Interim summary report

EURL-*Salmonella* Proficiency Test Primary Production Stage 2021

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

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1. Introduction

In September 2021, an EURL-*Salmonella* Proficiency Test (PT) for detection of *Salmonella* in samples from the Primary Production Stage (PPS) was organised for the National Reference Laboratories-*Salmonella* (NRLs-*Salmonella*). The matrix for this PT was chicken faeces adhering to boot socks (referred to as boot sock samples in this report).
In total 35 NRLs for *Salmonella* participated in this study: 27 NRLs PPS originating from 27 EU-Member States (MS), 7 NRLs from third countries within Europe (EU candidate or potential EU candidate MSs and members of the European Free Trade Association (EFTA)) and one NRL from a non-European country.

This interim summary report consists of two parts. One part contains the individual NRL results, which was sent separately to the NRLs-*Salmonella*; the other part contains the overall results of all NRLs-*Salmonella*, which is described here.

2. Materials & Methods

2.1 Samples

The samples in this PT consisted of chicken faeces adhering to boot socks with different concentrations of *Salmonella* Infantis (SI).

Each NRL-*Salmonella* had to analyse 16 samples in total:
- 4 boot sock samples with a high level of *Salmonella* Infantis (SI)
- 6 boot sock samples with a low level of *Salmonella* Infantis (SI)
- 4 negative boot sock samples (no *Salmonella* added)
- 1 procedure control (boot sock with BPW only)
- 1 positive control sample (laboratories' own *Salmonella* control strain)

The chicken faeces originated from a specific pathogen free farm (SPF farm) and absence of *Salmonella* was tested in ten different samples prior to the PT.
The boot socks were moistened with 15 ml of peptone saline solution (PS) and left

to saturate at room temperature for at least 30 minutes. 10 g of *Salmonella* free chicken faeces was added to the moistened boot socks. Next, one third of the total number of boot socks (with chicken faeces) was contaminated with a low level of SI, one third with a high level of SI and one third was not inoculated with *Salmonella* (negative samples). The artificially contaminated samples were stored at 5 °C until the day of transport. The decoding of these samples can be found in the tables of the individual NRL results.
On Monday 20 September 2021, the boot sock samples were packed with frozen cooling elements and sent to the NRLs. Upon arrival, the NRLs were requested to store the samples at 5 °C until the start of the analysis on Monday 27 September 2021.

The level of natural background flora in the chicken faeces was determined on the day of the performance of the PT (27 September 2021). Table 2.1 shows the number of aerobic bacteria and *Enterobacteriaceae* in the chicken faeces.

Table 2.1 Number of aerobic bacteria and Enterobacteriaceae *per gram 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

Table 2.2 shows the level of the diluted culture of *Salmonella* Infantis used to artificially contaminate the boot sock samples. Additionally, the number of *Salmonella* in the boot sock samples was determined using a five-tube Most Probable Number (MPN) test in the week of the Proficiency Test.

Table 2.2 Number of Salmonella *Infantis (SI) in the inoculum and in the inoculated boot sock samples*

Date of testing	Low level SI (cfu / sample)	High level SI (cfu / sample)
23 Sept 2021 Inoculation boot sock samples	12	31
5 Oct 2021[a] MPN of contaminated boot sock samples (95 % confidence limit)	2,3 (0,78-7)	35 (11-110)

[a] After storage at 5°C for 1 week

2.2 Analysis of samples following EN ISO 6579-1 / A1:2020

The prescribed method was EN ISO 6579-1:2017 including Amendment 1 (EN ISO 6579-1:2017/A1:2020), and the underlying EN ISO documents, e.g. the EN ISO 6887 series for preparation of test samples. EN ISO 6579-1:2017(/A1:2020) describes the technical steps for the detection of *Salmonella* in food, animal feed samples, environmental samples from the food production area, and samples from the primary production stage.

In summary, for samples from the primary production stage:

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

- selective enrichment on:
  Modified semi-solid Rappaport-Vassiliadis (MSRV) agar
- plating-out on two isolation media:
  first isolation medium: Xylose Lysine Deoxycholate agar (XLD);
  second isolation medium (obligatory): medium of choice;
- confirmation by means of:
  appropriate biochemical and serological tests (EN ISO 6579-1:2017(/A1:2020)) or reliable, commercially available identification kits.

