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

EURL-*Salmonella* Proficiency Test Primary Production Stage 2021

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

RIVM report 2021-0129
I. Pol-Hofstad | K. Mooijman

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Synopsis

EURL-*Salmonella* Proficiency Test Primary Production Stage, 2021
Detection of *Salmonella* in chicken faeces adhering to boot socks

In the annual EURL-*Salmonella* Proficiency Test performed in 2021, the National Reference Laboratories (NRLs) of the European (EU) Member States were able to detect *Salmonella* in chicken faeces adhering to boot socks. All laboratories were able to detect *Salmonella* in high and low concentrations in the contaminated boot sock samples. All but one laboratory achieved good results. This one laboratory had accidentally swapped control samples, resulting in a moderate performance score. This was the outcome of the Proficiency Test for the detection of *Salmonella* in samples from the primary production stage, organised by the coordinating European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*) in September 2021.

Since 1992, all NRLs in EU Member States are obliged to participate in the annual quality control proficiency tests for *Salmonella*. Each Member State has to appoint an NRL with responsibility for analysing *Salmonella* in samples taken from the animal primary production stage. In total, 35 NRLs participated in this study: 27 NRLs originating from all 27 EU Member States, seven NRLs based in other countries in Europe and one NRL based in a non-European country.

The EURL-*Salmonella* is based at the Dutch National Institute for Public Health and the Environment (RIVM). An important task of the EURL-*Salmonella* is to monitor and improve the performance of the NRLs in Europe.

Keywords: *Salmonella*, EURL, NRL, Proficiency Test, chicken faeces, boot socks, *Salmonella* detection method

Publiekssamenvatting

Het EURL-*Salmonella* ringonderzoek productiedieren (2021)

Detectie van *Salmonella* in kippenmest op overschoentjes

De Nationale Referentie Laboratoria (NRL's) van de Europese lidstaten waren in 2021 in staat om *Salmonella* aan te tonen in kippenmest op overschoentjes. Alle deelnemers konden hoge en lage concentraties van *Salmonella* aantonen. Op één na hebben alle laboratoria een goede score behaald. Dat ene laboratorium had de controlemonsters verwisseld en haalde daarom een matige score. Dit blijkt uit het jaarlijkse ringonderzoek dat het Europese Referentielaboratorium (EURL) voor *Salmonella* in september 2021 organiseerde.

Sinds 1992 zijn de NRL's van de Europese lidstaten verplicht om elk jaar mee te doen aan kwaliteitstoetsen. Dit zijn de zogeheten ringonderzoeken voor *Salmonella*. Elke lidstaat wijst hiervoor een laboratorium aan, het Nationale Referentie Laboratorium. Deze laboratoria zijn er namens dat land voor verantwoordelijk *Salmonella* aan te tonen in de leefomgeving van dieren die voor de voedselproductie worden gehouden. In totaal hebben 35 NRL's aan dit ringonderzoek deelgenomen: 27 NRL's afkomstig uit alle 27 EU-lidstaten, zeven NRL's uit andere Europese landen en één NRL uit een niet-Europees land.

Het EURL-*Salmonella* is gevestigd bij het Nederlandse Rijksinstituut voor Volksgezondheid en Milieu (RIVM). Een belangrijke taak van het EURL-*Salmonella* is toezien op de kwaliteit van de nationale referentielaboratoria voor deze bacterie in Europa.

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

Contents

Summary

In September 2021, the EURL-*Salmonella* Proficiency Test on the detection of *Salmonella* in samples from the primary production stage (PPS) was held. A total of 35 National Reference Laboratories (NRLs) for *Salmonella* participated in this study: 27 NRLs PPS from the 27 EU-Member States (MS), seven NRLs from third European countries (EU candidate MS or potential EU candidate MS and members of the European Free Trade Association (EFTA)), and one NRL from a non-European country.

In this study, chicken faeces adhering to boot socks contaminated with a diluted culture of *Salmonella* Infantis at the EURL-*Salmonella* laboratory, was used as a matrix (referred to as boot sock samples in this report).

Each NRL received 16 blindly coded samples consisting of 10 boot sock samples artificially contaminated with two different concentrations of *Salmonella* Infantis: six low-level contaminated samples (MPN concentration: 2,3 cfu/sample) and four high-level contaminated samples (MPN concentration: 35 cfu/sample). Additionally, four negative boot sock samples (no *Salmonella* added) and two control samples had to be analysed. The control samples consisted of a blank procedure control sample and a positive control sample. For the latter, the participants had to use their own positive control strain. The samples were stored at 5 °C until the day of transport. On Monday, 20 September 2021, the boot sock samples were packed and sent to the NRLs. The NRLs were asked to store the samples at 5 °C on arrival until the start of the analysis on Monday, 27 September 2021.

Method
All laboratories used the prescribed method EN ISO 6579-1:2017. The majority of the laboratories also indicated that they followed Amendment 1 of EN ISO 6579-1 (EN ISO 6579-1:2017/A1:2020). One laboratory reported to be NRL-*Salmonella* for samples from the primary production stage but used Rappaport Vassiliadis Soya (RVS) broth instead of modified semi-solid Rappaport-Vassiliadis (MSRV) agar for the selective enrichment. This is not in line with the prescribed method in EN ISO 6579-1:2017 for analysing PPS samples.
Six laboratories also used a PCR method as second detection method for analysing the samples. Not all laboratories found identical results using the PCR method compared to the results found with EN ISO 6579-1:2017(/A1:2020).

Results control samples
Of the 35 participating laboratories, 34 scored well, analysing both the procedure control sample as well as their own positive control sample correctly. One laboratory made an administrative error by accidentally reporting their positive control as tested negative for *Salmonella*. This laboratory scored a moderate performance (lab code 18).

Results for artificially contaminated boot sock samples
All laboratories detected *Salmonella* in the boot sock samples contaminated with a low level of *Salmonella*. Eight laboratories (lab codes 5, 7, 8, 9, 11, 20, 29 and 30) tested one of the six samples negative for *Salmonella.* Five laboratories (lab code 1, 21, 22, 26 and 36) tested two of the six samples negative for *Salmonella*. These results are still within the criteria for good performance, which permit three negative samples. The sensitivity rate for these samples was 91,4%.

Almost all laboratories detected *Salmonella* in all four high-level samples. Only four laboratories (lab code 1, 11, 17 and 21) scored one of the four high-level samples as negative. This is still within the criteria for good performance, which permit one negative sample. The sensitivity rate for these samples was 97,1%.

All negative samples were scored correctly as negative, resulting in a specificity rate of 100%.

Overall, the laboratories scored well in this Proficiency Test, with an accuracy of 95,5%. Thirty-four laboratories fulfilled the criteria of good performance. One laboratory scored a moderate performance due to an administrative error. It switched the results of the positive and the negative control samples.

1 Introduction

An important task of the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*), as laid down 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 held by EURL-*Salmonella* from 1995 onwards is summarised on the EURL-*Salmonella* website (EURL-*Salmonella*, 2021a).

In September 2021, the EURL-*Salmonella* held 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 boot sock samples. The results from PTs like these show 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 method prescribed for the detection of *Salmonella* spp. is set out in EN ISO 6579-1:2017(/A1:2020).

The design of this study was comparable to previous PTs held by EURL-*Salmonella* (Diddens & Mooijman, 2020; Pol-Hofstad & Mooijman, 2019 and Pol-Hofstad & Mooijman, 2020). For the current study, chicken faeces on boot sock samples were artificially contaminated with a diluted culture of *Salmonella* Infantis (SI) at the laboratory of the EURL-*Salmonella*.

