faeces samples (10 g each) which are naturally contaminated with *Salmonella* will be examined (no addition of capsules).

Finally, to obtain more detailed information on the temperatures and times during transport of the samples we will include again an electronic temperature recorder in the parcel. The amount of materials can not be packed in one parcel and will be divided over three parcels (one containing capsules, one containing *Salmonella* negative faeces and one containing *Salmonella* positive faeces). The three parcels, however, are packed in one box. We will include only one recorder and only in the parcel containing the capsules. The recorder will be packed in a plastic bag, which will also contain your labcode. **You are urgently requested to return this complete plastic bag with recorder and labcode to the CRL-*Salmonella*, immediately after receipt of the parcel**. For this purpose a return envelope with a preprinted address label of the CRL-*Salmonella* has been included.
The boxes (each containing 3 parcels) will be sent as diagnostic specimens by door-to-door courier service. Please contact CRL-*Salmonella* when the parcel has not arrived at your laboratory within 5 working days after the day of mailing (25th of October 2004)

Objectives

The main objective of the eighth interlaboratory comparison 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.
A derived objective of the study is to evaluate the influence of the incubation time of the pre-enrichment broth on the detection of *Salmonella* in naturally and artificially contaminated chicken faeces.

Outline of the study
Each participant will receive one box containing three separate parcels.
Parcel 1:
- 25 numbered vials; each containing one *Salmonella* Typhimurium, one *Salmonella* Enteritidis or blank capsule (numbered 1-25);
- 10 control vials; each containing one capsule with or without *Salmonella* (numbered C1-C10).

This parcel will contain the small electronic temperature recorder in a plastic bag with your labcode. **This recorder (in the plastic bag) should be returned to the CRL-*Salmonella* as soon as possible**.
Parcel 2:
- 300 g of poultry faeces (free from *Salmonella*).

Parcel 3:
- 250 g of naturally contaminated (with *Salmonella*) faeces.

The performance of the study will be in <u>week 46</u> (starting on 8 November 2004).
The documents necessary for performing the study are:
- Protocol Bacteriological Interlaboratory Comparison Study VIII (2004), on the detection of *Salmonella* spp.;
- SOP Bacteriological Interlaboratory Comparison Study VIII (2004), on the detection of *Salmonella* spp.
- Test Report Bacteriological Interlaboratory Comparison Study VIII (2004), on the detection of *Salmonella* spp.;
- ISO 6579 (2002). Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.;
- Draft Annex D of ISO 6579 (Oct. 2004)

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 interlaboratory comparison study will not be supplied by the CRL.

Time table of bacteriological interlaboratory comparison study VIII (2004)

Week	Date	Topic
42	11 – 15 October	Mailing of the protocol, standard operating procedure and test report to the NRLs
44	25 – 29 October	Mailing of the parcels to the NRLs as diagnostic specimens by door-to-door courier service. Immediately after arrival of the parcels at the laboratory: - Check for any serious damages (**do not accept damaged packages)**; - Check for completeness; - Remove the electronic temperature recorder from the parcel (leave it in the plastic bag with labcode) and **return it to CRL-*Salmonella* using the return envelope**; - **store all materials at -20°C ± 5°C**. If you did not receive the parcel at 29 October, do contact the CRL immediately.
45	1 – 5 November	Preparation of: 1. Non selective pre-enrichment medium (see SOP 6.1) 2. Selective enrichment media (see SOP 6.2) 3. Solid selective plating media (see SOP 6.3) 4. Confirmation media (see SOP 6.4)
46	8 - 12 November	Performance of the study, following the instructions as given in the protocol and the SOP of study VIII (2004).
48	22 – 26 November	Completion of the test report and faxing or e-mailing it to the CRL. The original test report will be sent to CRL.
49	29 November – 3 December	Data input at CRL-Salmonella and sending these data by CRL to NRLs for checking
50	6 – 10 December	Checking the results by the National Reference Laboratories.

