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EURL-*Salmonella* Proficiency Test Primary Production Stage, 2023

Detection of *Salmonella* in chicken faeces samples

RIVM report 2023-0342
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Colophon

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Synopsis

EURL-*Salmonella* Proficiency Test for Primary Production Stage, (2023)

Detection of *Salmonella* in chicken faeces samples

Since 1992, the National Reference Laboratories (NRLs) from the EU Member States have been obliged to take part in an annual quality control, which consists of conducting 'proficiency tests'. The objective of these proficiency tests is to detect *Salmonella* bacteria in samples taken from the living environment of animals, such as stables. In 2023, all NRLs from the EU Member States were able to detect *Salmonella* in chicken faeces samples. All participating laboratories were able to detect both low and high concentrations of *Salmonella*.

One laboratory needed a second attempt. Another laboratory initially obtained a poor score due to an administrative error. Using raw data, this laboratory was able to demonstrate that two samples had been switched. As a result, it became possible to upgrade its score to moderate performance. This was the outcome of the proficiency test organised by the European Union Reference Laboratory in October 2023.

In total, 37 NRLs took part in this proficiency test. They included the NRLs from the 27 EU Member States, nine NRLs from other European countries and one NRL from a non-European country. The laboratories used a mandatory, internationally recognised analytical method to detect *Salmonella* in chicken faeces samples. Each laboratory was sent a set of samples that had been artificially contaminated with two different concentrations of *Salmonella* Typhimurium, or had not been contaminated at all.

The proficiency test was organised by the European Union Reference Laboratory (EURL) for *Salmonella*, which is located at RIVM. One of EURL-*Salmonella*'s key tasks is to monitor the quality of the *Salmonella* NRLs in Europe.

Keywords: *Salmonella*, EURL, NRL, proficiency test, chicken faeces, *Salmonella* detection method

Publiekssamenvatting

Het EURL-*Salmonella* ringonderzoek productiedieren (2023)

Detectie van *Salmonella* in kippenmest

Sinds 1992 zijn de Nationale Referentie Laboratoria (NRL's) van de Europese lidstaten verplicht om elk jaar hun kwaliteit te laten toetsen met zogeheten ringonderzoeken. Het doel van een van de ringonderzoeken is *Salmonella* bacteriën opsporen in monsters uit de leefomgeving van dieren, zoals stallen. In 2023 waren alle NRL's uit de EU-lidstaten in staat om *Salmonella* aan te tonen in de kippenmest monsters. Alle deelnemers konden hoge en lage concentraties *Salmonella* aantonen.

Eén laboratorium had hiervoor een herkansing nodig. Een ander laboratorium haalde eerst een slechte score door een administratieve fout. Dit laboratorium kon daarna met ruwe data aantonen dat het twee monsters had verwisseld. Daardoor kon de score omhoog worden bijgesteld naar een matige score. Dit blijkt uit het ringonderzoek dat het overkoepelende Europese laboratorium in oktober 2023 organiseerde.

In totaal hebben 37 NRL's aan dit ringonderzoek meegedaan. Dat zijn de NRL's uit de 27 EU-lidstaten, negen NRL's uit andere Europese landen en één NRL uit een niet-Europees land. De laboratoria gebruikten een verplichte, internationaal erkende analysemethode om *Salmonella* in kippenmestmonsters aan te tonen. Elk laboratorium kreeg een pakket toegestuurd met monsters die kunstmatig waren besmet met twee verschillende concentraties *Salmonella* Typhimurium, of zonder deze bacterie.

Het ringonderzoek is georganiseerd door het Europese Referentie Laboratorium voor *Salmonella* (EURL). Dit is gevestigd bij het RIVM. Een belangrijke taak van het EURL-*Salmonella* is toezien op de kwaliteit van de NRL's-*Salmonella* in Europa.

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

Contents

Annex I Example of an individual laboratory, Performance Report of the EURL-*Salmonella* PT PPS 2023 — 37

Summary

In October 2023, the European Union Reference Laboratory for *Salmonella* (EURL) Proficiency Test on the detection of *Salmonella* in samples from the primary production stage (PPS) was conducted. Participation was mandatory for the National Reference Laboratories (NRLs) for *Salmonella* of all European Union (EU) Member States (MSs) that are responsible for the detection of *Salmonella* in PPS samples. A total of 37 NRLs-*Salmonella* participated in this study, originating from the 27 EU Member States, nine NRLs from other European countries (EU candidate MSs or potential EU candidate MSs, members of the European Free Trade Association (EFTA) and EU third countries), and one NRL from a non-European country.

Samples

In this study, the matrix under analysis was chicken faeces, artificially contaminated at the EURL-*Salmonella* laboratory with a diluted culture of *Salmonella* Typhimurium.

Each NRL-*Salmonella* had to analyse the following set of blindly coded samples:

- 4 chicken faeces samples with a high level of *S.* Typhimurium (inoculum 50 cfu/sample);
- 6 chicken faeces samples with a low level of *S.* Typhimurium (inoculum 17 cfu/sample);
- 4 negative chicken faeces samples (no *Salmonella* added);
- 1 procedure control (buffered peptone water only);
- 1 positive control sample (laboratories' own *Salmonella* control strain).

The samples were prepared at the EURL-*Salmonella* laboratory and stored at 5 °C for approximately one week until the day of dispatch. On Monday 25 September 2023, the chicken faeces samples were packed and sent to the NRLs-*Salmonella*. The NRLs were asked to store the samples at 5 °C on arrival until the start of the analysis on Monday 2 October 2023.

Method

All laboratories used the prescribed method EN ISO 6579-1:2017(/A1:2020) to test the samples. Only one participating laboratory was not accredited for this method (lab code 7).

Seven laboratories also reported results for a second method. The results of their alternative methods were identical to those obtained using EN ISO 6579-1:2017(/A1:2020).

Results

Nearly all 37 laboratories analysed both the procedure control and their own positive control sample correctly. One laboratory (lab code 21) made an administrative mistake, switching the two control samples, but

could prove their initial correct results using their raw data. The accuracy rate of the control samples was 100%.

Nearly all laboratories detected *Salmonella* in three or more out of the six chicken faeces samples contaminated with a low level of *Salmonella* Typhimurium (17 cfu/sample). One laboratory (lab code 1) scored an unsatisfactory performance as this laboratory tested four out of the six low level samples negative for *Salmonella*. These results are not within the criteria for good performance, which permit three negative samples. The sensitivity rate for these samples was 96,4%.

Nearly all laboratories detected *Salmonella* in all four high-level samples contaminated with *Salmonella* Typhimurium (50 cfu/sample). One laboratory (lab code 1) tested one out of the four high-level samples negative for *Salmonella*, which is still within the criteria for good performance. The sensitivity rate for these samples was 99,3%.

All four negative samples were scored correctly as negative by all laboratories. The specificity rate of the negative samples was 100%.

