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

**EU Interlaboratory comparison study
primary production XVII (2014)**
Detection of *Salmonella* in chicken faeces

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Colophon

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Abstract

EU Interlaboratory comparison study primary production XVII (2014)
Detection of *Salmonella* in chicken faeces

In 2014, it was shown that 34 out of 36 National Reference Laboratories (NRLs) in the European Union were able to detect high and low levels of *Salmonella* in chicken faeces. The performance of two laboratories was rated as 'moderate' because of a technical reporting mistake. The laboratories detected *Salmonella* in 99% of the contaminated samples. This is evident from the 17th interlaboratory comparison study of primary production samples (such as chicken faeces), which was organized by the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*).

European obligation
The study was conducted in March 2014. Participation was obligatory for all EU Member State NRLs that are responsible for the detection of *Salmonella* in samples from the primary production stage.
EURL-*Salmonella* is part of the Dutch National Institute for Public Health and the Environment (RIVM).

The laboratories detected the Salmonella in the chicken faeces by using the internationally prescribed method Modified Semi-solid Rappaport Vassiliadis (MSRV). Each laboratory received a package of 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.

Control samples
In a laboratory analysis, it is necessary for the participants to show that they carried out their analyses in a reliable manner by using 'control samples'. These control samples all gave the desired result.
Nonetheless, optimization of these control samples may be desirable.

Keywords: *Salmonella*, EURL, NRL, interlaboratory comparison study, *Salmonella* detection method, chicken faeces, control sample

Publiekssamenvatting

EU Ringonderzoek primaire productie XVII (2014)
Detectie van *Salmonella* in kippenmest

In 2014 waren 34 van de 36 Nationale Referentie Laboratoria (NRL's) in de Europese Unie in staat om zowel hoge als lage concentraties *Salmonella* in kippenmest aan te tonen. Twee NRL's behaalden een matig resultaat vanwege een technische rapportagefout. In totaal hebben de laboratoria in 99 procent van de besmette monsters *Salmonella* aangetoond, zo blijkt uit het zeventiende ringonderzoek met monsters van kippenmest. Dit ringonderzoek wordt jaarlijks georganiseerd door het referentielaboratorium van de Europese Unie voor *Salmonella* (EURL-*Salmonella*).

Europese verplichting
Het onderzoek is in maart 2014 gehouden. Alle NRL's van de Europese lidstaten die verantwoordelijk zijn voor de opsporing van *Salmonella* in monsters van de primaire productie van dieren, 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 kippenmest aan met behulp van de internationaal voorgeschreven analysemethode Modified Semi-solid Rappaport Vassiliadis (MSRV). Elk laboratorium kreeg een pakket toegestuurd met kippenmest dat ofwel besmet was met *Salmonella* in twee verschillende concentraties, of geen *Salmonella* bevatte. De laboratoria dienden de monsters volgens een protocol te onderzoeken op de aanwezigheid van *Salmonella*.

Controlemonsters
Bij een laboratoriumanalyse is het noodzakelijk dat de deelnemers via 'controlemonsters' aantonen dat zij hun analyses betrouwbaar hebben uitgevoerd. Deze controlemonsters gaven allemaal het gewenste resultaat. Enige optimalisatie van de controles is nog wenselijk.

Kernwoorden: *Salmonella*, EURL, NRL, ringonderzoek, kippenmest, *Salmonella*-detectiemethode, controlemonster

Contents

Summary

In March 2014 the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*) organized the 17th interlaboratory comparison study on the detection of *Salmonella* in samples from the primary production stage (XVII). The matrix of concern was chicken faeces.
The participants were 36 National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*): 29 NRLs from the 28 EU Member States (EU-MS), 5 NRLs from third countries within Europe (EU candidate MS or potential EU candidate MS, member of the European Free Trade Association (EFTA)) and, at the request of EC DG-Sanco, one NRL from a non-European country.

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 the primary production stage. For this purpose, chicken faeces samples of 25 grams that had been artificially contaminated with *Salmonella* Typhimurium (STM) 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, 2007a), using selective enrichment on Modified Semi-solid Rappaport-Vassiliadis (MSRV).

The samples consisted of chicken faeces that had been artificially contaminated with a diluted culture of *Salmonella* Typhimurium (STM) at a low level (approximately 10 CFU/25 g of faeces), at a high level (approximately 100 CFU/25 g of faeces) and with no *Salmonella* at all (blank samples). The samples were artificially contaminated at the laboratory of the EURL for *Salmonella*. Before the start of the study, several experiments were carried out to make sure that the samples were fit for use in an interlaboratory comparison study (e.g. choice of *Salmonella* serovar, stability at different storage temperatures and influence of background flora).

Eighteen individually numbered blind samples of chicken faeces had to be tested by the participants for the presence or absence of *Salmonella*. These samples consisted of six blank samples, six samples with a low level of STM (inoculum 14 CFU/sample) and six samples with a high level of STM (inoculum 67 CFU/sample). Additionally, three control samples had to be tested: two blank control samples (procedure control (BPW) and matrix control sample (chicken faeces) and one own (NRL) positive control sample (with *Salmonella*).

The laboratories found *Salmonella* in almost all (contaminated) samples after selective enrichment on MSRV, resulting in a sensitivity rate of 99%.
Forty-eight hours of incubation of the selective enrichment medium MSRV showed only 0.05% more positive results than 24 hours of incubation.

For the positive control, the majority of the participants (24 laboratories) used a diluted culture of *Salmonella*. The *Salmonella* serovars used for the positive control sample were *S.* Enteritidis (15) and *S.* Typhimurium (9). The concentration of the positive control varied between 8 to 10^6 CFU/sample. For the positive control, it is advisable to use a concentration close to the detection limit and a *Salmonella* serovar not often isolated from routine samples.

PCR was used as their own method by five participants, four of which found the same results as when the bacteriological culture method was used.

Thirty-four out of 36 laboratories achieved the level of good performance. Two NRLs made a technical mistake in reporting their own positive control sample. Those two laboratories were therefore indicated as 'moderate performance'. A follow-up study was considered unnecessary for those laboratories.

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*, 2015).
The first and most important objective of the study, which was organized by the EURL for *Salmonella* in March 2014, was to see whether the participating laboratories could detect *Salmonella* at different contamination levels in chicken faeces. 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 whether comparable results can be obtained by all NRLs-*Salmonella*.

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, 2007a).

