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

EURL-*Salmonella* Proficiency Test Food 2021

Detection of *Salmonella* in liquid whole egg

RIVM report 2021-0128

R.E. Diddens | K.A. Mooijman

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Synopsis

EURL-*Salmonella* Proficiency Test Food
Detection of *Salmonella* in liquid egg

In 2021, the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*) organised a Proficiency Test for *Salmonella* bacteria in liquid egg. All participating National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*) fulfilled the criteria of good performance for the EURL-*Salmonella* Proficiency Test Food 2021.

From 1992, NRLs for *Salmonella* of the European Member States are obliged to participate in an annual quality control, the so-called Proficiency Tests. One of these Proficiency Tests checks whether NRLs can detect *Salmonella* bacteria in food; in this report – in liquid egg.

The laboratories used an obligatory, internationally accepted method to detect the presence of *Salmonella* in liquid egg samples. Each laboratory received a package containing samples which were artificially contaminated with two different concentrations of *Salmonella* Enteritidis or without *Salmonella*. The samples were artificially contaminated with *Salmonella* at the EURL-*Salmonella* laboratory.

A total of 33 NRLs-*Salmonella* participated in this Proficiency Test: 28 NRLs from 27 EU Member States and five NRLs from other European countries. The EURL-*Salmonella* is part of the Dutch National Institute for Public Health and the Environment (RIVM).

Keywords: *Salmonella*, EURL, NRL, Proficiency Test, *Salmonella* detection method, food, liquid egg

Publiekssamenvatting

EURL-*Salmonella* ringonderzoek Voedsel 2021

Detectie van *Salmonella* in vloeibaar ei

In 2021 organiseerde het Europese Unie Referentie Laboratorium voor *Salmonella* (EURL-*Salmonella*) een ringonderzoek voor de *Salmonella*-bacterie in vloeibaar ei. Alle deelnemende Nationale Referentie Laboratoria voor *Salmonella* (NRL's-*Salmonella*) hebben een goede score behaald voor het EURL-*Salmonella* ringonderzoek Voedsel 2021.

Sinds 1992 zijn de NRL's voor *Salmonella* van de Europese lidstaten verplicht om elk jaar hun kwaliteit te laten toetsen met zogeheten ringonderzoeken. Met een van de ringonderzoeken wordt gecontroleerd of de NRL's de *Salmonella*-bacterie in voedsel kunnen aantonen; dit keer in vloeibaar ei.

De laboratoria hebben een verplichte, internationale erkende analysemethode gebruikt om *Salmonella* in monsters vloeibaar ei aan te tonen. Elk laboratorium kreeg een pakket toegestuurd met monsters die besmet waren met twee verschillende concentraties *Salmonella* Enteritidis of zonder *Salmonella*. De monsters zijn op het laboratorium van het EURL-*Salmonella* kunstmatig besmet met *Salmonella*.

In totaal deden 33 NRL's-S*almonella* mee aan dit ringonderzoek: 28 NRL's van 27 lidstaten van de Europese Unie en 5 NRL's van andere Europese landen. Het EURL-*Salmonella* is gevestigd bij het Nederlandse Rijksinstituut voor Volksgezondheid en Milieu (RIVM).

Kernwoorden: *Salmonella,* EURL, NRL, ringonderzoek, *Salmonella*-detectiemethode, voedsel, vloeibaar ei

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Summary

In March 2021, an EURL-*Salmonella* Proficiency Test (PT) for the detection of *Salmonella* in food was organised for the NRLs-*Salmonella.* The matrix under analysis was liquid whole egg. In total, 33 NRLs-*Salmonella* participated in this PT: 28 NRLs from 27 EU Member States (MS) and 5 NRLs from third countries (EU candidate MS, members of the European Free Trade Association (EFTA), and the United Kingdom).

The most important objective was to test the performance of the participating laboratories' detection of *Salmonella* Enteritidis (SE) in the artificially contaminated liquid whole egg samples. The prescribed method for detecting *Salmonella* spp. was EN ISO 6579-1:2017(/A1:2020). The participants were asked to report *Salmonella* 'detected' or 'not detected' for each sample (after confirmation).

Prior to starting the Proficiency Test, pre-tests were conducted to ensure the samples were fit for use, especially regarding the stability of the artificially contaminated samples at different storage temperatures (5 °C and 10 °C). Additionally, the concentration of the natural background flora (aerobic count and *Enterobacteriaceae*) in the liquid whole egg was measured. The aim was to prepare stable liquid whole egg samples with a low level of *Salmonella* Enteritidis (SE) of approximately 5-10 cfu/test portion as well as samples with a high level of *Salmonella* Enteritidis of approximately 50-100 cfu/test portion.

The results of the pre-tests showed that the artificially contaminated liquid whole egg samples containing 7 cfu SE/25 g were stable at 5 °C for up to three weeks. The same liquid whole egg samples were (slightly) less stable at 10 °C; after three weeks of storage four of the six samples were still positive for *Salmonella*. The number of aerobic bacteria in the liquid whole egg samples remained relatively stable when stored at 5 °C for up to three weeks. Storage at 10 °C showed an increase in the number of aerobic bacteria after three weeks of storage. The number of *Enterobacteriaceae* in the liquid whole egg samples remained <10 cfu/g during storage at 5°C and 10°C for up to three weeks. Based on these results, the aim was to inoculate the low level liquid whole egg samples with approximately 10 cfu SE/25 g.