Additionally, the NRLs-*Salmonella* were allowed to analyse the boot sock samples with a second detection method, if this is (routinely) used in their laboratories. However, only the results obtained with EN ISO 6579-1:2017(/A1:2020) were used to assess the performance of each NRL. From the results of all laboratories the specificity, sensitivity and accuracy rates were calculated, as follows:

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

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

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

2.3 Performance analysis

Criteria for good performance used in the current EURL-*Salmonella* PT for detection of *Salmonella* in the boot sock samples are shown in Table 2.3

Table 2.3 Criteria for good performance

Boot sock samples	Percentage positive	# pos samples / total # samples
Negative samples	0%[*]	0 / 4
Low level of *S.* Infantis	≥ 50%	≥ 3 / 6
High level of *S.* Infantis	≥ 75%	≥ 3 / 4
Control samples	**Percentage positive**	**# pos samples / total # samples**
Procedure control	0%	0 / 1
Positive control with *Salmonella*	100%	1 / 1

*100% Salmonella-free matrix cannot be guaranteed, 1 positive out of 4 negative samples is still considered as acceptable (25%).

3. Results

3.1 General

On Monday 20 September 2021 the boot sock samples were sent to the participating laboratories. One laboratory received the parcel within the same day of dispatch. Seventeen parcels were delivered after two days, eleven parcels after three days and two parcels after four days of dispatch. Laboratories 22, 27 and 30 received the samples after respectively six, eight and nine days of dispatch. One parcel arrived very late due to severe delay at the border, only after 22 days the laboratory could start the analysis (lab code 21).
The temperature during transport and storage was registered using a temperature probe. The temperature of the parcels during transport was predominantly between -3,5 °C and 5 °C. Some parcels experienced an elevated transport temperature while arriving already within two or three days after dispatch: parcels for laboratory 4, 8 and 35 had a temperature of 7, 16 and 14,5 °C respectively. The parcels had to be stored at 5 °C upon arrival at the laboratory. The storage temperature of the sample at the laboratories varied between -4,5 °C and 11,5 °C.
The temperature of the parcels arriving late were checked in more detail. Parcels for laboratories 21, 22, 27 and 30 were exposed to higher and/or lower temperatures compared to the preferred transport temperature of approximately 5 °C. Most laboratories started the analyses on the 27 September 2021. However, one laboratory started immediately after receipt of the parcel on 23 September 2021 and one laboratory started 1 day earlier on 26 September 2021. The laboratories receiving the parcels later than the official performance date (27 September 2021) started on the day the parcel arrived at their laboratory (lab codes 21, 22, 27 and 30).

All laboratories followed the prescribed method EN ISO 6579-1:2017. The majority of the laboratories also indicated that they follow the recently published amendment of EN ISO 6579-1 (EN ISO 6579-1:2017/A1:2020). One laboratory reported to be NRL-*Salmonella* for analysing PPS samples, but did not use MSRV agar for the selective enrichment, but used RVS broth. This is not in line with what is prescribed 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.

3.2 Boot sock samples

3.2.1 Negative samples

All laboratories correctly analysed the *Salmonella*-negative boot sock samples negative for *Salmonella*.

3.2.2 Samples with a low level of Salmonella *Infantis*

Almost all laboratories were able to detect *Salmonella* in all six low level boot sock samples. See Figure 3.1 for results. Eight laboratories (lab codes 5, 7, 8, 9, 11, 20, 29 and 30) could not detect *Salmonella* in one of the six low contaminated samples. Five laboratories (lab code 1, 21, 22, 26 and 36) could not detect *Salmonella* in two of the six low contaminated samples. The results of all laboratories are still within the limits of good performance, which allows for three out of the six low contaminated samples to be scored negative (see Table 2.3).

