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

EU Interlaboratory comparison study veterinary XIV (2011)

Detection of Salmonella in chicken faeces

RIVM report 330604023/2011

A.F.A. Kuijpers | K.A. Mooijman

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

EU Interlaboratory comparison study veterinary XIV (2011)

Detection of *Salmonella* in chicken faeces

RIVM Report 330604023/2011

Colophon

A.F.A. Kuijpers
K.A. Mooijman

Contact:
A.F.A. Kuijpers
Laboratory for Zoonoses and Environmental Microbiology (LZO)
Angelina.Kuijpers@rivm.nl

This investigation has been performed by order and for the account of European Commission, Health and Consumer Protection Directorate-General, within the framework of V/330604/11/CS by the European Union Reference Laboratory for *Salmonella*

Abstract

EU Interlaboratory comparison study veterinary XIV (2011)
Detection of *Salmonella* in chicken faeces

In 2011, from the 32 National Reference Laboratories (NRLs) for *Salmonella* in the European Union, 29 were able to detect both high and low levels of *Salmonella* in chicken faeces. The desired outcome was achieved on the first attempt. Of the remaining 3 laboratories, 1 scored a moderate performance. *Salmonella* was found in 98% of the samples tested by the laboratories.

These are the results of the fourteenth veterinary interlaboratory comparison study organized by the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella,* previously CRL). The study was conducted in March 2011, with a follow-up study in June 2011. All NRLs responsible for the detection of *Salmonella* in veterinary samples from all European Member States 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 moderate performance scored by one laboratory was most probably caused by a technical (electrical) failure during the performance of the proficiency test. Two NRLs scored an underperformance on the first attempt but obtained the desired outcome in a follow-up study. One of these NRLs found *Salmonella* in a blank sample (false positive); this was probably due to cross-contamination. The other laboratory had difficulty in detecting low levels of *Salmonella* in the chicken faeces. This laboratory still scored minimum results in the follow-up study. The cause of this was most likely the ready-to-use medium for detecting the presence or absence of *Salmonella* which seemed to be insufficiently sensitive. The laboratory will further investigate this.

The internationally prescribed method for demonstrating the presence of *Salmonella* in veterinary samples was used during the study. Each laboratory received a package containing chicken faeces (free from *Salmonella*) and reference materials containing no or different levels of *Salmonella*. The laboratories were instructed to spike samples of chicken faeces with reference material and then test all samples for the presence of *Salmonella*.

The laboratories themselves added the reference material to the samples of chicken faeces. This was done to ensure that the level of *Salmonella* remained stable during transport and storage. During this proficiency test, the so-called lenticule discs were used as reference material for the first time. Prior to this study, capsules were used which needed a more complicated preparation. The new procedure was so successful that it will be continued. The test samples made with this material were found to be more like the 'normal' samples that are analysed daily in the reference laboratories.

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

Rapport in het kort

EU Ringonderzoek veterinair XIV (2011)
Detectie van *Salmonella* in kippenmest

In 2011 waren 29 van de 32 Nationale Referentie Laboratoria (NRL's) in de Europese Unie in staat om hoge en lage concentraties *Salmonella* in kippenmest aan te tonen. Ze behaalden direct het gewenste niveau. Van de 3 overige behaalde 1 laboratorium een matig resultaat. In totaal hebben de laboratoria in 98 procent van de (besmette) monsters *Salmonella* gedetecteerd.

Dit blijkt uit het veertiende veterinair ringonderzoek dat het referentielaboratorium van de Europese Unie (EURL, voorheen CRL) voor *Salmonella* heeft georganiseerd. Het onderzoek is in maart 2011 gehouden, de herkansing was in juni 2011. Deelname aan het onderzoek is verplicht voor alle NRL's van de Europese lidstaten die verantwoordelijk zijn voor de opsporing van *Salmonella* in dierlijke mest. Het EURL-*Salmonella* is gevestigd bij het Nederlandse Rijksinstituut voor Volksgezondheid en Milieu (RIVM).

Het matige resultaat dat een laboratorium behaalde is waarschijnlijk veroorzaakt door een technische (elektriciteit) storing tijdens het ringonderzoek. Twee laboratoria scoorden aanvankelijk onvoldoende en behaalden het gewenste resultaat tijdens de herkansing. Een van deze NRL's had in de eerste ronde bij een blanco monster aangegeven dat het *Salmonella* bevatte (vals positief); waarschijnlijk door kruisbesmetting. Het andere laboratorium had moeite de lage concentraties *Salmonella* in kippenmest aan te tonen. Dit laboratorium behaalde minimale resultaten tijdens de herkansing, doordat het gebruikte kant-en-klaarmedium waarmee *Salmonella* al dan niet wordt aangetoond, onvoldoende gevoelig leek te zijn. Dit gaat het laboratorium verder onderzoeken.

Tijdens de onderzoeken hanteren de laboratoria de internationaal voorgeschreven methode om *Salmonella* aan te tonen in dierlijk mest. Elk laboratorium krijgt een pakket toegestuurd met kippenmest (vrij van *Salmonella*) en zogeheten referentiemateriaal, dat geen of verschillende besmettingsniveaus *Salmonella* bevat. De laboratoria dienen de kippenmest en het referentiemateriaal zelf samen te voegen en vervolgens te onderzoeken of er *Salmonella* aanwezig is. Dit om er zeker van te zijn dat de aangeleverde hoeveelheid *Salmonella* in tact blijft.

Bij dit ringonderzoek is voor het referentiemateriaal voor het eerst gebruik gemaakt van zogeheten *lenticule discs;* voorheen waren dat capsules die een ingewikkeldere voorbereiding vereisten. Deze werkwijze was dermate succesvol dat dit wordt voortgezet. De monsters die met dit materiaal worden gemaakt, lijken namelijk meer op de 'gewone' monsters die in de dagelijkse praktijk bij de laboratoria binnenkomen om te worden onderzocht.

Trefwoorden: *Salmonella*; EURL; NRL; ringonderzoek; kippenmest; *Salmonella* detectiemethode; lenticule disc

Contents

Summary

In March 2011 the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*) organised the fourteenth veterinary interlaboratory comparison study on detection of *Salmonella* in chicken faeces. Participants were 32 National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*): 28 NRLs from 27 EU Member States, 3 NRLs from member countries 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, chicken faeces samples of 25 grams each were analysed in the presence of reference materials 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 for the first time lenticule discs were used as reference materials. The change from capsules (former studies) to lenticule discs was especially made because of the easiness of handling of the lenticules. Furthermore, with lenticule discs it was better possible to use the normal routine procedures for sample treatment and therefore to mimic the daily routine analyses better.

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 chicken faeces: 5 lenticule discs contained approximately 6 colony forming units (cfu) of *Salmonella* Typhimurium (STM6), 5 lenticule discs contained approximately 61 cfu of *S*. Typhimurium (STM61), 5 lenticule discs contained approximately 6 cfu of *S.* Enteritidis (SE6), 5 lenticule discs contained approximately 57 cfu of *S.* Enteritidis (SE57) 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 STM6, 2 lenticule discs SE6, 1 lenticule disc SE57 and 2 blank lenticule discs.

On average the laboratories found *Salmonella* in 98% of the (contaminated) samples when using the prescribed veterinary method, with selective enrichment on MSRV.
48 hours of incubation of MSRV gave overall 10% more positive results. This was most obvious for the low level contaminated SE samples which gave 30% more positive results compared to 24 hours of incubation.

Twenty-nine NRLs fulfilled the criteria of good performance on the first attempt. One NRL scored a moderate performance; their sensitivity problem was most probably caused by an electricity failure during the performance of the study. Two laboratories needed a follow-up study to reach the desired level. One of them found a false positive blank sample (without matrix) most probably caused by cross-contamination. The other laboratory had difficulty in detecting low levels of *Salmonella* in the chicken faeces. This laboratory still found a minimum

score in the follow-up study. Their underperformance was most likely caused by ready-to-use MSRV which seemed to be insufficiently sensitive. The laboratory will perform further investigations on this medium.

The first EURL-*Salmonella* study organised with lenticule discs as reference material was successful.

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 2011, 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 is carried out uniformly and comparable results can be obtained by all National Reference Laboratories for *Salmonella* (NRL-*Salmonella*). The second objective was to compare the different methods for the detection of *Salmonella* in animal faeces.

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).

In this study, for the first time lenticule discs were used as reference materials. The change from capsules (former studies) to lenticule discs was especially made because of easiness of handling of the lenticule discs. Furthermore, with lenticule discs it was better possible to use the normal routine procedures for sample treatment and therefore to mimic the daily routine analyses better.

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 contamination level of the low level reference material was close to the detection limit of the method; the level of the high level samples was approximately 5-10 times above the detection limit. Seven control samples consisting of different reference materials, had to be tested without the addition of chicken faeces. These latter reference materials consisted of 2 lenticule discs with approximately 6 cfu of *Salmonella* Typhimurium (STM6), 2 lenticule discs with approximately 6 cfu of *Salmonella* Enteritidis (SE6), 1 lenticule disc with approximately 57 cfu of *Salmonella* Enteritidis (SE57) and 2 blank lenticule discs. Twenty-five samples of *Salmonella* negative chicken faeces spiked with 5 different reference materials had to be examined. For the latter samples the different reference materials consisted of 2 levels of *Salmonella* Typhimurium (STM6 and STM61), 2 levels of *Salmonella* Enteritidis (SE6 and SE57) and blank reference materials.

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	Ministry of Agriculture, Natural Resources and Environment Veterinary Services Laboratory for the Control of Foods of Animal Origin (LCFAO)
Czech Republic	Prague	State Veterinary Institute
Denmark	Esbjerg	Danish Veterinary and Food Administration Region West 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	Halkis	Veterinary Laboratory of Halkis Hellenic Republic Ministry of Rural Development and Food
Hungary	Budapest	Central Agricultural Office, Food and Feed Safety Directorate Central Food-Microbiological Diagnostic Laboratory
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	Veterinary Laboratories Agency (VLA) Weybridge Department of Food and Environmental Safety
United Kingdom	Belfast	Agri-Food and Bioscience Institute (AFBI) Veterinary Sciences Division Bacteriology

3 Materials and methods

3.1 Reference materials

3.1.1 Batches of lenticule discs

The reference material consisted of lenticule discs obtained from the Health Protection Agency (HPA) in Newcastle, UK. 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.
Five batches of lenticule discs were prepared by HPA:

- *S.* Typhimurium (STM) at a level of approximately 5 cfu per lenticule disc: batch 323-101021;
- *S.* Typhimurium (STM) at a level of approximately 50 cfu per lenticule disc: batch 523-100927;
- *S.* Enteritidis (SE) at a level of approximately 5 cfu per lenticule disc: batch 414-100514B;
- *S.* Enteritidis (SE) at a level of approximately 50 cfu per lenticule disc: batch 814-091203;
- Blank lenticule disc, containing no microorganisms: batch 000-100111.

3.1.2 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. For this the same homogeneity test was used as formerly used for the capsules. 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).

3.1.3 Test on the stability of lenticule discs and a new procedure

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*. Therefore, some additional stability tests were performed on the *Salmonella* lenticule discs at the EURL-*Salmonella* laboratory.
A limited test on the long-term stability was performed on lenticule discs containing *S.* Enteritidis (SE) and *S.* Typhimurium (STM), which were ordered in 2005 by the EURL. This concerned SE at a level of 92 cfu/lenticule disc (SE92 batch 214-050615) and STM at a level of 93 cfu/lenticule disc (STM93 batch 523-050615). The lenticule discs were stored at -20 °C for almost 5.5 years and the mean contamination level of 5 lenticule discs of each batch were compared to the mean contamination level originally indicated on the insert of the batch of lenticule discs (tested with 30 lenticule discs). The (long-term) stability of a SE at a low level of 7 cfu/lenticule disc (SE7 batch 414-100514) was tested after 5 months of storage at -20 °C.

To test the stability of the lenticule discs at elevated temperatures (as may occur during transport), a so-called challenge test was performed. For this 5 lenticule discs of SE92 and STM93 were tested at day 0, after 3 days and after 7 days of storage at 5 °C , 22 °C and at 30 °C . Additionally 5 lenticule discs of SE7 were tested.

For the counting of the lenticule discs in the different stability tests, 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.

