Interim summary report

EURL-*Salmonella* Combined Proficiency Test Primary Production Stage – Food 2022

Detection of *Salmonella* in hygiene swab samples

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1. Introduction

In September 2022, the combined EURL-*Salmonella* Proficiency Test (PT) for detection of *Salmonella* in samples from the Primary Production Stage (PPS) and Food was organised for the National Reference Laboratories (NRLs) for *Salmonella*. The matrix for this PT was hygiene swabs. This matrix is suitable to mimic samples from the Primary Production stage as well as the food production environment. Since EURL-*Salmonella* did not organise a PT for detection of *Salmonella* in food in 2022, this PT was obligatory for both the NRLs-*Salmonella* analysing PPS samples as well as for NRLs analysing food samples. In total 68 NRLs-*Salmonella* participated in this study: 34 NRLs for PPS and 34 NRLs for Food, originating from 35 countries. 56 participants originated from 27 EU-Member States (MS), 11 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 combined PT consisted of hygiene swab samples contaminated with background flora (*Enterobacter cloacae* and *Citrobacter freundii or Enterobacter cloacae* and *Citrobacter youngae*) and with different concentrations of two *Salmonella* strains: *Salmonella* Infantis (SI) and/or *Salmonella* Enteritidis (SE).

Each NRL-*Salmonella* had to analyse 16 blindly coded samples in total:

- 4 hygiene swab samples with a high level of *Salmonella* Infantis (SI) and *Salmonella* Enteritidis (SE), in combination with a mixture of *Enterobacter cloacae and Citrobacter freundii* (10^6 cfu/sample).

- 6 hygiene swab samples with a low level of *Salmonella* Infantis (SI), in combination with a mixture of *Enterobacter cloacae and Citrobacter freundii* (10^6 cfu/sample).
- 4 negative hygiene swab samples (no *Salmonella* added)
  - 2 samples: *Enterobacter cloacae* and *Citrobacter freundii* (10^6 cfu/sample);
  - *2 samples: Enterobacter cloacae* and *Citrobacter youngae* (10^6 cfu/sample).
- 1 procedure control (hygiene swab samples with PS only)
- 1 positive control sample (laboratories' own *Salmonella* control strain)

Each hygiene swab was packed in a plastic bag and moistened with 10 ml of peptone saline solution (PS) and left to saturate at room temperature for at least 30 minutes. Next, all hygiene swabs were artificially contaminated with a high concentration of background flora by adding 1 ml of a suspension containing an even mixture of *Enterobacter cloacae* and *Citrobacter freundii* (approx. 10^6 cfu/ml) or an even mixture of *Enterobacter cloacae* and *Citrobacter youngae* (approx. 10^6 cfu/ml). Additionally, the hygiene swab samples were contaminated with *S.* Infantis (low level samples) or a combination of *S*. Infantis *and S.* Enteritidis (high level samples). The artificially contaminated samples were stored at 5 °C ± 3 °C until the day of transport, approximately 5-6 days after inoculation. On Monday 26 September 2022, the artificially contaminated hygiene swab samples were packed with frozen cooling elements and sent to the NRLs-*Salmonella*. Upon arrival, the NRLs were requested to store the samples at 5 °C ± 3 °C until the start of the analysis on Monday 3 October 2022. The decoding of the samples can be found in the tables of the individual NRL results.

The level of background flora in the two cell suspensions used to contaminate the hygiene swab samples was determined on the day of inoculation of the samples (see Table 2.1).

Table 2.1 Number of background flora in the cell suspensions used to artificially contaminate the hygiene swabs (cfu per ml)

Date	***Enterobacter cloacae* and *Citrobacter freundii* (cfu / ml)**	***Enterobacter cloacae* and *Citrobacter youngae* (cfu / ml)**
21 Sept 2022	8,0 x 10^5	9,7 x 10^5

Table 2.2 shows the level of the diluted culture of *S.* Infantis and of the mixture of *S.* Infantis and *S.* Enteritidis used to artificially contaminate the hygiene swab samples. Additionally, the number of *Salmonella* in the hygiene swab 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) and the mixture of* Salmonella *Infantis and* Salmonella *Enteritidis (SE) in the inoculum and in the inoculated hygiene swab samples*