The set-up of this study was comparable to the interlaboratory comparison study organized in 2013 on the detection of *Salmonella* spp. in samples from the primary production stage (PPS) (Kuijpers and Mooijman, 2014a). For the current study, the samples (chicken faeces) were artificially contaminated with a diluted culture of *Salmonella* Typhimurium (STM) at the laboratory of the EURL-*Salmonella.*

Like in earlier studies, the contamination level of the low-level contaminated samples was close to the detection limit of the method and the level of the high-level contaminated samples was approximately 5-10 times above the detection limit. In total, 18 chicken faeces samples were tested, 6 samples per contamination level (blank, low level and high level) containing one *Salmonella* serovar (*Salmonella* Typhimurium). Additionally, three control samples (two blank control samples and one positive control sample) were tested. The number of samples and the level of contamination in the samples were in accordance with CEN 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 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
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	Chalikida	Veterinary Laboratory of Chalikida
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*
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

Country	City	Institute
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, Centre for Infectious Diseases (RIVM/Cib) 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 Chicken faeces from a laying hen flock

3.1.1 General

The matrix in this interlaboratory comparison study was chicken faeces from a *Salmonella*–free laying hen flock (SPF-farm). The chicken faeces was kindly provided by the Animal Health Service (GD) in Deventer, the Netherlands. For the pre-test, two batches of 5-10 kg chicken faeces were used. For the interlaboratory comparison study, a batch weighing approximately 30 kg was collected. This latter batch arrived at the EURL-*Salmonella* on 4 March 2014, where it was stored at 5 °C. Immediately after receipt of the chicken faeces, 5 samples (for the pre-test) or 10 samples (for the interlaboratory comparison study) of 25 g each were taken and checked for the absence of *Salmonella* following Annex D of ISO 6579 (Anonymous, 2007a). For this purpose, the 25-gram samples were each added to 225 ml of 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). Next, the suspect growth on MSRV plates was plated out on Xylose Lysine Deoxycholate agar (XLD) and Brilliance *Salmonella* Agar (BSA) and confirmed biochemically. After checking for the absence of *Salmonella*, the chicken faeces was repacked in portions of 25 g in Whirl-pak plastic bags and stored at 5 °C (see 3.3.1).

3.1.2 Total bacterial count in chicken faeces

The total number of aerobic bacteria in the chicken faeces was investigated by following ISO 4833 (Anonymous, 2003a). A portion of 20 g of the chicken faeces was homogenized in 180 ml of peptone saline solution in a plastic bag. The content was mixed by using a pulsifier (for 60 sec). Next, ten 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 of 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.

3.1.3 Number of Enterobacteriaceae in chicken faeces

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 chicken faeces was homogenized in 180 ml of peptone saline solution in a plastic bag. The contents were mixed using a pulsifier (for 60 sec). Next, ten 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 of 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.

3.2 Artificial contamination of chicken faeces samples

3.2.1 Pre-tests for the preparation of contaminated chicken faeces samples
The chicken faeces samples were artificially contaminated at the laboratory of the EURL-*Salmonella* with a diluted culture of *Salmonella.* Some experiments were performed prior to the start of the interlaboratory comparison study, especially in relation to the stability of *Salmonella* in the artificially contaminated chicken faeces samples when stored at different temperatures.
For the contamination of the samples, *Salmonella* Typhimurium (STM) ATCC 14028 obtained from the American Type Culture Collection (ATCC, Manassas, USA) was used.
The strain was inoculated in Buffered Peptone Water (BPW) and incubated at (37 ± 1) °C overnight. Next, the culture was diluted in peptone saline solution to make it possible to inoculate the chicken faeces with approximately 5–10 CFU/sample and 50–100 CFU/sample.
For the enumeration 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.
Samples of 25 g of chicken faeces were artificially contaminated with a dilution of a *Salmonella* culture (different levels of STM). Some control samples were also prepared without the addition of *Salmonella* (blank chicken faeces samples).
All chicken faeces samples were stored at 5 °C, 10 °C and 15 °C for a period of 0, 7, 14 and 21 days. After each storage period at the different temperatures, the artificially contaminated STM and blank chicken faeces samples were tested for the presence of *Salmonella* by following Annex D of ISO 6579 (Anonymous, 2007a), with selective enrichment on MSRV.
To obtain an indication of the amount of the background flora in the samples, the blank chicken faeces samples (without the addition of *Salmonella*) were tested for the number of aerobic bacteria (see 3.1.2) and *Enterobacteriaceae* (see 3.1.3).

3.2.2 Determination of the contamination level in chicken faeces samples by MPN
The level of contamination in the final chicken faeces samples, as used at the time of the study, was determined by using a five-tube most probable number (MPN) technique. For this, ten dilutions of five chicken faeces samples of each contamination level were tested, representing 25 g, 2.5 g and 0.25 g of the original sample. The presence of *Salmonella* was determined in each dilution by following Annex D of ISO 6579 (Anonymous, 2007a). From the number of confirmed positive dilutions, the MPN of *Salmonella* in the original sample was calculated by using an MPN program in Excel that is freely available on the Internet (Jarvis et al., 2010).

3.3 Design of the interlaboratory comparison study

3.3.1 Samples: chicken faeces from a laying hen flock
Approximately two weeks before the start of the study, a total of
810 chicken faeces samples were prepared. To accomplish this, the
following steps were performed:
labelling of each plastic bag;
adding 25 g of chicken faeces to each plastic bag;
adding approximately 0.1 ml of a diluted culture of *Salmonella*
Typhimurium ATCC 14028 to the faeces in each plastic bag. The
contamination levels aimed at were 10–15 CFU/25 g faeces,
50–100 CFU/25 g faeces and blank.
storing samples at 5 °C until transport on 24 March 2014.

On 24 March 2014 (one week before the start of the study), the chicken
faeces samples were prepared for shipment (see Section 3.3.2) and sent
to the participants via door-to-door courier service. After arrival at the
laboratories, the chicken faeces samples had to be stored at 5 °C until
the start of the study. Further details about the shipping and handling of
the samples and the reporting of the test results can be found in the
protocol (EURL-*Salmonella*, 2014a), in the Standard Operation
Procedure (SOP, EURL-*Salmonella*, 2014b) and in (a print-out from) the
web-based test report (EURL-*Salmonella*, 2014c). The protocol, SOP and
test report used during the study can be found on the EURL-*Salmonella*
website or can be obtained by corresponding with the author of this
report.

Eighteen chicken faeces samples (numbered B1–B18) and three control
samples (numbered C1-C3) had to be tested by each participant.
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 sample, the one they normally use when
analysing routine samples for the detection of *Salmonella*. In addition to
this, control samples of the BPW and of the matrix had to be analysed
(both blank controls).