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

Kirsten Mooijman (tel. number: + 31 30 274 3537) or
Hans Korver (tel. number: + 31 30 274 4263)
Fax. number: + 31 30 274 4434
E-mail: Kirsten.Mooijman@rivm.nl and Hans.Korver@rivm.nl

Annex 8. Standard Operation Procedure

INTERLABORATORY COMPARISON STUDY VIII (2004) ON THE DETECTION OF *SALMONELLA* spp. organised by CRL-*Salmonella*

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 (STM), *Salmonella* Enteritidis (SE) or *Salmonella* Panama (SPan) as prepared by the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*) are used. Furthermore poultry faeces is used. The application of this SOP is limited to the interlaboratory comparison 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 of a test strain in 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 List of abbreviations

BPW	Buffered Peptone Water
MSRV	Modified semi-solid Rappaport Vassiliadis medium
RM	Reference Material
SOP	Standard Operating Procedure
TSI	Triple sugar/iron agar
UA	Urea Agar
XLD	Xylose Lysine Deoxycholate agar

6 Culture media

For this study the 'prescribed' method (according to the draft Annex D of ISO 6579) is as follows:

Non selective pre-enrichment medium	BPW (beside incubation of 18 h also incubation of 4 h)
Selective enrichment medium	MSRV
Selective plating medium for first and second isolation	XLD and a second medium for choice (obligatory !)

Composition and preparation of the media and reagents of the 'prescribed' method are described in Annex B, and in draft Annex D of the ISO 6579: 2002(E). Complete ready-to-use media or dehydrated media are also allowed to be used, as long as the composition is in accordance with the information given below. Control the quality of the media before use.

Beside the 'prescribed'method it is allowed to use other methods, e.g. the one(s) routinely used in your laboratory ['Own' method(s)]. Prepare media for the 'own' method(s) according to the relevant instructions. Note all relevant information in the test report.

6.1 Non selective pre-enrichment medium

- Buffered Peptone water (BPW) (Annex B.1)
  Mind to distribute the BPW in portions of **90 ml** into suitable flasks before sterilisation.

6.2 Selective enrichment medium

- Modified Semi solid Rappaport Vassiliadis (MSRV) — Draft Annex D (October, 2004) of ISO 6579
  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 novobiocin has to be added.
  The final concentration of the novobiocin in the medium should be 0.02 g/l. Plates should be poured with a volume of 15 to 20 ml.
- Selective enrichment medium routinely used in your laboratory (optionally)

6.3 Solid selective media for first and second isolation

- 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 15-20 ml in 90 mm plates or 30-40 ml in 140 mm-plates.
- Second selective plating medium of choice (obligatory)
- Own medium (optionally)

6.4 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 (optional) — (Annex B.5)

7 Apparatus and glassware

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

7.1 Apparatus

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

7.2 Glassware

- Culture bottles or jars with nominal capacity of 200 ml;
- Culture tubes with approximate sizes: 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).

8 Procedure

8.1 General

Below the 'prescribed' method of the eighth interlaboratory comparison study of CRL- Salmonella is described. The different steps in the procedure are also summarised in Annex A of this SOP. Beside the 'prescribed'method it is also allowed to use one or more own methods. Please record all relevant data in the test report.
Mind that for the 'prescribed' method two incubation times of BPW is prescribed [(4 ± ½) h and (18 ± 2) h]. Use for your own method(s) the incubation time of BPW which is relevant for the method (note on the test report)

8.2 Prewarming BPW and thawing faeces

Take the frozen faeces out of the freezer at the end of the day (eg. on Monday) before you start testing and thaw the portions frozen faeces in the closed container **overnight at 5 °C.** Label 25 jars containing 90 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 g faeces is added (= C12). These control jars should further be handled in the same way as the other jars. Place all jars **overnight** at (37 ± 1) °C. Also place some extra non-labelled jars containing 90 ml of BPW at 37 °C. These extra jars can be used in case some jars might have been contaminated. Record in the test report (page 2) the requested data of BPW.

8.3 Pre-enrichment

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.
Shortly before adding the capsules, take the jars with BPW from the 37 °C incubator and inspect them for visual growth. Discard infected jars.
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 **45 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 45 minutes add the thawed faeces to the jars according to the following scheme:

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

- **Add 10 grams of faeces from portion 2 to jars labelled N1-N20.**

Do not shake the jars after adding the faeces.

Place all jars in the (37 ± 1) °C incubator for (4 ± ½) 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. After incubation and transferring the requested amount of material to the selective enrichment medium, incubate the jars further for a total of (18 ± 2) h at (37 ± 1) ^{o}C.

