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

EU Interlaboratory comparison study primary production XVI (2013)

Detection of *Salmonella* in chicken faeces adhering to boot socks

RIVM report 330604031/2014

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Colophon

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Abstract

EU Interlaboratory comparison study primary production XVI (2013)
Detection of *Salmonella* in chicken faeces adhering to boot socks

In 2013, all 36 National Reference Laboratories (NRLs) in the European Union were able to detect high and low levels of *Salmonella* in chicken faeces collected from stables with laying hens. The laboratories achieved the desired level of good performance immediately. The laboratories detected *Salmonella* in 96% of the contaminated samples. This is evident from the 16th interlaboratory comparison study of primary production samples (such as chicken faeces), which was organized by the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*). In this study, environmental material was collected by researchers walking through the stable wearing overshoes (boot socks).

Intertlaboratory comparison study obligatory for EU Member States
The study was conducted in March 2013. Participation was obligatory for all EU Member State NRLs which are responsible for the detection of *Salmonella* in samples from primary production. EURL-*Salmonella* is part of the Dutch National Institute for Public Health and the Environment (RIVM).

The laboratories used the internationally prescribed Modified Semi-solid Rappaport-Vassiliadis (MSRV) method to detect the presence of *Salmonella* in chicken faeces adhering to boot socks. Each laboratory received a package of boot socks containing chicken faeces with two different concentrations of *Salmonella*, or containing no *Salmonella* at all. The laboratories were required to analyse the samples for the presence of *Salmonella* in accordance with the study protocol.

New procedures
For the first time, the samples (matrix) were artificially contaminated with a diluted culture of *Salmonella* Typhimurium at the EURL-*Salmonella* laboratory. The EURL-*Salmonella* laboratory investigated the optimal sample delivery procedure for this type of study. The procedure used was positively received by the NRLs because they themselves were no longer required to combine the *Salmonella* samples, as was the case in previous studies. This procedure will therefore continue to be used in future studies, although its feasibility will be assessed for each study. A further innovation was that the participating laboratories were able to submit their findings via the Internet. This change was also positively received by the NRLs, and made it easier for the EURL to analyse the data. It was decided to optimize this procedure and to continue using it.

Keywords: *Salmonella*, EURL, NRL, interlaboratory comparison study, environmental material, *Salmonella* detection method, boot socks

Publiekssamenvatting

EU Ringonderzoek primaire productie XVI (2013)
Detectie van *Salmonella* in overschoenen met kippenmest

In 2013 waren alle 36 Nationale Referentie Laboratoria (NRL's) in de Europese Unie in staat om hoge en lage concentraties *Salmonella* in een stal met leghennen (kippenmest) aan te tonen. Ze behaalden direct het gewenste niveau. In totaal hebben de laboratoria in 96 procent van de besmette monsters *Salmonella* opgespoord. Dit blijkt uit het zestiende ringonderzoek met materiaal van de dieren (zoals uitwerpselen) dat werd georganiseerd door het referentielaboratorium van de Europese Unie voor *Salmonella* (EURL-*Salmonella*). Voor dit soort onderzoek zijn monsters van de uitwerpselen van kippen verzameld door met overschoenen door de stal te 'wandelen' (omgevingsmateriaal).

Ringonderzoek verplicht voor Europese lidstaten
Het onderzoek is in maart 2013 gehouden. Alle NRL's van de Europese lidstaten die verantwoordelijk zijn voor de opsporing van *Salmonella* in dierlijke mest, zijn verplicht om aan het onderzoek deel te nemen. Het EURL-*Salmonella* is gevestigd bij het Nederlandse Rijksinstituut voor Volksgezondheid en Milieu (RIVM).

De laboratoria toonden de *Salmonella*-bacterie in de overschoenen met kippenmest aan met behulp van de internationaal voorgeschreven analysemethode (MSRV). Elk laboratorium kreeg een pakket toegestuurd met overschoenen waaraan kippenmest zat met *Salmonella* in twee verschillende concentraties of zonder *Salmonella*. De laboratoria dienden de monsters volgens een protocol te onderzoeken op de aanwezigheid van *Salmonella*.

Nieuwe werkwijzen
Voor het eerst is het te onderzoeken materiaal (matrix) op het laboratorium van het EURL-*Salmonella* kunstmatig besmet met een verdunde cultuur van een *Salmonella* Typhimurium. Het laboratorium van het EURL-*Salmonella* heeft voor dit soort studies onderzocht hoe de monsters op deze wijze optimaal kunnen worden aangeleverd. De NRL's vinden deze werkwijze positief, omdat zijzelf niet meer de monsters met de *Salmonella* hoeven samen te voegen; dit was in eerdere studies wel het geval. Deze werkwijze wordt daarom voorgezet, al wordt per studie bekeken of het haalbaar is. Een andere vernieuwing is dat de deelnemende laboratoria hun bevindingen via internet konden aanleveren. De NRL's vonden ook dit een verbetering, en voor het analyserend EURL zijn de gegevens eenvoudiger te analyseren. Besloten is deze werkwijze te optimaliseren en voort te zetten.

Trefwoorden: *Salmonella*; EURL; NRL; ringonderzoek; kippenmest; omgevingsmateriaal; *Salmonella*-detectiemethode; overschoenen;

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Summary

In March 2013 the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*) organised the 16th interlaboratory comparison study on the detection of *Salmonella* in samples from primary production (XVI). The matrix of concern were boot socks, to which environmental material (mainly faeces) from a laying hen flock was attached.
This study is a combined study with the CEN mandate study (Validation of Annex D of EN ISO 6579). The data were differently treated for the CEN mandate (which tested the performance of the study method) and for this EURL study (which tested the performance of the laboratories). This report describes the results of the EURL-*Salmonella* study. The results of the CEN mandate study are described in a separate report (Mooijman et al., under preparation).
The participants were 36 National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*): 28 NRLs from the 27 EU Member States (EU-MS) and 8 NRLs from non-EU countries. The non-EU countries (Bosnia and Herzegovina, Croatia, Former Yugoslav Republic of Macedonia, Serbia, Switzerland, Norway, Iceland and Israel) included Candidate EU-Member States, Members of the European Free Trade Association (EFTA) and a country outside Europe.

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 matrix from primary production. For this purpose, boot socks with environmental material from a laying hen flock, artificially contaminated with *Salmonella* Typhimurium at various contamination levels, were analysed. The performance of the laboratories was compared with the criteria for good performance. The prescribed method was Annex D of ISO 6579 (Anonymous, 2007), using selective enrichment on Modified Semi-solid Rappaport-Vassiliadis (MSRV) agar.

Artificially contaminated boot sock samples had not been used in earlier studies. Therefore, some additional tests were performed at the laboratory of the EURL-*Salmonella* prior to the study. It was tested how well *Salmonella* could be detected in the boot sock samples after moistening the boot socks with different solutions or without moistening them; in the presence of different amounts of background flora in the chicken faeces; when the samples were artificially contaminated with different *Salmonella* serovars at different levels; and during storage at different temperatures.

Thirty individually numbered plastic bags with boot socks artificially contaminated with *Salmonella* Typhimurium or with a blank solution had to be tested by the participants for the presence or absence of *Salmonella*. To 24 of the boot sock samples, environmental material from a laying hen flock was added. Eight of these samples contained approximately nine colony-forming units (CFU) of *Salmonella* Typhimurium (STM low), eight samples contained approximately 81 CFU of *S*. Typhimurium (STM high) and eight samples contained no *Salmonella* at all (blanks). Six boot sock samples to which no environmental material had been added acted as control samples; two of these samples were artificially contaminated with STM low and two with STM high, while two were left blank. Before being artificially contaminated, each boot sock was moistened with 15 ml peptone saline solution.

On average, the participants found *Salmonella* in 96% of the contaminated samples using the prescribed method, i.e. selective enrichment on MSRV.

Nineteen of the 36 participants (53%) tested all boot socks with environmental material (chicken faeces) contaminated with *S*. Typhimurium positive.
Forty-eight hours of incubation of MSRV gave overall 3% more positive results than 24 hours of incubation.
PCR was used as an own method by nine participants, of which five found the same results as with the bacteriological culture method.

All NRLs fulfilled the criteria of 'good performance'.