In total, 14 boot sock samples had to be tested: four boot sock samples artificially contaminated with a high level of SI, six boot sock samples artificially contaminated with a low level of SI, and four negative boot sock samples (no *Salmonella* added). Additionally, two control samples had to be tested: one procedure control and one positive control. The number of samples and the contamination levels were based on information described in EN ISO 22117:2019.

2 Participants

Country	City	Institute
Austria	Graz	Austrian Agency for Health and Food Safety (AGES IMED/VEMI)
Belgium	Brussels	Sciensano
Bulgaria	Sofia	National Diagnostic and Research Veterinary Institute (NDRVMI), National Reference Centre of Food Safety
Croatia	Zagreb	Croatian Veterinary Institute
Cyprus	Nicosia	Cyprus Veterinary Services Pathology, Bacteriology, Parasitology Laboratory
Czech Republic	Praha	State Veterinary Institute
Denmark	Ringsted	Danish Veterinary and Food administration
Estonia	Tartu	Estonian Veterinary and Food Laboratory, Bacteriology-Pathology Department
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
Iceland	Reykjavik	Matís ohf, Food Safety and Analytical services
Ireland, Republic of	Celbridge, Co. Kildare	Central Veterinary Research Laboratory (CVRL/DAFM) Laboratories Backweston, Department of Agriculture, Food and the Marine, Bacteriology
Israel	Masmiya	Laboratory of the Israel Poultry and Egg Board
Italy	Padova Legnaro	Istituto Zooprofilattico Sperimentale delle Venezie, OIE

Country	City	Institute
Latvia	Riga	Institute of Food Safety, Animal Health and Environment BIOR Bacteriology and Parasitology Division
Lithuania	Vilnius	National Food and Veterinary Risk Assessment Institute, Laboratory of Microbiology and Pathology, Bacteriology Group
Luxembourg, Grand-Duchy of	Dudelange	Laboratoire de Médicine Vétérinaire de l"Etat, Bacteriologie
Malta	Valletta	Malta Public Health Laboratory (PHL), Evans Building
North Macedonia, Republic of	Skopje	Food Institute, Faculty of Veterinary Medicine
Netherlands, the	Bilthoven	National Institute for Public Health and the Environment (RIVM), Centre for Zoonosis and Environmental Microbiology (Z&O)
Northern Ireland	NRL tasks are carried out by NRL Ireland (PPS)	
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
Romania	Bucharest	Institute for Diagnosis and Animal Health
Serbia	Belgrade	NIVS-Scientific Veterinary Institute of Serbia
Slovak Republic	Bratislava	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	Institute for Food Safety and Hygiene, National reference Centre for Poultry and Rabbit Disease
United Kingdom	Addlestone	Animal and Plant Health Agency (APHA), Bacteriology Department
Turkey	Ankara	Veterinary control central research institute

3 Materials and methods

3.1 Preparation of artificially contaminated boot sock samples

3.1.1 General

The matrix used for this PT was boot socks (Sodibox, Nevez, France) to which chicken faeces from a broiler breeder flock were added. The boot sock samples were artificially contaminated with a diluted culture of *Salmonella* Infantis at the EURL-*Salmonella* laboratory.

3.1.2 Pre-tests for the preparation of boot sock samples

The batch of 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 19 April 2021. The faeces contained small flies which were inactivated by storing the faeces at -20 °C for one day. The next day, three samples of 25 gram of defrosted chicken faeces each were randomly taken from the batch and tested for the absence of *Salmonella* according to EN ISO 6579-1:2017/A1:2020. The boot socks were moisturised by adding 15 ml of peptone saline solution and left at room temperature for one to several hours to allow the fluid to thoroughly moisten them. Subsequently, 10 grams of chicken faeces was added to the boot socks. Some boot socks were artificially contaminated with various low concentrations (10-17 cfu) of a diluted culture of *Salmonella* Infantis (strain number 15-A7 from the EURL-*Salmonella*'s own collection).
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 10 °C for three weeks. After zero, one, two and three weeks of storage, five boot sock samples were tested at each time interval for the presence of *Salmonella* according to EN ISO 6579-1:2017/A1:2020. In addition, one boot sock sample was tested for the concentration of background flora according to EN ISO 21528-2:2017 for the number of *Enterobacteriaceae* and according to EN ISO 4833-1:2013 for the total aerobic count, also after zero, one, two, and three weeks of storage.

3.1.3 Preparation of boot sock samples for the Proficiency Test

A large batch (15 kg) of chicken faeces from the same flock as the pre-tests arrived at the EURL-*Salmonella* laboratory on Monday 1 September 2021. Five samples, each of 25 g, were tested for the absence of *Salmonella* according to EN ISO 6579-1:2017/A1:2020. After testing negative for *Salmonella*, 10 grams of chicken faeces were added to each pre-moistened boot sock sample (see 3.1.2). Additionally, almost half of the total number of boot sock samples was contaminated with a low level (approx. 12 cfu/sample) of SI and approximately a quarter of the samples with a high level (approx. 31 cfu/sample) of SI by adding 0,1 ml of the appropriate dilution of an overnight culture. Approximately a quarter of the samples was not inoculated with *Salmonella* (negative samples). The concentration of the inoculum used to contaminate the boot sock samples was determined by streaking the inoculum on XLD agar plates. Immediately after artificial contamination, the samples were stored at 5 °C until transport to the participating laboratories on Monday 20 September 2021. Due to delivery problems caused by the COVID-19

pandemic, the ordered boot socks were not delivered in time to prepare the PT samples. Instead, an old batch of boot socks was used, which was past its expiration date (30-7-2020). The EURL investigated if the 'old' boot socks were still sterile, and this proved to be the case. It was therefore concluded that the 'old' boot socks were still fit for use in this PT.

3.1.4 Determination of the level of background flora in boot sock samples

The total number of aerobic bacteria and the number of *Enterobacteriaceae* in the faeces adhering to the boot socks was investigated by following EN ISO 4833-1:2013 and EN ISO 21528-2:2017 respectively. The boot sock samples were homogenised (kneaded) in peptone saline solution and 10-fold dilutions were analysed on plate count agar (PCA) and violet red bile glucose (VRBG) agar.

3.1.5 Determination of the number of Salmonella *in boot sock samples by MPN*

The contamination level of *Salmonella* in the artificially contaminated boot sock samples was determined using a five-tube most probable number (MPN) technique. For this, 10-fold dilutions of five artificially contaminated boot sock 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/A1:2020. 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 boot sock 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 type of samples tested by the participants.

For the control samples, the laboratories were asked to use their own positive *Salmonella* control strain, which they normally use when analysing routine samples for the detection of *Salmonella*. In addition to this positive control (C2), a blank procedure control (C1) consisting of only buffered peptone water (BPW) had to be analysed. The protocol and test report can be found in Annexes I and II respectively.

Table 3.1 Overview of the number and type of samples tested per laboratory in the Proficiency Test PPS 2021

Contamination level	Boot sock samples (n=14)
S. Infantis low level	6
S. Infantis high level	4
Negative (no *Salmonella* added)	4
	Control samples (n=2)
C1: Blank procedure control (BPW only)	1
C2: Positive control with own *Salmonella* strain	1

3.2.2 Shipment of parcels and temperature recording during shipment

The 16 coded samples containing the contaminated and the negative boot sock samples plus the two control samples with boot socks were packed in 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) via a door-to-door courier service. The participants were asked to store the samples at 5 °C upon receipt. To monitor exposure to abusive temperatures during shipment and storage, a micro temperature logger was placed between the samples to record the temperature.

