Interim summary report

EURL-*Salmonella* Proficiency Test Live Bivalve Molluscs 2020

Detection of *Salmonella* in mussels

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Introduction

In March 2020, an EURL-*Salmonella* Proficiency Test for detection of *Salmonella* in Live Bivalve Molluscs was organised for the National Reference Laboratories-*Salmonella* (NRLs-*Salmonella*). The matrix under analysis were mussels. NRLs-*Salmonella* which analyse Live Bivalve Molluscs (LBM), were invited to participate in this Proficiency Test (PT). Due to the COVID-19 outbreak not all laboratories were able to participate during this PT in March. Therefore another round for this PT was organised in August 2020 for nine laboratories.
In total 23 NRLs-*Salmonella* participated in this PT: 20 NRLs from 20 EU-Member States (MS) and 3 NRLs from third countries (EU candidate MS and members 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.

Materials & Methods

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 material. The mussels and the *Salmonella* reference materials were both provided by the EURL-*Salmonella*.

Each NRL-*Salmonella* had to analyse six 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*)
- two control samples (procedure control and own positive control)

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. A batch of vials which contained *Salmonella* Typhimurium in a milk matrix, and a batch of vials which contained only milk (without *Salmonella*). The batch containing

Salmonella Typhimurium was labelled with A, B and D. The batch without *Salmonella* was labelled with C. Every NRL-*Salmonella* received four vials: A, B, C and D.

The reference materials were sent on Monday 9 March 2020, the week before the start of the PT. Due to unexpected circumstances, the parcels were kept on hold at the depot centre of the courier service until Thursday 12 March. During this time, the materials were kept frozen with dry ice. The parcels were retrieved and stored at -70 °C, until Monday 16 March 2020 and shipped again, packed with fresh dry ice.

On Monday 16 March 2020, 21 packages of 2 kg fresh mussels were obtained from a supermarket in the Netherlands and shipped to the participants. Two participants already indicated beforehand that they were not able to participate, because of the COVID-19 pandemic.
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, respectively 12-03-2020 and 19-03-2020.

In the protocol it was indicated that if both parcels would arrive the next day (17 March 2020), the NRLs were requested to store the *Salmonella* reference materials at -20 °C and the package with mussels at 3 ± 2 °C until the start of the analysis on Wednesday 18 March 2020. If the parcels would arrive on Wednesday 18 March 2020, the laboratories were requested to start with the PT immediately.

Eventually, seven more laboratories indicated that they were not able to participate in the PT of March 2020, due to the COVID-19 pandemic. These laboratories were asked to store the four vials of *Salmonella* reference materials between -70 °C and -86 °C. The mussels could not be stored and the laboratories were advised to destroy the package. In August 2020 a second round was organised and a new batch of mussels was obtained from a supermarket in the Netherlands. The mussels were purchased and shipped (with cooling elements) to the nine laboratories on 24 August 2020. The mussels were packed under modified atmosphere conditions and each laboratory received a package of 2 kg fresh mussels (like for the first round of the PT in March). All packages of mussels had an identical packing date and expiration date, respectively 19-08-2020 and 26-08-2020.
Two laboratories, which did not yet received the *Salmonella* reference materials in March, also received a parcel with four vials of *Salmonella* reference materials, packed with dry ice.

Both batches of mussels were tested for the level of natural background flora: number of *Enterobacteriaceae* and number of aerobic bacteria. Table 1 shows the number of *Enterobacteriaceae* and aerobic bacteria per gram mussels of the batches used by EURL-*Salmonella* for this PT LBM 2020.

Table 1. Number of Enterobacteriaceae *and aerobic bacteria per gram mussels flesh and intravalvular fluid*

Date	***Enterobacteriaceae*** **(cfu/g)**	**Aerobic bacteria (cfu/g)**
18 March 2020	<10	$7,3 \times 10^3$
26 August 2020 (round 2)	$2,5 \times 10^2$	$9,2 \times 10^2$

Table 2 shows the concentration of *Salmonella* in the reference materials, labelled with A, B and D.
The reference materials tested on 16 and 18 March experienced similar conditions as the materials used by the laboratories.
The reference materials tested on 16 March 2020 were also packed with dry ice and handed over to the courier service on Monday 9 March 2020 and picked up again from the depot centre of the courier service on Thursday 12 March. Next the reference materials were stored at -70 °C. This parcel was originally intended for one of the NRLs-*Salmonella*, but due to the COVID-19 pandemic this NRL was no longer able to participate in the first round of the PT in March 2020.
The reference materials tested on 18 March 2020 were packed in a parcel with dry ice, which was stored under the following conditions at the EURL-*Salmonella*:

- at room temperature from 9 March until 12 March 2020;
- at -70 °C from 12 March until 16 March 2020;
- at room temperature from 16 March until 18 March 2020.