Each laboratory received 14 samples, each containing 25 g of liquid whole egg. These samples consisted of four samples with a high level of SE (inoculum 69 cfu/sample), six samples with a low level of SE (inoculum 10 cfu/sample) and four negative samples (no *Salmonella* added). The Proficiency Test samples were artificially contaminated with a diluted culture of *Salmonella* Enteritidis at the EURL-*Salmonella* laboratory. In addition, each participating laboratory had to test two control samples: a procedure control (Buffered Peptone Water only) and a positive control with *Salmonella*.

All thirty-three laboratories fulfilled the criteria for good performance in the EURL-*Salmonella* Proficiency Test for detection of *Salmonella* in liquid whole egg samples.

The accuracy rate of all control samples was 100%. The sensitivity rates of the liquid whole egg samples artificially contaminated with *Salmonella* Enteritidis was 99,7%. The accuracy rate of all liquid whole egg samples for all participating laboratories was 99,8%. The specificity rate of the negative liquid whole egg samples was 100%.

The NRLs-*Salmonella* were given the opportunity to analyse the samples using a second detection method if this method was (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.

Twelve laboratories used a second method for detecting *Salmonella* in the liquid whole egg samples. Most laboratories used a PCR or a real-time PCR as second detection method. The results of the second detection methods were all equal to those obtained with EN ISO 6579-1:2017(/A1:2020).

1 Introduction

An important task of the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*), as laid down in Commission Regulation EC No 2017/625 (EC, 2017), is the organisation of Proficiency Tests to evaluate the performance of the National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*). The history of the Proficiency Tests on the detection of *Salmonella*, as organised by EURL-*Salmonella* from 1995, is summarised on the EURL-*Salmonella* website (EURL-*Salmonella*, 2019).

The objective of the current study was to test whether the participating laboratories could detect *Salmonella* in liquid whole egg. This is important in order to verify that the examination of samples is carried out uniformly in all EU Member States (MS), and that comparable results are 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 Proficiency Test (PT) was organised in March 2021 and the NRLs-*Salmonella* which analyse *Salmonella* in food samples were invited to participate.

For the current PT, the liquid whole egg samples were artificially contaminated with a diluted culture of *Salmonella* Enteritidis (SE) at the EURL-*Salmonella* laboratory.

Fourteen liquid whole egg samples were tested by each NRL-*Salmonella*: four samples were contaminated with a high level of SE, six samples with a low level of SE, and four negative samples (liquid whole egg samples without *Salmonella*).
Additionally, two control samples (procedure control and own positive control with *Salmonella*) had to be tested by the laboratories.

2 Participants

Country	City	Institute / NRL-*Salmonella*
Austria	Graz	AGES - Institute for Medical Microbiology and Hygiene, NRC *Salmonella*
Belgium	Brussels	Sciensano, Food Pathogens
Bulgaria	Sofia	National Diagnostic Research Veterinary Institute (NDRVMI), BFSA
Croatia	Zagreb	Croatian Veterinary Institute Zagreb, Laboratory for Food Microbiology, Dept VPH
Cyprus	Nicosia	Cyprus Veterinary Services, Laboratory for the Control of Food of Animal Origin
Czech Republic	Prague	Státní veterinární ústav Praha, dep. of Bacteriology
Denmark	Ringsted	Danish Veterinary and Food Administration, Microbiology Laboratory
Estonia	Tartu	Estonian Veterinary and Food Laboratory, department of food microbiology
Finland	Helsinki	Finnish Food Authority, Microbiology Unit
France	Ploufragan	Anses, Anses Ploufragan - Unité HQPAP
Germany	Berlin	German Federal Institute for Risk Assessment (BFR), Biological Safety
Greece	Chalkida	Veterinary Laboratory of Chalkis, NRL Greece - *Salmonella*
Hungary	Budapest	National Food Chain Safety Office, Food Chain Safety Laboratory Directorate, Microbiological NRL
Iceland	Reykjavik	Matís ohf, Food Safety and Analytical services
Ireland	Celbridge, Co Kildare	Department of Agriculture Food and the Marine Laboratories, *Salmonella* section
Italy	Legnaro Padova	Istituto Zooprofilattico Sperimentale delle Venezie, General and Experimental Microbiology
Latvia	Riga	Institute of Food Safety, Animal Health and Environment BIOR, Microbiology and Pathology Laboratory
Lithuania	Vilnius	National food and veterinary risk assessment institute, Food microbiology unit
Luxembourg	Dudelange	Laboratoire national de santé, surveillance alimentaire
Malta	Valletta	Malta Public Health Laboratory, Microbiology Section - EVANS Buildings

Country	City	Institute / NRL-*Salmonella*
Netherlands	Bilthoven	National Institute for Public Health and the Environment (RIVM), Centre for Zoonoses and Environmental Microbiology (Z&O)
Netherlands	Wageningen	Wageningen Food Safety Research (WFSR), BU 3 Microbiologie 2
Norway	Oslo	Norwegian Veterinary Institute, Bacteriology - animals
Poland	Pulawy	National Veterinary Research Institute (NVRI), Department of Hygiene of Food of Animal Origin
Portugal	Vairão	Instituto National de Investigação Agrária e Veterinária Unidade de Tecnologia e Segurança Alimentar (INIAV), Food Microbiology Laboratory
Romania	Bucharest	Hygiene and Veterinary Public Health Institute, Microbiology
Serbia	Belgrade	Institute of Veterinary Medicine of Serbia, Department of food and feed safety
Slovak Republic	Bratislava	State Veterinary and Food Institute, NRL for *Salmonella*
Slovenia	Ljubljana	UL, Veterinary faculty / NVI, Institute of Microbiology and Parasitology
Spain	Madrid, Majadahonda	Centro Nacional de Alimentación, Microbiología Alimentaria
Sweden	Uppsala	National Vetarinary Institute, Department of Microbiology
Switzerland	Zürich	Institute for Food Safety and Hygiene, Vetsuisse Faculty, University of Zurich
United Kingdom	London	Public Health England, Food, Water and Environmental Microbiology - London