The samples used in this study closely mimic routine samples, gave good results and were easier to use than the previously used samples, where the participants had to mix matrix and reference material themselves, shortly before analysis.
For the first time in an EURL-*Salmonella* detection study, the NRLs could deliver their findings via the Internet using a web-based test report. The NRLs considered this an improvement over the method of reporting used in previous studies. Furthermore, for the EURL the data were easier to analyse.

1 Introduction

An important task of the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*), as laid down in Commission Regulation No 882/2004 (EC, 2004), is the organization of interlaboratory comparison studies to test the performance of the National Reference Laboratories (NRLs) for *Salmonella*. The history of the interlaboratory comparison studies as organized by EURL-*Salmonella* (formerly called CRL-*Salmonella*) since 1995 is summarized on our website (EURL-*Salmonella*, 2014).
The first and most important objective of the study, organized by the EURL for *Salmonella* in March 2013, was to see whether the participating laboratories could detect *Salmonella* at different contamination levels in boot socks with environmental material from a laying hen flock. This information is important in order to ascertain whether the examination of samples in the EU Member States (EU-MS) is carried out uniformly and comparable results can be obtained by all NRLs-*Salmonella*.
This study was a combined study with the CEN mandate study (Validation of Annex D of EN ISO 6579). The data were differently treated for the CEN mandate (which tested the performance of the study method) and for this EURL study (which tested the performance of the laboratories). This report describes the results of the EURL-*Salmonella* study. The results of the CEN mandate study are described in a separate report (Mooijman et al., in preparation).

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

There were some differences between the set-up of this study and that of earlier interlaboratory comparison studies on the detection of *Salmonella* spp. in veterinary, food and feed samples. For the current study, the (boot sock) samples were artificially contaminated with a diluted culture of *Salmonella* Typhimurium (STM) at the laboratory of the EURL-*Salmonella*, while in previous studies the participants had to mix matrix and reference material themselves, prior to analysis. Furthermore, more samples were tested than the minimum number of samples as described in CEN ISO /TS 22117 (Anonymous, 2010), to make this study also useful for the validation of the method (CEN mandate study).
Where CEN ISO/TS 22117 prescribes a minimum of six samples per contamination level (blank, low and high), in this study, eight samples per level had to be tested. Additionally, six control samples were included.

2 Participants

Country	City	Institute
Austria	Graz	Austrian Agency for Health and Food Safety (AGES IMED/VEMI)
Belgium	Brussels	Veterinary and Agrochemical Research Centre (VAR) CODA-CERVA
Bosnia-Herzegovina	Sarajevo	Veterinary Faculty of Sarajevo Department for Health Care of Poultry
Bulgaria	Sofia	National Diagnostic and Research Veterinary Institute (NDRVMI), National Reference Centre of Food Safety
Croatia	Zagreb	Croatian Veterinary Institute Poultry Centre, Laboratory for Bacteriology
Cyprus	Nicosia	Cyprus Veterinary Services Pathology, Bacteriology, Parasitology Laboratory
Czech Republic	Prague	State Veterinary Institute
Denmark	Ringsted	Danish Veterinary and Food Administration Microbiology Laboratory
Estonia	Tartu	Estonia Veterinary and Food Laboratory, Bacteriology-Pathology Department
Finland	Kuopio	Finnish Food Safety Authority Evira Research Department, Veterinary Bacteriology
France	Ploufragan	Anses-site de Ploufragan-Plouzané HQPAP Laboratoire d'Etudes et de Recherches Avicoles, Porcines et Piscicoles Unité Hygiène et Qualité des Produits Avicoles et Porcins
Germany	Berlin	Federal Institute for Risk Assessment (BfR) National Veterinary Reference Laboratory for *Salmonella*
Greece	Chalikida	Veterinary Laboratory of Chalikida
Hungary	Budapest	National Food Chain Safety Office, Food and Feed Safety Directorate, food microbiology
Iceland	Reykjavik	University of Iceland Institute, Keldur Institute for Experimental Pathology
Ireland, Republic of	Kildare	Central Veterinary Research Laboratory (CVRL/DAFFM) Laboratories Backweston, Department of Agriculture, Food and the Marine, Bacteriology
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
Macedonia, FYR of	Skopje	Food Institute, Faculty of Veterinary Medicine
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 Centre for Zoonoses and Environmental Microbiology (cZ&O)

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
Serbia	Belgrade	Institute of Veterinary Medicine of Serbia
Slovak Republic	Bratislava	State Veterinary and Food Institute Reference Laboratory for *Salmonella*
Slovenia	Ljubljana	National Veterinary Institute, Veterinary Faculty
Spain	Madrid Algete	Laboratorio Central de Veterinaria
Sweden	Uppsala	National Veterinary Institute (SVA), Department of Bacteriology
Switzerland	Bern	National Centre for Zoonoses, Bacterial Animal Diseases and Antimicrobial Resistance (ZOBA), Institute of veterinary bacteriology, Vetsuisse faculty Berne
United Kingdom	Addlestone	Animal Health and Veterinary Laboratories Agency (AHVLA)Weybridge, Bacteriology Department
United Kingdom	Belfast	Agri-Food and Bioscience Institute (AFBI) Veterinary Sciences Division Bacteriology

3 Materials and methods

3.1 Artificial contamination of boot sock samples

3.1.1 Pre-tests for preparation of boot sock samples

The matrix in this interlaboratory comparison study was boot socks, to which environmental material (mainly faeces) from a laying hen flock was added. The boot socks (Sodibox, Nevez, France) were artificially contaminated at the laboratory of the EURL-*Salmonella* with a diluted culture of *Salmonella.* As artificial contamination of samples with a diluted culture was not used in earlier studies, some tests were performed before the start of the study. It was tested how well *Salmonella* could be detected in the boot sock samples after moistening the boot socks with different solutions or without moistening them; in the presence of different amounts of background flora in the chicken faeces; when the samples were artificially contaminated with different *Salmonella* serovars at different levels; and during storage at different temperatures.
For this, two *Salmonella* serovars were tested: *S*. Typhimurium (STM) ATCC 14028 and *S*. Enteritidis (SE) ATCC 13076. The strains were obtained from the American Type Culture Collection (ATCC, Manassas, USA). Each strain was inoculated in Buffered Peptone Water (BPW) and incubated at (37 ± 1) °C overnight. Next, each culture was diluted in peptone saline solution to be able to inoculate the boot sock samples with approximately 5–10 CFU/sample and 50–100 CFU/sample. For the calculation of the contamination level (CFU/ml), 0.1 ml of the diluted culture was spread over an XLD plate and incubated at 37 °C for 20–24 hours.
Boot socks are often moistened before use. Therefore, to mimic routine sampling, the effect of moistening was also tested by adding 15 ml of different solutions, or no solution at all, to one pair of boot socks. The tested solutions were Buffered Peptone Water (ISO 6579, Anonymous, 2002), peptone saline solution (per 1L: 1.0 g Peptone and 8.5 g Sodium Chloride) and distilled water. After the solution had been added, the boot socks were stored at room temperature for one to several hours, to allow the fluid to thoroughly moisten the socks. Next, 10 g environmental material from a laying hen flock and a dilution of a *Salmonella* culture (different levels of STM or SE) were added to each pair of boot socks. Some control boot sock samples were also prepared, without the addition of environmental material and/or without the addition of *Salmonella* (blank boot sock samples).

The boot sock samples were stored at 5 °C, 10 °C and 15 °C for a period of 0, 7, 14 and 21 days. After each storage time at the different temperatures, the artificially contaminated SE, STM, blank and control boot sock samples were tested for the presence of *Salmonella* according to Annex D of ISO 6579 (Anonymous, 2007), with selective enrichment on MSRV. To have an indication of the influence of background flora in the samples, the blank boot sock samples (with environmental material, but without the addition of *Salmonella*) were tested for the number of aerobic bacteria and *Enterobacteriaceae*. For this purpose the ISO procedures for establishing the total number of aerobic bacteria (ISO 4833: Anonymous, 2003a) and for analysing the *Enterobacteriaceae* count (ISO 21528-2: Anonymous, 2004) were followed.

