National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

EU Interlaboratory comparison study veterinary XV (2012)

Detection of Salmonella in pig faeces

RIVM report 330604028/2013

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Colophon

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This investigation has been performed by order and for the account of the European Commission, Directorate-General for Health and Consumer Protection (DG-Sanco), within the framework of RIVM Project number V/330604/12 European Union Reference Laboratory for *Salmonella* 2012.

Abstract

EU Interlaboratory comparison study veterinary XV (2012)
Detection of *Salmonella* in pig faeces

In 2012, from the 33 National Reference Laboratories (NRLs) for *Salmonella* in the European Union, 31 were able to detect both high and low levels of *Salmonella* in pig faeces. The desired outcome was achieved on the first attempt. Two NRLs scored an underperformance because they had difficulty in detecting *Salmonella* in the pig faeces. Both laboratories obtained the desired outcome in a repeat study.
Salmonella was found in 93% of the contaminated samples tested in the laboratories.

Interlaboratory comparison study obligatory for European Member States
This report presents he results of the fifteenth veterinary interlaboratory comparison study organized by the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*). The study was conducted in March 2012, with a follow-up study in June 2012. In all European Member States, the NRLs responsible for the detection of *Salmonella* in veterinary samples were required to participate in this study. The EURL-*Salmonella* is part of the Dutch National Institute for Public Health and the Environment (RIVM).

The laboratories identified the presence of *Salmonella* by using the internationally prescribed method in veterinary samples, MSRV. Each laboratory received a package containing pig faeces (free from *Salmonella*) and reference materials (lenticule discs) containing no or different levels of *Salmonella*. The laboratories were instructed to spike samples of pig faeces with reference material and subsequently test all samples for the presence of *Salmonella*.

Detection of two specific types of *Salmonella* in pig faeces
In the study, two types of *Salmonella* (serovars) found regularly in pigs were tested. It was shown some laboratories had more difficulty detecting low levels of *Salmonella* Typhimurium compared to *Salmonella* Derby at comparable concentrations. S. Derby was not used in earlier studies and some additional tests (interference with pig faeces, stability test of *S*. Derby in the lenticule discs) were performed at the laboratory of the EURL-*Salmonella* prior to the start of the comparison study.

Key words: *Salmonella*; EURL; NRL; proficiency test; pig faeces; *Salmonella* detection methods; lenticule disc.

Rapport in het kort

EU Ringonderzoek veterinair XV (2012)
Detectie van *Salmonella* in varkensmest

In 2012 waren 31 van de 33 Nationale Referentie Laboratoria (NRL's) in de Europese Unie in staat om hoge en lage concentraties *Salmonella* in varkensmest aan te tonen. Ze behaalden direct het gewenste niveau. Twee NRL's behaalden aanvankelijk onvoldoende resultaat, omdat ze problemen hadden om *Salmonella* in varkensmest aan te tonen. Beide laboratoria behaalden het gewenste resultaat tijdens de herkansing. In totaal hebben de laboratoria in 93 procent van de besmette monsters *Salmonella* opgespoord.

Ringonderzoek verplicht voor Europese lidstaten

Dit blijkt uit het vijftiende veterinair ringonderzoek dat het referentielaboratorium van de Europese Unie voor *Salmonella* (EURL-*Salmonella*) heeft georganiseerd. Het onderzoek is in maart 2012 gehouden, de herkansing was in juni 2012. Alle NRL's van de Europese lidstaten die verantwoordelijk zijn voor de opsporing van *Salmonella* in dierlijke mest, zijn verplicht om aan het onderzoek deel te nemen. Het EURL-*Salmonella* is gevestigd bij het Nederlandse Rijksinstituut voor Volksgezondheid en Milieu (RIVM).

De laboratoria toonden de *Salmonella*-bacterie in dierlijke mest aan met behulp van de internationaal voorgeschreven analysemethode (MSRV). Elk laboratorium kreeg een pakket toegestuurd met varkensmest, dat vrij was van *Salmonella*, en het zogeheten referentiemateriaal (*lenticule discs*), dat geen of verschillende niveaus *Salmonella* bevatte. De laboratoria dienden de varkensmest en het referentiemateriaal zelf volgens een protocol samen te voegen en te onderzoeken op de aanwezigheid van *Salmonella*.

Twee specifieke typen *Salmonella* aantonen in varkensmest

Tijdens de studie zijn twee typen *Salmonella* (serovars) die regelmatig bij varkens worden aangetroffen, onderzocht. Het blijkt dat sommige laboratoria de lage concentratie Salmonella Typhimurium minder goed kunnen aantonen dan vergelijkbare concentratie van Salmonella Derby. *S*. Derby was nog niet eerder in een studie gebruikt. Het laboratorium van het EURL-*Salmonella* heeft daarom voorafgaand aan de studie enkele extra onderzoeken uitgevoerd. Onder andere is gekeken of de temperatuur van invloed is op de aanwezigheid van *S*. Derby in het referentiemateriaal in combinatie met het te testen materiaal (varkensmest).

Trefwoorden: *Salmonella*; EURL; NRL; ringonderzoek; varkensmest; *Salmonella*-detectiemethode; lenticule disc

Contents

Summary

In March 2012 the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*) organised the fifteenth veterinary interlaboratory comparison study on detection of *Salmonella*. The matrix of concern was pig faeces. Participants were 33 National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*): 28 NRLs from 27 EU Member States and 1 candidate EU-MS, Croatia. 3 Members of the European Free Trade Association (EFTA): Switzerland, Norway and Iceland and on request of DG-Sanco 1 non-European NRL from a third country: Israel.

The most important objective of the study was to test the performance of the participating laboratories for the detection of *Salmonella* at different contamination levels in a veterinary matrix. To do so, pig faeces samples of 25 grams each were analysed in the presence of reference materials (being lenticule discs) containing *Salmonella* at various contamination levels. A proposal for good performance was made and the performance of the laboratories was compared to this proposal. The prescribed method was Annex D of ISO 6579 (Anonymous, 2007), with selective enrichment on Modified Semi-solid Rappaport Vassiliadis (MSRV) agar. Optionally, a laboratory could also use other, own media or procedures for the detection of *Salmonella*.

In this study two *Salmonella* serovars, regularly found in pigs, were tested being *Salmonella* Typhimurium and *Salmonella* Derby. *S*. Derby has not been used in earlier studies and some additional tests (interference with pig faeces, stability test of *S*. Derby in the lenticule discs) were performed at the laboratory of the EURL-*Salmonella* before the start of the study.

Thirty-two individually numbered lenticule discs had to be tested by the participants for the presence or absence of *Salmonella*. Twenty-five of the lenticule discs had to be examined in combination with each 25 grams of *Salmonella*-negative pig faeces: 5 lenticule discs contained approximately 6 colony forming units (cfu) of *Salmonella* Derby (SD6), 5 lenticule discs contained approximately 37 cfu of *S*. Derby (SD37), 5 lenticule discs contained approximately 10 cfu of *S.* Typhimurium (STM10), 5 lenticule discs contained approximately 58 cfu of *S.* Typhimurium (STM58) and 5 lenticule discs contained no *Salmonella* at all (blank lenticule discs). Seven lenticule discs, to which no faeces had to be added, were control samples, existing of 2 lenticule discs STM10, 2 lenticule discs SD6, 1 lenticule disc SD37 and 2 blank lenticule discs.

On average, the laboratories found *Salmonella* in 93% of the (contaminated) samples when using the prescribed method, with selective enrichment on MSRV. 48 hours of incubation of MSRV gave overall 4-5% more positive results. This was most obvious for the samples containing *S*. Typhimurium, which gave 7-8% more positive results compared to 24 hours of incubation.

Thirty-one NRLs fulfilled the criteria of good performance on the first attempt. Two laboratories needed a follow-up study to reach the desired level. Both laboratories had difficulty in detecting *Salmonella* in the pig faeces, possibly caused by deviations in their media. One laboratory changed from manufacturer for their media and one laboratory possibly added the wrong supplement to MSRV.

1 Introduction

An important task of the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*), as laid down in the Commission Regulation No 882/2004 (EC, 2004), is the organisation of interlaboratory comparison studies. The history of the interlaboratory comparison studies as organised by EURL-*Salmonella* since 1995 is summarised in Annex 1.
The first and most important objective of the study, organized by the EURL for *Salmonella* in March 2012, was to see if the participating laboratories could detect *Salmonella* at different contamination levels in animal faeces. This information is important to know whether the examination of samples in the EU Member States (MS) is carried out uniformly and comparable results can be obtained by all National Reference Laboratories for *Salmonella* (NRL-*Salmonella*).

The prescribed method for the detection of *Salmonella* spp. in animal faeces is Annex D of ISO 6579 (Anonymous, 2007), with selective enrichment on Modified Semi-solid Rappaport Vassiliadis (MSRV).

The set-up of this study was comparable to earlier interlaboratory comparison studies on the detection of *Salmonella* spp. in veterinary, food and feed samples. The reference material consisted of lenticule discs. Good experiences have been gained with the lenticule discs in the veterinary study as organized in March 2011 (Kuijpers and Mooijman, 2011) and in the food study organized in October 2011 (Kuijpers and Mooijman, 2012a). Like in the former studies the contamination level of the low level lenticule discs was close to the detection limit of the method and the level of the high level samples was approximately 5-10 times above the detection limit. Seven control samples consisting of different lenticule discs had to be tested without the addition of pig faeces. These latter samples consisted of 2 lenticule discs with approximately 6 cfu of *Salmonella* Derby (SD6), 2 lenticule discs with approximately 10 cfu of *Salmonella* Typhimurium (STM10), 1 lenticule disc with approximately 37 cfu of *Salmonella* Derby (SD37) and 2 blank lenticule discs (BL). Twenty-five samples of *Salmonella* negative pig faeces spiked with 5 different lenticule discs had to be examined. For the latter samples the different lenticule discs consisted of 2 levels of *Salmonella* Typhimurium (STM10 and STM58), 2 levels of *Salmonella* Derby (SD6 and SD37) and blank lenticule discs.

2 Participants

Country	City	Institute
Austria	Graz	Austrian Agency for Health and Food Safety (AGES IVET)
Belgium	Brussels	Veterinary and Agrochemical Research Center (VAR) General and Molecular Bacteriology CODA-CERVA
Bulgaria	Sofia	National Diagnostic and Research Veterinary Institute
Cyprus	Nicosia	Cyprus Veterinary Services Pathology, Bacteriology, Parasitology Laboratory
Croatia	Zagreb	Croatian Veterinary Institute Laboratory for general bacteriology and microbiology
Czech Republic	Prague	State Veterinary Institute
Denmark	Esbjerg	Danish Veterinary and Food Administration Microbiology Laboratory
Estonia	Tartu	Estonia Veterinary and Food Laboratory, Bacteriology-Pathology Department
Finland	Kuopio	Finnish Food Safety Authority Evira Research Department, Veterinary Bacteriology
France	Ploufragan	Anses-site de Ploufragan-Plouzané HQPAP Laboratoire d'Etudes et de Recherches Avicoles, Porcines et Piscicoles Unité Hygiène et Qualité des Produits Avicoles et Porcins
Germany	Berlin	Federal Institute for Risk Assessment (BfR) National Veterinary Reference Laboratory for *Salmonella*
Greece	Chalikida	Veterinary Laboratory of Chalikida
Hungary	Budapest	Central Agricultural Office, Food and Feed Safety Directorate
Iceland	Reykjavik	University of Iceland Institute, Keldur Institute for Experimental Pathology
Ireland Republic of	Kildare	Central Veterinary Research Laboratory (CVRL/DAFF) Department of Agriculture, Fisheries and Food
Israel	Kiryat Malachi	Southern Poultry Health Laboratory (Beer Tuvia)
Italy	Padova Legnaro	Istituto Zooprofilattico Sperimentale delle Venezie, OIE National Reference Laboratory for *Salmonella*
Latvia	Riga	Institute of Food Safety, Animal Health and Environment BIOR Animal Disease Diagnostic Laboratory
Lithuania	Vilnius	National Food and Veterinary Risk Assessment Institute
Luxembourg	Luxembourg	Laboratoire de Médecine Vétérinaire de l'Etat, Animal Zoonosis
Malta	Valletta	Public Health Laboratory (PHL) Evans Building
Netherlands the	Bilthoven	National Institute for Public Health and the Environment (RIVM/Cib) Centre for Infectious Diseases Control Laboratory for Zoonoses and Environmental Microbiology (LZO)

Country	City	Institute
Norway	Oslo	National Veterinary Institute, Section of Bacteriology
Poland	Pulawy	National Veterinary Research Institute (NVRI) Department of Microbiology
Portugal	Lisbon	Laboratório Nacional de Investigação Veterinária (LNIV)
Romania	Bucharest	Institute for Diagnosis and Animal Health, Bacteriology
Slovak Republic	Bratislava	State Veterinary and Food Institute Reference Laboratory for *Salmonella*
Slovenia	Ljubljana	National Veterinary Institute, Veterinary Faculty
Spain	Madrid Algete	Laboratorio de Sanidad Y Produccion Animal de Algete Central de Veterinaria
Sweden	Uppsala	National Veterinary Institute (SVA), Department of Bacteriology
Switzerland	Bern	National Centre for Zoonoses, Bacterial Animal Diseases and Antimicrobial Resistance (ZOBA), Institute of veterinary bacteriology, Vetsuisse faculty Berne
United Kingdom	Addlestone	Animal Health and Veterinary Laboratories Agency (AHVLA) Weybridge, Bacteriology Department
United Kingdom	Belfast	Agri-Food and Bioscience Institute (AFBI) Veterinary Sciences Division Bacteriology

3 Materials and methods

3.1 Reference materials

3.1.1 Pre-test with Salmonella Derby

The matrix in this interlaboratory comparison study was pig faeces contaminated with two *Salmonella* serovars, regularly found in pigs, being *Salmonella* Typhimurium (STM) and *Salmonella* Derby (SD). As lenticule discs containing *S*. Derby were not used in earlier studies, some tests were performed to determine the detection limit of this serovar in pig faeces.
For this, two strains of *S*. Derby were used: NCTC 5721 and NCTC 5722. The strains were obtained from the National Collection of type Cultures (NCTC) of the Health Protection Agency (HPA, Newcastle, UK). Each strain was inoculated in Buffered Peptone Water (BPW) and overnight incubated at (37 ± 1) °C. Next each culture was diluted in Peptone Saline Solution to be able to inoculate samples of 25 g pig faeces with levels of approximate 0.5, 5, 50 and 500 cfu. The dilutions were prepared in duplicate and to the duplicate dilutions a heat stress was applied (15 min at 50 °C) before inoculating the faeces. This was done to mimick the 'stress' of the strains in the lenticule discs. The contaminated faeces samples were tested for the presence of *Salmonella* according to Annex D of ISO 6579 (Anonymous, 2007) with selective enrichment on MSRV.

3.1.2 Batches of lenticule discs

The reference material consisted of lenticule discs obtained from the HPA. Lenticule discs are microbiological reference materials, which are plano-convex discs containing microorganisms at a defined number in a solid water-soluble matrix (HPA, 2011). They are supplied as a single unit supported on a silica gel insert in a small airtight plastic tube (see Annex 5). The discs are lens-shaped and coloured and therefore easily seen on top of the filter insert. The *Salmonella* strains used for preparation of the lenticules were originated from NCTC of the HPA.
Five batches of lenticule discs were prepared by HPA:

- *S.* Typhimurium (STM) at a level of approximately 10 cfu per lenticule disc: NCTC 12023 batch 323-111025;
- *S.* Typhimurium (STM) at a level of approximately 58 cfu per lenticule disc: NCTC 12023 batch 523-100927R;
- *S.* Derby (SD) at a level of approximately 6 cfu per lenticule disc: NCTC 5722 batch 624-111215;
- *S.* Derby (SD) at a level of approximately 37 cfu per lenticule disc: NCTC 5722 batch 634-111214;
- Blank lenticule disc containing no microorganisms: batch 000-110825.

3.1.3 Homogeneity of the lenticule discs

The mean number of organisms of each batch was counted by HPA before the lenticule discs were sent to the EURL-*Salmonella*. For this, the HPA tested 30 lenticules per batch. The data were reported on the insert of the batch of lenticules and subjected to a homogeneity test at the EURL. It was tested whether the variation in counts between the lenticule discs was less than two times a Poisson distribution, using the following formula: $T_2 / (I-1) \leq 2$. Where

T_2 is a measure for the variation between lenticule discs of one batch and I is the number of lenticule discs (see Annex 2).

3.1.4 Stability of the lenticule discs

In literature, information can be found on the stability of several types of lenticule discs during storage and transport (Boyd et al., 2006 and Desai et al., 2006), but there is no specific information for *Salmonella*. Tests on the long-term stability and challenge tests on lenticule discs containing *S.* Enteritidis (SE) and *S.* Typhimurium (STM) were performed earlier by the EURL-*Salmonella,* showing good results (Kuijpers and Mooijman, 2011 and 2012a).
For the current study, the contamination level of each batch of lenticule discs was verified at the EURL by testing 2 lenticule discs (containing STM) or 5 lenticule discs (containing SD) per batch after receipt and storage at -20 °C. As *S*. Derby lenticules were used for the first time, their stability at elevated temperatures was also tested. For this 3 lenticule discs of SD6 and 3 lenticules of SD37 were tested at day 0, after 3 days and after 7 days of storage at 4 °C, 22 °C and at 30 °C.
For the counting of the lenticule discs, each lenticule disc was placed onto Colombia agar plates with sheep blood (Oxoid PB5008A, Germany). After 10 minutes of rehydration of the lenticule disc, the resultant 'drop' was spread over the plate and incubated at 37 °C for 20-24 hours. This method is also used by HPA to count the mean number of organisms of each batch of lenticule discs.

