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

The 19th EU Interlaboratory comparison study in primary production (2016)

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

RIVM Report 2016-0044

I.E. Pol-Hofstad | K.A. Mooijman

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Colophon

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Synopsis

The 19th EU Interlaboratory comparison study in primary production (2016)
Detection of *Salmonella* in chicken faeces adhering to boot socks

In February 2016, the nineteenth EURL-*Salmonella* interlaboratory comparison study on the detection of *Salmonella* in samples taken from the primary production stage was organised. Participation was obligatory for all EU Member State National Reference Laboratories (NRLs) that are responsible for the detection of *Salmonella* in samples taken from the primary production stage. In total, 36 NRLs participated in this study: 29 NRLs from 28 EU-Member States (MS), 6 NRLs from other countries in Europe (EU candidate MS or potential EU candidate MS and MS of the European Free Trade Association (EFTA)) and, on request of European Commission, one NRL from a non-European country. EURL-*Salmonella* is a part of the Dutch National Institute for Public Health and the Environment (RIVM).

Results
Each laboratory received a package of boot sock samples containing chicken faeces with two different concentrations of *Salmonella* (high and low) and blank samples without *Salmonella*. The chicken faeces originated from a pathogen free (SPF) broiler breeder flock. The laboratories were asked to analyse the samples using Annex D of the ISO 6579 (Anonymous, 2007) for the detection of *Salmonella*.

All laboratories were able to detect *Salmonella* in all the boot sock samples with contaminated chicken faeces. This is reflected by the high scores for sensitivity, specificity and accuracy in this study. Both the blank control sample and the positive control sample were analysed correctly by all laboratories. One laboratory made a transcription error when copying the raw data onto the electronic reporting form and scored a ‘moderate performance’.
Blank samples containing chicken faeces not containing *Salmonella* were correctly analysed as negative by almost all laboratories. One laboratory found *Salmonella* present in 3 of the 6 blank samples and this was indicated as a ‘poor performance’.

Keywords: *Salmonella*, EURL, NRL, interlaboratory comparison study, *Salmonella* detection method, boot socks, chicken faeces

Publiekssamenvatting

Het 19e EU-ringonderzoek primaire productie 2016
Detectie van *Salmonella* in overschoenen met kippenmest

In februari 2016 vond het negentiende EURL *Salmonella*-ringonderzoek naar *Salmonella* plaats. Deze jaarlijkse kwaliteitstoets is verplicht voor alle Nationale Referentie Laboratoria (NRL's) van de Europese lidstaten die verantwoordelijk zijn voor het aantonen van *Salmonella* in dierlijke mest.

Resultaten
Alle deelnemers waren in staat om *Salmonella* te detecteren in de besmette overschoentjes met kippenmest. Ook hebben de laboratoria de meegestuurde controlemonsters correct geanalyseerd. Eén laboratorium heeft een fout gemaakt toen het de ruwe resultaten overnam in het elektronische rapportageformulier. Hiervoor kreeg dit laboratorium een matige score. Bijna alle laboratoria konden de monsters waar geen *Salmonella* aan was toegevoegd (blanco) als zodanig opsporen. Eén laboratorium vond echter *Salmonella* in drie van de zes blanco monsters en scoorde daardoor een onvoldoende.

Deelnemers
In totaal hebben 36 NRL's deelgenomen: 29 NRL's van 28 lidstaten in de EU (Noord-Ierland heeft een eigen NRL), zes NRL's uit kandidaatlanden voor het EU-lidmaatschap of lidstaten van de European Free Trade Association (EFTA), en één niet-Europees NRL dat op verzoek van de Europese Commissie is toegevoegd (Israël). Het Europese Referentielaboratirum (EURL) *Salmonella* is gevestigd bij het Nederlandse Rijksinstituut voor Volksgezondheid en Milieu (RIVM). De hoofdtaak van het EURL-*Salmonella* is toezien op de kwaliteit van de nationale referentielaboratoria voor deze bacterie in Europa.

Werkwijze
Elk laboratorium kreeg een pakket toegestuurd met daarin de monsters van overschoentjes met kippenmest. De kippenmest is op het EURL-laboratorium besmet met de *Salmonella*-bacterie in twee concentraties (hoog en laag). Ook zijn er onbesmette blanco monsters meegestuurd. De laboratoria dienden de monsters te analyseren volgens Annex D uit de internationaal voorgeschreven ISO-methode 6579 (Anonymous, 2007) op de aanwezigheid van *Salmonella*.

Kernwoorden: *Salmonella*, EURL, NRL, ringonderzoek, *Salmonella*-detectiemethode, overschoenen, kippenmest.

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Summary

In February 2016, the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*) organised the 19th interlaboratory comparison study on the detection of *Salmonella* in samples taken from the primary production stage. The matrix of concern involved boot socks to which chicken faeces from a pathogen-free broiler breeder flock was added.
The participants in the study were 36 National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*): 29 NRLs from the 28 EU Member States (MS), three NRLs from EU candidate MS or potential candidate MS, three NLRs from Member States of the European Free Trade Association (EFTA) and, on request of EC DG-Sante, one NRL from a non-European country.