3.1.2 Determination of the contamination level of the boot sock samples by MPN

The level of contamination of the final boot sock samples, as used at the time of the study, was determined by using a five-tube most probable number (MPN)

technique. For this, tenfold dilutions of five boot sock samples of each contamination level, were tested representing 10 g, 1 g and 0.1 g of the original sample. The presence of *Salmonella* was determined in each dilution by following Annex D of ISO 6579 (Anonymous, 2007). From the number of confirmed positive dilutions, the MPN of *Salmonella* in the original sample was calculated, by using an MPN program in Excel, freely available on the Internet (Jarvis et al., 2010) was used.

3.2 Environmental material from a laying hen flock

3.2.1 General

Environmental material from a laying hen flock (mainly chicken faeces) was collected by the Animal Health Service (GD) Deventer at a *Salmonella*-free farm (SPF-farm). As a large amount of environmental material (approximately 15 kg) was needed, the GD collected five batches from the same flock at different times. The environmental material arrived at the EURL-*Salmonella* on 22nd January 2013, where it was homogenized and stored at 5 °C. Immediately after receipt, ten samples each of 25 g were taken randomly from the homogenized batch and checked for the absence of *Salmonella* following Annex D of ISO 6579 (Anonymous, 2007). For this purpose the ten 25 g samples were each added to 225 ml Buffered Peptone Water (BPW). After pre-enrichment at (37 ± 1) °C for 16–20 hours, selective enrichment was carried out on Modified Semi-solid Rappaport-Vassiliadis (MSRV) agar. Next, the suspect growth on MSRV plates was plated out on Xylose Lysine Deoxycholate agar (XLD) and Brilliance *Salmonella* Agar (BSA) and confirmed biochemically.

3.2.2 Total bacterial count in environmental material

The total number of aerobic bacteria in the environmental material was investigated by following ISO 4833 (Anonymous, 2003a). A portion of 20 g of the environmental material was homogenized in 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 two 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 environmental material

In addition to the total number of aerobic bacteria, the *Enterobacteriaceae* count was determined by following ISO 21528-2 (Anonymous, 2004). A portion of 20 g of the environmental material was homogenized in 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 two 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: boot socks with environmental material from a laying hen flock

Approximately two weeks before the study, a total of 1200 boot sock samples were prepared. For this, the following steps were performed:

- Labelling of each plastic bag, containing one pair of boot socks;
- Addition of 15 ml peptone saline solution to each pair of boot socks and storage of samples at room temperature overnight;
- Addition of 10 g environmental material to 960 pairs of pre-moistened boot socks and storage of samples at 5 °C for 1 to 2 days.
- Addition of approximately 0.1 ml of a diluted culture of *Salmonella* Typhimurium ATCC 14028 to a selection of the boot sock samples. The contamination levels aimed at were 10–15 CFU/sample, 50–60 CFU/sample and blank.

On 4th March 2013 (one week before the study) the boot sock samples (each pair of boot socks packed in a separate numbered plastic bag) were packed (see Section 3.3.2) and sent by door-to-door courier service to the participants. After arrival at the laboratories, the boot sock samples had to be stored at 5 °C until the start of the study. Further details of the mailing and handling of the samples and the reporting of the test results can be found in the protocol (EURL-*Salmonella*, 2013a), in the Standard Operation Procedure (SOP, EURL-*Salmonella*, 2013b) and in a print-out from the web-based test report (EURL-*Salmonella*, 2013c). The protocol, SOP and test report used during the study can be found on the EURL-*Salmonella* website or can be obtained through the corresponding author of this report.

Six control boot sock samples without environmental material (numbered C1-C6) and 24 boot sock samples with environmental material (numbered B1–B24) had to be tested by each participant. Table 1 shows the number of boot sock samples with and without the addition of environmental material in combination with the (artificial) contamination level of *Salmonella.*

Table 1. Overview of the number of boot sock samples tested per laboratory in the interlaboratory comparison study

Contamination level	Control boot socks (n=6) No matrix added	Test samples (n=24) with environmental material (chicken faeces)
S. Typhimurium low level (STM9)	2	8
S. Typhimurium high level (STM81)	2	8
Blank (BL)	2	8

3.3.2 Sample packaging and temperature recording during shipment

Each pair of boot sock samples was packed in a plastic bag. Next, the 30 bags of boot sock samples destined for each NRL were distributed over two plastic safety bags.

Both safety bags 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 service to the participants. For the control of exposure to abusive temperatures during shipment and storage, micro temperature loggers were used to record the temperature during transport. These loggers are tiny units sealed in a 16 mm diameter and 6 mm deep stainless steel case. Each shipping box contained one logger, packed in one of the safety bags. The loggers were programmed by the

EURL-*Salmonella* to measure the temperature every hour. Each NRL had to return the temperature recorder to EURL-*Salmonella* on the day the laboratory started the study. At the EURL-*Salmonella* the loggers were read using a special computer program and all recorded temperatures from the start of the shipment until the start of the study were transferred to an Excel sheet.

3.4 Methods

The prescribed method for this interlaboratory comparison study was Annex D of ISO 6579 (Anonymous, 2007). In addition, the NRLs were free to perform a Polymerase Chain Reaction (PCR) method.

The prescribed method in summary:

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

Selective enrichment on:
- Modified Semi-solid Rappaport-Vassiliadis medium (MSRV) ;

Plating-out on the following isolation media:
- Xylose Lysine Desoxycholate agar (XLD);
- second plating-out medium of choice;

Confirmation:
- 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 boot sock samples. The specificity, sensitivity and accuracy rates were calculated according to the following formulae:

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

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

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

For the determination of 'good performance' per laboratory, the results found with the selective enrichment medium MSRV together with all combinations of isolation media used by the laboratory were taken into account. For example, if a laboratory found for the STM low level with matrix 6/8 samples positive with MSRV/BGA, but no positive samples with MSRV/XLD, this was still considered a good result. The opposite was used for the blank samples. Here also, all combinations of media used per laboratory were taken into account. If, for

example, a laboratory found 2/8 blank samples positive with MSRV/BGA but no positive samples with the other media, this was still considered a ‘no-good’ result. The results will therefore be presented for selective enrichment on MSRV in combination with the isolation medium (XLD or non-XLD) that gave the highest number of *Salmonella* isolations (MSRV/x).

Table 2. Criteria for testing good performance in the primary production study XVI (2013)

Minimum result		
Contamination level	Percentage positive	No. of positive samples/ total no. of samples
Control samples: boot socks, no matrix		
STM high	100%	2/2
STM low	50%	1/2
Blank control	0%	0/2
Samples: boot socks with environmental material		
STM high	80%	7/8
STM low	60%	5/8
Blank[1]	15% at max[1]	1/8 at max[1]

1. All should be negative. However, as no 100% guarantee of the *Salmonella* negativity of the matrix can be given, 1 positive out of 8 blank samples (15% pos.) is considered acceptable.

4 Results

4.1 Artificial contamination of boot sock samples

4.1.1 Pre-tests for preparation of boot sock samples

Seven sets of experiments were performed. During each set of experiments the stability of *Salmonella* in the boot sock samples was tested during storage of the samples at different temperatures, up to three weeks. During each set of experiments, different variables were tested in different combinations (see Section 3.1.1).

The major findings are summarized below:

Salmonella Typhimurium (STM) was shown to be more stable in the boot sock samples artificially contaminated with environmental material than *S.* Enteritidis (SE).
- All five low-contamination STM samples (5 CFU/boot sock) tested positive after 15 days of storage at 5 ºC.
- No positive results were found from the five low-contamination SE samples (5 CFU/boot sock) after 7 days of storage at 5 ºC.

All subsequent experiments were therefore performed with *S*. Typhimurium only.

Moistening of the boot sock samples prior to the addition of the environmental material and the *Salmonella* culture resulted in more stable samples but no differences were found between the tested solutions: peptone saline solution, BPW and distilled water.
- The background flora in the environmental material on the moistened boot socks was 1 log higher after 14 days of storage at 5 ºC or 15 ºC than the background flora in the environmental material on dry boot socks.
- A few more *Salmonella* positive samples were found after 21 days of storage at 5 ºC or 15 ºC in moistened boot socks than in dry boot socks.

All subsequent experiments were therefore performed with the addition of 15 ml peptone saline solution.