3 Materials and methods

3.1 Preparation of artificially contaminated liquid whole egg samples

3.1.1 General

The matrix used for this PT was liquid whole egg. Pasteurised liquid whole egg from the brand Eggstra (the Netherlands) was used for the pre-tests and the preparation of the PT samples.

In total, 16 packages of 1 kg pasteurised liquid whole egg were obtained on 12-02-2021. All packages had an identical expiration date: 02-05-2021. All packages were stored at 5 °C until sample preparation. After receipt at the EURL-*Salmonella*, the absence of *Salmonella* was checked by analysing a total of five 25 g test portions of the pasteurised liquid whole egg batch. To each test portion, 225 ml of Buffered Peptone Water (BPW) was added and mixed with a homogeniser. For preparation of the test portions, the procedures described in EN ISO 6887-1:2017 and EN ISO 6887-4:2017 were followed.

After pre-enrichment in BPW, selective enrichment was carried out in Muller-Kauffmann tetrathionate-novobiocin broth (MKTTn) and on Modified semi-solid Rappaport-Vassiliadis agar (MSRV) agar. The MKTTn tubes and the suspect growth on the MSRV plates were then plated out on Xylose Lysine Deoxycholate (XLD) agar and Brilliance *Salmonella* Agar (BSA). Suspect colonies were confirmed biochemically and serologically.

Salmonella Enteritidis (SE) isolated from a chicken product was used to artificially contaminate the 25 g liquid whole egg samples.

3.1.2 Pre-tests for the preparation of liquid whole egg samples

Two packages of 1 kg pasteurised liquid whole egg were used for the pre-test. The packages with different expiration dates (14-12-2020 and 20-12-2020) were mixed together and 25 g pooled samples were weighed.

Salmonella Enteritidis was inoculated in Brain Heart Infusion broth (BHI) and incubated at 34 °C to 38 °C for 18 h ± 2 h. Next, tenfold dilutions were prepared in peptone saline solution in order to inoculate the liquid whole egg samples with approximately 5 cfu/25 g and 10 cfu/25 g. The level of the inoculum was determined by streaking 0,1 ml of the diluted culture onto XLD agar and incubating the plates at 34 °C to 38 °C for 24 h ± 3 h.

To test the stability of *Salmonella* in the liquid whole egg samples during storage and transport, the inoculated samples were stored at 5 °C and at 10 °C. After storage at 0, 7, 14 and 21 days, six artificially contaminated samples were tested for the presence of *Salmonella* following EN ISO 6579-1:2017(/A1:2020) (see 3.3). This was done for each inoculation level and storage temperature.

In addition, negative liquid whole egg samples (no *Salmonella* added) were also stored at 5 °C and 10 °C. The level of the natural background

flora was determined in these samples on the same sampling days (t = 0, 7, 14 and 21 days), by analysing the number of aerobic bacteria and *Enterobacteriaceae* (see 3.1.4).

3.1.3 Preparation of liquid whole egg samples for the Proficiency Test

By the end of February 2021, the PT samples were prepared for 33 participating laboratories. Per laboratory, 14 liquid whole egg samples were prepared. For this, 462 subsamples of each 25 g liquid whole egg were weighed into (plastic) sample bags. In total, 132 subsamples were individually, artificially contaminated with a high level, and 198 subsamples with a low level of the diluted overnight culture of *S*. Enteritidis; 132 subsamples were not contaminated with *Salmonella* (negative samples).

The following set of samples were prepared for each participant:

- four samples, each containing 25 g of liquid whole egg with a high level of *Salmonella* Enteritidis, aimed at 100 cfu/25 g;
- six samples, each containing 25 g of liquid whole egg with a low level of *Salmonella* Enteritidis, aimed at 10 cfu/25 g;
- four negative samples, each containing 25 g of liquid whole egg (no *Salmonella* added);
- two control samples consisting of empty filter sample bags for the procedure control (BPW only) and own positive control.

After artificial contamination, the samples were mixed by hand and stored at 5 °C until transport to the NRLs-*Salmonella* on 1 March 2021.

The level of the inoculum used to artificially contaminate the 25 g liquid whole egg samples was determined by streaking 0,1 ml of the diluted culture onto XLD agar and incubating the plates at 34 °C to 38 °C for 24 h ± 3 h.

3.1.4 Determination of level of background flora in liquid whole egg

The total number of aerobic bacteria and the number of *Enterobacteriaceae* in the liquid whole egg were investigated by following EN ISO 4833-1:2013 and EN ISO 21528-2:2017 respectively. For this purpose, an initial suspension was prepared by adding 225 ml of peptone saline solution to 25 g of liquid whole egg. Next, tenfold dilutions of the initial suspension were analysed on Plate Count Agar (PCA) and on Violet Red Bile Glucose (VRBG) Agar.