3.3 Methods

The method prescribed for this PT was EN ISO 6579-1:2017, including A1:2020. This method begins with pre-enrichment in buffered peptone water (BPW), followed by selective enrichment in modified semi-solid Rappaport-Vassiliadis (MSRV) agar. Plating-out is carried out on xylose lysine deoxycholate agar (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 the participant. Results could be reported using EURL-*Salmonella* result form (EURL *Salmonella* 2021b). Participants received their individual laboratory performance in a performance report (See appendix II), in addition to the interim summary report (EURL *Salmonella*, 2021c).

3.4 Statistical analysis of the data

The specificity, sensitivity and accuracy rates were calculated for the boot sock samples. For the control samples, only the accuracy rates were calculated. The rates were calculated using 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 in Table 3.2 were used.

*Table 3.2 Criteria for good performance in the EURL-*Salmonella *PT PPS 2021*

Contamination level	% positive	# positive samples/ total # samples
Boot sock samples with chicken faeces		
***S*. Infantis high-level**	Min. 75 %	Min. 3/4
***S*. Infantis low-level**	Min. 50 %	Min. 3/6
Negative (no *Salmonella* added)	25 %[1]	1/4[1]
Control samples		
Procedure control (BPW only)	0 %	0 /1
Positive control with *Salmonella*	100 %	1 /1

1 All should be negative. However, as no 100% guarantee of the *Salmonella* negativity of the matrix can be given, one positive out of four negative samples (25% positive) is considered acceptable

4 Results and Discussion

4.1 Preparation of artificially contaminated boot sock samples

4.1.1 Pre-tests for the preparation of boot sock samples

The study design was based on the tests performed for the PT PPS held in 2018 by the *EURL-Salmonella* (Pol-Hofstad and Mooijman, 2019). To test whether the boot sock samples were stable during transport and storage, the samples were contaminated with a low concentration of approx. 15 cfu of *Salmonella* Infantis per boot sock sample, as described in 3.1.2.

The pre-test samples were stored at 5 °C to mimic storage conditions and at 10 °C to test the effect of temperature abuse during transport. The pre-test samples were stored for up to three weeks and analysed for the presence of *Salmonella* using EN ISO 6579:1-2017/A1:2020. In Figure 4.1 the stability results of boot sock samples artificially contaminated with two different levels of *Salmonella* Infantis are shown.

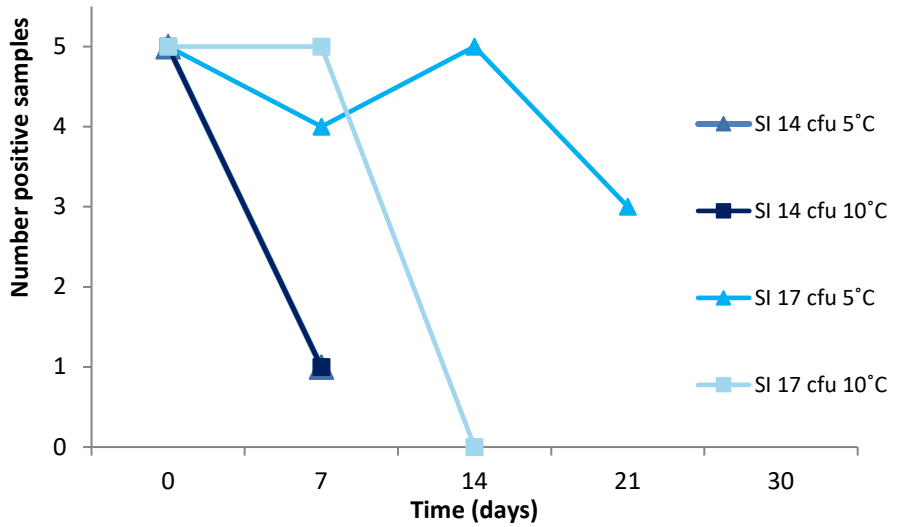

Figure 4.1 Stability tests of boot sock samples (n = 5) artificially contaminated with a low concentration of Salmonella *Infantis*

Figure 4.1 shows that *Salmonella* Infantis is inactivated to a large extent during storage of the pre-test samples. When 14 cfu was added to the boot sock samples, only one of the five samples tested positive for *Salmonella* after one week of storage at both temperatures. In a second experiment, a higher amount of *Salmonella* Infantis was added to the boot sock samples. When 17 cfu was added, three of the five samples still tested positive for *Salmonella* when stored for three weeks at 5 °C. A higher storage temperature led to inactivation of *Salmonella* in all five samples within two weeks of storage.

The effect of storage time and temperature on the number of background flora in the pre-test samples is shown in Figure 4.2. Surprisingly, no *Enterobacteriaceae* were detected in these boot sock samples with chicken faeces. The number of aerobic bacteria was found to be stable

during three weeks of storage at both 5 °C and 10 °C. Although only aerobic bacteria were detected in the chicken faeces, it was still considered sufficiently representative for real-life PPS samples.

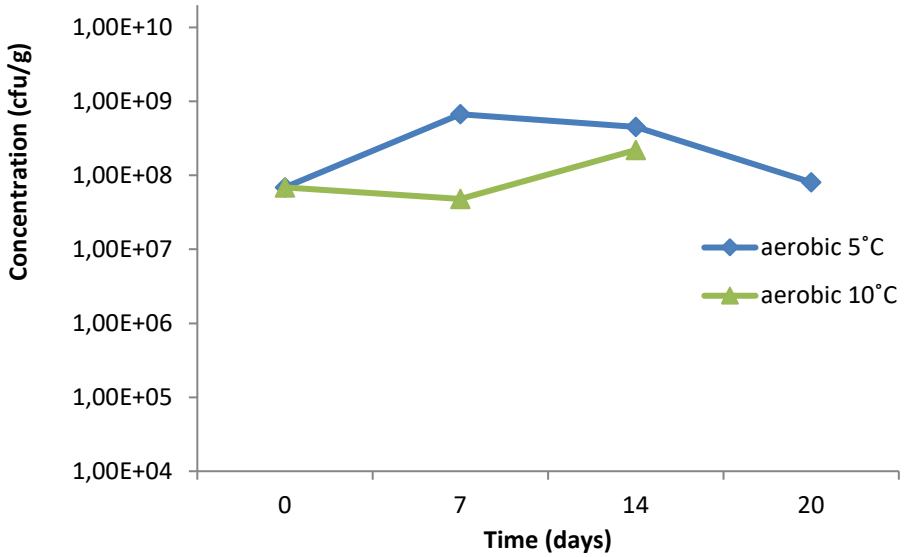

Figure 4.2 The effect of storage time and temperature on the number of aerobic bacteria in the boot sock samples

4.1.2 *Preparation of boot sock samples for the Proficiency Test*

Samples for the PT were prepared as described in 3.1.3. Samples were contaminated with approx. 17 cfu/sample and approx. 50 cfu/sample of *Salmonella* Infantis, representing low and high levels of contamination.

4.1.3 *Background flora in the boot sock samples*

The concentration of the background flora in the study samples was determined according to EN ISO 4833-1:2013 and EN ISO 21528-2:2017, as described in 3.1.4. Results are shown in Table 4.1. In the second batch of chicken faeces used for the PT samples *Enterobacteriacea* were found, up to levels of 1,0 x 10^7 cfu/g.