Date of testing	Low level SI (cfu / sample)	High level SI + SE (cfu / sample)
21 Sept 2022 Inoculation Hygiene swab samples	8	30 + 8
3 Oct 2022[a] MPN of artificially contaminated hygiene swab samples (95 % confidence limit)	2,15 (0,85-5,5)	17,25 (6,5-45)

[a] After storage at 5°C for approx. 1,5 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 or Food samples:

- pre-enrichment in:
  Buffered Peptone Water (BPW);
- selective enrichment on/in:
  PPS: Modified semi-solid Rappaport-Vassiliadis (MSRV) agar
  Food: Mueller-Kauffmann tetrathionate-novobiocin (MKTTn) broth;
  Modified semi-solid Rappaport-Vassiliadis (MSRV) agar and/or;
  Rappaport-Vassiliadis broth with Soya (RVS);
- 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 hygiene swab 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 hygiene swab samples are shown in Table 2.3.

Due to unexpected deviating results in approx. 10% of the total number of negative samples tested, the results of the negative samples were not evaluated.

Table 2.3 Criteria for good performance

Hygiene swab samples	Percentage positive	# pos. samples / total # samples
Negative samples	No evaluation	No evaluation
Low level of *SI*	≥ 50%	≥ 3 / 6
High level of *SI* + *SE*	≥ 75%	≥ 3 / 4
Control samples	**Percentage positive**	**# pos. samples / total # samples**
Procedure control	0%	0 / 1
Positive control with *Salmonella*	100%	1 / 1

3. Results

3.1 General

On Monday 26 September 2022, the hygiene swab samples were sent to the participating laboratories. Two parcels arrived within the same day of dispatch. Forty-nine parcels were delivered after one day, seven after two days, five after three days and four after four days. Laboratory 23 received the samples on 4 October 2022 and started the analyses the next day.
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 4 °C. The storage temperature of the samples at the laboratories varied between -0,5 °C and 7 °C. The temperature of the parcels arriving late were checked in more detail. The parcel for laboratory 23 experienced an elevated transport temperature during the second part of the transport time. The temperature remained at 1 °C until 30 September followed by a gradual increase in temperature until 10 °C on 4 October 2022 when it arrived at its destination.
Most laboratories started the analyses on 3 October 2022. However, three laboratory started immediately after receipt of their parcels on 28, 29 or 30 September 2022 and five laboratories started one day later on 4 October 2022 and one laboratory started two days later on 5 October 2022.

All laboratories followed 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). Laboratories 52 and 60 reported to be NRL-*Salmonella* for analysing PPS samples, but did not use MSRV agar for the selective enrichment. This is not in line with what is prescribed in EN ISO 6579-1:2017 for analysing PPS samples. Twenty laboratories also used a second detection method for analysing the samples. Not all laboratories found identical results using the alternative method compared to the results found with EN ISO 6579-1:2017/A1:2020. One NRL Food did

not report their results under their separate lab code 32 but mistakenly reported their food results combined with their PPS results under their lab code for NRL PPS.

3.2 Hygiene swab samples

3.2.1 Negative samples

The negative samples were artificially contaminated with background flora in two different combinations. Samples B1 and B8 contained a mixture of *Enterobacter cloacae* and *Citrobacter freundii* (10^6 cfu/sample), and samples B9 and B14 contained a mixture of *Enterobacter cloacae* and *Citrobacter youngae* (10^6 cfu/sample). Forty-three laboratories tested all four negative hygiene swab samples negative for *Salmonella* (see figure 3.1). However, in total 25 laboratories tested one or two negative samples positive for *Salmonella*. Eleven laboratories detected *Salmonella* in sample B1, four laboratories detected *Salmonella* in sample B8, five laboratories detected *Salmonella* in sample B9 and 7 laboratories detected *Salmonella* in sample B14. Two laboratories reported two negative samples positive for *Salmonella* (Lab code 1: samples B9 and B14; Lab code 31: B1 and B8). All these laboratories were requested to send information and raw data on these samples to investigate the results in more detail. Since 10% of the total amount of negative samples was scored positive for *Salmonella*, the EURL-*Salmonella* has decided not to evaluated the results of the negative samples. Investigations to the source of contamination is ongoing.