The aim of this study was to evaluate the performance of the participating laboratories in the detection of *Salmonella* at different contamination levels in a matrix from the primary production stage. For this purpose, boot socks with chicken faeces that had been artificially contaminated with *Salmonella* Typhimurium at various contamination levels were analysed. The performance of the laboratories was assessed on the basis of 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.

The boot socks with pathogen-free chicken faeces were artificially contaminated with a diluted culture of *Salmonella* Typhimurium (STM) at a low level (approximately 10 cfu/sample) and at a high level (approximately 95 cfu/sample) and with no *Salmonella* at all (blank samples) at the laboratory of the EURL. Before the start of the study, several experiments were carried out to confirm the stability of the samples at different storage conditions.

Each laboratory received a package containing 18 individually numbered, blind boot sock samples to be tested for the presence or absence of *Salmonella*. These samples consisted of six blank samples, six samples contaminated with a low level of *Salmonella* and six samples contaminated with a high level of *Salmonella*. In addition, two control samples had to be tested: one procedure control and one positive control sample for which the laboratories had to use their own positive control strain.

The participants detected *Salmonella* in 99.5% of the contaminated samples using the prescribed method. Thirty-four of the 36 participants found all boot sock samples with contaminated chicken faeces to test positive. Two laboratories found one out of the six samples with a low level of contamination to test negative. This is well above the agreed performance, whereby three of the six samples contaminated with a low level of *Salmonella* are allowed to be scored negative.
For the blank boot sock samples, almost all laboratories scored all six samples as negative. One laboratory (non-EU MS: lab code 28) found three of the six samples to test positive for *Salmonella*. With respect to

blank samples, one out of six of the samples are permitted to test positive. This laboratory, therefore, scored a 'poor performance'.

The control samples were scored correctly by all laboratories. One laboratory (lab code 32) made an error in copying the raw data onto the electronic report form, which resulted in a 'moderate performance' score.

PCR was used as an additional method by six participants. All six participants found identical results compared to the bacteriological culture method, including the laboratory (lab code 28) scoring three of the six blank samples as false positives.

For the positive control, the majority of the participants (23 laboratories) used a diluted culture of *Salmonella*. The *Salmonella* serovars most often used for the positive control sample were *S*. Enteritidis (16) and *S.* Typhimurium (7). Yet it is recommended that rarer serovars be used so that cross-contamination is easier to detect. The concentration of the positive control varied between 6 and 10^6 CFU/sample, whereas levels just above the detection limit are advised for the positive control samples of detection methods.

In conclusion: 34 NRLs have fulfilled the criteria of 'good performance', one NRL scored a 'moderate performance' as a result of a transcription error and one NRL received a 'poor performance' for detecting *Salmonella* in three of the six blank samples. A follow-up study was offered to this laboratory, but this offer could not be accepted due to lack of financial means.

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 since 1995, as organised by EURL-*Salmonella* (formerly called CRL-*Salmonella*), is summarized on the EURL-*Salmonella* website (http://www.eurlsalomonella.eu).

In February 2016, the EURL-*Salmonella* organised an interlaboratory study to test whether the NRLs for *Salmonella* could detect *Salmonella* at different contamination levels in chicken faeces adhering to boot socks. The results from interlaboratory studies like this show whether the examination of samples in the EU Member States (EU-MS) is being carried out uniformly and whether comparable results can be obtained by all NRLs-*Salmonella*.

The method prescribed 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).

The study design of this study was comparable to the interlaboratory study organised in 2015 (Pol-Hofstad, 2016) and the CEN-mandate study organised in 2013 (Kuijpers and Mooijman, 2013). For this latter study, the chicken faeces adhering to boot socks was artificially contaminated with a diluted culture of *Salmonella* Typhimurium (STM) at the laboratory of the EURL-*Salmonella*.
In total, 18 boot sock samples with chicken faeces were tested: six samples per contamination level (blank, low and high concentrations of *Salmonella* Typhimurium). Additionally, two control samples were tested: one procedure control and one positive control. The number and contamination level of the samples were in accordance with ISO/TS 22117 (Anonymous, 2010).

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 Laboratory for bacterial disease in 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 General Bacteriology and Microbiology
Cyprus	Nicosia	Cyprus Veterinary Services Pathology, Bacteriology, Parasitology Laboratory
Czech Republic	Prague	State Veterinary Institute
Denmark	Ringsted	Danish Veterinary and Food Administration
Estonia	Tartu	Estonia Veterinary and Food Laboratory, Bacteriology-Pathology Department
Finland	Kuopio	Finnish Food Safety Authority Evira Research Department, Veterinary Bacteriology
France	Ploufragan	Anses, Laboratoire de Ploufragan-Plouzané Unité Hygiène et Qualité des Produits Avicoles et Porcins (HQPAP)
Germany	Berlin	Federal Institute for Risk Assessment (BfR) National Veterinary Reference Laboratory for *Salmonella*
Greece	Chalkida	Veterinary Laboratory of Chalkida
Hungary	Budapest	National Food Chain Safety Office, Food and Feed Safety Directorate
Iceland	Reykjavik	Matís ohf, Icelandic Food and Biotech R&D
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*

Country	City	Institute
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 Laboratory for food and feed microbiology
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)
Norway	Oslo	Norwegian Veterinary Institute, Section of Bacteriology
Poland	Pulawy	National Veterinary Research Institute (NVRI) Department of Microbiology
Portugal	Lisbon	Instituto Nacional de Investigação Agrária e Veterinária (INIAV) Unidade de Produção e Saúde Animal Laboratorio de Bacteriologia
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 Preparation of boot sock samples with artificially contaminated chicken faeces

3.1.1 Pre-tests for the preparation of boot sock samples

The matrix in this interlaboratory comparison study was boot socks, to which faeces from a broiler breeder flock were added. The boot socks (Sodibox, Nevez, France) bearing the chicken faeces were artificially contaminated at the laboratory of the EURL-*Salmonella* with a diluted culture of *Salmonella*.