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

Date	Aerobic bacteria (cfu/g)	***Enterobacteriaceae*** **(cfu/g)**
27 September 2021[a]	2,6 x 10^8	1,0 x 10^7

[a] After storage at 5 °C for 1 week

4.1.4 *Number of* Salmonella *in boot sock samples*

The boot sock samples with chicken faeces were artificially contaminated at the EURL-*Salmonella* laboratory by adding the appropriate volume of a diluted *Salmonella* culture. Table 4.2 shows the contamination level of the diluted culture of *Salmonella* Infantis used as inoculum to contaminate the boot sock samples.

Table 4.2 Number of Salmonella *Infantis (SI) in the inoculum and in the contaminated boot sock samples*

Date of testing	Low level SI (cfu)	High level SI (cfu)
15 Sept 2021 **(inoculum level diluted culture)**	12	31
27 Sept 2021[a] **MPN contaminated boot sock samples (95 % confidence limit)**	2,3 (0,78-7)	35 (11-110)

[a] After storage at 5 °C for 1 week

After inoculation, the samples were stored at 5 °C for one week until being transported to the participants on Monday 20 September 2021. The final contamination level of *Salmonella* in the boot sock samples was determined by performing a five-tube most probable number (MPN) test in the week of the PT study (see Table 4.2).

4.2 Technical data for the Proficiency Test

4.2.1 General

A total of 35 NRLs *Salmonella* subscribed to this study: 27 NRLs PPS from 27 EU-MS, seven NRLs from third European countries (EU candidate MS or potential EU candidate MS and EFTA countries) and one NRL was based in a non-European country.

4.2.2 Accreditation and methods used

Almost all laboratories were accredited according to EN ISO/IEC 17025:2017 for EN ISO 6579-1:2017. Twenty-two laboratories indicated that they were also accredited for the amendment of EN ISO 6579-1 (EN ISO 6579-1:2017/A1:2020). One laboratory indicated that they were not accredited at all (lab code 36). In this PT, 21 laboratories used Amendment 1 of EN ISO 6579-1:2017. One laboratory reported it had used the selective enrichment medium RVS broth instead of the prescribed MSRV agar for analysing PPS samples.

4.2.3 Transport of samples

The samples were transported using a door-to-door courier service on Monday, 20 September 2021.

One laboratory received the parcel on the day of dispatch. Sixteen parcels were delivered after two days, 11 parcels after three days, two parcels after four days and four parcels arrived after six, seven, eight and nine days respectively. One parcel arrived very late due to border delays (lab code 21). It was delivered on 12 October 2021 and was analysed immediately after receipt. The temperature during transport and storage was registered using a temperature probe. The temperature of the parcels during transport was predominantly between -1,5 °C and 7,5 °C. The temperature of the parcels arriving late was checked more thoroughly. When the samples for laboratory 21 were placed in the box with cooling elements (at the laboratory of the EURL-*Salmonella*), the temperature of the samples decreased to -1 °C and remained at approx. 0 °C for almost

three days, followed by a slow increase to approximately 10°C on 26 September (Figure 4.3). The temperature remained at approx. 10 °C for 11 days. From 7 October the temperature dropped quickly to -3,5°C until the box reached the laboratory on 11 October and the analyses was started the next day.

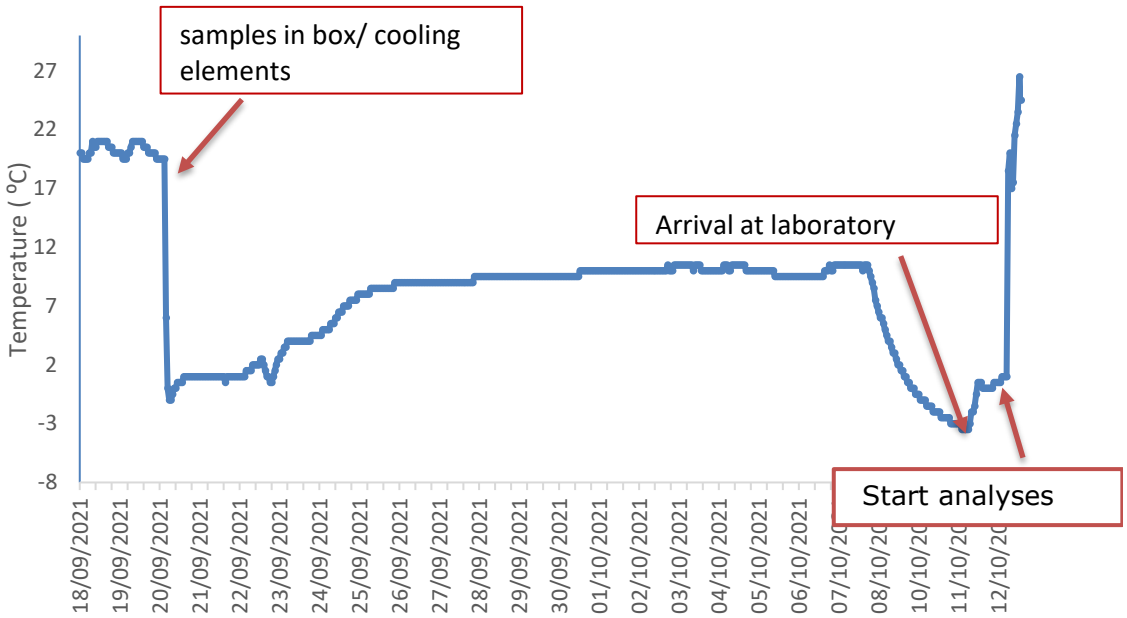

Figure 4.3 Temperature profile of the parcel for laboratory 21

The parcel for laboratory 22 arrived six days after dispatch, but was subject to high temperatures during transport, as can be seen in Figure 4.4. The temperature remained at approx. 3,5 °C for three days after dispatch, but in the three following days a sharp increase in temperature to 25 °C took place. The samples were analysed immediately after arrival on 26 September 2021.
The parcel for laboratory 27 was also exposed to high temperatures during transport (see Figure 4.5). The samples remained cool at 2 °C until 25 September 2021. The temperature rapidly rose to 20 °C on 26 September and stayed this high until the box arrived at the laboratory on 29 September 2021. The samples were analysed the next day.
The samples for laboratory 30 remained at approx. 4 °C for three days after dispatch (see Figure 4.6). From 23 September 2021, the temperature rose to 13,5 °C and even further to 24 °C, until the samples reached the laboratory on 28 September 2021. The samples were then stored cold, and the laboratory started their analyses on 29 September 2021.