3.1.5 Pre-tests for the interlaboratory comparison study

Before organising the interlaboratory comparison study it was tested whether *Salmonella* could still be detected after mixing a *Salmonella* lenticule disc with the matrix (pig faeces). For this 5 lenticules SD6, 2 lenticules SD37 and 1 lenticule STM10 were each added to 25 g pig faeces (free from *Salmonella*) in 225 ml BPW.
The artificially contaminated faeces samples were tested for the presence of *Salmonella* according the SOP of the study and Annex D of ISO 6579 (Anonymous, 2007), with selective enrichment on MSRV.

3.2 Pig faeces samples

3.2.1 General

Pig faeces were sampled by the Animal Health Service (GD) Deventer at a *Salmonella* free farm (SPF-farm). As a large batch of approximately 30 kg pig faeces was needed , the GD sampled on different days in one week at one farm in the same pig stable. A total batch of 33 kg of pig faeces arrived at the EURL-*Salmonella* on 30 January 2012 and was stored at 5 °C. Due to the different sample days, the batch consisted of different sub batches containing different amounts of moisture. At random 10 portions of each 25 g were taken from different sub batches of pig faeces and checked for the absence of *Salmonella.* For the testing for *Salmonella* Annex D of ISO 6579 (Anonymous, 2007) was followed. For this purpose 10 portions of 25 grams were each added to 225 ml Buffered Peptone Water (BPW). After pre-enrichment at (37 ± 1) °C for 16-20 hours, selective enrichment was carried out on MSRV. Next, the suspect plates were plated-out on Xylose Lysine Deoxycholate agar (XLD) and Brilliance *Salmonella* Agar (BSA) and confirmed biochemically.

3.2.2 Total bacterial count in pig faeces

The total number of aerobic bacteria was investigated in the pig faeces. The procedure of ISO 4833 (Anonymous, 2003a) was followed for this purpose. Portions of 20 grams of faeces 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 1 ml of each dilution was brought into 2 empty Petri dishes (diameter 9 cm). To each dish 15 ml of molten Plate Count Agar (PCA) was added. After the PCA was solidified, an additional 5 ml PCA was added to the agar. The plates were incubated at (30 ± 1) °C for (72 ± 3) hours and the total number of aerobic bacteria was counted after incubation.

3.2.3 Number of Enterobacteriaceae in pig faeces

In addition to the total number of aerobic bacteria, the *Enterobacteriaceae* count was determined. The procedure of ISO 21528-2 (Anonymous, 2004) was used for this purpose. Portions of 20 grams of faeces 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 1 ml of each dilution was brought into 2 empty Petri dishes (diameter 9 cm). To each dish, 10 ml of molten Violet Red Bile Glucose agar (VRBG) was added. After the VRBG was solidified, an additional 15 ml VRBG was added to the agar. These plates were incubated at (37 ± 1) °C for (24 ± 2) hours and the number of typical violet-red colonies was counted after incubation. Five typical colonies were tested for the fermentation of glucose and for a negative oxidase reaction. After this confirmation the number of *Enterobacteriaceae* was calculated.

3.3 Design of the interlaboratory comparison study

3.3.1 Samples: lenticule discs and pig faeces

On 13 February 2012 (2 weeks before the study) the reference materials (32 individually numbered lenticule discs) and 800 grams of *Salmonella* negative pig faeces were packed with cooling devices as biological substance category B (UN 3373) and sent by door-to-door courier service to the participants. After arrival at the laboratory the lenticule discs had to be stored at -20 °C and the faeces had to be stored at +5 °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 4) and Standard Operation Procedure (SOP, Annex 5). The test report which was used during the study can be found at the EURL-*Salmonella* website:
http://www.eurlsalmonella.eu/Proficiency_testing/Detection_studies_Faeces
or can be obtained through the corresponding author of this report.

Seven control lenticule discs had to be tested without faeces (numbered C1-C7). Twenty-five lenticule discs (numbered B1-B25) were each tested in combination with 25 grams of faeces (negative for *Salmonella*). Table 1 shows the types and the number of lenticule discs and faeces samples which had to be tested.

Table 1 Overview of the types and the number of lenticule discs tested per laboratory in the interlaboratory comparison study.

Lenticule discs	Control lenticule discs (n=7) No matrix added	Test samples (n=25) with 25 grams *Salmonella* negative pig faeces
S. Derby 6 (SD6)	2	5
S. Derby 37 (SD37)	1	5
S. Typhimurium 10 (STM10)	2	5
S. Typhimurium 58 (STM58)	-	5
Blank (BL)	2	5

3.3.2 Pre-treatment of the samples

The NRLs could use pre-treatment procedures of the samples as they normally use for daily routine analyses. To gain information on the different pre-treatment procedures (e.g. pre-warming of BPW, different ways of mixing the samples in BPW) and to check whether the different procedures did not influence the results, some additional questions were added to the test report.

3.3.3 Sample packaging and temperature recording during shipment

The lenticule discs and the pig faeces were packed in two plastic containers firmly closed with screw caps (biopacks). Both biopacks were placed in one large shipping box, together with three frozen (-20 °C) cooling devices. Each shipping box was sent as biological substances category B (UN3373) by door-to-door courier services. 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 shipping box contained one logger, packed in the biopack with capsules. The loggers were programmed by the EURL-*Salmonella* to measure the temperature every hour. Each NRL had to return the temperature recorder immediately after receipt of the parcel, to the EURL. At the EURL-*Salmonella* the loggers were read by using a computer and all recorded temperature from the start of the shipment until the arrival at each National Reference Laboratories were transferred into an Excel sheet.

3.4 Methods

The prescribed method of this interlaboratory comparison study was Annex D of ISO 6579 (Anonymous, 2007). In addition to the prescribed methods the NRLs were free to use their own methods. This could be different medium combinations and/or investigation of the samples with alternative methods, like Polymerase Chain Reaction (PCR) based methods.

In summary:

Pre-enrichment in:
- Buffered Peptone Water (BPW) (prescribed).

Selective enrichment on:
- Modified Semi-solid Rappaport Vassiliadis medium (MSRV) (prescribed);
- own selective enrichment medium (optional).

Plating-out on:
- Xylose Lysine Desoxycholate agar (XLD) (prescribed);
- second plating-out medium for choice (obligatory);
- own plating-out medium (optional).

Confirmation of identity:
- Confirmation by means of appropriate biochemical tests (ISO 6579, Anonymous, 2002) or by reliable, commercially available identification kits and/or serological tests.

3.5 Statistical analysis of the data

The specificity, sensitivity and accuracy rates were calculated for the control samples, and the artificially contaminated samples with pig faeces (negative for *Salmonella* spp.). The specificity, sensitivity and accuracy rates were calculated according to the following formulae:

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

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

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

3.6 Good performance

The criteria used for testing good performance in this study are given in Table 2. For determining good performance per laboratory, the results found with MSRV together with all combinations of isolation media used by the laboratory were taken into account. For example, if a laboratory found for the STM10 lenticule disc with matrix 3/5 positive with MSRV/BGA but no positives with MSRV/XLD, this was still considered as a good result. The opposite was done for the judgement of the results of the blank lenticule discs. Here also all combinations of isolation media used per laboratory were taken into account. If for example a laboratory found 2/5 blank lenticule discs positive with MSRV/BGA but no positives with the other isolation media, this was still considered as a 'no-good' result.

Table 2 Criteria for testing good performance in the veterinary study XV (2012).

Control samples (lenticule disc, no matrix)	Minimum result	
	Percentage positive	No. of positive samples / total no. of samples
SD37	100%	1/1
STM10 and SD6	50%	1/2
Blank control lenticule disc	0%	0/2

Samples: pig faeces (lenticule with matrix)	Minimum result	
	Percentage positive	No. of positive samples / total no. of samples
Blank[1]	20% at max[1]	1/5 at max[1]
STM58 and SD37	80%	4/5
STM10 and SD6	40%	2/5

1: All should be negative. However, as no 100% guarantees about the *Salmonella* negativity of the matrix can be given, 1 positive out of 5 blank samples (20% pos.) will still be considered as acceptable.

4 Results

4.1 Reference materials

4.1.1 Pre-test with Salmonella Derby

Table 3 gives the results of the experiments to determine the detection limit of S. Derby in pig faeces. In the Table the mean levels are indicated which were used to artificially contaminated the pig faeces samples (25 g each). For each strain and each level, with and without applied stress, it is indicated whether the sample was found positive for Salmonella after pre-enrichment in BPW, selective enrichment on MSRV and plating out on XLD.
There were no differences observed between the results of the two strains of *S*. Derby (NCTC 5721 and NCTC 5722), neither did heat stress of 15 minutes at 50 °C influence the results.

Table 3 Detection of S. Derby with and without stress (15 min. at 50 °C*) in 25 g pig faeces*.

Mean contamination level of *S*. Derby cfu/ml	**NCTC 5721**		**NCTC 5721 15 min. 50° C**		**NCTC 5722**		**NCTC 5722 15 min 50 ° C**	
0.1	-	+	-	-	-	-	-	+
4	+	+	+	+	+	+	+	+
53	+	+	+	+	+	+	+	+
> 500	+	+	+	+	+	+	+	+

4.1.2 Contamination level and homogeneity of the lenticule discs

Table 4 summarises the information on the contamination level of each batch of lenticule discs as tested by the HPA. The mean levels, as well as the lowest and highest counts (in cfu) found per batch are indicated. Additionally, the results of the homogeneity test of each batch as performed by the EURL are given. The results of the homogeneity test shows each batch fulfilled the criteria well (variation less than two times Poisson distribution).

Table 4 Level of contamination and homogeneity of SD and STM lenticule discs.

	SD6	**SD37**	**STM10**	**STM58**
Batch number	624-111215	634-111214	323-111025	523-100927B
Date testing lenticules*	3.1.2012	3.1.2012	14.11.2011	24.10.2011
Number of lenticules tested	30	30	30	30
Mean cfu per lenticule	6	37	10	58
Min-max cfu per lenticule	3-11	20-54	4-16	46-74
T_2 / (I-1)**	0.77	1.87	1.33	0.89

* Tested by HPA.

** Calculated by EURL-*Salmonella.*

cfu = colony forming units; min-max = enumerated minimum and maximum cfu.
formula T_2 see Annex 2; I is number of lenticule discs; Demand for homogeneity $T_2/(I-1) \leq 2$

4.1.3 *Stability of the lenticule discs*

Table 5 summarises the results of the verification of the contamination levels of the batches of lenticules after arrival at the EURL-*Salmonella.* The results of both batches of STM and SD showed values between the minimum and maximum cfu counted by HPA after preparation.

Table 5 Contamination level of SD and STM lenticule discs tested at HPA immediate after preparation and tested at the EURL-*Salmonella after transport and storage at -20 °C.*

	SD6	SD37	STM10	STM58
Batch number	624-111215	634-111214	323-111025	523-100927B
Date of testing at HPA	3.1.2012	3.1.2012	14.11.2011	24.10.2011
Number of lenticules tested*	30	30	30	30
Mean cfu per lenticule	6	37	10	58
Min-max cfu per lenticule	3-11	20-54	4-16	46-74
Date of testing at EURL-S	17.1.2012	17.1.2012	17.1.2012	17.1.2012
Number of lenticules tested**	5	5	2	2
Mean cfu per lenticule disc	9	46	9	55
Min-max cfu per lenticule	6-12	26-54	5,13	49,61

* Tested by HPA.

** Tested by EURL-*Salmonella.*

cfu = colony forming units.

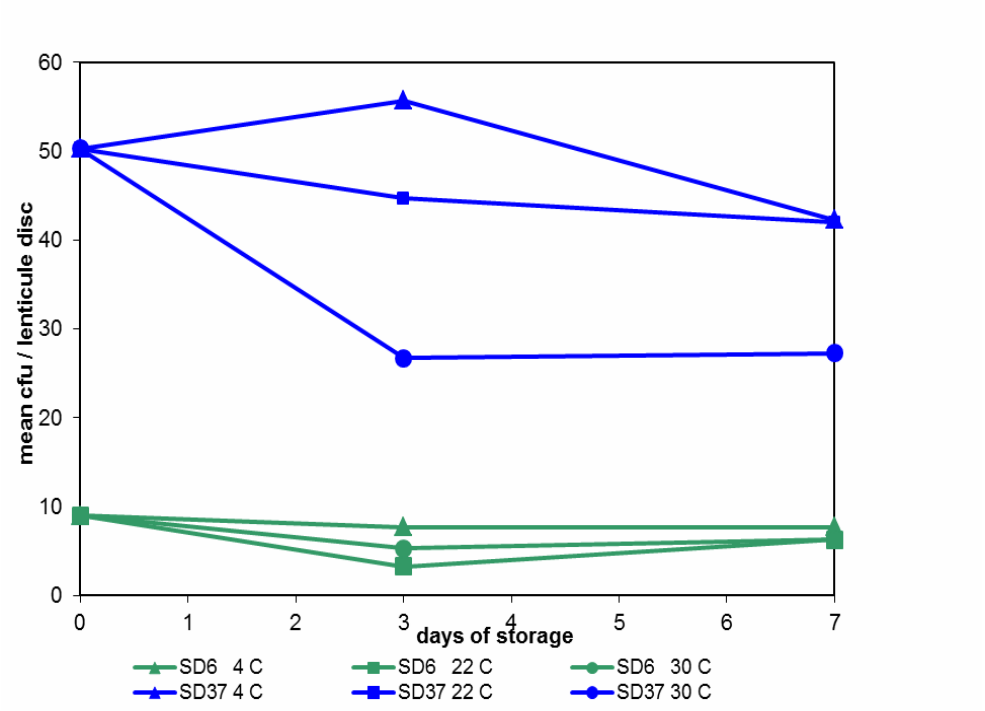


Figure 1 Challenge test of lenticule discs containing S. Derby, stored at different temperatures. Mean results of 3 lenticule discs per test are indicated.

Figure 1 shows the results of the challenge test of lenticules containing *Salmonella* Derby. Little or no effect was seen on the contamination level of the SD6 lenticules after storage at 4 °C, 22 °C or 30 °C for up to 7 days. The SD37

lenticules also showed to be stable after storage at 4 °C and 22 °C for one week. When stored at 30 °C an initial decrease in the mean contamination level was seen; after this decreased, the mean contamination level remained stable.

4.1.4 Pre-test for the interlaboratory comparison study

Table 6 shows the results of the pre-test of the interlaboratory comparison study performed at the EURL-*Salmonella.*

Table 6 Number of positive results of control lenticule discs and of pig samples artificially contaminated with S. Derby and S. Typhimurium.

	No of positive results / Total no. of samples	
Lenticule discs	**Control lenticule discs (n=3) No matrix added**	**Test samples (n=10) with 25 grams *Salmonella* negative pig faeces**
S. Derby 6 (SD6)	2/2	3/5
S. Derby 37 (SD37)	-	2/2
S. Typhimurium 10 (STM10)	-	1/3
Blank (BL)	0/1	-

The control lenticules of pig faeces samples artificially contaminated with low level lenticules were all scored correctly; approximately half of the number of tested samples were found negative. The high level samples were all tested positive.

4.2 Pig faeces samples

The faeces were tested negative for *Salmonella* and stored at 5 °C. On Monday 13 February 2012 the faeces were mailed to the NRLs. After receipt, the NRLs had to store the faeces at 5 °C. The number of aerobic bacteria and the number of *Enterobacteriaceae* were tested twice; firstly at the day the faeces arrived at the EURL (30/01/2012 and secondly, after storage at 5 °C, close to the planned date of the interlaboratory comparison study (20/02/2012). Table 7 shows the results, indicating the amount of background flora was relatively high in the pig faeces and which remained stable even after storage of one month.

Table 7 Number of aerobic bacteria and the number of Enterobacteriaceae per gram of pig faeces.

Date	**Aerobic bacteria cfu/g**	***Enterobacteriaceae* cfu/g**
30 January 2012	$2*10^9$	$5*10^5$
20 February 2012	$1*10^9$	$5*10^5$

4.3 Technical data interlaboratory comparison study

4.3.1 General

In this study 33 NRLs participated: 28 NRLs from 27 EU-MS, one NRL from a EU candidate country, 3 NRLs from member countries of the European Free Trade Association State (EFTA) and on request of DG-Sanco, 1 NRL from a third country (outside Europe).
Thirty-one laboratories performed the study on the planned date (week 8 starting on 20/02/2012). Two laboratories (lab codes 6 and 16) performed the study one week earlier and two laboratories (lab codes 20 and 21) started 7-10 days later. Laboratory 21 did make a mistake with the samples and received a new portion of faeces on 29/02/2012 after which they immediately started the study.
One laboratory (lab code 6) used a different procedure for the confirmation of the isolates. Instead of confirming at least one colony from each isolation medium (as indicated in the SOP), the laboratory confirmed one colony either from XLD, or from the second isolation medium (Rapid *Salmonella*).