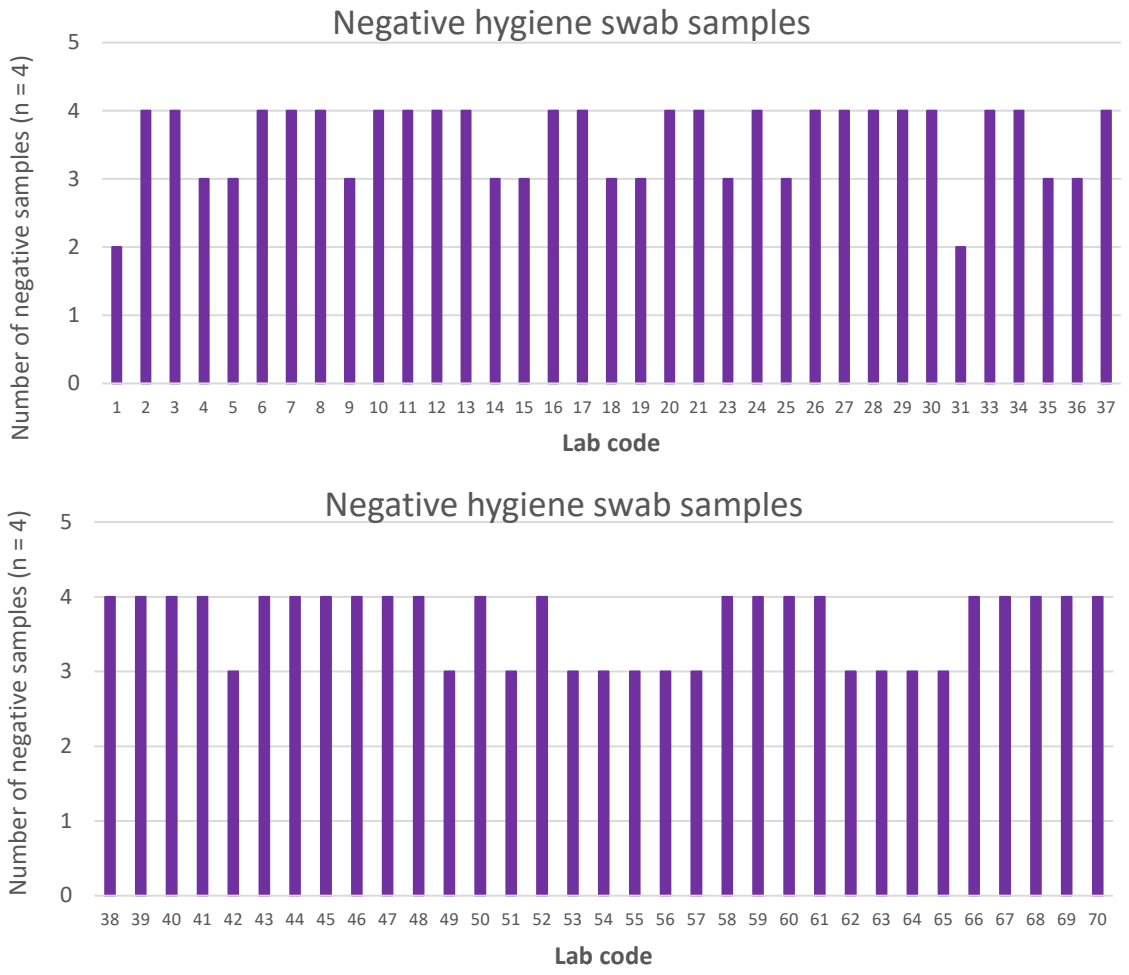

*Figure 3.1. Number of negative hygiene swab samples containing background flora (*Enterobacter cloacae/Citrobacter freundii *or* Enterobacter cloacae/Citrobacter youngae *(n=4)) tested negative for* Salmonella, *per participant.*

3.2.2 Samples with a low level of Salmonella *Infantis*

Almost all laboratories were able to detect *Salmonella* Infantis in all six low level hygiene swab samples. See Figure 3.2 for results. One laboratory (lab code 1) could not detect *Salmonella* Infantis in one of the six low contaminated samples. This result is 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).