Laboratory 1 was offered a follow-up study, which consisted of six low level samples (inoculum 18 cfu/sample), one high level sample (inoculum 200 cfu/sample) and three negative samples. In this follow-up study, laboratory 1 performed well and scored all samples correctly, resulting in good performance in the follow-up study.

The NRLs-*Salmonella* were given the opportunity to analyse the samples by means of a second detection method. The results of this second detection method were not used to assess the performance of the NRLs-*Salmonella*. Seven participants used a second detection method and the obtained results were similar to those of the prescribed EN ISO 6579-1:2017(/A1:2020).

Overall, the NRLs-*Salmonella* scored well in this Proficiency Test, with an accuracy of 98,3%. One laboratory (lab code 21) scored a moderate performance because of an administrative error, and one laboratory needed a follow-up study, in which it performed well.

1 Introduction

An important task of the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*), as laid out in Commission Regulation No 625/2017 (EC, 2017), is the organisation of Proficiency Tests (PTs) to evaluate the performance of the National Reference Laboratories (NRLs) for *Salmonella*. The history of the PTs organised by EURL-*Salmonella* from 1995 onwards is summarised on the EURL-*Salmonella* website (EURL-*Salmonella*, 2024).

In September 2023, the EURL-*Salmonella* conducted a PT to evaluate whether the NRLs responsible for the detection of *Salmonella* in samples from the Primary Production stage (PPS) could detect *Salmonella* at different contamination levels in chicken faeces samples. The results from PTs such as this one show whether the examination of samples in the EU Member States (EU-MSs) is carried out uniformly and whether all NRLs-*Salmonella* obtain comparable results.

The method prescribed for the detection of *Salmonella* species (spp.) is set out in EN ISO 6579-1:2017(/A1:2020).

The design of this study was comparable to previous PTs conducted by EURL-*Salmonella* (Diddens & Mooijman, 2022; Pol-Hofstad & Mooijman, 2021 and Pol-Hofstad & Mooijman, 2022). For the current study, chicken faeces was artificially contaminated with a diluted culture of *Salmonella* Typhimurium (STm) at the EURL-*Salmonella* laboratory.

In total, 16 samples had to be tested:

- 4 chicken faeces samples with a high level of STm (intended concentration: 50 cfu/sample);
- 6 chicken faeces samples with a low level of STm (intended concentration: 15 cfu/sample)*;*
- 4 negative chicken faeces samples (no *Salmonella* added);
- 1 procedure control (buffered peptone water – BPW- only);
- 1 positive control sample (laboratories' own *Salmonella* control strain).

The number of samples and the contamination levels were based on information described in EN ISO 22117:2019.

2 Participants

In table 2.1, the country, city and the name of the institute of the participating NRLs are displayed.

Table 2.1 List of participants NRLs Primary Production Stage

Country	City	Institute
Austria	Graz	Austrian Agency for Health and Food Safety (AGES/VEMI)
Belgium	Brussels	Sciensano
Bosnia and Herzegovinia	Sarajevo	Veterinary Institute - Veterinary Faculty
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	Praha	State Veterinary Institute
Denmark	Ringsted	Danish Veterinary and Food administration
Estonia	Tartu	National Centre for Laboratory Research and Risk Assessment
Finland	Kuopio	Finnish Food Authority, Laboratory and Research Division
France	Ploufragan	Anses, Laboratoire de Ploufragan-Plouzané Unité Hygiène et Qualité des Produits Avicoles et Porcins (HQPAP)
Germany	Berlin	German Federal Institute for Risk Assessment (BfR) Biological Safety Department
Greece	Chalkida	Veterinary Laboratory of Chalkis
Hungary	Budapest	National Food Chain Safety Office, Food and Feed Safety Directorate, Microbiological NRL
Iceland	Reykjavik	Matís ohf, Food Safety and Analytical services
Ireland, Republic of	Kildare	Central Veterinary Research Laboratory (CVRL/DAFFM) Laboratories Backweston, Department of Bacteriology
Israel	Masmiya	Laboratory of the Israel Poultry and Egg Board
Italy	Padova Legnaro	Istituto Zooprofilattico Sperimentale delle Venezie, OIE
Latvia	Riga	Institute of Food Safety, Animal Health and Environment BIOR Bacteriology and Parasitology Division

Country	City	Institute
Lithuania	Vilnius	National Food and Veterinary Risk Assessment Institute, Laboratory of Microbiology and Pathology, Bacteriology Group
Luxembourg, Grand-Duchy of	Diddeléng	Laboratoire de Médicine Vétérinaire de l'Etat, Bacteriologie
Malta	Valletta	Malta Public Health Laboratory (PHL), Evans Building
Netherlands, the	Bilthoven	National Institute for Public Health and the Environment (RIVM), Centre for Zoonosis and Environmental Microbiology (Z&O)
Northern Ireland	NRL tasks PPS are carried out by NRL Ireland	
Norway	Ås	Norwegian Veterinary Institute, Section of Microbiology
Poland	Pulawy	National Veterinary Research Institute, department of microbiology
Portugal	Vairão	Instituto Nacional de Investigação Agrária e Veterinária, Food Microbiology Laboratory
Republic of Moldova	Chisnau	Republican Centre for Veterinary Diagnostics
Republic of North Macedonia	Skopje	Food Institute, Faculty of Veterinary Medicine, Laboratory for food and feed microbiology
Romania	Bucharest	Institute for Diagnosis and Animal Health
Serbia	Novi Sad	Scientific Veterinary Institute "Novi Sad"
Slovak Republic	Dolny Kubin	State Veterinary and Food Institute
Slovenia	Ljubljana	National Veterinary Institute, Veterinary Faculty (UL, NVI)
Spain	Madrid Algete	Laboratorio Central de Veterinaria
Sweden	Uppsala	National Veterinary Institute
Switzerland	Zurich	National Reference Centre for Poultry and Rabbit Diseases (NRGK), Institute of Food Safety and Hygiene, University of Zurich
Türkiye	Ankara	Veterinary Control Central Research Institute
United Kingdom	Addlestone	Animal and Plant Health Agency

3 Materials and Methods

3.1 Preparation of artificially contaminated chicken faeces samples

3.1.1 General

The matrix used for this PT was chicken faeces. Chicken faeces was artificially contaminated with a diluted culture of STm at the EURL-*Salmonella* laboratory.

3.1.2 Pre-tests for the preparation of chicken faeces samples

The batch of chicken faeces was collected from a *Salmonella*-free broiler breeder flock by the Animal Health Service (GD, Deventer). The batch of faeces (2 kg) for the pre-tests arrived at the EURL on 11 April 2023. The faeces were stored at -20 °C for one day to inactivate small flies possibly present in the faeces. The next day, three 25 gram samples of defrosted chicken faeces, were randomly taken from the batch and tested for the absence of *Salmonella* according to EN ISO 6579-1:2017/A1:2020.