4.3.2 Accreditation/certification

31 laboratories mentioned to be accredited for their quality system according to ISO/IEC 17025 (Anonymous, 2005) and two laboratories (lab codes 20 and 27 EU-MSs) are planning to become accredited. 29 laboratories are accredited for annex D of ISO 6579; 18 are also accredited for ISO 6579 and 3 (lab codes 8, 20 and 27) are planning to become accredited for annex D within one year. One laboratory of an EU-MS (lab code 31) is not accredited for the analysis of *Salmonella* in samples from primary production (Annex D of ISO 6579) but is accredited for detection of *Salmonella* in food and feeding stuffs (ISO 6579). One non-EU laboratory (lab code 8) is planning to become accredited for the MSRV method and is currently accredited for a national standard method for food matrices (only RVS). One laboratory (lab code 19 EU-MS) did not mention for which method they are accredited.

4.3.3 Transport of samples

Table 8 gives an overview of the transport times and the temperatures during transport of the parcels. The NRLs returned the temperature recorders immediately after receipt to the EURL-*Salmonella.* The average transport time to the EU-MS was 30 hours. Twenty-three of the laboratories received the materials within 1 day. Two parcels (lab codes 2 and 28) were delayed at the customs (non-EU-MSs) and arrived after 4 days at the institute. For the majority of the parcels the transport temperature did not exceed 5 °C. For 13 NRLs the time of transport recorded on the test report did not correspond with the time reported by the courier. Presumably, the parcel arrived at the time reported by the courier at the institute, but due to internal logistics at the institute, the parcel arrived 1 to 2 hours later at the laboratory of the NRL.

Table 8 Overview of transport times and temperatures during shipment of the parcels to the NRLs.

Lab code	Transport time[1] Total in hours (h)	< 0 °C	0 °C - 5 °C	5 °C - 10 °C	> 15 °C	AdditionalStorage[2] time in hours (h)
1	48	25	23			1 h 0 - 4°C
2	70	41	29			24 h < 0 °C
3	24	14	10			
4	46	18	27		1	
5	24	24				3 h 1 °C
6	27	14	13			
7	46	10	36			1 h 1°C
8	24	14	10			1 h 2°C
9	24	14	10			
10	24	8	16			
11	25	14	9	2		
12	22	21	1			1 h
13	30	14	16			15 h 1°C
14	26	17	7		2	
15	1					
16	22	8	14			
17	26	14	12			
18	26	20	6			
19	27	15	11		1	
20	27	18	9			
21	24	14	10			
21*	49	5	44			
22	28	19	9			14 h 4 °C, 11h 17-
23	25	14	10		1	
24	23	16	7			2 h 0-1°C
25	44	18	26			
26	46	14	32			4 h 2°C
27	45	14	31			
28	94	12	82			1 h 23°C
29	50	24	26			1 h 1-3 °C
30	26	24	1		1	1 h 19°C
31	26	11	15			
32	23	21	2			
33	24	10	14			
Average	34					
Average EU[3]	30					

1 = Transport time according to the courier.
2 = Storage time of the samples at the institute before arriving at the laboratory of the NRL.
3 = Average Transport time to the countries of EU Member States.
* Laboratory 21 received an extra parcel

4.3.4 *Pre-treatment of the samples*

For testing the samples, the laboratories were asked to use the procedures and materials as normally used for routine samples as much as possible (see Annex 5, SOP of this study).
There was an almost equal distribution between the type of containers used: bags 36%, jars (30%) or bottles (27%). 51% of the laboratories used containers pre-filled with BPW. The majority of the laboratories pre-warmed the BPW at room temperature (63%), the others at 37 °C. The samples (BPW, lenticule disc and matrix) were mixed by most of the laboratories by shaking gently (63%), some by kneading (24%) or another way of mixing (13%), for example with a spoon. None of the laboratories used a pulsifier, stomacher or vortex to mix.

4.3.5 *Media*

Each laboratory was asked to test the samples with the prescribed method (Annex D of ISO 6579). All laboratories used the selective enrichment medium MSRV, the plating-out medium XLD and a second plating-out medium of own choice. Six laboratories used one or more additional selective enrichment media: RVS (lab codes 8, 13 and 17), MKTTn (lab codes 7 and 19), RVS and MKTTn (lab code 23). Three laboratories (lab codes 7, 13 and 22) used 3 isolation media. Table 9 shows the media used per laboratory. Details on the media which are not described in ISO 6579 (Anonymous, 1993 and 2002) are given in Annex 3. The Tables 10-13 give information on the composition of the media which were prescribed and on incubation temperatures and times. These Tables only indicate the laboratories who reported deviations from the prescribed compositions. One laboratory (lab code 8) reported a longer incubation time of the pre-enrichment in BPW. The laboratories 6 and 30 did not mention the pH of the media. Laboratory 3 did not mention the composition of MSRV and XLD used. Five laboratories (lab codes 10, 14, 23, 28 and 30) used MSRV with a higher concentration of Novobiocin than the prescribed 0.01 g/L. Four laboratories (lab codes 11, 22, 29 and 33) reported a deviating pH for the MSRV than the described pH of 5.2. Laboratory 11 used XLD with the addition of Novobiocin (1 ml of a 1,5% Novobiocin solution).

A second plating-out medium for choice was obligatory. Fifteen laboratories used modified BGA (Anonymous, 1993) as a second plating-out medium. Eight laboratories used Rambach agar, three laboratories used SM (ID) 2, three laboratories used Rapid *Salmonella* (RS) agar and two laboratories Brilliance Salmonella Agar (BSA). The following media were used only by one laboratory: BPLSA, BGA with Sulfadiazine (BGA ^{S}), ASAP, BxLH, Onoz and BxLH medium. The use of an extra plating agar between the 'isolation' and the 'confirmation' steps was optional. Eighteen laboratories performed this extra culture step on Nutrient agar (Anonymous, 2002) or another agar medium (e.g. Bromthymol blue lactose sucrose agar, Colombia Blood agar, Plate Count Agar).

Table 9 Media combinations used per laboratory.

Lab code	Selective enrichment media	Plating-out Media
1	MSRV	XLD RS
2	MSRV	XLD BGA mod
3	MSRV	XLD Rambach
4	MSRV	XLD BPLS= BGA mod
5	MSRV	XLD Rambach
6	MSRV	XLD RS
7	MSRV MKTTn	XLD Rambach RS
8	MSRV RVS	XLD BSA
9	MSRV	XLD BGA mod
10	MSRV	XLD BPLSA
11	MSRV	XLD+ Novobiocin BGA mod
12	MSRV	XLD BGA^{S}
13	MSRV RVS	XLD Rambach BGA mod
14	MSRV	XLD BGPA=BGA mod
15	MSRV	XLD BGA mod
16	MSRV	XLD SMID2
17	MSRV RVS	XLD BGA mod
18	MSRV	XLD SM2
19	MSRV MKTTn	XLD BGA mod
20	MSRV	XLD SM2
21	MSRV	XLD BxLH
22	MSRV	XLD BGA mod Rambach
23	MSRV RVS MKTTn	XLD BPLS=BGA mod
24	MSRV	XLD BGA mod
25	MSRV	XLD Rambach
26	MSRV	XLD Önöz
27	MSRV	XLD BGA mod
28	MSRV	XLD Rambach
29	MSRV	XLD BPLS=BGA mod
30	MSRV	XLD BSA
31	MSRV	XLD Rambach
32	MSRV	XLD BGA mod
33	MSRV	XLD ASAP

Explanations of the abbreviations are given in the 'List of abbreviations'.
Compositions of the media not described in ISO 6579 (Anonymous, 1993 and 2002) are given in Annex 3.

Table 10 Incubation time and temperature of BPW.

	Pre-enrichment in BPW	
Lab code	**Time (h:min)**	**Incubation temperature in °C (min-max)**
SOP & ISO 6579	**16 – 20**	**36-38**
8	23	36.5-37

Grey cell: deviating incubation time.

Table 11 Composition (in g/L) and pH of BPW.

Lab code	Enzymatic digest of casein (Peptone)	Sodium Chloride (NaCl)	Disodium hydrogen Phosphate dode-cahydrate* ($Na_2HPO_4.12H_2O$)	Potassium dihydrogen phosphate (KH_2PO_4)	pH
ISO 6579	**10.0**	**5.0**	**9.0**	**1.5**	**6.8 – 7.2**
1, 2, 24	10.0	5.0	3.5	1.5	7.3
6, 15, 30, 33	10.0	5.0	3.5	1.5	-
16	10.0	5.0	9.0	1.5	-

Grey cell: deviating from ISO 6579 = No information

* = 3.5 grams Disodium hydrogen phosphate (anhydrous) is equivalent to 9 grams disodium hydrogen phosphate dodecahydrate.

Table 12 Composition (in g/L) and pH of MSRV.

Lab code	Enzymatic digest of casein (Tryptose)	Casein hydro-lysate	Sodium chloride (NaCl)	Potassium Dihydrogen Phosphate (KH_2PO_4 K_2HPO_4)	Magnesium chloride anhydrous ($MgCl_2$)	Malachite green oxalate	Agar	Novo biocin	pH
Annex D ISO 6579	**4.6**	**4.6**	**7.3**	**1.5**	**10.9**	**0.04**	**2.7**	**0.01 (10mg/L)**	**5.1- 5.4**
3	-	-	-	-	-	-	-	-	5.2
5	8.3	0.9	7.3	1.5	12.4	0.04	2.6	0.01	5.2
6, 8, 16	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.01	-
10, 14	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.2
11,22	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.01	5.5
23	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.05	5.2
28	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.3
29, 33	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.01	5.0
30	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	-

Grey cell: deviating from Annex D of ISO 6579 - = No information

*Peptone /typtone mixture

Table 13 Composition (in g/L) and pH of XLD.

Lab code	Xyl-ose	L-lysine	Lact-ose	Sucrose (Saccharose)	Sodium chloride (NaCl)	Yeast Ex-tract	Phe-nol red	Agar	Sodium deoxy-cholate ($C_{24}H_{39}NaO_4$)	Sodium thio-sul-phate ($Na_2S_2O_3$)	Iron (III) Ammonium Citrate ($C_6H_8O_7 \cdot nFe \cdot nH_3N$)	pH
ISO 6579	**3.75**	**5.0**	**7.5**	**7.5**	**5.0**	**3.0**	**0.08**	**9-18**	**1.0**	**6.8**	**0.8**	**7.2 – 7.6**
2	3.5	5.0	7.5	7.5	5.0	3.0	0.08	13.5	2.5	6.8	0.8	7.2
3	-	-	-	-	-	-	-	-	-	-	-	7.4
5	3.5	5.0	7.5	7.5	5.0	3.0	0.08	13.5	2.5	6.8	0.8	6.6
6, 27, 33	3.75	5.0	7.5	7.5	5.0	3.0	0.08	13.5 12.5	1.0	6.8	0.8	-
16, 28	3.5	5.0	7.5	7.5	5.0	3.0	0.08	13.5	2.5	6.8	0.8	7.4
20	3.5	5.0	7.5	7.5	5.0	3.0	0.08	13.5	2.5	6.8	0.8	-
25	3.5	5.0	7.5	7.5	5.0	3.0	0.08	13.5	2.5	6.8	0.8	7.3
30	3.75	5.3	7.5	7.5	5.0	3.0	0.08	12.5	1.0	6.8	0.8	-

Grey cell: deviating from ISO 6579 = No information

All participating laboratories performed confirmation tests for *Salmonella*: biochemically, serologically or both. The majority of the laboratories used both biochemical and serological tests. Seven laboratories (lab codes 11, 15, 16, 20, 22 29 and 30) used only a biochemical test(s), three laboratories (lab code 5, 10 and 18) used only a serological test(s). Three laboratories showed a limited confirmation: laboratory 30 performed only one biochemical test (TSI), laboratory 9 used only a chromagar and one O antigen test and laboratory 18 performed only one antigen test. The Tables 14 and 15 summarise the confirmation media and tests.

Table 14 Serological confirmation of Salmonella.

Lab code	Serological	
	O antigens	**H antigens**
2,9,10,21,25,28,32,33	+	-
3,4,5,7,12,13,14,17,19,23,24,26,27,31	+	+
6	+	-
1,8,11,15,16,18,20,22,29,30	-	-

- = Not done/ not mentioned.

Table 15 Biochemical and other confirmation tests of Salmonella.

Lab code	TSI	UA	LDC	Gal	VP	Indole	Kit	Other
1	-	-	-	-	-	-	Enterotube II	Poly A-S Vi
2	-	-	-	-	-	+	HY Enterotest	-
3	+	+	+	+	-	+	-	semi-solid glucose agar
4, 13, 19, 25	+	+	+	+	+	+	-	-
5,10	-	-	-	-	-	-	-	-
6	+	-	+	-	-	-	-	-
7	+	-	+	+	+	-	-	Uréa indole
8	+	+	+	-	-	-	-	Lysine Iron Agar, Oxoid Latex Agglutination Test
9	-	-	-	-	-	-	-	Chromagar
11, 15, 20	+	+	+	-	-	-	-	-
12, 32	+	+	+	+	-	+	-	-
14	+	+	+	-	-	+	-	Glucose
16	-	-	-	-	-	-	Microbact 12A, Oxoid	-
17	+	+	+	-	-	+	-	MacConkey Agar No.3
18	-	-	-	-	-	-	-	Enteroclon- Anti-Salmonella Test
21	-	-	-	-	-	-	-	Kligler agar, urea and indol broth,ONPG and FDA medium, motility test
22	+	+	+	-	+	+	-	Citrate, Simmons
23	+	+	+	-	-	+	-	-
24	-	-	-	-	-	-	-	Kohns No1 medium (Mast Diagnostics)
26	-	-	-	-	-	-	API 20E	InvA-PCR
27	+	-	-	-	-	-	Microgen GN-ID A Panel	Automatic Identification System, Vitek 2-Compact, Biomerieux
28	+	-	-	-	-	+	API 32E	Lysine iron agar
29	+	+	+	-	-	-	API 20E	
30	+	-	-	-	-	-	-	-
31	+	+	+	-	-	-	Enterotest 24 (Lachema)	MALDI-TOF
33	-	-	-	-	-	-	API 20E	-

- = Not done/ not mentioned.

4.4 Control samples

4.4.1 General

None of the laboratories isolated *Salmonella* from the (blank) procedure control (C8: no lenticule disc/no faeces) nor from the faeces control (C9: no lenticule disc/negative pig faeces). All laboratories scored correct results for all the control lenticule discs. The results given in the Tables are the highest number of positive isolations found with MSRV in combination with any isolation medium (MSRV/x). Table 16 gives the results of all control samples (lenticule discs without faeces). All samples were scored correctly. Six laboratories used an additional selective enrichment medium (own method, see Table 9). Table A.6.1 in Annex 6 gives the results found with these own methods compared to the MSRV results; also with the own method all results were scored correctly.

Blank lenticule discs without addition of faeces (n=2)
All laboratories correctly analysed the blank lenticule disc negative for *Salmonella* with all used media.

S. Derby 6 lenticule discs (SD6) without addition of faeces (n=2)
All laboratories isolated *Salmonella* Derby at a mean level of approximately 6 cfu/lenticule disc from both lenticule discs.

S. Derby 37 lenticule discs (SD37) without addition of faeces (n=1)
All participating laboratories tested the one control lenticule disc containing SD37 positive.

S. Typhimurium 10 lenticule discs (STM10) without addition of faeces (n=2)
All 33 laboratories tested both lenticule discs containing STM10 positive.

The results were compared with the definition of 'good performance' (see section 3.6), as all samples were scored correctly, all laboratories fulfilled the criteria for the control samples.

Table 16 Total number of positive results of the control samples (lenticule disc without faeces) per laboratory.

Lab code	The highest number of positive isolations found with MSRV in combination with any isolation medium (MSRV/x)			
	Blank n=2	SD6 n=2	SD37 n=1	STM10 n=2
Good Performance	**0**	**≥ 1**	**1**	**≥ 1**
All laboratories	0	2	1	2

4.4.2 Specificity, sensitivity and accuracy rates of the control samples

Table 17 shows the specificity, sensitivity and accuracy rates for the control lenticule discs without the addition of faeces. The rates are calculated for the selective enrichment medium MSRV with plating-out medium XLD and 'non-XLD media'. The calculations were performed on the results of all participants and on the results of only the EU-MS (without the results of the EFTA States, candidate and third countries). No differences were found between these groups.

The laboratories scored an excellent result for the control samples with an accuracy rate of 100% for MSRV.

Table 17 Specificity, sensitivity and accuracy rates of the control samples (without the addition of faeces) for the selective enrichment on MRSV.

Control lenticule discs		**MRVS/x** n= 33
Blank n=2	No. of samples No. of negative samples **Specificity in %**	66 66 **100**
STM10 n=2	No. of samples No. of positive samples **Sensitivity in%**	66 66 **100**
SD6 n=2	No. of samples No. of positive samples **Sensitivity in%**	66 66 **100**
SD37 n=1	No. of samples No. of positive samples **Sensitivity in%**	33 33 **100**
All lenticule discs with *Salmonella*	No. of samples No. of positive samples **Sensitivity in%**	165 165 **100**
All lenticule discs	No. of samples No. of correct samples **Accuracy in%**	231 231 **100**

X= isolation medium (XLD or non-XLD) which gave the highest number of positives.