3.1.4 Pre-tests for the interlaboratory comparison study

To check the 'robustness' of the lenticule discs, it was tested whether *Salmonella* could still be detected after mixing a *Salmonella* lenticule disc with different matrices. Lenticule discs used for the experiment were: STM10 (batch 223-050615) and SE7 (batch 414-100514). Matrices (free of *Salmonella*) tested in this experiment: minced beef, minced meat (pork/beef) and chicken faeces.
To 10 portions of each 25 grams of minced meat a lenticule disc (STM10) was added. Five samples were placed at 3 °C and 5 were placed at -20 °C for 3 days. Five additional portions of each 25 grams of minced meat (no lenticule added) were stored at 5 °C and after 3 days of storage, a lenticule disc (STM10) was added to each portion and immediately tested. To each minced meat sample (with lenticule disc) 225 ml of Buffered Pepton Water (BPW) was added and mixed for 1 minute in a stomacher.
To 10 portions of each 25 grams of chicken faeces, a lenticule disc (STM10) was added. Next 225 ml of BPW was added. Five samples were mixed in a pulsifier for 1 minute and 5 samples were not mixed.
To 10 portions of each 25 grams of minced beef, a lenticule disc (SE7) was added. Five samples were stored at 5 °C for 3 days and 5 samples were tested immediately. To all minced beef samples (stored for 3 days and fresh prepared) 225 ml of BPW was added and mixed for 1 minute in a pulsifier.
To 10 portions of each 25 grams of chicken faeces, a lenticule disc (SE7) was added. Next 225 ml of BPW was added. Five portions were mixed in a pulsifier for 1 minute and 5 portions were not mixed.

All meat samples were tested for the presence of *Salmonella* according to ISO 6579 (Anonymous, 2002) and Annex D of ISO 6579 (Anonymous, 2007) with selective enrichment in RVS, MKTTn and on MSRV. The faeces samples were tested for the presence of *Salmonella* according Annex D of ISO 6579 (Anonymous, 2007) only, with selective enrichment on MSRV.

Because of the introduction of lenticule discs, the Standard Operating Procedure (SOP) for the analysis of the samples in the interlaboratory comparison study was amended (see Annex 5). The applicability of this SOP was tested at the laboratory of the EURL by following the full protocol of the interlaboratory comparison study with the same number and type of samples (see Table 1).

3.2 Chicken faeces samples

3.2.1 General

Chicken faeces were sampled by the Animal Health Service (GD) Deventer at a *Salmonella* free farm (SPF-farm). A large batch of 50 kilograms from this farm arrived at the EURL-*Salmonella* on 11 January 2011. The faeces were stored at 5 °C and checked for the absence of *Salmonella* by testing 10 portions of 25 grams of chicken faeces randomly picked from the large batch. 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 16-18 hours, selective enrichment was carried out on Modified Semi-solid Rappaport Vassiliadis (MSRV). Next, the suspect plates were plated-out on Xylose Lysine Deoxycholate agar (XLD) and Brilliant Green Agar (BGA; Anonymous, 1993) and confirmed biochemically.

3.2.2 Total bacterial count in chicken faeces

The total number of aerobic bacteria was investigated in the chicken 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 a 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 chicken faeces

In addition to the total count 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 chicken faeces

On 14 February 2011 (2 weeks before the study) the reference materials (35 individually numbered lenticule discs) and 700 grams of *Salmonella* negative chicken 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 (Annex 5). The test report which was used during the study can be found at the EURL-*Salmonella* website: http://www.rivm.nl/crlsalmonella/prof_testing/detection_stud/ 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 chicken faeces
S. Enteritidis 6 (SE6)	2	5
S. Enteritidis 57 (SE57)	1	5
S. Typhimurium 6 (STM6)	2	5
S. Typhimurium 61 (STM61)	-	5
Blank	2	5

3.3.2 Pre-treatment of the samples

In this study for the first time lenticule discs were used. As these lenticule discs were easier to dissolve and more robust than the formerly used capsules, the NRLs could use pre-treatment procedures of the samples as they normally use in 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 chicken 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 means of the computer and all data from the start of the shipment until the

arrival at the National Reference Laboratories were transferred to an Excel graph which shows all recorded temperatures.

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 also allowed 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 chicken 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 STM6 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 performed for 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 XIV (2011)

Control samples (lenticule disc, no matrix)	Minimum result	
	Percentage positive	No. of positive samples / total no. of samples
SE57	100%	1/1
STM6 and SE6	50%	1/2
Blank control lenticule disc	0%	0/1

Samples: chicken 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]
STM61 and SE57	80%	4/5
STM6 and SE6	50%	3/5

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 Contamination level and homogeneity of the lenticule discs

Table 3 summarises the information on the contamination level of each batch of lenticule discs as tested by 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 indicated The results of the homogeneity test show that each batch fulfilled well the criteria as originally set for the capsule reference materials (T_2 / (I-1)) ≤ 2).

Table 3 Level of contamination and homogeneity of SE and STM lenticule discs

	SE6	SE57	STM6	STM61
Batch number	414-100514B	814-091203	323-101021	523-100927
Date testing lenticules*	14.10.2010	17.12.2009	5.11.2010	11.10.2010
Number of lenticules tested	30	30	30	30
Mean cfu per lenticule	6	57	6	61
Min-max cfu per lenticule	2-10	40-70	1-10	48-77
T_2 / (I-1)**	0.77	0.97	0.86	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 $T_2/(I-1) \leq 2$

4.1.2 Testing stability of lenticule discs

Table 4 summarises the results of the (limited) test on the long-term stability of the lenticule discs. All batches showed a (small) decrease in the mean of cfu after storage at -20 °C.

Table 4 Level of contamination of SE and STM lenticule discs before and after storage

	SE7	SE92	STM93
Batch number	414-100514	214-050615	523-050615
Storage in -20 °C since	27.05.2010	01.05.2005	01.05.2005
Number of lenticules tested*	30	30	30
Mean cfu per lenticule	7	92	93
Min-max cfu per lenticule	3-13	70-110	73-121
Date testing lenticules	25.10.2010	25.10.2010	25.10.2010
Number of lenticules tested**	5	5	5
Mean cfu per lenticule disc	3.4	75.2	64.8
Min-max cfu per lenticule	1-5	73-86	58-76

* Tested by HPA.
** Tested by EURL-*Salmonella.*
cfu = colony forming units.

Figure 1 shows the results of the challenge test. No effect on the mean number of cfu was seen after storage of the batches SE7 at 22 °C and STM93 at 5 °C for 1 week. The decrease in the mean number of cfu was more obvious when the batches were stored at 30 °C for 3 to 7 days. The storage of the lenticule discs elevated temperatures seem to have a larger effect to the lenticule discs containing SE than to the materials containing STM.

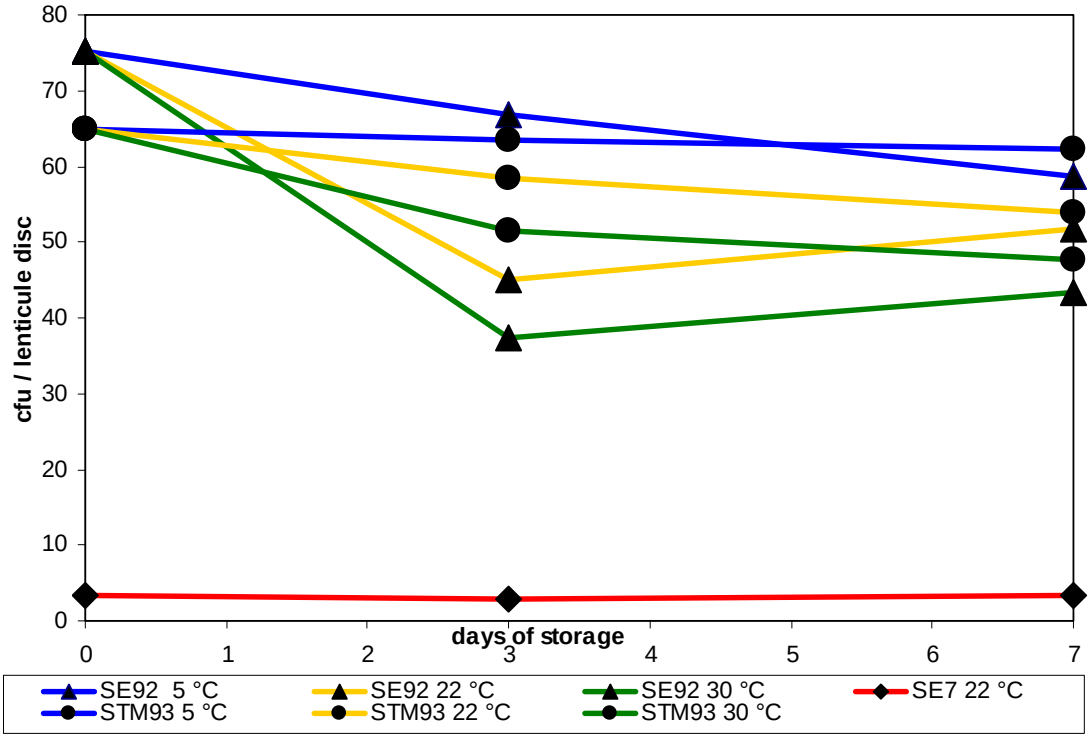


Figure 1 Challenge test of lenticule discs stored at different temperatures. Mean results of 5 lenticule discs per test are indicated.

4.1.3 Pre-test for the interlaboratory comparison study

Table 5 summarises the results of the 'robustness test' of lenticule discs. The different combinations of matrices, lenticule discs, storage and mixing did not show any effect on the results, that is, all samples were tested positive for *Salmonella*.

The pre-test of the full (new) procedure of the interlaboratory comparison study performed at the EURL-*Salmonella* showed good results. All samples of artificially contaminated chicken faeces and nine control samples were scored correctly.

Table 5 Results robustness test of the lenticule discs

Matrix (25g/sample)	Lenticule disc	Combination lenticule disc in matrix		Mixing 1 min Stomacher/pulsifier	No of positive results/ Total no of samples
		Storage temperature	Storage time		
Minced meat (pork/beef)	STM10	3 °C	3 days	yes	5/5
	STM10	-20 °C	3 days	yes	5/5
	STM10	3 °C (only meat)	3 days	yes	5/5
Minced beef	SE7	5 °C	3 days	yes	5/5
	SE7	-	-	yes	5/5
Chicken faeces	STM10	-	-	yes	5/5
	STM10	-	-	no	5/5
	SE7	-	-	yes	5/5
	SE7	-	-	no	5/5

4.2 Chicken faeces samples

The faeces were tested negative for *Salmonella* and stored at 5 °C. On Monday 14 February 2011 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 (12/01/2011) and secondly, after storage at 5 °C, close to the planned date of the interlaboratory comparison study (01/03/2011). Table 6 shows the results.

Table 6 Number of aerobic bacteria and the number of Enterobacteriaceae per gram of chicken faeces

Date	Aerobic bacteria cfu/g	*Enterobacteriaceae* cfu/g
12 January 2011	$4.9*10^8$	$6.6*10^4$
1 March 2011	$4.6*10^8$	$1.2*10^4$

4.3 Technical data interlaboratory comparison study

4.3.1 General

In this study 32 NRLs participated: 28 NRLs from 27 EU-Member States, three NRLs from member countries of the European Free Trade Association State and, on request of DG-Sanco, one NRL from a third country (outside-Europe). Thirty-one laboratories performed the study on the planned date (week 9 starting on 28/02/2011). One laboratory (lab code 18) performed the study one week earlier.

4.3.2 Accreditation/certification

30 laboratories mentioned to be accredited for their quality system according to ISO/IEC 17025 (Anonymous, 2005) and one laboratory (lab code 12) is planning to become accredited. 28 laboratories are accredited for annex D of ISO 6579; 16 are also accredited for ISO 6579 and 1 (lab code 12 an EU-MS) is planning to become accredited for annex D within 1 year. One laboratory of an EU-MS (lab

code 13) is not accredited for the analysis of samples from primary production (annex D of ISO 6579) but is accredited for food and feeding stuffs (ISO 6579). One non-EU laboratory (lab code 7) 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 23) did not mention for which method they are accredited. According to Commission Regulations (EC, 2004) each NRL has to be accredited for its relevant work field before 31 December 2009 (EC, 2005).

