Interim summary report

EURL-*Salmonella* Proficiency Test Live Bivalve Molluscs 2024

Detection of *Salmonella* in mussels

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1. Introduction

In February 2024, the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*) organised a Proficiency Test (PT) for the detection of *Salmonella* in live bivalve molluscs for the National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*). Mussels were used as matrix for this PT. NRLs-*Salmonella* which are responsible for the detection of *Salmonella* in bivalve molluscs were invited to participate in this PT.
Twenty-three NRLs-*Salmonella* registered for this PT, but one laboratory was not able to arrange the correct paperwork for the shipment (laboratory code 11). This laboratory, from a third country, had to cancel their participation at the last moment. The 22 participants in this PT: NRLs-*Salmonella* from 19 EU Member States and three NRLs-*Salmonella* from countries of the European Free Trade Association (EFTA).

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 preparation of the PT samples was done differently compared to other PTs of the EURL-*Salmonella* for detection of *Salmonella* in food, feed or samples from the primary production stage. During this PT the laboratories had to prepare the samples themselves and spike the samples with *Salmonella* reference materials. The design of this PT was comparable to the previous EURL-*Salmonella* PT for live bivalve molluscs in 2020. The mussels and the *Salmonella* reference materials were both provided by the EURL-*Salmonella*.

Each NRL-*Salmonella* had to analyse four samples in total:

- three positive samples of 25 g mussel flesh and intravalvular fluid (spiked with reference materials with *Salmonella*)
- one negative sample of 25 g mussel flesh and intravalvular fluid (spiked with reference materials without *Salmonella*)

Sample bags for control samples were not provided. Each NRL-*Salmonella* was expected to include (process) control samples according to its own Standard Operating Procedure and quality system. Still some information was requested about the laboratory control samples used.

The *Salmonella* reference materials were produced by Biosisto (the Netherlands), an organisation accredited for the production of (certified) reference materials. Two batches of reference materials were custom-made for this PT. One batch of vials which contained *Salmonella* Typhimurium in a milk matrix, and one batch of vials which contained only milk (without *Salmonella*). The vials containing *Salmonella* Typhimurium were labelled A, C and D. The vials without *Salmonella* were labelled B. All custom-made reference materials arrived on 9 January 2024 at the EURL-*Salmonella* and were stored at -70 °C until shipment on 26 February 2024. Each NRL-*Salmonella* received four vials: A, B, C and D, which were shipped with dry ice.

For the PT, packages of 2 kg fresh mussels were obtained from a supermarket in the Netherlands. On 26 February 2024, the packages of mussels were bought and shipped the same day to the participants. The package of mussels was shipped in a different parcel than the *Salmonella* reference materials. During transport the mussels were kept cool by using frozen cooling elements and the temperature during transport was registered by a temperature button. The mussels were packed under modified atmosphere conditions. All packages of mussels had an identical packing date and expiration date, being respectively 21-02-2024 and 28-02-2024.

The NRLs-*Salmonella* could start the analysis of the samples immediately after arrival of the parcel, or the following day. The latest date to start the analysis was by Wednesday 28 February 2024.
If the samples were not analysed immediately by the NRL-*Salmonella*, the (closed) package of 2 kg mussels had to be stored at 5 °C (± 3 °C), together with the temperature probe, and the four vials of *Salmonella* reference materials had to be stored at -20 °C.

The EURL-*Salmonella* tested the mussels for the level of natural background flora: number of aerobic bacteria and number of *Enterobacteriaceae*. Table 2.1 shows the number of *Enterobacteriaceae* and aerobic bacteria per gram mussels of the package of 2 kg mussels on Wednesday 28 February 2024, which was the latest date to start the analysis. The package of mussels had the same batch number and expiration date as sent to the participants.