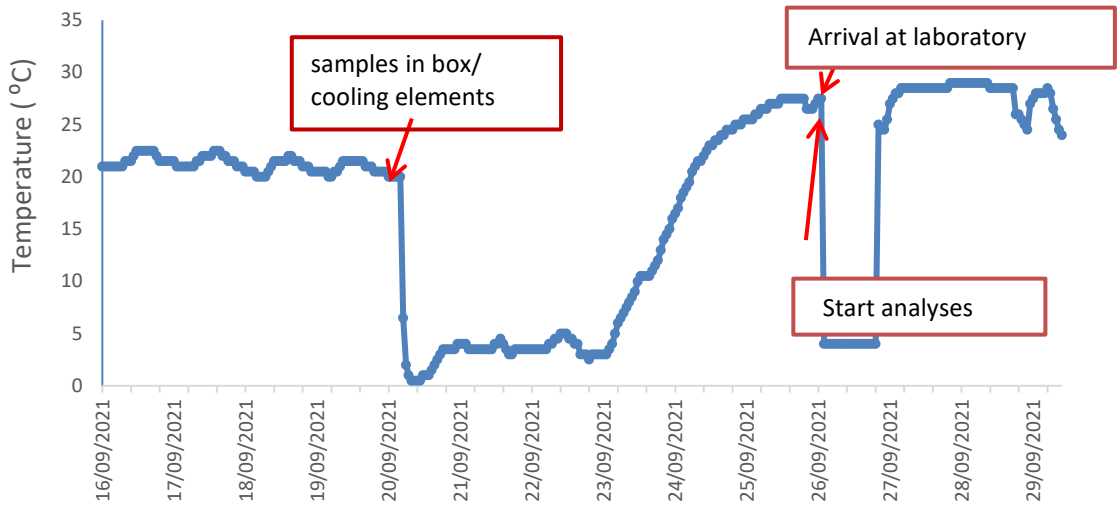

Figure 4.4 Temperature profile of the parcel for laboratory 22

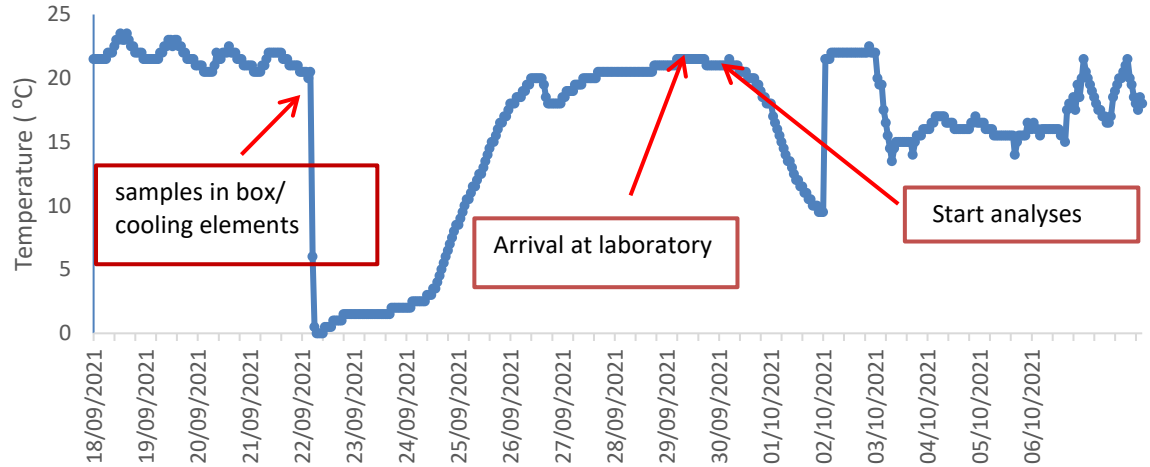

Figure 4.5 Temperature profile of the parcel for laboratory 27

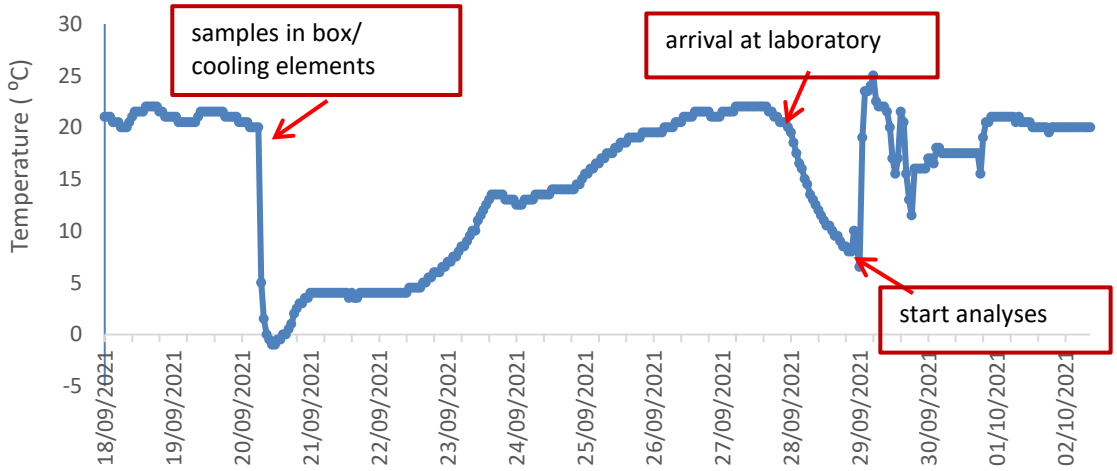

Figure 4.6 Temperature profile of the parcel for laboratory 30

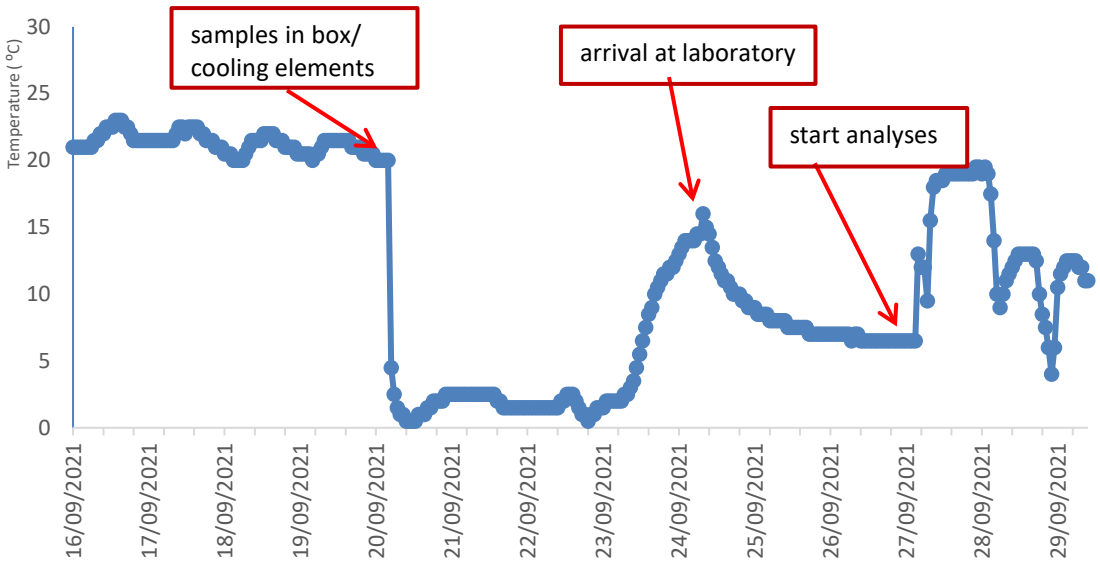

Figure 4.7 Temperature profile of the parcel for laboratory 35

The parcel for laboratory 35 arrived four days after dispatch, but the samples were exposed to elevated temperatures during transport (see Figure 4.7). The samples remained cool at 2 °C until 23 September 2021. The temperature then rose rapidly to 16 °C until the parcel arrived at the laboratory on 24 September. The samples were then stored cold and their temperature had dropped to 6,5 °C when the analyses started on 27 September 2021.

The participants were asked to store the parcels at 5 °C upon arrival at their laboratories. The storage temperatures at the receiving laboratories ranged from 0 to 10 °C. One laboratory (lab code 8) stored their samples at 17 °C.

Most laboratories began their analyses on 27 September 2021. However, three laboratories started later because their parcels did not arrive in time. One laboratory (lab code 23) already started their analyses on 23 September, the day the parcel was delivered. Two laboratories began a day early or late due to national holidays.