8.4 Selective enrichment

Allow the MSRV-plates 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 MSRV plates and if used, the data of the own selective enrichment broth in the test report. Label 25 plates of MSRV from 1 to 25. Also label 20 MSRV-plates from N1 to N20 and 12 plates from C1 to C12. All plates are incubated for (24 ± 3) h and later on for another (24 ± 3) h. **Please note that this study is divided into two parts: one with an incubation time of the BPW of (4 ± ½) h and the other with an incubation time of the BPW of (18 ± 2) h. So, subsequently all media will be inoculated and incubated accordingly.**

After equilibration: 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 ± 1) ^{o}C for (24 ± 3) h and later on another (24 ± 3) h. Record the temperature and time at the start and at the end of the incubation period and other requested data in the test report (page 4-7).

8.5 Isolation media (first and second isolation)

Note:

In the case that you do not have large dishes (140 mm) at your disposal use two small (90-100 mm) dishes, one after the other, using the same loop.
Record in the test report (page 8-13) 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 the suspect MSRV plates, the surface of an isolation medium in a large size Petri dish with the corresponding label number. The following isolation media will be used:

1) Xylose Lysine Desoxycholate agar (XLD)
   Place the Petri dishes with the bottom up in the incubator set at (37 ± 1) °C (note the temperature and time at the start and at the end of the incubation and other requested data on test report, page 8-9).

2) Second isolation agar plate for choice (obligatory!). Please note all relevant details on incubation temperature and time in the test report (page 10-11).

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

Second isolation after 48 h
After a total incubation time of two times (24 ± 3) h of the MSRV-plates, repeat the procedure described above (**First isolation after 24 h**).

8.6 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 ± 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 14-15) the requested data of the nutrient agar. Incubate the inoculated plates at (37 ± 1) °C for (24 ± 3) h.
If the selected colony is not confirmed as *Salmonella*, test at maximum another 5 typical colonies from the original isolation medium (stored at 5 °C). Report the number of colonies tested and the number of colonies confirmed as *Salmonella* for each dish in Table 1 [incubation time of BPW of (4 ± ½) h and selective enrichment on MSRV], Table 2 [incubation time of BPW of (18 ± 2) h and selective enrichment on MSRV] and Table 3 (isolation using own enrichment) on test report page 18-26. For the results of detection of *Salmonella* using PCR fill in Table 4 on test report page 27.

Biochemical confirmation
By means of a loop, inoculate the media specified below with the colony selected as described above (either directly from the isolation medium, or from nutrient agar). For each of the mentioned media follow the instructions in 9.5 of ISO 6579 (2002). Optionally inoculate other media which are routinely used for biochemical confirmation. Record in test report (page 20) the requested data of the media.

- TSI agar
- Urea agar
- l-Lysine decarboxylation medium

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

9 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.

Annex 9. Draft Annex D of ISO 6579 (Oct ’04)

Annex D
(normative)

Detection of *Salmonella* spp. in animal faeces and in samples of the primary production stage

D.1 Scope

ISO 6579 is primarily intended for the isolation of *Salmonella* spp. from food and feeding stuffs and is not always suitable for the detection of *Salmonella* spp. from other matrices.

This annex is applicable to the detection of *Salmonella* spp. in:
- Animal faeces (like poultry, pigs, cattle);
- Environmental samples in the area of the primary production stage (like dust).

The method in this annex is based upon ISO 6579, with a different selective enrichment. Therefore, where possible reference will be made to the full text of ISO 6579.

WARNING The selective enrichment medium as described in this annex (being Modified Semi-solid Rappaort Vassiliadis: MSRV) is less appropriate for the detection of non-motile Salmonellae. If non-motiles are expected it is advised to use beside MSRV also a liquid selective enrichment (see full text ISO 6579).

D.2 Normative references
See Ch. 2 of ISO 6579

D.3 Terms and definitions
See Ch. 3 of ISO 6579

D.4 Principle

D.4.1 General
The detection of *Salmonella* in animal faeces and in samples of the primary production stage necessitates four stages, like described in Ch. 4 of ISO 6579.

D.4.2 Pre-enrichment in non-selective liquid medium
Buffered Peptone Water (BPW) is inoculated at ambient temperature with the test portion, then incubated at (37 ± 1) °C for (18 ± 2) h.

NOTE Due to high concentrations of (disturbing) background flora in the faecal samples it may happen that *Salmonella* is not detected because it has been overgrown by the background flora during the pre-enrichment. For some type of samples it may therefore be advantage to shorten the incubation time of the non-selective pre-enrichment. In this respect an incubation time of 4-6 h may give good results.