Table 2.1 Number of aerobic bacteria and Enterobacteriaceae *per gram mussel flesh and intravalvular fluid*

Date	**Aerobic bacteria (cfu/g)**	***Enterobacteriaceae* (cfu/g)**
28 February 2024[a]	2,3 x 10^3	<10

[a] After storage at 5 °C for 2 days after arrival at the EURL-*Salmonella*

Table 2.2 shows the concentration of *Salmonella* Typhimurium in the customised reference materials labelled A, C and D, tested on three different dates. The vials originated from the same batch.

Table 2.2 Concentration of Salmonella *Typhimurium per millilitre customised reference materials (labelled A, C and D) used by the participants to artificially contaminate the mussel samples*

Date	**Concentration of *Salmonella* Typhimurium in the reference materials**
05 January 2024[a]	62 cfu/ml
16 January 2024[b]	48 cfu/ml
28 February 2024[c]	65 cfu/ml

[a] Tested by (C)RM producer Biosisto.
[b] After storage at -70 °C for one week (after arrival at the EURL-*Salmonella*).
[c] After storage at -70 °C for 6,5 weeks and at -20 °C for two days (after arrival at the EURL-*Salmonella*).

The NRLs-*Salmonella* were requested to artificially contaminate each test portion of 25 gram mussel flesh and intravalvular fluid with 200 µl reference material (see 2.2 for more details), in order to inoculate the mussels with approximately 10 cfu/sample.

Table 2.3 shows the contamination levels of *Salmonella* Typhimurium in the mussel samples after inoculation with 200 µl customised reference material (labelled A, C and D), tested at the EURL-*Salmonella* on Wednesday 28 February 2024.

Table 2.3 Contamination levels of Salmonella *Typhimurium in the mussel samples after inoculation with 200 µl customised reference material (labelled A, C and D), tested at the EURL-*Salmonella *at the latest date to start the PT*

Date of testing	***S.* Typhimurium (cfu per mussel sample)**
28 February 2024[a] Inoculation of mussels with 200 µl reference material at EURL-*Salmonella*	13

[a]After storage at -70 °C for 6,5 weeks and at -20 °C for two days (after arrival at the EURL-*Salmonella*)

The tested reference materials were all from the same batch, but only labelled differently: A, C and D. All laboratories received reference materials from the same batch.

The reference material labelled with B was sterile milk without *Salmonella*. This material was also tested and the concentration of *Salmonella* was 0 cfu/ml.

2.2 Analysis of samples following EN ISO 6579-1: 2017(/A1:2020)

The prescribed method was EN ISO 6579-1:2017(/A1:2020) and the underlying EN ISO documents, e.g., the EN ISO 6887 series, for the preparation of the test samples. Especially the procedure of EN ISO 6887-3:2017 had to be respected.

The laboratories had to prepare the mussel samples themselves and had to spike them with the *Salmonella* reference materials provided by the EURL-*Salmonella*. The laboratories were provided with the following instructions for the preparation and spiking of the mussel samples:

For the correct use of the *Salmonella* reference materials:
- Defrost the vials at room temperature for 30 minutes at the start of the analysis.
- Store in the refrigerator at 0 – 4 °C, until use.
- Mix well before use

Preparation of mussel samples (A, B, C and D):
- Per sample, open and pool at least 10 mussels.
- Weigh 25 gram of pooled sample in the supplied sample bag. Open more mussels, when needed.
- Repeat the preparation for the other three samples.

Spike the mussel samples with corresponding vial:
- Mix the *Salmonella* reference material well before use.
- All samples should be spiked with **200 µl** of the corresponding vial.
  - Sample A should be spiked with vial A
  - Sample B should be spiked with vial B
  - Sample C should be spiked with vial C
  - Sample D should be spiked with vial D

After sample preparation, the prescribed method had to be followed.
In summary:
- pre-enrichment in:
  Buffered Peptone Water (BPW);
- selective enrichment in/on:
  Muller-Kauffmann tetrathionate-novobiocin (MKTTn) broth;
  Modified semi-solid Rappaport-Vassiliadis (MSRV) agar and/or;
  Rappaport-Vassiliadis 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.

NRLs-*Salmonella* had to report the final confirmed results of the samples by indicating if *Salmonella* was 'detected' or 'not detected' per 25 g mussel sample (after confirmation).