The results of the different stability experiments are summarized in Figure 1. This figure shows relatively good stability of the artificially contaminated boot sock samples when stored at 5 ºC and at 10 ºC. After 14 days, 4–5/5 samples of both low- and high-contaminated samples were tested positive for *Salmonella*. A longer storage time (21 days) and/or storage at a higher temperature (15 ºC) resulted in a lower number of positive samples. This was most clear when the contamination level of the samples was below 10 CFU.

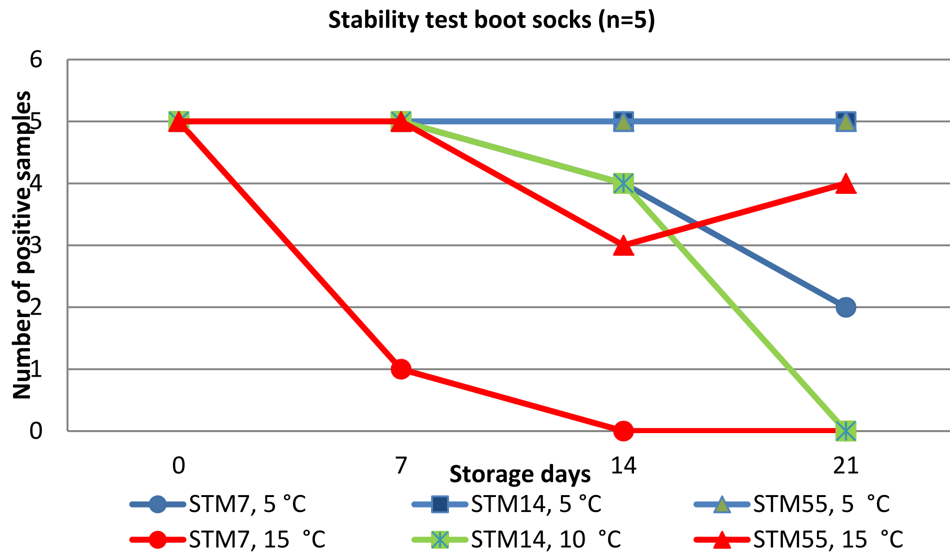


Figure 1. Stability test on boot sock samples artificially contaminated with Salmonella *Typhimurium (STM)*

From the results of the experiments, it was decided to use the following samples for the interlaboratory comparison study:
- One pair of boot socks in a plastic bag combined with 15 ml of peptone saline solution;
- 10 g of environmental material from a laying hen flock;
- artificially contaminated with a diluted culture of:
    - low-level STM (10–15 CFU/pair of boot socks)
    - high-level STM (50–100 CFU/pair of boot socks)
    - blank (0 CFU/pair of boot socks).

4.1.2 *Contamination level of the artificially contaminated boot sock samples*

Table 3 shows the contamination level of the low- and high contaminated boot sock samples. The inoculum level of the diluted STM culture (tested on XLD) as well as the contamination level in the boot sock samples after the inoculation with the diluted culture were tested. The latter was tested with a five-tube MPN test (see Section 3.1.2). The number of positive boot sock samples for 10 g, 1 g and 0.1 g were, respectively, for the low-level STM 5/5, 1/5 and 0/5 and for high-level STM 5/5, 5/5 and 4/5. The calculated MPN/pair of boot socks is given in Table 3.

Table 3. Number of Salmonella Typhimurium (STM)

Date of testing	Low-level STM CFU/pair of boot socks	High-level STM CFU/pair of boot socks
1st March 2013 (inoculum of boot socks)	9	81
11th March 2013 after storage at 5 °C MPN of boot socks with environmental material inoculated with STM (95% confidence limit)	3.3 (1.1–10)	160 (53–490)

4.2 Environmental material (from a laying hen flock)

The environmental material was tested negative for *Salmonella* and stored at 5 °C. On Monday 4th March 2013 the boot sock samples were sent to the NRLs. After receipt, the NRLs had to store the boot sock samples at 5 °C. The number of aerobic bacteria and the number of *Enterobacteriaceae* were tested twice; first on the day the environmental material arrived at the EURL (22/01/2013) and second, after storage at 5 °C, on the planned date of the interlaboratory comparison study (11/03/2013). Table 4 summarizes the results, showing that the amount of background flora remained stable even after storage of more than one month.

Table 4. Number of aerobic bacteria and number of Enterobacteriaceae per gram of environmental material from a laying hen flock

Date	Aerobic bacteria CFU/g	*Enterobacteriaceae* CFU/g
22nd January 2013	2×10^7	1×10^4
11th March 2013 after storage at 5 °C	1×10^7	1×10^4

4.3 Technical data: interlaboratory comparison study

4.3.1 General

In this study, 36 NRLs for *Salmonella* participated: 28 NRLs from 27 EU-MS and 8 NRLs from non-EU MSs. The non-EU MSs consisted of EU candidate countries, member countries of the European Free Trade Association State (EFTA) and, at the request of DG-Sanco, a country outside Europe.
Thirty-four laboratories performed the study on the planned date (week 11 starting on 11/03/2013). Two laboratories (lab codes 1 and 20) performed the study one week earlier.

4.3.2 Accreditation/certification

Thirty-three laboratories were accredited for their quality system according to ISO/IEC 17025 (Anonymous, 2005) and three EU-MS laboratories (16, 18 and 34) were in the process of accreditation. Thirty-three laboratories were accredited for Annex D of ISO 6579; 15 were accredited for ISO 6579.

4.3.3 Transport of samples

Twenty-nine participants received the samples within one day of dispatch and seven participants within two days. For five parcels (non-EU-MS) it was not possible to arrange door-to-door transport. The parcel for Laboratory 27 was delayed for one day at the airport because of bad weather. The parcel for

Laboratory 19 was delayed for three days at customs. For three participants the parcels were transported to an NRL in a neighbouring country (door-to-door). These parcels were picked up by the relevant NRL the day after arrival at the first NRL and needed some extra hours of transport. The majority of the NRLs returned the temperature recorders to the EURL-*Salmonella* at the time they started the study, as requested. Seven participants returned the temperature recorder immediately after the arrival of the material at their institute (as in earlier studies). For the majority of the parcels, the temperature did not exceed 5 °C during transport and storage at the NRL. The exceptions were Laboratory 19, where the sample was stored for two days at between 10 °C and 16 °C, and Laboratories 8, 10, 27 and 35, where the samples were stored for a few hours between 5 °C and 10 °C.

4.3.4 Media

Each laboratory was asked to test the samples using 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 their own choice.

Table 5 gives information on the pH, the concentration of Novobiocin and the incubation time that are prescribed for BPW and MSRV. The table lists only the deviations from the prescribed method that were reported.
Two laboratories (24 and 27) reported an excessive incubation time of the pre-enrichment in BPW.
Six laboratories (5, 19, 22, 24, 25 and 27) reported a pH of 7.3 instead of the prescribed maximum pH of 7.2 for BPW.
Three laboratories (6, 23 and 29) used MSRV with a higher concentration of Novobiocin than the prescribed 0.01 g/L.
Four laboratories (7, 17, 18 and 19) reported a higher pH (5.5–5.6) for the MSRV than the prescribed maximum pH of 5.4.
Laboratories 15 and 20 did not report the pH of the media.

Table 5. Reported technical deviations from the prescribed *procedure*

Lab code	BPW		MSRV	
	Incubation time (h:m)	pH	pH	Novobiocin
Prescribed in ISO 6579 Annex D	**16–20 h**	**6.8–7.2**	**5.1–5.4**	**10 mg/L**
5	19:00	7.3	5.1	10
6	19:36	7	5.1	20
7	18:15	-	5.6	10
15	18:00	-	-	10
17	18:00	6.9	5.5	10
18	18:15	7.2	5.5	10
19	20:00	7.3	5.5	10
20	17:20	-	-	10
22	20:00	7.3	5.3	10
23	19:30	7.2	5.2	50
24	21:55	7.3	5.4	10
25	19:30	7.3	5.2	10
27	21:30	7.3	5.0	10
29	18:27	7	5.2	20

Grey cells Deviating from ISO 6579 Annex D

- No information

A second plating-out medium of choice was obligatory. Table 6 shows the second isolation media used by the participants. Most laboratories used BGA (Anonymous, 1993) or a Chromogenic medium as a second plating-out medium.