4.5 Results faeces samples artificially contaminated with Salmonella

4.5.1 Results per type of lenticule disc and per laboratory

General
Table 18 gives the results of the *Salmonella* negative pig faeces samples artificially contaminated with lenticule discs. The results given in the Tables are the highest number of positive isolations found with MSRV in combination with any isolation medium (MSRV/x). Six laboratories used an additional selective enrichment medium (own method see Table 9). Annex 6, Table A.6.2 gives the results found with these own methods compared to the MSRV results. Two laboratories (lab codes 7 and 19) found different results with their own method in comparison to the MSRV method.

The majority of the laboratories (22/33) tested all artificially contaminated faeces samples positive for *Salmonella* with the prescribed method MSRV.

Blank lenticule discs with negative pig faeces (n=5)
32 laboratories correctly did not isolate *Salmonella* from the blank lenticule discs with the addition of negative pig faeces. Laboratory 6 found one blank sample added to negative faeces positive for *Salmonella.* All blanks should be tested negative**.** However, as no 100% guaranty about the *Salmonella* negativity of the pig faeces can be given, 1 positive out of 5 blank samples (80% neg.) will still be considered as acceptable. A false positive result for a blank sample may have been caused by cross-contamination or by misinterpretation of the results.

S. Derby 6 lenticule discs (SD6) with negative pig faeces (n=5)
Twenty-four laboratories were able to isolate *Salmonella* from all the five lenticule discs containing *Salmonella* Derby at a level of approximately 6 cfu/ lenticule disc in combination with pig faeces. Five laboratories (lab codes 6, 7, 10, 22 and 30) could not detect *Salmonella* in one or two lenticule discs on all of the used media. Three laboratories (lab codes 14, 21 and 29) found three lenticule discs negative with the prescribed method.
One laboratory (lab code 19) found four lenticule discs negative for all the media used. These lenticule discs contained SD at a low level (approximately 6 cfu/lenticule). Due to change, one out of five lenticule discs containing SD6 may be negative. However, it is not very likely to find four SD6 lenticule disc negative.

S. Derby 37 lenticule discs (SD37) with negative pig faeces (n=5)
Thirty-one laboratories isolated *Salmonella* from all the five lenticule discs containing *Salmonella* Derby at a level of approximately 37 cfu/ lenticule disc in combination with pig faeces. One laboratory (lab code 22) found one lenticule disc negative. Laboratory 19 found only three positive results with the prescribed selective enrichment medium MSRV but with their own selective enrichment medium (MKTTn) they found all five samples correctly positive.

S. Typhimurium 10 lenticule discs (STM10) with negative pig faeces (n=5)
Twenty-five laboratories isolated *Salmonella* from all five lenticule discs containing *Salmonella* Typhimurium at a level of approximately 10 cfu/ lenticule disc in combination with pig faeces. Five laboratories (lab codes 8, 14, 19, 21 and 24) could not detect *Salmonella* in one or two lenticule discs on all of the used media. Two laboratories (lab codes 22 and 29) found three lenticule discs negative with the prescribed method.

One laboratory (lab code 19) found four lenticule discs negative for all the media used. These lenticule discs contained STM at a low level (approximately 10 cfu/lenticule). Due to change, one out of five lenticule discs containing STM10 may be negative. However, it is not very likely to find four STM10 lenticule disc negative.

S. Typhimurium 58 lenticule discs (STM58) with negative pig faeces (n=5)
Thirty-one laboratories isolated *Salmonella* from all five lenticule discs containing *Salmonella* Typhimurium at a level of approximately 58 cfu/lenticule disc in combination with pig faeces. Two laboratories (lab code 19 and 30) could not detect *Salmonella* in two lenticule discs on all of the used media.

Table 18 Number of positive results found with the artificially contaminated pig faeces samples per laboratory.

Lab code	Highest number of positive isolations found with MSRV in combination with any isolation medium (MSRV/x)				
	Blank n=5	SD6 n=5	SD37 n=5	STM10 n=5	STM58 n=5
Good performance	**≤ 1**	**≥ 2**	**≥ 4**	**≥ 2**	**≥ 4**
1 - 5	0	5	5	5	5
6	**1**	**4**	5	5	5
7	0	**3**	5	5	5
8	0	5	5	**4**	5
9	0	5	5	5	5
10	0	**4**	5	5	5
11 - 13	0	5	5	5	5
14	0	**2**	5	**3**	5
15 - 18	0	5	5	5	5
19	0	**1**	**3**	**3**	**3**
20	0	5	5	5	5
21	0	**2**	5	**3**	5
22	0	**3**	**4**	**2**	5
23	0	5	5	5	5
24	0	5	5	**4**	5
25 - 28	0	5	5	5	5
29	0	**2**	5	**2**	5
30	0	**4**	5	**1**	**3**
31 - 33	0	5	5	5	5

Bold number: deviating result.
Grey cell: result is below good performance.

The results of the artificially contaminated pig faeces samples were compared with the definition of 'good performance' (see section 3.6) and 31 laboratories fulfilled these criteria for the prescribed method (MSRV). Two laboratories showed to have problems with the detection of *Salmonella* in the pig faeces and scored below the level of good performance. This concerned laboratory 30 for the STM samples, and laboratory 19 for both the STM and SD samples. Laboratory 19 found more negative results with their 'own' method MKTTn than with the prescribed method (see Annex 6).

4.5.2 *Results per medium, lenticule disc and per laboratory*

Figures 2-5 show the number of positive isolations per type of artificially contaminated pig faeces sample and per laboratory after pre-enrichment in BPW and selective enrichment on MSRV followed by isolation on selective plating agar.

The results of all artificially contaminated pig faeces samples were compared with the proposed definition of 'good performance' (see section 3.6). In Figures 2-5 the border of good performance is indicated with a black horizontal line.

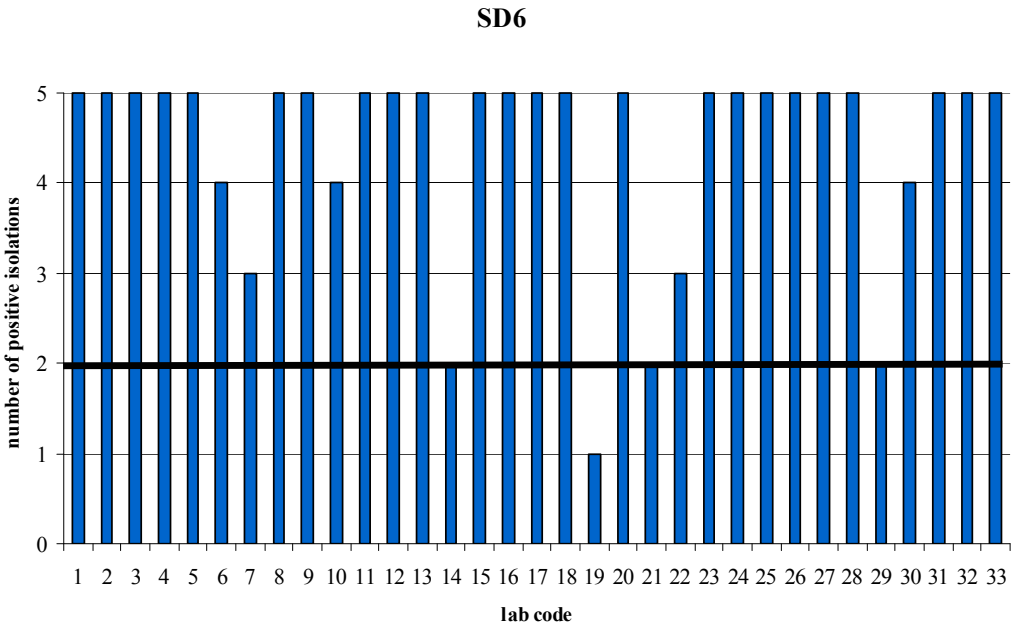
Table 19 presents the results of the number of positive isolations after 24 and 48 hours of incubation of the selective enrichment MSRV. On average, 5% more positive results were found after 48 hours of incubation, compared to 24 hours of incubation. However, for the low level STM samples 7-8% more positives were found after 48 hours of incubation while for the SD samples this difference was only 3%. The choice of isolation medium does not seem to give a significant difference in the number of positive isolations. Non-XLD plating-out media gave 2% more positive results compared to XLD. The majority of the laboratories used modified BGA as the second plating-out medium (see Table 9).

Table 19 Mean percentages of positive results found for the artificially contaminated pig faeces samples after 24 hours and 48 hours of incubation on MSRV.

Plating out medium	**Selective enrichment Medium MSRV 24 / 48 hours incubation**
	All lenticules with pig faeces
XLD	85 / 90%
Other (most often BGA)	87 / 92%

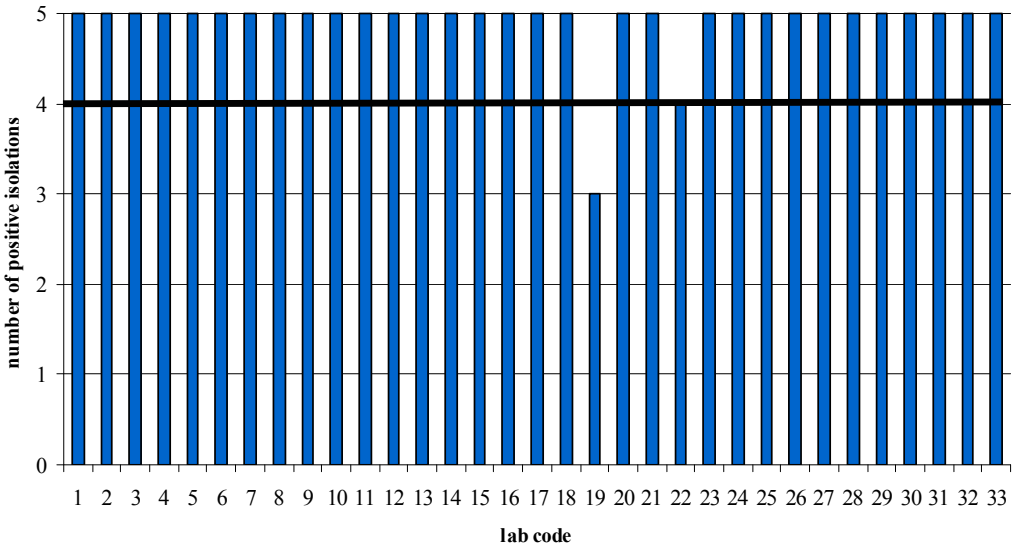
4.5.3 *Specificity, sensitivity and accuracy rates of the artificially contaminated samples*

Table 20 shows the specificity, sensitivity and accuracy rates for all types of artificially contaminated pig faeces samples. This Table gives the results for the different medium combinations: pre-enrichment in BPW, followed by selective enrichment on MSRV and isolation on selective plating agar showing the highest number of positives (MSRV/x). The calculations were performed on the results of all participants and on the results of the participants of the EU-MSs only (without the results of the participants of the EFTA States, candidate EU-MSs and third countries). No differences were found between these groups. The specificity rates (of the blank lenticule discs) were 99%. The high level SD37 and STM58 showed high sensitivity rates of 98%. For the low level artificially contaminated pig faeces samples (STM10 and SD6) the sensitivity rates were also relatively high: 88-89%.



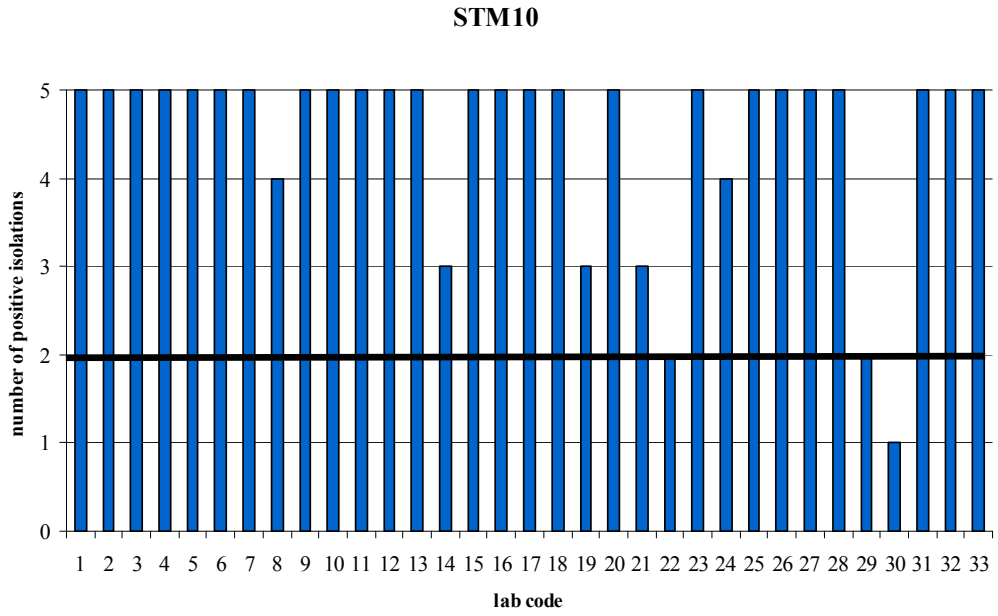
- = border of good performance

Figure 2 Results per laboratory of pig faeces samples artificially contaminated with SD6 lenticule discs (n=5) after selective enrichment on MSRV followed by isolation on the 'best' selective plating agar.



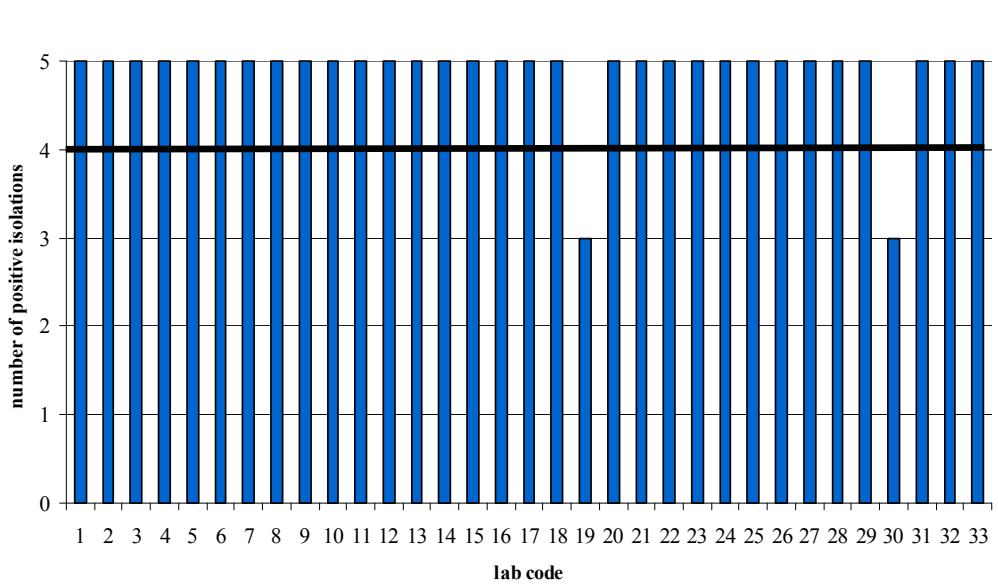
- = border of good performance

Figure 3 Results per laboratory of pig faeces samples artificially contaminated with SD37 lenticule discs (n=5) after selective enrichment on MSRV followed by isolation on the 'best' selective plating agar.



- = border of good performance

Figure 4 Results per laboratory of pig faeces samples artificially contaminated with STM10 lenticule discs (n=5) after selective enrichment on MSRV followed by isolation on the 'best' selective plating agar.



- = border of good performance

Figure 5 Results per laboratory of pig faeces samples artificially contaminated with STM58 lenticule discs (n=5) after selective enrichment on MSRV followed by isolation on the 'best' selective plating agar.

Table 20 Specificity, sensitivity and accuracy rates of the artificially contaminated pig faeces samples (each lenticule disc added to 25 grams pig faeces) after selective enrichment on MSRV.

Lenticule discs with pig faeces		**MRVS/X** n=33
Blank (n=5)	No. of samples No. of negative samples **Specificity in%**	165 164 **99**
STM6 (n=5)	No. of samples No. of positive samples **Sensitivity in%**	165 147 **89**
STM61 (n=5)	No. of samples No. of positive samples **Sensitivity in%**	165 161 **98**
SD6 (n=5)	No. of samples No. of positive samples **Sensitivity in%**	165 145 **88**
SD37 (n=5)	No. of samples No. of positive samples **Sensitivity in%**	165 161 **98**
All lenticule discs with *Salmonella*	No. of samples No. of positive samples **Sensitivity in%**	660 615 **93**
All lenticule discs	No. of samples No. of correct samples **Accuracy in%**	825 780 **95**

X= Isolation medium (XLD or non-XLD) which gave the highest number of positives.

4.6 PCR

Five laboratories (lab codes 2, 10, 18, 19 and 26) applied a PCR method as an additional detection technique. All laboratories except one tested the samples after pre-enrichment in BPW. Laboratory 10 started the DNA extraction after selective enrichment on MSRV. Laboratory 26 used an *InvA*-PCR normally used for confirmation of bacterial cultures and not for confirmation of pre-enrichment broths. The *InvA*-based PCR method is originally described by Rahn et al. (1992). Four laboratories used a PCR method which has been validated. The laboratories 10 and 19 used the PCR routinely for testing of respectively 300 and 8 samples per year. Table 21 gives further details on the used PCR techniques.

Table 21 Details on Polymerase Chain Reaction procedures, used as own method during the interlaboratory comparison study by six participants.