3.1.5 Determination of the number of Salmonella *in liquid whole egg samples by MPN*

The number of *Salmonella* was determined in the liquid whole egg samples at the start of the PT. This was done by using a five-tube, most probable number (MPN) technique. For this purpose, tenfold dilution of five artificially contaminated liquid whole egg samples of 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 by following EN ISO 6579-1:2017(/A1:2020). From the number of confirmed positive dilutions, the MPN of *Salmonella* in the original sample was calculated 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 (artificially contaminated) liquid whole egg samples, numbered A1 to A14. Additionally, two control samples (CTRL1 and CTRL2) had to be tested by the laboratories: a procedure control (CTRL1) consisting of only BPW and a positive control sample (CTRL2). The laboratories had to use their own positive control that they normally use when analysing routine samples for the detection of *Salmonella*.

Table 3.1 gives an overview of the number and type of samples tested by the participants.

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

Contamination level	**Liquid whole egg samples (n=14)**
S. Enteritidis high level (SE high)	4
S. Enteritidis low level (SE low)	6
Negative sample (no *Salmonella* added)	4
	Control samples (n=2)
Procedure control (BPW only)	1
Positive control with *Salmonella*	1

3.2.2 Shipment of parcels and temperature recording during shipment

The 14 PT samples were packed in a large safety bag and placed in a parcel with four frozen cooling elements. The parcel also included two empty sample bags (CTRL1 and CTRL2).

To monitor exposure to excessive temperatures during shipment and storage, temperature buttons were included in the large safety bag to record the temperature. These buttons are tiny units sealed in a stainless-steel case, 16 mm in diameter and 6 mm deep. One button was packed together with the PT samples in the large safety bag. The loggers were programmed by the EURL-*Salmonella* to measure the temperature every hour. Each NRL-*Salmonella* had to return the temperature recorder to the EURL-*Salmonella* on the day the laboratory started the PT. At the EURL-*Salmonella*, the loggers were read using a computer program, and all recorded temperatures from transport and storage were transferred to an Excel sheet.

The parcel was sent to the participants on 1 March 2021 as 'biological substances category B (UN3373)' (IATA, 2021) using a door-to-door courier service.

Further details about the shipping and handling of the samples and the reporting of the test results can be found in the protocol (EURL-*Salmonella*, 2021a) and in (a printout of) the result form (EURL-*Salmonella,* 2021b).

3.3 Methods

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. In 2020, Amendment 1 of EN ISO 6579-1:2017 was published (EN ISO 6579-1:2017/A1:2020), allowing incubation of selective media at a broader temperature range (34 °C to 38 °C instead of 37 °C ± 1 °C). The participants were free to choose the broader temperature range or to use 37 °C ± 1 °C.

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. For the analysis of food samples, EN ISO 6579-1(/A1:2020) prescribes the use of two selective enrichment media. In addition to Muller-Kauffmann tetrathionate-novobiocin broth (MKTTn) either Rappaport-Vassiliadis with Soya (RVS) broth or Modified semi-solid Rappaport-Vassiliadis agar (MSRV) agar shall be used. For the PT NRLs were also allowed to use all three selective enrichment media.

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 liquid whole egg.

Additionally, the NRLs-*Salmonella* were given the opportunity to analyse the samples using a second detection method if this method was (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.

3.4 Statistical analysis of the data

The specificity, sensitivity and accuracy rates were calculated for the liquid whole egg samples artificially contaminated with SE. For the control samples, only the accuracy rates were calculated. The rates were calculated using the following formulae:

Specificity rate

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

Sensitivity rate

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

Accuracy rate

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

3.5 Criteria for good performance

The criteria in Table 3.2 were used for the determination of 'good performance' for the EURL-*Salmonella* Proficiency Test Food 2021.

Table 3.2 Criteria for good performance in the Proficiency Test Food 2021

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

4 Results and discussion

4.1 Preparation of artificially contaminated liquid whole egg samples

4.1.1 Pre-tests for the preparation of liquid whole egg samples

Before performing the pre-test, five pooled samples of the two 1 kg liquid whole egg packages were tested for the absence of *Salmonella*. No *Salmonella* was detected in any of the five test portions (25 g each).

The subsamples of each 25 g liquid whole egg were artificially contaminated with two different concentrations of *Salmonella* Enteritidis. The actual inoculation levels were 4 cfu/25 g of liquid whole egg and 7 cfu/25 g of liquid whole egg.

Experiments were performed to test the stability of the liquid whole egg samples artificially contaminated with *Salmonella* Enteritidis during storage and transport. The samples were stored at 5 °C and at 10 °C to mimic storage conditions and the effect of temperature abuse during transport. The pre-test samples were stored for up to three weeks and analysed for the survival of *Salmonella* following EN ISO 6579-1:2017(/A1:2020). The results are presented in Figures 4.1 and 4.2.