4.3.3 Transport of samples

Table 7 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 33 hours. Twenty-four of the laboratories received the materials within 1 day. Two parcels (lab codes 13 and 20) were delayed and the latest parcel arrived after 9 days at the institute. The parcel of laboratory 6 was forwarded to another institute of the NRL and was delayed for 24 hours. For the majority of the parcels the transport temperature did not exceed 5 °C. The delayed parcel of laboratory 13 was stored for 1 week between 0 °C and -16 °C . The temperature recorder of laboratory 7 was broken when it arrived at the EURL, it was therefore not possible to read the results. For eleven 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.

4.3.4 Pre-treatment of the samples

For testing the samples, the laboratories were asked to use as much as possible the procedures and materials as normally used for routine samples (see Annex 5, SOP of this study).
There was an almost equal distribution between the used containers: bags, jars or bottles. 56% of the laboratories used containers pre-filled with BPW. The majority of the laboratories pre-warmed the BPW at room temperature (59%) the others at 37 °C. The samples (BPW, lenticule disc and matrix) were mixed gently in the laboratories by shaking (59%), kneading (19%) or another way of mixing (16%) for example with a spoon. None of the laboratories used a pulsifier, stomacher or vortex to mix. Only 6% of the laboratories did not mix the samples.

Table 7 Overview of temperatures during shipment of the parcels to the NRLs

		Time in hours (h) at				
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	22	13	9			
2	21	8	13			
3	24	13	11			
4	24	23	1			
5	47	40	7			2 h at 20 °C
6*	24 (+24)	3	21			
7	51	Temperature recorder was broken				
8	24	24				1 h at 0 °C
9	25	19	6			
10	26	25	1			1 h at 0 °C
11	26	14	12			
12	27	27				
13	190	187	2		1	
14	25	17	6		2	2 h at 20 °C
15	25	24			1	
16	27	27				
17	24	9	15			2 h at 19 °C
18	21	21				
19	45	22	22		1	
20	216	50	107	58	1	
21	46	22	24			1 h at 0 °C
22	26	26				2 h at - 24 °C
23	24	23	1			1 h at 0 °C
24	44	3	41			
25	50	4	46			
26	1	1				
27	23	23				
28	24	14	10			1 h at 0 °C
29	26	25	1			
30	27	25	2			
31	22	14	8			1 h at 0 °C
32	24	24				2 h at 0 °C
Average	39					
Average EU[3]	33					

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.
*The parcel of laboratory 6 had 24 hours extra transport time because the parcel was forwarded to another institute.

Table 8 Media combinations used per laboratory

Lab code	Selective enrichment media	Plating-out Media
1	MSRV	XLD BGA^{MOD}
2	MSRV	XLD BGA^{MOD}
3	MSRV	XLD Rambach
4	MSRV	XLD RS
5	MSRV	XLD Onoz
6	MSRV	XLD RS
7	MSRV RVS	XLD BSA
8	MSRV	XLD BPLSA
9	MSRV	XLD SM2
10	MSRV	XLD BGA^{S}
11	MSRV	XLD BPLS=BGA^{MOD}
12	MSRV	XLD SM2
13	MSRV	XLD BGA^{E}
14	MSRV MKTTn	XLD BGA SS
15	MSRV	XLD BGA^{MOD}
16	MSRV	XLD BGA^{MOD}
17	MSRV	XLD Rambach
18	MSRV	XLD SM2
19	MSRV	XLD BGA^{MOD}
20	MSRV	XLD BGA^{MOD}
21	MSRV MKTTn	XLD Rambach
22	MSRV	XLD BGA^{MOD}
23	MSRV	XLD Rambach
24	MSRV RVS MKTTn	XLD BGA^{MOD}
25	MSRV	XLD BGA^{MOD}
26	MSRV	XLD BGA^{MOD}
27	MSRV	XLD XLT4
28	MSRV	XLD BGA^{MOD}
29	MSRV MKTTn	XLD BGA^{MOD} BSA
30	MSRV RVS	XLD Rambach
31	MSRV	XLD Rambach
32	MSRV	XLD BxLH

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.

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 an additional selective enrichment medium: RVS (lab codes 7 and 30), MKTTn (lab codes 14, 21 and 29), RVS and MKTTn (lab code 24). Two laboratories (lab codes 14 and 29) used more than two isolation

media. Table 8 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 9-12 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. Three laboratories (lab codes 2, 7 and 15) reported a longer incubation time or a lower temperature of the pre-enrichment in BPW. Laboratory 4 did not mention the pH of the media. Two laboratories (lab codes 4 and 23) did not mention the composition of the media used. Two laboratories (lab codes 9 and 23) used MSRV without novobiocin and four laboratories used MSRV with a higher concentration of novobiocin than the prescribed 0.01 g/L. Four laboratories (lab codes 9, 16, 22 and 31) reported a deviating pH for the MSRV than the described pH of 5.2.

Table 9 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**
2	19	34.6-37
7	23.5	36.5-37
15	25	37

Grey cell: deviating times and temperatures.

Table 10 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	10	5	3.5*	1.5	7.3
4, 27	10	5	3.5*	1.5	-
11	10	5	3.5*	1.5	7.3
16	10	5	3.5*	1.5	7.3
18, 26	10	5	9	1.5	-
29	10	5	3.5*	1.5	7.4

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 11 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**
4	4.6	4.6	7.3	1.5	10.9	0.04	2.7	10	-
7	4.6	4.6	7.3	1.5	10.9	0.04	2.7	10	-
8	4.6	4.6	7.3	1.5	10.9	0.04	2.7	20	5.2
9	8.25*	0.9**	7.3	1.5	12.4	0.04	2.6	-	5.7
13	4.6	4.6	7.3	1.5	10.9	0.04	2.7	20	5.2
15	4.6	4.6	7.3	1.5	10.9	0.04	2.7	50	5.3
16	4.6	4.6	7.3	1.5	10.9	0.04	2.7	10	5.6
18	4.6	4.6	7.3	1.5	10.9	0.04	2.7	10	-
22	4.6	4.6	7.3	1.5	10.9	0.04	2.7	10	5.5
23	-	-	-	-	-	-	-	-	5.2
29	4.6	4.6	7.3	1.5	10.9	0.04	2.7	20	5.4
31	8.25*	0.9**	7.3	1.5	12.4	0.04	2.6	10	4.9

Grey cell: deviating from Annex D of ISO 6579 - = No information
*Pepton mixture **Yeast extract.

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

Lab code	Xylose	L-lysine	Lactose	Sucrose (Saccharose)	Sodium chloride (NaCl)	Yeast extract	Phenol red	Agar	Sodium deoxy-cholate ($C_{24}H_{39}NaO_4$)	Sodium thio-sulphate ($Na_2S_2O_3$)	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**
3, 31	3.5	5	7.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	7.4
4, 27	3.75	5	7.5	7.5	5	3	0.08	13.5	1	6.8	0.8	-
12	3.5	5	7.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	-
13	-	-	-	-	-	-	-	-	-	-	-	-
18	3.5	5	7.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	-
20	3.5	5	7.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	7.3
22	3.5	5	7.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	7.5
23	-	-	-	-	-	-	-	-	-	-	-	7.4
24	-	5	3.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	7.2
29	3.75	5.3	7.5	7.5	5	3	0.08	12.5	1	6.8	0.8	-

Grey cell: deviating from ISO 6579 - = No information

A second plating-out medium for choice was obligatory. Thirteen laboratories used BGA modified (ISO 6579: Anonymous, 1993) as a second plating-out medium. Six laboratories used Rambach agar, three laboratories used BGA agar, three laboratories used SM (ID) 2, two laboratories BSA and two laboratories used Rapid *Salmonella* (RS) agar. The following media were used only by one laboratory: BPLSA, Onoz, XLT4, BxLH, and SS medium.
The use of an extra plating agar between the 'isolation' and the 'confirmation' steps was optional and was performed by 16 laboratories. A total of 16 laboratories performed this extra culture step on a Nutrient agar (e.g. Nutrient agar (ISO 6579: Anonymous, 2002) or another agar (Bromthymol blue lactose sucrose agar, Colombia blood agar, Plate Count Agar).

Table 13 Biochemical confirmation of Salmonella

Lab code	**TSI**	**UA**	**LDC**	Gal	VP	Indole	Kit	Other
1	-	-	-	-	-	-	-	Kohns (Mast diagnostics)
2	+	+	+	+	-	+	-	
3	+	+	+	+	+	+	API RapID 20E	
4	+	-	+	-	-	+	-	Sorbitol
5, 27	-	-	-	-	-	-	API 20E	PCR
6	-	-	-	-	-	-	Enterotube II	
7	+	+	-	-	-	-	Lysine Iron agar	
8, 9	-	-	-	-	-	-	-	PCR
10	+	+	+	+	-	+	-	Agar Tryptose
11, 12, 16, 26	+	+	+	-	-	-	-	
13	+	-	-	-	-	-	GN-ID Panel (Microgen)	
14, 30	+	+	+	+	+	+	-	PCR
15	+	+	+	-	-	+	-	PCR
17	+	+	+	-	-	-	Enterotest 24 (Lachema)	MALDI-TOF
18	-	-	-	-	-	-	Microbact (Oxoid)	
19, 22	+	+	+	+	+	+	-	
20	-	-	-	-	-	+	Enterotest (HY Lab. LTD)	PCR
21	+	-	+	+	-	-	Urea/Indole	
23	+	+	+	+	-	+	-	Semi-solid Glucose agar
24	+	+	+	-	-	+	-	
25	+	+	+	-	-	+	Glucose	
28, 31	-	-	-	-	-	-	-	
29	+	-	-	-	-	-	-	
32	-	-	-	-	-	-	Kligler, urea, indol broth, Manitol and nitrate broth, ONPG and FDA medium	

- = Not done/ not mentioned.
Explanations of the abbreviations are given in the 'List of abbreviations'.

Table 14 Serological confirmation of Salmonella

Lab code	Serological			
	O antigens	**H antigens**	**Vi Antigens**	**Other**
1, 5, 16, 17, 21, 22, 23, 24, 25, 27, 30, 31	+	+	-	
2, 3, 8, 10, 14, 15, 18, 20, 28, 32	+	-	-	
4, 11, 12, 19, 26, 29	-	-	-	
13	+	-	+	
6	-	-	-	Poly A-S Vi
7	-	-	-	Latex Agglutination Test Oxoid
9	-	-	-	Anti-*Salmonella* Enteroclon

- = Not done/ not mentioned.

All participating laboratories performed confirmation tests for *Salmonella*: biochemically, serologically or both. The majority of the laboratories used both biochemical and serological tests. Six laboratories (lab codes 4, 11, 12, 19, 26, and 29) used only a biochemical test(s), four laboratories (lab code 8, 9, 28 and 31) used only a serological test(s). Four laboratories showed a limited confirmation: laboratory 29 performed only one biochemical test (TSI), laboratory 28 performed only one antigen test and the laboratories 8 and 9 performed additional to the one serological test a PCR test. The Tables 13 and 14 summarise the confirmation media and tests.

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 chicken faeces). Thirty laboratories scored correct results for all the control lenticule discs. Table 15 gives the results of all control samples (lenticule discs without faeces). This Table gives the highest number of positive isolations found with MSRV in combination with any isolation medium per laboratory. Six laboratories used an additional selective enrichment medium (own method see Table 8). Annex 6 Table A.6.1 gives the results found with these own methods, which are the same as found with the MSRV method.

Blank lenticule discs without addition of faeces (n=2)
Thirty-one laboratories correctly analysed the blank lenticule disc negative for *Salmonella* with all used media. Laboratory 27 found one blank control lenticule disc positive on both isolation media inoculated from the same MSRV. All blanks should be tested negative. Possible causes for finding a blank sample positive may be cross-contamination. This laboratory is asked to check their procedures.

S. Enteritidis 6 lenticule discs (SE6) without addition of faeces (n=2)
Thirty-one laboratories isolated *Salmonella* Enteritidis at a mean level of approximately 6 cfu/lenticule disc from both lenticule discs. Only laboratory 23 could not detect *Salmonella* in one out of two SE6 lenticule discs. These lenticule discs contained SE at a low level (approx 6 cfu/lenticule). Due to change one out of two lenticule discs containing SE6 may be negative.