The batch of faeces was collected by the Animal Health Service (GD) Deventer from a *Salmonella*-free broiler breeder flock (GD, Deventer, NL). The batch of faeces (2 kg) for the pre-test arrived at the EURL on 26 October 2015 and was stored at 5 °C. Immediately after receipt, three samples of 25 grams were taken randomly from the batch and tested for the presence of *Salmonella* according to Annex D of ISO 6579 (Anonymous, 2007).

Boot socks were moistened with 15 ml of peptone saline solution (per 1 l: 1.0 g peptone and 8.5 g sodium chloride) and left at room temperature for one to several hours to allow the fluid to thoroughly moisten the boot socks. Subsequently, 10 grams of chicken faeces was added to the boot socks. Some of the boot sock samples were artificially contaminated with different concentrations of a diluted culture of *Salmonella* Typhimurium ATCC 14028 culture, resulting in high-concentrated samples (65 cfu/sample), low-concentrated samples (9.5 cfu/sample) and blank samples.

To test the stability of the prepared samples, the boot sock samples were stored at 5 °C and 10 °C for a period of 0, 7, 14 and 21 days and tested for the presence of *Salmonella* according to Annex D of ISO 6579 (Anonymous, 2007).

3.1.2 Preparation of the boot sock samples bearing chicken faeces for the interlaboratory comparison study

A large batch (15 kg) of chicken faeces from the same flock as the pre-tests arrived at the EURL-*Salmonella* laboratory on Monday, 1 February 2016. Five samples, 25 g each, were tested for the presence of *Salmonella* according to Annex D of ISO 6579 (Anonymous, 2007). After testing negative, 10 grams of chicken faeces was added to each pre-moistened boot sock sample (see 3.1.1) and subsequently artificially contaminated with *Salmonella* Typhimurium ATCC 14028 by adding 0.1 ml of the appropriate dilution of an overnight culture. Three concentration levels were used; blank, low (11 cfu/sample) and high (95 cfu/sample). The concentration of the inoculum used to contaminate the samples of boot socks bearing chicken faeces was confirmed by testing on XLD agar plates. Immediately after artificial contamination, the samples were stored at 5 °C until transport to the participating laboratories on Monday, 15 February 2016.

3.1.3 Determination of background flora

To obtain an indication of the amount of background flora in the samples, the number of aerobic bacteria and the number of Enterobacteriaceae were determined in the samples of blank boot socks with chicken faeces using ISO 4833 (Anonymous, 2003) and ISO 21528-2 (Anonymous, 2004), respectively. The chicken faeces were homogenised in peptone saline solution. Serial dilutions were analysed on PCA (Plate Count Agar) and VRBG (Violet, Red Bile Glucose Agar) to obtain the total number of aerobic bacteria and Enterobacteriaceae present in the samples.

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

The level of contamination in the artificially contaminated boot sock samples was determined by using a five-tube most probable number (MPN) technique. For this, ten-fold dilutions of five boot sock samples at 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, using an MPN program in Excel (Jarvis et al., 2010).

3.2 Design of the interlaboratory comparison study

3.2.1 Number and type of samples

Each participant received 18 boot sock samples with artificially contaminated chicken faeces that were numbered B1 to B18. In addition, the laboratories had to test two control samples (C1 and C2). Table 1 gives an overview of the number and type of samples tested by the participants.

For the control samples, the laboratories were asked to use their own positive *Salmonella* control strain, which they normally use when analysing routine samples for the detection of *Salmonella*. In addition to this positive control (C2), a procedure control (C1) consisting of boot socks and 15 ml of only peptone saline solution had to be analysed. The protocol and test report used during the study can be found on the EURL-*Salmonella* website or can be obtained from the author of this report (EURL-*Salmonella* 2016a and 2016b).

Table 1. Overview of the number and type of samples tested per laboratory in the interlaboratory comparison study

Contamination level	Test samples chicken faeces (n=18)
STM low-level in boot socks + chicken faeces	6
STM high-level in boot socks + chicken faeces	6
Blank (BL) boot socks + chicken faeces	6
	Control samples (n=2)
Boot socks + 15 ml BPW* (C1)	1
Boot socks + 15 ml BPW* + Laboratories own pos. control strain (C2)	1

3.2.2 *Sample packaging and temperature recording during shipment*

Each NRL received 20 coded samples containing the boot socks with the contaminated chicken faeces, the blank samples and the control samples. The 20 bags were placed in two safety bags. The safety bags were placed in one large shipping box, together with two frozen (-20 °C) cooling devices. The shipping boxes were sent to the participants as 'biological substances category B (UN3373)' via a door-to-door courier service. The participants were asked to store the samples at 5 °C upon receipt. To monitor exposure to abusive temperatures during shipment and storage, a micro temperature logger was placed in between the samples and used to record the temperature.

