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EU Interlaboratory comparison study IX (2005) on bacteriological detection of *Salmonella* spp.

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Abstract

EU Interlaboratory comparison study IX (2005) on bacteriological detection of *Salmonella* spp.

In 2005 the ninth interlaboratory comparison study on bacteriological detection of *Salmonella* spp. was organised 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) and 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 dust (containing *Salmonella* Virchow and *Salmonella* Livingstone) 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) and a second selective medium chosen by the laboratory. 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 when compared to 4 h incubation of BPW. The accuracy rates for the artificially contaminated samples were 57 % and 98 % after respectively 4 and 18 h of incubation in BPW. The results for the naturally contaminated dust samples revealed also significant more positive results after 18 h of incubation. The accuracy rates for these samples were respectively 81 % and 99 % after 4 and 18 h of incubation in BPW. All NRLs achieved the level of good performance which was defined during the CRL-*Salmonella* workshop 2005; only two NRLs had small problems with one of the controls.

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

Rapport in het kort

EU Ringonderzoek IX (2005) voor bacteriologische detectie van *Salmonella* spp.

In 2005 werd door het Communautair Referentie Laboratorium voor *Salmonella* (CRL-*Salmonella*, Bilthoven, Nederland) het negende bacteriologische ringonderzoek georganiseerd. Deelnemers van 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 besmet stof (bevattende *Salmonella* Virchow en *Salmonella* Livingstone) 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 en een tweede selectief medium gekozen door de laboratoria.
Significant meer positieve isolaties werden gevonden met de kunstmatig besmette monsters (negatieve kippenfeces, kunstmatig besmet met referentiematerialen) na 18 uur incubatie in BPW in vergelijking met 4 uur incubatie in BPW. De waarden voor nauwkeurigheid ("accuracy rates") van de kunstmatig besmette monsters waren 57 % en 98 % na respectievelijk 4 en 18 uur incubatie in BPW. De resultaten van de natuurlijk besmette stofmonsters lieten ook significant meer positieve isolaties zien na 18 uur incubatie. De waardes voor nauwkeurigheid ("accuracy rates") voor deze monsters waren respectievelijk 81 % en 99 % na 4 en 18 uur incubatie in BPW. Alle NRLs voldeden aan de "good performance" die gedefinieerd was tijdens de CRL-*Salmonella* workshop 2005; slechts 2 NRLs hadden enkele problemen met één van de controles.

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

Contents

Summary

In fall 2005 the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*) organised the ninth 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. During the CRL-*Salmonella* workshop 2005 a proposal was made to define "good performance" of 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) and a second selective medium. Optionally, a laboratory could also use other, own media of procedures for the detection of *Salmonella* in addition to the prescribed procedure. 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. In this study the faeces was not mixed with any preservation medium as earlier studies have shown a negative effect of glycerol on the growth of *Salmonella* (Veenman *et al.,* in preparation). The 25 capsules were divided over the following groups: 5 capsules with *circa* 10 colony forming particles (cfp) of *Salmonella* Typhimurium (STM10), 5 capsules with *circa* 100 cfp *S*. Typhimurium (STM100), 5 capsules with *circa* 100 cfp *S*. Enteritidis (SE100), 5 capsules with *circa* 500 cfp *S*. Enteritidis (SE500) and 5 blank capsules. The other 10 capsules, to which no faeces had to be added, were control samples, existing of 3 capsules with *circa* 10 cfp *S*. Typhimurium, 2 capsules with *circa* 100 cfp *S*. Enteritidis, 1 capsule with *circa* 500 cfp *S*. Enteritidis, 2 capsules with *circa* 5 cfp *S*. Panama and 2 blank capsules. Beside the reference materials, also 10 dust samples (10 g each) naturally contaminated with *Salmonella* Virchow and *Salmonella* Livingstone were examined.

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 when compared with 4 h incubation in BPW. The accuracy rates for the artificially contaminated samples were 57 % and 98 % after respectively 4 and 18 h of incubation in BPW. The results for the naturally contaminated samples revealed also significant more positive results after 18 h of incubation. The accuracy rates for these samples were respectively 81 % and 99 % after 4 and 18 h of incubation in BPW. In contrast

to earlier studies the faeces was not mixed with any preservation medium, since studies at the CRL-*Salmonella* have shown a negative effect of glycerol on the growth of *Salmonella*. The high accuracy rates compared to earlier studies confirm the negative effect of glycerol. All NRLs achieved the level of good performance which was defined during the CRL-*Salmonella* workshop 2005. Only two NRLs had small problems with the control samples. Since the samples were tested well by all NRLs, no further actions were taken.

List of abbreviations

BGA	Brilliant Green Agar
BPLS	Brilliant Green Phenol-Red Lactose Sucrose agar
BPW	Buffered Peptone Water
BxLH	Brilliant Green, Xylose, Lysine, Sulphonamide
cfp	colony forming particles
CI	Confidence interval
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
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
R Sal	Rapid *Salmonella* Agar
RV	Rappaport Vassiliadis
RVS	Rappaport Vassiliadis Soya broth
SC	Selenite Cystine
SE	*Salmonella* Enteritidis
SM(ID)2	*Salmonella* Detection and Identification-2
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*) organises 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 ISO Technical Committee for 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 2005, 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 a short incubation time of BPW (4 h) beside the “normal” incubation time (18 h). These two incubation times of BPW were prescribed in the study of 2004 as well as of 2005.
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 *circa* 10 cfp *Salmonella* Typhimurium (STM10), 2 capsules with *circa* 100 cfp *Salmonella* Enteritidis (SE100), 1 capsule with *circa* 500 cfp *Salmonella* Enteritidis (SE500), 2 capsules with *circa* 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). In this study the faeces was not mixed with any preservation medium as earlier studies have shown a negative effect of glycerol on the growth of *Salmonella.* Furthermore, 10 naturally contaminated samples of dust containing *Salmonella* Virchow and *Salmonella* Livingstone were also examined.

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.
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) min;
- 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 and dust samples

3.2.1 General

Salmonella negative chicken faeces was obtained from *Salmonella* free poultry flocks. The dust samples were obtained from *Salmonella* positive poultry flocks. The faeces and the dust were tested for the presence or obsence of *Salmonella*. For this purpose 10 portions of 10 g were each added to 90 ml BPW. After pre-enrichment at 37 ^{o}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 dust were isolated on TSI agar and sent for serotyping to the Diagnostic Laboratory for Infectious Diseases and Perinatal Screening (LIS/RIVM). The faeces, bacteriologically negative for *Salmonella*, was used for the artificially contaminated samples. Both the faeces and dust were stored at 5 ºC. In this study the faeces was not mixed with any preservation medium as earlier studies have shown a negative effect of glycerol on the growth of *Salmonella* (Chung *et al.,* 1972 and Veenman *et al.,* in preperation).

3.2.2 Total bacterial count in faeces and in dust

The total number of aerobic bacteria was investigated for the faeces as well as for the dust. The procedure of ISO 4833 (Anonymous, 2003) was used for this purpose. Portions of 10 gram were homogenised into 90 ml peptone saline solution in a plastic bag. The content was mixed by using a pulsifier (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.

3.2.3 Enterobacteriaceae count in faeces and in dust

In addition to the total count of aerobic bacteria the *Enterobacteriaceae* count was determined. The procedure of ISO 21528-2 was used for this purpose. Portions of 20 gram were homogenized into 180 ml peptone saline solution in a plastic bag. The content was mixed by using a pulsifier (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 15 ml of molten Violet Red Bile Glucose agar (VRBG) was added. After the VRBG was solidified an additional 10-15 ml VRBG was added to the agar. These plates were incubated at (37 ± 1) ^{o}C for (24 ± 2) h for the enumeration of the *Enterobacteriaceae* count.

3.2.4 MPN of *Salmonella* in naturally contaminated dust

To semi-quantify the number of Salmonellae in the *Salmonella* positive dust, a Most Probable Number (MPN) method was used. For this purpose, ten gram of dust was added to 90 ml of buffered peptone water (BPW). Next tenfold dilutions were prepared in BPW until a concentration of 0.01 mg dust per 100 ml BPW. This procedure was repeated five times. The BPW jars with concentrations of 1000 mg till 0.01 mg dust (per 100 ml BPW) were incubated and handled according to the same procedure as described in subsection 3.2.1. After completion of the test the MPN was calculated using a MPN table (e.g. ISO 16649-3).

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 150 grams of positive dust for *Salmonella* were mailed (with cooling devices) as diagnostic specimens by courier service to the participants. After arrival at the laboratory the capsules had to be stored at –20 ^{o}C and the faecal and dust samples had to be stored at +5 ^{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 (numbered C1-C10). 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 10 samples (numbered D1 – D10) of 10 grams each of naturally contaminated dust samples (containing *S.* Virchow and *S.* Livingstone) were analysed. The types and the number of capsules and faeces or dust 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=10) with 10 g *Salmonella*-positive dust
S. Panama 5	2	---	---
S. Enteritidis 100	2	5	---
S. Enteritidis 500	1	5	---
S. Typhimurium 10	3	5	---
S. Typhimurium 100	---	5	---
Blank	2	5	---
No capsules	---	---	10

3.3.2 Methods

During the CRL-*Salmonella* workshop of April 2005 it was decided that the prescribed method of this interlaboratory comparison study would be draft Annex D of ISO 6579 (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.

Three biopacks and six cooling devices were placed in one large shipping box. In one of the three biopacks (the one containing the reference materials), a temperature recorder was enclosed.

3.4 Accreditation/certification

Twenty laboratories mentioned to be accredited for their quality system according to EN-ISO/IEC 17025 (labcodes 1, 2, 3, 4, 5, 6, 7, 9, 11, 12, 13, 14, 15, 16, 17, 18, 20, 22, 24 and 27) as is the CRL-*Salmonella*. Three laboratories (labcodes 8, 26 and 28) mentioned that they were not accredited or certified to any system and mentioned no planning to do so in the near future. Five laboratories (labcodes 10, 19, 21, 23 and 25) 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 dust samples.

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

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

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

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

3.6 Good performance

During the tenth CRL-*Salmonella* workshop in April 2005 a proposal was made to define "good performance" of the NRLs.

The following was suggested:

- Positive control capsules should all be positive, except for SPan5 of which 1 out of 2 may be negative;
- Blank control capsules should all be negative;
- Of blank capsules + faeces, 80 % should be negative;
- Of STM100 and SE500 + faeces, 80 % should be positive;
- Of STM10 and SE100 + faeces, 50 % should be positive

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 section 3.1. The enumerated minimum and maximum levels within each batch of capsules are also given in the table. 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	14-10-05	02-11-05	01-06-05	08-07-05	16-06-05
Number of capsules tested	25	20	25	25	25
Mean cfp per capsule	115	548	7	12	111
Min-max cfp per capsule	93-138	400-600	4-12	6-16	81-135
T_2 / (I-1)	1.54	1.93	0.83	0.65	1.69
Final batch; Test 1					
Date testing capsules	27-10-05	04-11-05	03-06-05	15-07-05	14-07-05
Number of capsules tested	50	25	50	50	50
Mean cfp per capsule	118	538	7	9	91
Min-max cfp per capsule	90-165	420-740	3-12	4-15	74-127
T_2 / (I-1)	1.93	1.44	1.10	0.98	1.10
Final batch; Test 2					
Date testing capsules	02-12-05	06-12-05	29-11-05	30-11-05	01-12-05
Number of capsules tested	25	25	25	25	25
Mean cfp per capsule	122	441	7	9	86
Min-max cfp per capsule	82-150	340-570	2-14	4-16	76-111
T_2 / (I-1)	2.05	1.05	1.59	0.96	1.40

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

4.2 Dust and faeces samples

The dust samples were received at 29-10-2005 and the faeces samples were received at 29-10-2005 and 13-10-2005 at the CRL-*Salmonella.* The dust contained *Salmonella* Livingstone and *Salmonella* Virchow. The number of *Salmonella* in the dust was determined by performing the MPN procedure (see subsection 3.2.4) on 22-11-2005. The MPN result of this positive dust was 34 cfp per gram (95 % confidence interval: 10-100 cfp per gram).

In Table 3 the total number of aerobic bacteria and Enterobacteriaceae are shown for the positive dust and the negative chicken faeces samples

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

	Aerobic count cfp/g	**Enterobacteriaceae cfp/g**
Naturally contaminated dust with *Salmonella*	$1.39*10^8$	$6.62*10^5$
Negative chicken faeces without *Salmonella*	$1.19*10^9$	$1.05*10^7$

4.3 Technical data interlaboratory comparison study

4.3.1 Media

Each laboratory was asked to test the samples with the selective enrichment medium MSRV and the plating out medium XLD and a plating out medium of own choice. The media used are shown in Table 4. All NRLs tested the samples with the prescribed medium combinations. Laboratory 14 did use XLD and another plating out medium (BGA), but only confirmed colonies of BGA when suspected colonies were found on both XLD and BGA. Three laboratories used RVS next to MSRV as selective enrichment medium; two laboratories used RV, two MKTTn, one SC and one DIASSALM. One laboratory used MSRV at both pH 5.2 (as prescribed in this study) and at pH 5.4 (as prescribed by the manufacturer). Small differences were found after 4 h incubation of BPW between the MSRV with pH 5.2 and 5.4, but no differences were found after 18 h incubation of BPW (see Annex 3).

Table 4 Media used per laboratory

Labcode	***Selective enrichment***	***Isolation medium***	***Labcode***	***Selective enrichment***	***Isolation medium***
1	MSRV	XLD Onoz	15	MSRV	XLD BGA
2	MSRV SC	XLD MacConkey	16	MSRV	XLD BGA
3	MSRV	XLD BxLH	17	MSRV	XLD Rambach
4	MSRV RVS	XLD R Sal BGA	18	MSRV	XLD SMID2
5	MSRV	XLD Rambach	19	MSRV	XLD Rambach BGA
6	MSRV RV	XLD BG	20	MSRV MKTTn	XLD BPLS
7	MSRV	XLD BGA	21	MSRV	XLD Rambach
8	MSRV RVS	XLD MLCB BGA	22	MSRV MKTTn	XLD BGA
9	MSRV	XLD BGA	23	MSRV	XLD SM2
10	MSRV	XLD XLT4	24	MSRV	XLD BGA
11	MSRV MSRV (pH 5.4)	XLD BGA	25	MSRV DIASSALM RVS	XLD BGA
12	MSRV RV	XLD Rambach XLT4	26	MSRV MKTTn	XLD SMI
13	MSRV	XLD BGA	27	MSRV RVS	XLD BGA
14	MSRV	BGA	28	MSRV	XLD Hektoen

4.3.2 Pre-warming time and temperature of BPW

Before adding the capsules, faeces and/or dust to the BPW, all jars had to be pre-warmed at (37 ± 1) ºC overnight. All laboratories met the criteria as set in the standard operation procedure.

4.3.3 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) ^{o}C for 45 min. Twenty-three laboratories dissolved the capsules in exactly forty-five minutes. One laboratory (labcode 11) reported a dissolving time of 5 min. Four laboratories used a dissolving time of more than 45 min, i.e. 50 min (laboratories 2 and 12) and 60 min (laboratories 17 and 24). One laboratory (labcode 26) started the dissolving with an incubator temperature of 34.7 ^{o}C and a final temperature of 34.7 ^{o}C. The NRL with labcode 19 reported a final temperature of the incubator of 35 ^{o}C. Laboratory 23 reported that the capsules were not completely dissolved after 45 min at 37 ºC.

4.3.4 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 24) incubated the BPW for the prescribed short incubation time (4 ± ½) h (see Table 4). Laboratory 24 incubated the samples for 1 h. Eight laboratories (labcodes 6, 10, 12, 13, 17, 19, 20 and 26) incubated the samples less than the prescribed long incubation time (less than 16 h). One laboratory (labcode 4) exceeded the prescribed long incubation time (more than 20 h).
The prescribed temperature for the incubation of BPW is (37 ± 1) ^{o}C. All laboratories except two (labcode 19 and 26) incubated the BPW at the prescribed temperature (Table 5).

Table 5 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	03:40	36.3-36.7	17:28	36.2-36.7
2	04:15	37	16:15	37
3	04:00	36.8-37.1	19:50	37.1-37.2
4	04:12	36.7-37.1	21:15	36.5-37.1
5	03:45	37.0-37.1	18:20	37.0-37.1
6	04:00	36.8-37.3	15:00	37.1
7	04:05	36.9-37.0	19:40	36.9-37.2
8	04:00	36.7-37.0	20:00	36.5-37.0
9	04:00	36.4-36.7	20:00	36.2-36.4
10	04:05	37	15:00	36.7-37
11	03:50	36.5-37.1	19:05	37.1-37.2
12	03:45	37.1-37.5	15:05	37.4-38
13	03:50	37.1	14:50	36.2-37.3
14	03:30	37	16:30	37
15	04:00	37.1	18:00	37.1-37.2
16	04:00	36.8-37.0	20:00	36.9-37.1
17	04:00	36.9-37.0	15:05	36.9-37.0
18	04:00	36.9-37.0	18:00	36.8-36.9
19	03:30	34.5-35.0	15:50	34.9
20	04:00	38	15:45	38
21	04:10	36.0-36.2	17:30	36.0-36.2
22	04:00	37.2-37.4	19:30	37.3
23	04:00	36.0-37.0	17:30	36.0-37.0
24	01:00	36.8-37.3	18:30	36.9-37.2
25	04:30	37.2	20:00	37.1-37.2
26	04:00	35.8-36.5	14:00	35.4-36.6
27	04:00	36.7-37.0	16:30	36.7-37.0
28	04:06	36-38	18:00	36-38

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

4.3.5 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). Laboratory 5 did not report the composition of the MSRV. All other laboratories except two (labcodes 8 and 24) 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. Four laboratories (labcodes 4, 15, 24 and 26) used a concentration of 0.02 g/l novobiocin in their selective enrichment medium MSRV. The NRL with labcode 18 did not add novobiocin to their MSRV and three laboratories (labcode 8, 9 and 28) did not report the concentration of novobiocin used.

4.3.6 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. Two laboratories (labcodes 4 and 24) used a total incubation time outside the prescribed range of 42 – 54 h. Most laboratories however complied with the required incubation time (see Table 6). All NRLs except six (labcodes 4, 6, 10, 11, 19 and 24) met the prescribed temperature of (41.5 ± 1) ^{o}C. In all these laboratories the starting temperature was too low.

Table 6 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	41.2-41.5	48:55	41.2-41.5
2	48:00	41.5	49:20	41.5
3	45:00	41.9-42.3	46:55	41.9-42.3
4	40:35	39.6-41.8	36:15	39.5-41.5
5	44:15	41.5	47:30	41.5
6	44:40	41.0-41.5	48:40	39.9-41.6
7	47:21	41.2-42.1	46:58	41.7-42.0
8	46:15	41.5-42.0	48:00	41.5-42.0
9	42:00	41.5-41.8	47:00	41.4-41.6
10	47:30	41.0-41.5	50:20	36-41.5
11	45:00	40.4-40.8	46:40	39.3-41.0
12	42:55	40.9-41.8	42:05	40.9-41.4
13	44:20	41.3-41.7	45:15	41.2-41.6
14	46:30	41.5	46:30	41.5
15	46:30	41.1-41.2	47:45	41.2-41.3
16	45:30	41.5-41.7	46:30	41.5-41.7
17	43:25	40.8-41.7	45:20	40.8-41.2
18	46:45	41.8	47:50	41.8
19	44:55	38.0-41.1	46:30	39.1-43.8
20	45:40	41.9-42.0	48:00	42.0
21	42:10	41.3-41.7	46:50	41.4-41.5
22	44:30	41.5	45:15	41.5
23	44:00	41.0-42.0	46:30	41.0-42.0
24	24:30	41.0-41.5	41:05	36.6-41.5
25	42:05	41.5-41.7	45:55	41.5
26	47:00	42.2-42.5	45:50	41.6-42.5
27	47:30	41.3-41.6	47:30	41.1-41.7
28	48:30	41-43	49:30	41-43

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

4.4 Control samples

General
None of the laboratories isolated *Salmonella* from the procedure control (C11: no capsule/no faeces) and one laboratory (labcode 8) isolated *Salmonella* from the faeces control (C12: no capsule/negative faeces).

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 7).

Table 7 Number of positive isolations per laboratory for blank capsules (n=2) without addition of faeces

	Laboratory codes													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
BPW 4 hrs	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BPW 18 hrs	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Laboratory codes													
	15	16	17	18	19	20	21	22	23	24	25	26	27	28
BPW 4 hrs	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BPW 18 hrs	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Salmonella Panama 5 capsules (n=2) without addition of faeces
One laboratory (labcode 10) was able to isolate *Salmonella* from both capsules containing *S*. Panama at a level of circa 5 cfp/capsule after both 4 h and 18 h incubation of BPW. Four laboratories (labcodes 15, 18, 24 and 25) isolated *Salmonella* from only one of the two capsules after 4 h incubation of BPW and isolated *Salmonella* from both capsules after 18 h incubation of BPW. Twenty-three laboratories failed to isolate *Salmonella* after 4 h incubation of BPW, but isolated *Salmonella* from both capsules after 18 h incubation of BPW. One laboratory (labcode 28) was not able to isolate *Salmonella* from any of the *S*. Panama capsules and therefore did not achieve the good performance level as proposed during the CRL-*Salmonella* workshop 2005 (see Table 8).