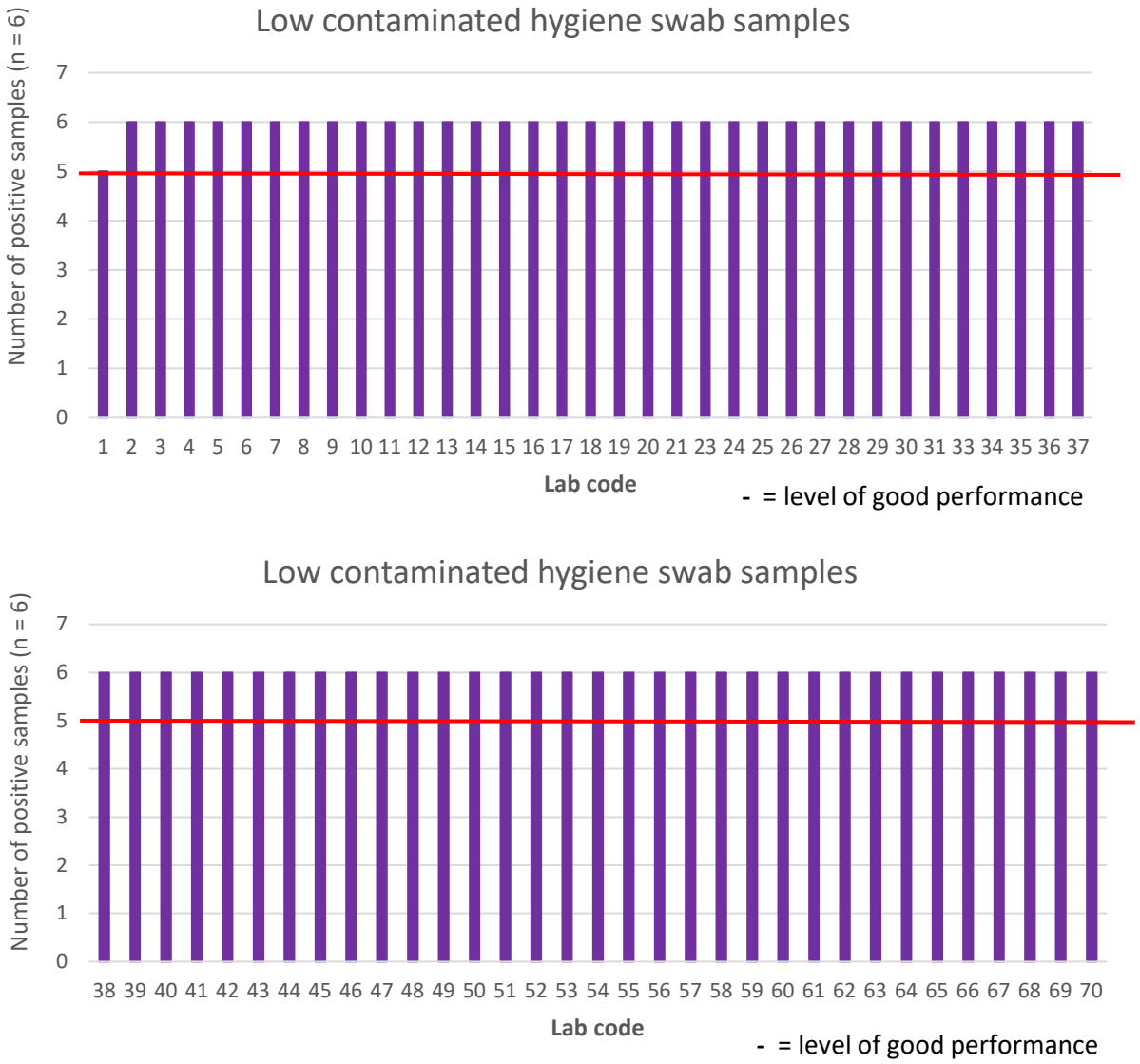

Figure 3.2. Number of positive Salmonella *isolations per laboratory found in the hygiene swab samples artificially contaminated with a low level of* Salmonella *Infantis (n=6).*

3.2.3 Samples with a high level of Salmonella *Infantis and* Salmonella *Enteritidis*

All laboratories were able to detect *Salmonella* in all four high level hygiene swab samples. See Figure 3.3 for results.