S. Enteritidis 57 lenticule discs (SE57) without addition of faeces (n=1)
All participating laboratories tested the one control lenticule disc containing SE57 positive.

S. Typhimurium 6 lenticule discs (STM6) without addition of faeces (n=2)
All 32 laboratories tested both lenticule discs containing STM6 positive.

The results of all control samples were compared with the definition of 'good performance' (see section 3.6). One laboratory (lab code 27) scored below these criteria.

Table 15 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			
	Blank n=2	SE6 n=2	SE57 n=1	STM6 n=2
Good Performance	**0**	**≥ 1**	**1**	**≥ 1**
23	0	**1**	1	2
27	**1**	2	1	2
Other laboratories 1-22; 24-26; 28-32	0	2	1	2

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

4.4.2 Specificity, sensitivity and accuracy rates of the control samples

Table 16 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 European Free Trade Association States, candidate and third countries). No differences were found between these groups.
As expected the high level control (SE57) showed a sensitivity rate of 100% for MSRV. Also the STM6 lenticule discs showed a rate of 100%. The sensitivity rate of SE6 and the specificity rate of the blank lenticule discs were 98%.

Table 16 Specificity, sensitivity and accuracy rates of the control samples (lenticule discs without the addition of (faeces) for the selective enrichment on MSRV and plating-out on XLD or non-XLD)

Control lenticule discs		**MRVS/ XLD**		**MSRV/ non-XLD***	
		All n= 32	EU MS n=28	All n= 32	EU MS n=28
Blank n=2	No. of samples No. of negative samples **Specificity in%**	64 63 **98**	56 55 **98**	68 67 **99**	60 59 **98**
STM6 n=2	No. of samples No. of positive samples **Sensitivity in%**	64 64 **100**	56 56 **100**	68 68 **100**	60 60 **100**
SE6 n=2	No. of samples No. of positive samples **Sensitivity in%**	64 63 **98**	56 55 **98**	68 67 **99**	60 59 **98**
SE57 n=1	No. of samples No. of positive samples **Sensitivity in%**	32 32 **100**	28 28 **100**	34 34 **100**	30 30 **100**
All lenticule discs with *Salmonella*	No. of samples No. of positive samples **Sensitivity in%**	160 159 **99**	140 139 **99**	170 169 **99**	150 149 **99**
All lenticule discs	No. of samples No. of correct samples **Accuracy in%**	224 222 **99**	196 194 **99**	238 236 **99**	210 208 **99**

* Two laboratories used more than one non XLD isolation medium.
All = results/of all laboratories.
EU-MS = results of only the laboratories of the EU Member States.

4.5 Results faeces samples artificially contaminated with *Salmonella*

4.5.1 Results per type of lenticule disc and per laboratory

General
Table 17 gives the results of the *Salmonella* negative chicken faeces samples artificially contaminated with lenticule discs. This table gives the highest number of positive isolations found with MSRV in combination with any isolation medium per laboratory. Six laboratories used an additional selective enrichment medium (own method see Table 8). Annex 6, Table A.6.2 gives the results found with these own methods. Two laboratories (lab codes 7 and 14) found different results with their own method in comparison to the MSRV method.

In general less positive results were found for samples containing *S*. Enteritidis when compared to the ones containing *S.* Typhimurium.

Blank lenticule discs with negative chicken faeces (n=5)
All laboratories correctly did not isolate *Salmonella* from the blank lenticule discs with the addition of negative chicken faeces. Laboratory 7 made a mistake and lost one blank lenticule disc.

S. Enteritidis 6 lenticule discs (SE6) with negative chicken faeces (n=5)
Twenty-two laboratories were able to isolate *Salmonella* from all the five lenticule discs containing *Salmonella* Enteritidis at a level of approximately 6 cfu/ lenticule disc in combination with chicken faeces. Eight laboratories could not detect *Salmonella* in one or two lenticule discs on all of the used media. Laboratory 14 found two lenticule discs negative with the prescribed method MSRV but three negative results with their own method, MKTTn.
Two laboratories (lab codes 4 and 19) found three lenticule discs negative for all the media used. These lenticule discs contained SE at a low level (approximately 6 cfu/lenticule). Due to change one out of five lenticule discs containing SE6 may be negative. However, it is not very likely to find more than one SE6 lenticule disc negative.

S. Enteritidis 57 lenticule discs (SE57) with negative chicken faeces (n=5)
Thirty-one laboratories isolated *Salmonella* from all the five lenticule discs containing *Salmonella* Enteritidis at a level of approximately 57 cfu/ lenticule disc in combination with chicken faeces. One laboratory (lab code 4) found one lenticule disc negative. Laboratory 14 correctly found all five SE57 lenticule discs positive with the prescribed method MSRV but found only four positive results with their own method, MKTTn.

S. Typhimurium 6 lenticule discs (STM6) with negative chicken faeces (n=5)
Thirty-one laboratories isolated *Salmonella* from all the five lenticule discs containing *Salmonella* Typhimurium at a level of approximately 6 cfu/ lenticule disc in combination with chicken faeces. One laboratory (lab code 14) found one lenticule disc negative with the prescribed method MSRV and also with their own method MKTTn. These lenticule discs contained STM at a low level (approximately 5 cfu/lenticule disc). Due to the variation between lenticule discs, one out of five lenticule discs containing STM5 may be negative. Laboratory 1 correctly found all five STM5 lenticule discs positive with the prescribed method MSRV but found only one positive result with their own method, MKTTn.

S. Typhimurium 61 lenticule discs (STM61) with negative chicken faeces (n=5)
All laboratories isolated *Salmonella* from all five lenticule discs containing *Salmonella* Typhimurium at a level of approximately 61 cfu/lenticule disc in combination with chicken faeces.

Table 17 Number of positive results found with the artificially contaminated chicken faeces samples per laboratory

Lab code	Highest number of positive isolations found with MSRV in combination with any isolation medium				
	Blank n=5	SE6 n=5	SE57 n=5	STM6 n=5	STM61 n=5
Good performance	**≤ 1**	**≥ 3**	**≥ 4**	**≥ 3**	**≥ 4**
1 – 3	0	5	5	5	5
4	0	**2**	**4**	5	5
5	0	**4**	5	5	5
6, 7*	0	5	5	5	5
8	0	**4**	5	5	5
9	0	**3**	5	5	5
10 – 13	0	5	5	5	5
14	0	**3**	5	**4**	5
15 – 17	0	5	5	5	5
18	0	**4**	5	5	5
19	0	**2**	5	5	5
20	0	**4**	5	5	5
21 – 24	0	5	5	5	5
25	0	**4**	5	5	5
26 – 29	0	5	5	5	5
30	0	**4**	5	5	5
31, 32	0	5	5	5	5

Bold number: deviating result.
Grey cell: result is below good performance.
*Laboratory 7 lost one blank lenticule disc (n=4).

The results of the artificially contaminated chicken faeces samples were compared with the definition of 'good performance' (see section 3.6) and 30 laboratories fulfilled these criteria for the prescribed method MSRV. The Laboratories 4 and 19 scored below the level of good performance with the SE6 samples. Laboratory 14 found more negative results with their 'own' method MKTTn as with the prescribed method (see Annex 6). If the same criteria as used for MSRV are followed for testing the performance of the MKTTn, these results would not fulfil the criteria of good performance.

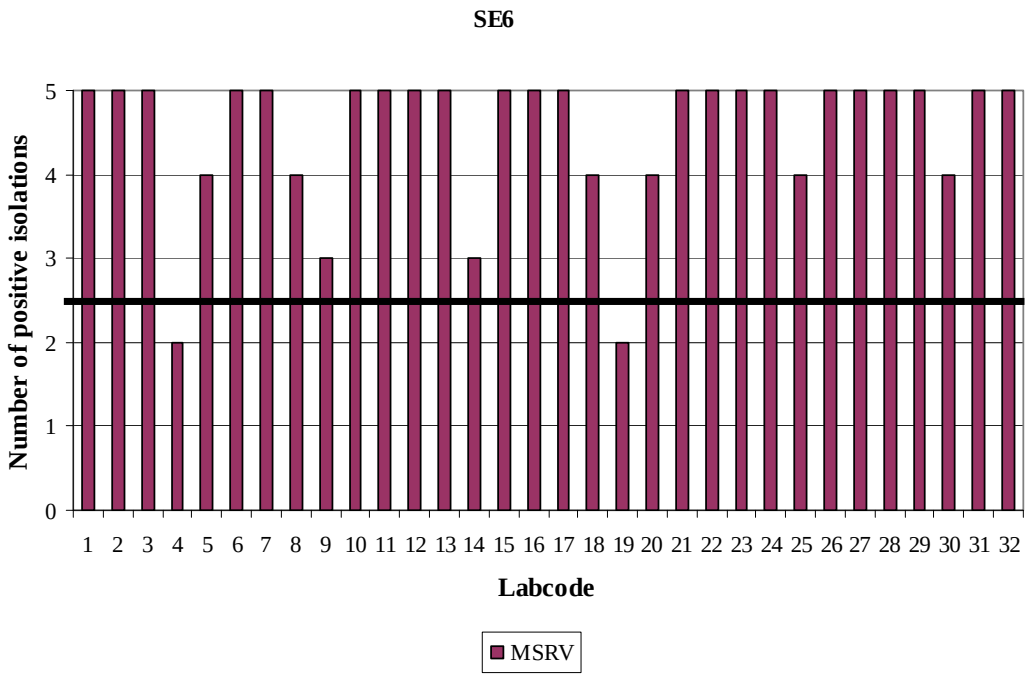


Figure 2 Results per laboratory of chicken faeces samples artificially contaminated with SE6 lenticule discs (n=5) after selective enrichment on MSRV followed by isolation on selective plating agar XLD.