D.4.3 Enrichment on selective semi-solid medium

Modified Semi-solid Rappaport Vassiliadis (MSRV) agar plates are inoculated with the culture obtained in D.4.2.
The MSRV is incubated at (41,5 ± 1) °C for (24 ± 3) h. If a plate is negative after 24 h it will be incubated for a further (24 ± 3) h.

D.4.4 Plating-out and identification

From the culture obtained in D.4.3, two selective solid media are inoculated:
- Xylose Lysine Deoxycholate (XLD) agar;
- Any other solid selective medium complementary to XLD agar (see 4.4 of ISO 6579).

The XLD agar is incubated at (37 ± 1) °C and examined after (24 ± 3) h.
The second selective agar is incubated in accordance with the manufacturer's instructions.

D.4.5 Confirmation of identity

Colonies of presumptive *Salmonella* are subcultured, then plated-out as described in D.4.4, and their identity is confirmed by means of appropriate biochemical and serological tests.

D.5 Culture media, reagents and sera

D.5.1 General

All media and reagents needed for this annex are described in Annex B, accept for Modified Semi-solid Rappaport Vassiliadis (MSRV) medium, which is described in this annex in D.5.2.
Alternatively, dehydrated complete media or diluents may be used. Follow, in that respect, the manufacturer's instructions.
For the preparation of the media use glass-distilled or demineralised water, free from substances that might affect growth of micro-organisms under the test conditions.

D.5.2 Modified Semi-solid Rappaport Vassiliadis medium (MSRV)

D.5.2.1 Base medium

Composition

Tryptose	4,59	g
Caseine hydrolysate	4,59	g
Sodium chloride (NaCl)	7,34	g
Potassium dihydrogen phosphate (KH_2PO_4)	1,47	g
Magnesium chloride anhydrous ($MgCL_2$)	10,93	g
Malachite green oxalate	0,037	g
Agar	2,7	g
Water	1000	ml

Preparation

Suspend the ingredients into the water.
Heat to boiling with agitation. **Do not autoclave**.
After boiling, the medium should be transparent blue. Do not hold the medium at high temperatures longer than necessary.
Cool the medium to approximately 50 °C.

D.5.2.2 Novobiocin solution

Composition

Novobiocin sodium salt	0,02	g
Water (D.5.1)	2	ml

Preparation

Dissolve the novobiocin sodium salt in the water.
Sterilise by filtration through a filter with a pore size of 0,22 µm.

The solution may be stored in small portions (e.g. of 2 ml) at -20 °C for up to one year.

D.5.2.3 Complete medium

Composition

Base medium (D.5.2.1)	1000	ml
Novobiocin solution (D.5.2.2)	2	ml

Preparation

Aseptically add 2 ml of the novobiocin solution (D.5.2.2) to 1000 ml of base medium (D.5.2.1) at 50 °C. Mix carefully.
The final pH should be 5,2 ± 0,2 at 20-25 °C.
Pour carefully into plates up to a final volume of 15-20 ml in Petri dishes with a diameter of 90 mm. Omit the formation of air bubbles.
Allow the medium to solidify and handle with care.
Store the plates, **with surface upwards**, for up to 2 weeks at (5 ± 3) °C in the dark.
Do not invert the plates, as the semi-solid agar is too sloppy to do so.
Any plates in which the semi-solid agar has liquefied or fragmented should not be used.
Immediately before use, dry the surface of the agar plates carefully, e.g. by placing them with the lids off and the agar surface **upwards** in a Laminar Air Flow cabinet.

D.6 Apparatus and glassware

See Ch. 6 of ISO 6579

D.7 Sampling

See Ch. 7 of ISO 6579

D.8 Preparation of test sample

See Ch. 8 of ISO 6579

For **dust** samples the following procedure can be followed:
- Weigh 50 g of dust into an equal weight of Buffered Peptone Water (BPW) and mix gently;
- Allow the sample to soften for 10-15 min then mix gently by hand, ensuring that the dust is fully saturated;
- Immediately after mixing remove 50 g of the mixture and add to 200 ml BPW (pre-warmed to room temperature) for the pre-enrichment (see D.9.1).