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

	Laboratory codes													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
BPW 4 hrs	0	0	0	0	0	0	0	0	0	2	0	0	0	0
BPW 18 hrs	2	2	2	2	2	2	2	2	2	2	2	2	2	2

	Laboratory codes													
	15	16	17	18	19	20	21	22	23	24	25	26	27	28
BPW 4 hrs	1	0	0	1	0	0	0	0	0	1	1	0	0	0
BPW 18 hrs	2	2	2	2	2	2	2	2	2	2	2	2	2	0

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

One laboratory (labcode 10) was able to isolate *Salmonella* from both capsules containing *Salmonella* Typhimurium at a mean level of circa 10 cfp/capsule after both 4 h and 18 h incubation of BPW. Two laboratories (labcodes 2 and 7) isolated *Salmonella* from only one of the three capsules after 4 h incubation of BPW and isolated *Salmonella* from all three capsules after 18 h incubation of BPW. Ten laboratories (labcodes 4, 11, 14, 15, 18, 23, 24, 25, 26 and 27) isolated *Salmonella* from two of the three capsules after 4 h incubation of BPW and isolated *Salmonella* from all three capsules after 18 h incubation of BPW. Fifteen laboratories (labcodes 1, 3, 5, 6, 8, 9, 12, 13, 16, 17, 19, 20, 21, 22 and 28) failed to isolate *Salmonella* after 4 h incubation of BPW, but isolated *Salmonella* from both capsules after 18 h incubation of BPW (see Table 9).

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

	Laboratory codes													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
BPW 4 hrs	0	2	0	1	0	0	2	0	0	3	1	0	0	1
BPW 18 hrs	3	3	3	3	3	3	3	3	3	3	3	3	3	3

	Laboratory codes													
	15	16	17	18	19	20	21	22	23	24	25	26	27	28
BPW 4 hrs	1	0	0	1	0	0	0	0	1	1	1	1	1	0
BPW 18 hrs	3	3	3	3	3	3	3	3	3	3	3	3	3	3

Salmonella Enteritidis 100 capsules (n=2) without addition of faeces
Two laboratories (labcodes 10 and 26) were able to isolate *Salmonella* from both capsules containing *Salmonella* Enteritidis at a mean level of circa 100 cfp/capsule after both 4 h and 18 h incubation of BPW. Eight laboratories (labcodes 1, 2, 4, 7, 12, 18, 24 and 25) isolated *Salmonella* from only one of the two capsules after 4 h incubation of BPW and isolated *Salmonella* from all three capsules after 18 h incubation of BPW. Eighteen laboratories (labcodes 3, 5, 6, 8, 9, 11, 13, 14, 15, 16, 17, 19, 20, 21, 22, 23, 27 and 28) failed to isolate *Salmonella* after 4 h incubation of BPW, but isolated *Salmonella* from both capsules after 18 h incubation of BPW (see Table 10).

Table 10 Number of positive isolations per laboratory for SE 100 (n=2) without addition of faeces

	Laboratory codes													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
BPW 4 hrs	1	1	0	1	0	0	1	0	0	2	0	1	0	0
BPW 18 hrs	2	2	2	2	2	2	2	2	2	2	2	2	2	2

	Laboratory codes													
	15	16	17	18	19	20	21	22	23	24	25	26	27	28
BPW 4 hrs	0	0	0	1	0	0	0	0	0	1	1	2	0	0
BPW 18 hrs	2	2	2	2	2	2	2	2	2	2	2	2	2	2

Salmonella Enteritidis 500 capsules (n=1) without addition of faeces
Nineteen laboratories (labcodes 1, 3, 4, 5, 7, 9, 10, 11, 12, 14, 15, 18, 19, 20, 23, 25, 26, 27 and 28) were able to isolate *Salmonella* from the capsule containing *Salmonella* Enteritidis at a mean level of circa 500 cfp/capsule after both 4 h and 18 h incubation of BPW. The other nine laboratories (labcodes 2, 6, 8, 13, 16, 17, 21, 22 and 24) failed to isolate *Salmonella* after 4 h incubation of BPW, but isolated *Salmonella* from the capsule after 18 h incubation of BPW (see Table 11).

Table 11 Number of positive isolations per laboratory for SE 500 (n=1) without addition of faeces

	Laboratory codes													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
BPW 4 hrs	1	0	1	1	1	0	1	0	1	1	1	1	0	1
BPW 18 hrs	1	1	1	1	1	1	1	1	1	1	1	1	1	1

	Laboratory codes													
	15	16	17	18	19	20	21	22	23	24	25	26	27	28
BPW 4 hrs	1	0	0	1	1	1	0	0	1	0	1	1	1	1
BPW 18 hrs	1	1	1	1	1	1	1	1	1	1	1	1	1	1

In Table 12 the specificity, sensitivity and accuracy for the control capsules without the addition of faeces are shown.
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 9 %, 20 %, 23 % and 68 %. The sensitivity after 18 h of incubation in BPW was for SPan 5 capsules 96 % and for all other three kinds of samples 100 % (see Table 11). The sensitivity for all capsules containing *Salmonella* was after 4 h of incubation of the BPW 24 % and after 18 h of incubation in BPW 99 %. The accuracy rate for all capsules (blank and capsules containing *Salmonella*) was 39 % after 4 h of incubation of BPW and 99 % after 18 h of incubation of BPW.

Table 12 Specificity, sensitivity and accuracy for all participating laboratories (n = 28) with all control capsules and all medium combinations without addition of faeces

		4 hr BPW	**18 hr BPW**
Blank (n =2 per lab)	number of samples	56	56
	negative samples	56	56
	Specificity in %	***100.0***	***100.0***
SPan 5 (n = 2 per lab)	number of samples	56	56
	positive samples	5	54
	Sensitivity in %	***8.9***	***96.4***
STM 10 (n = 3 per lab)	number of samples	84	84
	positive samples	17	84
	Sensitivity in %	***20.2***	***100.0***
SE 100 (n = 2 per lab)	number of samples	56	56
	positive samples	13	56
	Sensitivity in %	***23.2***	***100.0***
SE 500 (n = 1 per lab)	number of samples	28	28
	positive samples	19	28
	Sensitivity in %	***67.9***	***100.0***
All capsules with Salmonella	number of samples	224	224
	positive samples	54	222
	Sensitivity in %	***24.1***	***99.1***
All capsules	number of samples	280	280
	correct samples	110	278
	Accuracy in %	***39.3***	***99.3***

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

4.5.1 Results per type of capsule and per laboratory

General

Two laboratories (labcode 12 and 24) received two capsules with the same number and one capsule was missing. The results of both the double and the missing capsule numbers are not included in the results. Therefore n = 4 for laboratory 12 for STM 100 and SE100 and for laboratory 24 for SE100 and blank capsules.

Blank capsules with negative faeces

No *Salmonella* spp. were reported after 4 h incubation of BPW.

Laboratory 2 reported the isolation of *Salmonella* spp. of two of the five capsules and therefore did not achieve the level of "good performance" as suggested during the CRL-*Salmonella* workshop 2005. Laboratories 9 and 18 reported the isolation of *Salmonella* spp. of one of the five capsules after 18 h of incubation of the BPW (Table 13).

Table 13 Number of isolations per laboratory for blank capsules (n=5) with the addition of 10 g Salmonella negative chicken faeces

	Laboratory codes													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
BPW 4 hrs	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BPW 18 hrs	0	2	0	0	0	0	0	0	1	0	0	0	0	0

	Laboratory codes													
	15	16	17	18	19	20	21	22	23	24	25	26	27	28
BPW 4 hrs	0	0	0	0	0	0	0	0	0	0*	0	0	0	0
BPW 18 hrs	0	0	0	1	0	0	0	0	0	0*	0	0	0	0

** Only 4 samples were investigated*

S. *Typhimurium 10 capsules (STM10) with negative faeces*

In Table 14 the results are summarized of the *Salmonella*-negative faeces samples artificially contaminated with capsules containing STM10. Twelve laboratories (labcode 4, 8, 13, 21, 23 and 25) did not isolate *Salmonella* from any of the five samples after 4 h incubation of BPW. No laboratory found the maximum number (5) of positive isolations after 4 h incubation of BPW. After 18 h of incubation of BPW the maximum number of positives were found by 23 laboratories. Four laboratories (labcodes 3, 9, 10 and 21) reported the isolation of *Salmonella* of four of the five capsules and one laboratory (labcode 28) reported the isolation of *Salmonella* of three of the five capsules after 18 h incubation of BPW.

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

	Laboratory codes													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
BPW 4 hrs	1	2	1	3	0	4	2	1	0	0	1	4	2	0
BPW 18 hrs	5	5	4	5	5	5	5	5	4	4	5	5	5	5

	Laboratory codes													
	15	16	17	18	19	20	21	22	23	24	25	26	27	28
BPW 4 hrs	3	0	0	0	0	0	0	0	1	3	4	3	3	0
BPW 18 hrs	5	5	5	5	5	5	4	5	5	5	5	5	5	3

S. *Typhimurium 100 (STM100) with negative faeces*

In Table 15 the results are summarized of the *Salmonella*-negative faeces samples artificially contaminated with capsules containing STM100. All laboratories isolated *Salmonella* out of at least one of the five capsules after 4 h incubation of BPW. Thirteen laboratories (labcodes 3, 4, 6, 7, 10, 11, 12, 13, 15, 23, 25, 26 and 27) found the maximum number of positive isolations after 4 h incubation of BPW. After 18 h of incubation of BPW the maximum number of positives was found by all laboratories except for laboratory 3, who isolated *Salmonella* out four of the five samples.

Table 15 Number of positive isolations per laboratory for STM 100 (n=5) with the addition of 10 g Salmonella negative chicken faeces

	Laboratory codes													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
BPW 4 hrs	3	1	5	5	2	5	5	3	1	5	5	4*	5	1
BPW 18 hrs	5	5	4	5	5	5	5	5	5	5	5	4*	5	5

	Laboratory codes													
	15	16	17	18	19	20	21	22	23	24	25	26	27	28
BPW 4 hrs	5	1	3	4	3	3	2	1	5	2	5	5	5	2
BPW 18 hrs	5	5	5	5	5	5	5	5	5	5	5	5	5	5

** 4 samples were investigated instead of 5*

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

In Table 16 the results are summarized of the *Salmonella*-negative faeces samples artificially contaminated with capsules containing SE100. Twelve laboratories (labcode 2, 5, 7, 9, 10, 11, 14, 16, 17, 21 and 22) did not isolate *Salmonella* from any of the five samples after 4 h incubation of BPW. No laboratory found the maximum number (5) of positive isolations after 4 h incubation of BPW. After 18 h of incubation of BPW the maximum number of positives was found by 26 laboratories. Two laboratories (labcodes 21 and 22) reported the isolation of *Salmonella* of four of the five capsules after 18 h incubation of BPW.

Table 16 Number of positive isolations per laboratory for SE 100 (n=5) with the addition of 10 g Salmonella negative chicken faeces

	Laboratory codes													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
BPW 4 hrs	2	0	2	3	0	1	0	1	0	0	0	2*	1	0
BPW 18 hrs	5	5	5	5	5	5	5	5	5	5	5	4*	5	5

	Laboratory codes													
	15	16	17	18	19	20	21	22	23	24	25	26	27	28
BPW 4 hrs	1	0	0	1	0	3	0	0	2	2*	2	1	4	2
BPW 18 hrs	5	5	5	5	5	5	4	4	5	4*	5	5	5	5

** 4 samples were investigated instead of 5*

S. Enteritidis 500 (SE500) with negative faeces

One laboratory (labcode 22) did not isolate *Salmonella* from any of the five samples after 4 h incubation of BPW. Fourteen laboratories (labcode 3, 4, 6, 10, 11, 12, 13, 14, 15, 17, 18, 23, 25, 26 and 27) found the maximum of number (5) of positive isolations after 4 h incubation of BPW. After 18 h of incubation of BPW the maximum number of positives was found by all 28 laboratories (Table 17).

Table 17 Number of positive isolations per laboratory for SE 500 (n=5) with the addition of 10 g Salmonella negative chicken faeces

	Laboratory codes													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
BPW 4 hrs	4	1	5	5	3	5	4	1	1	5	5	5	5	2
BPW 18 hrs	5	5	3	5	5	5	5	5	5	5	5	5	5	5

	Laboratory codes													
	15	16	17	18	19	20	21	22	23	24	25	26	27	28
BPW 4 hrs	5	2	5	5	2	4	2	0	5	4	5	5	5	2
BPW 18 hrs	5	5	5	5	5	5	5	5	5	5	5	5	5	5

In Figure 1 the percentages of positive isolations per laboratory for all capsules (n = 20) containing *Salmonella* with the addition of 10 g *Salmonella* negative chicken faeces after 4 h incubation of BPW are presented.

In Figure 2 the percentages of positive isolations per laboratory for all capsules (n = 20) containing *Salmonella* with the addition of 10 g *Salmonella* negative chicken faeces after 18 h incubation of BPW are presented.

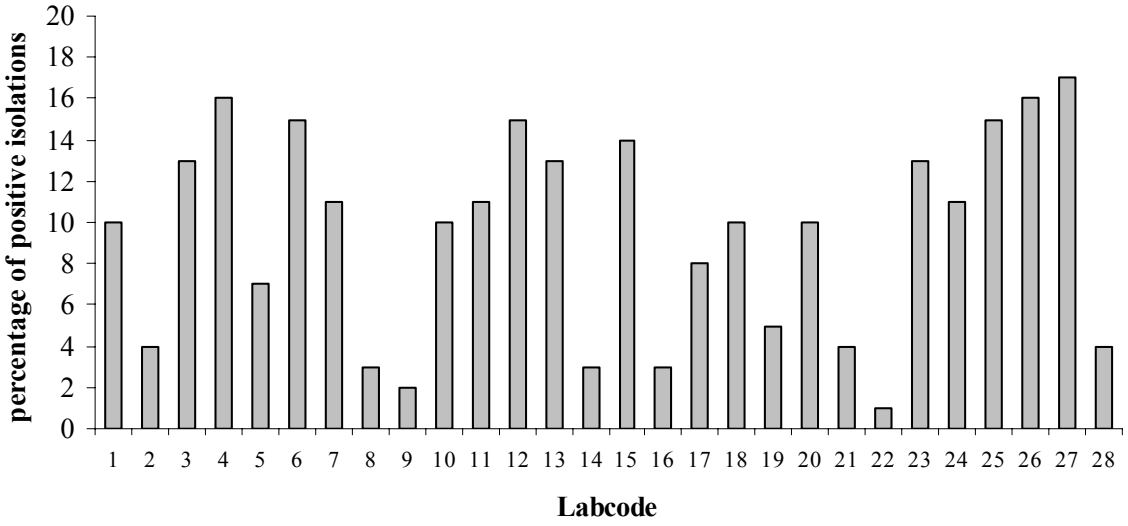

Figure 1 *Percentages of positive isolations per laboratory for all capsules (n = 20) with the addition of 10 g Salmonella negative chicken faeces after 4 h incubation of BPW. For laboratories 12 and 24 n = 18.*

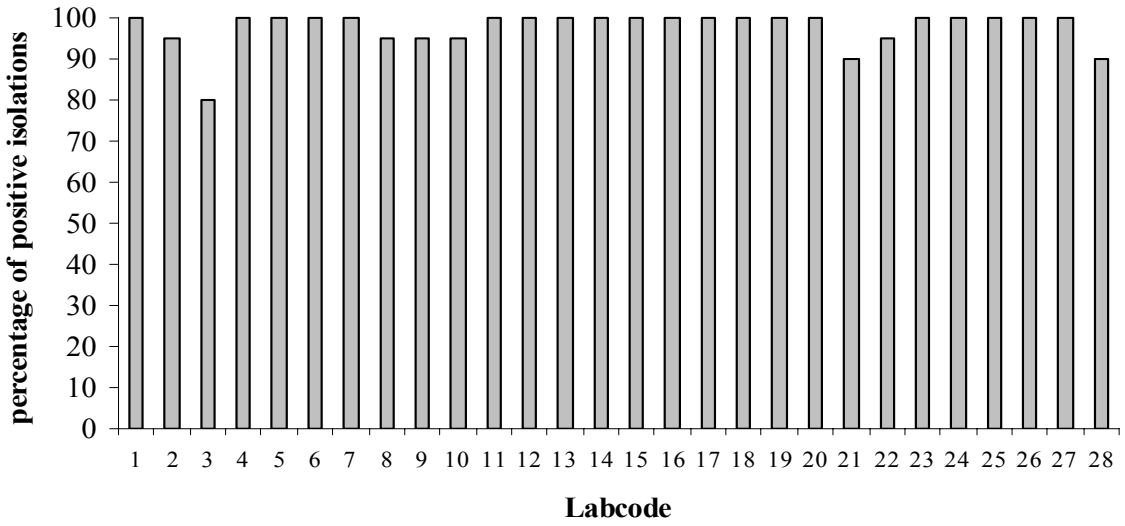

Figure 2 *Percentages of positive isolations per laboratory for all capsules (n = 20) with the addition of 10 g Salmonella negative chicken faeces after 18 h incubation of BPW. For laboratories 12 and 24 n = 18.*

4.5.2 Specificity, sensitivity and accuracy rates of artificially contaminated samples

The specificity, sensitivity and accuracy rates for all types of capsules with the addition of *Salmonella*-negative faeces are shown in Table 18. The specificity rate (of the blank samples) after 4 h of incubation of BPW was 100 %, and after 18 h of incubation 97 %. After 4 h of incubation of BPW the sensitivity was 27, 68, 21 and 71 % for respectively STM 10, STM 100, SE 100 and SE 500 capsules. After 18 h of incubation of BPW the sensitivity was more than 95 % in all cases. The sensitivity rate for all capsules containing *Salmonella* was 47 % after 4 h of incubation of BPW and 98 % after 18 h of incubation of BPW. The accuracy rate was 57 % and 98 % after 4 h and 18 h of incubation of BPW, respectively.

Table 18 Specificity, sensitivity and accuracy rates for all participating laboratories (n = 28) and all capsules with the addition of 10 g Salmonella negative faeces

		4 h BPW	**18 h BPW**
Blank (n = 5 per lab*)	number of samples	139	139
	negative samples	139	135
	Secificity in %	***100.0***	***97.1***
STM 10 (n = 5 per lab)	number of samples	140	140
	positive samples	38	134
	Sensitivity in %	***27.1***	***95.7***
STM 100 (n = 5 per lab*)	number of samples	139	139
	positive samples	94	138
	Sensitivity in %	***67.6***	***99.3***
SE 100 (n = 5 per lab*)	number of samples	138	138
	positive samples	29	136
	Sensitivity in %	***21.0***	***98.6***
SE 500 (n = 5 per lab)	number of samples	140	140
	positive samples	100	138
	Sensitivity in %	***71.4***	***98.6***
All capsules with Salmonella	number of samples	557	557
	positive samples	261	546
	Sensitivity in %	***46.9***	***98.0***
All capsules	number of samples	696	696
	correct samples	400	681
	Accuracy in %	***57.5***	***97.8***

** For laboratory 12, n = 4 for STM100 and SE100 and for laboratory 23, n = 4 for SE100 and blank capsules*

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

The results in Table 19 and Figures 3 and 4 shows that eleven laboratories (labcodes 1, 4, 6, 11, 13, 18, 20, 23, 25, 26 and 27) were able to recover *Salmonella* from all dust samples after 4 h incubation of BPW, whereas 26 laboratories were able to recover *Salmonella* from all dust samples after 18 h incubation of BPW. One laboratory (labcode 21) tested only 2 samples after 18 h incubation of BPW. Both of these samples were tested positive for *Salmonella*. Another laboratory (labcode 28) tested only 5 dust samples after 18 h incubation of BPW, of these 5 samples 3 were tested positive for *Salmonella.*

Table 19 Number of positive isolations per medium combination and per laboratory for naturally contaminated dust (n=20)

	Laboratory codes													
Medium combination	1	2	3	4	5	6	7	8	9	10	11	12	13	14
BPW 4 hrs	10	4	8	10	6	10	8	9	9	9	10	9	10	8
BPW 18 hrs	10	10	10	10	10	10	10	10	10	10	10	10	10	10

	Laboratory codes													
Medium combination	15	16	17	18	19	20	21	22	23	24	25	26	27	28
BPW 4 hrs	8	7	8	10	1	10	8	5	10	5	10	10	10	3
BPW 18 hrs	10	10	10	10	10	10	2*	10	10	10	10	10	10	5*

** Laboratory 21 investigated only 2 samples after 18 h in BPW and laboratory 28 investigated only 5 samples after 18 h in BPW*

Six laboratories (labcode 1, 2, 11, 12, 13 and 27) reported a strain in the dust samples that was yellow in LDC (meaning a positive reaction, whereas a negative purple color is normally expected). Two laboratories (labcode 1 and 13) confirmed the samples with API 20E as *Salmonella*. Laboratory 1 used a InvA-PCR and typed the strain as *S.* Virchow. Four laboratories (labcode 2, 11, 20 and 27) serotyped all samples (including artificially contaminated, control samples and dust). Laboratory 1 found *S.* Virchow, *S.* Kortrijk and *S.* Isangi in the dust samples. Laboratory 11 found *S.* Virchow, *S.* Livingstone and *S.* Infantis. Laboratory 20 typed all strains as *S.* Livingstone. Laboratory 25 and 27 only determined the O group. Laboratory 25 found both group B and C and laboratory 27 typed the strains O 6,7 group C.