3.3.2 Sample packaging and temperature recording during shipment
To each NRL, 21 plastic bags were sent, containing the chicken faeces
artificially contaminated with *Salmonella*, blank faeces samples or no
faeces at all (controls). The 21 bags were packed in two plastic safety
bags. The safety bags were placed in one large shipping box, together
with three frozen (-20 °C) cooling devices. Each shipping box was sent
to the participants as 'biological substances category B (UN3373)' using
a door-to-door courier service. To monitor 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 stainless steel case 16 mm in diameter and
6 mm deep. 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.

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

Contamination level	Test samples with chicken faeces (n=18)
S. Typhimurium low level (STM)	6
S. Typhimurium high level (STM)	6
Blank (BL)	6
	Control samples (n=3)
Own positive control sample with *Salmonella*	1
Chicken faeces (blank matrix control sample)	1
BPW (blank procedure control sample)	1

3.3.3   *Sample packaging and temperature recording during shipment*
To each NRL, 21 plastic bags were sent, containing the chicken faeces artificially contaminated with *Salmonella*, blank faeces samples or no faeces at all (controls). The 21 bags were packed in two plastic safety bags. The safety bags were placed in one large shipping box, together with three frozen (-20 °C) cooling devices. Each shipping box was sent to the participants as 'biological substances category B (UN3373)' using a door-to-door courier service. To monitor 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 stainless steel case 16 mm in diameter and 6 mm deep. 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 NRLs could follow the pre-treatment procedures for the faeces samples that they normally use in daily routine analysis (e.g. pre-warming of BPW, different ways of mixing the samples in BPW). The prescribed method for detection of *Salmonella* in this interlaboratory comparison study was Annex D of ISO 6579 (Anonymous, 2007a). In addition, the NRLs could use their own method, such as a Polymerase Chain Reaction (PCR) procedure.

The prescribed method in summary:

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

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

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

Confirmation:
Confirmation by means of appropriate biochemical tests (ISO 6579,
Anonymous, 2002) or using 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
artificially contaminated chicken faeces samples. For the control
samples, only the accuracy rates were calculated. The specificity,
sensitivity and accuracy rates were calculated according to the following
formulae:

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

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

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

3.6 Good performance

For the determination of good performance, the criteria indicated in
Table 2 were used. 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. If, for example, in respect of the
STM at low level with matrix, a laboratory found 4/6 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 2/6 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 XVII (2014)

Minimum result		
Contamination Level	Percentage positive	No. of positive samples/ total no. of samples
Samples Chicken faeces artificially contaminated		
S. Typhimurium high level (STM)	80%	5/6
S. Typhimurium low level (STM)	50%	3/6
Blank (BL)[1]	20% at max[1]	1/6 at max[1]
Control samples		
Positive control (Own control with *Salmonella)*	100%	1 /1
Procedure control (BPW)	0%	0 /1
Matrix control (Chicken faeces)	0%	0 /1

1.All should be negative. However, since no 100% guarantee of the *Salmonella* negativity of the matrix can be given, 1 positive out of 6 blank samples (20% pos.) is considered acceptable.

4 Results

4.1 Chicken faeces (from a laying hen flock)

All batches chicken faeces were tested negative for *Salmonella* and stored at 5 °C. For the interlaboratory comparison study, the faeces samples were sent to the NRLs-*Salmonella* on Monday 24 March 2014. After receipt the NRLs had to store the chicken faeces samples at 5 °C. The number of aerobic bacteria and the number of *Enterobacteriaceae* were tested twice at the laboratory of the EURL-*Salmonella*; firstly, on the day the chicken faeces arrived at the EURL (05/03/2014) and, secondly, after storage at 5 °C, close to the planned date of the interlaboratory comparison study (01/04/2014). Table 3 summarizes the results, showing that the amount of background flora remained stable even after storage for 4 weeks at 5 °C.

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

Date	Aerobic bacteria CFU/g	*Enterobacteriaceae* CFU/g
5 March 2014	2×10^9	4×10^3
1 April 2014 after storage at 5 °C	3×10^8	8×10^4

4.2 Artificial contamination of chicken faeces samples

4.2.1 Pre-tests for the preparation of contaminated faeces samples

Two sets of experiments were performed. During each set of experiments, the stability of *Salmonella* in the chicken faeces samples was tested during storage of the samples at different temperatures for up to three weeks. During each set of experiments, different variables were tested in different combinations (see Section 3.1.1). Figure 1 shows the results of all tested samples.

The major findings are summarized below:
An inoculation level of 6 CFU/25 g of chicken faeces was shown to be too low. Three out of six faeces samples artificially contaminated with *Salmonella* Typhimurium (STM) were negative after one week of storage at 5 °C.
When the faeces samples were artificially contaminated with *Salmonella* Typhimurium (STM) at a level of 10 CFU/25 g or higher, 4-6 of the six samples tested were still positive for *Salmonella* after 2-3 weeks of storage at 5 °C or at 10 °C.
The amount of background flora in the chicken faeces was relatively stable during storage at 5 °C and 10 °C for 3 weeks. The levels varied between 10^3 -10^5 CFU/g for the number of *Enterobacteriacea* and was stable at 10^8 CFU/g for the total number of aerobic bacteria.
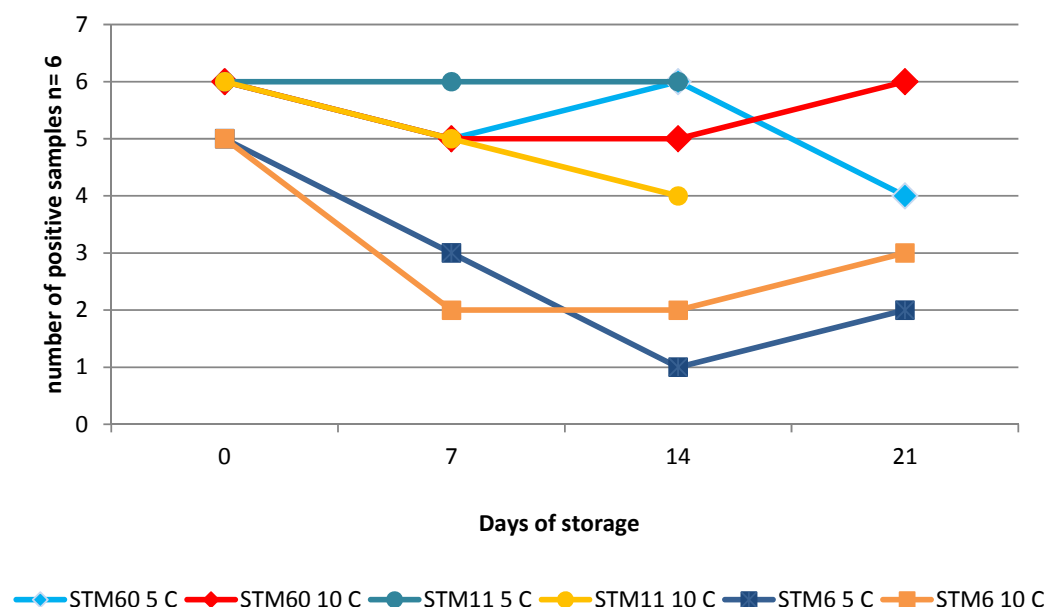
Figure 1. Stability tests on chicken faeces samples artificially contaminated with Salmonella Typhimurium (STM)