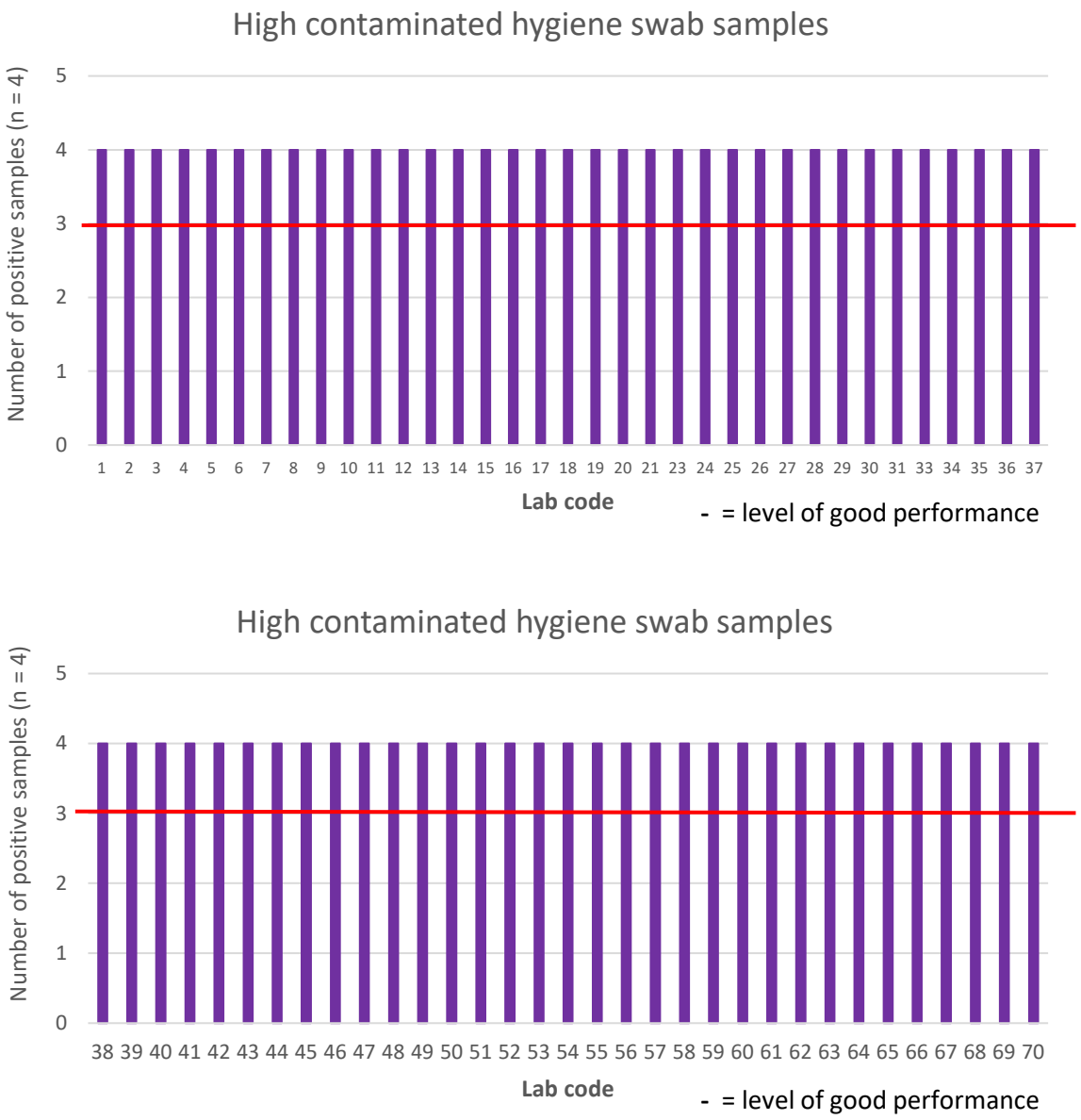

Figure 3.3. Number of positive Salmonella *isolations per laboratory found in the hygiene swab samples artificially contaminated with a high level of* Salmonella *Infantis and Salmonella Enteritidis (n=4).*