Lab code	PCR method : Reference
2	Real time PCR, Malorny et al., 2004 Not validated, Non commercial
8	Real-time PCR, Malorny et al., 2004; Josefsen 2007 Not validated Non commercial
10	Real time PCR, Malorny et al., 2004 Validated* Non commercial
18	Real-time PCR, Malorny et al., 2007 Validated Commercial
19	PCR no further information, Validated* Non commercial
26	*Inva* PCR, Validated* Non commercial

*Participants indicated the PCR method to be validated. However, it is not clear whether the method has been validated in accordance with ISO 16140 (Anonymous, 2003b) and no information on certificate number has been given.

Table 22 gives the results of both the PCR and the bacteriological cultivation (BAC) methods. Laboratory 8 did not report the results from the PCR method before the deadline; therefore these results are not mentioned in the Table. Only laboratory 18 found the same results with the PCR technique as with the bacteriological culture method (MSRV). The other laboratories (2, 10, 19 and 26) found more samples negative with the PCR technique than with the bacteriological detection method. Laboratory 19 mentioned the DNA extraction, performed with a kit (Invitek Bacterial DNA) which is routinely used in their laboratory, gave problems with the pig faeces samples.

Table 22 Number of positive results found for the control samples and for the artificially contaminated pig faeces samples by using a PCR technique and the bacteriological culture technique.

Lenticule Discs	Lab 2		Lab 10		Lab 18		Lab 19		Lab 26	
	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR
Control samples without matrix (n=7)										
SD6 (n=2)	2	2	2	2	2	2	2	2	2	2
SD37(n=1)	1	1	1	1	1	1	1	1	1	1
STM10 (n=2)	2	2	2	2	2	2	2	2	2	2
Blank (n=2)	0	0	0	0	0	0	0	0	0	0
Test samples with pig faeces (n=25)										
SD6 (n=5)	5	**4**	**4**	**4**	5	5	**1**	**1**	5	**3**
SD37 (n=5)	5	5	5	5	5	5	**3**	**3**	5	5
STM10 (n=5)	5	5	5	**4**	5	5	**3**	**0**	5	**2**
STM58 (n=5)	5	5	5	**4**	5	5	**3**	**2**	5	**2**
Blank (n=5)	0	0	0	0	0	0	0	0	0	0

BAC: bacteriological culture results (selective enrichment on MSRV).
Bold numbers: unexpected results.
Grey cells: different results found with the PCR method compared to the Bacteriological culture technique (BAC).

4.7 Performance of the NRLs

4.7.1 General

Thirty-one NRLs fulfilled the criteria of good performance for the prescribed method MSRV.
Two laboratories (lab codes 19 and 30) showed to have problems with the detection of *Salmonella* in pig faeces.
Both laboratories were contacted by the EURL-*Salmonella* in April 2012 to ask for any explanations for their deviating results. Both laboratories checked their procedures and analysed possible causes for their underperformance. For laboratory 19 a possible explanation was the change of manufacturer for their media, especially in case of MSRV. Although the routine media controls were scored correctly, many samples did grow atypical and showed no spreading on MSRV. Laboratory 30 did not measure the pH of the medium, did not perform a batch control of MSRV and did not perform quality control with *Salmonella* strains. The lack of controls made it more complicate to find possible clarifications for underperformance. After some discussion the laboratory suggested as explanation that they may have mixed up the supplements of the *Campylobacter* medium (Trimethoprim) with the supplement of the MSRV (Novobiocin). To check this theory, the lab performed an additional control with own SE lenticule samples cultured on the old batch of MSRV used in the full study and cultured on a new batch of MSRV with the correct pH. The new batch of MSRV gave better results.

To check whether the actions taken have been successful, laboratory 19 and 30 participated in a follow-up study organised by the EURL-*Salmonella* in June 2012.

Laboratory 14 did not find comparable results with the prescribed method (MSRV) and their 'own' method (MKTTn). When using MKTTn they could not detect *Salmonella* spp. in 5 out of 20 samples with low and high level contaminated SE and STM lenticule discs with matrix (faeces). If the same criteria as used for MSRV were followed for the performance of the MKTTn method, these results would not have fulfilled the criteria of good performance. Laboratory 14 mentioned the problems are most likely caused by problems with the preparation of MKTTn (potency of Novobiocin solution). The results found with the prescribed method (MSRV) fulfilled the criteria of good performance and no further actions were deemed necessary.

4.7.2 Follow-up study

The set-up of the follow-up study was the same as the full interlaboratory comparison study as organised in March 2011, but with a lower number of samples. Table 23 gives an overview of the samples used in the follow-up study.

Table 23 Overview of the types and the number of lenticule discs tested by the laboratories 19 and 30 in the follow-up interlaboratory comparison study.

Lenticule discs	Control lenticule discs (n=6) no matrix added	Test samples (n=15) with 25 grams *Salmonella* negative faeces
S. Derby 6 (SD6)	2	5
S. Typhimurium 10 (STM10)	2	5
S. Typhimurium 58 (STM58)	1	3
Blank	1	2

On Monday 4 June 2012, 1 parcel with 2 plastic containers was sent to laboratories 19 and 30 containing 6 control lenticule discs (numbered C1 – C6), 15 lenticule discs (numbered B1 – B15), 400 grams of pig faeces and 1 temperature recorder.
The performance of this follow-up study started on 11 June 2012 (week 42). The laboratory had to follow the same SOP and protocol as in the study of March 2012 (see Annexes 4 and 5). The test report was different from the March study (see Annex 8). For the media used, only the differences with the March study needed to be indicated.
For the incubation times and temperatures, no differences were observed in comparison with the full study.

Laboratory 19 tested the samples in the follow-up study differently to find a possible clarification for their deviations in the full study:

- The pre-treatment of the samples was done by kneading plastic bags instead of shake bottles as done in the full study.
- In the full study the laboratory used all media from lab M. The follow-up was performed with media from different manufacturers. The differences in compositions are indicated in Annex 3.
- MSRV from three different manufacturers (Becton Dickson Company (BBL), Oxoid and lab M) was used.
- Additionally, two own methods were performed: selective enrichment in MKTTn (Oxoid) and a PCR technique (same as in the full study) were used.
- The pre-enrichment medium BPW was obtained from Oxoid.
- The isolation media (XLD and BGA) were obtained from Oxoid and lab M.

The laboratory scored all control and blank samples correctly (only lenticule discs and no matrix added) on all used media. For the samples tested with matrix only small differences between the media were observed. In one out of five STM10 samples tested with MSRV from the manufacturer BBL no *Salmonella* was detected, all other samples were tested correctly. The samples analysed with media from Oxoid were all scored correctly. One STM58 sample with isolation on BGA was tested negative, but correctly found positive on XLD. The samples analysed with media from lab M were all scored correctly. The laboratory observed differences in growth on MSRV from different manufacturers: Oxoid showed the largest spreading on the plates and lab M was more solid with the smallest diameter of growth. The composition of the MSRV from the different manufacturers did not show differences and the pH was correctly between 5.13-5.19.
After selective enrichment in MKTTn (Oxoid), all pig faeces samples contaminated with STM lenticules were scored negative. MKTTn was used as an own method and not the prescribed method in this study.
All samples were scored correctly with the PCR. In the full study they used the same PCR method but scored only 6 out of 20 samples positive.
With these results, the laboratory fulfilled the criteria of good performance (see section 3.6) for the prescribed method MSRV in this follow-up study.

Laboratory 30 used the same media from the same manufacturer as in the full study, but they took good care the correct supplement for preparing MSRV was used. Additionally they performed a quality control for the MSRV and measured the pH. They could not detect one out of two STM10 control lenticules and one out of five STM10 samples with pig faeces. The Blank, SD6 and high level STM samples with and without pig faeces were all scored correctly. With these results, the laboratory fulfilled the criteria of good performance (see section 3.6) in this follow-up study.

5 Discussion

Reference materials
In this interlaboratory comparison study two serovars were tested: *S*. Typhimurium and *S.* Derby. The detection limit of *S*. Derby in pig faeces determined with a (stressed) culture was higher when determined with *S*. Derby lenticules. This difference was most likely caused by the fact that the strains in the lenticule discs were more stressed than in cultures. Half of the samples of pig faeces samples were positive when artificially contaminated with low level lenticule discs (SD6 and STM10) against all samples positive when artificially contaminated with (stressed) cultures at similar contamination levels. However, the number of positives was still sufficient for the use in the interlaboratory comparison study.

The homogeneity tests performed by HPA and by EURL-*Salmonella* on the original data of the HPA showed good homogeneity of the batches of lenticules. The variation on counts between lenticules of all four batches (SD6, SD37, STM10 and STM58) were close to a Poisson distribution.

The verification of the mean contamination levels of the batches of lenticules performed at the EURL-*Salmonella* showed no differences with the mean contamination levels determined immediately after preparation by HPA. This indicates sufficient stability of all four batches of lenticules when stored at -20 ºC.
Furthermore, the challenge test with the *S*. Derby lenticules also showed sufficient stability of these materials when stored at 4 ºC or 22 ºC for one week. When stored at 30 ºC an obvious decrease in the contamination level was observed.
However, as the transport temperature during transport of the parcel generally does not exceed 20 ºC for long times, the stability of *S.* Derby lenticules were considered to be sufficient.
No challenge test for the STM lenticule discs was performed, as this was already done in an earlier studies (Kuijpers and Mooijman, 2011and 2012a), showing comparable results to the current SD challenge test.

To prevent the batches of lenticule discs for a decrease in the mean level during transport, the materials were packed with frozen cooling elements and transported by courier service. The information of the temperature recorders, which were included in the parcels, showed the temperature in the parcels remained below 5 ºC for most of the transport time. Therefore it can be assumed that transport would not have negatively affected the mean level of the samples. This was confirmed by the fact that the laboratories with the longest transport times (lab codes 2 and 28) still found good results.

Performance of the laboratories
According to EC regulations (EC, 2004), each NRL should have been accredited for their relevant work field before 31 December 2009 (EC, 2005). All laboratories are currently accredited with the exception of two participants (EU-MSs) who are in the process to become accredited.
The prescribed method (Annex D of ISO 6579: MSRV) was used by all laboratories. Six laboratories used additionally an ‘own’ selective enrichment medium (RVS and/or MKTTn). For four laboratories the results with MSRV and

the own selective enrichment media in combination with all used isolation media gave the same results. Laboratory 19 found a lower number of positive results with selective enrichment in MKTTn in comparison to the prescribed method MSRV.

For determining 'good performance' per laboratory, the best performing isolation medium after selective enrichment on MSRV was taken into account (being the medium with the highest number of positive isolations).
The matrix used in this study (pig faeces) contained a high and stable level of disturbing background flora. Preliminary tests at the laboratory of the EURL-*Salmonella* showed the detection of *Salmonella* in pig faeces contaminated with low level lenticule discs (SD6 and STM10) was more difficult than for matrices used in earlier studies. Furthermore, the consistence of the portions of pig faeces sent to the participants was not homogenous in terms of moisture content. Due to this combination of facts it was decided to slightly adjust the criteria of good performance for the low level artificially contaminated pig faeces: at least 40% of the samples positive, instead of at least 60% as used in earlier studies.
Thirty-one out of 33 laboratories scored 'good performance'. Two laboratories (lab codes 19 and 30) showed to have problems with the detection of *Salmonella* in pig faeces and scored an underperformance for the prescribed method MSRV. They fulfilled the criteria of good performance in a follow-up study.

Explanations for the underperformances of laboratories19 and 30 were most likely the used media. Laboratory 19 used media from different manufacturers in the follow-up study and found better results in the follow-up study. In the full study the MSRV plates did not show the typical halo growth which may have been caused by the fact that the used MSRV was 'too solid'. Furthermore, BPW of another manufacturer was used in the follow-up study, resulting in better results with the PCR technique and possibly also in better results with the bacteriological detection method.
Laboratory 30 probably used the wrong supplement for preparing MSRV in the full study. They paid extra attention to this in the follow-up study, resulting in a good performance.

The media necessary to perform the studies are not delivered by the EURL-*Salmonella*. The participants prepare or order their own media and use their own (routine) supplier, as this is also an important aspect for the performance testing. Some laboratories may have problems with stock and/or quality of media or equipment. Causes for these problems can be lack of resources, but also the fact that material can, in some laboratories, only be ordered at a few manufacturers which may not always deliver the right quality (Kuijpers and Mooijman, 2012b).

Specificity, sensitivity and accuracy rates
The calculations were performed on the results of all participants and on the results of only the EU-MSs (without the results of participants from the EFTA countries, candidate EU-MSs and third countries). No differences were found between these groups.
The accuracy rate sensitivity rates and the specificity rates of the control samples was 100%, showing the NRLs were well able to detect *Salmonella* at different levels.
The accuracy rate of the samples with pig faeces was 95%. The high level contaminated (STM58 and SD37) pig-faeces samples was high (98%). The

sensitivity rate of the low level (SD6 and STM10) contaminated pig-faeces samples was lower (88-89%), indicating the level of this type of sample (6-10 cfu) became close to the detection limit of the method for this matrix.

Pre-treatment of the samples
In this study the participants could use their routinely used procedures for the pre-treatment of the samples, and different methods were used to mix the samples in BPW, like shaking, kneading or no mixing at all. No effect of any or no pre-treatment of the samples was seen on the results, which confirms the robustness of the lenticule disc reference materials. The same was seen in earlier studies (Kuijpers and Mooijman, 2012a)

Media and incubation
Deviations in media compositions or incubation temperatures were reported but minor effects were seen on the results.
The increase in the number of positive results after 48 hours of incubation of the selective enrichment on MSRV was 4-5%. This was most clear for the samples contaminated with STM, which showed 7-8% more positive results after 48 hours of incubation.

PCR
Five laboratories used a PCR technique additional to the prescribed method and only one of them found the same results as with the bacteriological detection method. The other laboratories found more negative results with their PCR method than with the bacteriological detection method.
One laboratory mentioned the kit they used for the DNA extraction gave problems with the pig faeces samples. They did not have this problem in previous proficiency tests and neither with the control samples containing only lenticule discs. However, this laboratory scored excellent results with the same PCR technique in the follow-up study in which they participated.

Evaluation of this study
It was more difficult to detect *Salmonella* in the matrix, pig faeces, used in this study compared to former studies organised by the EURL. The detection of *Salmonella* (SD and STM) at a low level and the detection of STM in the pig faeces caused more difficulties.
The background flora present in the pig faeces was very high and stable. The high number of *Enterobacteriaceae* and aerobic bacteria indicates there was a high level of competitive bacteria present in the pig faeces. This may have caused difficulties for *Salmonella* to grow and/or may have caused problems with reading of the isolation media and with the isolation of *Salmonella*. This may be a possible clarification for the relatively higher number of negative results found with the low level (STM10 and SD6) contaminated pig faeces in this study.

6 Conclusions

- All NRLs for *Salmonella* were able to detect high and low levels of *Salmonella* in pig faeces with the prescribed method MSRV. Two of the laboratories needed a follow-up study to fulfil the criteria of good performance.

- The accuracy, specificity and sensitivity rates of the control samples (without faeces) after selective enrichment on MSRV was 100%

- The specificity rate of the pig faeces samples artificially 'contaminated' with blank lenticule discs was 99% when tested with the prescribed method (MSRV).

- The sensitivity rates of the artificially contaminated pig faeces with high level *S.* Typhimurium and *S.* Derby lenticule discs were 98% for the prescribed method MSRV.

- The sensitivity rates of the artificially contaminated pig faeces with low level contaminated *S.* Typhimurium and *S.* Derby lenticule discs was 10% lower than the rates of the high contaminated samples.

- 48 hours incubation of selective enrichment medium MSRV showed 4-5% more positive results compared to 24 hours of incubation. This was most obvious for the STM samples (7-8% more positive results).

- *Salmonella* Derby was easier to detect in the low-level contaminated pig faeces (SD6) than *Salmonella* Typhimurium (STM10).

- The accuracy rate of the artificially contaminated pig faeces samples was 93% after selective enrichment on MSRV.

- The different pre-treatment procedures as used by the participants to mix the matrix and lenticule disc in BPW did not influence the ability to detect *Salmonella* in the samples of this interlaboratory comparison study.

- Compared to former studies it was more difficult to detect *Salmonella* in the low level contaminated matrix samples (pig faeces). This was more obvious for *S*. Typhimurium than for *S*. Derby.

List of abbreviations

ASAP	AES *Salmonella* Agar Plate
BAC	Bacteriological Culture technique
BGA(mod)	Brilliant Green Agar (modified)
BPLSA	Brilliant green Phenol-red Lactose Sucrose Agar
BPW	Buffered Peptone Water
BSA	Brilliance *Salmonella* Agar
BxLH	Brilliant green, Xylose, Lysine, Sulphonamide
cfu	colony forming units
EFTA	European Free Trade Association
EU	European Union
EURL(S)	European Union Reference Laboratory (*Salmonella*)
Gal	Galactosidase
HPA	Health Protection Agency
ISO	International Standardisation Organisation
LDC	Lysine Decarboxylase
MKTTn	Mueller Kauffmann Tetrathionate novobiocin broth
MS	Member State
MSRV	Modified Semi-solid Rappaport Vassiliadis
NCTC	National Collection of Type Cultures (HPA)
NRL	National Reference Laboratory
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
RIVM	Rijksinstituut voor Volksgezondheid en het Milieu (National Institute for Public Health and the Environment)
RS	Rapid *Salmonella*
RV(S)	Rappaport Vassiliadis (Soya) broth
SD	*Salmonella* Derby
SM (ID)2	*Salmonella* Detection and Identification-2
SOP	Standard Operating Procedure
STM	*Salmonella* Typhimurium
TSI	Triple Sugar Iron agar
UA	Urea Agar
VP	Voges-Proskauer
VRBG	Violet Red Bile Glucose agar
XLD	Xylose Lysine Deoxycholate agar

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Kuijpers AFA and Mooijman KA (2012b). Detection of *Salmonella* in food, feed and veterinary samples by EU laboratories. Food Research International (2012) Vol. 45 pp. 885-890.