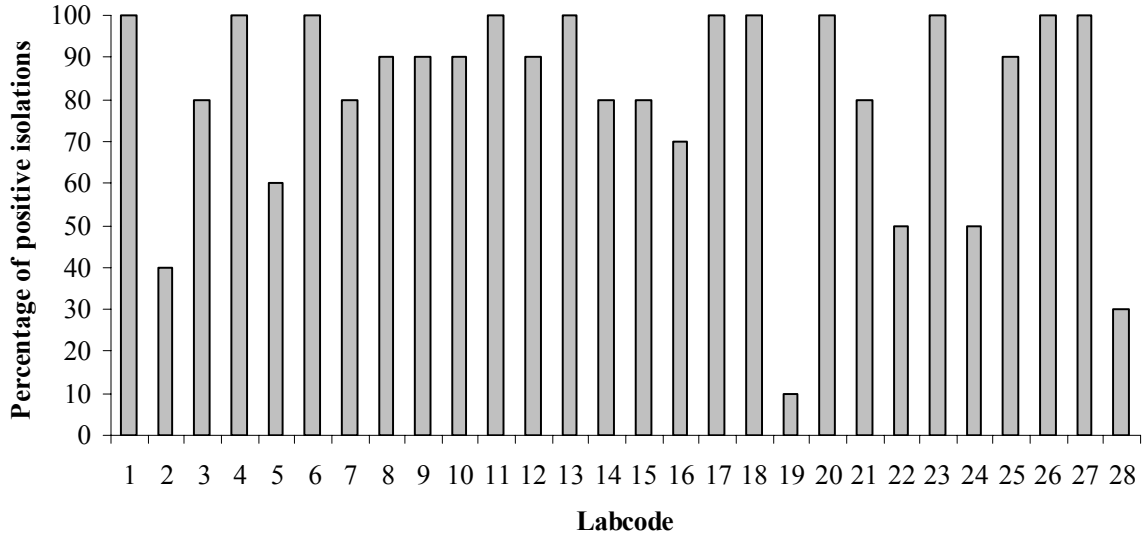

Figure 3 *Percentages of positive isolations (n = 10) per laboratory when analysing 10 g Salmonella positive dust after 4 h incubation of BPW.*

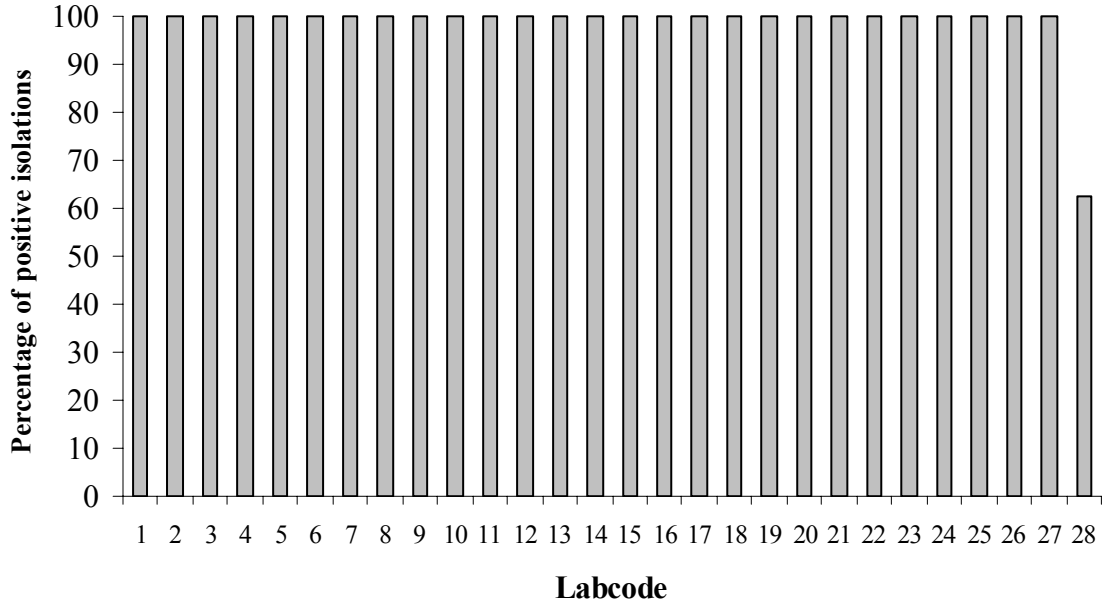

Figure 4 *Percentages of positive isolations (n = 10*) per laboratory when analysing 10 g Salmonella positive dust after 18 h incubation of BPW. * n = 2 for laboratory 21 and n = 5 for laboratory 28.*

The sensitivity rates for 28 participating laboratories for the naturally contaminated samples per medium combination are given in Table 20. The isolation of *Salmonella* from the naturally contaminated dust showed less positive isolations after 4 h of incubation of BPW than after 18 h of incubation of BPW, resulting in a sensitivity of 81 % and 99 %, respectively.

Table 20 *Sensitivity rates of all participating laboratories (n = 28) after 4 and 18 h incubation of BPW for the naturally contaminated dust with Salmonella (n=10)*

	4 hr BPW	**18 hr BPW**
number of samples (n = 10*)	280	270
positive samples	226	267
Sensitivity[1] in %	***80.7***	***98.9***

** n = 2 for laboratory 21 and n = 5 for laboratory 28 after 18 h incubation of BPW*

[1] No negative samples were tested; therefore the accuracy rate is equal to the sensitivity rate

4.7 PCR

Six laboratories (labcodes 1, 2, 6, 16, 20 and 22) applied the PCR as additional detection technique. Laboratory with labcode 16 only carried out PCR on 15 artificially contaminated samples, the other laboratories for all artificially contaminated, control and dust samples. Laboratory 1 carried out a PCR after both 4 and 18 h incubation in BPW. All other laboratories only tested the samples after 18 h incubation of BPW.

Table 21 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)
1	1000	150	5
2	1000	150	5
16	1000	150	5
20	1000	300	5
22	1000	200	5

Laboratory 6 used a touch of a loop of a MSRV plate.

Three laboratories (labcodes 2, 16 and 22) used a PCR procedure which was commercially available. The PCR results of all laboratories are shown in the Tables 5.1 - 5.3 in Annex 5.

For the control samples no samples were tested positive after 4 h incubation of BPW by laboratory 1 while with the culture method two samples were tested positive. After 18 h incubation of BPW laboratory 1, 6, 20 and 22 tested all samples correct, while with the culture method laboratory 1 tested one blank capsule positive. Laboratory 2 tested one blank capsule positive in the PCR, but negative in the culture method.
For the naturally contaminated samples all samples were tested positive after 18 h incubation of BPW as well in the PCR as in the culture method for all labs. After 4 h however, in the culture method all samples were tested positive by laboratory 1 while all samples were tested negative in the PCR.
No positive results were found in the PCR after 4 h incubation of BPW by laboratory 1 while 10 samples were found positive in the culture method. Also after 18 h of BPW more positive results (20) were obtained with the culture method than with the PCR (16) by laboratory 1. Laboratory 2 tested two blank samples positive in the culture method and one in the PCR. Laboratories 6, 16 and 20 found the same results in the PCR method as in the culture method. One positive sample was found negative in the culture method by laboratory 22, in the PCR the same sample was tested negative and one additional positive sample was tested negative.

4.8 Transport of samples

The temperature recorders were returned immediately after receipt to CRL-*Salmonella* by all NRLs. 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 laboratories 12 and 28 the transport time was 3 days. The average number of transport time was 1.5 days. The temperature of the content of the parcel was below 5 ^{o}C in all cases.

5. Discussion

Performance of the laboratories
Due to a mistake of the CRL-*Salmonella*, laboratory 12 and 24 received two capsules with the same number and one capsule was missing. The results of both the double and missing capsule numbers were not included in the results. In general all laboratories performed very well this year. Only two NRLs did not achieve the level of "good performance" as was suggested during the CRL-*Salmonella* workshop in April 2005. The NRL with labcode 2 isolated *Salmonella* out of 2 of the 5 blank capsules with the addition of faeces and the NRL with labcode 28 did not isolate *Salmonella* out of both capsules containing SPan5. Since all NRLs (including the NRLs with labcode 2 and 28) tested all the samples correctly no further actions were taken.
In this study more positive isolations were found than in the former study of 2004. For both the artificially contaminated samples as well as for the naturally contaminated dust, more positive isolations were found after 18 h incubation of BPW than after 4 h incubation. After 18 h incubation of BPW almost all samples were found positive for *Salmonella*. Incubation of BPW for 4 h therefore seems to be too short to isolate all *Salmonellas* present in the samples.

Transport of the samples
For this study the samples were shipped by courier service from door-to-door as diagnostic specimens, resulting in short transport times. All NRLs received the packages within 3 days. The temperature of the contents of the packages did not exceed 5 ºC.

Dust samples
The dust samples contained *S.* Livingstone and *S.* Virchow. For the *S.* Virchow present in this dust sample the conformation on LDC was yellow instead of purple (thus positive instead of negative). Six laboratories reported a strain in the dust samples that was yellow in LDC. Five laboratories further typed the strains in the dust. One laboratory only found *S.* Livingstone, one laboratory found *S.* Isangi and *S.* Kortrijk next to *S.* Virchow and one laboratory found *S.* Infantis next to *S.* Virchow and *S.* Livingstone. Another laboratory typed only the O-antigens and found strains from group C as well from group B. It is possible that these strains (*S.* Isangi, *S.* Kortrijk, *S.* Infantis and group B) were also present in the dust; however it could also be that the strains are typed incorrectly.

Artificially contaminated faecal samples
In this study the faeces was not mixed with any preservation medium, since studies carried out by the CRL-*Salmonella* revealed that glycerol has a negative effect on the growth of *Salmonella*. In this interlaboratory comparison study the results by the NRLs confirmed this effect of glycerol since the accuracy rates in this study were higher than in earlier studies were the faeces was mixed with peptone/glycerol (49 % and 77 % for 4 h and 18 h incubation of BPW in 2004 respectively and 57 % and 98 % in this study respectively). However, 4 h incubation in BPW seems to be too short for sufficient growth of *Salmonella* even without glycerol added to the faeces.

Control samples
All, but one NRL, found perfect results with the control samples (without faeces added) after 18 h incubation of BPW. After 18 h incubation of BPW one laboratory could not isolate *Salmonella* from either one of the SPan 5 control samples. Statistically it is possible that at low contamination levels occasionally a negative capsule can be found. However the changes are very small to find both SPan 5 capsules negative. An incubation time of 4 h of the pre-enrichment broth (BPW) seems to be too short for sufficient growth of *Salmonella*, resulting in low percentages of positive isolations (24 % sensitivity for all capsules containing *Salmonella* after 4 h incubation of BPW versus 99 % after 18 h).

Media
The capsules had to be dissolved in BPW for 45 minutes at 37 ºC. One laboratory reported an incubation time of only 5 minutes, which can result in not completely dissolved capsules and another laboratory reported that the capsules were not completely dissolved after 45 minutes. Also two laboratories reported a temperature below 37 ºC. A low temperature or not completely dissolved capsules can result in less positive isolations. However, in this study no effect on the positive isolations was found.
One laboratory incubated the samples for only 1 h instead of 4 h in BPW. Eight laboratories incubated the BPW less than 16 h instead of 16-20 h. Two laboratories reported an incubation temperature of less than 36 ºC. A short incubation time or a low incubation temperature can result in less positive isolations, although no effect was found in this study.
According to draft Annex D of ISO 6579 the concentration of novobiocin should be 0.01 g/l. Six laboratories reported the use of 0.02 g/l novobiocin and two laboratories did not use novobiocin. Five laboratories incubated the MSRV at a temperature below 40.5 ºC. The lack of novobiocin in the MSRV or a low incubation temperature may result in less selectivity of MSRV and easier outgrowth of disturbing background flora. The effect novobiocin or the low incubation temperature on the results of the study was not clear. Two laboratories reported a total incubation time for MSRV of less than 42 h. A short incubation time can result in less positive isolations. However, in this study no effect on the positive isolations was found.

PCR

With the PCR method all samples incubated 18 h in BPW were tested correctly, except for one blank control sample, which was found positive by laboratory 2. No direct explanation could be found. After 4 h incubation of BPW no artificially contaminated sample, control sample or dust sample was tested positive, while with the culture method some samples were. Overall the culture method gave better results than the PCR method.

Specificity, sensitivity and accuracy rates

The specificity for the blank capsules was 100 % for the control samples (without addition of faeces) after both 4 h and 18 h incubation in BPW. For the artificially contaminated samples the specificity was 100 % after 4 h incubation of BPW and 97 % after 18 h incubation of BPW. For all samples (artificially contaminated, naturally contaminated and controls) the sensitivity rates after 18 h incubation of BPW was for all different capsules higher than 95 %. The accuracy rates were higher after 18 h incubation of BPW than after 4 h incubation of BPW for the control samples (respectively 99 % and 39 %), the artificially contaminated samples (respectively 98 % and 57 %) and for the naturally contaminated dust samples (respectively 99 % and 81 %).

Future studies

In the following interlaboratory comparison studies, the faeces will no longer be mixed with peptone/glycerol solution since this study showed better results than earlier studies.

6. Conclusions

- All NRLs performed very well. All NRLs achieved the level of "good performance" as was suggested during the CRL-*Salmonella* workshop 2005 for the tested samples. Only two NRLs experienced small problems with the controls; therefore no further actions were taken.

- Significant more positive isolations were obtained from the artificially contaminated samples, the control samples and the naturally contaminated samples after 18 h incubation in the pre-enrichment broth BPW, when compared to 4 h incubation.

- The accuracy rates for the artificially contaminated samples were 57 % and 98 % after respectively 4 h and 18 h incubation of BPW.

- The accuracy rates for the naturally contaminated dust samples were 81 % and 99 % after respectively 4 h and 18 h incubation of BPW.

- After 4 h incubation of BPW it was easier to isolate *Salmonella* from capsules with a high level of *Salmonella* Typhimurium (STM100), combined with faeces than, in declining order, from capsules with a high or low level of *Salmonella* Enteritidis (SE500 and SE100) and from capsules with a low level of *Salmonella* Typhimurium (STM10).

- After 18 h incubation of BPW it was easier to isolate *Salmonella* from capsules with a high level of *Salmonella* Enteritidis (SE500), combined with faeces than, in declining order, from capsules with a high or low level of *Salmonella* Typhimurium (STM100 and STM10) and from capsules with a low level of *Salmonella* Enteritidis (SE100).

- The absence of glycerol in the chicken faeces has positively affected the results of this study.

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Annex 1. History of bacteriological studies

Table 1.1 History of bacteriological studies

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 1.1 History of bacteriological studies (continued)

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	Korver *et al*., 2005 (report 330300008)
		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**			
IX	2005	5	STM10		10 gram[2]	MSRV	XLD and own	This report
		5	STM100		10 gram			
		5	SE100		10 gram			
		5	SE500		10 gram			
		5	Blank		10 gram			
		3	STM10		No			
		2	SE100		No			
		1	SE500		No			
		2	SPan5		No			
		2	Blank		No			
		10	None		10 gram***			

*** = Naturally contaminated chicken faeces with Salmonella*

**** = Naturally contaminated dust with Salmonella*

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

[2] Faeces not mixed with any preservation medium

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 and per medium combination for all control samples after 4 h of incubation of BPW

	Medium combination		Capsules				
Labcode	Selective enrichment	Isolation medium	STM 10 (n=3)	SE 100 (n=2)	SE 500 (n=1)	Span 5 (n=2)	blank (n=2)
1	MSRV	XLD	0	1	1	0	0
	MSRV	Onoz	0	1	1	0	0
2	MSRV	XLD	2	1	0	0	0
	MSRV	MacConkey	2	1	0	0	0
	SC	XLD	1	1	0	0	0
	SC	MacConkey	1	1	0	0	0
3	MSRV	XLD	0	0	1	0	0
	MSRV	BxLH	0	0	1	0	0
4	MSRV	XLD	1	1	1	0	0
	MSRV	R Sal	1	1	1	0	0
	MSRV	BGA	1	1	1	0	0
	RVS	XLD	0	2	1	0	0
	RVS	R Sal	0	2	1	0	0
	RVS	BGA	0	2	1	0	0
5	MSRV	XLD	0	0	1	0	0
	MSRV	Rambach	0	0	1	0	0
6	MSRV	XLD	0	0	0	0	0
	MSRV	BG	0	0	0	0	0
7	MSRV	XLD	2	1	1	0	0
	MSRV	BGA	2	1	1	0	0
8	MSRV	XLD	0	0	0	0	0
	MSRV	BGA	0	0	0	0	0
	MSRV	MLCB	0	0	0	0	0
	RVS	XLD	0	0	0	0	0
	RVS	BGA	0	0	0	0	0
	RVS	MLCB	0	0	0	0	0
9	MSRV	XLD	0	0	1	0	0
	MSRV	BGA	0	0	1	0	0
10	MSRV	XLD	3	2	1	2	0
	MSRV	XLT4	3	2	1	2	0
11	MSRV	XLD	1	0	1	0	0
	MSRV	BGA	1	0	1	0	0
	MSRV (pH 5.4)	XLD	0	0	1	0	0
	MSRV (pH 5.4)	BGA	0	0	1	0	0
12	MSRV	XLD	0	1	1	0	0
	MSRV	Rambach	0	1	1	0	0
13	MSRV	XLD	0	0	0	0	0
	MSRV	BGA	0	0	0	0	0
14	MSRV	BGA	1	0	1	0	0

Table 3.1 Number of positive isolations per laboratory and per medium combination for all control samples after 4 h of incubation of BPW (continued)

	Medium combination		Capsules				
Labcode	Selective enrichment	Isolation medium	STM 10 (n=3)	SE 100 (n=2)	SE 500 (n=1)	Span 5 (n=2)	blank (n=2)
15	MSRV	XLD	1	0	1	1	0
	MSRV	BGA	1	0	1	1	0
16	MSRV	XLD	0	0	0	0	0
	MSRV	BGA	0	0	0	0	0
17	MSRV	XLD	0	0	0	0	0
	MSRV	Rambach	0	0	0	0	0
18	MSRV	XLD	1	1	1	1	0
	MSRV	SMID2	1	1	1	1	0
19	MSRV	XLD	0	0	1	0	0
	MSRV	Rambach	0	0	1	0	0
	MSRV	BGA	0	0	1	0	0
20	MSRV	XLD	0	0	1	0	0
	MSRV	BPLS	0	0	1	0	0
21	MSRV	XLD	0	0	0	0	0
	MSRV	Rambach	0	0	0	0	0
22	MSRV	XLD	0	0	0	0	0
	MSRV	BGA	0	0	0	0	0
23	MSRV	XLD	1	0	1	0	0
	MSRV	SM2	1	0	1	0	0
24	MSRV	XLD	1	1	0	1	0
	MSRV	BGA	1	1	0	1	0
25	MSRV	XLD	0	0	0	1	0
	MSRV	BGA	1	0	0	1	0
	DIASSALM	XLD	1	1	1	1	0
	DIASSALM	BGA	1	1	1	1	0
	RVS	XLD	1	0	1	0	0
	RVS	BGA	1	0	1	0	0
26	MSRV	XLD	1	1	1	0	0
	MSRV	SMI	1	1	1	0	0
	MKTTn	XLD	0	2	1	0	0
	MKTTn	SMI	0	2	1	0	0
27	MSRV	XLD	1	0	1	0	0
	MSRV	BGA	1	0	1	0	0
28	MSRV	XLD	0	0	1	0	0
	MSRV	Hektoen	0	0	1	0	0

Table 3.2 Number of positive isolations per laboratory and per medium combination for all control samples after 18 h of incubation of BPW

	Medium combination		Capsules				
Labcode	Selective enrichment	Isolation medium	STM 10 (n=3)	SE 100 (n=2)	SE 500 (n=1)	Span 5 (n=2)	blank (n=2)
1	MSRV	XLD	3	2	1	2	0
	MSRV	Onoz	3	2	1	2	0
2	MSRV	XLD	3	2	1	2	0
	MSRV	MacConkey	3	2	1	2	0
	SC	XLD	3	2	1	2	0
	SC	MacConkey	3	2	1	2	0
3	MSRV	XLD	3	2	1	2	0
	MSRV	BxLH	3	2	1	2	0
4	MSRV	XLD	3	2	1	2	0
	MSRV	R Sal	3	2	1	2	0
	MSRV	BGA	3	2	1	2	0
	RVS	XLD	3	2	1	2	0
	RVS	R Sal	3	2	1	2	0
	RVS	BGA	3	2	1	2	0
5	MSRV	XLD	3	2	1	2	0
	MSRV	Rambach	3	2	1	2	0
6	MSRV	XLD	3	2	1	2	0
	MSRV	BG	3	2	1	2	0
	RV	XLD	3	2	1	2	0
	RV	BG	3	2	1	2	0
7	MSRV	XLD	3	2	1	2	0
	MSRV	BGA	3	2	1	2	0
8	MSRV	XLD	3	2	1	2	0
	MSRV	BGA	3	2	1	2	0
	MSRV	MLCB	3	2	1	2	0
9	MSRV	XLD	3	2	1	2	0
	MSRV	BGA	3	2	1	2	0
10	MSRV	XLD	3	2	1	2	0
	MSRV	XLT4	3	2	1	2	0
11	MSRV	XLD	3	2	1	2	0
	MSRV	BGA	3	2	1	2	0
	MSRV (pH 5.4)	XLD	3	2	1	2	0
	MSRV (pH 5.4)	BGA	3	2	1	2	0
12	MSRV	XLD	3	2	1	2	0
	MSRV	Rambach	3	2	1	2	0
	RV	XLD	3	2	1	2	0
	RV	Rambach	3	2	1	2	0
	RV	XLT4	3	2	1	2	0
13	MSRV	XLD	3	2	1	2	0
	MSRV	BGA	3	2	1	2	0
14	MSRV	BGA	3	2	1	2	0

Table 3.2 Number of positive isolations per laboratory and per medium combination for all control samples after 18 h of incubation of BPW (continued)