3.3 Methods

The method prescribed for this interlaboratory comparison study was Annex D of ISO 6579 (Anonymous, 2007), which consists of a pre-enrichment in Buffered Peptone Water (BPW) and selective enrichment on Modified Semi-solid Rappaport-Vassiliadis (MSRV) agar, followed by plating-out on Xylose Lysine Deoxycholate agar (XLD) and a second medium of choice. Confirmation was performed using the appropriate biochemical tests (ISO 6579; Anonymous, 2002) or using reliable, commercially available identification kits and/or serological tests. In addition to the ISO method, the NRLs were free to use their own method, such as a Polymerase Chain Reaction (PCR) procedure.

3.4 Statistical analysis of the data

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

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

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

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

3.5 Good performance

For the determination of 'good performance' per laboratory, selection criteria have been set up (see Table 2). In respect of the high-level and low-level contaminated samples, a score of 20% and 50% negative, respectively, for *Salmonella* is allowed. In respect of the blank samples, it cannot be guaranteed absolutely that the whole batch of matrix is negative for *Salmonella*, so 1 positive sample is considered acceptable.

Table 2. Criteria for testing good performance in the interlaboratory comparison study.

Contamination level	% Positive	# Pos samples/ total # samples
Boot sock samples with chicken faeces		
***S*. Typhimurium high-level (STM)**	Min. 80 %	Min. 5/6
***S*. Typhimurium low-level (STM)**	Min. 50 %	Min. 3/6
Blank (BL)	Max. 20 %	Max. 1/6
Control samples		
Positive control (Own control strain of *Salmonella*)	100 %	1 /1
Procedure control (BPW)	0 %	0 /1

4 Results

4.1 Preparation of boot sock samples with artificially contaminated chicken faeces

4.1.1 Pre-tests for the preparation of boot sock samples with chicken faeces

The design of the study was based on the design used in the CEN mandate study, which was organised in 2013 by the EURL *Salmonella* (Kuijpers and Mooijman, 2013). The method used for the preparation of the samples was tested for reproducibility in the pre-tests.
The pre-test samples were stored at 5 °C to mimic storage conditions and at 10 °C to test the effect of temperature abuse during transport. The pre-test samples were stored for up to three weeks and analysed for survival of *Salmonella* using Annex D from ISO 6579 (Anonymous, 2007)

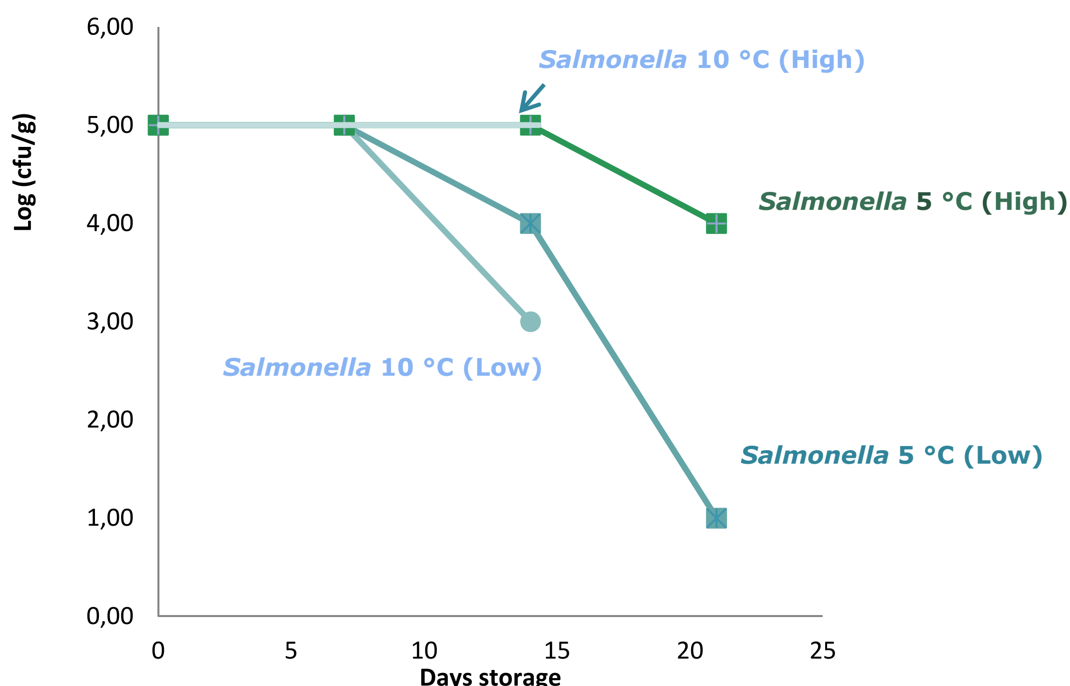

Figure 1. The number of boot sock samples with artificially contaminated chicken faeces that tested positive for Salmonella after storage for three weeks at 5 °C and two weeks at 10 °C.