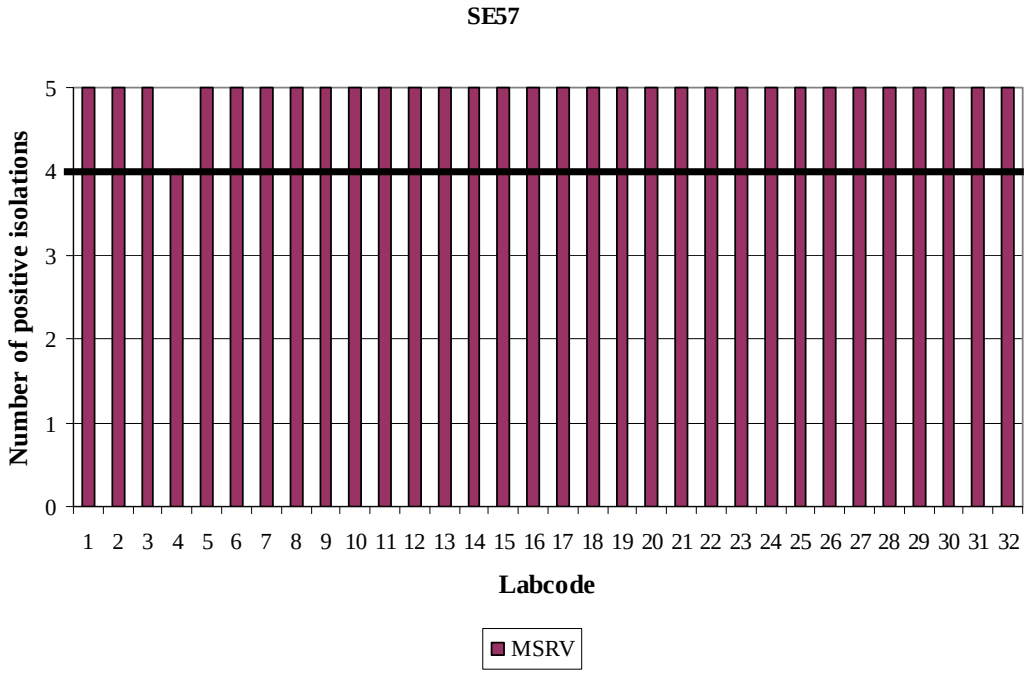
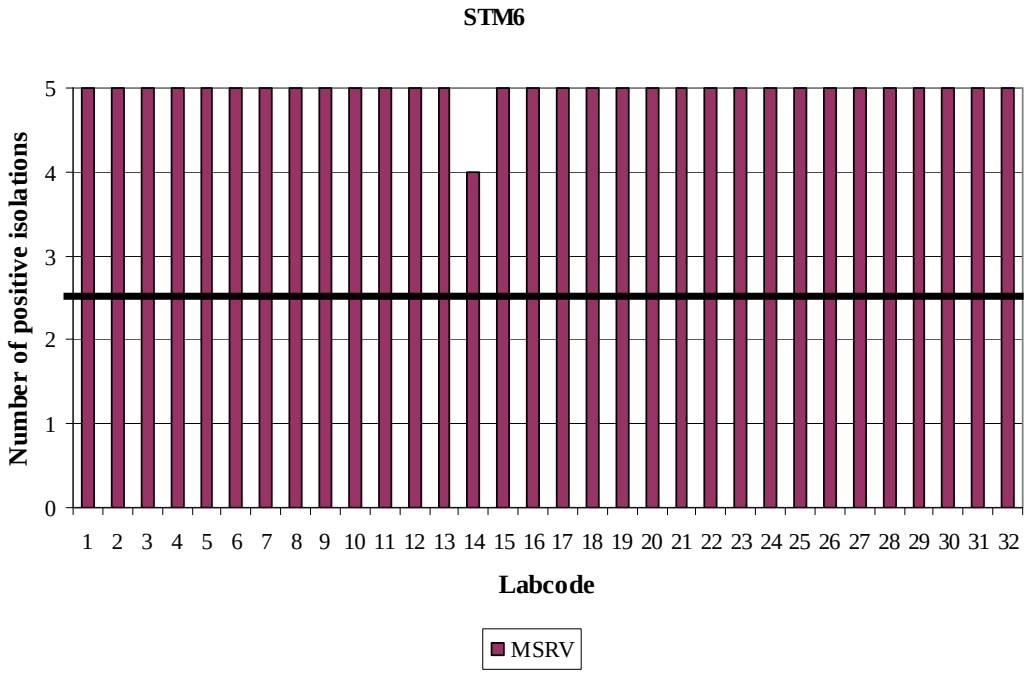
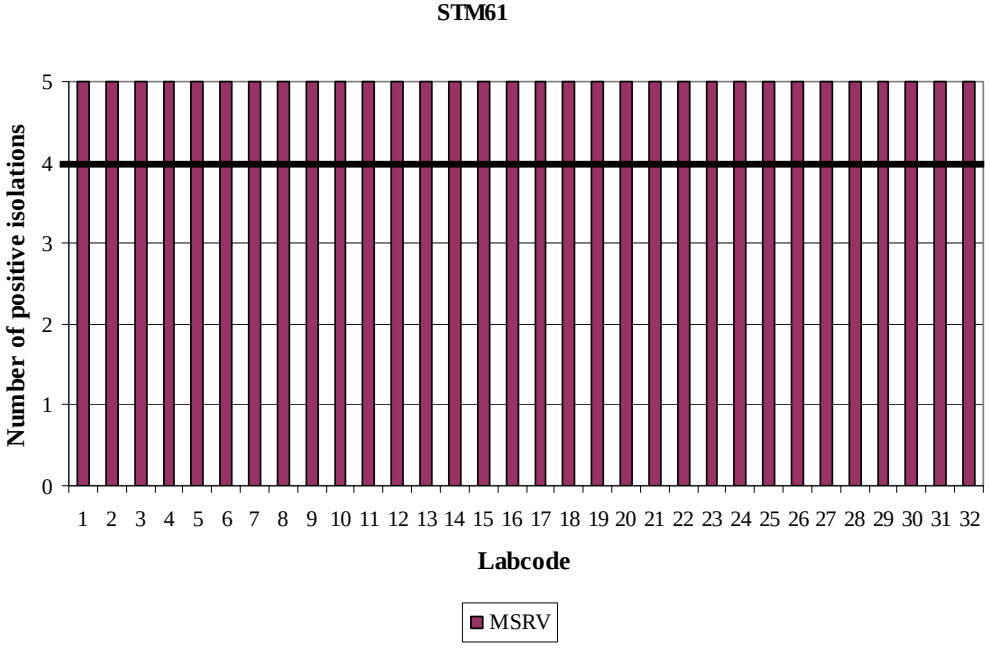


Figure 3 Results per laboratory of chicken faeces samples artificially contaminated with SE57 lenticule discs (n=5) after selective enrichment on MSRV followed by isolation on selective plating agar XLD.



- = border of good performance

Figure 4 Results per laboratory of chicken faeces samples artificially contaminated with STM6 lenticule discs (n=5) after selective enrichment on MSRV followed by isolation on selective plating agar XLD.



- = border of good performance

Figure 5 Results per laboratory of chicken faeces samples artificially contaminated with STM61 lenticule discs (n=5) after selective enrichment on MSRV followed by isolation on selective plating agar XLD.

4.5.2 Results per medium, lenticule disc and per laboratory

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

The results of all artificially contaminated chicken faeces samples were compared with the proposed definition of 'good performance' (see section 3.6). In Figures 1-4 the border of good performance is indicated with a black horizontal line.
Table 18 presents the results of the number of positive isolations after 24 and 48 hours of incubation of the selective enrichment MSRV. On average, 10% more positive results were found after 48 hours of incubation, compared to 24 hours of incubation. However, for the low level SE samples 30% more positives were found after 48 hours of incubation. The choice of isolation medium does not seem to have affected the number of positive isolations, XLD and other plating-out media gave the same results. The majority of the laboratories used BGA as the second plating-out medium (see Table 8).

Table 18 Mean percentages of positive results found for the artificially contaminated chicken 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 faeces)	SE6 lenticules (with faeces)
XLD	87 / 97%	61 / 90%
Other (most often BGA)	87 / 97%	60 / 90%

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

Table 19 shows the specificity, sensitivity and accuracy rates for all types of lenticule discs added to the chicken faeces. 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 XLD and on other selective isolation agar media (non-XLD). The calculations were performed on the results of all participants and on the results of the participants of the EU Member States only (without the results of the participants of the European Free Trade Association States, candidate and third countries). Only small differences (if any) were found between these groups. The specificity rates (of the blank lenticule discs) were 100%. As expected the high level SE57 and STM61 showed sensitivity rates of 100% or very close to 100%. For the low level materials STM6 the rates were also very high: 99%. The low level SE6 showed sensitivity rates around 90%.

Table 19 Specificity, sensitivity and accuracy rates of the artificially contaminated chicken faeces samples (each lenticule disc added to 25 grams chicken faeces) for the selective enrichment on MSRV and plating-out on XLD or non-XLD

Lenticule discs with chicken faeces		**MRVS/XLD**		**MSRV/non-XLD***	
		All n=32	EU MS n=28	All n=32	EU MS n=28
Blank (n=5)	No. of samples No. of negative samples **Specificity in%**	159** 159 **100**	140 140 **100**	169 169 **100**	150 150 **100**
STM6 (n=5)	No. of samples No. of positive samples **Sensitivity in%**	160 159 **99**	140 139 **99**	170 168 **99**	150 148 **99**
STM61 (n=5)	No. of samples No. of positive samples **Sensitivity in%**	160 160 **100**	140 140 **100**	170 169 **99**	150 149 **99**
SE6 (n=5)	No. of samples No. of positive samples **Sensitivity in%**	160 144 **90**	140 125 **89**	170 151 **89**	150 132 **88**
SE57 (n=5)	No. of samples No. of positive samples **Sensitivity in%**	160 159 **99**	140 139 **99**	170 169 **99**	150 149 **99**
All lenticule discs with *Salmonella*	No. of samples No. of positive samples **Sensitivity in%**	640 622 **97**	560 543 **97**	680 657 **97**	600 578 **96**
All lenticule discs	No. of samples No. of correct samples **Accuracy in%**	799 781 **98**	700 683 **98**	849 826 **97**	750 728 **97**

* Two laboratories used more than one non XLD isolation medium.
** One NRL made a mistake with one sample.
All = results/of all laboratories.
EU-MS = results of only the laboratories of the EU Member States.

4.6 PCR

Eight laboratories (lab codes 5, 8, 9, 14, 15, 20, 27 and 30) applied a PCR method as an additional detection technique. All laboratories except one tested the samples after pre-enrichment in BPW. Laboratory 8 started the extraction after selective enrichment on MSRV. Laboratory 5 used an *InvA*-PCR normally used for confirmation of bacterial cultures and not for pre-enrichment broths. The *InvA*-based PCR method is originally described by Rahn et al. (1992). Five PCR methods were validated. Only laboratories 8 and 14 used the PCR routinely. Table 20 gives further details on the used PCR techniques.

Table 20 Details on Polymerase Chain Reaction procedures, used as own method during the interlaboratory comparison study by eight participants.

Lab code	Volume of BPW (µl)	Volume of DNA sample (µl)	Volume of DNA / PCR mix (µl)	PCR method : Reference
5	1000	150	5/10	*Inva* PCR, Validated*, Non commercially
8	-	300	5/25	Real time PCR, Malorny et al., 2004, Validated* Non commercially
9	-	-	-	Realtime PCR, No further information
14	1000	100	5/25	Validated* Non commercially
15	1000	200	1/50	Conventional PCR, Aabo et al. (1993), Not validated, Commercially
20	1000	50	5/25	Real time PCR, Malorny et al., (2004), Not validated, Non commercially
27	10000	100	3/-	Real time PCR, Hein et al., (2006), Validated*, Non commercially
30	1000	150	5/50	Real time PCR (iQ-Check), Validated for biological samples (organs, faeces) by AFNOR (2004), Commercially

*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 21 gives the results of both the PCR and the bacteriological cultivation (BAC). Three laboratories (14, 15 and 20) found the same results with the PCR technique as with the bacteriological culture method (MSRV). One laboratory (lab code 30) found one blank control sample positive with PCR while they correctly found this sample negative on MSRV. The other laboratories (5, 8, 9 and 27) found more samples negative with the PCR technique than with the bacteriological detection method.

Table 21 Number of positive results found for the control samples and for the artificially contaminated chicken faeces samples by using a PCR technique and the bacteriological culture technique

Lenticule Discs	Lab 5		Lab 8		Lab 9		Lab 14		Lab 15		Lab 20		Lab 27		Lab 30	
	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR
Control samples without matrix (n=7)																
SE6 (n=2)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
SE57(n=1)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
STM6 (n=2)	2	2	2	**1**	2	2	2	2	2	2	2	2	2	2	2	2
Blank (n=2)	0	0	0	0	0	0	0	0	0	0	0	0	**1**	**1**	0	**1**
Test samples with chicken faeces (n=25)																
SE6 (n=5)	**4**	**0**	**4**	**4**	**3**	**2**	**3**	**3**	5	5	**4**	**4**	5	5	**4**	**4**
SE57 (n=5)	5	**4**	5	5	5	5	5	5	5	5	5	5	5	**4**	5	5
STM6 (n=5)	5	**4**	5	5	5	**4**	**4**	**4**	5	5	5	5	5	5	5	5
STM61 (n=5)	5	5	5	5	5	5	5	5	5	5	5	5	5	**4**	5	5
Blank (n=5)	0	0	0	0	0	0	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 Bacteriological culture technique (BAC) or with PCR.

4.7 Performance of the NRLs

General
Twenty-nine NRLs fulfilled the criteria of good performance for the prescribed method MSRV.
Two laboratories (lab codes 4 and 19) showed to have a sensitivity problem for the prescribed method and one laboratory (lab code 27) found one blank control lenticule disc (without faeces) positive.
One laboratory (lab code 14) found the minimum score with the prescribed method (MSRV), but still fulfilled the criteria of good performance. However they showed to have difficulties with the detection of *Salmonella* with their own method (MKTTn).
The four laboratories were contacted by the EURL-*Salmonella* in April 2011 to ask for any explanations for their deviating results.