4.2.4 Methods

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

Table 4.3 Second plating-out media used by the NRLs

Media	No. of users
ASAP	0
BGA	8
BGA mod	5
BPLS	3
BSA	3
BxLH	1
Sm (ID)2	1
Rambach	8
Chromo *Salmonella*	2
RAPID'*Salmonella*	2
BSA Oxoid	3
RSAL	2

Explanations of the abbreviations used can be found in the 'List of abbreviations'

Technical details that deviated from the prescribed EN ISO method 6579-1:2017(/A1:2020) are listed in Table 4.4 (in bold). Only three laboratories reported some deviations. Two laboratories (lab codes 21 and 30) incubated their BPW solution for too many hours; 24 hours and a range of 18-24 hours respectively. One laboratory (lab code 27) used RVS broth instead of the prescribed MSRV agar for analysing samples from the primary production stage. In addition, 5 ml novobiocin (details on concentration was not reported) was added to the RVS.

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

Lab code	BPW		MSRV		
	Incubation time	T (°C)	T (°C)	pH	Novobiocin
EN ISO 6579-1	**16–20 h**	**37**	**41,5**	**5,1–5,4**	**10 mg/l**
21	**24**	37	42	5.1	**-**
27	18	37	41,5	0	**1000**
30	**18-24**	37	41,5	5,2	**5ml**

Deviations from EN ISO 6579-1:2017(/A1:2020) are indicated in bold

All participating laboratories performed one or several confirmation tests for *Salmonella*. Table 4.5 summarises all reported combinations. Twenty-four laboratories performed a biochemical test. Four laboratories used only one confirmation test. Most participants used a combination of two or more confirmation methods, namely a biochemical test in combination with a serological test, serotyping or a PCR test. Other methods used were MALDI-TOF and Chromogenic agar method.

Table 4.5 Number of laboratories using the different confirmation methods

Number of labs	Bio-chemical	Sero-logical	Sero-typing	PCR	Other
2	x				
7	x	x			
6	x		x		
0	x			x	
2	x				MALDI-TOF
3	x	x	x		
1	x	x		x	
1	x		x	x	
1	x		x		Chromo-genic agar
2		x			
1		x			MALDI-TOF
2			x		
5			x		MALDI-TOF
1					MALDI-TOF

4.3 Control samples

4.3.1 General

Two control samples (moistened boot socks) were also sent to the laboratories. One was used for the blank procedure control (C1). The other was used for the positive control to which the laboratories had to add their own positive control strain (C2), normally used in their routine analysis for *Salmonella* detection.

Procedure control (BPW only)

Almost all laboratories analysed the procedure control correctly as being negative for *Salmonella* and scored good results for this control sample. Laboratory 18 reported this sample as positive for *Salmonella.*

Positive control with Salmonella

Almost all laboratories correctly scored their own *Salmonella* positive control sample as positive. Laboratory 18 reported this sample as negative for *Salmonella*. This laboratory explained they had made an administrative error by accidentally reporting the C2 sample as negative, while their raw data proved it to be positive for *Salmonella*. This laboratory scored a moderate performance.

The *Salmonella* serovars used for the positive control sample are shown in Table 4.6. Most of the NRLs-*Salmonella* used *S*. Enteritidis or *S*. Typhimurium for their positive control samples. However, the use of a less common *Salmonella* serovar in routine samples may be advisable 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	11
S. Typhimurium	10
S. Nottingham	5
S. Tennessee, *S.* Tranaroa, *S.* Harleystreet, *S*. Bongori, *S.* Regent, *S.* Alachua, *S*. Blegdam, *S.* Abaetetuba, *S.* Zanzibar	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 and for the EU-MS only. The data have been corrected for the administrative error of laboratory 18. No differences were found between these two groups. All laboratories showed correct results, resulting in accuracy rates of 100%.

Table 4.7 Correct scores found with the control samples by all participants and by the EU NRLS PPS only

Control samples		**All labs n = 35**	**EU NRLs PPS n = 27**
Procedure control n=1	No. of samples	35	27
	No. of negative samples	35	27
	Specificity in %	**100%**	**100%**
Positive control (own *Salmonella*) n=1	No. of samples	35	27
	No. of positive samples	35	27
	Sensitivity in %	**100%**	**100%**
All control samples n=2	No. of samples	70	54
	No. of correct samples	70	54
	Accuracy in %	**100%**	**100%**

4.4 Boot sock samples

4.4.1 General

Boot sock samples contaminated with two different concentrations of *Salmonella* Infantis - low (MPN concentration 2,3 cfu/sample) and high (MPN concentration 35 cfu/sample) - as well as negative samples were analysed for the presence of *Salmonella* by the participants. Table 4.8 shows the overall results found by the participants.

Table 4.8 Number of positive Salmonella *isolations found in the boot sock samples at each laboratory*

	Number of positive isolations		
	Negative n=4	**SI low n=6**	**SI high n=4**
Criteria for good performance	0	≥3	≥3
Lab code 1 and 21	0	4	3
Lab code 11	0	5	3
Lab code 17	0	6	3
Lab code 22, 26 and 36	0	4	4
Lab code 5, 7, 8, 9, 20, 29 and 30	0	5	4
All other NRLs (n = 21)	0	6	4

Boot sock samples contaminated with a low level of Salmonella *Infantis*
Most of the participating laboratories were able to detect *Salmonella* in all six boot sock samples that were contaminated with a low inoculum level of approximately 3 cfu *S.* Infantis. Eight laboratories (lab codes 5, 7, 8, 9, 11, 20, 29 and 30) reported one of the six samples as negative for *Salmonella*. Five laboratories reported two 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, so these laboratories met the criteria for a good performance score. The results of all participants are shown in Figure 4.8.

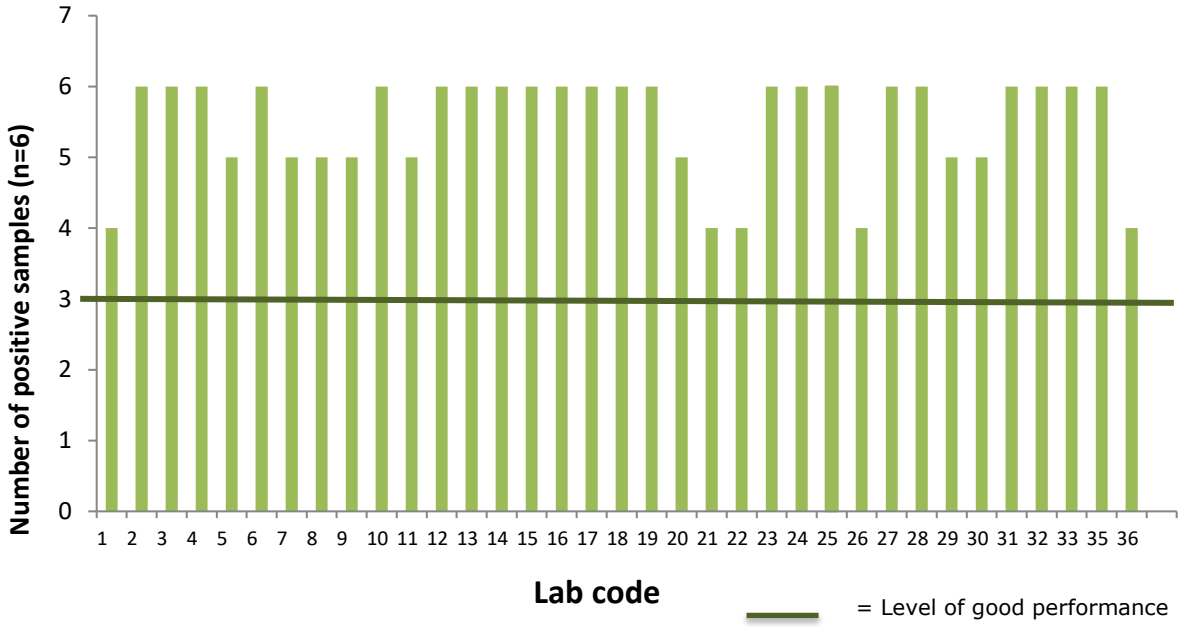

Figure 4.8 Number of positive Salmonella *isolations per laboratory, found in the boot sock samples contaminated with a low level of* Salmonella *Infantis (n=6)*