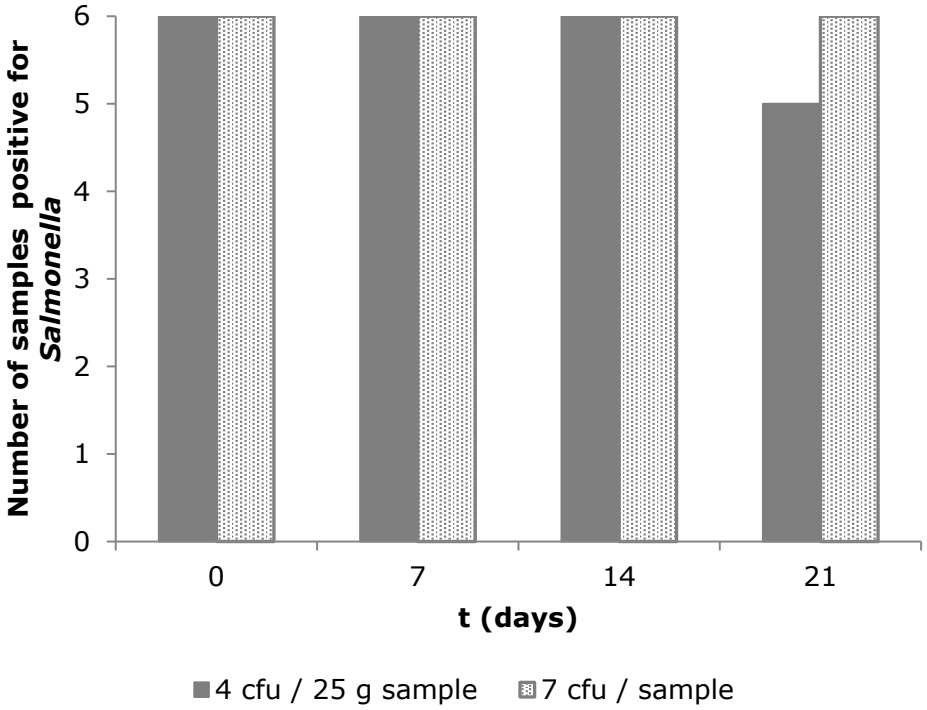

Figure 4.1 Stability test of liquid whole egg samples (n=6) artificially contaminated with S. *Enteritidis at levels of 4 cfu/25 g and 7 cfu/25 g, stored at 5 °C*

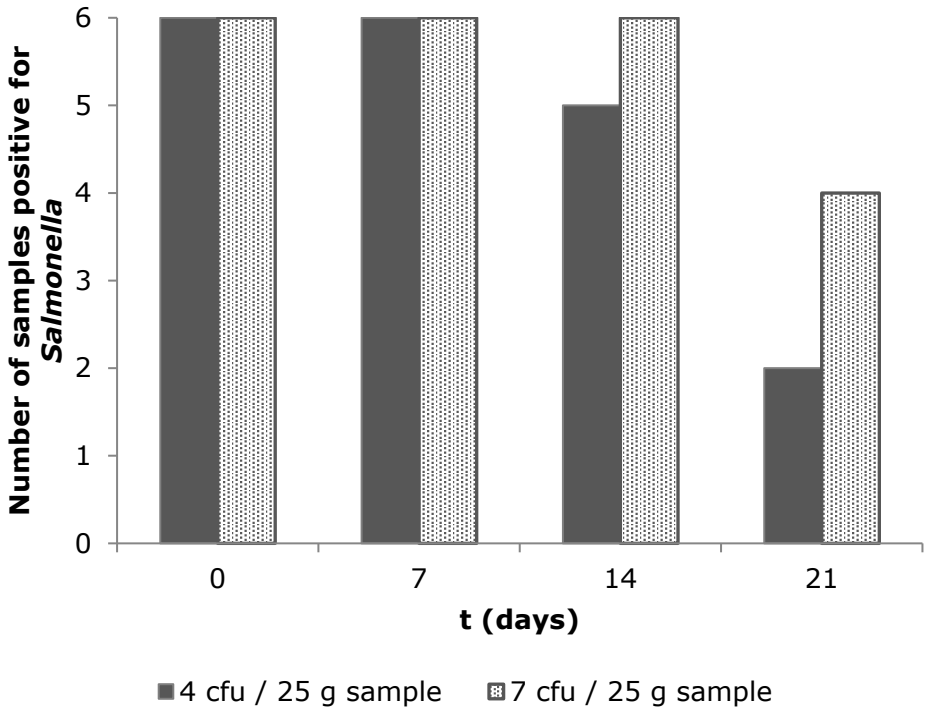

Figure 4.2 Stability test of liquid whole egg samples (n=6) artificially contaminated with S. *Enteritidis at levels of 4 cfu/25 g and 7 cfu/25 g, stored at 10 °C*

Figure 4.1 shows that the liquid whole egg samples artificially contaminated with the two low concentrations of *Salmonella* Enteritidis were stable during three weeks of storage at 5 °C.
Figure 4.2 shows that the same liquid whole egg samples were less stable at 10 °C, as after 21 days of storage only two and four samples were still positive for *Salmonella*, for the levels of 4 cfu/25 g and 7 cfu/25 g respectively. Based on these results, the aim was to inoculate the low level liquid whole egg samples with approximately 10 cfu SE/25 g.
Figure 4.3 shows the number of aerobic bacteria in the liquid whole egg samples during storage at 5 °C and at 10 °C. At 5 °C the level remained relatively stable for up to three weeks. At 10 °C the number of aerobic bacteria increased during storage.

The number of *Enterobacteriaceae* remained <10 cfu/g in the liquid whole egg samples during storage at 5 °C and at 10 °C.