Laboratory 27 found one blank control lenticule disc (without faeces) positive for *S.* Typhimurium on both isolation media used after selective enrichment on MSRV. In trying to clarify this false positive result they were asked to perform additional typing procedures, like serotyping and phagetyping. The laboratory investigated possible reasons and checked their procedures. They did not observe any differences between the false positive sample and the other samples with STM in the confirmation tests for *Salmonella* (identification kit API20E and serotyping with O and H antigens) performed during the ringtrial. The laboratory does not perform phagetyping. Additional to the confirmation tests, the laboratory performed PFGE on *S*. Typhimurium isolated from samples of the interlaboratory comparison study (including the false positive isolate) and isolated from some routine samples. Only one strain isolated from a routine

sample showed a different pattern, which did not help in clarifying the false positive sample. During the ringtrial the laboratory performed a PCR as an own method for all the samples and the CT value of the false positive sample was much higher than of the rest of positive controls. This could indicate a low level (cross) contamination of the BPW. The negative control (only BPW) was correctly negative. Therefore, the most likely clarification of the false positive result seems to be a problem with the handling of the samples. This may be especially the case for the lenticule discs which, like for the majority of the participants, were used for the first time. The laboratory took some measures in trying to prevent similar problems in the future. For this they reviewed their protocols concerning filling of the jars with BPW and observation of the BPW for turbidity before inoculation of the sample and regarding inoculation of the samples.
Furthermore they intend to change their second isolation medium XLT4 by a chromogenic medium which may be more complementary to XLD.

The laboratories 4 and 19 scored below the criteria of good performance for the SE6 lenticule discs added to faeces. Laboratory 19 mentioned in their test report (before knowing the outcome of the study) that there was a 'break down' of the refrigerator where the faeces were stored (25 hours at 18 ºC) and the freezer where the lenticules were stored (29 hours at 1 ºC). Due to the storage at elevated temperatures, the background flora might have increased in the faeces. This may have caused problems with the detection of *Salmonella* in the low level lenticule discs when added to the faeces and can be a clarification for the underperformance of the laboratory. Although the recommended storage temperature of the lenticule discs is -20 ºC, it can be concluded from tests performed at the EURL that storage of the lenticule discs at 1 ºC for 29 hours may have had only a minor effect on the viability of the *Salmonella* strains in the lenticule disc. This is supported by the results of this NRL found with the control lenticule discs SE6, STM6 and SE57 which were all scored positive. As it seems to be likely that the electricity disruption may have affected the results of laboratory 19, the EURL considered that a follow-up study was not necessary and the results of laboratory 19 were indicated as a 'moderate performance'.
Laboratory 4 did check their procedures but did not find a clear clarification for the deviating results with the SE6-faeces samples. The laboratory analyses only a few routine samples per month and uses ready-to-use media. After finding the low number of positives with the SE samples in the ringtrial, the laboratory indicated to have doubts about the sensitivity of the ready-to-use MSRV medium.

To check whether the actions taken have been successful, laboratory 4 and 27 participated in a follow-up study organised by the EURL-*Salmonella* in June 2011.

Laboratory 14 did not find comparable results with the prescribed method (MSRV) and their 'own' method (MKTTn). The laboratory scored the minimum results for the SE6 samples with MSRV. 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 that 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.

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 (see section 4.1 'Reference materials'). In this follow-up study, blank samples and low level samples containing *S.* Enteritidis were tested, as these type of samples caused most of the problems. Table 22 gives an overview of the samples used in the follow-up study.

Table 22 Overview of the types and the number of lenticule discs tested by the laboratories 4 and 27 in the follow-up interlaboratory comparison study

Lenticule discs	**Control lenticule discs (n=5) no matrix added**	**Test samples (n=12) with 25 grams *Salmonella* negative faeces**
S. Enteritidis 6 (SE6)	2	6
S. Enteritidis 57 (SE57)	1	2
Blank	2	4

On Monday 30 May 2011, 1 parcel with 2 plastic containers was sent to laboratory 4 and 27 containing: 5 control lenticule discs (numbered C1 – C5), 12 lenticule discs (numbered B1 – B12), 400 grams of chicken faeces and 1 temperature recorder.
The performance of this follow-up study started in week 23-24 (6-13 June 2011). The laboratory had to follow the same SOP and protocol as in the study of March 2011 (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 media compositions, incubation times and temperatures, no differences were observed in comparison with the full study.
Laboratory 4 performed the follow up study with two extra selective enrichment media: RVS and MKTTn in addition to the prescribed method MSRV. They scored all control and blank samples correctly (only lenticule discs and no matrix added) on MSRV, RVS and MKTTn. For the samples tested with matrix, three out of six SE6 and the two SE57 samples showed suspected colonies on MSRV after 48 hours of incubation. With the MKTTn method all SE samples with matrix were correctly scored positive after 24 hours. With the RVS method only one out of eight SE samples with matrix was found positive. After they found the samples positive with the MKTTn method, the laboratory inoculated isolation media from the non-suspected MSRV plates as well and found three more SE samples positive. With these results, laboratory 4 (just) fulfilled the criteria of good performance. However, like for the full interlaboratory comparison study, the MSRV medium of this laboratory showed a sensitivity problem. The laboratory performed some additional tests with the ready to use MSRV medium and had some doubts about the quality (sensitivity) of the medium. They are in contact with the manufacturer and continue further investigations on the medium.
Laboratory 27 used a third isolation medium additional to XLD and XLT4, being ASAP. They correctly scored all blank samples negative and detected *Salmonella* in all the samples artificially contaminated with SE lenticules. With these results, the laboratory fulfilled the criteria of good performance (see section 3.6) in this follow-up study.

5 Discussion

Reference materials
After many years of using 'capsule-reference materials' to artificially contaminate the matrix in interlaboratory comparison studies of the EURL-*Salmonella*, it was decided to change to lenticule discs. The main reason for this change was the easiness of handling and the robustness of the lenticule discs, which could help to better mimic 'real-life' routine samples. The different tests performed at the EURL confirmed this. The original criterion for the variation in counts between capsules within one batch was that it should be less than twice a Poisson distribution. The same homogeneity test was applied to the batches of lenticule discs as ordered for the interlaboratory comparison study. The test showed that the variation between lenticule discs of all four batches was even less than once a Poisson distribution. It could therefore be concluded that the batches of lenticule discs were well homogeneous.

A limited long-term stability test was performed on 3 batches of lenticule discs stored at -20 ºC. For all batches a decrease in the mean number of cfu was seen. However several factors may have influenced the tests. The analyses at the start and at the end of the study were performed in 2 different laboratories (HPA and the EURL-*Salmonella*). Furthermore, the mean level of the batches of lenticule discs was determined by HPA before they were shipped to the EURL. This latter may also have (negatively) influenced the mean level. Therefore, it may have been the case that the mean levels were somewhat lower when received at the EURL, but this was not tested. Additionally, the HPA determined the mean levels on 30 lenticule discs per batch, the EURL on only 5 lenticule discs. This also hampers the comparison of the results because of the differences in variation in results around the mean level. However, for the interlaboratory comparison studies it is more important to know the stability of the reference materials after a short time of storage, especially under 'abuse circumstances', which may be the case during transport. The results of the challenge test show little or no effect on the mean contamination levels of the batches of lenticule discs with SE and STM after 1 week of storage at 5 ºC. When stored at higher temperatures like 22 ºC or 30 ºC a decrease in the mean number of cfu was seen after 3 to 7 days. Still a well countable mean level remained in the batches. In the worst situation the mean level after storage was half the mean level before storage (SE92 stored at 30 ºC).

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. From the information of the temperature recorders, which were included in the parcels, it can be seen that the temperature in the parcel remained below 5 ºC for most of the transport time. It can therefore be assumed that transport would not have negatively affected the mean level of the batches. This was confirmed by the fact that the laboratories with the longest transport times (lab codes 13 and 20) and with the highest transport temperature (lab code 20) still found good results.

Performance of the laboratories
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 all laboratories except one, the results with

MSRV and the own selective enrichment media in combination with all used isolation media gave the same scores. Laboratory 14 found a lower number of positive results with selective enrichment in MKTTn in comparison to MSRV. This deviating result was most probably caused by problems with the preparation of the MKTTn medium.

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). Twenty-nine out of in total 31 laboratories scored 'good performance'. Two laboratories (lab codes 4 and 27) scored an underperformance for the prescribed method MSRV and participated in a follow-up study. One laboratory (lab code 19) scored a 'moderate performance' as an electricity disruption most likely affected their results. Laboratory 14 scored an 'underperformance' with their own method (MKTTn) but they fulfilled the criteria for the prescribed method MSRV.

The most likely explanation for the sensitivity problems of laboratory 4 (only 2/5 SE6-faeces samples tested positive), was the quality of MSRV. Ready-for-use MSRV bottles were used, which needed to be heated before pouring into Petri dishes. In fact this means that the MSRV medium is heated twice (firstly when prepared by the manufacturer and secondly when poured into Petri dishes), which most likely has a negative effect on the quality of the medium. In the follow-up study the laboratory tested the samples additionally with the selective enrichment media RVS and MKTTn. Correct results were found with MKTTn, but with the MSRV again sensitivity problems were seen. None of the samples were found suspect after 24 hours of incubation of MSRV. Only after 48 hours of incubation the minimum score of good performance was obtained. The laboratory will further investigate the quality of their MSRV to prevent similar problems in the future.
Laboratory 27 may have had problems with (cross) contamination of a blank control lenticule disc. These problems seemed to have been solved in the follow-up study where they scored all samples correctly.

Specificity, sensitivity and accuracy rates
The calculations were performed on the results of all participants and on the results of only the EU Member States (without the results of participants from the European Free Trade Association States, candidate and third countries). Only minor differences (if any) were found between these groups.
The sensitivity rates and the specificity rates of the majority of the samples (control samples as well as artificially contaminated samples) were close to 100%, showing that the NRLs were well able to detect *Salmonella* at different levels. Only the sensitivity rate of the SE6-faeces samples was lower (90%), indicating that the level of this type of sample (6 cfu) became close to the detection limit of the method.

Pre-treatment of the samples
In this study the participants could use their routinely used procedures to pre-treat the samples, especially for the mixing of the samples in BPW. In the former studies this was not possible as the capsules needed a special pre-treatment for dissolution. The participating laboratories indeed used different methods 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.

Media and incubation
Deviations in media compositions or incubation temperatures were reported but no effects were seen on the results. One laboratory used a ready-to-use medium (MSRV) which did not fulfil the quality (see earlier discussion).
The increase in the number of positive results after 48 hours of incubation of the selective enrichment on MSRV was 10%. This was most clear for the low level contaminated SE samples which showed 30% more positive results after 48 hours of incubation. An explanation for this latter result may be the fact that the level of contamination of 6 cfu SE per lenticule disc in combination with the used matrix (chicken faeces) was close to the detection limit.

PCR
Eight laboratories used a PCR technique additional to the prescribed method and three of them found the same results as with the bacteriological detection methods. One laboratory found a blank control sample (without matrix) positive while the same sample was correctly scored negative on MSRV. Four laboratories found more negative results with their PCR method than with the bacteriological detection method.

Evaluation of this study
Overall it was concluded that the first EURL-*Salmonella* study organised with lenticule discs was successful.
The use of lenticule discs as reference materials that artificially contaminate a matrix showed some advantages as well as some disadvantages compared to the capsule reference materials.
Advantages:

- It is possible to treat the samples in the same way as routine samples, especially in relation to mixing of the samples. For example there is no problem to use a stomacher for mixing of the samples with lenticule discs.
- The risk of cross-contamination is reduced compared to the situation when capsules were used, as the lenticule disc is added to BPW after addition of the matrix instead of before.

Disadvantages:

- *Salmonella* Enteritidis in the lenticule discs gave atypical colonies on Rambach isolation medium.

6 Conclusions

- 29 NRLs for *Salmonella* were able to detect high and low levels of *Salmonella* in chicken faeces with the prescribed method MSRV. Two of the laboratories needed a follow-up study to fulfil the criteria of good performance. One laboratory scored a 'moderate performance'.

- The accuracy, specificity and sensitivity rates for the control samples (without faeces) of MSRV were > 98%

- The specificity rate of the chicken faeces samples artificially 'contaminated' with blank lenticule discs was 100% when tested with the prescribed method (MSRV).

- The sensitivity rates of artificially contaminated chicken faeces with low and high level STM lenticule discs and with high level SE lenticule discs were > 99% for the prescribed method MSRV.

- The sensitivity rate of artificially contaminated chicken faeces with low level SE lenticule discs was 10% lower than the rates of the other samples.