Table 6 Media used as second plating-out medium

Media	Number of users	Lab code
BGA^{mod} (ISO 6579, 1993)*	16	2, 3, 5, 6, 7, 8, 11, 12, 16, 19, 23, 24, 25, 31, 32
BGA	6	4, 13, 28, 29, 30, 33
SM(ID)2 (=Chrom ID Salm)	4	10, 20, 34, 36
Rambach	3	14, 17, 35
BSA (=OSCM)	3	1, 22, 27
RS	3	9, 15, 18
ASAP	1	26
BxLH	1	21

Explanations of the abbreviations are given in the 'List of abbreviations'.

* BGA^{mod} is also called BPLS or BGPA.

The use of an extra non-selective plating agar between the 'isolation' and 'confirmation' steps was optional. A total of 21 laboratories performed this extra step (e.g. by using Nutrient agar ISO 6579: Anonymous, 2002).

All participating laboratories performed confirmation tests for *Salmonella*: biochemically, serologically or both. Tables 7 and 8 summarize the confirmation media and tests. Four laboratories (17, 21, 31 and 36) performed serological tests only and eight laboratories (1, 5, 6, 7, 20, 22, 24 and 34) performed only a biochemical test.

Table 7. Biochemical and other confirmation tests for Salmonella

Lab code	TSI	UA	LDC	Gal	VP	Indole	Kit	Other
1, 7, 25, 34	+	+	+	-	-	-		
2	+	-	+	-	-	-	RapiD 20E	
3, 9, 13, 28, 35	+	+	+	+	+	+		
4	+	-	-	-	-	-	Enterotest	MALDI-TOF
5	-	-	-	-	-	-		Kohns No1 Medium
6	+	+	+	-	-	+		Glucose
8	-	-	-	-	-	+	Enterotest	PCR
10	+	-	-	-	-	-		
11	+	+	+	-	-	+		MacConkey
12	+	+	+	+	-	+		
14, 26	-	-	-	-	-	-	API20E	PCR
15	+	-	+	-	-	-		Sorbitol-Mobility
16	+	-	-	-	-	-	Microgen GN – ID A Panel	Automatic Identification System Vitek
17, 21, 31, 36	-	-	-	-	-	-		
18	-	-	-	-	-	-	API20E	
19	+	+	+	+	-	-		PCR
20	-	-	-	-	-	-	MICROBACT 12A	
21	-	-	-	-	-	-		Kligler agar, urea, indol mannitol, nitrate broth, ONPG FDA medium, motility test
22	-	-	-	-	-	-		MALDI-TOF
23	+	+	+	-	-	+		
24	+	+	+	-	-	+		SIM medium, Simmons Citrate ag.
27	+	+	-	-	-	-		PCR, LIA agar
28, 30	+	+	+	+	+	+		PCR
29	+	+	+	+	-	+		semi-solid glucose agar
32	-	-	-	-	-	-		Chromagar
33	+	+	+	+	-	+		PCR

Table 8. Serological confirmation of Salmonella

Lab code	Serological		
	O antigens	**H antigens**	**Vi antigens**
1, 5, 6, 7, 20, 22, 24, 30, 34	-	-	-
3, 4, 9, 10, 11, 14, 16, 17, 23, 26, 29, 31, 33, 35	+	+	-
8, 12, 13, 15, 19, 21, 25, 32	+	-	-
18, 36	+	-	+
28	+	+	+
2	Polyvalent somatic A-E group, Vi antisera		
27	Latex agglutination		

4.4 Control samples

4.4.1 General

Table 9 gives the results of all control samples (boot socks without the addition of environmental material). The results given in the table are the highest number of positive isolations found with MSRV in combination with any isolation medium (MSRV/x). There was no difference between the scores of the different isolation media used: XLD or non-XLD (e.g. BGA).

Table 9. Total number of positive results of the control samples (boot socks without the addition of environmental material) per laboratory

Lab code	The highest number of positive isolations found with MSRV in combination with any isolation medium (MSRV/x)		
	Blank n=2	STM Low n=2	STM High n=2
Good performance	**0**	**≥ 1**	**2**
35	0	**1**	2
1–34, 36	0	2	2

Bold number = deviating result.

Blank boot sock samples, without the addition of environmental material (n=2)
All laboratories correctly analysed the blank boot sock samples negative for *Salmonella* irrespective of the media used.

S. Typhimurium (STM low) boot sock samples without addition of environmental material (n=2)
All laboratories except one tested all low-contamination control boot sock samples positive for *Salmonella*. Laboratory 35 (non-EU-MS country) did not detect *Salmonella* in one of the two low level contaminated control samples.

S. Typhimurium (STM high) boot sock samples without addition of environmental material (n=2)
All participating laboratories tested the two control boot sock samples containing *Salmonella* Typhimurium at an inoculum level of approximately 81 CFU/sample positive.

The results were compared with the definition of ‘good performance’ (see section 3.6). All laboratories fulfilled the criteria for the control samples.

4.4.2 *Specificity, sensitivity and accuracy rates of the control samples*

Table 10 shows the specificity, sensitivity and accuracy rates for the control boot sock samples without the addition of environmental material. 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. Only minor differences were found between these groups.
The laboratories scored an excellent result for the control samples with an accuracy rate of 99.5%.

Table 10. Specificity, sensitivity and accuracy rates of the control samples (without the addition of environmental material) for the selective enrichment on MSRV

Control boot sock samples		MSRV/X All participants n=36	MSRV/X EU-MS n=28
Blank n=2	No. of samples No. of negative samples **Specificity in %**	72 72 **100**	56 56 **100**
STM low n=2	No. of samples No. of positive samples **Sensitivity in %**	72 71 **98.6**	56 56 **100**
STM high n=2	No. of samples No. of positive samples **Sensitivity in %**	72 72 **100**	56 56 **100**
All boot sock samples with *Salmonella*	No. of samples No. of positive samples **Sensitivity in %**	144 143 **99.3**	112 112 **100**
All boot sock samples	No. of samples No. of correct samples **Accuracy in %**	216 215 **99.5**	168 168 **100**

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

4.5 Results for boot sock samples with environmental material artificially contaminated with *Salmonella*

4.5.1 *Results per level of* Salmonella *and per laboratory*

General

Table 11 gives the results of the boot sock samples to which artificially contaminated (with STM*)* environmental material from a laying hen flock was added (10 g/pair of boot socks). The results given in this table are the highest number of positive isolations found with MSRV in combination with any isolation medium (MSRV/x). There was no difference between the scores of the different isolation media used: XLD or non-XLD (e.g. BGA).

The majority of the laboratories (19/36) found all boot sock samples with artificially contaminated environmental material positive for *Salmonella* using the prescribed method, MSRV.

Blank boot sock samples with environmental material (n=8)
All laboratories except one correctly found the blank boot sock samples with environmental material negative for *Salmonella*. Laboratory 16 found one blank sample positive for *Salmonella.* All blanks should test negative. However, as no 100% guarantee of the *Salmonella* negativity of the environmental material could be given, 1 positive out of 8 blank samples (85% neg.) was considered acceptable.

S. Typhimurium (STM low) boot sock samples with environmental material (n=8)
Twenty-four laboratories were able to isolate *Salmonella* from all the eight boot sock samples containing *Salmonella* Typhimurium at an inoculum level of approximately 9 CFU/pair of boot socks with environmental material. Twelve laboratories (5, 6, 7, 8, 13, 15, 22, 29, 30, 31, 33 and 34) did not detect *Salmonella* in one of the eight low level contaminated boot sock samples with environmental material and two laboratories (10 and 18) missed *Salmonella* in two of the eight low-level samples.

S. Typhimurium (STM high) boot sock samples with environmental material (n=8)
Thirty-one laboratories isolated *Salmonella* from all the eight boot sock samples containing *Salmonella* Typhimurium at an inoculum level of approximately 81 CFU/pair of boot socks with environmental material. Five laboratories (3, 20, 22, 25 and 30) did not detect *Salmonella* Typhimurium in one of the eight highlevel contaminated boot sock samples with environmental material.

The results of the artificially contaminated boot sock samples with environmental material were compared with the definition of ‘good performance’ (see Section 3.6) and all laboratories fulfilled these criteria for the prescribed method (MSRV).