Figure 1 shows that the storage of the pre-test samples at 5 °C or 10 °C for two weeks had a relatively small effect on the survival of *Salmonella*. The high-level contaminated samples were all positive. For the low-level contaminated samples stored at 10 °C, two samples scored negative for *Salmonella and* when stored at 5 °C only one sample scored negative. Storing the samples for a longer period at 5 °C resulted in one negative sample when the concentration of *Salmonella* was high and four negative samples when the concentration was low. Storing at 10 °C for longer than two weeks is not a relevant condition in this ring trial and was therefore not tested. The effect of storage and temperature on the background flora is shown in Figure 2. The light coloured lines show the effect of 10 °C and

the dark coloured lines show the effect of storage at 5 °C. Figure 2 shows little difference in the number of aerobic bacteria between storage at 5 °C or at 10 °C. The number of aerobic bacteria remained approximately at the same level (log 10 cfu/g) for up to three weeks. The Enterbacteriaceae were more sensitive to storage temperatures. At both temperatures, the number of Enterobacteriaceae decreased, 3.5 log units at 5 °C and 4.4 log units at 10 °C. However, there was still sufficient flora left to represent a real life sample.

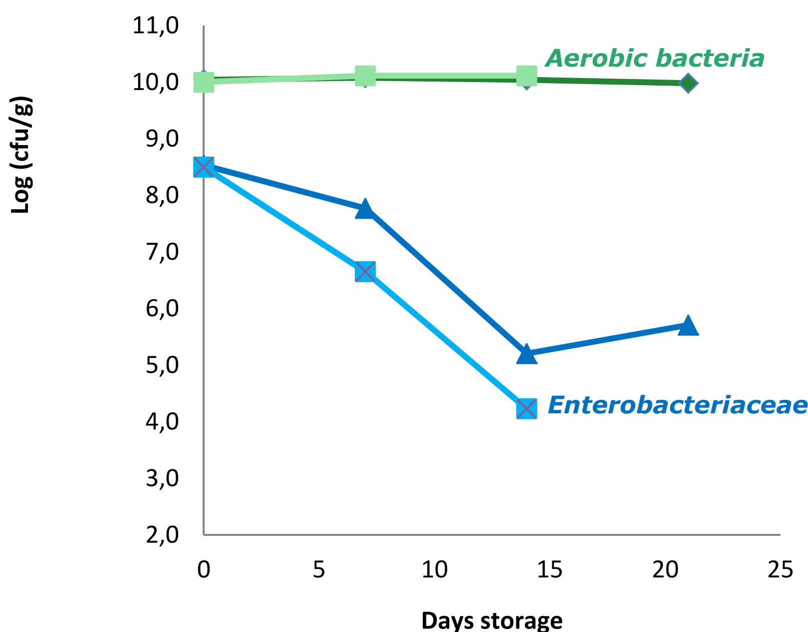

Figure 2. The effect of temperature and storage time on the number of aerobic bacteria and Enterobacteriaceae in chicken faeces (dark colour = 5 °C, light colour = 10 °C).

4.1.2 *Contamination level of the boot sock samples with artificially contaminated chicken faeces*

Samples for the interlaboratory comparison study were prepared in the same way as was done for the pre-test. The chicken faeces came from the same flock of parent animals as the batch of faeces used in the pre-test. Immediately after the arrival of the new batch of chicken faeces (15 kg), the faeces was tested for the presence of *Salmonella* and for the amount of background flora by analysing the number of aerobic bacteria and Enterobacteriaceae. The results are presented in Table 3. The amount of background flora in the fresh faeces was 7.1 x 10^9 cfu/g for the aerobic bacteria and 1.5 x 10^6 cfu/g for Enterobacteriaceae. Storage of the faeces at 5 °C for a week had hardly any effect on the aerobic count, as expected. The number of Enterobacteriaceae decreased slightly by 1 log unit.

Table 3. Number of aerobic bacteria and Enterobacteriaceae per gram of chicken faeces

Date of testing	Aerobic bacteria cfu/g	Enterobacteriaceae cfu/g
2 February 2016	$7.1x10^7$	**$3.5x10^6$**
9 February 2016, after storage at 5 °C	**$1.5x10^7$**	**$2.8x10^5$**

The boot sock samples with chicken faeces were artificially contaminated at the EURL laboratory by adding 100 µl of a diluted *Salmonella* culture. Table 2 shows the contamination level of the diluted culture of *Salmonella* Typhimurium used as inoculum to contaminate the chicken faeces samples. The boot sock samples with chicken faeces contaminated at a low level were inoculated with 11 cfu, while the samples contaminated at a high level were inoculated with 95 cfu. After inoculation, the samples were stored at 5 °C for almost one week until transport to the participants on the 15th of February 2016. The final contamination level of *Salmonella* in the boot sock samples was determined by performing a five-tube Most Probable Number (MPN) test in the week of the interlaboratory comparison study.

Table 4. Salmonella Typhimurium (STM) concentration in inoculum culture and in boot sock samples with contaminated chicken faeces.

Date of testing	Low-level STM cfu/25 g chicken faeces	High-level STM cfu/25 g chicken faeces
9 February 2015 (Inoculum level diluted culture)	11	**95**
22 February 2016 (5 °C) MPN of boot socks with artificially contaminated chicken faeces (95 % confidence limit)	**5 (1.5-16.3)**	**>> (65 - >>)**

4.2 Technical data from interlaboratory comparison study

4.2.1 General

In this study, 36 NRLs for *Salmonella* participated: 29 NRLs from 28 EU-MS and six third countries within Europe (EU candidate MS or potential EU candidate MS and members of the European Free Trade Association (EFTA)) and, at the request of DG-Sante, one NRL from a non-European country.