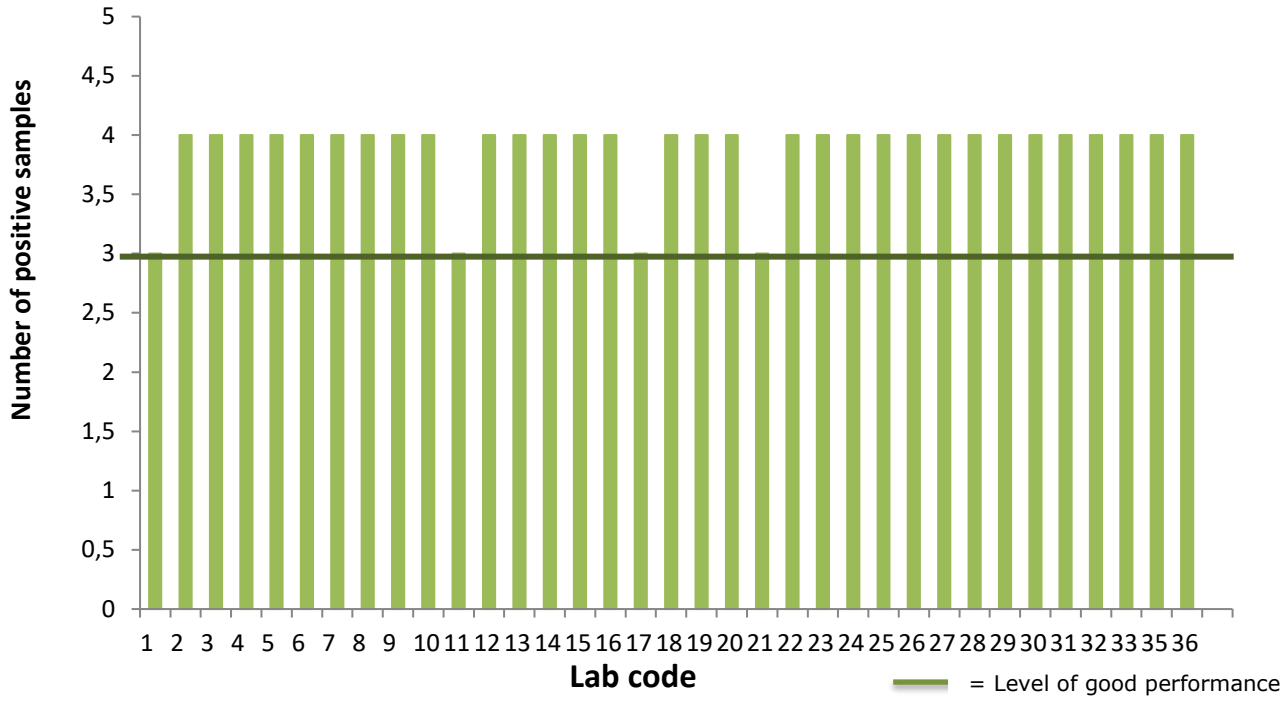

Figure 4.9 Number of positive Salmonella *isolations per laboratory, found in the boot sock samples contaminated with a high level of* Salmonella *Infantis (n=4)*

Boot sock samples contaminated with a high level of Salmonella *Infantis*
Most of the participating laboratories were able to detect *Salmonella* in all four samples inoculated with a high concentration of *S. Infantis*. Four laboratories (lab codes 1, 11, 17 and 21) reported one of four high-level samples as negative for *Salmonella*. This is still in accordance with the criteria for good performance. The results are shown in Figure 4.9.

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

Table 4.9 shows the specificity, sensitivity and accuracy rates for all boot sock samples. The calculations were performed on the results of all participants and on the results of the EU NRLs PPS only. All participants performed well in this study: the specificity rate (100%), the sensitivity rates (low level: 91,4%; high level 97,1%) and the accuracy rate (95,5%) were very high. Hardly any differences were found between all participants and the EU NRLs PPS, as shown in Table 4.9.

Table 4.9 Specificity, sensitivity and accuracy rates found by the participating laboratories (all participants and EU-MS only) when analysing the boot sock samples

Boot sock samples		All participants n=35	EU NRLs PPS n=27
Negative samples n=4	No. of samples	140	108
	No. of negative samples	140	108
	Specificity in %	**100**	**100**
Low level SI n=6	No. of samples	210	162
	No. of positive samples	192	151
	Sensitivity in %	**91,4**	**93,2**
High level SI n=4	No. of samples	140	108
	No. of positive samples	136	105
	Sensitivity in %	**97,1**	**97,2**
All boot sock samples with SI n=10	No. of samples	350	270
	No. of positive samples	328	256
	Sensitivity in %	**93,7**	**94,8**
All boot sock samples (pos. and neg.) n=14	No. of samples	490	378
	No. of correct samples	468	364
	Accuracy in %	**95,5**	**96,3**

4.4.3 *Second detection method*

In this PT, 6 laboratories also used a second method to analyse the boot sock samples. An overview of the methods used per laboratory can be found in Table 4.10.

*Table 4.10 Details on the second detection methods used by NRLs-*Salmonella *during the Proficiency Test PPS 2021*

Lab code	Second detection method	Validated (by)	Reference	Routinely # per year
4	Real time PCR iQ-Check Salmonella II	AFNOR	BRD 07/06-07/04	895
5	qPCR	AFNOR	ABI 29/02-09/10	20
7	PCR	in house	Josefsen et al.(2007) Malorny et al.(2004) O.I.E. Chapter 2.2.3	No
10	PCR	§64 of the National Food and Feed Code	Malorny et al.(2004)	
21	OIE Manual Diagnostic Tests and Vaccines for Terrestrial Animals 2021 Chapter 3.10.7.	No	-	
22	Real Time PCR 7500	National Laboratory Accreditation Authority	ISO 22119:2011(E)	3000

All laboratories used a PCR method as a second method. Only one laboratory (lab code 21) used a non-validated PCR method. Three laboratories routinely use this second method for sample analysis. The majority of NRLs found identical results with their second method compared to the prescribed bacteriological culture method. One laboratory (lab code 5) found one high-level sample to be negative for *Salmonella* using their second method, but positive using the bacteriological culture method. Laboratory 7 tested one low-level sample as negative for *Salmonella* using their second method, in contrast to their results using the bacteriological culture method.

4.5 Performance of the NRLs

4.5.1 General

All laboratories were able to detect *Salmonella* in high and low concentrations in boot sock samples. Of the 35 laboratories, 34 fulfilled the criteria for good performance. One laboratory (lab code 18) reported their positive control sample as negative for *Salmonella*. This laboratory made an administrative error and accidently reported the positive control sample as negative and the other way around. Using their raw data, this laboratory could prove that in fact they tested the positive control sample to be positive for *Salmonella*. For this reason, this laboratory scored a moderate performance in this study. No follow-up study was deemed necessary, as only administrative errors were made.

5 Conclusions

All NRLs for *Salmonella* were able to detect high and low levels of *Salmonella* in boot sock samples.

Thirty-four NRLs scored a 'good performance'. One laboratory (lab code 18) scored a moderate performance due to an administrative error and accidently reporting the positive control sample as negative for *Salmonella*.