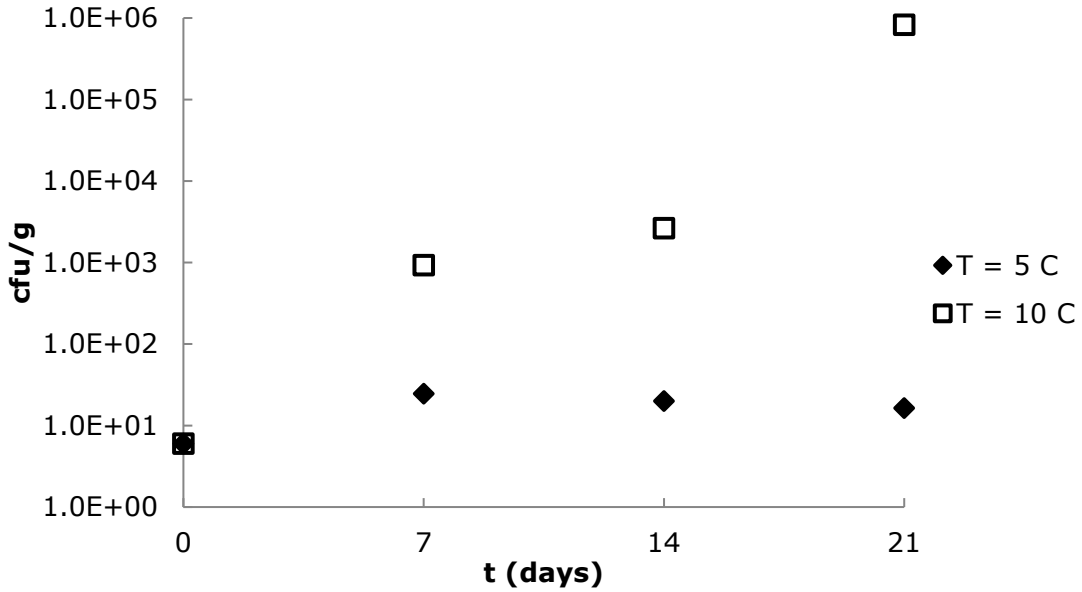

Figure 4.3 Number of aerobic bacteria per gram of liquid whole egg (negative for Salmonella*) after storage at 5 °C and 10 °C*

4.1.2 Preparation of liquid whole egg samples for Proficiency Test

From five different 1 kg pasteurised liquid whole egg packages, a 25 g test portion was taken and tested for the absence of *Salmonella* following EN ISO 6579-1:2017(/A1:2020). *Salmonella* was not detected in any of the five samples.

The samples were then prepared as described in 3.1.3. All samples were stored at 5 °C until shipment.

4.1.3 Background flora in liquid whole egg

The level of natural background flora in the liquid whole egg was tested on 16 February 2021 (shortly after receipt of the liquid whole egg) and on 9 March 2021 (during the PT). Table 4.1 shows the number of aerobic bacteria and *Enterobacteriaceae* in the liquid whole egg.

Table 4.1 Number of aerobic bacteria and Enterobacteriaceae *per gram liquid whole egg*

Date	Aerobic bacteria (cfu/g)	*Enterobacteriaceae* (cfu/g)
16 February 2021	$9{,}6 \times 10^2$	<1
9 March 2021[a]	$3{,}2 \times 10^3$	<1

a After storage at 5 °C for 3 weeks

4.1.4 Number of Salmonella *in liquid whole egg samples*

Table 4.2 shows the inoculation levels of the diluted culture of *Salmonella* Enteritidis used to artificially contaminate the liquid whole egg samples. Additionally, this table shows the results of the five tube MPN test performed on the artificially contaminated PT samples with low and high level SE at the start of the PT.

Table 4.2 Number of Salmonella *Enteritidis in the inoculum for artificial contamination of the liquid whole egg samples and after storage at 5 °C for 1,5 weeks*

Date	Low level SE in cfu per sample	High level SE in cfu per sample
25 February 2021 Inoculation of liquid whole egg	10	69
8 March 2021[a] MPN of liquid whole egg samples, inoculated with SE (95% confidence limit)	3,3 (1,1-10,3)	160 (52,5-500)

a After storage at 5 °C for 1,5 week

The results show that the intended low and high contamination levels of *Salmonella* Enteritidis in the liquid whole egg samples were reached.

4.2 Technical data for the Proficiency Test

4.2.1 General

In total, 33 NRLs-*Salmonella* participated in this PT: 28 NRLs from 27 EU Member States and five NRLs from third countries (EU candidate MS, members of the European Free Trade Association (EFTA), and the United Kingdom).
Thirty-one laboratories performed the Proficiency Test on 8 March 2021. One participant started the PT on 9 March 2021 and one laboratory started on 11 March 2021 due to a delay in parcel transportation.

4.2.2 Accreditation

All laboratories are accredited for EN ISO 6579-1:2017. Two laboratories also indicated they were accredited for the amendment of EN ISO 6579-1:2017(/A1:2020).
Nine laboratories also have one or more other *Salmonella* methods under accreditation: AFNOR BRD 07/06 - 07/04 (iQ-Check *Salmonella* II); BAX system; NF U 47-100, NF U 47-101, NF U 47-102; NMKL 71, 1999; NMKL 187, 2016; SureTect real-time PCR; Vidas.

4.2.3 Transport of samples

On 1 March 2021, the liquid whole egg samples were sent to 33 laboratories. Thirty-two of the parcels were delivered at the NRLs within one or two days. The parcel of laboratory 23 was held at customs and arrived after nine days of transport, therefore this laboratory started the PT on 11 March 2021.

The temperature during transport and storage of the parcels with PT samples was registered using a temperature probe. The temperature of 32 parcels was 2 °C or lower during transport. The parcel with samples intended for laboratory 23 was delivered after nine days. In the parcel a maximum temperature of 11,5 °C was measured. Figure 4.4 shows the temperature record of the parcel for laboratory 23, until receipt and start of the PT on 11 March 2021.