Malorny B, Paccassoni E, Fach P, Bunge C, Martin A and Helmuth R (2004). Diagnostic real-time PCR for detection of *Salmonella* in food. Appl. Environ. Microbiol. 70:7046-7052.

Malorny B., Mäde D, Teufel P, Berghof-Jäger C, Huber I, Anderson A and Helmuth R (2007). Multicenter validation study of two blockcycler- and one capillary-based real-time PCR methods for the detection of Salmonella in milk powder. Int. Journal Food Microbiol. 117, 211-218.

Rahn K, De Grandis SA, Clarke RC, McEwan SA, Galan JE, Ginocchio C, Curtiss R 3rd and Gyles CL (1992). Amplification of an invA gene sequence of *Salmonella* typhimurium by polymerase chain reaction as a specific method of detection of *Salmonella*. Mol. Cell. Probes 6, 271–279.

Annex 1 History of EURL-*Salmonella* interlaboratory comparison studies on the detection of *Salmonella*

Study Year Reference[1]	Number of samples	RM[2]	Actual number of cfu/RM	Matrix amount | type		Selective enrichment medium	Plating-out medium
I 1995 Voogt et al., 1996 RIVM Report 284500003	26 4	STM5 Blank	6 0	No No		RV and SC	BGA and own
II 1996 Voogt et al., 1997 RIVM Report 284500007	15 15 2 1 1	STM100 STM1000 SPan5 STM100 Blank	116 930 5 116 0	1 gram 1 gram No No No	Chicken faeces mixed with Glycerol[3]	RV, SC and own	BGA and own
III 1998 Raes et al, 1998 RIVM Report 284500011	14 14 7 14 4 2 5	STM10 STM100 STM100S E100 STM10 SPan5 Blank	11 94 94 95 11 5 0	1 gram 1 gram 1 gram* 1 gram No No No	Chicken faeces mixed with Glycerol[3]	RV and own	BGA and own
IV 1999 Raes et al, 2000 RIVM Report 284500014	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	Chicken faeces mixed with Glycerol[3]	RV or RVS, MSRV and own	BGA and own
V 2000 Raes et al, 2001 RIVM Report 284500018	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**	Chicken faeces mixed with Glycerol[3]	RV or RVS, MSRV and own	BGA and XLD

Table A1.1 History of EURL-Salmonella interlaboratory comparison studies on detection of Salmonella in veterinary samples.Table A1.1 (continued)

Study Year Reference[1]	Number of samples	RM[2]	Actual number of cfu/RM	Matrix amount	Matrix type	Selective enrichment medium	Plating-out medium
VI	5	STM10	11	10 gram	Chicken	RVS,	BGA,
2002	5	STM100	139	10 gram	faeces	MSRV,	XLD
	5	SE100	92	10 gram	mixed	MKTTn	and own
	5	SE500	389	10 gram	with	and own	
	5	Blank	0	10 gram	Glycerol[3]		
Korver et al.,	3	STM10	11	No			
2003	3	SE100	92	No			
RIVM Report	2	SPan5	5	No			
330300001	2	Blank	0	No			
	20	None	-	25 gram**			
VII	5	STM10	12	10 gram	Chicken	RVS,	BGA,
2003	5	STM100	96	10 gram	faeces	MSRV,	XLD
	5	SE100	127	10 gram	mixed	MKTTn	and own
	5	SE500	595	10 gram	with	and own	
	5	Blank	0	10 gram	Glycerol[3]		
Korver et al.,	3	STM10	12	No			
2005	3	SE100	127	No			
RIVM Report	2	SPan5	9	No			
330300004	2	Blank	0	No			
	20	None	-	10 gram**			
VIII	7	STM10	13	10 gram	Chicken	MSRV	XLD
2004	4	STM100	81	10 gram	faeces	and own	and own
	7	SE100	74	10 gram	mixed		
	4	SE500	434	10 gram	with		
	3	Blank	0	10 gram	Glycerol[3]		
	3	STM10	13	No			
Korver et al.,	2	SE100	74	No			
2005	1	SE500	434	No			
RIVM Report	2	SPan5	7	No			
330300008	2	Blank	0	No			
	20	None	-	10 gram**			
IX	5	STM10	9	10 gram	Chicken	MSRV	XLD
2005	5	STM100	86	10 gram	faeces[4]	and own	and own
	5	SE100	122	10 gram			
	5	SE500	441	10 gram			
	5	Blank	0	10 gram			
	3	STM10	9	No			
Berk et al.,	2	SE100	86	No			
2006	1	SE500	441	No			
RIVM Report	2	SPan5	7	No			
330300011	2	Blank	0	No			
	10	None	-	10 gram***			

Table A1.1 (continued)

Study Year Reference[1]	Number of samples	RM[2]	Actual number of cfu/RM	Matrix amount	type	Selective enrichment medium	Plating-out medium
X	5	STM10	9	10 gram	Pig	MSRV	XLD
2006	5	STM100	98	10 gram	faeces[4]	and own	and own
	5	SE100	74	10 gram			
	5	SE500	519	10 gram			
	5	Blank	0	10 gram			
Kuijpers et al.,	3	STM10	9	No			
2007	2	SE100	98	No			
RIVM Report	1	SE500	519	No			
330604004	2	SPan5	5	No			
	2	Blank	0	No			
XI	5	STM5	6	10 gram	Chicken	MSRV	XLD
2008	5	STM50	47	10 gram	faeces[4]	and own	and own
	5	SE10	9	10 gram			
	5	SE100	90	10 gram			
	5	Blank	0	10 gram			
Kuijpers et al.,	3	STM5	6	No			
2008	2	SE10	9	No			
RIVM Report	1	SE100	90	No			
330604011	2	SPan5	5	No			
	2	Blank	0	No			
XII	5	STM5	6	10 gram	Chicken	MSRV	XLD
2009	5	STM50	53	10 gram	faeces[4]	and own	and own
	5	SE20	18	10 gram			
	5	SE100	84	10 gram			
	5	Blank	0	10 gram			
Kuijpers et al.,	3	STM5	6	No			
2009	2	SE20	18	No			
RIVM Report	1	SE100	84	No			
330604014	2	SPan5	7	No			
	2	Blank	0	No			
XIII	5	STM5	5	10 gram	Chicken	MSRV	XLD
2010	5	STM50	56	10 gram	faeces[4]	and own	and own
	5	SE20	13	10 gram			
	5	SE100	78	10 gram			
	5	Blank	0	10 gram			
Kuijpers et al.,	4	SE20	22	10 gram*			
2010	2	STM5	8	No			
RIVM Report	2	SE20	13	No			
330604018	1	SE100	78	No			
	1	Blank	0	No			

Table A1.1 (continued)

Study Year Reference[1]	Number of samples	RM[2]	Actual number of cfu/RM	Matrix amount	type	Selective enrichment medium	Plating-out medium
XIV	5	STM6	6	25 gram	Chicken	MSRV	XLD
2011	5	STM61	61	25 gram	faeces[4]	and own	and own
	5	SE6	6	25 gram			
	5	SE57	57	25 gram			
Kuijpers and	5	Blank	0	25 gram			
Mooijman 2011	2	STM6	6	No			
RIVM Report	2	SE6	6	No			
330604023	1	SE57	57	No			
	2	Blank	0	No			
XV	5	STM10	6	25 gram	Pig	MSRV	XLD
2012	5	STM58	58	25 gram	faeces[4]	and own	and own
	5	SD6	6	25 gram			
	5	SD37	37	25 gram			
	5	Blank	0	25 gram			
This report	2	STM10	10	No			
	2	SD6	6	No			
	1	SD37	37	No			
	2	Blank	0	No			

[1] The report of each study can be obtained through the corresponding author of this report or can be found at the EURL-*Salmonella* website: http://www.eurlsalmonella.eu/Publications.

[2] In the studies organised from 1995 to 2010 the RMs existed of gelatine capsules containing artificially contaminated milk powder. In the studies organised from 2011 the RMs existed of lenticule discs (HPA, UK).

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

[4] Faeces not mixed with any preservation medium.

* =With antibiotics

** =Naturally contaminated chicken faeces with *Salmonella*

*** =Naturally contaminated dust with *Salmonella*

Table A1.2 EURL-Salmonella interlaboratory comparison study on the detection of Salmonella in food samples.

Study Year Reference[1]	Number of samples	RM[2]	Actual number of cfu/RM	Matrix amount	Matrix type	Selective enrichment Medium	Plating-out medium
I	5	STM10	9	10 gram	Minced beef	RVS,	XLD
2006	5	STM100	98	10 gram		MKTTn,	and own
	5	SE100	74	10 gram		MSRV	
	5	SE500	519	10 gram		and own	
	5	Blank	0	10 gram			
Kuijpers et al.,	3	STM10	9	No			
2007	2	SE100	98	No			
RIVM Report	1	SE500	519	No			
330604003	2	SPan5	5	No			
	2	Blank	0	No			
II	5	STM5	4	10 gram	Minced beef	RVS,	XLD
2007	5	STM50	40	10 gram		MKTTn,	and own
	5	SE10	7	10 gram		MSRV	
	5	SE100	71	10 gram		and own	
	5	Blank	0	10 gram			
Kuijpers et al.,	3	STM5	4	No			
2008	2	SE10	7	No			
RIVM Report	1	SE100	71	No			
330604010	2	SPan5	7	No			
	2	Blank	0	No			
III	5	STM5	6	10 gram	Minced	RVS,	XLD
2009	5	STM50	54	10 gram	chicken meat	MKTTn,	and own
	5	SE20	12	10 gram		MSRV	
	5	SE100	50	10 gram		and own	
	5	Blank	0	10 gram			
Kuijpers et al.,	3	STM5	6	No			
2010	2	SE20	12	No			
RIVM Report	1	SE100	50	No			
330604017	2	SPan5	6	No			
	2	Blank	0	No			
IV	8	STM5	6	25 gram	Minced	RVS,	XLD
2010	8	STM50	55	25 gram	pork/beef	MKTTn,	and own
	8	Blank	0	25 gram	meat	MSRV	
Kuijpers et al.,	3	STM5	6	No		and own	
2011	1	STM50	55	No			
RIVM Report	1	Blank	0	No			
330604020							

Table A1.2 (continued)

Study Year Reference[1]	Number of samples	RM[2]	Actual number of cfu/RM	Matrix amount	Matrix type	Selective enrichment Medium	Plating-out medium
V	5	STM6	6	25 gram	Minced	RVS,	XLD
2011	5	STM61	61	25 gram	pork/beef	MKTTn,	and own
	5	SE8	8	25 gram	meat	MSRV	
	5	SE51	51	25 gram		and own	
Kuijpers et al.,	5	Blank	0	25 gram			
2012	2	STM6	6	No			
RIVM Report	2	SE8	8	No			
330604025	1	SE51	51	No			
	2	Blank	0	No			

[1] The report of each study can be obtained through the corresponding author of this report or can be found at the EURL-*Salmonella* website: http://www.eurlsalmonella.eu/Publications.

[2] In the studies organised from 1995 to 2010 the RMs existed of gelatine capsules containing artificially contaminated milk powder. In the studies organised from 2011 the RMs existed of lenticule discs (HPA, UK).

Table A1.3 EURL-Salmonella interlaboratory comparison study on the detection of Salmonella in animal feed samples.

Study Year Reference[1]	Number of samples	RM[2]	Actual number of cfu/capsule	Matrix amount	Matrix type	Selective enrichment medium	Plating-out medium
I	5	STM5	5	25 gram	Chicken feed (mixed grains)	RVS, MKTTn, MSRV and own	XLD and own
2008	5	STM50	43	25 gram			
	5	SE20	15	25 gram			
	5	SE100	48	25 gram			
	5	Blank	0	25 gram			
Kuijpers et al.,	3	STM5	5	No			
2009	2	SE20	15	No			
RIVM Report	1	SE100	48	No			
330604012	2	SPan5	5	No			
	2	Blank	0	No			

[1] The report of each study can be obtained through the corresponding author of this report or can be found at the EURL-*Salmonella* website: http://www.eurlsalmonella.eu/Publications.

[2] In the studies organised from 1995 to 2010 the RMs existed of gelatine capsules containing artificially contaminated milk powder. In the studies organised from 2011 the RMs existed of lenticule discs (HPA, UK).

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 1. For the variation between capsules of one batch, the Poisson distribution is the theoretical smallest possible variation which could be achieved. However, over-dispersion 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 $T_2/(I-1) \leq 2$.

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

Annex 3 Composition of the media used

MKTTn (Oxoid CM 1048 Hampshire, United Kingdom) (Biokar BK 169 HA, Beauvais, France)
Composition of MKTTn: according ISO 6579, 2002

MKTTn (LAB M, LAB 202, Bury, United Kingdom)
Composition of MKTTn: the concentration of the compounds in g/L water: Meat extract 4.3, Enzymatic digest of casein 8.6, Sodium chloride 2.6, Calcium carbonate 38.7, Sodium thiosulfate pentahydrate 30.45, Ox bile for bacteriological use 4.78, Brilliant green 0.0096, Iodine 4.0, Potassium iodide 5.0, Novobiocin 0.04

MKTTn (Merck, Muller-Kauffmann Tetrathionate Novobiocin enrichment broth 1.05878.0500, Darmstadt, Germany)
Composition of MKTTn: the concentration of the compounds in g/L water: Meat extract 4.3, Enzymatic digest of casein 8.6, Sodium chloride 2.6, Calcium carbonate 38.7, Sodium thiosulfate pentahydrate 30.5, Ox bile 4.78, Brilliant green 0.0096, Iodine 4.0, Novobiocin sodium salt 0.04

RVS (Oxoid CM 0866, Hampshire, United Kingdom) (Scharlau Chemie SA 02-379, Barcelona, Spain)
Composition of RVS: according ISO 6579, 2002

RVS (Merck Salmonella enrichment broth acc. To RAPPAPORT and VASSILIADIS/ RVS broth, 107700.0500, Darmstadt, Germany)
Composition of RVS: the concentration of the compounds in g/L water: Peptone from soy meal 4.5, Magnesium chloride hexahydrate 28.6, NaCl 7.2, di-Potassium hydrogen phosphate 0.18, Potassium dihydrogen phosphate 1.26, Malachite green oxalate 0.036.

AES, Brilliant Green Agar/VBRP EDEL (AEB 521500, Combourg, France)
Composition of AES: the concentration of the compounds in g/L water: Peptone 10, Beef extract 5, Lactose 10, Sucrose 10, Yeast extract 3, Disodium hydrogen phosphate 1, Sodium dihydrogen phosphate 0.6, Phenol red 0.09, Brilliant green 0.0047, Agar 13.

ASAP (AEB 520090, Combourg, France)
Composition of ASAP medium: the concentration of the compounds in g/L water: Peptone 10, Opaque agent 10, Chromogen mixture and inhibitor 13, agar 15, pH 7.2
Vanessa S, Mallinson ET, Bülte M 2008 A comparison of standard cultural methods for the detection of foodborne *Salmonella* species including three new chromogenic plating media. Int J Food Micr 123 (2008) 61–66.