4.2.2 Accreditation

Thirty-five laboratories were accredited for Annex D of ISO 6579; 15 were accredited for ISO 6579 and one EU-MS laboratory (11) is in the process of accreditation.

4.2.3 *Transport of samples*

The samples were transported using a door- to- door courier on the 15th of February 2016. Ten laboratories received the parcels within one day of dispatch and seventeen participants within two days. Seven laboratories received the parcels after three days, one laboratory after four days and one laboratory after six days, due to problems at the border. Participants were asked to store the parcel at 5 °C upon arrival in their laboratories. The temperature during transport and storage was recorded using a temperature recorder placed between the samples in the sample bag. The temperature during transport was predominantly between -1 °C and 5 °C. The storage temperature at the receiving laboratories ranged from 2 °C to 6 °C. Two laboratories had a storage temperature above 20 °C for several days (lab codes 7 and 17).

4.2.4 *Media*

Each laboratory was asked to test the samples using the prescribed method (Annex D of ISO 6579) while using MSRV as selective enrichment medium and XLD and a second plating-out medium of their own choice.

4.3 Control samples

4.3.1 *General*

Two control samples consisting of boot socks moistened with 15 ml of peptone saline solution only were sent to the laboratories. One was used as a procedure control. The other was used for the positive control and the laboratories had to add their own positive control strain normally used in their routine analysis for the detection of *Salmonella*. All laboratories scored good results for both control samples (see Table 5). One laboratory made a mistake in copying the raw data onto the electronic report sheet and scored a 'moderate performance' (lab code 32) as a result.

Table 5. Summary of results found by the laboratories with the control samples

Control	Number of labs with compliant results	
	compliant	non-compliant
Positive control own *Salmonella* n=1	36	0
Procedure control BPW n=1	36	0

For the positive control, the majority of the participants used a diluted culture of *Salmonella* (23 laboratories). Others used a lenticule disc (7), a freeze-dried ampoule (2) or a Culti-Loop (1) with *Salmonella*. The *Salmonella* serovars used for the positive control sample were *Salmonella* Enteritidis (16), *Salmonella* Typhimurium (7), *Salmonella* Nottingham (3) and *Salmonella* Poona (1) and others (9).

4.3.2 *Specificity, sensitivity and accuracy rates for the artificially contaminated samples*

Table 6 shows the specificity, sensitivity and accuracy rates for the control samples. All laboratories scored good results for the control samples with an accuracy rate of 100 %.

Table 6. Specificity, sensitivity and accuracy rates of the control samples for all participants and EU Members States only

Control samples		All participants n=36	EU-MS n=29
Procedure control Blank (BPW) n=1	No. of samples	36	29
	No. of negative samples	36	29
	Correct score in %	**100**	**100**
Positive control (Own *Salmonella*) n=1	No. of samples	36	29
	No. of positive samples	36	29
	Correct score in %	**100**	**100**
All control samples n=2	No. of samples	72	58
	No. of correct samples	72	58
	Accuracy in %	**100**	**100**

4.4 Artificially contaminated chicken faeces adhering to boot socks

4.4.1 *General*

Boot sock samples with chicken faeces artificially contaminated with two different levels of *Salmonella* Typhimurium, low (approx. 11 cfu/25 g) and high (approx. 95 cfu/25 g), as well as blank samples, were analysed for the presence of *Salmonella* by the participants.

Blank boot sock samples with chicken faeces (n=6)
All but one laboratory correctly analysed the blank boot sock samples with chicken faeces negative for *Salmonella*. Laboratory 28 found three of the six blank samples to test positive for *Salmonella* and scored a 'poor performance' as a result.

Boot sock samples with chicken faeces containing a low concentration of Salmonella *Typhimurium (n=6)*
Almost all laboratories were able to detect *Salmonella* in all six boot sock samples with faeces contaminated with a low inoculum level of approximately 11 cfu/25g. Only two laboratories (lab codes 6 and 20) found one of the six samples to test negative for *Salmonella*. In respect of low level samples, it is acceptable to score a maximum of three of the six samples as negative, so these two laboratories scored well above the criteria for good performance. Results are shown in Figure 3.