- 48 hours incubation of selective enrichment medium MSRV showed 10% more positive results compared to 24 hours of incubation. This was most clear for the low level SE samples which showed 30% more positive results after an incubation time of 48 hours.

- The low-level materials of *S*. Typhimurium (STM6) were easier to detect than the low-level materials of *S*. Enteritidis (SE6).

- The accuracy rates of the artificially contaminated chicken faeces samples were > 97% for 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.

- The first EURL-*Salmonella* study organised with lenticule discs as reference material to artificially contaminate a matrix was successful.

List of abbreviations

ASAP	AES *Salmonella* Agar Plate
BGA	Brilliant Green Agar
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
CRL	Community Reference Laboratory (new name EURL)
EFTA	European Free Trade Association
EU	European Union
EURL	European Union Reference Laboratory
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
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
SE	*Salmonella* Enteritidis
SM (ID)2	*Salmonella* Detection and Identification-2
SOP	Standard Operating Procedure
SS	*Salmonella Shigella* medium
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
XLT4	Xylose Lysine Tergitol 4 agar

References

Aabo S, Rasmussen OF, Rossen L, Sorensen PD and Olsen JE (1993). *Salmonella* identification by the polymerase chain reaction. Molecular and Cellular Probes, 7, 171-178.

AFNOR (2004). Association Française de Normalisation (AFNOR) Validation PCR iQ-Check Certificate 07/06-07/04, 01-07-2004 http://www.afnor-validation.com/afnor-validation-validated-methods/salmonella.html http://www.afnor-validation.com/Certificates/BIORAD/BRD%2007-06%2007-04%20(en).pdf (15 July 2011).

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

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

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

Anonymous (2003b). ISO 16140. Microbiology of food and animal feeding stuffs – Protocol for the validation of alternative methods. International Organisation for Standardisation, Geneva, Switzerland.

Anonymous (2004). ISO 21528-2. Microbiology of food and animal feeding stuffs – Horizontal methods for the detection and enumeration of *Enterobacteriaceae* – Part 2: Colony-count method. International Organisation for Standardisation, Geneva, Switzerland.

Anonymous (2005). ISO 17025. General requirements for the competence of testing and calibration laboratories. International Organisation for Standardisation, Geneva, Switzerland.

Anonymous (2007). Amendment of ISO 6579:2002/Amd1 Annex D: Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage. International Organisation for Standardisation, Geneva, Switzerland.

Boyd P, Eaton S and Magee J (2006). The stability of micro-organisms preserved in LENTICULE discs, demonstrated by repeat sample distributions of the EQUAL Scheme for Indicator Organisms (water microbiology). Journal of Applied Microbiology 100, 272–278.

Desai M, Russell JE and Gharbia S (2006). Genetic stability of strains preserved on LENTICULE discs and by freeze-drying: a comparison using fluorescent amplified fragment length polymorphism analysis. FEMS Microbiol Lett 257, 158–162.

EC (2004). Commission Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules. Official Journal of the European Union L 165 of 30 April 2004.

EC (2005). Commission Regulation (EC) No 2076/2005 of the European Parliament and of the Council of 5 December 2005 laying down transitional arrangements for the implementation of Regulations (EC) No 853/2004, (EC) No 854/2004 and (EC) No 882/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004.

Hein I, Flekna G, Krassnig M and Wagner M (2006). Real time PCR for the detection of *Salmonella* spp. in food: An alternative approach to a conventional PCR system suggested by the FOOD-PCR project. Journal of Microbiological Methods 66, 538-547.

HPA Health Protection Agency. Lenticule Disc Handling Information. HPA Culture Collection, Salisbury, United Kingdom.
http://www.hpacultures.org.uk/products/lenticulediscs/index.jsp
http://www.hpacultures.org.uk/media/C4B/10/LENT_Handling_Website_V.pdf
(21 April, 2011)

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.

Rahn K, De Grandis SA, Clarke RC, McEwan SA, Galan JE, Ginocchio C, Curtiss R 3rd, 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*

Table A1.1 History of EURL-Salmonella interlaboratory comparison studies on detection of Salmonella in veterinary 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 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 STM100 SE100 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 (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	Matrix 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			
	5	Blank	0	25 gram			
	2	STM6	6	No			
	2	SE6	6	No			
This study	1	SE57	57	No			
	1	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.rivm.nl/crlsalmonella/publication/
[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							

[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.rivm.nl/crlsalmonella/publication/

[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.rivm.nl/crlsalmonella/publication/

[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 Information on the media used

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

MKTTn (Oxoid CM 343 Hampshire, United Kingdom)
Composition of MKTTn medium: the concentration of the compounds in g/L water: Meat extract 7, Enzymatic digest of casein 2.3, Sodium chloride 2.3, Calcium carbonate 25, Sodium thiosulfate pentahydrate 40.7, Ox bile for bacteriological use 4.75, Brilliant green 0.1/100mL, Iodine 4, Potassium iodide 5, Novobiocine 0, pH 7.8

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

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) (HImedia Laboratories M971, Mumbai, India) (Biolife 4012562, Milan, Italy) (Hy Laboratories Ltd. DD074, Rehovot, Israel) (SIFIN TN 1110, Berlin, Germany)
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^{E} (AES 004235 Cranbury, USA)
Composition of BGA medium: not mentioned

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 (previous OSCM) (Oxoid CM 1092/ PO598A, 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)
Onoz 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 (Merck 107500.0002/07500.0001, Darmstadt, Germany)
(CHROMagar RR703-25, Paris, France)
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 3563961/ 3564705, 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

SS medium (Becton Dickinson 211597, Ontario, Canada)
Rose, H. M., and M. H. Kolodny. 1942. The use of SS (*Shigella-Salmonella*) Agar for the isolation of Flexner Dysentery bacilli from the feces. J. Lab. Clin. Med. 27:1081-1083.

Composition of SS agar: the concentration of the compounds in g/L water: Beef extract 5, Pancreatic digest of casein 2.5, peptic digest annual tissue 2.5, Lactose 10, Bile salts 8.5, Sodium citrate 8.5, Sodium thiosulphate 8.5, Ferric citrate 1, Brilliant green 0.00033, Neutral red 0.025, Agar 13.5, pH 7.1

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) 6.3, Tris 0.2, Lactose 6, Ox bile (bovine and swine) 1.5, Chromogenic mix 9.6, Sodium chloride 5, Selective mix 0.03, Agar 14 pH 6.7- 7.3

XLT4 (Oxoid PO5116A, Hampshire, United Kingdom)
Miller, R.G., C.R. Tate. 1990. XLT4: A highly selective plating medium for the isolation of *Salmonella*. The Maryland Poultryman, April: 2-7 (1990).
Composition of XLT4 medium: the concentration of the compounds in g/L water: Peptone 1.6, Yeast extract 3, L-Lysine 5, Lactose 7.5, Saccharose 7.5, Xylose 3.75, Sodium Chloride 5, Sodium Thiosulphate 6.8, Ferric Ammonium Citrate 0.8, 7-ethyl-2 methyl-4-undecanol hydrogen (Tergitol 4) 4.6 ml, Phenol Red 0.08, Agar 18 pH 7.4

Annex 4 Protocol

INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN CHICKEN FAECES organised by EU-RL-*Salmonella*

STUDY XIV - 2011

Introduction

This protocol describes the procedures for the 14th 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 numbers of samples to be tested will be comparable to earlier studies on the detection of *Salmonella* spp. in veterinary samples, but the type of reference materials will be different. 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 chicken faeces samples (*Salmonella*-negative) artificially contaminated with reference materials. The reference materials (RMs) consist of lenticule discs containing *Salmonella* strains at different contamination levels. Each laboratory will examine 25 faeces samples (25 g each) in combination with a *Salmonella* lenticule and 7 control samples (lenticules 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 chicken faeces. The container with the lenticules 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 EU-RL-*Salmonella*, immediately after receipt of the parcel**. For this purpose a return envelope with a pre-printed address label of the EU-RL-*Salmonella* will be included. Do not forget to note your lab code before returning it to the EU-RL. **Each box will be sent as biological substance category B (UN3373) by door-to-door courier service. Please contact EU-RL-*Salmonella* when the parcel has not arrived at your laboratory at 17th of February 2011 (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 poultry faeces.

Outline of the study

Each participant will receive (in week 7 of 2011) one box containing 2 plastic containers, packed with cooling elements. The containers contain:

Container 1:
One plastic bag with 34 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 EU-RL-*Salmonella* as soon as possible.**
Store container 1 at (-20 ± 5) ºC immediately after receipt.

Container 2:
One plastic bag with 900 g of chicken faeces (free from *Salmonella*).
Store container 2 at (5 ± 3) ºC immediately after receipt.

The performance of the study will be in week 9 (starting on 28 February 2011).

The documents necessary for performing the study are:

- Protocol Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in animal faeces XIV (2011) (this document);
- SOP Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in animal faeces XIV (2011);
- Test report Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in animal faeces XIV (2011);
- 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 EU-RL.

All data have to be reported in the test report and sent to the EU-RL-*Salmonella* before **18 March 2011.** The EU-RL 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:

Angelina Kuijpers (Tel. number: + 31 30 274 2093)
Kirsten Mooijman (Tel. number: + 31 30 274 3537)
RIVM / LZO (internal Pb 63)EU-RL *Salmonella*
P.O. Box 1
3720 BA Bilthoven, The Netherlands
http://www.rivm.nl/crlsalmonella
Fax. number: + 31 30 274 4434
E-mail : Angelina.Kuijpers@rivm.nl or Kirsten.Mooijman@rivm.nl

Timetable of interlaboratory comparison study
ANIMAL FAECES XIV (2011)

Week	Date	Topic
4	24 - 28 January	Mailing of the protocol, standard operating procedure and test report to the NRLs-*Salmonella*
7	14 – 18 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 EU-RL-*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 17 February, do contact the EU-RL 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)
9	28 February – 4 March	Performance of the study, following the instructions as given in the protocol and the SOP of study Animal faeces XIV (2011).
11	Before 18 March	Completion of the test report. Send the test report, preferably by e-mail to the EU-RL *Salmonella* (Angelina.Kuijpers@rivm.nl)*
12	21 -25 March	Data input at EU-RL-*Salmonella* and sending these data to NRLs. Checking these results by the National Reference Laboratories.
	May - June 2011	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 EU-RL, it is not necessary to send the original test report as well, unless it is not legible (to be indicated by EU-RL-*Salmonella*).

Annex 5 Standard Operating Procedure (SOP)

INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN CHICKEN FAECES organised by EU-RL-*Salmonella*
STUDY XIV - 2011

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 chicken faeces. For this purpose Reference Materials (RMs) containing *Salmonella* Typhimurium (STM) or *Salmonella* Enteritidis (SE) as prepared by the Health Protection Agency (HPA, United Kingdom) are used. As matrix, chicken 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.

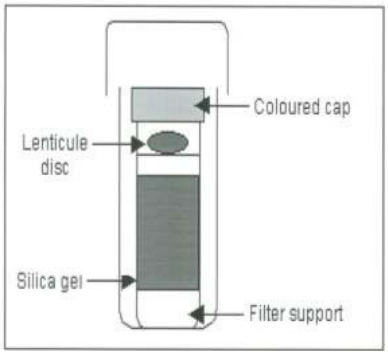
Lenticule Disc Handling Information. HPA Culture Collection, Salisbury, United Kingdom.
More information on the reference materials (lenticule discs) as produced by the HPA can be found on:
http://www.hpacultures.org.uk/products/lenticulediscs/index.jsp

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) (ISO6579 Annex 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) (ISO6579 Annex B.5).

7 Apparatus and glassware

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

7.1 Apparatus

- -oven (for dry sterilisation) or autoclave (for wet sterilisation);
- 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 fourteenth interlaboratory comparison study in chicken faeces of EU-RL-*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 & 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 follows:
  - 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 pre filled 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 chicken 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 follows:
  - 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 from 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.
Before confirmation (see below), optionally, streak the typical colonies onto the surface of nutrient agar plates with the corresponding label numbers, in a manner which allows to develop well isolated colonies. Record on 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).

<u>Confirmation of identity</u>
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.