Table 11. Number of positive results found with the artificially contaminated boot sock samples (10 g environmental material/pair of boot socks) per laboratory

Lab code	Highest number of positive isolations found with MSRV in combination with any isolation medium (MSRV/x)		
	Blank n=8	STM Low n=8	STM High n=8
Good performance	**≤1**	**≥5**	**≥7**
1, 2	0	8	8
3	0	8	**7**
4	0	8	8
5–8	0	**7**	8
9	0	8	8
10	0	**6**	8
11, 12	0	8	8
13	0	**7**	8
14	0	8	8
15	0	**7**	8
16	**1**	8	8
17	0	8	8
18	0	**6**	8
19	0	8	8
20	0	8	**7**
21	0	8	8
22	0	**7**	**7**
23, 24	0	8	8
25	0	8	**7**
26–28	0	8	8
29	0	**7**	8
30	0	**7**	**7**
31	0	**7**	8
32	0	8	8
33, 34	0	**7**	8
35, 36	0	8	8

Bold number = deviating result.

4.5.2 *Results per medium, per level of contamination and per laboratory*

Figures 2 and 3 show the number of positive isolations per type of artificially contaminated boot sock sample (with environmental material) and per laboratory after pre-enrichment in BPW and selective enrichment on MSRV followed by isolation on selective plating agar.

The results of all artificially contaminated boot sock samples with environmental material were compared with the proposed definition of 'good performance' (see Section 3.6). In Figures 2 and 3 the border of good performance is indicated by a black horizontal line.

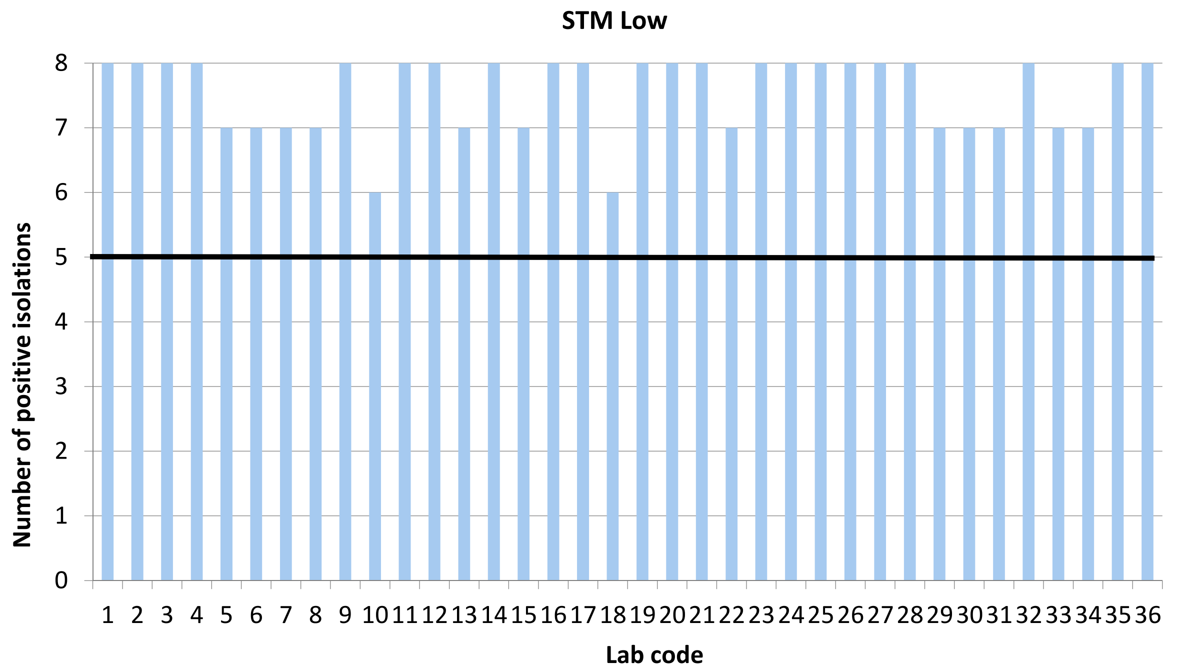
Table 12 presents the results of the number of positive isolations after 24 and 48 hours of incubation of the selective enrichment medium, MSRV. Depending on the level of contamination, 2–3% more positive results were found after 48 hours of incubation than after 24 hours of incubation. Laboratory 15 found 50% more positive results after 48 hours of incubation (24 h: 8 positive samples; 48 h: 16 positive samples). If the results of this laboratory are not taken into account, the overall increase in positive results after 48 hours of incubation was only 1–2%.

Table 12. Number and percentages of positive results found for the artificially contaminated boot sock samples after 24 hours and 48 hours of incubation on MSRV

	Selective enrichment medium MSRV	
Level of contamination	**Number of positive samples after 24/48 h incubation**	**% of positive samples after 24/48 h incubation**
Blank	286/287	0/0.3%
STM low	263/272	91/94%
STM high	277/283	96/98%

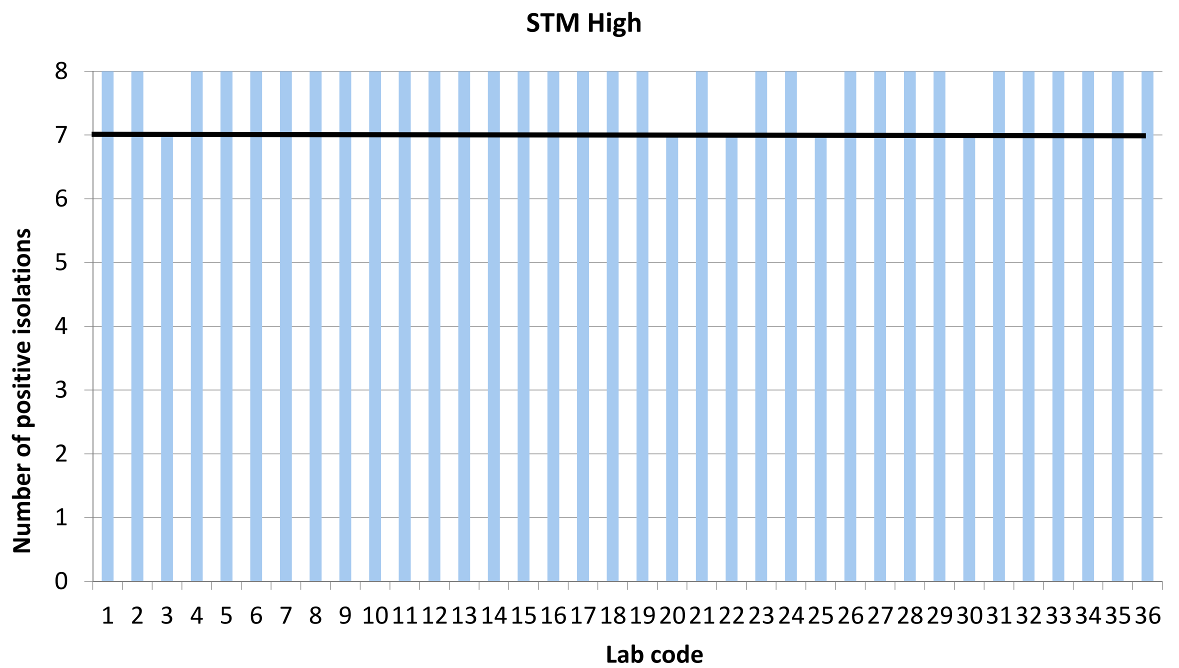
4.5.3 *Specificity, sensitivity and accuracy rates of the artificially contaminated samples*

Table 13 shows the specificity, sensitivity and accuracy rates for all types of artificially contaminated boot sock samples with environmental material. This table gives the results for the different medium combinations: pre-enrichment in BPW, followed by selective enrichment on MSRV and isolation on selective plating agar showing the highest number of positives (MSRV/x). The calculations were performed on the results of all participants and on the results of the participants of the EU-MS only. Only minor differences were found between these groups. The specificity rate (almost 100%) and the sensitivity rates (low level: 94%; high level 98%) were high for the whole group of participants.