In table 3.1 the specificity, sensitivity and accuracy rates are given for the hygiene swab samples. The laboratories have scored good results with the high and low levels of *Salmonella,* as shown by the high rates for sensitivity. The values for specificity and accuracy were calculated but no evaluation on this values will be performed because of the relatively high number of negative samples being found positive for *Salmonella.*

Table 3.1. Specificity, sensitivity and accuracy rates of the hygiene swab samples, artificially contaminated with Salmonella *Infantis (SI) and/or* Salmonella *Enteritidis (SE)*

Hygiene swab samples		Total no of labs n = 68	EU NRLs only n=56	EU NRLs PPS n =28	EU NRLs Food n=28
Negative n=4	No. of samples	272	224	112	112
	No. of neg. samples	245	204	101	103
	Specificity in %	**90,1%**	**91,1%**	**90.2%**	**92.0%**
Low level (SI) n=6	No. of samples	408	336	168	168
	No. of pos. samples	407	336	168	168
	Sensitivity in %	**99,8%**	**100%**	**100%**	**100%**
High level (SI and SE) n=4	No. of samples	272	224	112	112
	No. of positive samples	272	224	112	112
	Sensitivity in %	**100%**	**100%**	**100%**	**100%**
All hygiene swabs with *Salmonella* (SI and/or SE)	No. of samples	680	560	280	280
	No. of positive samples	679	560	280	280
	Sensitivity in %	**99,9%**	**100%**	**100%**	**100%**
All hygiene swabs (pos. and neg.)	No. of samples	952	784	392	392
	No. of correct samples	924	764	381	383
	Accuracy in %	**97,1%**	**97,4%**	**97.2%**	**97,7%**

3.3 Control samples

The laboratories were asked to use their own positive control normally used in their routine analysis for the detection of *Salmonella*. Additional to this own control, a procedure control sample (PS only) had to be analysed. All laboratories scored both control samples correct.

For the positive control, the majority of the participants used *Salmonella* Enteritidis (18) as their positive control, followed by *Salmonella* Typhimurium (16) and *Salmonella* Nottingham (9). Twenty-five 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 100%.

Table 3.2 Correct scores of the control samples

Control samples		Total no of labs n = 68	EU NRLs PPS n = 28	EU NRLs Food n = 28
Procedure control (PS only) n = 1	No. of samples	68	28	28
	No. of negative samples	68	28	28
	Correct score in %	**100%**	**100%**	**100%**
Positive control (Own *Salmonella* control) n = 1	No. of samples	68	28	28
	No. of positive samples	68	28	28
	Correct score in %	**100%**	**100%**	**100%**
All control samples n = 2	No. of samples	136	56	56
	No. of correct samples	136	56	56
	Accuracy in %	**100%**	**100%**	**100%**

4. Good performance

The results of the 68 participating laboratories were evaluated and fulfilled the criteria of good performance. All laboratories were able to detect *Salmonella* in high and low concentrations in the hygiene swab samples. Only one laboratory (lab code 1) was not able to detect *Salmonella* in one low level sample, but this is still well within the criteria for good performance.
Since almost 10% of the negative samples was found positive for *Salmonella*, the EURL-*Salmonella* has decided not to include the results of these samples into the evaluation of the performance.

5. List of abbreviations

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
MKTTn	Mueller-Kauffmann tetrathionate-novobiocin broth
MSRV	Modified semi-solid Rappaport-Vassiliadis
NRL	National Reference Laboratory
PPS	Primary Production Stage
PS	Peptone Saline solution
PT	Proficiency Test
RVS	Rappaport-Vassiliadis broth with Soya
SE	*Salmonella* Enteritidis
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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