Before deciding on the *Salmonella* serovar to artificially contaminate the chicken faeces for this PT, a stability test was performed for three different *Salmonella* serovars. Faeces was weighed in portions of 25 gram and artificially contaminated with low concentrations of diluted cultures of *Salmonella* Enteritidis (strain number from EURL-*Salmonella*'s own collection: Salm 532), *Salmonella* Infantis (strain number from EURL-*Salmonella*'s own collection: 15A-7) or *Salmonella* Typhimurium (ATCC 14028).

To test the stability of the Proficiency Test samples during transport and in storage conditions, the pre-test samples were stored at 5 °C and at 10 °C for three weeks. After zero, one, two and three weeks of storage, five faeces samples were tested at each time interval for the presence of *Salmonella* according to EN ISO 6579-1:2017/A1:2020. In addition, one (non-contaminated) faeces sample was tested for the concentration of background flora according to section 3.1.4 (number of *Enterobacteriaceae* and total aerobic count), likewise after zero, one, two, and three weeks of storage.

3.1.3 Preparation of chicken faeces samples for the Proficiency Test

A large batch of chicken faeces was collected from a *Salmonella*-free broiler breeder flock by the Animal Health Service (GD, Deventer) on Monday 4 September and was treated in the same way as described in section 3.1.2. The chicken faeces samples were artificially contaminated with a suspension of STm according to the following scheme:

- 4 chicken faeces samples with a high level of STm (intended concentration 50 cfu/sample)*;*
- 6 chicken faeces samples with a low level of STm (intended concentration 15 cfu/sample)*;*
- 4 negative chicken faeces samples (no *Salmonella* added);
- 1 procedure control (BPW) only);
- 1 positive control sample (laboratories' own *Salmonella* control strain).

The concentration of the inoculum used to contaminate the chicken faeces was confirmed by streaking the inoculum on xylose lysine deoxycholate (XLD) agar plates. Immediately following artificial contamination, the high, low and negative samples were stored at 5 °C until dispatch to the participating laboratories on Monday 25 September 2023.

3.1.4 Determination of the level of background flora in chicken faeces samples

The total number of aerobic bacteria and the number of *Enterobacteriaceae* in chicken faeces was assessed by following EN ISO 4833-1:2013 and EN ISO 21528-2:2017, respectively. The chicken faeces samples were homogenised (kneaded) in peptone saline solution and ten-fold dilutions were analysed on plate count agar (PCA) and violet red bile glucose (VRBG) agar.

3.1.5 Determination of the level of Salmonella *in chicken faeces samples by MPN*

The contamination level of *Salmonella* in the artificially contaminated chicken faeces samples was determined using a five-tube most probable number (MPN) technique. Ten-fold dilutions of five artificially contaminated chicken faeces samples at 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 following EN ISO 6579-1:2017. The MPN of *Salmonella* in the original sample was calculated from the number of confirmed positive dilutions, using freely available Excel-Based MPN software (Jarvis et al., 2010).

3.2 Design of the Proficiency Test

3.2.1 Number and type of samples

Each participant received 14 artificially contaminated chicken faeces samples, numbered B1 to B14. In addition, the laboratories had to test two control samples (C1 and C2). Table 3.1 gives an overview of the number and types 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 only buffered peptone water (BPW) had to be analysed. The protocol and result form can be found on the EURL-*Salmonella* website (EURL-*Salmonella* 2023a, 2023b).

Table 3.1 Overview of the number and types of samples tested per laboratory in the Proficiency Test PPS 2023

Strain and contamination level	No of chicken faeces samples (n=14)
***S.* Typhimurium low-level**	6
***S.* Typhimurium high-level**	4
Negative (no *Salmonella* added)	4

Strain and contamination level	No of control samples (n=2)
C1: Blank procedure control (BPW only)	1
C2: Positive control (own control with *Salmonella*)	1

3.2.2 *Shipment of parcels and temperature recording during shipment*

The 16 blindly coded samples, containing the contaminated and the negative chicken faeces samples plus the two control sample bags, were packed into two safety bags. These were placed in one large shipping box, together with four frozen (-20 °C) cooling elements. The shipping boxes were sent to the participants as 'biological substances category B', (UN3373) using 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 between the samples to record the temperature.

3.3 Methods

The method for detection of *Salmonella* prescribed for this PT was EN ISO 6579-1:2017 including A1:2020. The method starts with pre-enrichment in BPW, and selective enrichment is carried out on modified semi-solid Rappaport-Vassiliadis (MSRV) agar. Plating-out is carried out on XLD and a second isolation medium of choice. Confirmation is performed using the appropriate biochemical and serological tests as prescribed in EN ISO 6579-1:2017 or using reliable, validated identification kits.

In addition to the EN ISO method, the NRLs were free to use their own method, such as a polymerase chain reaction (PCR) procedure. Only the results obtained with the prescribed method, EN ISO 6579-1:2017(/A1:2020), were used to assess the performance of each participant. Results had to be reported using the EURL-*Salmonella* result form (EURL *Salmonella,* 2023b). Participants received their individual laboratory performance results in a performance report (See the example in Annex I), in addition to the interim summary report (Pol-Hofstad and Mooijman, 2023).

3.4 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 rates were calculated with the following formulae:

Specificity rate:

$$\frac{\text{Number of negative results}}{\text{Total number of (expected) negative samples}} \text{ x } 100\%$$

Sensitivity rate:

$$\frac{\text{Number of positive results}}{\text{Total number of (expected) positive samples}} \text{ x } 100\%$$

Accuracy rate:

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

3.5 Criteria for good performance

For the determination of 'good performance', the criteria indicated in Table 3.2 were used.

*Table 3.2 Criteria for good performance in the EURL-*Salmonella *PT PPS 2023*

Artificially contaminated samples	% positive	# positive samples/ total # samples
High level of *S*. Typhimurium	≥ 80 %	3/4
Low level of *S*. Typhimurium	≥ 50 %	3/6
Negative samples	0%	0/4

3.6 Follow-up study

A follow-up study was set up for laboratory 1. The study design was tailored to the problems this laboratory faced with scoring high- and low-level samples negative for *Salmonella*.

Table 3.3 Overview of the number and type of samples tested by laboratory 1 in the follow-up study, February 2024

Strain and contamination level	No of chicken faeces samples (n=9)
***S*. Typhimurium low level**	6
***S*. Typhimurium high level**	1
Negative (no *Salmonella* added)	2

4 Results and Discussion

4.1 Preparation of artificially contaminated chicken faeces samples

4.1.1 Pre-tests for the preparation of chicken faeces samples

The study design was based on the tests performed for the PT PPS organised in 2021 by the EURL-*Salmonella* (Pol-Hofstad and Mooijman, 2021). The pre-test samples were prepared according to section 3.1.2 and stored at 5 °C to mimic storage and transport conditions for up to three weeks. At day 0, day 7, day 13, and day 21, the samples were analysed for the presence of *Enterobacteriaceae*, total aerobic count and *Salmonella* using EN ISO 4833-1:2013, EN ISO 21528-2:2017 and EN ISO 6579:1-2017, respectively (see section 3.1.2 and 3.1.4). The results are presented in Figure 4.1 and Figure 4.2.