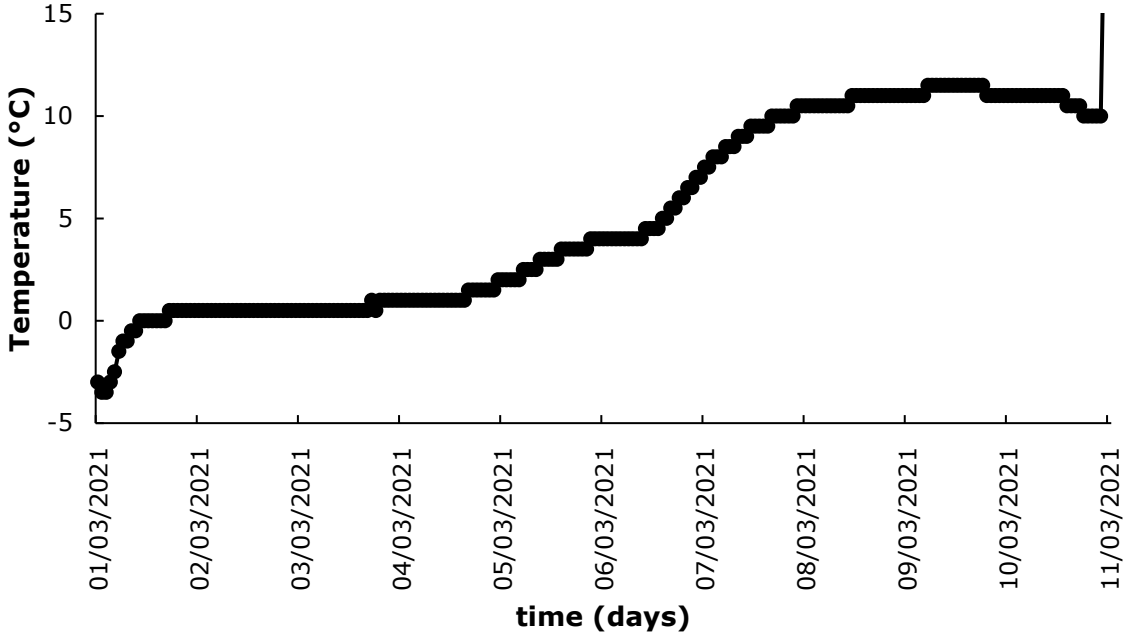

Figure 4.4 Temperature record of the parcel with PT samples for laboratory 23

4.2.4 Media

For this PT, the prescribed method for the detection of *Salmonella* in liquid whole egg was EN ISO 6579-1:2017(/A1:2020), which prescribes the use of MKTTn and RVS and/or MSRV as selective enrichment media. Fourteen laboratories used MKTTn and MSRV as selective enrichment media (laboratories 2, 3, 4, 7, 11, 15, 19, 23, 25, 26, 27, 29, 32 and 33). Twelve laboratories used MKTTn and RVS as selective enrichment media (laboratories 1, 5, 6, 8, 9, 10, 14, 16, 17, 18, 24 and 31). Six laboratories used all three prescribed selective enrichment media: MKTTn, MSRV and RVS (laboratories 12, 13, 20, 21, 22 and 28). Laboratory 30 used RVS and MSRV (and not the prescribed MKTTn) as selective enrichment media.
Table 4.3 shows the reported values of the incubation times, the concentrations of novobiocin, pH, and the incubation temperatures of the different media. Only those laboratories are shown which reported deviating values from EN ISO 6579-1:2017(/A1:2020).

Table 4.3 Reported technical deviations from prescribed method EN ISO 6579-1:2017(/A1:2020)

	BPW	MKTTn			RVS		MSRV		
Laboratory code	**Incubation time (h)**	**Concentration novobiocin (mg/L)**	**pH**	**Temperature (°C)**	**pH**	**Temperature (°C)**	**Concentration novobiocin (mg/L)**	**pH**	**Temperature (°C)**
EN ISO 6579-1 (/A1:2020)	18 h ± 2 h	40 mg/L	7 – 8,2	34 °C - 38 °C	5,2 ± 0,2	41,5 °C ± 1 °C	10 mg/L	5,1 – 5,4	41,5 °C ± 1 °C
9	24	40	-	37	-	41,5			
13	19	10	8,02	37	5,42	41,5	10	5,42	41,5
14	18	40	6,6	36	5,2	41,5			
18	18	40	6,80	37,0	5,40	41,5			
29	18	40	8,08	37			-	5,38	37
30	18				5,2	42	10	5,3	42
31	19	0	7,80	41,2	5,30	41,2			

Grey cells are deviations from EN ISO 6579-1:2017(/A1:2020)

- : no information reported

One laboratory (laboratory 9) used a longer incubation time than prescribed for the pre-enrichment in BPW.
Two laboratories (laboratories 13 and 31) reported a lower concentration of novobiocin in MKTTn than prescribed. Laboratory 31 also incubated MKTTn at a higher temperature than prescribed. According to EN ISO 6579-1:2017(/A1:2020), the pH of the base medium of MKTTn broth should be 7,8 – 8,2. In addition, it indicates that the complete medium should no longer be used if, after storage, the pH is <7. Two laboratories (laboratories 14 and 18) reported a pH lower than 7. Laboratory 9 did not measure the pH of MKTTn nor did they measure the pH of RVS.
The prescribed incubation temperature for MSRV is 41,5 °C ± 1 °C. Laboratory 29 reported an incubation temperature of 37 °C. This laboratory neither reported the pH of MSRV.