	Medium combination		Capsules				
Labcode	Selective enrichment	Isolation medium	STM 10 (n=3)	SE 100 (n=2)	SE 500 (n=1)	Span 5 (n=2)	blank (n=2)
15	MSRV	XLD	3	2	1	2	0
	MSRV	BGA	3	2	1	2	0
16	MSRV	XLD	3	2	1	2	0
	MSRV	BGA	3	2	1	2	0
17	MSRV	XLD	3	2	1	2	0
	MSRV	Rambach	3	2	1	2	0
18	MSRV	XLD	3	2	1	2	0
	MSRV	SMID2	3	2	1	2	0
19	MSRV	XLD	3	2	1	2	0
	MSRV	Rambach	3	2	1	2	0
	MSRV	BGA	3	2	1	2	0
20	MSRV	XLD	3	2	1	2	0
	MSRV	BPLS	3	2	1	2	0
	MKTTn	XLD	3	2	1	1	0
	MKTTn	BPLS	3	2	1	2	0
21	MSRV	XLD	3	2	1	2	0
	MSRV	Rambach	3	2	1	2	0
22	MSRV	XLD	3	2	1	2	0
	MSRV	BGA	3	2	1	2	0
	MKTTn	XLD	3	2	1	2	0
	MKTTn	BGA	3	2	1	2	0
23	MSRV	XLD	3	2	1	2	0
	MSRV	SM2	3	2	1	2	0
24	MSRV	XLD	3	2	1	2	0
	MSRV	BGA	3	2	1	2	0
25	MSRV	XLD	3	2	1	2	0
	MSRV	BGA	3	2	1	2	0
	DIASSALM	XLD	3	2	1	2	0
	DIASSALM	BGA	3	2	1	2	0
	RVS	XLD	3	2	1	2	0
	RVS	BGA	3	2	1	2	0
26	MSRV	XLD	3	2	1	2	0
	MSRV	SMI	3	2	1	2	0
	MKTTn	XLD	3	2	1	2	0
	MKTTn	SMI	3	2	1	2	0
27	MSRV	XLD	3	2	1	2	0
	MSRV	BGA	3	2	1	2	0
	RVS	XLD	3	2	1	2	0
	RVS	BGA	3	2	1	2	0
28	MSRV	XLD	3	2	1	0	0
	MSRV	Hektoen	3	2	1	0	0

Table 3.3 Number of positive isolations per laboratory and per medium combination for all samples after 4 h of incubation of BPW

	Medium combination		Capsules				
Labcode	Selective enrichment	Isolation medium	STM 10 (n = 5)	STM 100 (n = 5)	SE 100 (n = 5)	SE 500 (n = 5)	Blank (n = 5)
1	MSRV	XLD	1	3	2	4	0
	MSRV	Onoz	1	3	2	4	0
2	MSRV	XLD	2	1	0	1	0
	MSRV	MacConkey	2	1	0	1	0
	SC	XLD	0	0	0	1	0
	SC	MacConkey	0	0	0	1	0
3	MSRV	XLD	1	5	2	5	0
	MSRV	BxLH	1	5	2	5	0
4	MSRV	XLD	2	5	1	5	0
	MSRV	R Sal	2	5	1	5	0
	MSRV	BGA	2	5	1	5	0
	RVS	XLD	3	5	3	5	0
	RVS	R Sal	3	5	3	5	0
	RVS	BGA	3	5	3	5	0
5	MSRV	XLD	0	2	0	3	0
	MSRV	Rambach	0	2	0	3	0
6	MSRV	XLD	4	5	1	5	0
	MSRV	BG	4	5	1	5	0
7	MSRV	XLD	2	5	0	4	0
	MSRV	BGA	2	5	0	4	0
8	MSRV	XLD	1	0	1	1	0
	MSRV	BGA	1	0	1	1	0
	MSRV	MLCB	1	0	1	1	0
	RVS	XLD	0	3	0	1	0
	RVS	BGA	0	3	0	1	0
	RVS	MLCB	0	3	0	1	0
9	MSRV	XLD	0	1	0	1	0
	MSRV	BGA	0	1	0	1	0
10	MSRV	XLD	0	5	0	5	0
	MSRV	XLT4	0	5	0	5	0
11	MSRV	XLD	1	3	0	4	0
	MSRV	BGA	1	3	0	4	0
	MSRV (pH 5.4)	XLD	1	5	0	5	0
	MSRV (pH 5.4)	BGA	1	5	0	5	0
12	MSRV	XLD	4	4*	2*	5	0
	MSRV	Rambach	4	4*	2*	5	0
13	MSRV	XLD	2	5	1	5	0
	MSRV	BGA	2	5	1	5	0
14	MSRV	BGA	0	1	0	2	0

** 4 samples were investigated instead of 5*

Table 3.3 Number of positive isolations per laboratory and per medium combination for all control samples after 4 h of incubation of BPW (continued)

	Medium combination		Capsules				
Labcode	Selective enrichment	Isolation medium	STM 10 (n = 5)	STM 100 (n = 5)	SE 100 (n = 5)	SE 500 (n = 5)	Blank (n = 5)
15	MSRV	XLD	3	5	1	5	0
	MSRV	BGA	3	5	1	5	0
16	MSRV	XLD	0	1	0	2	0
	MSRV	BGA	0	1	0	2	0
17	MSRV	XLD	0	3	0	5	0
	MSRV	Rambach	0	3	0	5	0
18	MSRV	XLD	0	4	1	5	0
	MSRV	SMID2	0	4	1	5	0
19	MSRV	XLD	0	3	0	2	0
	MSRV	Rambach	0	3	0	2	0
	MSRV	BGA	0	3	0	2	0
20	MSRV	XLD	0	3	3	4	0
	MSRV	BPLS	0	3	3	4	0
21	MSRV	XLD	0	2	0	2	0
	MSRV	Rambach	0	2	0	2	0
22	MSRV	XLD	0	1	0	0	0
	MSRV	BGA	0	1	0	0	0
23	MSRV	XLD	1	5	2	5	0
	MSRV	SM2	1	5	2	5	0
24	MSRV	XLD	3	2	2*	4	0*
	MSRV	BGA	3	2	2*	4	0*
25	MSRV	XLD	0	5	2	5	0
	MSRV	BGA	0	5	2	5	0
	DIASSALM	XLD	4	5	3	5	0
	DIASSALM	BGA	4	5	3	5	0
	RVS	XLD	3	5	2	5	0
	RVS	BGA	3	5	2	5	0
26	MSRV	XLD	0	3	1	4	0
	MSRV	SMI	0	3	1	4	0
	MKTTn	XLD	1	5	3	5	0
	MKTTn	SMI	3	5	3	5	0
27	MSRV	XLD	3	5	4	5	0
	MSRV	BGA	3	5	4	5	0
28	MSRV	XLD	0	2	0	2	0
	MSRV	Hektoen	0	1	0	2	0

** 4 samples were investigated instead of 5*

Table 3.4 Number of positive isolations per laboratory and per medium combination for all samples after 18 h of incubation of BPW

	Medium combination		Capsules				
Labcode	Selective enrichment	Isolation medium	STM 10 (n = 5)	STM 100 (n = 5)	SE 100 (n = 5)	SE 500 (n = 5)	Blank (n = 5)
1	MSRV	XLD	5	5	5	5	0
	MSRV	Onoz	5	5	5	5	0
2	MSRV	XLD	5	5	5	4	2
	MSRV	MacConkey	5	5	5	4	2
	SC	XLD	4	5	5	5	2
	SC	MacConkey	4	5	5	5	2
3	MSRV	XLD	4	4	5	3	0
	MSRV	BxLH	4	4	5	3	0
4	MSRV	XLD	5	4	5	5	0
	MSRV	R Sal	5	4	5	5	0
	MSRV	BGA	5	4	5	5	0
	RVS	XLD	4	4	5	5	0
	RVS	R Sal	5	5	5	5	0
	RVS	BGA	4	5	5	5	0
5	MSRV	XLD	5	5	5	5	0
	MSRV	Rambach	5	5	5	5	0
6	MSRV	XLD	5	5	5	5	0
	MSRV	BG	5	5	5	5	0
	RV	BG	5	5	5	5	0
	RV	XLD	5	5	5	5	0
7	MSRV	XLD	5	5	5	5	0
	MSRV	BGA	5	5	5	5	0
8	MSRV	XLD	5	5	5	5	0
	MSRV	MLCB	5	5	5	5	0
	MSRV	BGA	5	5	4	5	0
	RVS	XLD	5	5	5	5	0
	RVS	MLCB	5	5	5	5	0
	RVS	BGA	5	4	3	3	0
9	MSRV	XLD	4	5	5	5	1
	MSRV	BGA	4	5	5	5	1
10	MSRV	XLT4	4	5	5	5	0
	MSRV	XLD	4	5	5	5	0
11	MSRV	XLD	5	5	5	5	0
	MSRV	BGA	5	5	5	5	0
	MSRV (pH 5.4)	XLD	5	5	5	5	0
	MSRV (pH 5.4)	BGA	5	5	5	5	0
12	MSRV	XLD	5	4*	4*	5	0
	MSRV	Rambach	5	4*	4*	5	0
	RV	XLT4	5	4*	4*	5	0
	RV	XLD	5	4*	4*	5	0
	RV	Rambach	5	4*	4*	5	0
13	MSRV	XLD	5	5	5	5	0
	MSRV	BGA	5	5	5	5	0
14	MSRV	BGA	5	5	5	5	0

** 4 samples were investigated instead of 5*

Table 3.4 Number of positive isolations per laboratory and per medium combination for all samples after 18 h of incubation of BPW (continued)

	Medium combination		Capsules				
Labcode	Selective enrichment	Isolation medium	STM 10 (n = 5)	STM 100 (n = 5)	SE 100 (n = 5)	SE 500 (n = 5)	Blank (n = 5)
15	MSRV	XLD	5	5	5	5	0
	MSRV	BGA	5	5	5	5	0
16	MSRV	XLD	5	5	5	5	0
	MSRV	BGA	5	5	5	5	0
17	MSRV	XLD	5	5	5	5	0
	MSRV	Rambach	5	5	5	5	0
18	MSRV	XLD	5	5	5	5	1
	MSRV	SMID2	5	5	5	5	1
19	MSRV	XLD	5	5	5	5	0
	MSRV	Rambach	5	5	5	5	0
	MSRV	BGA	5	5	5	5	0
20	MKTTn	XLD	2	3	0	1	0
	MKTTn	BPLS	3	5	0	3	0
	MSRV	XLD	5	5	5	5	0
	MSRV	BPLS	5	5	5	5	0
21	MSRV	XLD	4	5	4	5	0
	MSRV	Rambach	4	5	4	5	0
22	MKTTn	XLD	4	5	3	5	0
	MKTTn	BGA	4	5	3	4	0
	MSRV	XLD	5	5	4	5	0
	MSRV	BGA	5	5	4	5	0
23	MSRV	XLD	5	5	5	5	0
	MSRV	SM2	5	5	5	5	0
24	MSRV	XLD	5	5	4*	5	0*
	MSRV	BGA	5	5	4*	5	0*
25	DIASSALM	XLD	5	5	5	5	0
	DIASSALM	BGA	5	5	5	5	0
	MSRV	XLD	5	5	5	5	0
	MSRV	BGA	5	5	5	5	0
	RVS	XLD	5	5	5	5	0
	RVS	BGA	5	5	5	5	0
26	MKTTn	XLD	5	5	5	5	0
	MKTTn	SMI	5	5	5	5	0
	MSRV	XLD	5	5	5	5	0
	MSRV	SMI	5	5	5	5	0
27	MSRV	XLD	5	5	5	5	0
	MSRV	BGA	5	5	5	5	0
	RVS	XLD	4	5	5	5	0
	RVS	BGA	4	5	5	5	0
28	MSRV	XLD	2	5	5	5	0
	MSRV	Hektoen	3	4	5	5	0

** 4 samples were investigated instead of 5*

Table 3.5 Number of positive isolations per laboratory with all medium combinations for naturally contaminated dust (n=10) after 4 h and 18 h incubation of BPW

	Medium combination		4 h BPW	8 h BPW
Labcode	Selective enrichment	Isolation medium		
1	MSRV	XLD	10	10
	MSRV	Onoz	10	10
2	MSRV	XLD	4	10
	MSRV	MacConkey	4	10
	SC	XLD	3	9
	SC	MacConkey	3	9
3	MSRV	XLD	8	10
	MSRV	BxLH	8	10
4	MSRV	XLD	9	10
	MSRV	R Sal	10	10
	MSRV	BGA	9	10
	RVS	XLD	10	10
	RVS	R Sal	10	10
	RVS	BGA	10	10
5	MSRV	XLD	6	10
	MSRV	Rambach	6	10
6	MSRV	XLD	10	10
	MSRV	BG	10	10
	RV	XLD	-	10
	RV	BG	-	10
7	MSRV	XLD	8	10
	MSRV	BGA	8	10
8	MSRV	XLD	8	10
	MSRV	BGA	4	10
	MSRV	MLCB	9	10
	RVS	XLD	8	10
	RVS	BGA	8	10
	RVS	MLCB	8	10
9	MSRV	XLD	9	10
	MSRV	BGA	9	10
10	MSRV	XLD	9	10
	MSRV	XLT4	9	10
11	MSRV	XLD	9	10
	MSRV	BGA	9	10
	MSRV (pH 5.4)	XLD	10	10
	MSRV (pH 5.4)	BGA	10	10
12	MSRV	XLD	9	10
	MSRV	Rambach	9	10
	MK	XLD	-	10
	MK	Rambach	-	10
	MK	XLT4	-	10
13	MSRV	XLD	10	10
	MSRV	BGA	10	10
14	MSRV	BGA	8	10

Table 3.5 Number of positive isolations per laboratory with all medium combinations for naturally contaminated dust (n=10) after 4 h and 18 h incubation of BPW (continued)

	Medium combination		4 h BPW	8 h BPW
Labcode	Selective enrichment	Isolation medium		
15	MSRV	XLD	8	10
	MSRV	BGA	8	10
16	MSRV	XLD	7	10
	MSRV	BGA	7	10
17	MSRV	XLD	8	10
	MSRV	Rambach	8	10
18	MSRV	XLD	10	10
	MSRV	SMID2	10	10
19	MSRV	XLD	1	10
	MSRV	Rambach	1	10
	MSRV	BGA	1	10
20	MSRV	XLD	10	10
	MSRV	BPLS	10	10
	MKTTn	XLD	-	4
	MKTTn	BPLS	-	2
21	MSRV	XLD	8	2*
	MSRV	Rambach	8	2*
22	MSRV	XLD	5	10
	MSRV	BGA	5	10
	MKTTn	XLD	-	10
	MKTTn	BGA	-	8
23	MSRV	XLD	10	10
	MSRV	SM2	10	10
24	MSRV	XLD	5	10
	MSRV	BGA	5	10
25	MSRV	XLD	10	10
	MSRV	BGA	10	10
	DIASSALM	XLD	10	10
	DIASSALM	BGA	10	10
	RVS	XLD	9	10
	RVS	BGA	9	10
26	MSRV	XLD	10	10
	MSRV	SMI	10	10
	MKTTn	XLD	10	10
	MKTTn	SMI	10	10
27	MSRV	XLD	10	10
	MSRV	BGA	10	10
	RVS	XLD	-	8
	RVS	BGA	-	7
28	MSRV	XLD	3	5*
	MSRV	Hektoen	1	5*

- not determined

** after 18 h incubation in BPW n=2 for laboratory 21 and n=8 for laboratory 28*

Annex 4. Information on the media used

Table 4.1 Manufacturer of BPW

Labcode	Kind of medium	Name	Code	Batch
1	Dehydrated	Oxoid	CM0509	391319
2	Dehydrated	Oxoid	CM0509	346349
3	Dehydrated	Oxoid	CM1049	405665
4	Ready-to-use	Biorad	3554179	5D0482
5	Individual Ingredients	-	-	-
6	Dehydrated	Oxoid	CM0509	410247
7	Dehydrated	Merck	1.06404	K33800104
8	Dehydrated	Lab M	Lab 46	084016298
9	Dehydrated	Oxoid	CM0509	272066
10	Dehydrated	Merck	1.07228	VM232428
11	Dehydrated	Merck	1.07228	VM181928
12	Dehydrated	AES	AEB140302	513937
13	Dehydrated	Oxoid	CM0509	398599
14	Ready-to-use	Biotrading	0386	0530716866
15	Dehydrated	Oxoid	CM0509	387206
16	Dehydrated	Oxoid	CM1049	422588
17	Dehydrated	Merck	1.07228	VM463828531
18	Dehydrated	Merck	1.07228	VM452128527
19	Dehydrated	Merck	1.07228	VM389928
20	Dehydrated	Merck	1.07228	VL701928124
21	Dehydrated	Merck	1.07228	VM304428436
22	Dehydrated	Oxoid	CM1049	391380
23	Dehydrated	Merck	1.07228	VM389928
24	Dehydrated	Oxoid	CM0509	305195
25	Dehydrated	Oxoid	CM0509	381407
26	Ready-to-use	Biomerieux	42042	796998801
27	Dehydrated	Oxoid	CM0509	401491
28	Dehydrated	Oxoid	CM0509	335025

- not reported

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

Labcode	Peptone	Sodium Chloride	Disodium phophate	Potassium dihydrogen phosphate	pH after preperation	pH at day of use
ISO 6579	10.0	5.0	3.5/9.0	1.5	7.0±0.2	7.0±0.2
1	10.0	5.0	3.5	1.5	7.2	7.2
2	10.0	5.0	3.5	1.5	7.2	7.2
3	10.0	5.0	3.5	1.5	7.1	7.1
4	10.0	5.0	3.5	1.5	-	-
5	10.0	5.0	9.0	1.5	7.0	7.0
6	10.0	5.0	3.5	1.5	7.2	-
7	10.0	5.0	3.5	1.5	7.0	-
8	-	-	-	-	7.2	7.2
9	10.0	5.0	3.5	1.5	7.2	7.2
10	10.0	5.0	9.0	1.5	7.1	7.1
11	10.0	5.0	9.0	1.5	7.2	-
12	10.0	5.0	3.5	1.5	7.1	7.1
13	10.0	5.0	3.5	1.5	7.2	-
14	10.0	5.0	9.0	1.5	-	7.0
15	10.0	5.0	3.5	1.5	7.1	7.1
16	10.0	5.0	3.5	1.5	6.9	6.9
17	10.0	5.0	9.0	1.5	7.1	7.1
18	10.0	5.0	9.0	1.5	7.1	7.2
19	10.0	5.0	9.0	1.5	7.0	-
20	10.0	5.0	10.5*		7.2	7.2
21	10.0	5.0	9.0	1.5	7.0	6.9
22	10.0	5.0	3.5	1.5	7.0	7.1
23	10.0	5.0	9.0	1.5	7.1	7.2
24	10.0	5.0	3.5	1.5	7.2	7.3
25	10.0	5.0	3.5	1.5	7.3	7.3
26	10.0	5.0	9.0	1.5	-	-
27	10.0	5.0	9.0	1.5	7.1	7.1
28	10.0	5.0	3.5	1.5	-	-

** phosphate buffer, grey cells are deviating from ISO 6579*

Table 4.3 Manufacturer of MSRV

Labcode	Kind of medium	Name	Code	Batch
1	Dehydrated	Oxoid	CM0910	400827
2	Dehydrated	Oxoid	CM0910	417485
3	Dehydrated	Merck	1.09878	VM309178
4	Ready-to-use	Biorad	3556139	K2511
5	Dehydrated	Biorad	3564325	077275
6	Dehydrated	Oxoid	CM0910	384304
7	Dehydrated	Biolife	4019822	3L1701
8	Dehydrated	LAB M	Lab 150	081178/080
9	Dehydrated	Liofilchem	610018	5035206
10	Dehydrated	Difco	218681	4208808
11	Dehydrated	Oxoid	CM0910	393456
12	Dehydrated	AES	AEB140672	523525
13	Dehydrated	Difco	218681	4208808
14	Dehydrated	Difco	218681	5126853
15	Dehydrated	Oxoid	CM0910	409269
16	Dehydrated	Difco	218681	4138799
17	Dehydrated	Biokar	4C757	4M969
18	Dehydrated	Oxoid	CM0910	388805
19	Dehydrated	Difco	218681	4168799
20	Dehydrated	Oxoid	CM0910	388805
21	Dehydrated	Biolife	4019822	4C4601
22	Dehydrated	Oxoid	CM0910	409269
23	Dehydrated	Merck	1.09878	VM309178
24	Dehydrated	TEKIA	TM 7511A	100.56
25	Dehydrated	Merck	1.09878	VM309178
26	Dehydrated	Oxoid	CM0910	417485
27	Dehydrated	Oxoid	CM0910	409269
28	Dehydrated	Oxoid	CM0910	417485

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

Labcode	Tryp-tose	Casein hydro-lysate	NaCl	Potass. Digydrogen phosphate	$MgCl_2$ anhy-drous	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	5.35	5.4
2	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.3	5.34
3	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.59	5.58
4	9.2*		7.3	1.5	10.9	0.037	2.7	-	-
5	-	-	-	-	-	-	-	5.2	5.2
6	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.6	-
7	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.2	-
8	2.3	4.6	7.34	1.5	10.9	0.037	2.5	5.2	-
9	4.5	4.5	7.3	1.4	10.9	0.037	2.7	5.2	5.4
10	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.29	-
11	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.2	-
12	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.7	-
13	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.33	-
14	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.29	5.21
15	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.4	5.3
16	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.1	5.1
17	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.2	5.2
18	4.95	4.95	7.34	1.47	10.93	0.037	2.7	5.5	5.5
19	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.05	-
20	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.4	5.4
21	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.21	5.22
22	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.39	-
23	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.6	-
24	4.59	4.59	7.34	1.35	10.93	0.037	2.7	5.6	5.65
25	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.56	-
26	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.4	5.4
27	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.41	5.41
28	4.59	4.59	7.34	1.47	10.93	0.037	2.7	5.51	5.54