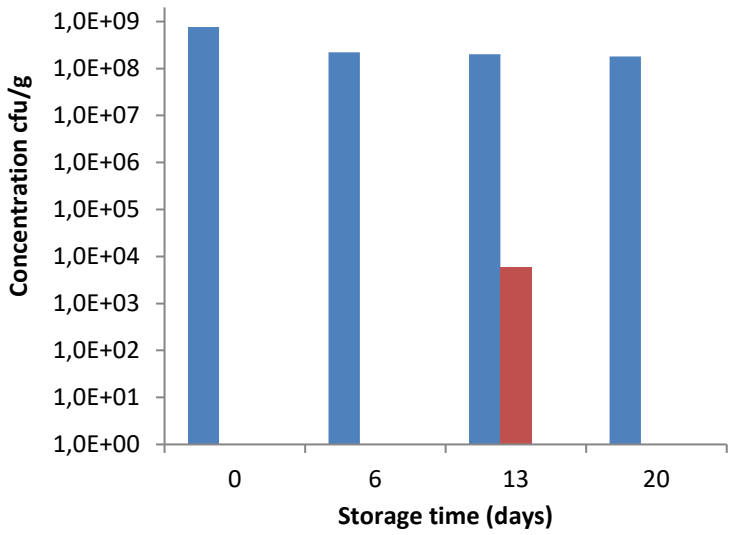

Figure 4.1 The effect of storage time on the number of total aerobic count (blue bars) and Enterobacteriaceae (red bar) in chicken faeces samples

The background flora, represented by the total aerobic count, remained stable in the chicken faeces samples at approximately 10^8 cfu/g, when stored at 5 °C for at least three weeks. The number of *Enterobacteriaceae* seemed to fluctuate between the samples; some chicken faeces samples did not contain *Enterobacteriaceae*, one sample in week two contained 10^4 cfu/g. Although only aerobic bacteria were detected in most chicken faeces samples, it was still considered sufficiently representative for real-life PPS samples.

The results in Figure 4.2 show that *Salmonella* Enteritidis (SE) as well as *Salmonella* Infantis (SI) were both inactivated to a large extent during storage of the pre-test samples at 5 °C and at 10 °C. In contrast to data from previous studies, even at concentrations as high as 17 cfu for both SE and SI, hardly any positive samples were left after 13 days of storage (data not shown). Therefore, it was decided not to use these two serovars for artificially contaminating the PT samples, but to use

Salmonella Typhimurium (STm), since it is known from previous studies that this strain survives in chicken faeces for a relatively long time.

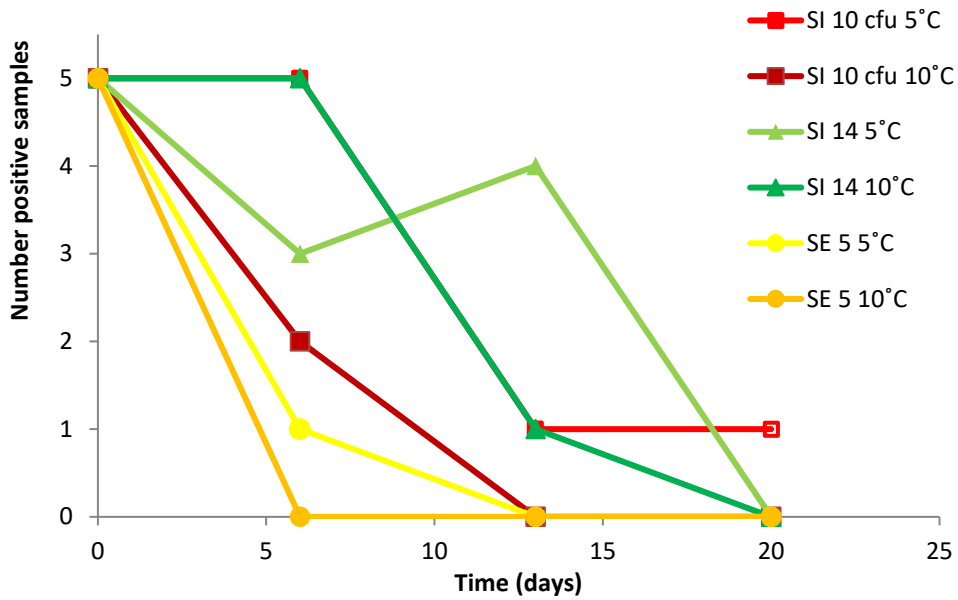

Figure 4.2 Stability tests of chicken faeces samples (n = 5) artificially contaminated with a low concentration of Salmonella *Enteritidis or* Salmonella *Infantis*

A third pre-test was carried out with chicken faeces samples artificially contaminated with *S.* Typhimurium. Test portions of 25 g chicken faeces samples from a second batch (GD, Deventer, the Netherlands, 4 September 2023) were artificially contaminated with 7 cfu *S*. Typhimurium and were analysed for the presence of *Salmonella* after one, two and three weeks of storage at 5 °C and 10 °C (Figure 4.3).

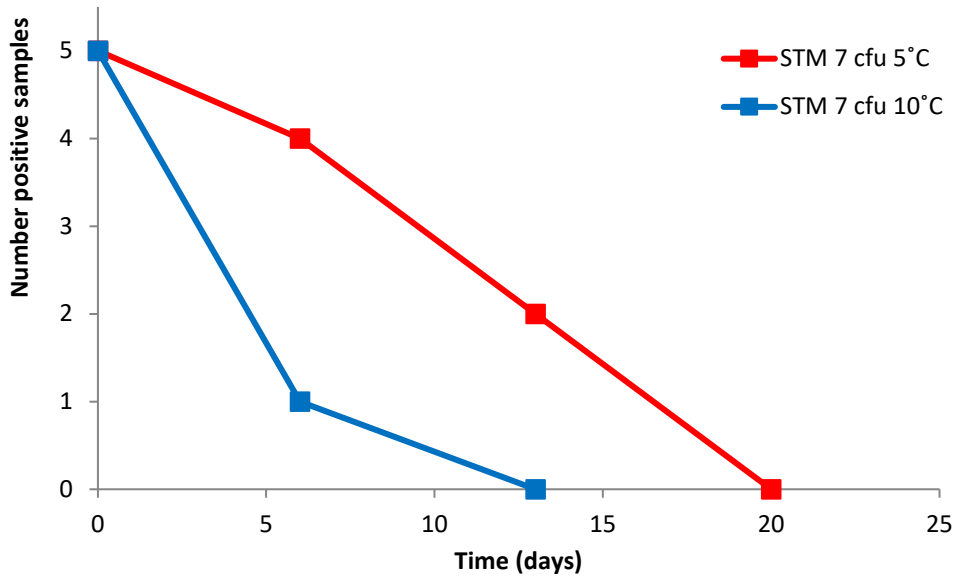

Figure 4.3 Stability tests of chicken faeces samples (n = 5) artificially contaminated with a low concentration of Salmonella *Typhimurium*

Unfortunately, *S*. Typhimurium was also inactivated during the storage period, both at 5 °C and 10 °C. After two weeks of storage at 5 °C, only two out of the five samples were still positive for *Salmonella*. This is not sufficient to be suitable for use as PT samples. It was decided to increase the intended concentration in the PT samples to 15 cfu *S.* Typhimurium per test portion (25 g).