Additionally, the NRLs-*Salmonella* were allowed to analyse the samples with a second detection method, if this is (routinely) used in their laboratories. These results could also be reported, but only the results obtained with EN ISO 6579-1:2017(/A1:2020) were used to assess the performance of each NRL-*Salmonella*.

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 mussels, using EN ISO 6579-1:2017(/A1:2020) are shown in Table 2.4.

*Table 2.4 Criteria for good performance for the EURL-*Salmonella *PT LBM 2024 using EN ISO 6579-1:2017(/A1:2020)*

Artificially contaminated samples	Percentage positive	# pos samples/ total # samples
Negative samples	0%	0 / 1
Positive samples	> 65%	≥ 2 / 3

3. Results

3.1 General

On Monday 26 February 2024 two parcels with mussels and *Salmonella* reference materials were sent to each participants. Twenty NRLs-*Salmonella* received their parcels within one day. Two NRLs-*Salmonella* (laboratory codes 6 and 16) received their parcels after two days of transport.

Fourteen NRLs-*Salmonella* started the analysis of the samples immediately after arrival of the parcel on 27 February 2024. Eight laboratories started the PT on Wednesday 28 February 2024.

The temperature during transport and storage of the mussels was registered using a temperature probe. The temperature of all parcels during transport was below 4,5 °C. Six laboratories stored the mussels for one day after arrival of the parcel, until starting the PT on 28 February 2024. The measured storage temperature of the mussels in these laboratories was also below 4,5 °C.
All laboratories indicated that the reference materials were still frozen after receipt, except for laboratory 14, which indicated that the materials were not frozen anymore.

Twenty-one laboratories used the prescribed method EN ISO 6579-1:2017(/A1:2020). Laboratory 19 used only a validated alternative method: Rapid *Salmonella* method (AFNOR BRD 7/11-12/05), instead of EN ISO 6579-1:2017(/A1:2020).
Five laboratories used MKTTn and MSRV as selective enrichment media. Twelve laboratories used MKTTn and RVS as selective enrichment media. Four laboratories used three enrichment media: MKTTn, MSRV and RVS.

Five laboratories also reported results of a second detection method, in addition to the results of EN ISO 6579-1:2017(/A1:2020), for analysing the samples. The results of this second method were not used to assess the performance of these laboratories. Three laboratories used a real-time PCR as additional method, and two laboratories a PCR method. The results of the second detection methods were all similar to the reported results obtained with EN ISO 6579-1:2017(/A1:2020).

3.2 Mussel samples with reference materials

Negative samples

During this PT only one negative sample was included. The 21 laboratories analysing the samples with the prescribed method EN ISO 6579-1:2017(/A1:2020) scored the negative sample correctly: *Salmonella* was not detected. The one laboratory analysing the samples only with an alternative method (laboratory code 19) also scored this sample correctly negative for *Salmonella*.