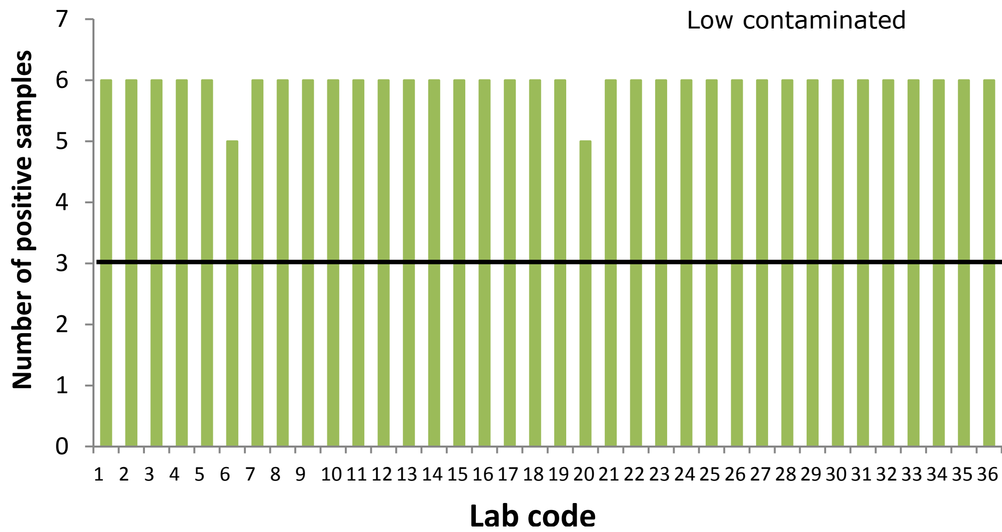

Figure 3. The number of boot sock samples with chicken faeces artificially contaminated with low levels of Salmonella Typhimurium (n = 6) that tested positive per laboratory

- = line of good performance

Boot sock samples with chicken faeces containing a high concentration of Salmonella *Typhimurium (n=6)*
In this study, all laboratories were able to detect *Salmonella* in all six boot sock samples inoculated with approximately 95 cfu/25 g. Results are shown in Figure 4.

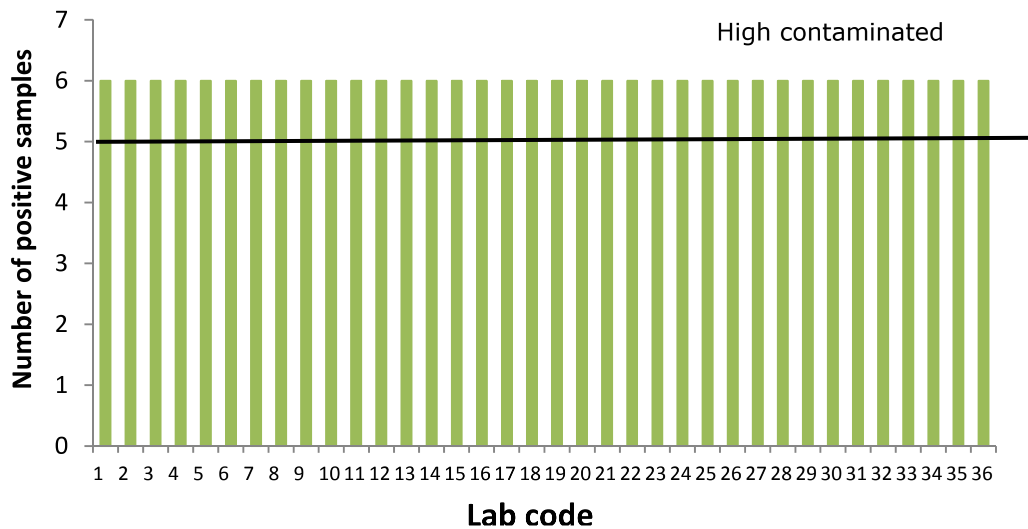

Figure 4. The number of boot sock samples with chicken faeces artificially contaminated with high levels of Salmonella Typhimurium (n = 6) that tested positive per laboratory

- = line of good performance

4.4.2 *Specificity, sensitivity and accuracy rates for the artificially contaminated samples*

Table 7 shows the specificity, sensitivity and accuracy rates for all artificially contaminated faeces samples adhering to boot socks. 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 (99.5 %) and the sensitivity rates (low level: 99 %; high level 100 %) were high for the whole group of participants.

Table 7. Specificity, sensitivity and accuracy rates of the boot sock samples with chicken faeces artificially contaminated with Salmonella *Typhimurium*

Boot socks with chicken faeces		All participants n=36	EU-MS n=29
Blank n=6	No. of samples	216	174
	No. of negative samples	213	174
	Specificity in %	**99**	**100**
Low level STM n=6	No. of samples	216	174
	No. of positive samples	214	174
	Sensitivity in %	**99**	**100**
High level STM n=6	No. of samples	216	174
	No. of positive samples	216	174
	Sensitivity in %	**100**	**100**
All boot sock samples with STM	No. of samples	432	348
	No. of positive samples	430	348
	Sensitivity in %	**99.5**	**100**
All boot socks (pos. and neg.)	No. of samples	648	522
	No. of correct samples	643	522
	Accuracy in %	**99**	**100**

4.5 PCR (own method)

Six laboratories (lab code 23, 28, 30, 31, 32 and 35) also performed a PCR method on the boot sock samples as an additional detection technique (see Table 8). All these laboratories except for one tested the samples after pre-enrichment in BPW. Laboratory 28 started the DNA extraction before pre-enrichment in BPW. All laboratories used a real-time PCR except laboratory 28, which used a commercially available three-step PCR with reference to G.G. Stone et al. Clin. Microbiol. 1994. 32 (7): 1742. In four laboratories, the PCR method is validated and three laboratories use the PCR method for their routine testing. All six laboratories found identical results using their PCR method compared to those achieved with the bacteriological culture method, including the false positive results for the three blank samples (lab code 28). One laboratory (lab code 31) did not report the PCR result for sample B4 (without further explanation) and one laboratory (lab code 30) did not perform PCR on the two control samples.