From the results of the experiments, it was decided to use the following samples for the interlaboratory comparison study:
for each participant, 18 x 25 g of chicken faeces from a SPF laying hen flock;
each sample individually inoculated with a diluted culture of Salmonella Typhimurium at the following levels:
- low-level STM: 10–15 CFU/25 g of chicken faeces;
- high-level STM: 50–100 CFU/25 g of chicken faeces;
- blank: 0 CFU/25 g of chicken faeces.

4.2.2   Contamination level of the artificially contaminated chicken faeces samples
Table 4 shows the contamination level of the chicken faeces samples at low levels and high levels of contamination. The inoculum level of the diluted STM culture (tested on XLD), as well as the contamination level in the chicken faeces samples after the inoculation with the diluted culture, were tested. The latter was tested using a five-tube MPN test (see Section 3.1.2). The number of positive chicken faeces samples for 25 g, 2.5 g and 0.25 g were, respectively, 5/5, 1/5 and 0/5 for the low-level STM and 5/5, 5/5 and 1/5 for high-level STM. The calculated MPN/25 g of chicken faeces is shown in Table 3.

Table 4. Number of Salmonella *Typhimurium (STM)*

Date of testing	Low-level STM CFU/25 g chicken faeces	High-level STM CFU/25 g chicken faeces
20 March 2014 (inoculum of chicken faeces)	14	67
30 March 2014 after storage at 5 °C MPN of chicken faeces inoculated with STM (95% confidence limit)	3.3 (1.1–10)	35 (11–110)

4.3 Technical data of interlaboratory comparison study

4.3.1 General

Thirty-six NRLs for *Salmonella* participated in this study: 29 NRLs from 28 EU Member States (MS) and 7 NRLs from non-EU MSs. The non-EU MSs consisted of EU candidate MSs or potential EU candidate MSs, member countries of the European Free Trade Association (EFTA) and, at the request of DG-Sanco, a country outside Europe.
Thirty-five laboratories performed the study on the planned date (week 14, starting on 31/03/2014). Laboratory 21 performed the study one week earlier.

4.3.2 Accreditation/certification

Thirty-two laboratories are accredited for their quality system according to ISO/IEC 17025 (Anonymous, 2005), three EU MS laboratories (2, 16 and 19) were in the process for accreditation (2014 or 2015) and one EU MS laboratory (lab code 9) has no plan for accreditation. Thirty-two laboratories are accredited for Annex D of ISO 6579 and 13 of them are also accredited for ISO 6579.

4.3.3 Transport of samples

Seven participants received the samples within one day after dispatch, seven-teen participants within two days and eight participants after three days of transport. For two parcels (non-EU MSs) it was not possible to arrange door-to-door transport. The parcel for laboratory 36 was retained by customs officials and arrived only after 6 days of transport at the participating laboratory. For laboratory 23, the parcel was transported to an NRL in a neighbouring country (door-to-door). This parcel was picked up by the relevant NRL after arrival at the neighbouring NRL and required 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. Three participants (lab codes 25, 28 and 30) returned the temperature recorder immediately after the arrival of the samples at their institute (as in earlier studies). Two temperature recorders (lab codes 34 and 36) were broken. For the majority of the parcels, the temperature did not exceed 5 °C during transport and storage at the NRL. The exceptions were the laboratories 6, 7, 22, 23, 27, 31, 32 and 33, where the samples were stored for a few days between 5 °C and 11 °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 provides information on the reported pH, the concentration of Novobiocin, the incubation time and temperature that deviated from the prescribed method.
Three laboratories (3, 7 and 27) reported a (slightly) longer incubation time for the pre-enrichment in BPW. Four laboratories (7, 10, 22, 27 and 36) reported a higher pH than the prescribed maximum pH of 7.2 for BPW.
Five laboratories (4, 22, 28, 33 and 34) used MSRV with a higher or lower concentration of Novobiocin than the prescribed 10 mg/L.
Laboratory 7 reported a higher pH of 5.5 for the MSRV than the prescribed maximum pH of 5.4.
Laboratory 34 reported an incubation temperature of 36.4 °C instead of the prescribed 40.5-42.5 °C.
Laboratories 14, 18 and 21 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	Incubation temperature in °C (min-max)
Prescribed in ISO 6579 Annex D	**16–20 h**	**6.8–7.2**	**5.1–5.4**	**10 mg/L**	**40.5 – 42.5**
3	20:25	7.06	5.07	10	41.7
4	20:00	6.9	5.1	20	41.5
7	24:00	7.3	5.5	10	41.5
10	18:10	7.29	5.1	10	41.5
14	18:45	7.9	-	10	41.9
18	17:45	-	-	10	41.5
21	18:40	-	-	10	41.1
22	20:00	7.3	5.2	0.05	41.5
27	21:00	7.3	5.3	10	41.5
28	18:00	7.13	5.29	25	41.5
33	20:00	7	5.4	2	41.5
34	18:10	7.0	5.25	1	36.4
36	18:20	7.23	5.23	10	41.1

Grey cells   Deviating from ISO 6579 Annex D
-              No information

Table 6 Media used as second plating-out medium

Media	Number of users	Lab code
BGA^{mod} (ISO 6579, 1993)	6	4, 5, 6, 10, 11, 17
BGA	9	1, 2, 7, 8, 9, 28, 32, 33, 36
Rambach (Merck)	7	13, 23, 26, 30, 31, 34, 35
SM(ID)2 (Biomerieux)	4	14, 19, 20, 21
RS (Bio-rad)	3	16, 18, 24
BPLS = BGAP (Merck)	3	3, 22, 25
BSA (=OSCM)	1	15
Salmonella Chromogenic Medium II (Oxoid)	1	27
BxLH (Home-made)	1	12
ASAP (BioMerieux-AES)	1	29

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

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.
The use of an extra non-selective plating agar between the 'isolation' and 'confirmation' steps was optional. A total of 25 laboratories performed this extra step (e.g. by using Nutrient agar; Anonymous, 2002).