4.1.2 Preparation of chicken faeces samples for the Proficiency Test

Samples for the PT were prepared as described in section 3.1.3. Samples were artificially contaminated with *S*. Typhimurium to reach the intended concentration of 15 cfu STm/sample or 50 cfu STm/sample, representing low and high levels of contamination in the chicken faeces samples.

4.1.3 Background flora in the chicken faeces samples

The concentration of the background flora in the chicken faeces samples was determined according to EN ISO 4833-1:2013 and EN ISO 21528-2:2017, as described in section 3.1.4. Results are shown in Table 4.1. In the second batch of chicken faeces used for the PT samples, *Enterobacteriaceae* were found up to a level of $2{,}5 \times 10^5$ on 5 September 2023. The level of aerobic bacteria was $2{,}6 \times 10^8$ cfu/g. Storage at 5 °C for 4 weeks did not affect the number of background flora.

Table 4.1 Number of aerobic bacteria and Enterobacteriaceae per gram chicken faeces

Date	***Aerobic bacteria* (cfu/g)**	***Enterobacteriaceae* (cfu/g)**
5 September 2023	$1{,}3 \times 10^8$	$2{,}5 \times 10^5$
2 October 2023[a]	$5{,}4 \times 10^8$	$1{,}5 \times 10^6$

[a] After storage at 5 °C for 4 weeks

4.1.4 Level of Salmonella *in the chicken faeces samples*

The chicken faeces samples were artificially contaminated at the EURL-*Salmonella* laboratory by adding an appropriate volume of a diluted STm culture to obtain the desired contamination level. Table 4.2 shows the contamination levels of the diluted culture of *Salmonella* used as an inoculum to contaminate the chicken faeces samples, as well as the contamination level in the samples after storage at 5 °C.

Following inoculation, the samples were stored at 5 °C for 13 days until they were dispatched to the participants on 2 October 2023. The final contamination level of *Salmonella* in the chicken faeces samples was determined by performing a five-tube Most Probable Number (MPN) test in the week of the PT study and 1 week later (see Table 4.2).

Table 4.2 Level of Salmonella *Typhimurium (STm) in the inoculums for artificial contamination of the chicken faeces samples and in the samples after storage at 5 °C for 13 days and 20 days*

Date of testing	Low level STm (cfu/sample)	High level STm (cfu/sample)
19 Sept 2023 (inoculum level diluted culture)	17	50
2 Oct 2023[a] MPN contaminated chicken faeces samples (95% confidence limit)	8 (2,47 - 25)	8 (2,47 - 25)
9 Oct 2023[b] MPN contaminated chicken faeces samples (95% confidence limit)	0 (0 – 0,675)	1,7 (0,725 – 4)

[a] After storage of the inoculated samples at 5 °C for 13 days
[b] After storage of the inoculated samples at 5 °C for 20 days

4.2 Technical data of the Proficiency Test

4.2.1 General

A total of 37 NRLs-*Salmonella* subscribed to this study originating from 37 countries. The participants originated from 27 EU-MSs, nine NRLs from other European countries (EU candidates or potential EU candidate MSs, members of the EFTA countries, and EU third countries), and one NRL was based in a non-European country. All 37 NRLs-*Salmonella* reported their results.

4.2.2 Accreditation and Methods used

Six laboratories were accredited for EN ISO 6579-1:2017, 24 laboratories were accredited for EN ISO 6579-1:2017/A1:2020 and six laboratories were accredited for both EN ISO 6579-1:2017 and the amendment A1:2020. One laboratory was not accredited at all. Out of the 37 laboratories, 28 used EN ISO 6579-1:2017/A1:2020 for the detection of *Salmonella* in this study, the other nine laboratories used EN ISO 6579-1:2017.

4.2.3 Transport of samples

The samples were transported using a door-to-door courier service on Monday 25 September 2023. Two laboratories received the parcel on the day of dispatch. Twenty-two parcels were delivered after one day, ten parcels after two days and one parcel after four days of dispatch. Two parcels experienced some delay at the border. One parcel (lab code 37) arrived after seven days and one parcel arrived only 11 days following dispatch (lab code 17).

The samples had to be stored at 5 °C upon arrival at the laboratory. The temperature during transport and storage was registered using a temperature probe. The temperature of the parcels during transport was predominantly between -4,5 °C and +4,5 °C, and for storage between 0,5 and 8,5 °C. The temperature of the parcels arriving late were checked in greater detail. The parcel meant for laboratory 17 was held up at the border for quite some time. The temperature can only stay low for as long

as the cooling elements stay frosted. The staff at customs was very helpful keeping the parcel in cold storage until all necessary papers were approved and the parcel was cleared for further transport to the laboratory. The samples arrived at the laboratory on 10 October 2023 at a temperature of 8,5 °C (see Figure 4.3). The samples were analysed immediately following arrival.

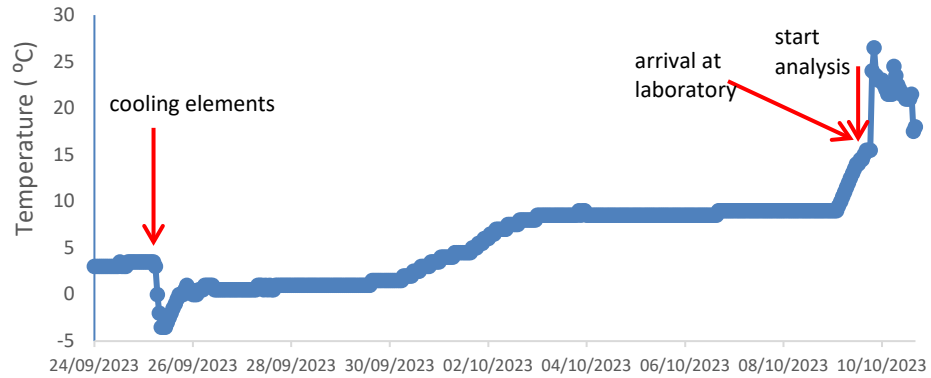

Figure 4.3 Temperature profile of the parcel for Laboratory 17

The parcel for laboratory 37 arrived after 7 days. Figure 4.4 shows the temperature profile of that parcel during transport and storage. The temperature stayed stable at around 1,5 °C until 2 October, when the parcel arrived at the laboratory. The temperature rose to 20 °C until the analyses started on 3 October 2023.