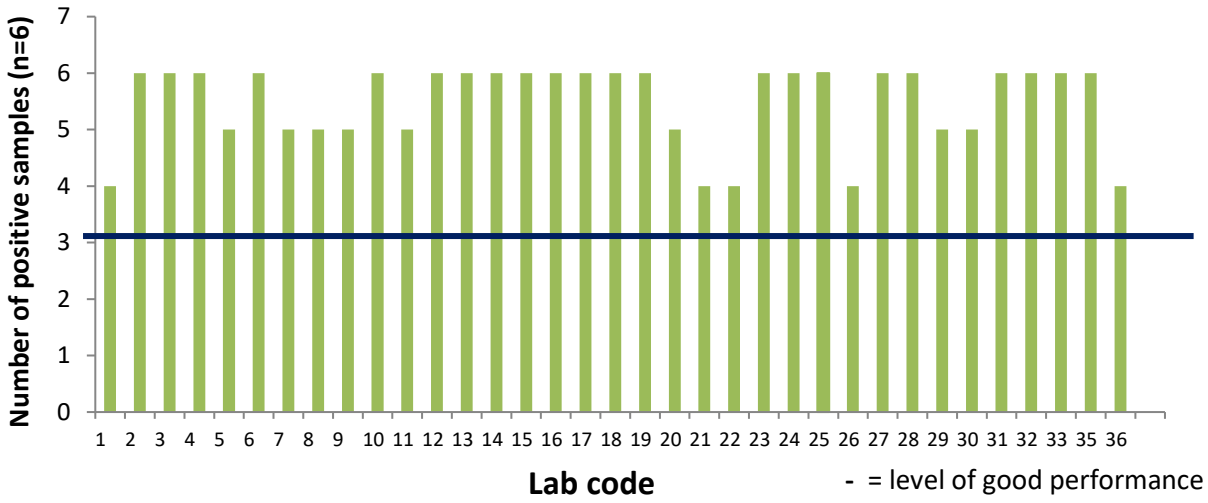

Figure 3.1. Number of positive Salmonella *isolations per laboratory found in the boot sock samples contaminated with a low level of* Salmonella Infantis *(n=6).*

3.2.3 Samples with a high level of Salmonella *Infantis*

Almost all laboratories detected *Salmonella* in all four high level boot sock samples. See Figure 3.2 for results. Four laboratories (lab code 1, 11, 17 and 21) scored one sample out of the four high contaminated samples negative. This is still within the level of good performance which allows for one sample out of the four high level contaminated samples to be scored negative (see Table 2.3).