** peptone, grey cells are deviating from draft Annex D*

Table 4.5 Manufacturer of own selective medium

Labcode	Kind of medium	Name of medium	Manufacturer	Code number	Batch	pH after preparation	pH at day of use
2	Dehydrated	SC	Oxoid	CM0699	355993	7.12	7.02
4	Ready-to-use	RVS	Biorad	3555773	5G0151	-	-
6	Dehydrated	RVS	Oxoid	CM0866	401738	5.4	-
8	Dehydrated	RVS	Oxoid	CM0866	382648	5.2	5.2
12	Dehydrated	MKTTn	AES	AEB140702	504736	8.3	-
20	Dehydrated	MKTTn	Oxoid	CM1048	302083	8.2	8.2
22	Dehydrated	MKTTn					
24	Dehydrated	RV	Oxoid	CM0669	373692	5.2	5.25
25	Dehydrated	DIASSALM	Lab M	Lab 537	081453/227	4.98	-
25	Dehydrated	RVS	Oxoid	CM0866	408959	5.2	5.2
26	Ready-to-use	MKTTn	Biomerieux	42114	796896601	-	-
27	Dehydrated	RVS	Biomark	B386	526904	5.21	5.21

- not reported

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

Labcode	name medium	Composition	
2	SC	Tryptone	5.0
		Lactose	4.0
		Disodium phosphate	10.0
		L-Cystine	0.01
		Sodium hydrogen selenite	4.0
4	RVS	Soja peptone	4.5
		Sodium Chloride	7.2
		Dihydrogenphophate	1.26
		Dipotassium hydrogenphophate	0.18
		Mg Chloride deshydrate	28.6
6	RVS	Soya peptone	4.5
		Sodium Chloride	7.2
		Potassium dihydrogen phosphate	1.26
		Dipotassium dihydrogen phosphate	0.18
		Mg Chloride anhydrose	13.58
		Malachite green	0.036
8	RVS	Soya peptone	4.5
		Sodium Chloride	7.2
		Potassium dihydrogen phosphate	1.26
		Dipotassium dihydrogen phosphate	0.18
		Mg Chloride anhydrose	13.58
		Malachite green	0.036
12	MKTTn	Tryptone	7.0
		Peptone soja	2.3
		Sodium chloride	2.3
		Calcium carbonate	25.0
		Sodium thisulfate	40.7
		Bile de boeuf	4.75
20	MKTTn	Meat extract	4.3
		Encymatic digest of casein	8.6
		Sodium Chloride	2.6
		Calcium carbonate	38.7
		Sodium thisulphate (anhydrous)	30.5
		Ox-bile	4.78
		Brilliant green	0.0096
24	RV	Soya peptone	5.0
		Sodium chloride	8.0
		Potassium dihydrogen phosphate	1.6
		Magnesium chloride	40.0
		Malachite green	0.04

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

Labcode	name medium	Composition	
25	DIASSALM	Tryptone	20.0
		Meat peptone	6.1
		Ferrous Ammonium Sulphate	0.2
		Sodium thiosulphate 5	5.0
		Sucrose	7.5
		Lactose	0.5
		Bromocresol purple	0.08
		Malachite green	0.037
		Magnesium chloride	11.0
		Agar	2.8
25	RVS	Soya peptone	4.5
		Sodium chloride	7.2
		Potassium digydrogen phosphate	1.26
		di-potassium hydrogen phosphate	0.18
		Magnesium chloride (anhydrous)	13.58
		Malachite green	0.036
26	MKTTn	Enzymatic digest of meat extract	4.23
		Enzymatic digest of casein	8.45
		Sodium chloride	2.54
		Calcium carbonate	38.04
		sodium thiosulphate	30.27
		Ox bile	4.75
27	RVS	Papaic digest of soybean meal	4.5
		Sodium chloride	7.2
		Monopotassium phosphate	1.44
		Magnesium chloride	36.0
		Malachite green	0.036

Table 4.7 Manufacturer and pH of XLD

Labcode	Kind of medium	Manu-facturer	Code Number	Batch	pH after preparation	pH at day of use
Iso 6579	--	--	--	--	7.4±0.2	7.4±0.2
1	Dehydrated	Lab M	Lab 32	081417/126	7.26	7.3
2	Dehydrated	Oxoid	CM0469	406673	7.04	7.08
3	Dehydrated	Oxoid	CM0469	355657	7.32	7.32
4	Ready-to-use	Biorad	41741	2144B	-	-
5	Dehydrated	Lab M	Lab 32	084012/272	7.4	7.4
6	Dehydrated	Lab M	Lab 32	082681/188	7.4	-
7	Ready-to-use	Beckton D.	-	5263812	-	-
8	-	Cruinn	XLD-10	3K9N	7.4	-
9	Dehydrated	Oxoid	CM0469	370146	7.4	7.2
10	Ready-to-use	Oxoid	P05057A	929541	-	-
11	Ready-to-use	Oxoid	CM 469	374958	7.5	-
12	Dehydrated	AES	AEB153402	513238	7.6	-
13	Dehydrated	Oxoid	CM0469	374958	7.35	7.4
14	Dehydrated	Oxoid	CM0469	314459	7.42	7.5
15	Dehydrated	Oxoid	CM0469	374958	7.3	7.4
16	Dehydrated	Lab M	Lab 32	069663	7.2	7.2
17	Dehydrated	Merck	1.05287	VM497887546	7.4	7.4
18	Ready-to-use	Oxoid	CM0469	400477	7.38	7.36
19	Dehydrated	Oxoid	CM0469	406673	7.52	-
20	Dehydrated	Merck	1.05287	VM497887546	7.4	7.4
21	Dehydrated	Merck	1.05287	VM388187507	7.47	7.1
22	Dehydrated	Difco	278850	5061893	7.43	-
23	Ready-to-use	Biomerieux	43563	798735201	-	-
24	Dehydrated	Oxoid	CM0469	305175	7.4	7.56
25	Dehydrated	Oxoid	CM0469	378415	6.9	-
26	Ready-to-use	Biomerieux	43563	798392301	-	-
27	Dehydrated	Merck	1.05287	VM388187507	7.42	7.42
28	Dehydrated	Oxoid	CM0469	335144	7.16	7.14

- not reported; grey cells = deviating from ISO 6579

Table 4.8 Composition of XLD in g/l

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	3.75	5.0	7.5	7.5	5.0	3.0
4	3.5	5.0	7.5	7.5	5.0	3.0
5	-	-	-	-	-	-
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	-	-	-	-	-	-
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	3.5	5.0	7.5	7.5	5.0	3.0
13	3.75	5.0	7.5	7.5	5.0	3.0
14	3.75	5.0	7.5	7.5	5.0	3.0
15	3.75	5.0	7.5	7.5	5.0	3.0
16	3.75	5.0	7.5	7.5	5.0	3.0
17	3.75	5.0	7.5	7.5	5.0	3.0
18	3.5	5.0	7.5	7.5	5.0	3.0
19	3.75	5.0	7.5	7.5	5.0	3.0
20	3.75	5.0	7.5	7.5	5.0	3.0
21	3.75	5.0	7.5	7.5	5.0	3.0
22	3.75	5.0	7.5	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	3.5	5.0	7.5	7.5	5.0	3.0
27	3.75	5.0	7.5	7.5	5.0	3.0
28	3.75	5.0	7.5	7.5	5.0	3.0

- not reported, grey cells are deviating from ISO 6579

Table 4.8 Composition of XLD in g/l (continued)

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	13.0	1.0	6.8	0.8	
2	0.08	12.5	1.0	6.8	0.8	-
3	0.08	12.5	1.0	6.8	0.8	-
4	0.08	13.5	2.5	6.8	0.8	7.5
5	-	-	-	-	-	-
6	0.08	13.0	1.0	6.8	0.8	-
7	0.08	12.5	1.0	6.8	0.8	-
8	-	-	-	-	-	-
9	0.08	12.5	1.0	6.8	0.8	-
10	0.08	12.5	1.0	6.8	0.8	-
11	0.08	12.5	1.0	6.8	0.8	-
12	0.08	13.5	2.5	6.8	0.8	7.5
13	0.08	12.5	1.0	6.8	0.8	-
14	0.08	12.5	1.0	6.8	0.8	-
15	0.08	12.5	1.0	6.8	0.8	-
16	0.08	13.0	1.0	6.8	0.8	-
17	0.08	14.5	1.0	6.8	0.8	-
18	0.08	13.5	2.5	6.8	0.8	-
19	0.08	12.5	1.0	6.89	0.8	-
20	0.08	14.5	1.0	6.8	0.8	-
21	0.08	14.5	1.0	6.8	0.8	-
22	0.08	15.0	2.5	6.8	0.8	-
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	12.5	1.0	6.8	0.8	-
26	0.08	13.5	2.5	6.8	0.8	7.5
27	0.08	14.5	1.0	6.8	0.8	-
28	0.08	12.5	1.0	6.8	0.8	-

- not reported, grey cells are deviating from ISO 6579

Table 4.9 Manufacturer of own isolation medium

Labcode	Name medium	Prescribed incubation temp.	Kind of medium	Manufacturer	Code number	Batch number
1	Önöz	37	Dehydrated	Merck	15034	VM 224234415
2	MacConkey	37	Dehydrated	Oxoid	CM0115	368884
3	BxLH	37	Individual ingredients	-	-	-
4	BGA	37	Ready-to-use	Oxoid	PO5033A	935732
	R Sal	37	Ready-to-use	Biorad	3563961	5K0013
5	Rambach	35-37	Dehydrated	Merck	1.07500	OC526200
6	BG		Dehydrated	Oxoid	CM0329	351324
7	BGA	37	Dehydrated	Biogenetics	BM730	4P1004
8	BGA	37	-	Cruinn	BGA-10	CK9H
	MLCB	37	-	Cruinn	MLC-10	MJ9H
9	BGA	37	Dehydrated	Oxoid	CM0329	387441
10	XLT4	37	Ready-to-use	Reactivos Diagnóstico	PA 0003	38022
11	BGA	37	Ready-to-use	Oxoid	CM329	363859
12	Rambach	37	Dehydrated	Merck	1.07500	OC526200
	XLT4	37	Dehydrated	Biokar	BK156HA	5F896
13	BGA	37	Dehydrated	Merck	1.10747	VM055547 / VM325547
14	BGA	37	Ready-to-use	Biotrading	-	0528700843
15	Rambach	35-37	Dehydrated	Merck	1.075	OC526200
16	BGA	37	Dehydrated	Lab M	Lab 34	079924
17	Rambach	35-37	Dehydrated	Merck	1.075	00526200
18	SMID2	36.5	Ready-to-use	Biomerieux	43629	797554201
19	Rambach	41.5	Dehydrated	Merck	1.07500	OC526200
	BGA	37	Dehydrated	Oxoid	CM0329	397535
20	BPLS	37	Dehydrated	Merck	1.07237	VM324937440
21	Rambach	37	Dehydrated	Merck	1.07500	OC400310
22	BGA	37	Dehydrated	Difco	228530	5102815
	Brolac	37	Dehydrated	Merck	1.01639	226
23	SMID2	37	Ready-to-use	Biomerieux	43621	798922701
24	BGA	37	Dehydrated	Oxoid	CM0329	B 256433
25	BGA	37	Dehydrated	Oxoid	CM0329	397535
26	SMI	37	Ready-to-use	Biomerieux	43621	798736401
27	BGA	37	Dehydrated	Biomark	B439	3980605
28	Hektoen	37	Dehydrated	Oxoid	CM0419	343064

Table 4.10 Manufacturer and pH of nutrient agar

Labcode	Kind of medium	Manufacturer	Code number	Batch	pH after preparation	pH at day of use
ISO 6579	--	--	--	--	7.0±0.2	7.0±0.2
3	Dehydrated	Biomed SIS	-	10204	7.25	7.25
5	Individual Ingredients	-	-	-	7.2	7.2
6	Individual Ingredients	-	-	-	-	-
9	Dehydrated	Oxoid	CM3	261000	7.3	7.2
10	Dehydrated	Difco	213000	4237960	6.8	-
11	Ready-to-use	Oxoid	CM0329	363859	-	-
15	Dehydrated	IMUNA	-	850204	7.2	7.2
16	Dehydrated	Sifin	TN1168	0650304	7	7
22	Dehydrated	Oxoid	CM1	237111	7.31	-
24	Dehydrated	B&L	KM 1073	B00626	7.3	7.5
27	Individual Ingredients	-	-	-	7.05	7.05

- not reported; grey cells are deviating from ISO 6579

Table 4.11 Manufacturer and pH of TSI

Labcode	Name manufacturer	Code number	Batch	pH of medium
ISO 6579	--	--	--	7.4±0.2
1	Merck	3915	VM 327515442	7.36
2	Oxoid	CM0277	397536	7.6
5	Own ingredients	-	-	7.4
6	BBL	11749	3007215	7.2
7	Lab M	59	77785257	7.4
8	Oxoid	CM0277	-	7.4
9	Oxoid	CM0277	370297	7.4
10	Merck	1-03915	WM174515	-
12	AES	AEB151252	407532	7.6
13	Difco	226540	1034003	7.19-7.27
14	Oxoid	TV 5074 D	934732	7.44
15	Oxoid	CM0277	348376	7.4
16	Biorad	64384	160109	7.2
17	Biokar	BK059HA	1K756	7.4
21	Merck	1.03915	VM010515311	7.6
22	Difco	211749	4006519	7.25
23	Beckton D.	226540	3210447	7.4
24	Oxoid	CM0277	259231	7.4
27	Biomark	B1116	6240705	7.4
28	Oxoid	CM 33	274110	6.69

- not reported, grey cells are deviating from ISO 6579

Table 4.12 Manufacturer and pH of ureum agar

Labcode	Name manufacturer	Code number	Batch	pH of medium
ISO 6579	--	--	--	6.8±0.2
1	Oxoid	CM0053	326371	6.71
2	Oxoid	CM0053	369759	6.8
3	Own ingredients	-	-	-
5	Own ingredients	-	-	6.8
6	SVA	321660	051117	6.7
7	Oxoid	CM0053	304569	6.8
9	Oxoid	CM0053	326371	6.8
10	Merck	1.08492	VL 864392214	6.8
12	AES	AEB111545	435579	-
13	Oxoid	CM0053	326371/401740	6.67-6.64
14	NVI	E6475A	240652	7.09
15	Oxoid	CM0053	259099	6.7
16	Oxoid	TN1143	2330903	6.8
17	Own ingredients	-	-	6.9
21	Merck	1.08492	VL864392214	6.75
22	Difco	211795	31289749	6.83
23	Beckton D.	211795	4245764	6.8
24	Merck	1.08492	VL 8643292	6.8
27	Own ingredients	-	-	6.92
28	Oxoid	CM0053	293716	6.5

- not reported; grey cells are deviating from ISO 6579

Table 4.13 Manufacturer and pH of LDC

Labcode	Name manufacturer	Code number	Batch	pH of medium
ISO 6579	--	--	--	6.8±0.2
2	INCDMI	622	05121472	6.4
4	Oxoid	TV 5028 N	934717	-
5	Own ingredients	-	-	6.8
7	Difco	21175	3237358	6.8
9	Oxoid	CM 381	326442	6.7
10	BD	211759	3237358	6.9
12	Difco	211759	4257207	6.8
13	-	-	-	6.69
14	Oxoid	TV 5028 N	936529	6.97
15	Own ingredients	-	-	6.8
16	Sifin	TN1154	2330903	6.8
17	Own ingredients	-	-	7
21	Own ingredients	-	-	6.77
22	Oxoid	CM0308	13178001	6.2
23	Becton D.	211759	3210447	6.8
24	Oxoid	CM0308	12560601	5.99
27	Own ingredients	-	-	6.87

- not reported; grey cells are deviating from ISO 6579

Table 4.14 Manufacturer and pH of other confirmation media

Labcode	Name media or otherwise	Name manufacturer	Code number	Batch	pH of medium
3	Kligler agar	Own Ingredients	-	-	-
3	ONPG and FDA	Own Ingredients	-	-	-
3	mannitol and nitrate reduction broth	Own Ingredients	-	-	-
4	Hg enterotest	Audit Diagnostics Benelux	TT146	3411	-
7	SIM	Oxoid	CM435B	380624	7.3
8	Mac Conkey	Cruinn	MWS-10	019A	7.4
10	Indol test	Scharlau Chemie S.A.	064-TA0132	34786	7.3
11	Confirmation by serotyping	-	-	-	-
17	Drigalski agar	Own Ingredients			7.6
19	Confirmation by serotyping	-	-	-	-
22	SCA	Oxoid	CM0155	343726	7.02
25	Mast Diganostics	Kohns	DM138-1A	168139/157072	6.62
27	API 20 E	API	REF20100	790842001	-

- not reported

Annex 5. Results of alternative methods

Table 5.1 PCR results of artificially contaminated samples of laboratories 1, 2, 6, 16, 20 and 22 after 4 and 18 h of incubation in BPW

Nr	Lab 1				Lab 2		Lab 6		Lab 16		Lab 20		Lab 22	
	4 h BPW		18 hr BPW		18 h	18 h	18 h	18 h	18 h	18 h	18 h	18 h	18 h	18 h
	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR
1	-	-	+	-	+	+	+	+	+	+	+	+	+	+
2	-	I	+	+	+	+	+	+	+	+	+	+	+	+
3	+	-	+	+	+	+	+	+	+	+	+	+	+	+
4	-	-	+	+	+	+	+	+	+	+	+	+	+	+
5	-	-	+	+	+	+	+	+	+	+	+	+	+	+
6	+	-	+	+	+	+	+	+	+	+	+	+	+	+
7	+	-	+	+	+	+	+	+	+	+	+	+	+	+
8	-	-	+	-	+	+	+	+	+	+	+	+	+	-
9	+	-	+	+	+	+	+	+	+	+	+	+	+	+
10	-	-	+	+	+	+	+	+	+	+	+	+	+	+
11	+	-	+	+	+	+	+	+	+	+	+	+	+	+
12*	-	-	-	I	-	-	-	-	-	-	-	-	-	-
13	-	-	+	+	+	+	+	+	+	+	+	+	+	+
14*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	+	+	+	+	+	+	+	+	+	+	+	+
16	-	-	+	+	+	+	+	+	+	nt	+	+	+	+
17	+	-	+	I	+	+	+	+	+	nt	+	+	+	+
18	+	-	+	+	+	+	+	+	+	nt	+	+	+	+
19	+	-	+	+	+	+	+	+	+	nt	+	+	+	+
20*	-	-	-	I	+	-	-	-	-	nt	-	-	-	-
21*	-	-	-	-	-	-	-	-	-	nt	-	-	-	-
22	-	-	+	+	+	+	+	+	+	nt	+	+	+	+
23	+	-	+	+	+	+	+	+	+	nt	+	+	+	+
24	+	-	+	I	+	+	+	+	+	nt	+	+	-	-
25*	-	-	-	-	+	+	-	-	-	nt	-	-	-	-
Total	10	0	20	16	22	21	20	20	20	13	20	20	19	18

*BAC = bacteriological culture results; * = Blank capsule; + = positive; - = negative;*
I = inhibition; grey cells = unexpected results, nt = not tested

Table 5.2 PCR results of dust samples of laboratories 1, 2, 6, 20 and 22 after 4 and 18 h of incubation in BPW

Nr	Lab 1				Lab 2		Lab 6		Lab 20		Lab 22	
	4 h BPW		18 hr BPW		18 h	18 h	18 h	18 h	18 h	18 h	18 h	18 h
	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR
D1	+	-	+	+	+	+	+	+	+	+	+	+
D2	+	-	+	+	+	+	+	+	+	+	+	+
D3	+	-	+	+	+	+	+	+	+	+	+	+
D4	+	-	+	+	+	+	+	+	+	+	+	+
D5	+	-	+	+	+	+	+	+	+	+	+	+
D6	+	-	+	+	+	+	+	+	+	+	+	+
D7	+	-	+	+	+	+	+	+	+	+	+	+
D8	+	I	+	+	+	+	+	+	+	+	+	+
D9	+	I	+	+	+	+	+	+	+	+	+	+
D10	+	-	+	+	+	+	+	+	+	+	+	+
Total	10	0	10	10	10	10	10	10	10	10	10	10

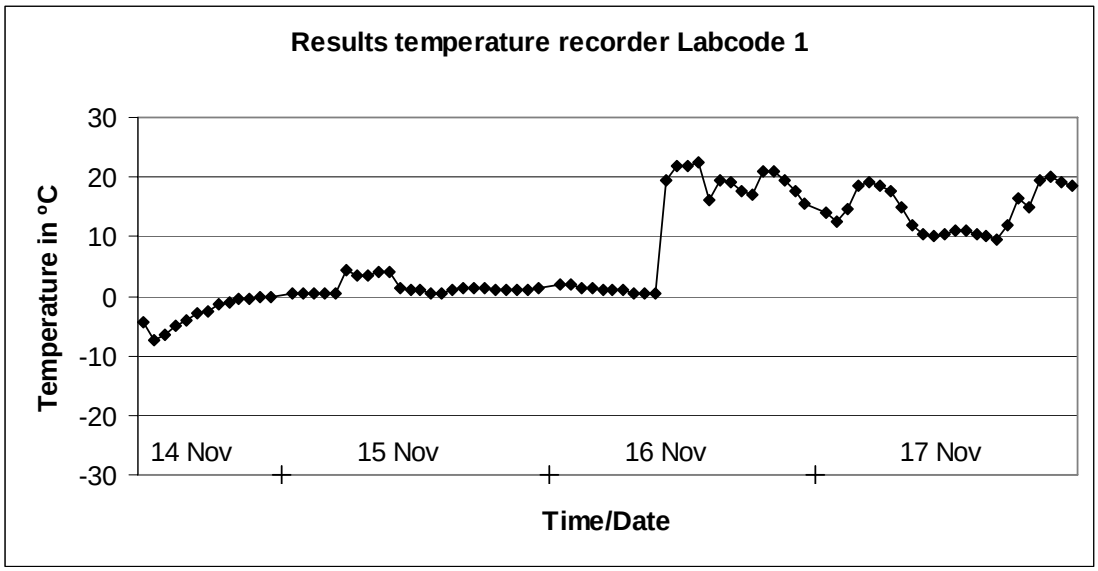
*BAC = bacteriological culture results; * = Blank capsule; + = positive; - = negative;*
I = inhibition; grey cells = unexpected results, nt = not determined