This was done to mimic the shipping of the parcel with reference materials to the NRLs.

The remaining reference materials were stored at -70 °C at the EURL-*Salmonella* and tested on 13 July 2020 for the second round of the PT.

Table 2. Concentration of Salmonella *Typhimurium in the reference materials (labelled with A, B and D) used by the participants to artificially contaminate the mussel samples*

Date of testing	**Concentration of *Salmonella* in the reference materials (cfu/ml)**
12 February 2020	$1,32 \times 10^2$
11 March 2020	$1,27 \times 10^2$
16 March 2020[a]	$1,29 \times 10^2$
18 March 2020[b]	$1,22 \times 10^2$
13 July 2020	$1,20 \times 10^2$

a. After the materials were sent with dry ice for three days, retrieved by EURL-*Salmonella* and stored at -70 °C.
b. After mimicking shipment of the reference materials by storage of the parcel with reference materials and dry ice successively at room temperature from 09-03-2020 until 12-03-2020, at -70 °C from 12-03-2020 until 16-03-2020 and at room temperature from 16-03-2020 until 18-03-2020.

The NRLs-*Salmonella* were requested to artificially contaminate the mussels with 100 µl per reference material, in order to inoculate the mussels with approximately 10 cfu/sample. Table 3 shows the number of cfu of *Salmonella* Typhimurium after artificially contamination of the mussel at the EURL-*Salmonella* at the start of the PT.

Table 3. Number of Salmonella *Typhimurium in the mussel samples after artificial contamination with 100 µl reference material at the EURL-*Salmonella *at the start of both rounds of the PT*

Date of testing	***S.*** **Typhimurium (cfu per mussel sample)**
18 March 2020[a] Inoculation of mussels with 100 µl reference material at EURL-*Salmonella*	13
26 August 2020[b] Inoculation of mussels with 100 µl reference material at EURL-*Salmonella*	12

a. After mimicking shipment of the reference materials by storage of the parcel with reference materials and dry ice successively at room temperature from 09-03-2020 until 12-03-2020, at -70 °C from 12-03-2020 until 16-03-2020 and at room temperature from 16-03-2020 until 18-03-2020.
b. EURL-*Salmonella* PT LBM 2020 – round 2.

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

The reference material labelled with C was sterile milk without *Salmonella*. This material was also tested and the concentration of *Salmonella* in these vials was always 0 cfu/ml.

Analysis of samples following EN ISO 6579-1

The prescribed method was 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.) and the underlying EN ISO documents, e.g. the EN ISO 6887 series for preparation of test samples.
EN ISO 6579-1:2017 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. For the selective enrichment of *Salmonella* from food, animal feed samples, and environmental samples from the food production area, EN ISO 6579-1 prescribes the use of two selective enrichment media. In addition to Muller-Kauffmann TetraThionate-novobiocin broth (MKTTn) either Rappaport Vassilliadis with Soya (RVS) broth or Modified Semi-solid Rappaport Vassilliadis agar (MSRV) agar shall be used. For the PT it was also allowed to use all three selective enrichment media.

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 correct use of the *Salmonella* reference materials:
- Defrost the vials at room temperature for 30 minutes on the day of the start of the Proficiency Test.
- 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 the content of at least 10 mussels.
- Weigh 25 g 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 the corresponding vial:
- Mix the *Salmonella* reference material well before use.
- All samples should be spiked with 100 µl of the corresponding vial.

- Sample A should be spiked with 100 µl of vial A.
- Sample B should be spiked with 100 µl of vial B.
- Sample C should be spiked with 100 µl of vial C.
- Sample D should be spiked with 100 µl of vial D.

Sample bags for sample A, B, C and D were also provided by the EURL-*Salmonella*. In addition two sample bags were provided for the procedure control (only Buffered Peptone Water) and for the positive control (BPW spiked with own *Salmonella* control strain), respectively coded with CTRL 1 and CTRL 2.

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.

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 were used to assess the performance of the NRL.

From the results of all laboratories the specificity, sensitivity and accuracy rates were calculated, as follows:

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

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

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

Performance analysis

Criteria for good performance used in the current EURL-*Salmonella* PT for detection of *Salmonella* in mussels are shown in Table 4.

Table 4. Criteria for good performance

Contaminated samples	**Percentage positive**	**# pos samples/ total # samples**
Negative samples	0%	0 / 1
Positive samples	>50%	2 / 3
Control samples	**Percentage positive**	**# pos samples/ total # samples**
BPW	0%	0 / 1
Own positive control	100%	1 / 1

Results

General

During the preparation of the PT, the COVID-19 outbreak started in Europe. Initially two laboratories indicated that they were not able to participate during this PT in March 2020 (lab codes 5 and 20). Therefore 21 parcels were shipped to the remaining participants. All parcels were delivered at the NRLs within 1-2 days.