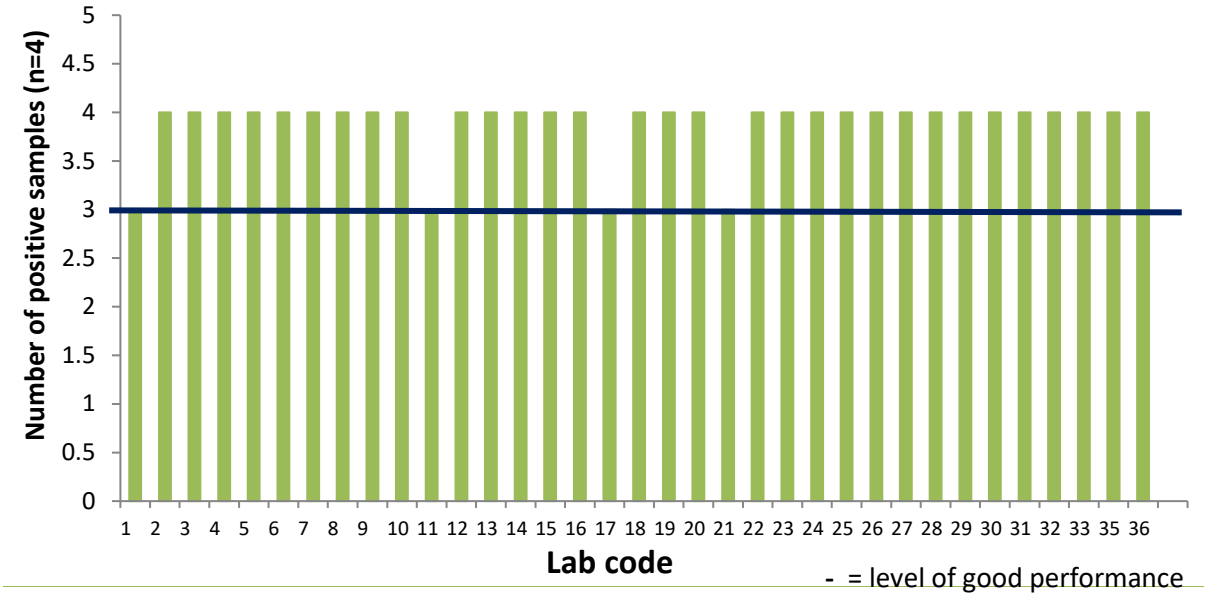

Figure 3.2. Number of positive Salmonella *isolations per laboratory found in the boot sock samples contaminated with a high level of* Salmonella *Infantis (n=4).*

In table 3.1 the specificity, sensitivity and accuracy rates are given for the boot sock samples. The laboratories have scored good results with all boot sock samples (negative, low and high contaminated) as shown by the high rates for specificity, sensitivity and accuracy (> 91%).

Table 3.1. Specificity, sensitivity and accuracy rates of the boot sock samples, artificially contaminated with Salmonella *Infantis (SI)*

Boot sock samples		**Total no of labs n = 35**
Negative n=4	No. of samples No. of negative samples **Specificity in %**	140 140 **100%**
Low level (SI) n=6	No. of samples No. of positive samples **Sensitivity in %**	210 192 **91,4%**
High level (SI) n=4	No. of samples No. of positive samples **Sensitivity in %**	140 136 **97,1%**
All boot sock samples with SI	No. of samples No. of positive samples **Sensitivity in %**	350 328 **93,7%**
All boot sock samples (positive and negative)	No. of samples No. of correct samples **Accuracy in %**	490 468 **95,5%**

3.3 Control samples

The laboratories were asked to use their own positive control strain normally used in their routine analysis for the detection of *Salmonella*. Additional to this own control, a procedure control sample (BPW only) had to be analysed. All but one laboratory scored both control samples correct. Laboratory 18 scored an unsatisfactory performance by reporting their positive control sample negative for *Salmonella*.

For the positive control, the majority of the participants used as their positive control *Salmonella* Enteritidis (11), followed by *Salmonella* Typhimurium (10) and *Salmonella* Nottingham (5). Nine participants used other *Salmonella* serovars.

Table 3.2 shows the correct scores for each control sample and the accuracy rate for both control samples. The laboratories scored good results for the control samples with an accuracy rate of 97,1%.

Table 3.2 Correct scores of the control samples

Control samples		Total no of labs n = 35
Procedure control (BPW only) n = 1	No. of samples No. of negative samples **Correct score in %**	35 34 **97,1%**
Positive control (Own *Salmonella* control) n = 1	No. of samples No. of positive samples **Correct score in %**	35 34 **97,1%**
All control samples n = 2	No. of samples No. of correct samples **Accuracy in %**	70 68 **97,1%**

4. Good performance

The results of 34 of the 35 participating laboratories fulfilled the criteria of good performance.
One laboratory (lab code 18) scored their own positive control negative and their procedure control positive for *Salmonella*; resulting in an unsatisfactory performance. This laboratory will be contacted for additional explanation of this result.

5. List of abbreviations

PS	Peptone Saline Solution
BPW	Buffered Peptone Water
cfu	colony forming units
EFTA	European Free Trade Associations
EU	European Union
EURL	European Union Reference Laboratory
ISO	International Standardisation Organisation
MPN	Most Probable Number
MS	Member State
MSRV	Modified semi-solid Rappaport-Vassiliadis
NRL	National Reference Laboratory
PPS	Primary Production Stage
PT	Proficiency Test
RVS	Rappaport-Vassiliadis medium with Soya
SI	*Salmonella* Infantis
XLD	Xylose Lysine Deoxycholate agar

6. References

EN ISO 6579-1: 2017. Microbiology of the food chain – Horizontal method for the detection, enumeration and serotyping of *Salmonella* spp. Part 1: Detection of *Salmonella* spp.

EN ISO 6579-1/A1:2020. Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of *Salmonella* - Part 1: Detection of *Salmonella* spp. - Amendment 1 Broader range of incubation temperatures, amendment to the status of Annex D, and correction of the composition of MSRV and SC (ISO 6579-1:2017/Amd 1:2020).

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