All participating laboratories performed one or several confirmation tests for *Salmonella*, see Tables 7 and 8. Four laboratories (1, 10, 14 and 34) performed serological tests only and nine laboratories (4, 8, 15, 18, 19, 21, 27, 28 and 33) performed only a biochemical test. Two laboratories (13 and 27) used the Maldi-Toff test and six laboratories (7, 25, 28, 31, 35 and 36) a PCR method for confirmation in addition to biochemical tests (although laboratory 35 did not mention their PCR results for this study).

Table 7. Biochemical and other confirmation tests for Salmonella

Lab code	TSI	UA	LDC	Gal	VP	Indole	Kit	Other
1	-	-	-	-	-	-		Chromogenic Agar
10, 14, 34	-	-	-	-	-	-		
2	+	-	-	-	-	-	Microgen GN - ID A	Vitek 2
3, 9, 15, 19	+	+	+	-	-	-		
4	+	+	+	-	-	+		Glicose
5, 23, 32	+	+	+	+	+	+		
6, 22	+	+	+	-	-	+		
7	+	+	+	+	+	+	API-20E	PCR
8	+	-	+	-	-	-	Wellcolex	
11, 26	+	+	+	+	-	+		
12	-	-	-	-	-	-		Kligler agar, urea and indol broth, mannitol and nitrate broth, ONPG and FDA medium, motility test
13	-	-	-	-	-	-	Enterotest 24	MALDI TOF
16, 29	-	-	-	-	-	-	API-20E	
17	+	-	+	-	-	-	Rapid 20E	
18	+	-	+	-	-	-		sorbitol mobility
20	+	-	-	-	-	-	API ID32E	
21	-	-	-	-	-	-	Microbact 12A	
24	+	+	+	+	+	+		Malonate
25	-	-	-	-	-	-	Microbact 24E	PCR
27	-	-	-	-	-	-		MALDI TOF
28, 31	+	+	+	+	+	+		PCR
30	+	+	+	+	-	+		semi-solid glucose agar
33	+	+	+	-	-	+		Simmons, Citrate
35	-	-	-	-	-	-	API-20E	InvA-PCR
36	-	-	-	-	-	+	HY Enterotest	PCR

Table 8. Serological confirmation of Salmonella

Lab code	Serological		
	O antigens	**H antigens**	**Vi antigens**
2, 17, 20, 22	+	+	+
5, 6, 9, 10, 13, 23, 24, 25, 30, 31, 32, 34, 35	+	+	-
16	+	-	+
1, 3, 7, 11, 12, 26, 29, 36	+	-	-
4, 8, 15, 18, 19, 21, 27, 28, 33	-	-	-
14	Enteroclon - Anti-*Salmonella* Tests		

4.4 Results of control samples in interlaboratory comparison study

4.4.1 General

Table 9 shows the results of all control samples. 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), with the exception for one laboratory (lab code 13).

Table 9. Total number of positive results from the control samples per laboratory

Lab code	Number of positive isolations found with MSRV in combination with isolation medium XLD/2nd		
	Own control With *Salmonella* n=1	**Procedure control BPW n=1**	**Matrix control Chicken faeces 25g n=1**
Good performance	**1**	**0**	**0**
4 and 7	**0**	0	0
13	1/**0**	0	0
1-3, 5, 6, 8-12, 14-36	1	0	0

Bold number: deviating result

Grey cell: result is below good performance

When only one number is mentioned, both isolation media gave the same results.

Positive control with Salmonella

Thirty-four laboratories scored good results with their own *Salmonella* positive control sample and detected *Salmonella* with all media used. The laboratories 4 and 7 could not detect *Salmonella* after selective enrichment on MSRV in combination with any isolation medium. Laboratory 13 could not detect *Salmonella* after selective enrichment on MSRV in combination with isolation on Rambach, but scored the same sample as positive on isolation medium XLD.

For the positive control samples, the majority of the participants used a diluted culture of *Salmonella* (24 laboratories). Others used a lenticule disc (7), a Freeze-dried ampoule (2), capsule (1), cryobank (1) or a frozen culture (1) with *Salmonella.* Table 10 shows the *Salmonella* serovars used for the positive control samples. Most often, *Salmonella* Enteritidis (15) and *Salmonella* Typhimurium (9) were used. The concentration of *Salmonella* in the positive control samples used by the different participants varied between 8 and 10^6 CFU/sample.

Table 10. Salmonella serovars used by the participants for the positive control samples

Salmonella serovar	Number of users
S. Enteritidis	15
S. Typhimurium	9
S. Nottingham	3
S. Goldcoast, *S.* Alachua	2
S. Infantis *S.* Bongori, *S.* Abony, *S.* Dublin, *S.* Tennessee	1

Procedure control Blank (only BPW)
All laboratories analysed the one procedure control sample (no matrix, only BPW) correctly as negative for *Salmonella*.

Matrix control Blank (chicken faeces)
All laboratories analysed the one chicken faeces control sample (25 g of matrix) correctly as negative for *Salmonella,* irrespective of the media used.

The results were compared with the definition of 'good performance' (see Section 3.6). Laboratories 4 and 7 did not fulfil these criteria for the control samples, as they scored their own positive control as being negative for *Salmonella*.

Table 11. Correct scores found with the control samples by all laboratories ('All') and by the laboratories of the EU member states only ('EU')

Control samples		MSRV/X	
		All n=36	EU n=29
Procedure control Blank (BPW) n=1	No. of samples	36	29
	No. of negative samples	36	29
	Correct score in %	**100**	**100**
Matrix control Blank Blank chicken faeces 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	34	28
	Correct score in %	**94.4**	**96.6**
All Control samples	No. of samples	108	87
	No. of correct samples	106	86
	Accuracy in %	**98.1**	**98.9**

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

4.4.2   *Correct scores of the control samples*
Table 11 shows the correct scores found with the control samples. The rates are calculated for the selective enrichment medium MSRV in combination with the plating-out medium giving the highest number of positives (XLD and 'non-XLD'). The calculations were performed on the results of all participants, as well as on the results of the EU MS only. Almost no differences were found between these groups.
The laboratories scored an excellent result for the control samples, with an accuracy rate of 98% or higher.