Positive samples with *Salmonella* Typhimurium

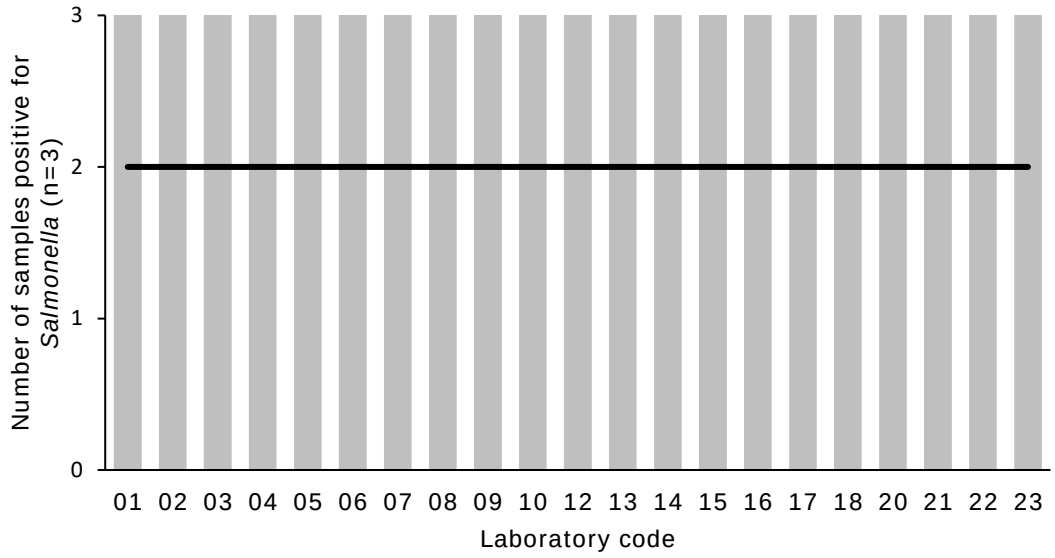
The 21 laboratories analysing the samples with the prescribed method EN ISO 6579-1:2017(/A1:2020) detected *Salmonella* correctly in all three mussel samples spiked with *Salmonella* reference materials. The one laboratory analysing the samples only with an alternative method (laboratory code 19) also correctly scored these three mussel samples positive for *Salmonella*.

In Table 3.1 the specificity, sensitivity and accuracy rates are given for the artificially contaminated mussel samples.

Table 3.1 Specificity, sensitivity, and accuracy rates for the artificially contaminated mussel samples analysed with EN ISO 6579-1:2017(/A1:2020)

Mussel samples		**Participants n = 21[a]**
Negative samples n=1	No. of samples	21
	No. of negative samples	21
	Specificity in %	**100%**
Positive samples with *Salmonella* Typhimurium n=3	No. of samples	63
	No. of positive samples	63
	Sensitivity in %	**100%**
All mussel samples	No. of samples	84
	No. of correct samples	84
	Accuracy in %	**100%**

[a] *Laboratory 19 was excluded. Laboratory 19 did not use EN ISO 6579-1:2017(/A1:2020), but only a validated alternative method.*



:level of good performance

Figure 3.1 Number of positive Salmonella *isolations per laboratory found in the mussel samples artificially contaminated with* Salmonella *Typhimurium reference material (n=3), using EN ISO 6579-1:2017(/A1:2020). Laboratory 19 did not use EN ISO 6579-1:2017(/A1:2020), but only a validated alternative method.*

3.3 Control samples

Sample bags for control samples were no longer provided by the EURL-*Salmonella*. Each NRL-*Salmonella* was expected to include (process) control samples according to its own Standard Operating Procedure and quality system. The laboratories reported the use of different *Salmonella* serovars (e.g. *S.* Enteritidis, *S.* Nottingham and *S.* Typhimurium) for their positive control with a concentration varying from 3 cfu/sample to 2500 cfu/sample. Eight laboratories also used a matrix with their positive control.

4. Performance of the participants

Twenty-one laboratories fulfilled the criteria of good performance for the EURL-*Salmonella* Proficiency Test for the detection of *Salmonella* in mussel samples. The performance of laboratory 19 was not assessed, because the laboratory did not used the prescribed method (EN ISO 6579-1:2017(/A1:2020)).

5. List of abbreviations

BPW	Buffered peptone water
cfu	colony-forming units
(C)RM	(Certified) Reference Material
EFTA	European Free Trade Associations
EU	European Union
EURL	European Union Reference Laboratory
ISO	International Organization for Standardization
MKTTn	Muller-Kauffmann tetrathionate-novobiocin broth
MSRV	Modified semi-solid Rappaport-Vassiliadis
NRL	National Reference Laboratory
PCR	Polymerase Chain Reaction
PT	Proficiency Test
RVS	Rappaport-Vassiliadis medium with Soya
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 6579-1:2017/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).

EN ISO 6887-1 & -3:2017. Microbiology of the food chain - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination - series for preparation of test samples. Part 1: General rules for the preparation of the initial suspension and decimal dilutions. Part 3: Specific rules for the preparation of fish and fishery products.

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