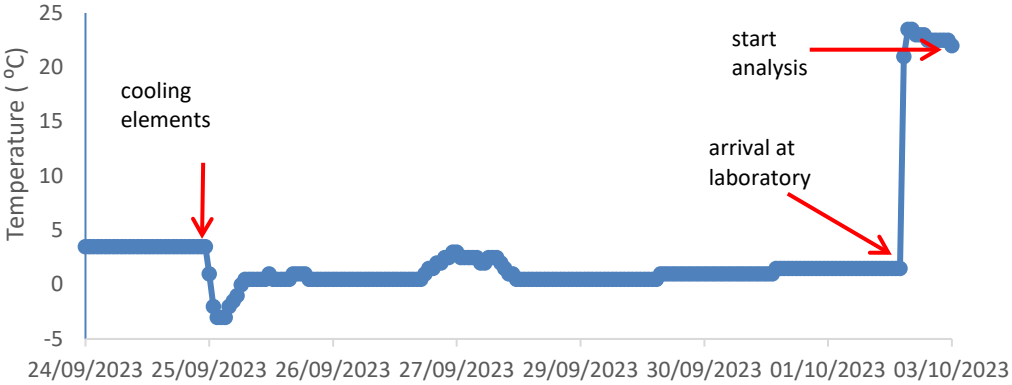

Figure 4.4 Temperature profile of the parcel for Laboratory 37

The majority of the laboratories started the analyses on 2 October 2023. Three laboratories started earlier on 27 (lab code 20) and 29 September (lab code 3) and 1 October (lab code 10). Five laboratories started 1 (lab code 37), 6 (lab code 19) and 7 days later (lab code 17).

4.2.4 Methods

The prescribed method was EN ISO 6579-1:2017(/A1:2020), in which MSRV agar has to be used as selective enrichment media, and XLD agar and a second medium of choice for plating out. Table 4.3 shows which second plating-out media were used by the participants.

Table 4.3 Second plating-out media used by the NRLs-Salmonella

Media	No. of users
ASAP	1
BGA	10
BGA mod	5
BSA	2
BxLH	1
SM2	1
Rambach	11
ChromID *Salmonella* agar	1
Chromo	1
Rapid *Salmonella*	3
RSAL	1
Iris *Salmonella* agar	1
***Salmonella* differential agar**	1
BPLS	2

Explanations of the abbreviations used are given in the list of abbreviations.

Technical details on the method that deviated from the prescribed method (EN ISO 6579-1:2017(/A1:2020)) are listed in Table 4.4 (grey-shaded cells); nine laboratories reported details of deviations. Two laboratories (lab codes 17 and 37) incubated the BPW pre-enrichment for too many hours. Three laboratories (lab codes 9, 25, and 33) used MSRV with a higher novobiocin concentration than prescribed (10 mg/l) and one laboratory (lab code 34) with too low a concentration of Novobiocin. Two laboratories (lab codes 1 and 26) did not report the novobiocin concentration at all.

Table 4.4 Reported technical deviations from the prescribed method (EN ISO 6579-1:2017(/A1:2020)

Lab code	BPW		MSRV		
	T (ºC)	Incubation time	T (ºC)	pH	Novo-biocin
EN ISO 6579-1(/A1:2020)	**34 - 38**	**16–20 h**	**41,5 ± 1 °C**	**5,1–5,4**	**10 mg/l**
1	37	18	42	5,3	-
9	37	20	41,5	5,3	40 mg/L
17	37	24	41,5	5,23	10
25	37	20	41,5	5,2	20 mg/L
26	37	19	41,5	5,23	-
33	37	18,5	41,5	5,4	18 mg/L
34	36,5	18+/-2h	41,5	5,2	0,01 mg/L
37	37	24	41,5	5,2	10

1 Deviations from EN ISO 6579-1:2017 are indicated in grey
-: no information

All participating laboratories performed one or several confirmation tests for *Salmonella*. Table 4.5 summarises all reported combinations. The majority of the participants (26) used a biochemical test in combination with other confirmation methods, such as serological testing, serotyping, PCR testing or MALDI-TOF. Six laboratories used only one confirmation test.

Table 4.5 Number of laboratories using the various confirmation methods

Number of labs	Bio-chemical	Sero-logical	Sero-typing	PCR	Other
1	X				
7	X	x			
7	X		X		
3	X				MALDI-TOF
2	X	x	X		
1	X	x	X	x	
2	X	x			MALDI-TOF
4	X		X		MALDI-TOF
1		x			
1		x	X		
1		x		X	
1			X		
2			X		MALDI-TOF
3					MALDI-TOF
1				x	MALDI-TOF

4.3 Control samples

4.3.1 General

Two sterile empty bags for the control samples were sent to the laboratories. One was used for the procedure control (C1). The other was used for the positive control to which the laboratories had to add their own positive control strain (C2), which they normally use in their routine analysis for *Salmonella* detection.

Procedure control (BPW only)
All but one laboratory analysed the procedure control correctly as being negative for *Salmonella* and scored good results for this control sample.

Positive control with *Salmonella*
All but one laboratory correctly scored their own *Salmonella*-positive control sample as positive. The *Salmonella* serovars used for the positive control sample are shown in Table 4.6. The majority of the NRLs-*Salmonella* used *S.* Enteritidis or *S*. Typhimurium for their positive control samples. However, the use of a less common *Salmonella* serovar as a control strain may be advisable in order to make the detection of possible cross-contamination easier.

Table 4.6 Salmonella *serovars used by participants for the positive control samples*

***Salmonella* serovar**	**Number of users**
***S*. Enteritidis**	10
***S*. Typhimurium**	9
***S*. Nottingham**	5
***S.* Infantis**	2
***S.* Alachua, *S*. Blegdam, Harleystreet, *S.* Tranaroa, *S.* Agbeni, *S.* Abaetetuba, *S.* Tennessee, *S.* Regent, *S.* Weltevreden, *S*. Hadar, *S*. Bongori, serovar 66:z_{41}:-**	1 (per serovar)

4.3.2 *Correct scores of the control samples*

Table 4.7 shows the number of correctly analysed control samples for all participants. One laboratory (lab code 21) reported their procedure control positive and their positive control negative and, as a result, scored an unsatisfactory performance. Following inquiries, this laboratory could prove, by means of their raw data, that they made an administrative error in mixing up both control samples when reporting the results. Therefore, the performance of this laboratory was amended to moderate performance. Subsequently, all laboratories showed correct results, resulting in an accuracy rate of 100%.

Table 4.7 Correct scores found with the control samples by all participants.