D.9 Procedure

D.9.1 Non-selective pre-enrichment

Pre-warm the BPW to room temperature on the day before use. Discard any jars of BPW with turbidity. Label jars appropriately.
Mix samples well by the most suitable means for the sample type and add 25 g to 225 ml BPW, swirl gently to ensure the sample is mixed and submerged in the BPW.
If the specified mass of test portion is other than 25 g, use the necessary quantity of BPW to yield a 1/10 dilution (e.g. add 10 g of sample to 90 ml BPW).
Incubate the jars at (37 ± 1) °C for (18 ± 2) h.
Remove the incubated jars carefully from the incubator so as not to stir up solids.

D.9.2 Selective enrichment

Allow the MSRV plates to equilibrate at room temperature if they were stored at a lower temperature.
If the surfaces of the MSRV plates are wet, carefully dry the plates in a Laminar Air Flow cabinet or incubator.
Label lids of MSRV plates to correspond with pre-enrichment cultures. Ensure that MSRV plates are **never** inverted. Sides of plates can be marked to correspond with lids if required.
Carefully withdraw 0,1 ml of incubated BPW broth from the interface between the fluid and the jar close to the surface of the liquid. Do not disturb the solids in the jar and avoid including solid particulates in the inoculum taken.
Inoculate the MSRV plates with 3 drops of incubated BPW culture. The 3 drops should total 0,1 ml and be placed separately and equally spaced on the surface of the medium.
Incubate the inoculated MSRV plates at (41,5 ± 1) °C for (24 ± 3) h.
Do not invert the plates.

Positive plates will show a halo of growth originating from the inoculation spot. The halo of growth is characterized by a white sediment with a clear edge.

If the plates are negative after 24 h, they should be incubated for a further (24 ± 3) h.

D.9.3 Plating-out

Allow the Xylose Lysine Deoxycholate agar (XLD) plates and the second selective plating-out medium (see 5.2.4.2 of ISO 6579) to equilibrate at room temperature if they were stored at a lower temperature. Ensure that the surface of plates is dry before use.
Observe the MSRV plate on a clear white surface or light box. Determine where the furthest point of spread of opaque growth from the inoculation points is and dip a 10 µl loop just inside the border of the opaque growth.
If there has been no spread from the initial inoculation spot dip the loop into each of the inoculation spots where there has been non-motile growth.
Withdraw the loop ensuring that no large lumps of MSRV are extracted. Inoculate the surface of an XLD plate so that well-isolated colonies will be obtained. Proceed in the same way with the second selective plating-out medium using a new sterile loop.
For obtaining isolated colonies the following procedure can be followed. Streak with the loop a zig-zag streak pattern on the plating-out medium, starting with a very fine streak to remove most of the inoculum from the loop and create a ‘well’ in the first quarter of the plate and a narrow streak in the second quarter of the plate. Take a new 10 µl loop and pass through the

streak in the second quarter of the plate to streak out a wider zig-zag pattern in the third quarter of the plate, turn over the loop and pass through the third quarter streak to create a final quarter streak which will result in individual colonies.

Incubate the XLD-plates inverted at (37 ± 1) °C for (24 ± 3) h.
Incubate the second plating-out medium in accordance with the manufacturer's instructions.

Return negative MSRV plates to the 41,5^{o} C incubator and incubate for a further (24 ± 3) h.
Repeat the plating-out after 48 h of incubation of MSRV.

Typical colonies of *Salmonella* grown on XLD-agar have a black centre and a lightly transparent zone of reddish colour due to the colour change of the indicator.

NOTE *Salmonella* H_2S negative variants (e.g. *Salmonella* Paratyphi A) grown on XLD agar are pink with a darker pink centre. Lactose-positive *Salmonella* grown on XLD agar are yellow with or without blackening (also see 9.4.4 of ISO 6579).

D.9.4 Confirmation

For confirmation of the typical colonies, isolated on the plating-out media, follow the instructions as given in Ch. 9.5 of ISO 6579.
If well isolated (typical) colonies are available on the plating-out media it is possible to perform the biochemical confirmation directly on these colonies without the intermediate step of streaking the colonies on nutrient agar (see 9.5.2. of ISO 6579).

D.10 Expression of results

See Ch. 10 of ISO 6579

D.11 Test report

See Ch. 11 of ISO 6579

D.12 Quality assurance

See Ch. 12 of ISO 6579