BGA modified (Oxoid CM 0329/PO5033A, Hampshire, United Kingdom) (BPLS, Merck 1.10747, Darmstadt, Germany) (Lab M, lab 34 Bury, United Kingdom) (BGPA, Biolife 4012562, Milan, Italy) (Hy Laboratories Ltd. DD074, Rehovot, Israel) (AES CHEMUNEX, AEB. 521500, Cranbury, USA)
Watson and Walker 1978 A modification of brilliant green agar for improved isolation of *Salmonella*. J. Appl.Bact. 45 195-204
Composition of BGA modified: Edel and Kampelmacher; according ISO 6579, 1993

BGA (Oxoid CM 0263, Hampshire, United Kingdom)
Composition of BGA medium: the concentration of the compounds in g/L water: Proteose peptone 10, Yeast extract 3, Lactose 10, Sucrose 10, Sodium chloride 5, Phenol red 0.08, Brilliant green 0.0125, Agar 12, pH 6.8-7.0

BGA^{S} with Sulfadiazine (Conda laboratories 136600, Madrid, Spain)
Composition of BGA medium: the concentration of the compounds in g/L water: Yeast extract 3, Tryptone 5, Peptic digest of animal tissue 5, Lactose 10, Saccharose 10, Sodium chloride 5, Phenol red 0.08, Sulfadiazine 0.08, Agar 20, pH 7.4

BPLSA (Merck 107237.0500, Darmstadt, Germany)
Adam D., Zusatz von Natriumdesoxycholat zum Brilliantgrün-Phenolrot-Agar nach Kristensen-Kauffmann zur Hemmung des Schwärmvermögens von Proteuskeimen, 1966 Ärztl. Lab. 12, 245.
Composition of BPLSA medium: the concentration of the compounds in g/L water: Peptone from meat 5, Peptone from casein 5, Meat extract 5, Sodium chloride 3, di-sodium hydrogen phosphate 2, Lactose 10, Sucrose 10, Phenol red 0.08, brilliant green 0.0125, Agar agar 12, pH 7

BSA Brilliance Salmonella Agar (previous OSCM) (Oxoid CM 1092/ PO5098A, Hampshire, United Kingdom)
Schönenbrücher V, Mallinson ET, Bülte M. A comparison of standard cultural methods for the detection of foodborne *Salmonella* species including three new chromogenic plating media. Int J Food Microbiol. 2008 Mar 31;123(1-2):61-66.
Composition of BSA agar: the concentration of the compounds in g/L water: *Salmonella* Growth mix 14, Chromogen mix 25, Agar 15, Cefsulodin 0.012, Novobiocin 0.05, pH 7.2

BxLH
Composition of BxLH : not mentioned
Home made 12 ingredients, the medium is patented, pH 7.2

Onöz (Merck 115034, Darmstadt, Germany)
Onöz E, Hoffmann K. 1978 [Experience with a new culture medium for S*almonella* diagnosis (author's transl)] Zentralbl Bakteriol [Orig A]. 1978 Jan;240(1):16-21. German.
Composition of Onöz medium: the concentration of the compounds in g/L water: Yeast 3, Meat extract 6, Pepton from meat 6.8, Lactose 11.5, Sucrose 13, Bile salt mixture 3.825, Tri-Sodium nitrate 5,5-Hydrate 9.3, Sodium Thiosulfate 5-Hydrate 4.25, L-Phenylalanine 5, Iron(III) Citrate 0.5, Magnesiumsulfate 0.4, Brilliant Green 0.00166, Neutral Red 0.002, Aniline Blue 0.25, Metachrome Yellow 0.47, di-Sodium Hydrogen Phosphate2-Hydrate 1, Agar-Agar 15, pH 7.1-7.2

Rambach (Merck107500.003/1 107500.0002/07500.0001, Darmstadt, Germany)
Rambach, A.: New Plate Medium far Facilitated Differentiation of *Salmonella* spp. from Proteus sac. and Other Enteric Bacteria, Appl. Environm. Microbiol., 1990, 56; 301-303.
Composition of Rambach medium: the concentration of the compounds in g/L water: Peptone 8, NaCl 5, Sodium deoxycholate 1.0, Chromogenic mix 1.5, Propylene glycol 10.5, Agar-agar 15, Rambach agar supplement 10 ml, pH 7.1-7.3

RS (Biorad 356-3961/ 356-4705, Marnes-La-Coquette, France)
Lauer W and Martinez F. 2009. RAPID'*Salmonella*TM Chromogenic Medium. Journal of AOAC Int. Vol. 92, No 6: 1871-1875
Composition of Rapid *Salmonella* agar: the concentration of the compounds in g/L water: Casein Peptone 5, Meat extract 5, Selective agents 14, Chromogenic mixture 0.31, Agar 12, pH 7.2

SM(ID)2 = Chrom ID (bioMérieux SM2 43621/43629, Marcy l' Etoile, France)
Pignato, S., G. Giammanco, and G. Giammanco. 1995 Rambach agar and SM-ID medium sensitivity for presumptive identification of *Salmonella* subspecies I to VI. J. Med. Microbiol., Vol 43, Issue 1, 68-71
Composition of SM ID2 medium: the concentration of the compounds in g/L water: Peptones (swine and bovine) 6.25, Tris 0.16, Lactose 6, Ox bile (bovine and swine) 1.5, Chromogenic mix 9.63, Sodium chloride 5, Selective mix 0.03, Agar 14 pH 6.7- 7.3

Annex 4 Protocol

INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN PIG FAECES organised by EURL-*Salmonella* STUDY XV - 2012

Introduction

This protocol describes the procedures for the 15th interlaboratory comparison study on the detection of *Salmonella* spp. in animal faeces amongst the National Reference Laboratories (NRLs) for *Salmonella* in the EU. In this study the number of samples to be tested and the type of reference material (lenticule discs) will be comparable to the last study on the detection of *Salmonella* spp. in veterinary samples. The prescribed method is, like in earlier studies, the procedure as described in Annex D of ISO 6579 (EN-ISO 6579:2002/Amd1: 2007: Amendment 1: Annex D: Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage). Furthermore, laboratories who are interested can also perform PCR on the samples and/or use additional methods (routinely) used in their laboratories.

The samples will consist of pig faeces samples (*Salmonella*-negative) artificially contaminated with reference materials. The reference materials (RMs) consist of lenticule discs containing different *Salmonella* strains at different contamination levels. Each laboratory will examine 25 faeces samples (25 g each) in combination with a *Salmonella* lenticule disc and 7 control samples (lenticule discs only).

The samples will be packed in 2 plastic containers in one large box together with cooling elements. One container will contain the lenticule discs; the other container will contain the pig faeces. The container with the lenticule discs will also contain a temperature recorder to measure the temperature during transport of the samples. The recorder will be packed in a plastic bag, which will also contain your lab code. **You are urgently requested to return this complete plastic bag with recorder and lab code to the EURL-*Salmonella*, immediately after receipt of the parcel**. For this purpose a return envelope with a preprinted address label of the EURL-*Salmonella* will be included. Do not forget to note your lab code before returning it to the EURL.
Each box will be sent as biological substance category B (UN3373) by door-to-door courier service. Please contact EURL-*Salmonella* when the parcel has not arrived at your laboratory at 16th of February 2012 (this is 4 working days after the day of mailing).

Objective

The main objective of the interlaboratory comparison study is to evaluate the performance of the NRLs for *Salmonella* for their ability to detect *Salmonella* spp. at different contamination levels in faeces.

Outline of the study

Each participant will receive (in week 7 of 2012) one box containing 2 plastic containers, packed with cooling elements. The containers contain:

Container 1:
One plastic bag with 32 numbered vials each containing one lenticule disc with or without *Salmonella*
-25 vials numbered B1-B25;
-7 vials numbered C1-C7.

This container will also contain the small electronic temperature recorder in a plastic bag with your lab code. **This recorder (in the plastic bag) should be returned to the EURL-*Salmonella* as soon as possible.**
Store container 1 at (-20 ± 5) ºC immediately after receipt.

Container 2:
One plastic bag with 750 g of pig faeces (free from *Salmonella*).
Store container 2 at (5 ± 3) ºC immediately after receipt.

The performance of the study will be in week 8 (starting on 20 February 2012).

The documents necessary for performing the study are:

- Protocol Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in animal faeces XV (2012) (this document);
- SOP Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in animal faeces XV (2012);
- Test report Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in animal faeces XV (2012);
- ISO 6579 (2002). Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.;
- ISO 6579:2002/Amd 1: 2007 Amendment 1 Annex D: Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage.

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

All data have to be reported in the test report and sent to the EURL-*Salmonella* before **9 March 2012.** The EURL will prepare a summary report soon after the study to inform all NRLs on the overall results.
Results which will be received after the deadline can not be used in the analyses for the interim summary report.

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

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Timetable of interlaboratory comparison study
ANIMAL FAECES XV (2012)

Week	Date	Topic
5	2 – 3 February	Mailing of the protocol, standard operating procedure and test report to the NRLs-*Salmonella*
7	13 – 17 February	Mailing of the parcels to the NRLs as biological substance category B (UN3373) 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 container (leave it in the plastic bag with lab code) and return it to EURL-*Salmonella* using the return envelope; **- Store the lenticules at - 20°C ± 5 °C** - **Store the faeces at + 5°C ± 3 °C** **If you did not receive the parcel at 16 February, do contact the EURL immediately.** **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)
8	20 – 24 February	Performance of the study, following the instructions as given in the protocol and the SOP of study Animal faeces XV (2012).
10	Before 9 March	Completion of the test report. Send the test report, preferably by e-mail to the EURL-*Salmonella* (Angelina.Kuijpers@rivm.nl)*
	March	Data input at EURL-*Salmonella* and sending these data to NRLs. Checking these results by the National Reference Laboratories.
	April - May 2012	Sending of the final results to the NRLs together with an interim summary. As a follow-up, actions will be undertaken in case of poor performance.

* If the test report is e-mailed to the EURL, it is not necessary to send the original test report as well, unless it is not legible (to be indicated by EURL-*Salmonella*).

Annex 5 Standard Operating Procedure (SOP)

INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN PIG FAECES organised by EURL-*Salmonella*
STUDY XV - 2012

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 in faeces. For this purpose Reference Materials (RMs) containing *Salmonella* spp. as prepared by the Health Protection Agency (HPA, United Kingdom) are used. As matrix, pig faeces negative for *Salmonella* 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.

ISO 6579:2002/Amd 1 2007. Amendment 1 Annex D: Detection of *Salmonella* spp. in animal faces and in environmental samples from the primary production stage.

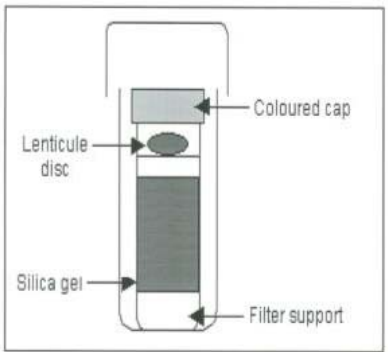
Lenticule Disc Handling Information. HPA Culture Collection, Salisbury, United Kingdom.
More information on the reference materials (lenticule discs) as produced by the HPA can be found on:
http://www.hpacultures.org.uk/products/lenticulediscs/index.jsp

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.
- *Reference Material*: a lenticule disc containing microorganism at a defined number in a water soluble matrix.

Note: Each lenticule is individually packed in small vials as indicated in the figure below.



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
XLD Xylose Lysine Deoxycholate agar

6 Culture media

For this study the prescribed method is the procedure as described in Annex D of ISO 6579, for which the following media are needed.

Non selective pre-enrichment medium	BPW
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 are described in Annex B and in Annex D of the ISO 6579: 2002. 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. Check the quality of the media before use.

In addition to the prescribed method (Annex D of ISO 6579) it is possible to use other methods, e.g. the one(s) routinely used in your laboratory ['Own' method(s)]. This can vary from another culture method to 'a PCR technique'. If necessary prepare media for the 'own' method(s) according to the relevant instructions. Record all relevant information in the test report.

6.1 Non selective pre-enrichment medium

- buffered Peptone water (BPW) (ISO6579Annex B.1).

6.2 Selective enrichment medium

- Modified Semi solid Rappaport Vassiliadis (MSRV) (ISO6579 Annex D) ;
- Own selective enrichment medium routinely used in your laboratory (optional)

6.3 Solid selective media for first and second isolation

- Xylose-Lysine-Desoxycholate (XLD) agar (90 mm plates) (ISO6579 Annex B.4)
- Second isolation medium of choice (obligatory)
- Own medium used in your laboratory (optional)

6.4 Confirmation media

- Biochemical confirmation as described in ISO 6579 Annex B.6-B.11 or by reliable, commercially available identification kits.
- Nutrient agar (optional) (ISO6579Annex 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);
- Water bath or incubator, capable of operating at 37 °C ± 1 °C;
- Water bath or incubator, capable of operating at 41.5 °C ± 1 °C;
- Sterile loops of 1 μl;
- pH-meter; having an accuracy of calibration of ± 0.1 pH unit at 25 °C.

7.2 Glassware

- Culture containers (bottles, jars or plastic bags) with nominal capacity of approximately 400 ml
- Culture tubes with approximate sizes: 8 mm in diameter and 160 mm in length
- Micro-pipettes; nominal capacity 0.1 ml
- Petri dishes; standard size (diameter 90 mm to 100 mm)

8 Procedure

Below the prescribed method of the fifteenth interlaboratory comparison study in pig faeces of EURL-*Salmonella* is described. The different steps in the procedure are also summarized in Annex A of this SOP. In addition to this method it is also allowed to use one or more own methods. Please record all relevant data in the test report. Details of the method can be found in ISO 6579 and Annex D of ISO 6579. For testing the samples use as much as possible the materials you are normally using for your routine samples. For example, either use bags or jars for the pre-enrichment in BPW depending on what you routinely use. Bottles bags or jars for the pre-enrichment in BPW are further mentioned as containers.

8.1 Pre-enrichment (day 1)

Use BPW equilibrated to at least room temperature (follow your routine procedure).
Record in the test report (pages 2 and 3) the requested data on BPW.

Take the numbered vials with the *Salmonella* lenticules out of the freezer, 10-15 minutes before they are added to the BPW to allow them to equilibrate to room temperature.

- Label 34 containers as follow:
  - 25 containers from B1 to B25
  - 9 containers from C1 to C9 (control lenticules)

- Add 25 g of faeces to each container labelled B1–B25 and C9.
- Add 225 ml BPW to each container (B1-B25 and C1-C9).

When your containers are already prefilled with 225 ml BPW, add 25 g of faeces to the BPW.

Add no faeces to the containers labelled C1 – C8.

One container is a procedure control to which no lenticule or faeces is added (= C8).
One container is the negative faeces control to which only 25 g faeces is added (= C9).
These control containers should be handled in the same way as the other containers.

- Add to the 32 labelled containers (containing BPW with or without faeces) a lenticule disc from the vial with the corresponding label number
  (B1- B25 and C1 – C7).

No lenticules are added to C8 and C9.

- Leave all the containers for 10 – 15 minutes at room temperature to re-hydrate the lenticule. Before proceeding, ensure that the disc is completely dissolved. As the disc is coloured, it may be visible when it is re-hydrated. Even when it is not visible whether the lenticule is re-

hydrated, proceed with the next steps of the procedure after 15 minutes standing at room temperature.

- Gently mix the samples: shake carefully when your samples are in a jar or knead shortly when the samples are in a plastic bag. (The use of a pulsifier or stomacher is not advisable as the pig faeces may contain sharp particles).

- Incubate all samples at 37 °C ± 1 °C for 18 h ± 2 h. Record the temperature and time at the start and at the end of the incubation period and other requested data on page 3 of the test report.

If PCR is performed, record all requested data on pages 16-17 and 22 of the test report.

8.2 Selective enrichment (day 2)

Allow the MSRV plates to equilibrate to room temperature, if they were stored at a lower temperature. Dry the surface of the MSRV plates in a Laminair Air Flow cabinet if necessary. Record (pages 4-7) the requested data of the MSRV and own selective enrichment media (if used) in the test report.

- Label 34 MSRV plates as follow:
  - 25 plates from B1 to B25
  - 9 plates from C1 to C9 (control)

If other selective enrichment media are used, label them in the same way as described for MSRV.

After equilibration of the media:
Prescribed method:

- Inoculate the MSRV plates with three drops of BPW culture, with a total volume of 0.1 ml. Incubate **(not upside down)** at 41.5 °C ± 1 °C for 24 h ± 3 h and if negative for another 24 h ± 3 h;

Optional method:

- Inoculate the routinely used selective medium/media (other than those **mentioned above),** with the corresponding BPW culture (note the inoculation volume of BPW used and the volume of the selective medium/media on the test report). Incubate at the temperature and for the time routinely used.

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

8.3 Isolation media (first and second isolation) (day 3 and 4)

Record in the test report (pages 8-13) the requested data of the isolation media used. Label 38 (standard size) Petri dishes of each isolation medium from B1 to B25 and C1 to C9.

First isolation after 24 h

Inoculation:
Inoculate from suspect MSRV plates, the surface of an isolation medium in one standard size Petri dish with the corresponding label number in such a way that well isolated colonies will be obtained. 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 °C (record temperature and time and other requested data in the test report, pages 8 and 9).

2) Second isolation medium. Follow the instructions of the manufacturer (record temperature and time and other requested data in the test report, pages 10 and 11).

3) Optional: selective isolation medium/media routinely used in your laboratory. Incubate the medium/media at the temperature and for the time routinely used (record temperature and time and other requested data in the test report, pages 12 and 13).

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 48 h ± 3 h of the MSRV and, if relevant of own selective enrichment media, repeat the procedure described above (**First isolation after 24 h**). Repeat the full procedure only when the first isolation after 24 h of MSRV and, if relevant of the own selective enrichment media, is negative.

8.4 Confirmation of colonies from first and second isolation (day 4 and day 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 plates at 5 °C ± 3 °C.
Optionally, before confirmation (see below) streak the typical colonies onto the surface of nutrient agar plates with the corresponding label numbers, in a manner which allows to develop well isolated colonies. Record on the test report (page 14) the requested data of the nutrient agar. Incubate the inoculated plates at 37 °C ± 1 °C for 24 h ± 3 h.
If the selected colony is not confirmed as *Salmonella*, test a further 4 typical colonies from the original isolation medium (stored at 5 °C). Report the number of colonies tested (in the column named 'col') and the number of colonies confirmed as *Salmonella* (in the column 'sal') for each dish in Table 1 (isolation using MSRV) and Table 2 (isolation using own enrichment) on the test report (pages 18-21).
If a PCR method has been used, report the results in Table 3 of the test report (page 22).

Confirmation of identity
The identity from the colony selected above (either directly from the isolation medium or from nutrient agar) is confirmed by means of appropriate biochemical and serological tests. Follow the instructions of ISO 6579. Note in the test report (page 15) which media/tests have been used for confirmation. The interpretation of the biochemical tests is given in Table 1 of ISO 6579:2002 on page 9. Optionally inoculate other media which are routinely used for confirmation. Record in the test report (page 15) the requested data.