Control samples		**Total no of labs n = 37**
Procedure control (BPW only) n = 1	No. of samples No. of negative samples **Correct score in %**	37 37 **100%**
Positive control (Own *Salmonella* control) n = 1	No. of samples No. of positive samples **Correct score in %**	37 37 **100%**
All control samples n = 2	No. of samples No. of correct samples **Accuracy in %**	74 74 **100%**

4.4 Artificially contaminated chicken faeces samples

4.4.1 *General*

The participants analysed the chicken faeces samples artificially contaminated with two different concentrations of STm (low: 17 cfu/sample; high: 50 cfu/sample) as well as negative samples were analysed for the presence of *Salmonella*. Table 4.8 shows the overall results found by the participants.

Table 4.8 Number chicken faeces samples tested positive for Salmonella *by each participant*

	Number of positive samples		
	Negative n=4	**STm low n=6**	**STm high n=4**
Criteria good performance	0	≥3	≥3
Lab code 1	0	2	3
Lab codes 12, 14, 21 and 31	0	5	4
All other NRLs (n = 32)	0	6	4

grey cell = result below level of good performance

Negative chicken faeces samples
All NRLs correctly scored the negative samples negative for *Salmonella*.

Chicken faeces samples contaminated with a low level of Salmonella *Typhimurium*
Most of the participating laboratories were able to detect *Salmonella* in all six chicken faeces samples that were contaminated with a low inoculum level of 17 cfu *S.* Typhimurium per sample. Four laboratories (lab codes 12, 14, 21, 31) reported one out of the six samples as negative for *Salmonella*. One laboratory (lab code 1) reported four out of the six samples as negative for *Salmonella.* With respect to low-level samples, a negative score for a maximum of three out of six samples is regarded as acceptable; therefore, laboratory 1 did not meet the criteria for a good performance. The results of all participants are shown in Figure 4.5.

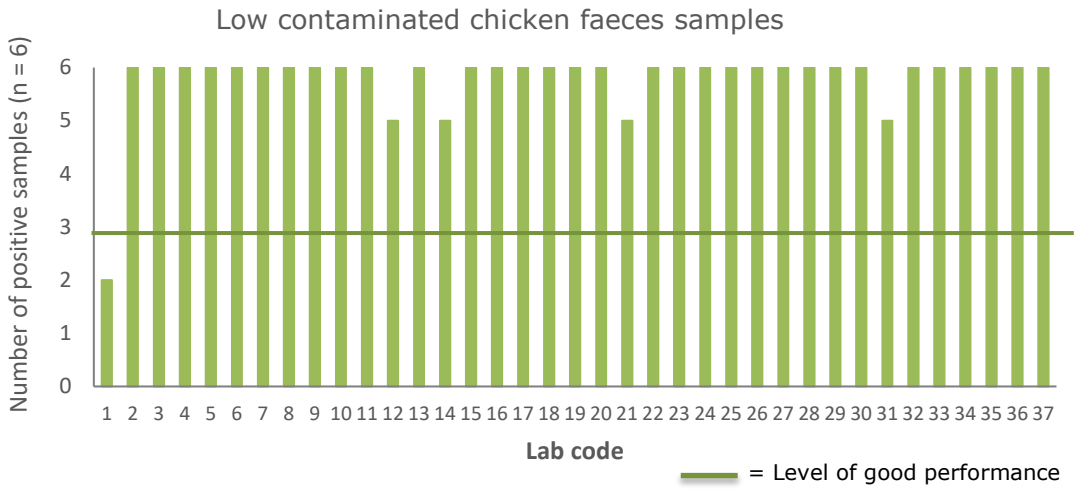

Figure 4.5 Number of positive Salmonella *isolations per laboratory found in the chicken faeces samples contaminated with a low level of* Salmonella *Typhimurium*

Chicken faeces samples contaminated with a high level of Salmonella *Typhimurium*

Nearly all participating laboratories were able to detect *Salmonella* in all four chicken faeces samples that were contaminated with a high inoculum level of 50 cfu STm per sample. One laboratory (lab code 1) reported one out of the four samples as negative for *Salmonella*. With respect to high-level samples, a negative score for at maximum one out of four samples is regarded as acceptable. Therefore, alle laboratories scored a good performance for the high-level samples. The results are shown in figure 4.6.

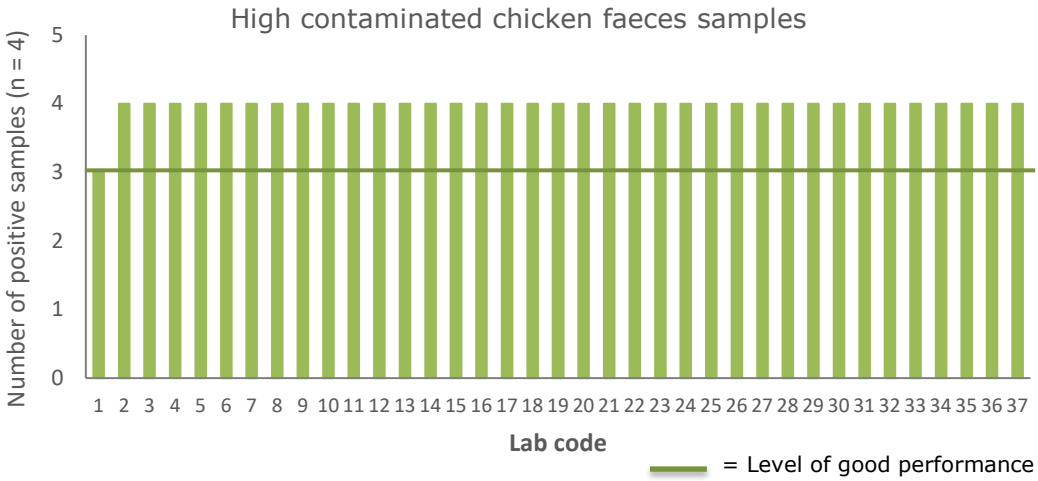

Figure 4.6 Number of positive Salmonella *isolations per laboratory found in the chicken faeces samples contaminated with a high level of* Salmonella *Typhimurium.*

Table 4.9 Specificity, sensitivity and accuracy rates found by the participating laboratories (all participants and EU-MSs only) for the chicken faeces samples

Chicken faeces samples		All participants n=37	EU MSs only n=27
Negative samples n=4	No. of samples	148	108
	No. of negative samples	148	108
	Specificity in %	**100**	**100**
Low level STm n=6	No. of samples	222	162
	No. of positive samples	214	155
	Sensitivity in %	**96,4**	**95,7**
High level STm n=4	No. of samples	148	108
	No. of positive samples	147	105
	Sensitivity in %	**99,3**	**99,3**
All chicken faeces samples with STm n=10	No. of samples	370	270
	No. of positive samples	361	262
	Sensitivity in %	**97,6**	**97**
All chicken faeces samples (pos. and neg.) n=14	No. of samples	518	378
	No. of correct samples	509	370
	Accuracy in %	**98,3**	**97,9**

Specificity, sensitivity and accuracy rates of the artificially contaminated samples

Table 4.9 shows the specificity, sensitivity and accuracy rates for all artificially contaminated chicken faeces samples. The calculations were performed on the results of all participants and on the results of the EU MSs only. All participants but one performed well in this study: the sensitivity rates (low-level: 96,4%; high-level 99,3%) were very high. Hardly any differences were found between all participants and the EU MSs as shown in Table 4.9. The specificity of the negative samples was 100%. The accuracy calculated on the basis of all the chicken faeces samples was 98,3%.