4.5   **Results of artificially contaminated chicken faeces samples in interlaboratory comparison study**

4.5.1   *Results per contamination level and per laboratory*
General
Table 12 gives the results found in the interlaboratory comparison study with the chicken faeces samples artificially contaminated with *Salmonella* Typhimurium (STM). The results given in this table are the highest number of positive isolations found with MSRV in combination with any isolation medium (MSRV/x). No differences in scores were seen between the different isolation media used: XLD or non-XLD (e.g. BGA), with the exception of two laboratories (4 and 13).

The majority of the laboratories (31/36) found all *Salmonella* chicken faeces samples to test positive when using the prescribed method (selective enrichment on MSRV).

Blank samples (n=6)
All laboratories correctly found the blank chicken faeces samples to be negative for *Salmonella*.

Low-level contaminated Salmonella *Typhimurium (STM low) samples (n=6)*
Thirty-one laboratories detected *Salmonella* in all six samples containing *Salmonella* Typhimurium at an inoculum level of approximately 14 CFU/ 25 g of chicken faeces. Five laboratories (7, 13, 14, 21 and 26) could not detect *Salmonella* in one of the six chicken faeces samples contaminated at a low level. These samples contained S. Typhimurium at a low level (3 MPN/sample at the day of performance), so that, due to change, 1 out of 6 low-level samples may test negative.
Laboratory 13 could not detect *Salmonella* with their second isolation medium (Rambach), but scored five samples correctly as positive after isolation on XLD inoculated from the same MSRV plates.
High-level contaminated Salmonella *Typhimurium (STM high) samples (n=6)*
All laboratories detected *Salmonella* in all six samples containing *Salmonella* Typhimurium at an inoculum level of approximately 67 CFU/ 25 g of chicken faeces. Laboratory 13 could not detect *Salmonella* with their second isolation medium (Rambach), but scored all samples correctly as positive after isolation on XLD inoculated from the same MSRV plates. Laboratory 4 also found fewer positive results (4/6) with their second isolation medium (BGA).
The results of the artificially contaminated chicken faeces samples were compared with the definition of 'good performance' (see Section 3.6) and all laboratories fulfilled these criteria for the prescribed method (MSRV).

Table 12. Number of positive results found by the participating laboratories with the artificially contaminated chicken faeces samples (25 g) after selective enrichment on MSRV and per isolation medium

Lab code	Number of positive isolations found with MSRV in combination with isolation medium XLD/2nd		
	Blank **n=6**	**STM Low** **n=6**	**STM High** **n=6**
Good performance	**≤1**	**≥3**	**≥5**
1-3	0	6	6
4	0	6/**4**	6
5, 6	0	6	6
7	0	**5**	6
8-12	0	6	6
13	0	**5/0**	6/**0**
14	0	**5**	6
15-20	0	6	6
21	0	**5**	6
22-25	0	6	6
26	0	**5**	6
27-36	0	6	6

Bold number = deviating result.

When only one number is mentioned, both isolation media gave the same results.

4.5.2   *Results per medium, per contamination level and per laboratory*
Figures 2 and 3 show the number of positive isolations per type of artificially contaminated chicken faeces and per laboratory after pre-enrichment in BPW, selective enrichment on MSRV and isolation on selective plating agars.
In both figures, the border of good performance is indicated by a black horizontal line.

Table 13 presents the percentages of samples tested positive for *Salmonella* after selective enrichment on MSRV in combination with isolation on XLD or non-XLD. Depending on the level of contamination, 3–4% more positive results were found after isolation on XLD agar, compared with isolation on a non-XLD isolation medium (most often BGA).
An extra incubation time of 24 hours for the MSRV did not result in more positive samples. The overall increase in positive results after 48 hours of incubation was only 0.05%.

Table 13. Mean percentages of faeces samples tested positive for Salmonella after selective enrichment on MSRV, followed by isolation on different plating-out media

| Plating-out medium	Selective enrichment medium MSRV	
	Low-level contaminated	High-level contaminated
XLD	98%	100%
Other (often BGA)	94%	97%
Difference XLD/other	4%	3%

4.5.3 *Specificity, sensitivity and accuracy rates of the artificially contaminated faeces samples*
Table 14 shows the specificity, sensitivity and accuracy rates for all types of artificially contaminated chicken faeces samples. This table gives the results for the different medium combinations: pre-enrichment in BPW, followed by selective enrichment on MSRV and isolation on a selective plating agar showing the highest number of positive results (MSRV/x). The calculations were performed on the results of all participants and on the results of the participants from the EU MS only. No differences were seen between these groups. The specificity rate (100%) and the sensitivity rates (low-level: 98%; high-level 100%) were high for the whole group of participants.

Table 14. Specificity, sensitivity and accuracy rates of the artificially contaminated chicken faeces samples after selective enrichment on MSRV in combination with 'the best' isolation medium

Chicken faeces samples		MSRV/X all participants n=36	MSRV/X EU-MS n=29
Blank n=6	No. of samples	216	174
	No. of negative samples	216	174
	Specificity in %	**100**	**100**
STM low n=6	No. of samples	216	174
	No. of positive samples	211	170
	Sensitivity in %	**97.9**	**97.7**
STM high n=6	No. of samples	216	174
	No. of positive samples	216	174
	Sensitivity in %	**100**	**100**
All chicken faeces samples with *Salmonella*	No. of samples	432	348
	No. of positive samples	427	344
	Sensitivity in %	**98.8**	**98.9**
All chicken faeces samples	No. of samples	648	522
	No. of correct samples	643	518
	Accuracy in %	**99.2**	**99.2**