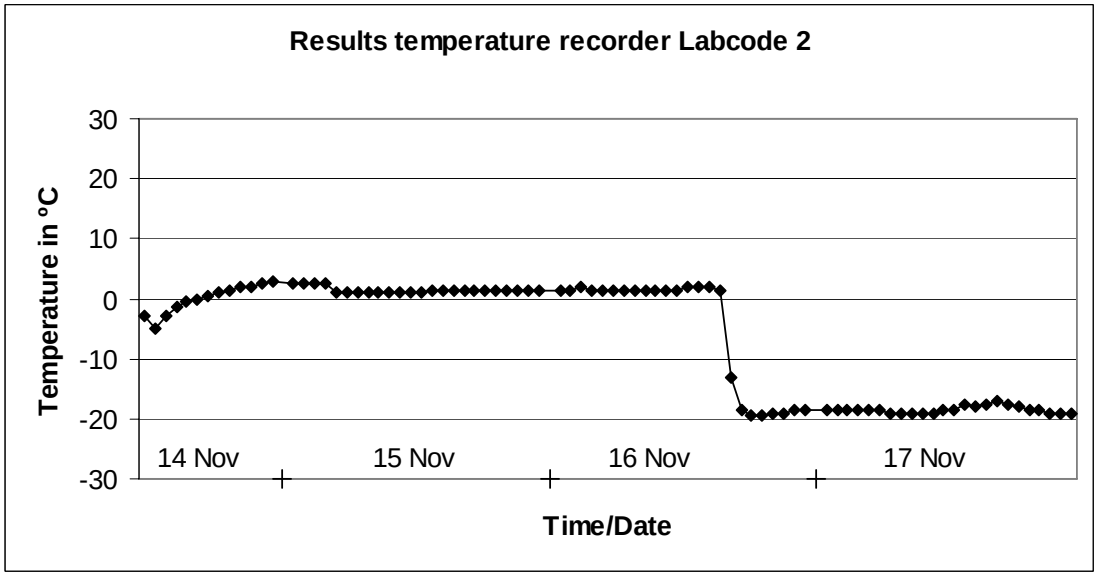
Table 5.3 PCR results of control samples of laboratories 1, 2, 6, 20 and 22 after 4 and 18 h of incubation in BPW

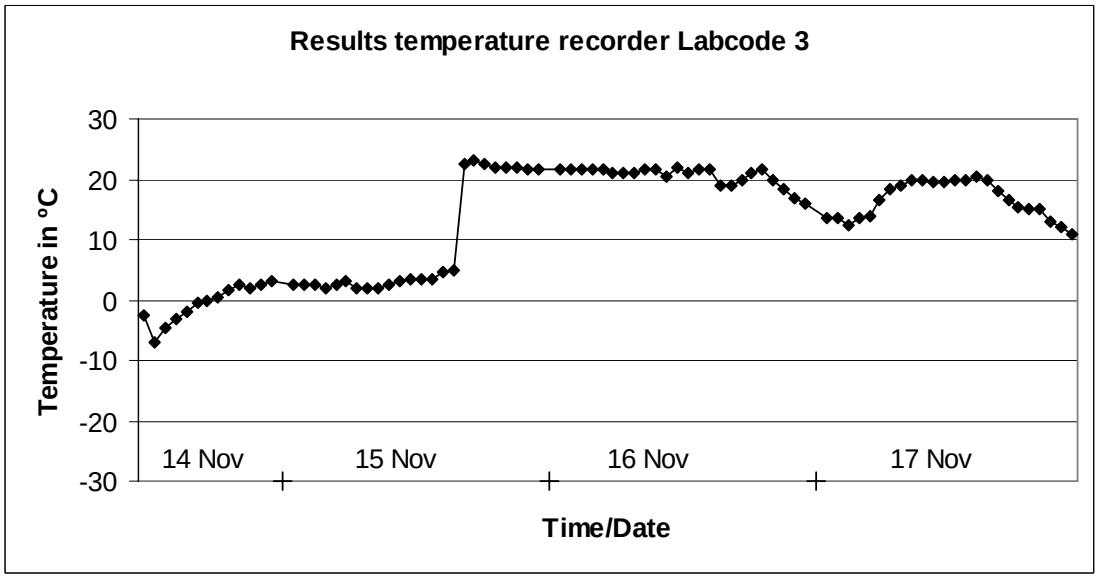
Nr	Lab 1				Lab 2		Lab 6		Lab 20		Lab 22	
	4 h BPW		18 hr BPW		18 h	18 h	18 h	18 h	18 h	18 h	18 h	18 h
	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR
C1	-	-	+	+	+	+	+	+	+	+	+	+
C2	+	-	+	+	+	+	+	+	+	+	+	+
C3*	-	-	-	-	-	+	-	-	-	-	-	-
C4	-	-	+	+	+	+	+	+	+	+	+	+
C5	-	-	+	+	+	+	+	+	+	+	+	+
C6	-	-	+	+	+	+	+	+	+	+	+	+
C7	-	-	+	+	+	+	+	+	+	+	+	+
C8*	-	-	+	-	-	-	-	-	-	-	-	-
C9	+	-	+	+	+	+	+	+	+	+	+	+
C10	-	-	+	+	+	+	+	+	+	+	+	+
C11	-	-	-	-	-	-	-	-	-	-	-	-
C12	-	-	-	-	-	-	-	-	-	-	-	-
Total	2	0	9	8	8	9	8	8	8	8	8	8

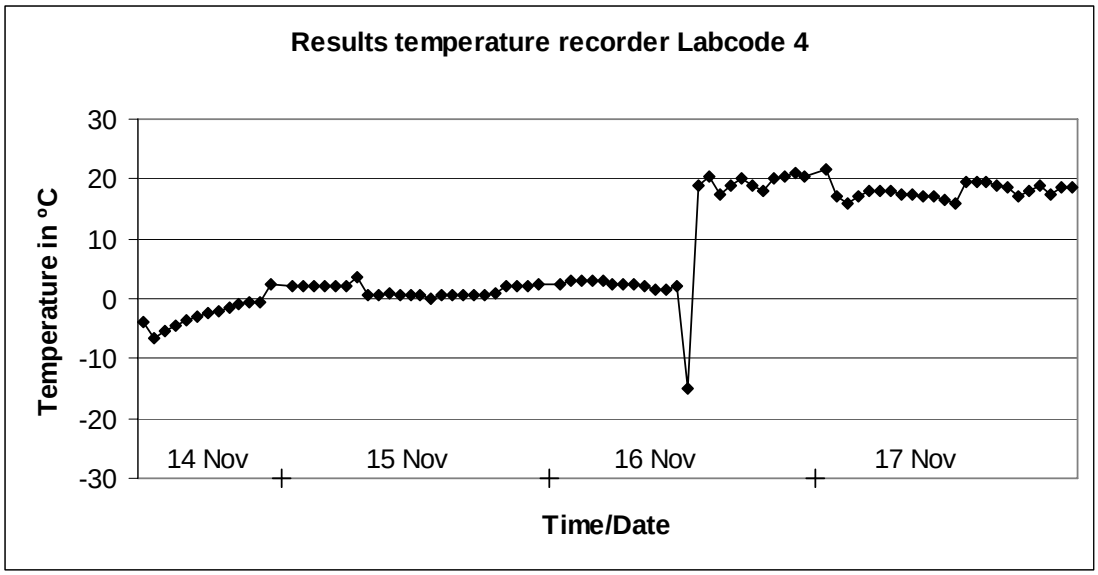
*BAC = bacteriological culture results; * = Blank capsule; + = positive; - = negative;*
I = inhibition; grey cells = unexpected results