4.5 Second detection method

In the current PT, seven laboratories also used a second method to analyse the chicken faeces samples. An overview of the methods used per laboratory can be found in Table 4.10. All laboratories used a PCR method as a second method. Not all methods were validated or routinely used by the participants. All NRLs found identical results with their second method compared to the prescribed bacteriological culture method.

*Table 4.10 Details on the second detection methods used by NRLs-*Salmonella *in the PT PPS 2023*

Lab code	Second detection method	Validated (by)	Reference	Routinely # per year
1	IQ Check Salmonella	AFNOR	BRD 07/06-07/04	0
4	Home method SOP B.2	National Accreditation Bureau	LA.01.139	0
8	Real-time PCR	§64 of the national Food and Feed Code	Malorny et al., (2004) AEM 70:7046-7052	190
10	Real-Time PCR	National Laboratory Accreditation Authority	ISO 22119:2011(E)	1000
17	PCR - detection invA gene	No	-	0
27	NF U 47-100	No	-	300
28	PCR: in-house method using Josefsen et al., 2007 primers	LABRIS; in-house validation	not applicable	0

4.6 Follow-up study

Laboratory 1 experienced problems with the high number of background flora and could not detect *Salmonella* in four out of the six low-level chicken faeces samples and in one out of the four high-level samples. For this laboratory, a follow-up study was set up with a study design tailored to this problem of detection of *Salmonella* in the presence of high numbers of background flora. Chicken faeces from the same batch as the one used for the full PT was re-tested for the absence of *Salmonella* and for the number of background flora, according to section 3.1.4 and 3.1.5. The test results showed that the chicken faeces was *Salmonella*-free and that the number of aerobic bacteria was still

sufficiently high for use in this follow-up study (number of aerobic bacteria: 6,0 x 10^7 cfu/g, *Enterobacteriaceae* < 10 cu/g). In total, nine samples were prepared for the follow-up study, consisting of two negative samples, six low-level (27 cfu) samples, and one high-level (203 cfu) sample. The samples were sent with a DHL courier as UN3373 parcel, including cooling elements, on 13 February 2024. The parcel arrived on 19 February 2024 at laboratory 1, and the samples were analysed immediately. Laboratory 1 analysed all samples correctly and scored a good performance in the follow-up study.

4.7 Performance by the NRLs

Nearly all laboratories were able to detect *Salmonella* in high- and low-level concentrations in the chicken faeces samples. Out of the 37 laboratories, 35 fulfilled the criteria for good performance in this study. One laboratory (lab code 21) reported their positive control sample as negative and their negative control sample as positive for *Salmonella*. This laboratory accidentally made an administrative error when reporting the results of the control samples. However, the raw data of this laboratory showed the correct results for both control samples. As a result, the performance of this laboratory was amended to a moderate performance.
One laboratory tested four out of the six low-level chicken faeces samples negative for *Salmonella* and scored an unsatisfactory performance. This laboratory participated in a follow-up study, in which it scored a good performance after all.

5 Conclusions

Thirty-five laboratories fulfilled the criteria of good performance in the EURL-*Salmonella* Proficiency Test for the Detection of *Salmonella* in chicken faeces samples.

One laboratory (lab code 21) scored a moderate performance by making an administrative error in mixing up the two control samples.

One laboratory (lab code 1) scored an unsatisfactory performance. This laboratory tested four out of the six low-level samples negative for *Salmonella* due to problems with the high level of background flora. In the follow-up study, this laboratory tested all samples correctly and eventually scored a good performance.

The accuracy rate of the control samples amounted to 100%

The specificity rate of the negative chicken faeces samples amounted to 100%

The sensitivity rate of the chicken faeces samples artificially contaminated with a low level of STm amounted to 96,4%.

The sensitivity rate of the chicken faeces samples artificially contaminated with a high level of STm amounted to 99,3%.

The accuracy rate of all matrix samples amounted to 98,3%

Seven participants used a second method in addition to the prescribed bacteriological culture method. All seven laboratories reported identical results for both methods.

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List of abbreviations

AFNOR	Association Française de Normalisation
ASAP	AES *Salmonella* agar plate
ATCC	American Type Culture Collection
BGA	Brilliant green agar
BGA (mod)	Brilliant green agar (modified)
BPLS	Brilliant green phenol-red lactose sucrose
BPW	Buffered peptone water
BSA	Brilliance *Salmonella* agar
BxLH	Brilliant green, xylose, lysine, sulphonamide
cfu	Colony-forming units
DG-SANTE	Directorate-General for Health and Consumer Protection
EFTA	European Free Trade Association
EN	European Standard
EU	European Union
EURL	European Union Reference Laboratory
ISO	International Organization for Standardization
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization – Time Of Flight
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
PS	Peptone saline solution
PT	Proficiency Test
RIVM	Rijksinstituut voor Volksgezondheid en Milieu (National Institute for Public Health and the Environment)
RSAL	Rapid*Salmonell*a
SE	*Salmonella* Enteritidis
SI	*Salmonella* Infantis
SM (ID)2	*Salmonella* detection and identification-2
SOP	Standard operation procedure
STm	*Salmonella* Typhimurium
TSA	Tryptone soya agar
VRBG	Violet red bile glucose
XLD	Xylose lysine deoxycholate

Annex I Example of an individual laboratory,
Performance Report of the EURL-*Salmonella* PT PPS 2023

Performance

EURL-*Salmonella* PT PPS 2023

Number of positive samples/Total number of samples per level

	chicken faeces samples			control samples	
Lab code	High	Low	Negative	BPW	pos control
	4/4	6/6	0/4	0/1	1/1

Evaluation: Good performance

Number	Level	Your result	Second method	Media choices:
B1	Low	Detected	Detected	MSRV
B2	Negative	Not detected	Not detected	XLD/ *Salmonella* differential agar
B3	Low	Detected	Detected	
B4	Low	Detected	Detected	
B5	Low	Detected	Detected	
B6	Low	Detected	Detected	
B7	High	Detected	Detected	
B8	Negative	Not detected	Not detected	
B9	Negative	Not detected	Not detected	
B10	High	Detected	Detected	
B11	High	Detected	Detected	
B12	High	Detected	Detected	
B13	Low	Detected	Detected	
B14	Negative	Not detected	Not detected	
C1	Negative	Not detected	Not detected	
C2	Positive	Detected	Detected	

Negative = Negative chicken faeces (no *Salmonella* added)
Low = Low concentration of *Salmonella* Typhimurium
High = High concentration of *Salmonella* Typhimurium
BPW = Buffered Peptone Water
Pos control = own positive control

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