▬ = border of good performance

Figure 2. Results per laboratory of boot sock samples with environmental material artificially contaminated with STM low (n=8) after selective enrichment on MSRV followed by isolation on the 'best' selective plating agar



▬ = border of good performance

Figure 3. Results per laboratory of boot sock samples with environmental material artificially contaminated with STM high (n=8) after selective enrichment on MSRV followed by isolation on the 'best' selective plating agar

Table 13. Specificity, sensitivity and accuracy rates of the artificially contaminated boot sock samples with environmental material after selective *enrichment on MSRV*

Boot sock samples with environmental material (10 g/pair of boot socks)		**MSRV/X All participants n=36**	**MSRV/X EU-MS n=28**
Blank n=8	No. of samples	288	224
	No. of negative samples	287	223
	Specificity in %	**99.7**	**99.6**
STM low n=8	No. of samples	288	224
	No. of positive samples	272	213
	Sensitivity in %	**94.4**	**95.1**
STM high n=8	No. of samples	288	224
	No. of positive samples	283	221
	Sensitivity in %	**98.3**	**98.7**
All boot sock samples with *Salmonella*	No. of samples	576	448
	No. of positive samples	555	434
	Sensitivity in %	**96.4**	**96.9**
All boot sock samples	No. of samples	864	672
	No. of correct samples	828	657
	Accuracy in %	**95.8**	**97.8**

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

4.6 PCR (own method)

Nine laboratories (8, 14, 19, 26, 27, 28, 30, 31 and 33) applied a PCR method as an additional detection technique. All these laboratories except two tested the samples after pre-enrichment in BPW. Laboratories 19 and 31 started the DNA extraction after selective enrichment on MSRV. All laboratories used a real-time PCR, except two (14 and 19), which used a (conventional) PCR with reference to Rahn et al. (1992). Six of the nine laboratories used a validated PCR method. Reference was made to certificate numbers and/or to ISO 16140 (Anonymous, 2003b). Four of the laboratories used the PCR routinely for testing of 40 to 550 samples per year. Table 14 gives further details of the PCR techniques used.

Table 14. Details of Polymerase Chain Reaction procedures used as own method during the interlaboratory comparison study by nine participants

Lab code	Real-time PCR	Conventional PCR	Validated	Commercially available	Routinely used number/year	Reference
8	+		+	-	550	Malorny et al., 2004; Lofstrom et al., 2010; Lofstrom and Hoorfar, 2012
14		+	+	-	-	Rahn et al., 1992
19		Three steps	-	+	50	Rahn et al., 1992
26	+		+	-	-	Hein et al., 2006
27	+		-	-	-	Malorny et al., 2007
28	+		+	+	-	Lauer et al., 2009
30	+		+	-	40	
31	+		+	-	89	Malorny et al., 2004
33	+		-	-	-	

Table 15. Number of positive results found for the artificially contaminated boot sock samples with environmental material by using a PCR technique and the bacteriological culture technique (n=24)

	Lab 8, 31		Lab 14		Lab 19, 26		Lab 28		Lab 30		Lab 33	
	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR	BAC	PCR
STM low (n=8)	**7**	**7**	8	**3**	8	8	8	**7**	**7**	**7**	**7**	**7**
STM high (n=8)	8	8	8	**7**	8	8	8	8	**7**	**7**	8	**7**
Blank (n=8)	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 PCR method in comparison with the bacteriological culture technique (BAC)

Note: Laboratory 27 did not report the PCR results.

Table 15 gives the results of both the PCR method and the bacteriological culture technique (BAC). Laboratory 27 did not report the results from the PCR method. Five laboratories (8, 19, 26, 30 and 31) found the same results with the PCR method as with the bacteriological culture method (MSRV). The other laboratories (14, 28 and 33) found more samples negative with the PCR method than with BAC. Laboratory 33 found different low level contaminated STM samples negative for the PCR and the bacteriological detection method.

4.7 Performance of the NRLs

All NRLs fulfilled the criteria of good performance for the prescribed MSRV method.

5 Discussion

Artificial contamination of samples with a diluted culture
After many years of using 'capsule' or 'lenticule disc' reference materials to artificially contaminate the matrix in the interlaboratory comparison studies of the EURL-*Salmonella*, it was decided to change to artificial contamination of the samples with a diluted culture at the laboratory of the EURL. The main reason for this change was to better mimic 'real life' routine samples and to enable easier handling of the study-samples for the participants.

As this type of sample had not been used before, several experiments were performed prior to the study to test the stability of the contaminated samples. Stability was tested at storage temperature (5 ºC) and at higher temperatures (10–15 ºC) to check the effect of possibly abusive temperatures during transport. Experiences from earlier studies had shown that in general the transport time of the parcels to the NRLs is 1–2 days at temperatures that remain most of the time below 10 ºC. Just occasionally, the temperature of a parcel during transport may be at ≥15 ºC for a few hours. The pre-tests in this study showed that artificial contamination of the boot sock samples with a diluted culture of *S*. Typhimurium resulted in sufficiently stable samples for use in the interlaboratory comparison study. As the samples inoculated with approximately 7 CFU STM/pair of boot socks showed a rapid decrease in the number of positives after one week of storage at 15 ºC, it was decided to increase the inoculation level of the low contaminated samples. Boot sock samples with environmental material, inoculated with a diluted culture of *S*. Typhimurium of 10–15 CFU proved to be best suited to the study. MPN determination of the mean contamination level in the samples indicated that this higher inoculum level was necessary to retain a sufficient number of *S*. Typhimurium in the samples until the time of the study. The MPN calculated for the low level contaminated samples was 1.1–10 MPN/per boot sock at the day the study. Although an MPN calculation gives only a rough estimation of the contamination level (Jarvis et al., 2010), it suggested that the final level of STM was somewhat lower than the inoculum of 9 CFU/pair of boot socks and was close to the detection limit.

Transport of the samples
To prevent the level of *Salmonella* Typhimurium decreasing during transport, the materials were packed with frozen cooling elements and transported by courier service. The information provided by the temperature recorders included in the parcels showed that the temperature in the parcels remained below 5 ºC for most of the transport time. Therefore, it can be assumed that transport did not negatively affect the mean contamination level of the samples. This was confirmed by the fact that the laboratory with the longest transport time in combination with the highest temperatures (lab code 19) still found all contaminated samples positive.

According to EC regulations 882/2004 (EC, 2004) and 2076/2005 (EC, 2005), each NRL should have been accredited in their relevant field before 31st December 2009. Thirty-three laboratories were accredited. Three (EU-MS) participants (lab codes 16, 18 and 34) were still in the process of accreditation, which is relatively late.

Performance of the laboratories
For the evaluation of the laboratories in terms of 'good performance', the best performing isolation medium after selective enrichment on MSRV (being the medium with the highest number of positive isolations) was taken into account.

Only one participant (Laboratory 16) scored a positive result for *Salmonella* in one blank boot sock sample with environmental material. This was considered acceptable, as no 100% guarantee of the *Salmonella* negativity of the matrix could be given. An explanation for this one false positive sample may be cross-contamination or misinterpretation of the results. The high number of background flora (especially *Enterobacteriaceae*) in the matrix may have caused problems reading the isolation media. In combination with a limited confirmation, the *Enterobacteriaceae* present in the matrix can be misinterpreted as *Salmonella*, resulting in a false positive blank result.
Only two laboratories (10 and 18) missed *Salmonella* in two out of eight low-level contaminated samples. As the contamination level in the final samples was close to the detection limit, this was considered an acceptable result.

According to the pre-set criteria, all laboratories scored 'good performance'.

Specificity, sensitivity and accuracy rates
The calculations were performed on the results of all participants and on the results of only the EU-MS. Minor differences (if any) were found between these groups.
All rates were high (varying between 94% and 100%).

The sensitivity rates may be influenced by the contamination level of the target organism, as well as by the level of disturbing background flora. For example, in the veterinary study of 2012 the level of background flora was 10 times higher than in the current study and the contamination level of the low-level STM samples was comparable to the current study, resulting in a sensitivity rate of 89% (Kuijpers and Mooijman, 2013) compared with 94% in the current study.

Media and incubation
Deviations in media composition or incubation temperature were reported but no or minor effects were seen on the results.
The increase in the number of positive results after 48 hours of incubation of the selective enrichment on MSRV was 2–3%. The majority of the laboratories found all samples positive after 24 hours of incubation. Only one NRL found a strong increase: 50% more positives after 48 hours of incubation.