The specificity, sensitivity and accuracy rates of the control samples were all 100%.

The sensitivity rate of all laboratories that tested the boot sock samples artificially contaminated with a low level of *S*. Infantis was 91,4%.

The sensitivity rate of all laboratories that tested the boot sock samples artificially contaminated with a high level of *S*. Infantis was 97,1%.

The accuracy rate of all laboratories for the detection of *Salmonella* in the artificially contaminated boot sock samples was 95,5%.

Six participants used a second method in addition to the prescribed bacteriological culture method. Four laboratories reported identical results for both methods. One laboratory (lab code 5) found one high-level sample negative, and one laboratory (lab code 7) found a low-level sample to be negative for *Salmonella,* in contrast to the positive results found for the same samples when the bacteriological culture method was used.

List of abbreviations

ASAP	AES *Salmonella* agar plate
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
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
PT	Proficiency Test
RIVM	Rijksinstituut voor Volksgezondheid en Milieu (National Institute for Public Health and the Environment)
RSAL	unknown abbreviation
RVS	Rappaport Vassiliadis soya broth
SI	*Salmonella* Infantis
SM (ID)2	*Salmonella* detection and identification-2
VRBG	Violet red bile glucose
XLD	Xylose lysine deoxycholate

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Annex I Documents sent to participants for EURL *Salmonella* PT PPS 2021

Protocol

EURL-*Salmonella* Proficiency test PPS 2021
Detection of *Salmonella* in chicken faeces adhering to boot socks

Introduction
This protocol describes the procedures for the Proficiency Test (PT) PPS 2021 on the detection of *Salmonella* spp. in matrix amongst the National Reference Laboratories (NRLs) for *Salmonella* in the EU. The samples consist of chicken faeces adhering to boot socks contaminated with different concentrations of a *Salmonella* serovar.

Note that the samples are transported with cooling packs and need to be stored at 5°C upon arrival.
The prescribed method is EN ISO 6579-1:2017 (Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of *Salmonella* - Part 1: Detection of *Salmonella* spp.). Additionally, laboratories (who are interested) can also perform a second detection method to analyse the sample, if this is (routinely) used in their laboratories. Only the results obtained with EN ISO 6579-1:2017 are used to assess the performance of the NRL. Please report relevant details of the method(s) used in the result form.

Objective
The main objective of the Proficiency Test is to evaluate the performance of the NRLs for *Salmonella* on their ability to detect *Salmonella* spp. at different contamination levels in chicken faeces adhering to boot socks.

Outline of the study
Each participant will receive one box containing two large plastic safety bags, packed with cooling elements. The plastic safety bags contain 16 numbered plastic bags, consisting of:

- 14 samples with chicken faeces adhering to boot socks samples artificially contaminated with different levels of a *Salmonella* serovar (coded B1-B14);
- 2 (empty) sample bags to be used for the control samples, being only BPW (coded C1), and the (own) positive control of the participating laboratory (coded C2).
- 1 sample bag containing a small electronic temperature recorder (coded with lab code)

Upon arrival: all samples have to be stored at 5 °C (± 3 °C) until the day of analyses (27 September 2021).

The sample bag containing the small electronic temperature recorder will measure the temperature during transport to the laboratory and storage of the samples at the laboratory. The sample bag with the recorder is coded with your lab code. You are urgently requested to return this complete plastic bag with recorder and lab code to the EURL-*Salmonella*, on the day your laboratory starts the study (27 September 2021). For this purpose, a return envelope with a pre-printed address label of the EURL-*Salmonella* is included.

Each box will be sent as a biological substance category B (UN3373) by door-to-door (for non-EU-MS sometimes door-to-airport) courier service DHL. Please contact EURL-*Salmonella* if the parcel has not arrived at your laboratory by 23 September 2021 (this is three working days after the day of dispatch).

The performance of the study will start on Monday 27 September 2021.

The documents necessary for performing the study are:
Protocol EURL *Salmonella* Proficiency test PPS 2021. Detection of *Salmonella* spp. in chicken faeces adhering to boot socks (this document);
Short instructions on electronic submission of data in the result form for the EURL *Salmonella* Proficiency Test on the detection of *Salmonella* spp. in chicken faeces adhering to boot socks;
EN ISO 6579-1:2017. Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of *Salmonella* - Part 1: Detection of *Salmonella* spp.

All data have to be reported through the result form. The link, which will also become available on the EURL-*Salmonella* website will be sent by email to the participants. Submission of data has to be finalised on 29 October 2021 (23:59 h CET) at the latest. Remember that the electronic result form is no longer accessible after this deadline! If you foresee problems with meeting the deadline, please contact us beforehand. The EURL will prepare a summary report soon after the study to inform all NRLs about the overall results.

Timetable

EURL- *Salmonella* Proficiency Test Primary Production Stage 2021
Detection of *Salmonella* in chicken faeces adhering to boot socks

Week	Date	Subject
27	Week of 26 July	E-mailing the link to the registration form for the detection study. Please register by 30 August 2021 at the latest.
38	Week of 20 September	Shipment of the parcels to the participants as Biological Substance Category B (UN 3373).
38	Week of 20 September	E-mailing the link for the result form to the participants. E-mailing the protocol and instructions for the result form to the NRLs. Preparation of media by the NRLs.
39	Monday 27 September	Performance of the Proficiency Test.
43	29 October 2021 at the latest	Deadline for completing the result form: 29 September 2021 (23:59h CET) After this deadline the result form will be closed
	December 2021	Interim Summary report

If you have questions or remarks about this Proficiency Test, or in case of problems,
please contact:

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Tel. number: + 31 88 6895 649 (work mobile: + 31 6 29646897)
RIVM / Z&O (internal Pb 63) EURL- *Salmonella*
P.O. Box 1, 3720 BA Bilthoven, the Netherlands
http://www.eurlsalmonella.eu/

Sample information

EURL-*Salmonella* Proficiency test PPS 2021
Detection of *Salmonella* in chicken faeces adhering to boot socks

General information on Samples
Inside this box you will find 14 samples for the Proficiency Test (PT) PPS 2021 on the detection of *Salmonella* spp. in chicken faeces adhering to boot socks. Due to delivery problems caused by the Covid-19 pandemic, we did not receive our boot socks order in time. Therefore, we were forced to use an older batch of boot socks which were past their expiration date. The EURL has investigated the sterility and the 'older' boot socks were found to be sterile and fit to be used in this PT.

EURL *Salmonella*
Irene Pol-Hofstad

Annex II Example of an individual laboratory Performance report of the EURL-*Salmonella* PT PPS 2021

Performance

EURL-*Salmonella* PT PPS 2021

Number of positive samples/Total number of samples per level

	chicken faeces on boot socks			control samples	
Lab code	High	Low	Negative	PS	pos control
#	4/4	6/6	0/4	1/1	0/1

Evaluation: performance

Number	Level	Your result	Media choices:
B1	Low	Detected	MSRV
B2	High	Detected	BGA and XLD
B3	High	Detected	
B4	Negative	Not detected	
B5	Negative	Not detected	
B6	Low	Detected	
B7	High	Detected	
B8	Low	Detected	
B9	High	Detected	
B10	Negative	Not detected	
B11	Negative	Not detected	
B12	Low	Detected	
B13	Low	Detected	
B14	Low	Detected	
C1	neg	Not Detected	
C2	pos	Detected	

Low = Low concentration *Salmonella* Infantis
High = High concentration *Salmonella* Infantis
Negative= Negative chicken faeces on boot socks sample (no *Salmonella* added)
PS = Peptone Saline solution

Pos control = own positive control

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