Table 8. Details of Polymerase Chain Reaction (PCR) procedures used by participants

Lab code	Type of PCR	Validated	Commercial available	# samples 2015	Reference
23	Real Time	Lofstrom et al	-	1894	Malorny *et al.* 2004
28	Conventional : Three steps	-	+	150	Stone *et al.* 1994
30	Real-time	Internally	+		
31	Real-time	-	-		Hein *et al*. 2006
32	Real-time	Nationally	-	119	Josefsen *et al*. 2007; Malorny *et al*. 2004
35	Real-time	NF validation : AOAC-RI	+		Journal of AOAC International, 92, 2009.

4.6 Performance of the NRLs

All the laboratories were able to detect *Salmonella* in high and low concentrations in boot sock samples with chicken faeces. Thirty-four of the 36 laboratories fulfilled the criteria of good performance for the prescribed MSRV method. One laboratory (non-EU-MS) scored a 'poor performance' for falsely detecting *Salmonella* in three blank boot sock samples with chicken faeces. One laboratory scored a 'moderate performance' for making an error in copying the raw data onto the electronic reporting form.

5 Discussion

Preparation of the boot sock samples with chicken faeces
In this study, boot sock samples with chicken faeces taken from a broiler breeder flock were artificially contaminated with a diluted culture of *Salmonella* Typhimurium at two different levels of contamination (high and low). The stability of the samples was verified in pre-tests showing good survival of *Salmonella* in both high-level and low-level contaminated samples after being stored for two to three weeks at 5 °C and 10 °C.

Transport of the samples
To prevent the concentration of *Salmonella* Typhimurium in the boot sock samples from 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. It can be assumed therefore that transport did not negatively affect the mean contamination level of the samples. This was confirmed by the fact that all laboratories detected *Salmonella* even in the boot socks samples that had chicken faeces with a low level of contamination, despite transport time or transport/storage temperature differences.

Accreditation
According to EC regulations 882/2004 (EC, 2004) and 2076/2005 (EC, 2005), each NRL should have been accredited in their relevant field before 31 December 2009. Thirty-five laboratories were accredited. One (EU-MS) participant (lab code 11) is still in the process of accreditation, which is rather late.

Performance of the laboratories
The performance of the laboratories was evaluated based on pre-set criteria for 'good performance'. In this study, most of the laboratories scored very well with respect to detecting *Salmonella* even in the boot sock samples with chicken faeces contaminated at a low level. Two laboratories found one of the six boot sock samples with chicken faeces contaminated at a low level to test negative. This is well above the limit for good performance, which allows a maximum of 50% of the samples with low-level contamination to be found negative. Thirty-four laboratories scored a 'good performance' for their reported results. One laboratory scored a 'moderate performance' because of a transcription error in copying raw data onto the electronic reporting form. One laboratory (non EU-MS) scored a 'poor performance' for detecting *Salmonella* in three of the six blank samples. Unfortunately, communication with this laboratory did not reveal an explanation for these findings. This laboratory could not accept the invitation for a follow-up study for financial reasons.

Specificity, sensitivity and accuracy rates
The calculations were performed on the results of all participants and on the results of only the EU-MS. Differences (if any) were very small

between these groups. Specificity, sensitivity and accuracy rates were high, ranging between 99 and 100 %

PCR
Six laboratories used a PCR technique in addition to the prescribed method and results reported were identical to results found using the bacteriological culture technique (BAC). No differences were found between the different kinds of PCR techniques, nor between DNA extraction from a BPW culture or from an MSRV culture.

6 Conclusions

- All NRLs for *Salmonella* were able to detect high and low levels of *Salmonella* in boot sock samples bearing chicken faeces using the prescribed MSRV method.

- Thirty-four NRLs scored a 'good performance' and one laboratory scored a 'moderate performance' due to a transcription error in copying raw data onto the electronic reporting form. One laboratory scored a 'poor performance' for falsely detecting *Salmonella* in three of the six blank boot sock samples with chicken faeces.

- The accuracy, specificity and sensitivity rates of the NRLs with respect to the control samples (without chicken faeces) after selective enrichment on MSRV were all 100 %.

- The sensitivity rate for the boot sock samples with chicken faeces artificially contaminated with a high level of *S*. Typhimurium was 100 % for the prescribed MSRV method.

- The sensitivity rate for the boot sock samples with chicken faeces artificially contaminated with a low level of *S*. Typhimurium was 99 %.

- The accuracy rate of the NRLs in detecting the boot sock samples with artificially contaminated chicken faeces was 99 % after selective enrichment on MSRV.

- At a relatively large number of the participating laboratories, the contamination level of the positive control is rather high, which may provide little information on the sensitivity of the method.

- The majority of the NRLs-*Salmonella* use *S*. Enteritidis or *S*. Typhimurium for their positive control samples. But the use of a *Salmonella* serovar that is rarer in terms of detection may be advisable in order to make the detection of possible cross contamination easier.

- Six participants used a PCR technique in addition to the prescribed MSRV method. All six laboratories reported identical results for both methods.

List of abbreviations

ATCC	American Type Culture Collection
BAC	Bacteriological Culture technique
BPW	Buffered Peptone Water
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
ISO	International Organization for Standardization
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)
SPF	Specific Pathogen Free
SOP	Standard Operating Procedure
STM	*Salmonella* Typhimurium
VRBG	Violet Red Bile Glucose agar
XLD	Xylose Lysine Deoxycholate agar

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