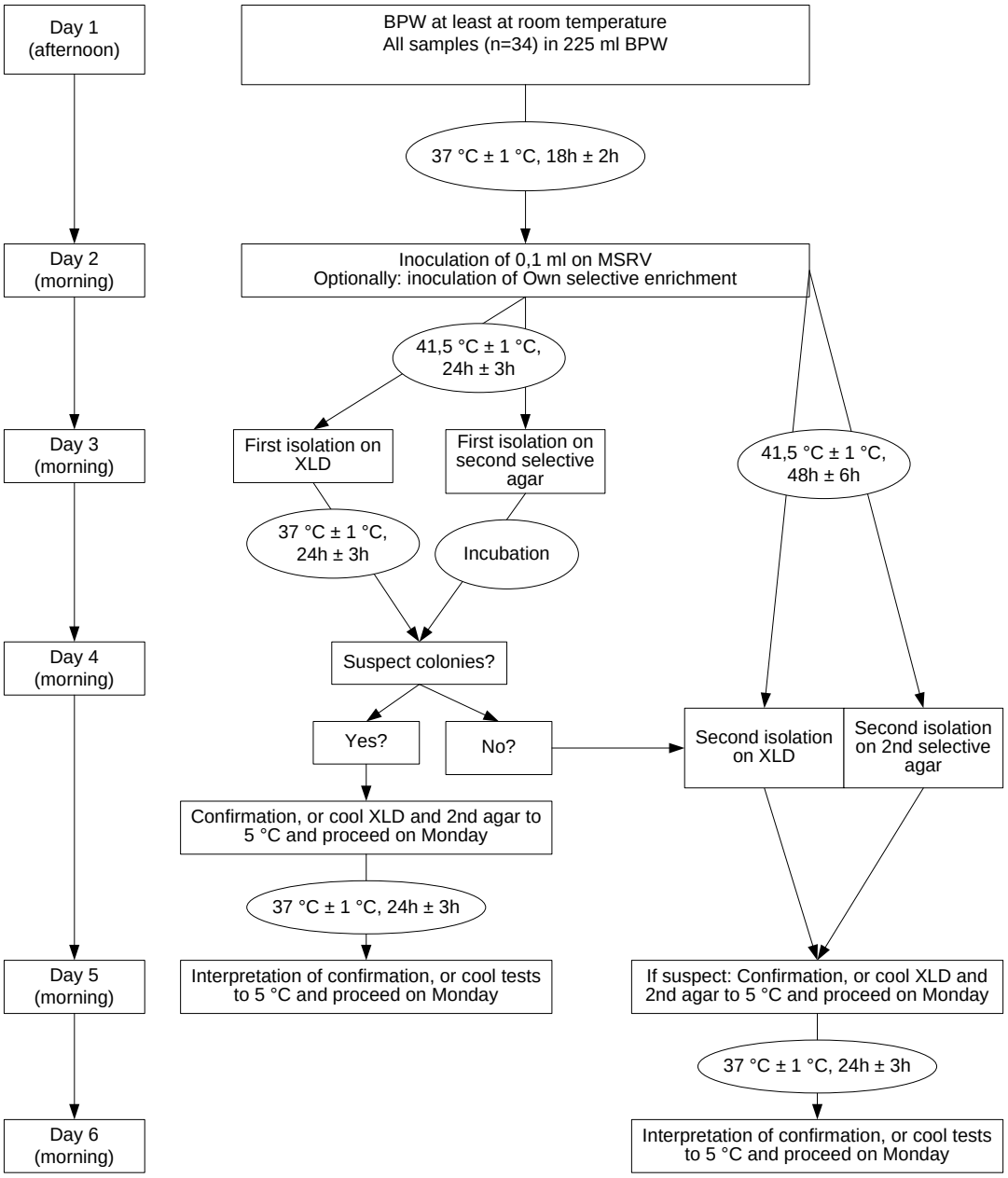
9 Test report

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.

Scheme of Bacteriological Interlaboratory Comparison Study ANIMAL FAECES XIV (2011) on the detection of *Salmonella* spp. in chicken 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 XIV
on the detection of *Salmonella* spp.
EU-RL-*Salmonella* 2011



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	**SE6 n=2**	**SE57 n=1**	**STM6 n=2**	**Blank n=2**	**SE6 n=2**	**SE57 n=1**	**STM6 n=2**
Good Performance	**0**	**≥ 1**	**1**	**≥ 1**	**0**	**≥ 1**	**1**	**≥ 1**
	MKTTn				**MSRV**			
14	0	2	1	2	0	2	1	2
24	0	2	1	2	0	2	1	2
29	0	2	1	2	0	2	1	2
	RVS				**MSRV**			
7	0	2	1	2	0	2	1	2
21	0	2	1	2	0	2	1	2
24	0	2	1	2	0	2	1	2
30	0	2	1	2	0	2	1	2

Table A6.2 Results 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	**SE6 n=5**	**SE57 n=5**	**STM6 n=5**	**STM61 n=5**	**Blank n=5**	**SE6 n=5**	**SE57 n=5**	**STM6 n=5**	**STM61 n=5**
Good Performance	**≤ 1**	**≥ 3**	**≥ 4**	**> 2**	**≥ 3**	**≤ 1**	**≥ 3**	**≥ 4**	**≥ 3**	**≥ 4**
	MKTTn					**MSRV**				
14	0	**2**	**4**	**4**	5	0	**3**	5	**4**	5
24	0	5	5	5	5	0	5	5	5	5
29	0	5	5	5	5	0	5	5	5	5
	RVS					**MSRV**				
7	0	5	5	5	5	0	5	5	5	5
21	0	5	5	5	5	0	5	5	5	5
24	0	5	5	5	5	0	5	5	5	5
30	0	**4**	5	5	5	0	**4**	5	5	5

Bold numbers: Deviating results.

Grey cells: Results below the level of good performance.

Annex 7 Test report Follow-up study

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

STUDY XIV - 2011

FOLLOW-UP STUDY June 2011

Laboratory code This is the same code as in FAECES XIV 2011	
Laboratory name (NRL)	
Address	
Country	
Date of arrival of the parcels	Date: …… - ……. – 2011 time: ……... h ……..…… min
Start time of storage at -20 °C (lenticule discs) Start time of storage at +5 °C (faeces)	Date: …… - ……. – 2011 time: ……... h ……..…… min Date: …… - ……. – 2011 time: ……... h ……..…… min
Parcels damaged?	☐ Yes ☐ No
Starting date testing	…… - ……. – 2011

PRE-ENRICHMENT – Buffered Peptone Water (BPW)

Medium information BPW
Was the composition of BPW the same as used in BRO FAECES XIV 2011 ?
☐ Yes ☐ No please give more details in an annex :

Preparation of BPW	
Date of preparation	…… - ……. – 2011
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: …… - ……. - 2011 time: …….. h ……..…… min temperature incubator: ……..…… °C
End at	Date: …… - ……. - 2011 time: …….. h ……..…… min temperature incubator: ……..…… °C

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

Medium information MSRV
Was the composition of MSRV the same as used in BRO FAECES XIV 2011 ?
☐ Yes ☐ No please give more details in an annex :

Preparation of MSRV	
Date of preparation	…… - ……. - 2011
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: …… - ……. - 2011 time: …….. h ……..…… min temperature incubator: ……..…… °C
End of the first period (first 24 h)	Date: …… - ……. - 2011 time: …….. h ……..…… min temperature incubator: ……..…… °C
Start of the second period (48 h)	Date: …… - ……. - 2011 time: …….. h ……..…… min temperature incubator: ……..…… °C
End of the second period (48 h)	Date: …… - ……. - 2011 time: …….. h ……..…… min temperature incubator: ……..…… °C

OWN SELECTIVE 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 XIV 2011 ?
☐ Yes
☐ No please give more details in an annex :

Preparation of the medium	
Date of preparation	…… - ……. - 2011
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: …… - ……. - 2011 time: …….. h ……..…… min temperature incubator: ……..…… °C
End of the first period (first 24 h)	Date: …… - ……. - 2011 time: …….. h ……..…… min temperature incubator: ……..…… °C
Start of the second period (48 h)	Date: …… - ……. - 2011 time: …….. h ……..…… min temperature incubator: ……..…… °C
End of the second period (48 h)	Date: …… - ……. - 2011 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 XIV 2011 ?
☐ Yes
☐ No please give more details in an annex :

Preparation of XLD	
Date of preparation	…… - ……. - 2011
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: …… - ……. - 2011 time: …….. h ……..…… min temperature incubator: ……..…… °C
End incubation of XLD, inoculated from 24 h MSRV	Date: …… - ……. - 2011 time: …….. h ……..…… min temperature incubator: ……..…… °C
Start incubation of XLD, inoculated from 48 h MSRV	Date: …… - ……. - 2011 time: …….. h ……..…… min temperature incubator: ……..…… °C
End incubation of XLD, inoculated from 48 h MSRV	Date: …… - ……. - 2011 time: …….. h ……..…… min temperature incubator: ……..…… °C

FIRST AND SECOND ISOLATION – Second Isolation medium.

Give 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 XIV 2011 ?
☐ Yes
☐ No please give more details in an annex :

Preparation of the second isolation medium	
Date of preparation	…… - ……. - 2011
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: …… - ……. - 2011 time: …….. h ……..…… min temperature incubator: ……..…… °C
End incubation of second medium, inoculated from 24 h MSRV	Date: …… - ……. - 2011 time: …….. h ……..…… min temperature incubator: ……..…… °C
Start incubation of second medium, inoculated from 48 h MSRV	Date: …… - ……. - 2011 time: …….. h ……..…… min temperature incubator: ……..…… °C
End incubation of second medium, inoculated from 48 h MSRV	Date: …… - ……. - 2011 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 °C	

Medium information OWN second isolation medium
Was the composition of media the same as used in BRO FAECES XIV 2011 ?
☐ Yes
☐ No please give more details in an annex :

Preparation of your own medium	
Date of preparation	…… - ……. - 2011
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: …… - ……. - 2011 time: …….. h ……..…… min temperature incubator: ……..…… °C
End incubation of own medium, inoculated from 24 h selective enrichment medium	Date: …… - ……. - 2011 time: …….. h ……..…… min temperature incubator: ……..…… °C
Start incubation of own medium, inoculated from 48 h selective enrichment medium	Date: …… - ……. - 2011 time: …….. h ……..…… min temperature incubator: ……..…… °C
End incubation of own medium, inoculated from 48 h selective enrichment medium	Date: …… - ……. - 2011 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 XIV 2011 ?
☐ 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 XIV BRO 2011, please give more information in an annex.	

*Table 1: Results of isolation using **MSRV** (dish numbers B1-B12)*

	MSRV 24 hours						MSRV 48 hours					
Sam-ple no.	XLD		Second isolation medium		Own isolation medium		XLD		Second isolation medium		Own isolation medium	
	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												

*Table 1 (continued): Results of isolation using **MSRV** (dish numbers C1- C5, C8 and C9)*

	MSRV 24 hours						MSRV 48 hours					
Sam-ple no.	XLD		Second isolation medium		Own isolation medium		XLD		Second isolation medium		Own isolation medium	
	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												
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-B12) * = fill in the name of the medium used*

	Own * 24 hours						Own * 48 hours					
Sam-ple 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]
B1												
B2												
B3												
B4												
B5												
B6												
B7												
B8												
B9												
B10												
B11												
B12												

*Table 2 (continued): Results of isolation using **Own** selective enrichment medium(dish numbers C1-C5, C8 and C9)*

	Own * 24 hours						Own * 48 hours					
Sam-ple 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												
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-B12 & C1-C5, C8 and C9)

PCR + or -			
Sample no.		Sample no.	
B1		C1	
B2		C2	
B3		C3	
B4		C4	
B5		C5	
B6			
B7			
B8		C8	
B9		C9	
B10			
B11			
B12			

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

Name of person (s) carrying out the follow-up of the fourteenth veterinary interlaboratory Comparison study (2011).	
Is the person(s) carrying out the follow-up of the fourteenth veterinary interlaboratory Comparison study (2011) 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 4 July 2011, by email to EURL-*Salmonella*. 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*).
Use the address below:

Angelina Kuijpers
E-mail : Angelina.Kuijpers@rivm.nl
EURL *Salmonella* (internal Pb 63)
RIVM / 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

National Insitute for Public Health
and the Environment
P.O. Box 1 | 3720 BA Bilthoven
www.rivm.com