Conserve one positive isolate (*Salmonella* strain) from each sample.
After the interlaboratory comparison study it may be necessary to perform some additional testing (in case of deviating results). Therefore it is requested to conserve one *Salmonella* confirmed colony from one of the used isolation media of each of the used selective enrichment medium from the samples B1-B25 and C1-C9.

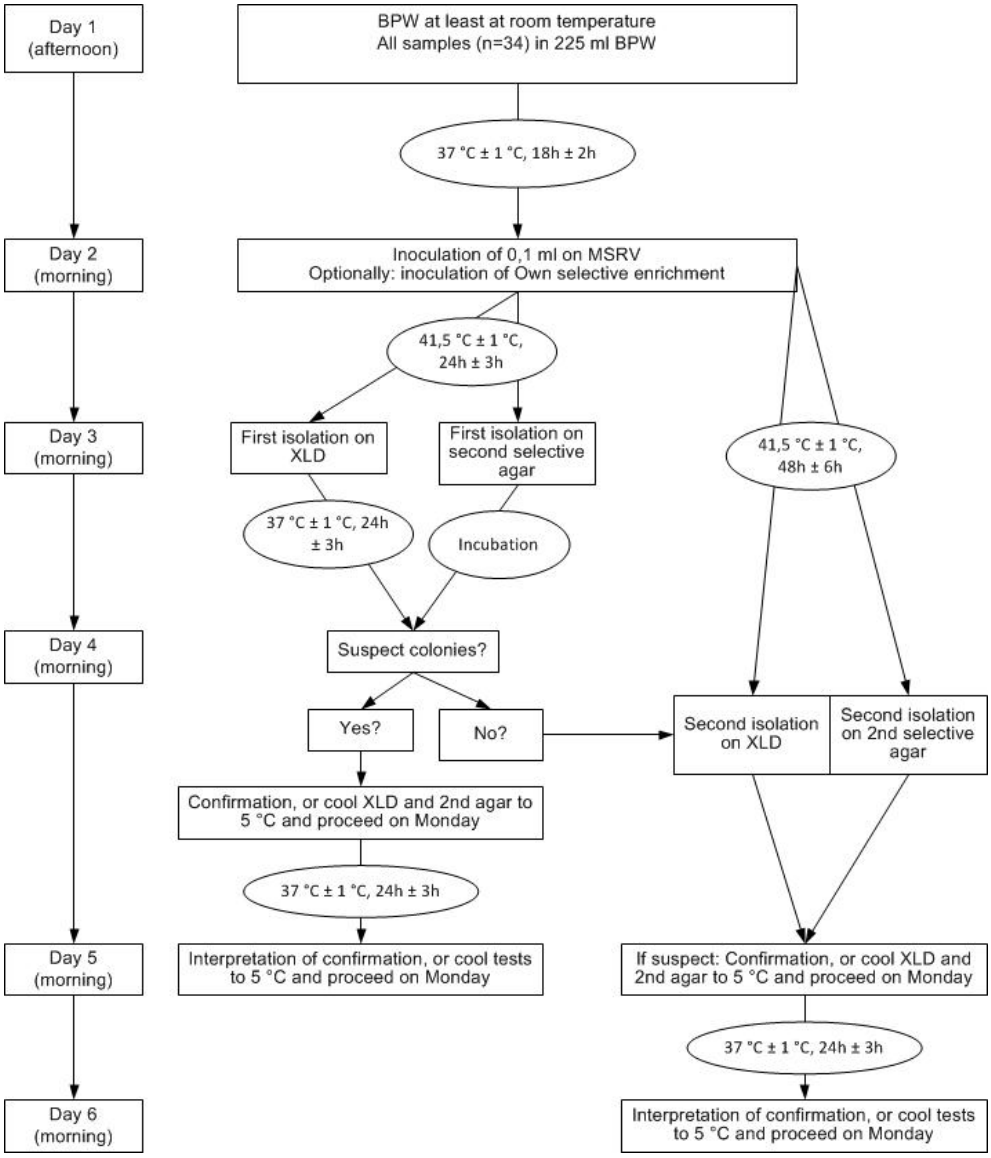
9 Test report
The test report should contain all information that might influence the results and is not mentioned in this SOP. Incidents or deviations from the specified procedures should also be recorded. The test report should include the name of the person in charge of the NRL and the names of the persons who are carrying

out the work. If the study was carried out by another laboratory than the NRL, please also give the details of this laboratory in the test report.

Overview of Interlaboratory Comparison Study ANIMAL FAECES XV (2012) on the detection of *Salmonella* spp. in pig faeces		
Day	Topic	Description
1	**Pre-enrichment**	Allow the BPW to equilibrate to at least room temperature Add 25 g faeces to container (jar or plastic bag) Add 225 ml BPW to faeces (or add 25 faeces directly to 225 ml BPW) Add 1 lenticule disc to BPW Leave 10- 15 minutes at room temperature Mix or shake gently Incubate (18 h ± 2) h at (37 °C ± 1) °C
2	**Selective enrichment**	0.1 ml BPW culture on MSRV plate, incubate at (41.5 ± 1) °C for (24 ± 3) h Own selective enrichment medium/ media
3	**First isolation after 24 h**	Inoculate from suspect MSRV (24h) plates and from Own selective medium/ media – XLD agar, incubate at (37 ± 1) °C for (24 ± 3) h – Second isolation medium* (obligatory) – Own selective medi(um)(a)* (optional) *=Incubate for specified time at the specified temperature
3	**Continue selective enrichment**	Incubate MSRV medium and if necessary Own medium/ media another 24 (± 3) hours at the relevant temperatures
4	**Second isolation after 48 h**	If the first isolation was negative, inoculate from suspect MSRV (48h) plates and if relevant from Own medium/ media – XLD agar, incubate at (37 ± 1) °C for (24 ± 3) h – Second isolation medium* – Own selective medium/ media* *=Incubate for specified time at the specified temperature
4	**Confirmation of identity**	Confirm the identity of the *Salmonella* suspect colonies from the first isolation media (day 3).
5	**Confirmation of identity**	Confirm the identity of the *Salmonella* suspect colonies from the second isolation media (day 4).

Annex A of SOP

Interlaboratory comparison study animal faeces XV
on the detection of *Salmonella* spp.
EURL-*Salmonella* 2012



Annex 6 Results found with 'own methods'

Table A6.1 Results control samples, analysed with an 'own method'.

Highest number of positive isolations found with the given selective enrichment medium in combination with any isolation medium								
Lab code	**Other than MSRV 'own method'**				**MSRV**			
	Blank n=2	**SD6 n=2**	**SD37 n=1**	**STM10 n=2**	**Blank n=2**	**SD6 n=2**	**SD37 n=1**	**STM10 n=2**
Good Performance	**0**	**≥ 1**	**1**	**≥ 1**	**0**	**≥ 1**	**1**	**≥ 1**
	MKTTn				**MSRV**			
7	0	2	1	2	0	2	1	2
19	0	2	1	2	0	2	1	2
23	0	2	1	2	0	2	1	2
	RVS				**MSRV**			
8	0	2	1	2	0	2	1	2
13	0	2	1	2	0	2	1	2
17	0	2	1	2	0	2	1	2
23	0	2	1	2	0	2	1	2

Table A6.2 Results pig faeces samples artificially contaminated with Salmonella, analysed with an 'own method'.

Highest number of positive isolations found with the given selective enrichment medium in combination with any isolation medium										
Lab code	**Other than MSRV 'own method'**					**MSRV**				
	Blank n=5	**SD6 n=5**	**SD37 n=5**	**STM10 n=5**	**STM58 n=5**	**Blank n=5**	**SD6 n=5**	**SD37 n=5**	**STM10 n=5**	**STM58 n=5**
Good Performance	**≤ 1**	**≥ 2**	**≥ 4**	**> 2**	**≥ 4**	**≤ 1**	**≥ 2**	**≥ 4**	**≥ 2**	**≥ 4**
	MKTTn					**MSRV**				
7	0	5	5	**4**	5	0	**3**	5	5	5
19	0	**2**	5	**1**	**3**	0	**1**	**3**	**3**	**3**
23	0	5	5	5	5	0	5	5	5	5
	RVS					**MSRV**				
8	0	5	5	**4**	5	0	5	5	**4**	5
13	0	5	5	5	5	0	5	5	5	5
17	0	5	5	5	5	0	5	5	5	5
23	0	5	5	5	5	0	5	5	5	5

Bold numbers: Deviating results.

Grey cells: Results below the level of good performance.

Annex 7 Test report Follow-up study

TEST REPORT

INTERLABORATORY COMPARISON STUDY ON THE

DETECTION OF *SALMONELLA* spp. IN PIG FAECES

organised by EURL-*Salmonella*

STUDY XV- 2012

FOLLOW-UP STUDY June 2012

Laboratory code This is the same code as in FAECES XV 2012	
Laboratory name (NRL)	
Address	
Country	
Date of arrival of the parcels	Date: …… - ……. – 2012 time: ……... h ……..…… min
Start time of storage at - 20 °C (lenticule discs) Start time of storage at +5 °C (faeces)	Date: …… - ……. – 2012 time: ……... h ……..…… min Date: …… - ……. – 2012 time: ……... h ……..…… min
Parcels damaged?	☐ Yes ☐ No
Starting date testing	…… - ……. – 2012

PRE-ENRICHMENT – Buffered Peptone Water (BPW)

Medium information BPW	
Was the composition of BPW the same as used in BRO FAECES XV 2012 ?	
☐ Yes ☐ No please give more details in an annex :	
Preparation of BPW	
Date of preparation	…… - ……. – 2012
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control of BPW?	☐yes ☐no

Containers with BPW	
Did you use containers with pre filled BPW ?	☐ yes ☐ no
What kind of containers did you use for the pre-enrichment in BPW ?	☐ plastic bags ☐ jars ☐ bottles ☐ ……

Equilibration of the BPW	
At which temperature did you equilibrate the BPW ?	☐ at 37 °C ☐ at room temperature ☐ ……. °C
For how long did you equilibrate the BPW ?	…….. h

Mix the samples (BPW, lenticules, faeces)	
How did you mix the samples ?	☐ shake ☐ knead ☐ vortex ☐ pulsifier ☐ stomacher ☐ ……
☐ did not mix the samples	

Incubation time and temperature for pre-enrichment (18 ± 2) hrs after adding faeces and lenticules	
Start at	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
End at	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C

SELECTVE ENRICHMENT - Modified Semi solid Rappaport Vassiliadis medium (MSRV)

Medium information MSRV
Was the composition of MSRV the same as used in BRO FAECES XV 2012 ?
☐ Yes
☐ No, please give more details in an annex :

Preparation of MSRV	
Date of preparation	…… - …….. - 2012
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control of MSRV?	☐yes ☐no

Incubation time and temperature for selective enrichment	
Start of the first period (first 24 h)	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
End of the first period (first 24 h)	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
Start of the second period (48 h)	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
End of the second period (48 h)	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C

OWN SELECTVE ENRICHMENT - Selective medium, routinely used in your laboratory (optional)

If you use more selective media, please give relevant information in an annex.	
Medium:	

Medium information OWN
Was the composition of own media the same as used in BRO FAECES XV 2012 ?
☐ Yes
☐ No, please give more details in an annex :

Preparation of the medium	
Date of preparation	…… - ……. - 2012
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control of the medium?	☐yes ☐no

Incubation time and temperature for own selective enrichment	
Start of the first period (first 24 h)	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
End of the first period (first 24 h)	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
Start of the second period (48 h)	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
End of the second period (48 h)	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C

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

Medium information XLD
Was the composition of XLD media the same as used in BRO FAECES XV 2012 ?
☐ Yes
☐ No, please give more details in an annex :

Preparation of XLD	
Date of preparation	…… - ……. - 2012
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control of XLD ?	☐yes ☐no

Incubation time and temperature for isolation	
Start incubation of XLD, inoculated from 24 h MSRV	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
End incubation of XLD, inoculated from 24 h MSRV	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
Start incubation of XLD, inoculated from 48 h MSRV	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
End incubation of XLD, inoculated from 48 h MSRV	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C

FIRST AND SECOND ISOLATION – Second Isolation medium.

GVe information on the second isolation medium.	
Name of the medium	
Prescribed incubation temperature in °C	

Medium information second isolation medium
Was the composition of media the same as used in BRO FAECES XV 2012 ?
☐ Yes
☐ No, please give more details in an annex :

Preparation of the second isolation medium	
Date of preparation	…… - ……. - 2012
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control?	☐yes ☐no

Incubation time and temperature for isolation	
Start incubation of second medium, inoculated from 24 h MSRV	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
End incubation of second medium, inoculated from 24 h MSRV	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
Start incubation of second medium, inoculated from 48 h MSRV	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
End incubation of second medium, inoculated from 48 h MSRV	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C

FIRST AND SECOND ISOLATION – Own Isolation medium routinely used In your laboratory (optional)

If you use more selective media, please give relevant information in an annex.	
Name of the medium	
Prescribed incubation temperature in ^{o}C	

Medium information OWN second isolation medium
Was the composition of media the same as used in BRO FAECES XV 2012 ?
☐ Yes
☐ No, please give more details in an annex :

Preparation of your own medium	
Date of preparation	…… - ……. - 2012
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control?	☐yes ☐no

Incubation time and temperature for isolation	
Start incubation of own medium, inoculated from 24 h selective enrichment medium	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
End incubation of own medium, inoculated from 24 h selective enrichment medium	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
Start incubation of own medium, inoculated from 48 h selective enrichment medium	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
End incubation of own medium, inoculated from 48 h selective enrichment medium	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C

CONFIRMATION – Nutrient agar

Did you streak the colonies on Nutrient agar before starting confirmation?		
☐yes	☐no	If yes give further information on nutrient agar below

Medium information Nutrient medium
Was the composition of media the same as used in BRO FAECES XV 2012 ?
☐ Yes
☐ No, please give more details in an annex :

CONFIRMATION of *Salmonella* suspect colonies

What media/test did you use for confirmation ?		
☐	Biochemical:	☐ Triple sugar/iron agar (TSI) ☐ Urea Agar (UA) ☐ L-Lysine decarboxylation medium (LDC) ☐ Galactosidase ☐ Voges-Proskauer (VP) ☐ Indole ☐ Identification kit name of the kit : …….. ☐ Other : …….
☐	Serotyping:	☐ O antigen ☐ H antigen ☐ Vi antigen ☐ Other : ……..
☐	Other confirmation test : …….	

DETECTION BY PCR (I)	
General questions	
Did you use PCR ?	☐ Yes ☐ No
If yes and when different from PCR-technique used during FAECES XV BRO 2012, please give more information in an annex .	

*Table 1: Results of isolation using **MSRV** (dish numbers B1-B15).*

sample no.	MSRV 24 hours						MSRV 48 hours					
	XLD		Second		Own		XLD		Second		Own	
	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]
B1												
B2												
B3												
B4												
B5												
B6												
B7												
B8												
B9												
B10												
B11												
B12												
B13												
B14												
B15												

*Table 1 (continued): Results of isolation using **MSRV** (dish numbers C1- C6, C8 and C9).*

sample no.	MSRV 24 hours						MSRV 48 hours					
	XLD		Second		Own		XLD		Second		Own	
	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]
C1												
C2												
C3												
C4												
C5												
C6												
C8												
C9												

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

*Table 2 Results of isolation using **OWN** selective enrichment medium (dish numbers B1-B15).*

	Own * 24 hours						Own * 48 hours					
sample no.			*		*				*		*	
	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]
B1												
B2												
B3												
B4												
B5												
B6												
B7												
B8												
B9												
B10												
B11												
B12												
B13												
B14												
B15												

* = Fill in the name of the medium used.

*Table 2 (continued): Results of isolation using **Own** selective enrichment medium (dish numbers C1-C6, C8 and C9).*

	Own * 24 hours						Own * 48 hours					
sample no.	XLD		*		*		XLD		*		*	
	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]
C1												
C2												
C3												
C4												
C5												
C6												
C8												
C9												

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

Table 3: Results of detection using PCR (sample numbers B1-B15 & C1-C6, C8 & C9).

PCR + or -			
Sample		samp	
B1		C1	
B2		C2	
B3		C3	
B4		C4	
B5		C5	
B6		C6	
B7			
B8		C8	
B9		C9	
B10			
B11			
B12			
B13			
B14			
B15			

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

Name of person(s) carrying out the follow-up of the fifteenth veterinary interlaboratory Comparison study (2012).	
Is the person(s) carrying out the follow-up of the fifteenth veterinary interlaboratory Comparison study (2012) working in the laboratory of the NRL mentioned on page 1?	☐YES ☐NO, give more information of the laboratory carrying out the study : Laboratory name ……. Address …… Is this laboratory accredited for the determination of *Salmonella*. ☐YES ☐NO

Date and signature	

Name of person in charge of the NRL. When not NRL (see page 1) mention also the name of the laboratory.	
Date and signature	

Please send the completed test report before 1 July 2012, by email to EURL-*Salmonella*. If the test report is e-mailed to the EURL it is not necessary to sent the original test report as well, unless it is not legible (to be indicated by EURL-*Salmonella*).
Use the address below:

Angelina Kuijpers
E-mail : Angelina.Kuijpers@rivm.nl
EURL *Salmonella* (internal Pb 63)
RVM / LZO
P.O. Box 1
3720 BA Bilthoven
The Netherlands

Tel. number: + 31 30 274 2093
Fax. number: + 31 30 274 4434
http://www.rivm.nl/crlsalmonella

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