PCR
Nine laboratories used a PCR technique in addition to the prescribed method. Five found the same results as with the bacteriological culture technique (BAC). Three laboratories found more results negative with their PCR method than with BAC. One of them did not report the results from the PCR method. The PCR results from these eight participants were not affected by the choice of PCR technique. No relation was seen between the sensitivity of the PCR technique and whether the technique was proprietary and/or had been validated. Nor was a difference visible between DNA extraction from a BPW culture and from an MSRV culture.
The best results were found by the laboratories that use a PCR technique routinely.
In comparison with former EURL-*Salmonella* interlaboratory comparison studies for the detection of *Salmonella* in samples from primary production (chicken faeces and pig faeces), a small increase was seen in the number of NRLs using a PCR technique as own method. In earlier studies, four or five laboratories used a PCR technique in addition to the prescribed method compared with nine laboratories in the current study (Kuijpers and Mooijman, 2011 & 2013).

Evaluation of this study
Artificial contamination of the matrix with a diluted culture at the laboratory of the EURL-*Salmonella* was successful. The samples were easier for the participants to handle and mimicked 'real life' samples more closely than the samples used in earlier studies by the EURL-*Salmonella*. Although the

preparation of this kind of sample is more complicated for the EURL, the advantages for the participants are significant. Therefore, it will be investigated whether the same method of contaminating samples can be used for other (future) detection studies.
The reporting of the results in the form of a web-based test report was used for the first time in a detection study and was well received by the participants. Furthermore, the data were easier for the EURL to analyse. Continuation of this method of reporting will be considered and, as necessary, it will be optimized for future studies.

6 Conclusions

- All NRLs for *Salmonella* were able to detect high and low levels of *Salmonella* in boot sock samples with environmental material (from a laying hen flock) with the prescribed MSRV method.
- The accuracy, specificity and sensitivity rates of the control samples (without environmental material) after selective enrichment on MSRV of the NRLs from the EU-MS were 100%.
- The specificity rate of boot sock samples with environmental material was almost 100% when tested according to the prescribed method (MSRV) by all participants.
- The sensitivity rate of the boot sock samples with environmental material artificially contaminated with high-level *S*. Typhimurium was 98% to 99% for the prescribed MSRV method.
- The sensitivity rates of the boot sock samples with environmental material artificially contaminated with low-level *S*. Typhimurium was only approximately 4% lower than the rates of the high-contaminated samples.
- 48 hours of incubation of the selective enrichment medium MSRV showed overall 2–3% more positive results than 24 hours of incubation. One participant found 50% more positive results after 48 hours of incubation. When the results of this laboratory are not taken into account, the increase is only 1–2%.
- The accuracy rate of the artificially contaminated boot sock samples with environmental material of the NRLs from the EU-MS was 98% after selective enrichment on MSRV.
- Samples artificially contaminated with a diluted culture mimicked ‘real life’ routine samples more closely and were easier for participants to use than the previously used mixtures of matrix and reference materials.
- The use by the participants of a web-based test report for reporting the results was successful. No (major) problems were indicated by the participants with this method of reporting, which was used for the first time in a detection study. Furthermore, digital reporting lowers the risk of transcription errors during analysis of the results.

List of abbreviations

ASAP	AES *Salmonella* Agar Plate
ATCC	American Type Culture Collection
BAC	Bacteriological Culture technique
BGA(mod)	Brilliant Green Agar (modified)
BGPA	Brilliant Green Phenol Agar
BPLS	Brilliant Green Phenol-red Lactose Sucrose
BPW	Buffered Peptone Water
BSA	Brilliance *Salmonella* Agar (OSCM)
BxLH	Brilliant green, Xylose, Lysine, Sulphonamide
CEN	Comité Européen de Normalisation (European Committee for Standardization)
CFU	Colony-Forming Units
EC	European Commission
EFTA	European Free Trade Association
EU	European Union
EURL	European Union Reference Laboratory
Gal	Galactosidase
ISO	International Organization for Standardization
LDC	Lysine Decarboxylase
MPN	Most Probable Number
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*
SE	*Salmonella* Enteritidis
SM (ID)2	*Salmonella* Detection and Identification-2
SPF	Specific Pathogen Free
SOP	Standard Operating Procedure
STM	*Salmonella* Typhimurium
TSI	Triple Sugar Iron agar
UA	Urea Agar
VP	Voges-Proskauer
VRBG	Violet Red Bile Glucose agar
XLD	Xylose Lysine Deoxycholate agar

References

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

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

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

Anonymous (2010). ISO 22117. Microbiology of food & animal feeding stuffs - Specific requirements & guidance for Proficiency Testing (PT) by interlaboratory comparison. International Organization for Standardisation, Geneva, Switzerland.

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. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2004R0882:20060525:EN:PDF (30 December, 2013)

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. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2005:338:0083:0088:EN:PDF (30 December, 2013)

EURL-*Salmonella* (2013a). Protocol Interlaboratory Comparison Study on the detection of *Salmonella* spp. in samples from the primary production organised by EURL-*Salmonella* STUDY XVI – 2013. http://www.eurlsalmonella.eu/Proficiency_testing/Detection_studies_Faeces (10 December, 2013)

EURL-*Salmonella* (2013b). Standard operating procedure (SOP) Interlaboratory Comparison Study on the detection of *Salmonella* spp. in samples from the primary production organised by EURL-*Salmonella* STUDY XVI – 2013. http://www.eurlsalmonella.eu/Proficiency_testing/Detection_studies_Faeces (10 December, 2013)

EURL-*Salmonella* (2013c). Testreport Interlaboratory Comparison Study on the detection of *Salmonella* spp. in samples from the primary production organised by EURL-*Salmonella* STUDY XVI – 2013. http://www.eurlsalmonella.eu/Proficiency_testing/Detection_studies_Faeces (10 December, 2013)

EURL-*Salmonella* (2014). History of EURL-*Salmonella* interlaboratory comparison studies on the detection of *Salmonella*. http://www.eurlsalmonella.eu/Publications/Inter-laboratory_comparison_study_Reports (10 January, 2014)

Hein I, Flekna G, Krassnig M, 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 (2006) 538-547.

Jarvis, B., Wilrich, C., Wilrich, P.-T. (2010) Reconsideration of the derivation of most probable numbers, their standard deviations, confidence bounds and rarity values. J. Appl. Microbiol. 109:1660–1667.
Link to MPN calculation programme: http://www.wiwiss.fu-berlin.de/institute/iso/mitarbeiter/wilrich/index.html (10 January, 2014)

Josefsen MH, Krause M, Hansen F, and Hoorfar J (2007) Optimaization of a 12-Hour TagMan PCR-Based Method for Detection of *Salmonella* Bacteria in Meat. Appl. Environ. Microbiol. May 2007 vol. 73 no. 9 3040-3048.

Kuijpers AFA and Mooijman KA (2011). EU Interlaboratory comparison study veterinary XIV (2011); Detection of *Salmonella* in chicken faeces. RIVM Report 330604023, Bilthoven, the Netherlands.

Kuijpers AFA and Mooijman KA (2013). EU Interlaboratory comparison study veterinary XIV (2012); Detection of *Salmonella* in pig faeces. RIVM report 330604028, Bilthoven, the Netherlands.

Lauer WF, Sidi CD and Tourniaire JP (2009). iQ-Check *Salmonella* II: real-time polymerase chain reaction test kit. Performance Tested Method 010803. Journal of AOAC International 92(6):1865

Lofstrom, C, Hansen F and Hoorfar J (2010). Validation of a 20-h real-time PCR method for screening of *Salmonella* in poultry faecal samples. Veterinary Microbiology 144:511-514.

Lofstrom, C.; Hoorfar, J (2012). Validation of an open-formula, diagnostic real-time PCR method for 20-h detection of *Salmonella* in animal feeds. Veterinary Microbiology 158: 431-435.

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.

Mooijman, KA, Kuijpers AFA, Wijnands, LM and Pielaat, A (under preparation) Validationof EN ISO 6579, Detection of *Salmonella* in the food chain. RIVM report 330554001/2014, Bilthoven, The Netherlands.

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

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