After sending the samples, seven more NRLs (lab codes 1, 4, 9, 15, 16, 21 and 22) indicated not to be able to start the EURL-*Salmonella* PT LBM 2020, due to measurements the countries had taken against COVID-19. These laboratories were asked to store the *Salmonella* reference materials at -70 °C to -86 °C (instead of -20 °C). The mussels could be destroyed (not possible to store).

The other laboratories started the analysis for the PT on 18 March 2020, except laboratory 7. This laboratory started the PT on Thursday 19 March, after consulting the EURL-*Salmonella*, because of measurements taken against COVID-19 in their laboratory.

Nine laboratories (lab codes 1, 4, 5, 9, 15, 16, 20, 21 and 22) participated in the second round of this PT in August 2020. On 24 August 2020, packages of 2 kg fresh mussels (packed with frozen cooling elements) were sent to each participant. Additionally, *Salmonella* reference materials packed with dry ice were sent to laboratories 5 and 20. All parcels arrived within one day at the laboratories, except for laboratory 1. Laboratory 1 received the parcel with mussels after three days of transport and started the PT one day later than planned (on 27 August 2020). The other eight laboratories started the PT on 26 August 2020.

During transport of the mussels, the temperature was measured using an electronic temperature probe. The temperature during transport (and for some laboratories also one day of storage) remained for all parcels between -2,5 °C and 7,5 °C.

In total eighteen laboratories received their parcels one day before the start of the PT and had to store their mussels and reference materials. The temperature logger was stored together with the mussels. The temperature during storage of the mussels varied between 0,5 °C and 8 °C. Four laboratories received their parcels on the starting day of the PT and did not store their materials, but started with the analysis immediately. Laboratory 1 received the parcel with mussels after three days of transport and the temperature reached at maximum 13 °C. All laboratories indicated that the reference materials were still frozen after receipt, except laboratory 3, which indicated that the materials were not frozen anymore.

Twenty-two laboratories used the prescribed method EN ISO 6579-1:2017. One laboratory did not used EN ISO 6579, but an alternative method: the Rapid *Salmonella* method (laboratory 16).
Twenty-one laboratories used MKTTn and RVS and/or MSRV as selective enrichment media. One laboratory used RVS and MSRV as selective media (laboratory 7). This laboratory did not use MKTTn as selective enrichment medium, which is prescribed in addition to MSRV and/or RVS in EN ISO 6579-1:2017 for analysis of food and feed samples. Laboratory 16 used the Rapid *Salmonella* method.

Seven laboratories also used a second detection method for analysis of the samples (laboratories 2, 4, 9, 12, 14, 15 and 20). The methods used were qPCR, Rapid *Salmonella* method and Bax system (standard PCR system). The results of the second detection method were all similar to the reported results obtained with EN ISO 6579-1:2017.

Mussel samples spiked with the reference materials

Negative samples
During this PT only one negative sample was included. Twenty-two laboratories correctly did not detect *Salmonella* in this negative sample. Only laboratory 8 wrongly detected *Salmonella* in the negative sample.

Positive samples with *Salmonella* Typhimurium
Twenty-three laboratories detected *Salmonella* in all three positive samples.

In Table 4 the specificity, sensitivity and accuracy rates are given for the artificially contaminated mussel samples.

Table 4. Specificity, sensitivity and accuracy rates of the mussel samples

Samples	Percentage positive	Total laboratories n = 23
Negative samples n=1	No. of samples	23
	No. of negative samples	22
	Specificity in %	95,7%
Positive samples with *Salmonella* Typhimurium n=3	No. of samples	69
	No. of positive samples	69
	Sensitivity in %	100%
All mussel samples	No. of samples	92
	No. of correct samples	91
	Accuracy in %	98,9%

Control samples

Procedure control (only BPW)
Twenty-two laboratories analysed the procedure control sample (no matrix, only BPW) correctly, *Salmonella* was not detected. Only laboratory 12 reported detection of *Salmonella* in their procedure control.

Own positive control with *Salmonella*
The laboratories were also asked to use their own positive control normally used in their routine analysis for the detection of *Salmonella*.
Twenty-two laboratories detected *Salmonella* in their own *Salmonella* positive control sample. Only laboratory 12 did not detect *Salmonella* in their positive control sample.

The *Salmonella* serovars used by the majority of the participants for the positive control sample were: *S.* Typhimurium (10), *S.* Nottingham (4), *S.* Enteritidis (2), and seven participants used other *Salmonella* serovars.

Table 5 gives the correct scores for the control samples with an accuracy rate of 95,7%.