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

Low STM
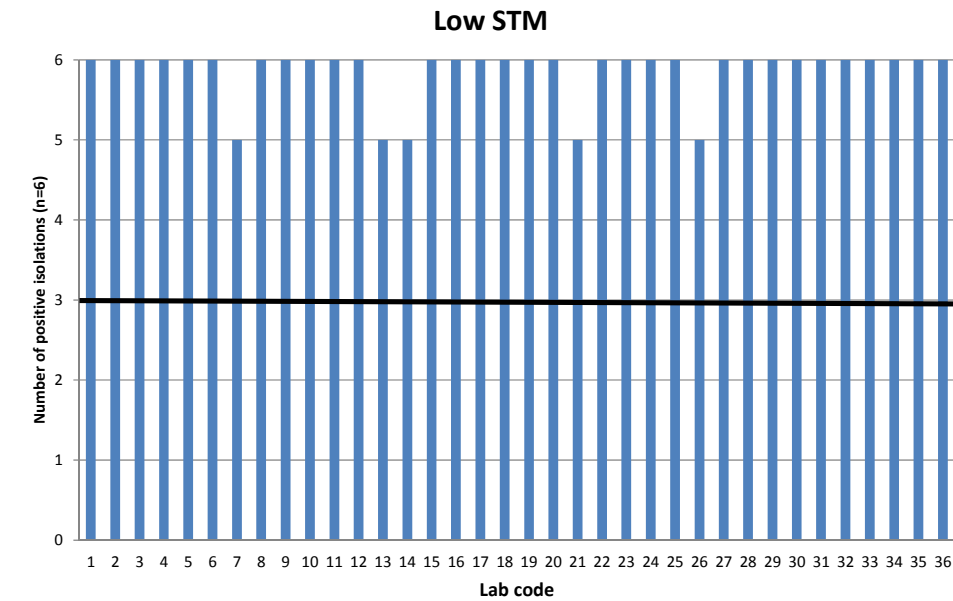
\- = border of good performance

Figure 2. Results found per laboratory with chicken faeces (25 g) samples artificially contaminated with STM low (n=6) after selective enrichment on MSRV followed by isolation on the 'best' selective plating agar

High STM
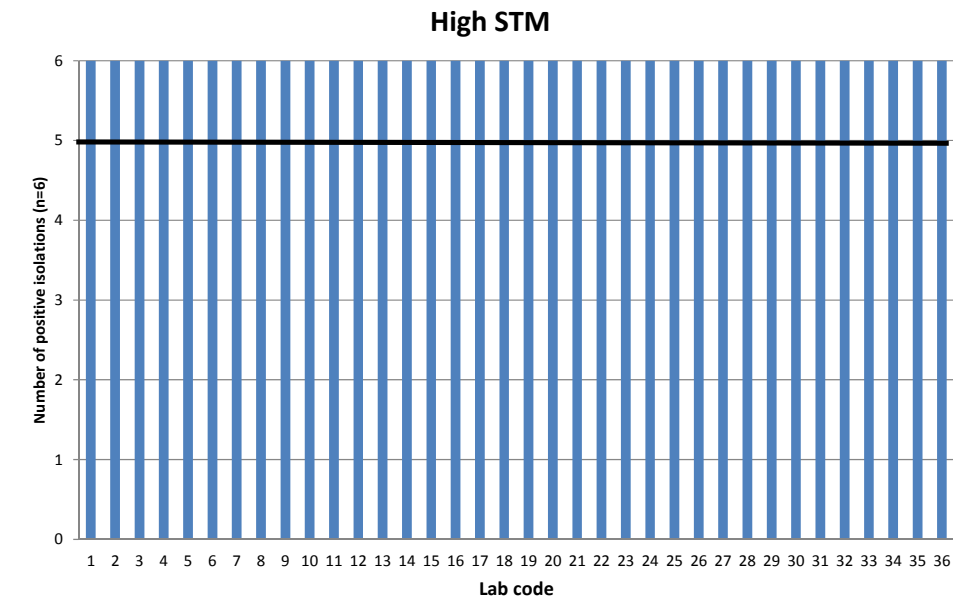
\- = border of good performance

Figure 3. Results found per laboratory with chicken faeces (25 g) samples artificially contaminated with STM high (n=6) after selective enrichment on MSRV, followed by isolation on the 'best' selective plating agar

4.6 **PCR (own method)**

Five laboratories (7, 25, 28, 31 and 36) applied a PCR method as an additional detection technique. All of these laboratories, except for two, tested the samples after pre-enrichment in BPW. Laboratories 7 and 25 started the DNA extraction after selective enrichment on MSRV or after culturing on an isolation medium. All the laboratories used a real-time PCR, except for laboratory 7. This laboratory used a three-step PCR with reference to Gregory et al. (1994). Four of the five laboratories used a validated PCR method. Reference was made to certificate numbers and/or to ISO 16140 (Anonymous, 2003b). Four laboratories used the PCR technique routinely for testing 11 to 699 samples per year.
Table 15 gives further details of the PCR techniques used.

Table 15. Details of Polymerase Chain Reaction procedures used as own method during the interlaboratory comparison study by five participants

Lab code	PCR method	Validated	Commer-cially available	Routinely used number of samples / year	DNA ex-traction after	Reference
7	Three step	-	-	60	Isolation medium	Gregory et al. (1994)
25	Real-time	+	-	89	MSRV	Malorny et al. (2004)
28	Real-time	+	-	11	BPW	
31	Real-time	+	+	-	BPW	Lauer et al. (2009)
36	Real-time	+	-	699	BPW	Malomy et al. (2004) Lofstrom et al. (2010 and 2012)

Table 16. Number of positive results found with the artificially contaminated faeces samples after using a PCR technique or the bacteriological culture technique (BAC)

Lab code	7		25, 28, 36		31	
	BAC	PCR	BAC	PCR	BAC	PCR
STM low (n=6)	**5**	**5**	6	6	6	**5**
STM high (n=6)	6	6	6	6	6	6
Blank (n=6)	0	0	0	0	0	0

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

Table 16 gives the results found using both the PCR method and the bacteriological culture technique (BAC). Four laboratories (7, 25, 28 and 36) found the same results using the PCR method as they did using the bacteriological culture method (MSRV). Laboratory 31 found one sample to test negative (STM low-level) using the PCR method, while the same

sample tested correctly as positive using the bacteriological culture method (BAC).

4.7 Performance of the NRLs

4.7.1 General

Thirty-four NRLs fulfilled the criteria of good performance and two laboratories scored below these criteria. For the determination of good performance, the results of all media were taken into account. Laboratory 13 could not detect *Salmonella* in any of the samples (including the positive control) with their second isolation medium, Rambach. However, with the isolation medium XLD (inoculated from the same MSRV), they tested all samples correctly, resulting in an overall good performance. The EURL-*Salmonella* advised this NRL to check their Rambach isolation medium.

The two deviating laboratories (4 and 7) were contacted by the EURL-*Salmonella* in April 2014 and asked for possible explanations for their deviating results. They both reported their own positive control sample as testing negative.
The laboratories 4 and 7 both indicated that they made a technical mistake. They used their own positive control sample, but reported this as an additional sample in their own data but did not report this result in the web-based test report of the study. After providing the raw data, it was decided that no further actions were considered necessary for those laboratories and their results were indicated as a 'moderate performance'.