Annex 6. Temperature recording





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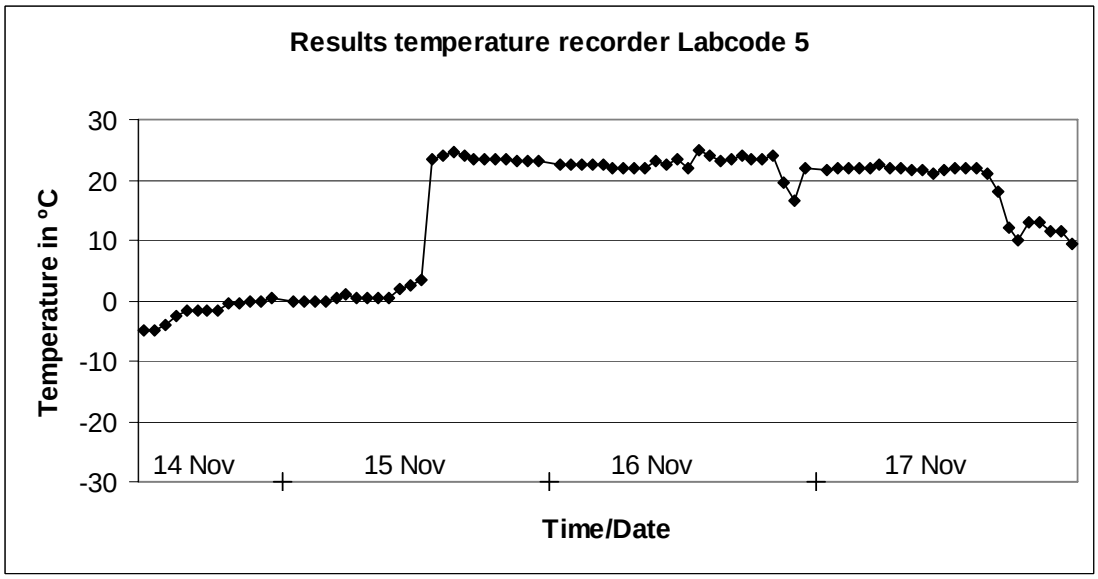
0
-10
-20
-30
14 Nov
15 Nov
16 Nov
17 Nov
Time/Date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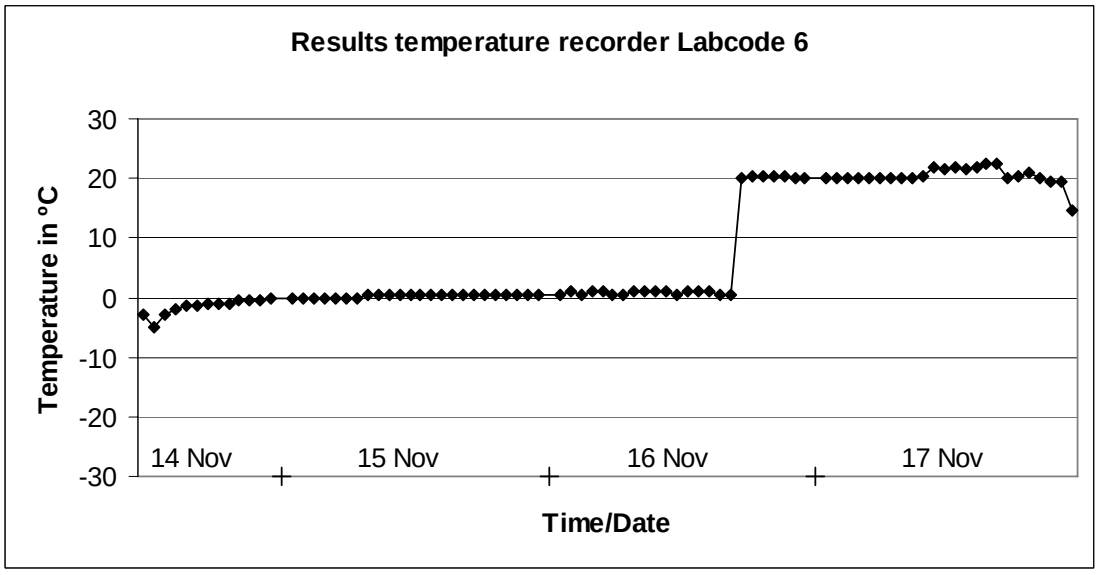
10
0
-10
-20
-30
14 Nov
15 Nov
16 Nov
17 Nov
Time/Date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0
-10
-20
-30
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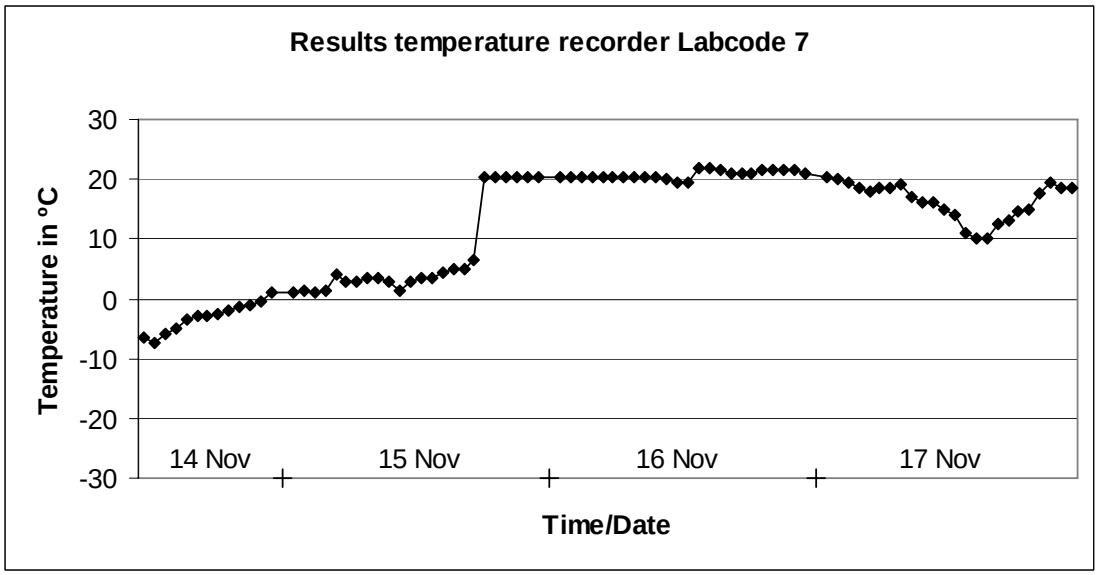
15 Nov
16 Nov
17 Nov
Time/Date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-30
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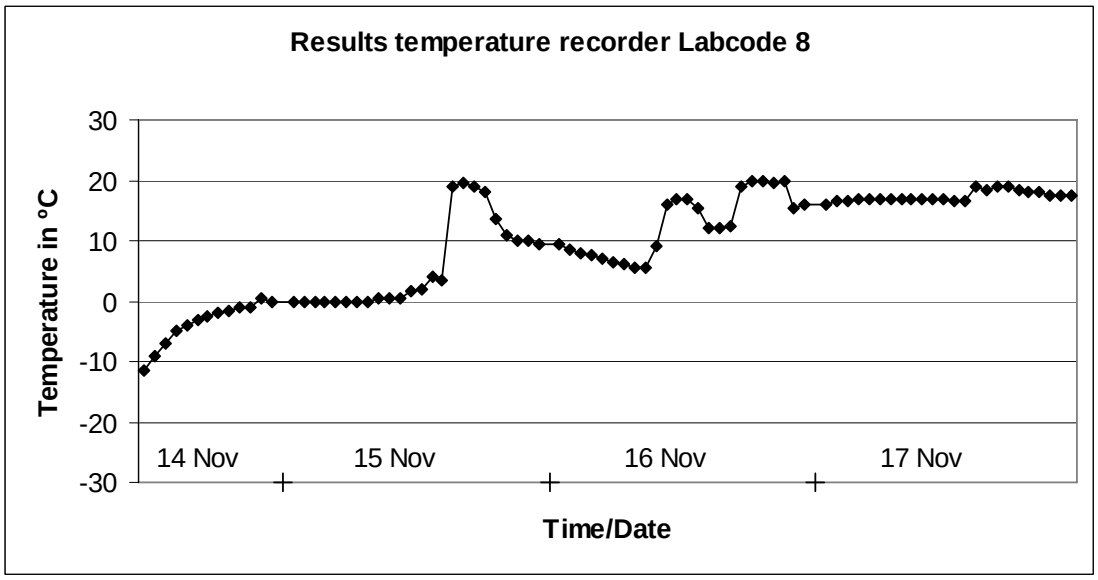
16 Nov
17 Nov
Time/Date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-30
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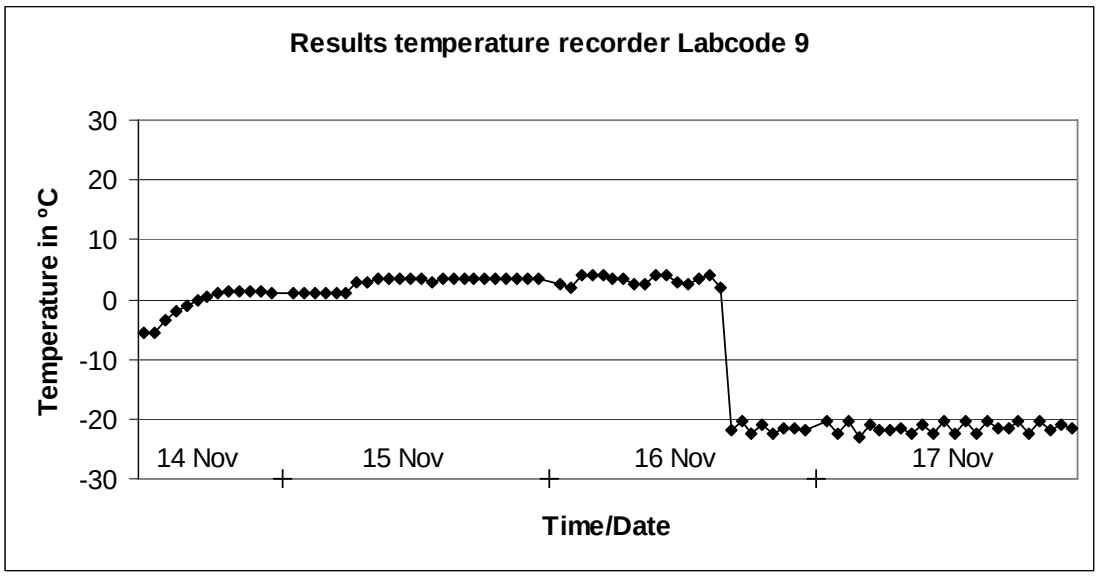
16 Nov
17 Nov
Time/Date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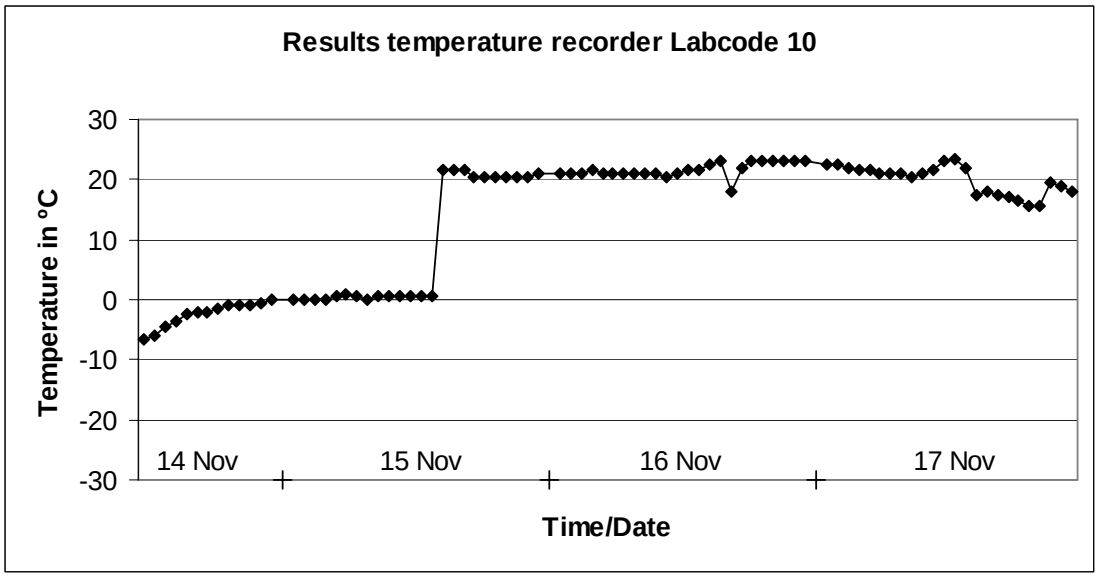
17 Nov
Time/Date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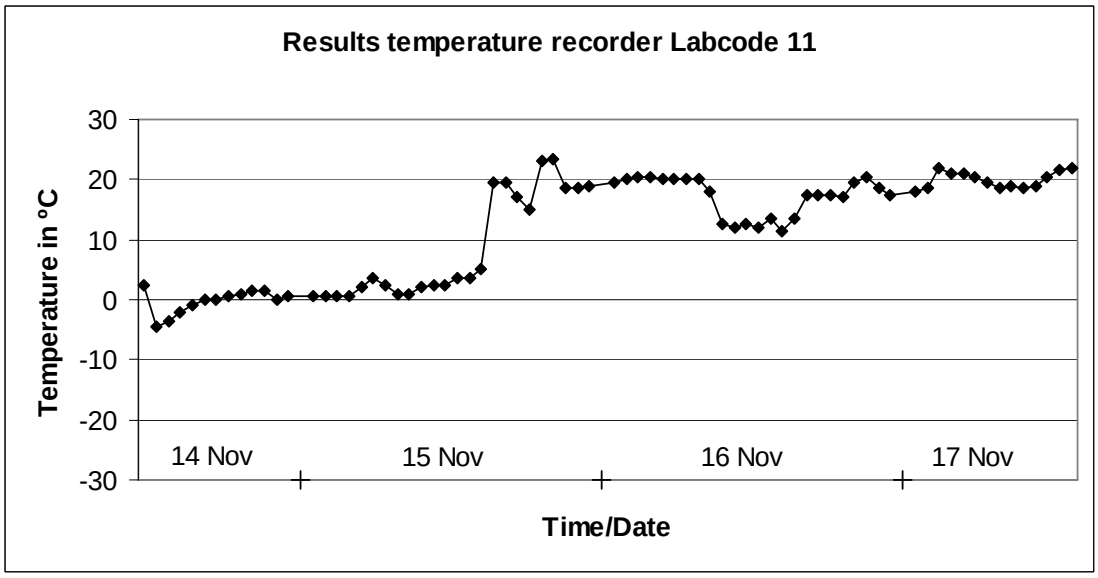
Time/Date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-20
-30
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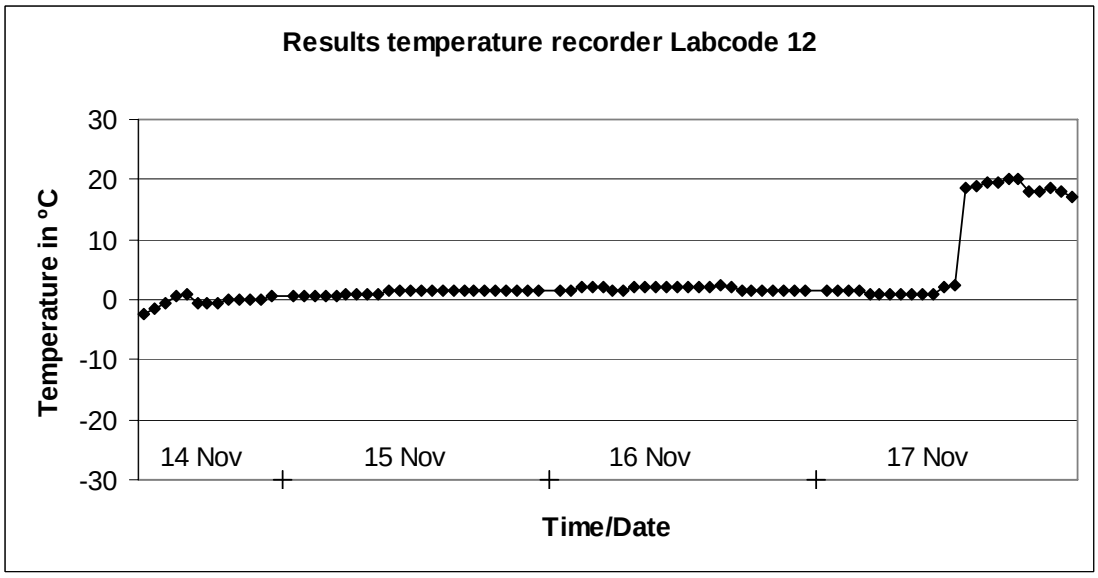
Time/Date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-10
-20
-30
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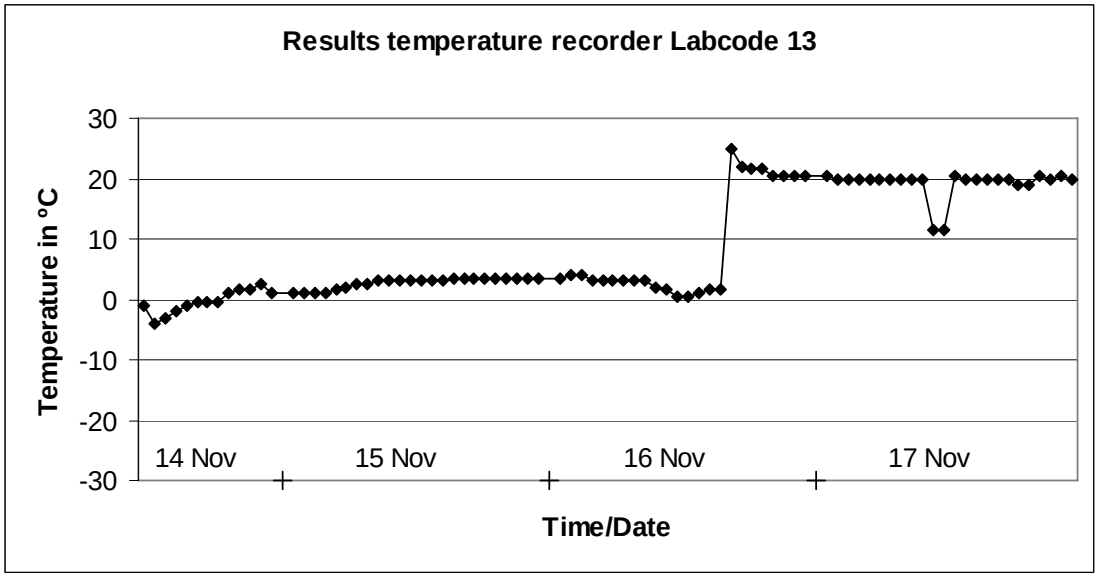
16 Nov
17 Nov
Time/Date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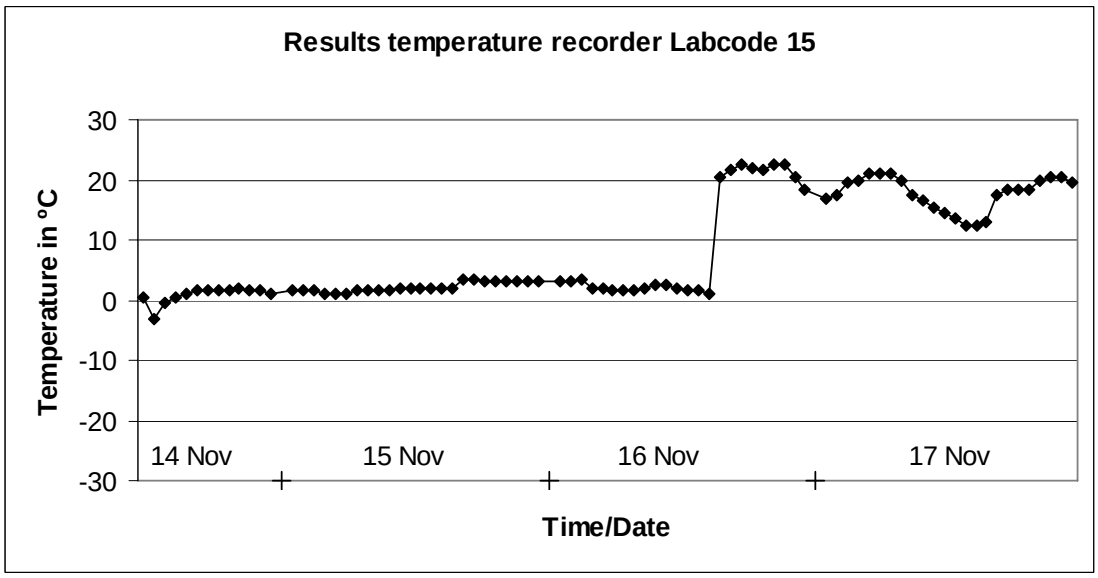
17 Nov
Time/Date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-10
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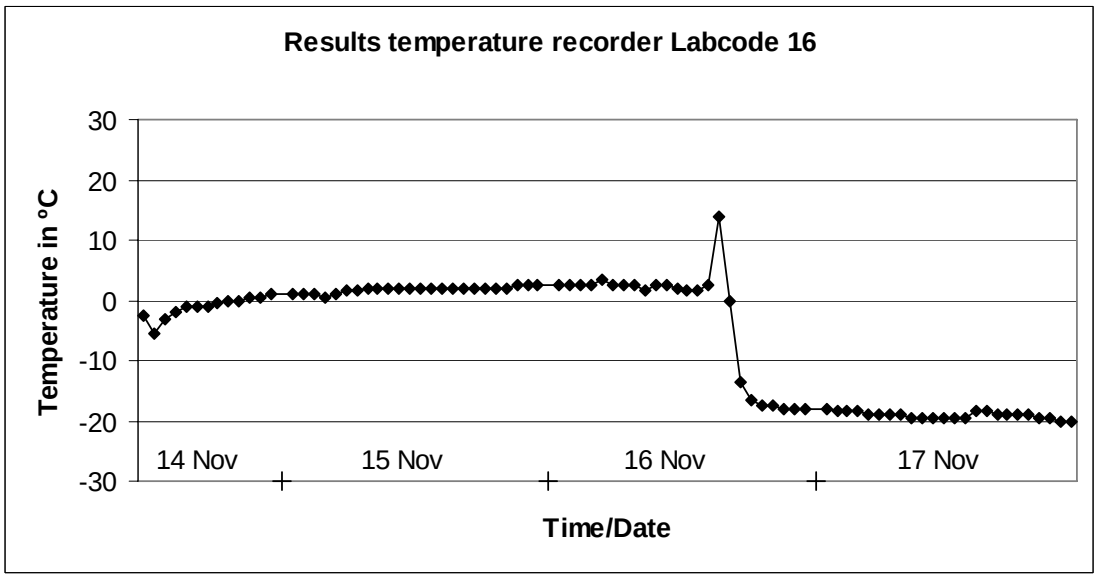
15 Nov
16 Nov
17 Nov
Time/Date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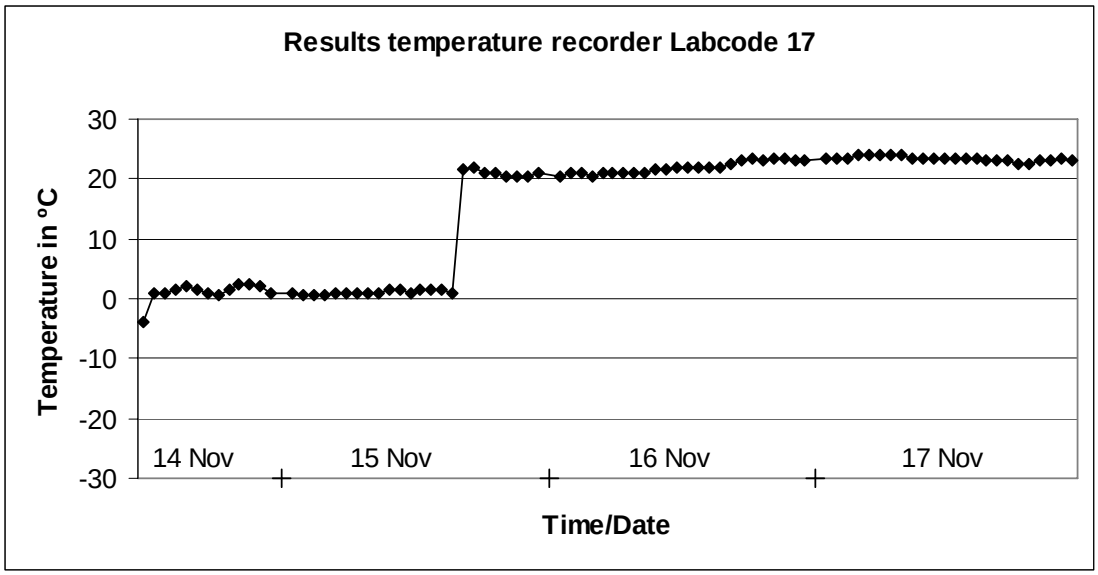
16 Nov
17 Nov
Time/Date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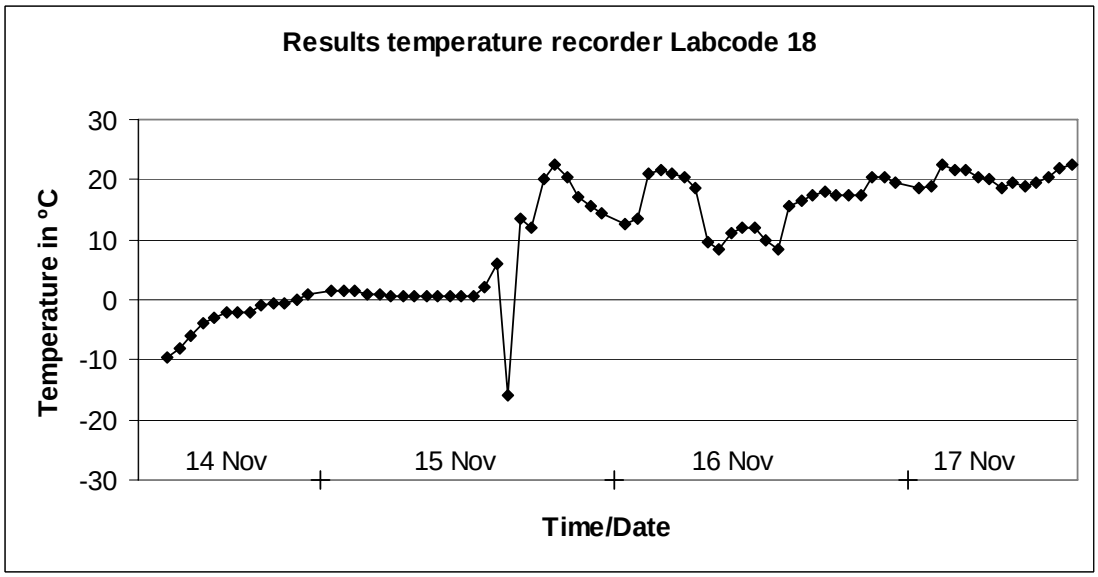
17 Nov
Time/Date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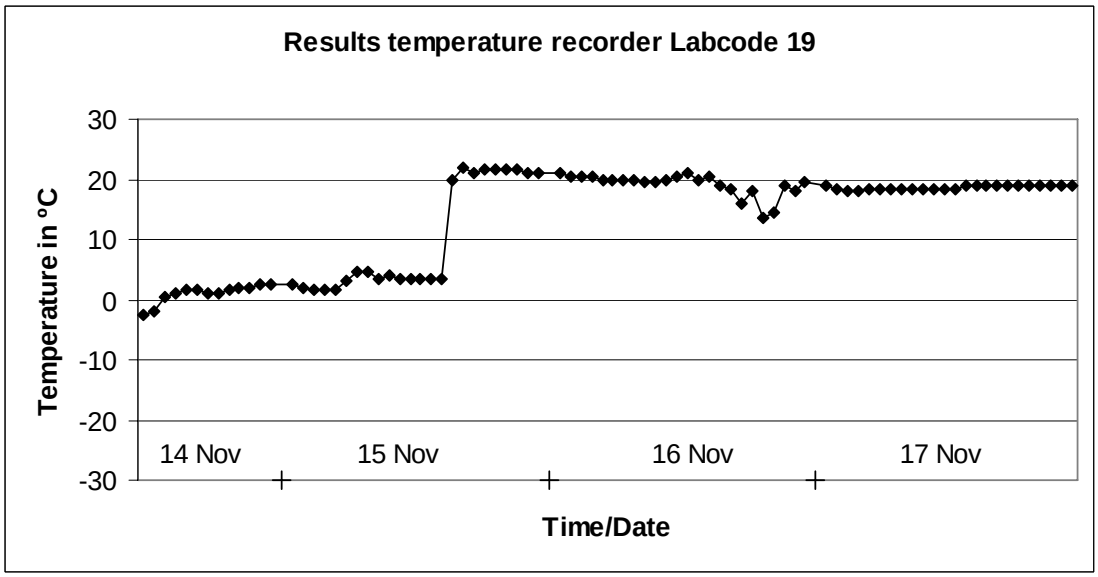
17 Nov
Time/Date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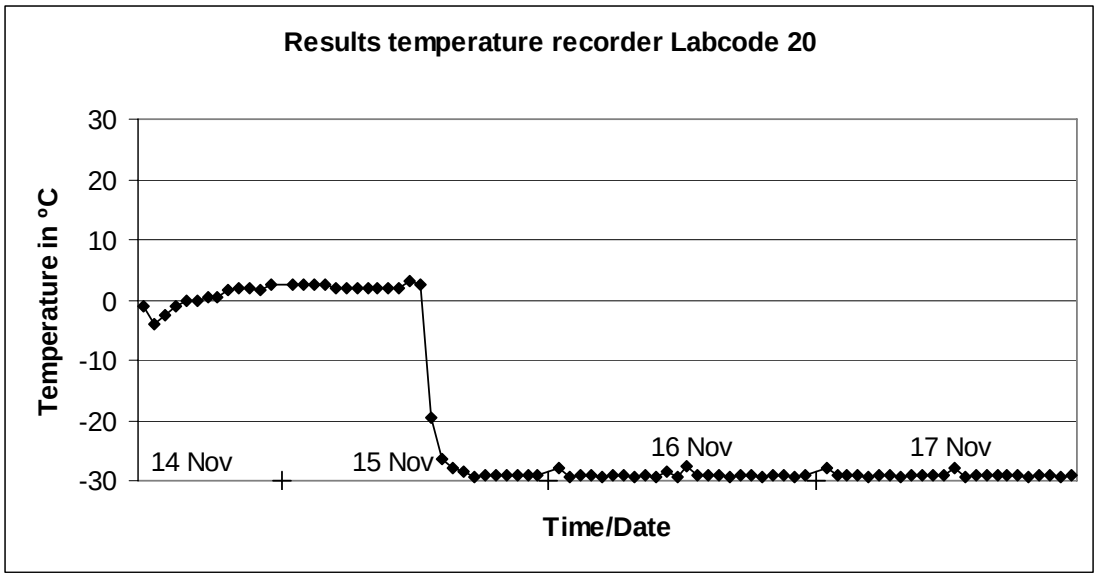
Time/Date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0
-10
-20
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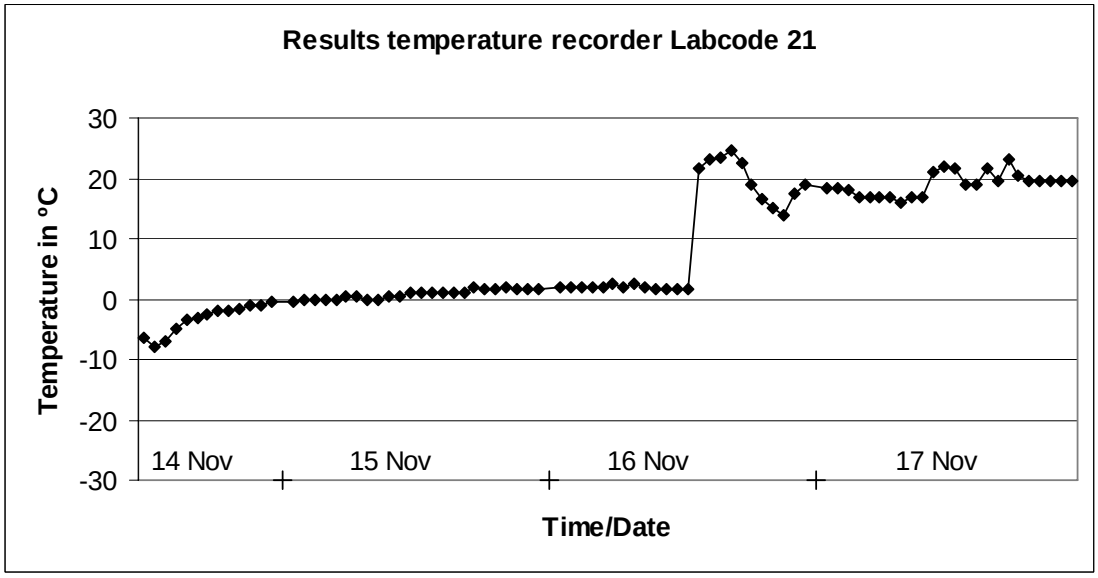
Time/Date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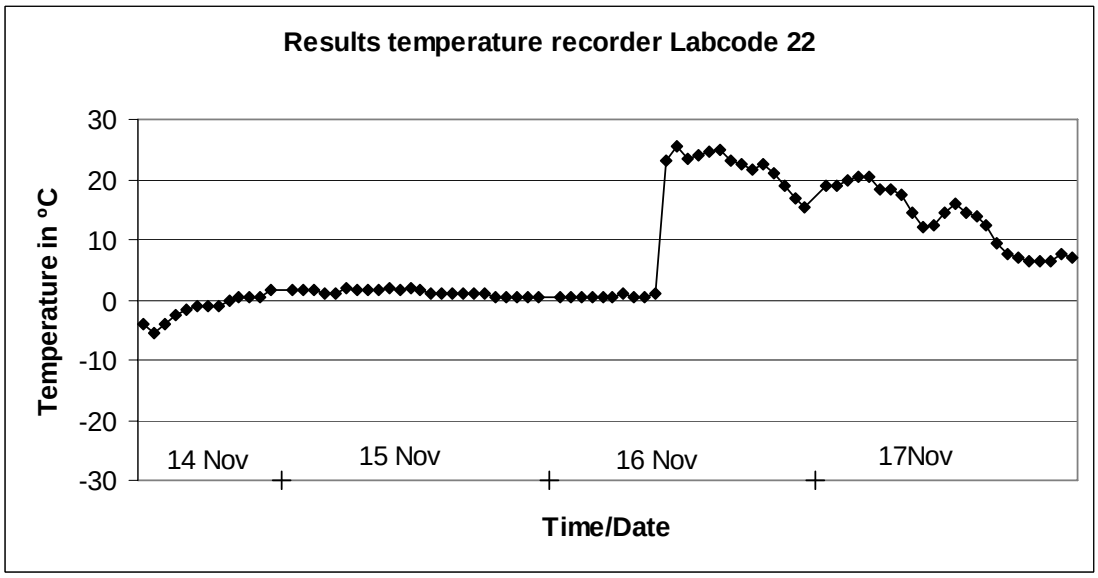
Time/Date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-30
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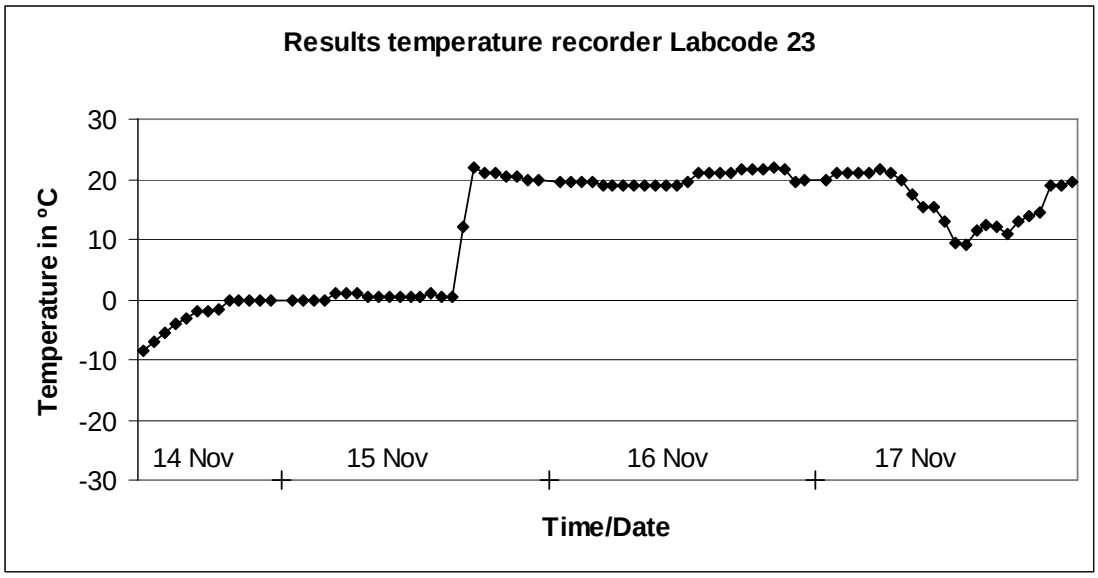
Time/Date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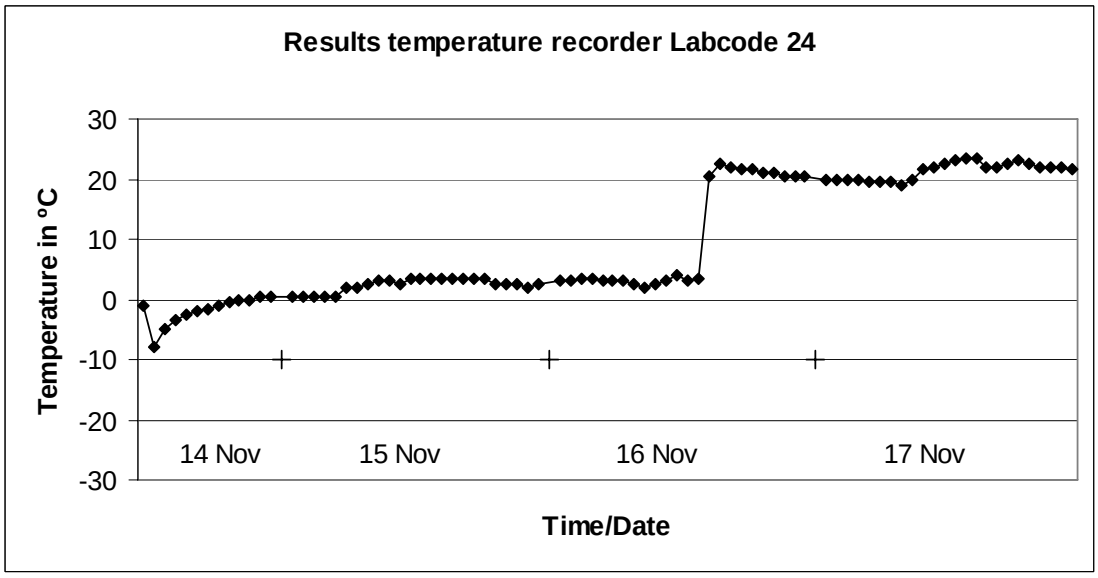
17 Nov
Time/Date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-30
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16 Nov
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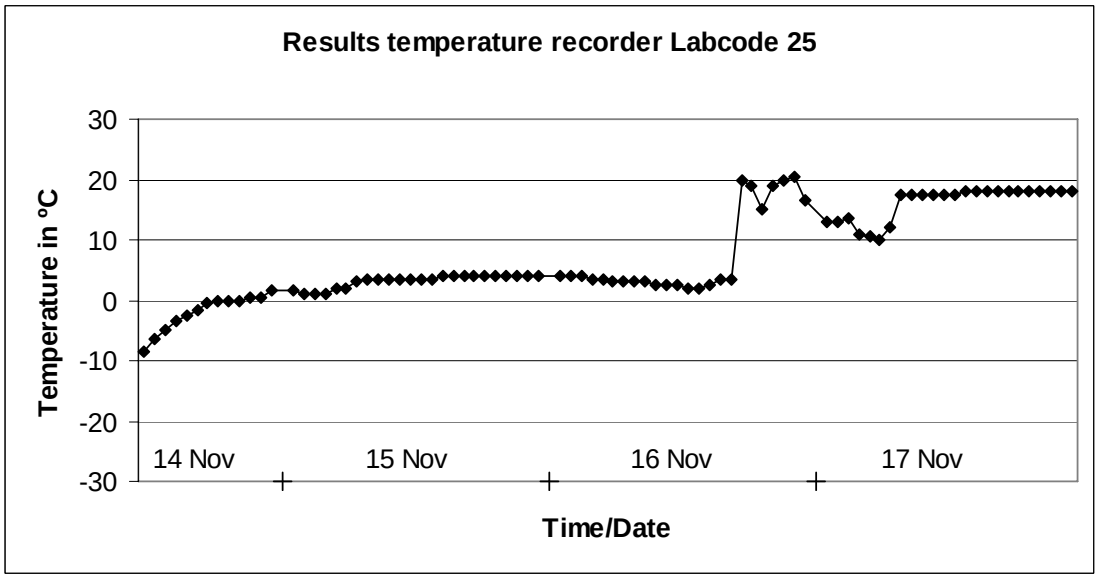
Time/Date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Temperature in °C
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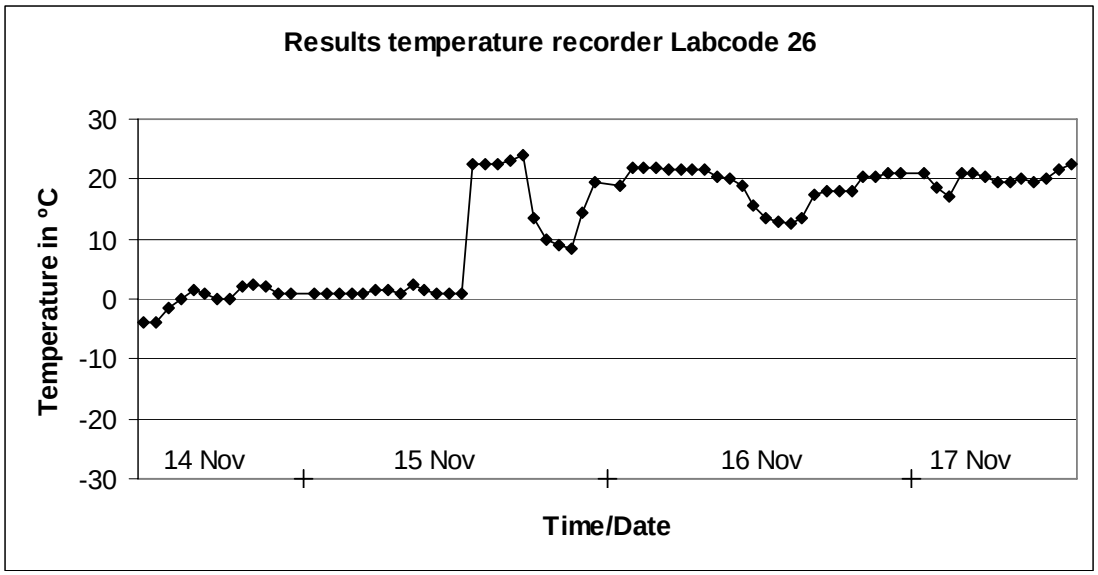
Time/Date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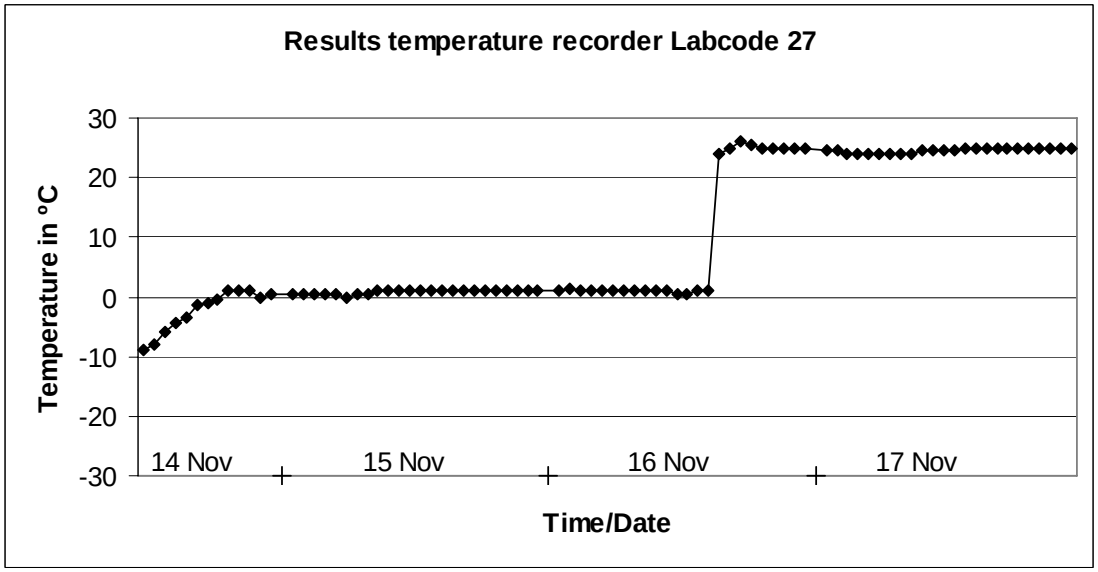
Time/Date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-10
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-30
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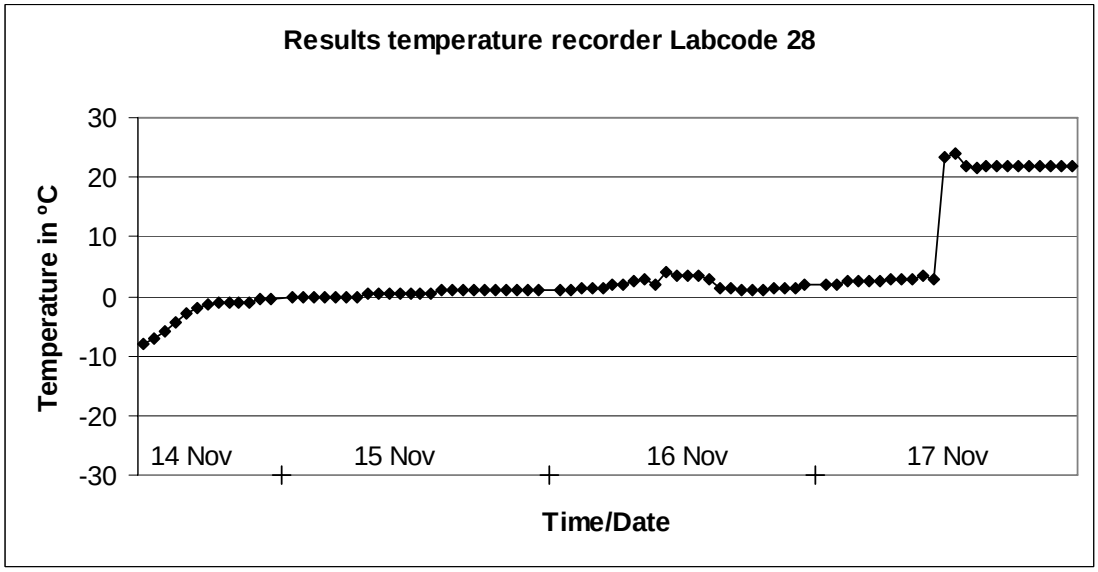
Time/Date