Performance of the participants

Twenty-one laboratories fulfilled the criteria of good performance.

Laboratory 8 wrongly detected *Salmonella* in a negative sample and therefore scored an unsatisfactory performance.

Laboratory 12 initially also scored an unsatisfactory performance, because they reported to have detected *Salmonella* in the procedure control while *Salmonella* was not detected in their own positive control sample.
Raw data showed that the analysis of the control samples were performed the other way around and therefore also reported the other way around. The results of their control samples were correct and for that reason laboratory 12 scored a moderate performance instead of an unsatisfactory performance.

The summary results can be found in Table 6.

Table 5. Correct scores of the control samples

Control samples	Percentage positive	Total laboratories n = 23
Procedure control (only BPW) n=1	No. of samples	23
	No. of negative samples	22
	Correct score in %	95,7%
Positive control with *Salmonella* n=1	No. of samples	23
	No. of positive samples	22
	Correct score in %	95,7%
All control samples n=2	No. of samples	46
	No. of correct samples	44
	Accuracy in %	95,7%

Follow-up

Laboratory 8 was asked for a possible technical explanation for their deviating result. The serotyping results were shared and showed that all positive samples were serotyped as *Salmonella* Typhimurium. Additionally the vials and mussels, which were stored frozen, were tested again by laboratory 8. *Salmonella* was not detected in vial C and in the mussels. Therefore, cross-contamination during the PT is the most likely explanation of the false positive result.

A follow-up was organised in August 2020, at the same time of the second round of the PT. The same batch of mussels used for round 2 of this PT was also sent to laboratory 8. The follow-up had almost a similar Proficiency Test design: four mussel samples had to be spiked with *Salmonella* reference material. The same reference materials were used, but renumbered. Two vials contained *Salmonella* Typhimurium and two vials contained sterile milk. The laboratory was asked to spike each mussel sample with 500 µl reference material (instead of 100 µl in the first PT), which was expected to result in 55 cfu *Salmonella* Typhimurium per mussel sample.

Laboratory 8 analysed all samples correctly and scored a good performance in the follow-up study. The results can be found in Table 7.

*Table 6. Summary results of all laboratories in EURL-*Salmonella *PT LBM 2020, including round 2*

Labcode	Number of positive samples / Total number of samples per level			
	Mussel samples spiked with reference material		Control samples	
	Positive	Negative	Procedure control	Positive control
1	3 / 3	0 / 1	0 / 1	1 / 1
2	3 / 3	0 / 1	0 / 1	1 / 1
3	3 / 3	0 / 1	0 / 1	1 / 1
4	3 / 3	0 / 1	0 / 1	1 / 1
5	3 / 3	0 / 1	0 / 1	1 / 1
6	3 / 3	0 / 1	0 / 1	1 / 1
7	3 / 3	0 / 1	0 / 1	1 / 1
8	3 / 3	1 / 1	0 / 1	1 / 1
9	3 / 3	0 / 1	0 / 1	1 / 1
10	3 / 3	0 / 1	0 / 1	1 / 1
11	3 / 3	0 / 1	0 / 1	1 / 1
12	3 / 3	0 / 1	1 / 1	0 / 1
13	3 / 3	0 / 1	0 / 1	1 / 1
14	3 / 3	0 / 1	0 / 1	1 / 1
15	3 / 3	0 / 1	0 / 1	1 / 1
16	3 / 3	0 / 1	0 / 1	1 / 1
17	3 / 3	0 / 1	0 / 1	1 / 1
18	3 / 3	0 / 1	0 / 1	1 / 1
19	3 / 3	0 / 1	0 / 1	1 / 1
20	3 / 3	0 / 1	0 / 1	1 / 1
21	3 / 3	0 / 1	0 / 1	1 / 1
22	3 / 3	0 / 1	0 / 1	1 / 1
23	3 / 3	0 / 1	0 / 1	1 / 1

In red: deviating results

*Table 7. Results of the follow-up study of the EURL-*Salmonella *PT LBM 2020*

Labcode	Number of positive samples / Total number of samples per level			
	Mussel samples spiked with reference material		Control samples	
	Positive	Negative	Procedure control	Positive control
8	2 / 2	0 / 2	0 / 1	1 / 1

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 Organization for Standardization
LBM	Live Bivalve Molluscs
MKTTn	Muller-Kauffmann TetraThionate-novobiocin broth
MS	Member State
MSRV	Modified Semi-solid Rappaport Vassilliadis agar
NRL	National Reference Laboratory
PT	Proficiency Test
RVS	Rappaport Vassilliadis with Soya

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 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.

EN ISO 22117:2019. Microbiology of the food chain — Specific requirements and guidance for proficiency testing by interlaboratory comparison.

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