5   Discussion

Artificial contamination of samples with a diluted culture
This is the third study in which the samples were artificially
contaminated with a diluted culture at the laboratory of the EURL. The
studies of 2013 for the detection of *Salmonella* in boot socks (Kuijpers
and Mooijman, 2014a) and for the detection of *Salmonella* in minced
chicken meat (Kuijpers et al. 2014b) were successful. The samples
mimic 'real life' routine samples and were easy to handle for the
participants. As each matrix and *Salmonella* serovar combination may
behave differently, the samples of the current study were tested prior to
the study for their stability at storage and transport temperatures (5 ºC
and 10 ºC).
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 below 10 ºC most of the time. Only 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 chicken faeces 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 6 CFU STM/ 25 g of chicken faeces showed a rapid
decrease in the number of positives after one week of storage at 5 ºC
and 10 ºC, it was decided to increase the inoculation level of the low-
contaminated samples to 10 – 15 CFU STM/25 g of faeces. 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 at the time of the study. The
MPN calculated for the low-level contaminated samples was
1.1–10 MPN/ 25 g of chicken faeces on the day of 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 14 CFU in 25 g of chicken faeces
and was close to the detection limit.

Transport of the samples
To prevent the level of *Salmonella* Typhimurium in the 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 period. It can therefore 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
period in combination with the highest temperatures (lab code 33) still
found all contaminated samples to be positive.

According to EC regulations 882/2004 (EC, 2004) and 2076/2005 (EC,
2005), each NRL should have been accredited in their relevant working
field before 31 December 2009. Thirty-two laboratories indicated that
they were accredited. Four (EU MS) participants (lab codes 2, 9, 16 and

19) are still in the process of becoming accredited, which is relatively late.

Performance of the laboratories
For the evaluation of the results 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.
Two laboratories (lab codes 4 and 7) scored an 'underperformance'. Both laboratories made a (technical) mistake in reporting their own positive control sample. In cases of reporting the results of routine samples, a transcription error may result in unwanted situations, such as 'incorrect non-compliance' of a food product. The results of laboratories 4 and 7 were therefore indicated as 'moderate performance'. A follow-up study was considered unnecessary for those laboratories.

According to the used criteria, 34 laboratories scored 'good performance' and two laboratories scored 'moderate 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 98% and 100%).

Positive control samples
The participants were asked to use the positive control sample(s) routinely used in their laboratory. *S.* Enteritidis and *S.* Typhimurium were the most frequently used *Salmonella* serovars and the concentration varied between 8 – 10^6 CFU/sample.
The intention of a positive control is that it demonstrates that the method is performed properly. In case of a qualitative method, such as the detection of *Salmonella*, it is also important to have information on the fact that the method is able to detect low numbers of the target organism. For this purpose, it is advisable to choose a contamination level for the positive control sample that is close to the detection limit of the method. For the choice of the organism in the positive control sample, it seems logical to choose a *Salmonella* serovar frequently found in routine analysis so that it is demonstrated that the method performs well for the main target organisms. On the other hand, it can also be of advantage to use a serovar rarely isolated from routine samples, as in this way, possible cross-contamination with the positive control sample can be detected more easily.
In ISO 7218 (Anonymous, 2007b) some general information is given on the quality assurance of results, but the conditions which a suitable positive control should fulfil are not prescribed. The choice of the *Salmonella* serovar, as well as the contamination level of *Salmonella* in the positive control sample for the detection of *Salmonella,* is to large extent left for the laboratory to decide.
Laboratory 13 scored a false negative result for their positive *Salmonella* control sample with their 'second' isolation medium (Rambach). The result of this control is a possible indication of problems with the relevant medium, as the laboratory also found no positive results for the

same medium with respect to the chicken faeces samples contaminated with *Salmonella,* although the same samples tested positive after isolation on XLD.

Media and incubation
During the study, small deviations in the prescription of the media (e.g. in pH or the concentration of novobiocin) or in incubation temperature and/or times were reported. The influence of these deviations on the results is not always clear. For instance, laboratory 7 reported deviations in their BPW (incubation time and pH) and MSRV (pH) and scored a lower number of positive chicken faeces samples. Whether this lower score was caused by those deviations is hard to trace.
One laboratory mentioned 36.4 ºC for the incubation temperature of all their media (BPW, MSRV and both isolation media). Whether this is a transcription error or a real deviation for MSRV, for which another temperature is prescribed, is not clear. The laboratory scored a good performance and additional information on this deviating incubation temperature was not requested.

The increase in the number of positive results after 48 hours of incubation of the selective enrichment on MSRV was nil. The majority of the laboratories found all samples to test positive after 24 hours of incubation.

PCR
Five laboratories used a PCR technique in addition to the prescribed method. Four laboratories found the same results as they did with the bacteriological culture technique (BAC), while one laboratory found one result negative using their PCR method, yet positive using the bacteriological culture technique.
The PCR results from these participants did not seem to be affected by the choice of the PCR technique, but rather by the skills of the laboratory in using the method. The best results were found by the laboratories that use a PCR technique routinely. This was also observed in the food study of 2013 on the detection of *Salmonella* in minced chicken meat (Kuijpers et al. 2014b).

6   Conclusions

Thirty-four out of 36 NRLs for *Salmonella* were able to detect high and low levels of *Salmonella* in chicken faeces samples. Two laboratories scored a 'moderate performance'.

High rates for the specificity, sensitivity and accuracy of the artificially contaminated chicken faeces were found (blank, low level and high level): 98 - 100%

The accuracy rate of the control samples after selective enrichment on MSRV by the NRLs from the EU MS was 99%.

Some participants may take the optimization of the positive control sample used in their daily routine analysis into consideration with respect to the choice of the *Salmonella* serovar and/or contamination level.

48 hours of incubation of the selective enrichment medium MSRV showed only 0.05% more positive results than 24 hours of incubation.

For the PCR technique used by five NRLs as their own method, the best results were found by the four laboratories that used a PCR technique routinely.

List of abbreviations

ASAP	AES *Salmonella* Agar Plate
ATCC	American Type Culture Collection
BAC	Bacteriological Culture technique
BGA(mod)	Brilliant Green Agar (modified)
BPLS	Brilliant Green Phenol-red Lactose Sucrose (BGAP)
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
DG-Sanco	Directorate-General for Health and Consumer Protection
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
PPS	primary production stage
RIVM	Rijksinstituut voor Volksgezondheid en het Milieu (National Institute for Public Health and the Environment)
RS	Rapid *Salmonella*
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

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