Results temperature recorder Labcode 28
Temperature in °C
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20
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0
-10
-20
-30
14 Nov
15 Nov
16 Nov
17 Nov
Time/Date

Annex 7. Protocol

INTERLABORATORY COMPARISON STUDY IX (2005) ON THE DETECTION OF *SALMONELLA* spp. organised by CRL-*Salmonella*

Introduction

The set-up of this 9th interlaboratory comparison study on the detection of *Salmonella* spp. is comparable to the set-up of the eighth study. Artificially contaminated (*Salmonella* negative) chicken faeces samples are tested by using reference materials. The reference materials (RMs) consist of gelatine capsules containing sublethally injured *Salmonella* Typhimurium (STM), *Salmonella* Enteritidis (SE) or *Salmonella* Panama (SPan) at different contamination levels. Each laboratory will 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). The prescribed method is the procedure as described in the 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). As in the eighth study two incubation times of the pre-enrichment broth (Buffered peptone water (BPW)) will be used, being (4 ± ½) h and (18 ± 2) h. Further, similar to former studies, laboratories can also use their own method(s) besides the prescribed method, including PCR techniques.
Different from the eighth study is the fact that faeces will no longer be mixed with peptone/glycerol solution and will therefore be more representative to routine samples. The faeces samples should therefore be **stored at 5 ºC** before use (and not at -20 ºC). Also different from the former study is that the naturally polluted faces samples are replaced by naturally polluted dust samples (gathered at poultry flocks). For this, 10 dust samples of each 10 g 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 dust). 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.
Each box (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 (14th of November 2004)

Objectives

The main objective of the ninth 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 artificially contaminated chicken faeces and in naturally contaminated dust.
Finally by comparing the results of this study with the results of the eighth study, further information may be obtained on the (negative) influence of peptone/glycerol on the detection of *Salmonella.* As the faeces of the eighth study was mixed with peptone/glycerol and the faeces of this ninth study is not mixed at all.

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:

- circa 300 g of poultry faeces (free from *Salmonella*).

Parcel 3:

- circa 150 g of naturally contaminated (with *Salmonella*) dust.

Parcel 1 should be stored at (-20 ± 5)ºC immediate after receipt.
Parcel 2 and 3 should be stored at (5 ± 3)ºC immediate after receipt.

The performance of the study will be in week 48 (starting on 28 November 2005).

The documents necessary for performing the study are:

- Protocol Interlaboratory Comparison Study IX (2005), on the detection of *Salmonella* spp.;
- SOP Interlaboratory Comparison Study IX (2005), on the detection of *Salmonella* spp.
- Test Report Interlaboratory Comparison Study IX (2005), 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 Amendment ISO 6579:2002/DAmd 1 (2005) Amendment 1 Annex D: Detection of *Salmonella* spp. in animal faeces and in samples from the primary production stage.

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

All data will be reported in the test report and send to the CRL-*Salmonella* and will be used for (statistical) analysis.
In the time table of the interlaboratory comparison study IX (see next page) on the bacteriological detection of *Salmonella*, to be organised in fall 2005, a **strict deadline** for sending the results to the CRL-*Salmonella* is indicated.
The reason for setting this strict deadline (on 16 December 2005) is that we want to prepare a short report to inform all NRLs within 1 to 2 months after the study on the overall results. In earlier studies the NRLs received only their own results immediately after the study. The information on how they performed in comparison with the other NRLs was given ca half a year after the study (at the workshop). This is considered very late and with this short report we try to improve the information on the study. We will start the first overall analyses immediately after the deadline. Results which will be received after the deadline can not be used in the analyses for the short report.
It may still be possible to use late results in the analyses for the final report but results received after writing the short report will not be incorporated in the final report.. However, this final (draft) report will not be available before ca half a year after the study.

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

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Time table of bacteriological interlaboratory comparison study IX (2005)

Week	Date	Topic
44	31 October – 4 November	Mailing of the protocol, standard operating procedure and test report to the NRLs
46	14 – 18 November	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 the dust and faeces samples at +5°C ± 3°C** - **Store the capsules at -20°C ± 5°C**. If you did not receive the parcel at 18 November, do contact the CRL immediately.
47	21 – 25 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)
48	28 November – 2 December	Performance of the study, following the instructions as given in the protocol and the SOP of study IX (2005).
50	Before 16 December	Completion of the test report and faxing or e-mailing it to the CRL. The original test report will be sent to CRL. **The deadline for sending the test report to CRL is 16 December 2005**
51	19 December – 6 January	Data input at CRL-*Salmonella* and sending these data by CRL to NRLs for checking
2	9 – 13 January	Checking the results by the National Reference Laboratories
	January/February 2006	Sending of the final results to the NRLs together with a short report. As a follow-up, actions will be undertaken for those NRLs which scored below the average results of all NRLs.

Annex 8. Standard Operating Procedure

INTERLABORATORY COMPARISON STUDY IX (2005) 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.

Draft Amendment ISO 6579:2002/DAmd 1, 2005. Amendment 1 Annex D: Detection of *Salmonella* spp. in animal faces and in samples from the primary production stage.

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). In the list of media given in 6.1 up to 6.4, reference is made to the relevant part of ISO 6579. 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) (ISO 6579, 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 2005
  This medium must be boiled to dissolve (instructions of manufacturer). After boiling the medium must be transparent blue. After cooling down to 50 °C the supplement or the novobiocin has to be added. ISO 6579)
  The final concentration of the novobiocin in the medium should be 0.01 g/l. Plates should be poured with a volume of 15 to 20 ml.
- Selective enrichment medium routinely used in your laboratory (optionally)

6.3 Solid selective media for first and second isolation

- Xylose-Lysine-Desoxycholate (ISO 6579, 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

e) Biochemical confirmation

- Triple sugar/iron agar (TSI agar) (ISO 6579, Annex B.6)
- Urea agar (ISO 6579, Annex B.7)
- l-Lysine decarboxylation medium (ISO 6579, Annex B.8)
- Nutrient agar (optional) (ISO 6579, 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 ninth 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. Details of the prescribed method can be found in draft Annex D of ISO 6579.
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 (day 0)

Label 25 jars containing 90 ml of BPW from 1 to 25 (eg. on Monday). For the dust samples number 10 jars of BPW from D1 to D10. 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 will be added and one jar is a negative faeces control to which only 10 g faeces will be added (= C12). These control jars should further be handled in the same way as the other jars. Place all jars with BPW **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 (day 1)

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 min** 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 min add the faeces and dust samples to the jars according to the following scheme:

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

! Do not shake the jars after adding the faeces.

- **Add 10 g of dust to jars labelled D1-D10.**

Shake the jars containing dust to make sure the dust isn't floating on the BPW.

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 (day 1 and day 2)

Please note that in this study two incubation times of the BPW are used: (4 ± ½) h and (18 ± 2) h. Therefore MSRV plates should be inoculated on day 1 and on day 2.

Day 1

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 Laminar Air Flow cabinet if necessary. Record (page 4-7) the requested data of the MSRV plates and if used, the data of the own selective enrichment medium in the test report. Label 25 plates of MSRV from 1 to 25. Also label 10 MSRV-plates from D1 to D10 and 12 plates from C1 to C12. If necessary also indicate on the plates that they are inoculated with the 4h BPW cultures. If you have other means to distinguish between the plates inoculated with 4h BPW cultures from 18h BPW cultures it is not necessary to label each individual plate.
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 if negative for 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).

Day 2

Repeat the procedure of day 1 for inoculation of the MSRV plates with the 18h BPW cultures.

8.5 Isolation media (first and second isolation; day 2, 3 and 4)

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 10 large Petri dishes from D1 to D10 and label 12 large Petri dishes from C1 to C12. If necessary also note the relevant incubation time of BPW on the plates. If you have other means to distinguish between the plates inoculated with 4h BPW cultures from 18h BPW cultures it is not necessary to label each individual plate.

First isolation after 24 h
Inoculate, by means of a loop, from the suspect MSRV plates (see draft Annex D of ISO 6579), 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 the 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 at first isolation after 24 h.

8.6 Confirmation of colonies from first and second isolation (day 3, 4 and 5)

For confirmation take from each Petri dish of each isolation medium at least 1 colony considered to be typical or suspect (use only well isolated colonies).
Store the isolation plates at (5 ± 3) ^{o}C.
Before biochemical confirmation (see below), optionally, streak each typical colony onto the surface of a nutrient agar plate with the corresponding label number, in a manner which allows to
develop well isolated colonies. Record on the test report (page 14-15) the requested data of the nutrient agar. Incubate the inoculated plates at (37 ± 1) °C for (24 ± 3) h.

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 the test report (page 16) 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).

- 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

If the initial 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 the test report page 27.

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