The selective enriched culture had to be plated-out on two isolation media: XLD and an obligatory second isolation medium. The choice of the second isolation medium for the different laboratories can be found in Table 4.4. Most laboratories used Rambach as a second isolation medium.

Table 4.4 Second isolation media used by the laboratories

Media	**No. of users**
BGA	5
BGA(mod)	5
BPLS	4
BSA	1
Chromogenic *Salmonella* Agar	3
Compass *Salmonella*	2
Rambach	8
Rapid *Salmonella* Agar	5
SM(ID)2	2

Explanations of the abbreviations used are given in the 'List of abbreviations'.

Three laboratories (laboratories 8, 19 and 25) did not use XLD as prescribed by EN ISO 6579-1:2017(/A1:2020).

The last step in the procedure for *Salmonella* detection is the confirmation step. All participating laboratories performed one or several confirmation tests for *Salmonella*. An overview can be found in Table 4.5.

Twenty-seven laboratories performed a biochemical test and performed one or more additional confirmation test(s). Eleven laboratories (also) used another confirmation test, such as MALDI-TOF.

Table 4.5 Number of laboratories using different confirmation methods

Number of labs	Bio-chemical	Serological	Serotyping	PCR	Other
9	x	x			
1	x	x	x		
3	x	x		x	
2	x	x			x
4	x		x		
1	x		x	x	
2	x		x		x
1	x			x	x
4	x				x
1		x	x		
1			x		
1			x		x
2				x	
1					x

4.3 Control samples

4.3.1 General

Two empty safety bags were sent to each participating NRL-*Salmonella* to be used for the control samples, being:

- a procedure control consisting only of BPW (CTRL1);
- a positive control with the laboratories' own *Salmonella* control strain (CTRL2).

Procedure control (BPW only)
All laboratories reported the procedure control sample (no matrix, BPW only) correctly negative for *Salmonella*.

Positive control with Salmonella
The laboratories were asked to use their own, normally used positive control in their routine analysis for the detection of *Salmonella*. All laboratories reported the detection of *Salmonella* in their positive control sample.

The *Salmonella* serovars used by the participants for the positive control sample were: *S.* Enteritidis (nine participants), *S.* Typhimurium (eight participants), *S.* Nottingham (five participants), *S*. Abaetetuba (three participants) and eight participants used another *Salmonella* serovar. More details are given in Table 4.6.

Table 4.6 Salmonella *serovars used by participants for the positive control samples*

***Salmonella* serovar**	**Number of participants**
S. Enteritidis	9
S. Typhimurium	8
S. Nottingham	5
S. Abaetetuba	3
S. Agona	1
S. Alachua	1
S. Blegdam	1
S. Derby	1
S. Harleystreet	1
S. Poona	1
S. Tranoroa (*Salmonella enterica* subsp. *salamae*)	1
Salmonella bongori serovar 66 : z41: -	1

The concentration of *Salmonella* in the positive control samples used by the different participants varied between 1 and 10^5 cfu/sample (see Table 4.7). Two laboratories did not determine the concentration of *Salmonella* added to their positive control sample.

Table 4.7 Concentration of Salmonella *in the positive control samples*

Concentration *Salmonella* (cfu/sample)	**Number of laboratories**
1 - 10	10
11 - 130	15
131 - 500	2
10^3 – 10^5	4
Not determined	2

A positive control sample for a detection method should demonstrate that media are capable of supporting the growth of the target organisms in low numbers. To obtain information on the sensitivity of a method, the concentration of a positive control sample should preferably be just above the detection limit of the method. Additionally, for a positive control, it may be advisable to use a rarely isolated serovar from the routine samples analysed in the laboratory. In this way, possible cross-contamination can be detected more easily.
Additionally, a more realistic control of the procedure is obtained when the positive control is added to a *Salmonella*–free matrix similar to the tested samples.
Seven laboratories (laboratories 11, 15, 20, 23, 24, 29 and 32) also used a matrix with their positive control. The matrices used were: minced meat, fishmeal, 'foodstuff', egg, chicken, milk, and 'mixed food'.

4.3.2 Correct scores of the control samples

Table 4.8 shows the number of correct scores found with the control samples. The calculations were performed for the results of all participants and for the EU MS only.

Table 4.8 Correct scores found with the control samples by all participants ('All') and by the laboratories of the EU Member States only ('EU MS')

Control samples		All n = 33	EU MS n = 28
Procedure control (BPW only) n=1	No. of samples	33	28
	No. of negative samples	33	28
	Correct score in %	100%	100%
Positive control with *Salmonella* n=1	No. of samples	33	28
	No. of positive samples	33	28
	Correct score in %	100%	100%
All control samples n = 2	No. of samples	66	56
	No. of correct samples	66	56
	Accuracy in %	100%	100%

4.4 Artificially contaminated liquid whole egg samples

4.4.1 General

Table 4.9 shows the results of the tested liquid whole egg PT samples. It shows that the temperature abuse of the parcel for laboratory 23, as well as the technical deviations (see section 4.2.4), did not influence the final results.

Table 4.9 Number of (artificially contaminated) liquid whole egg samples tested positive for Salmonella *at each laboratory*

Laboratory code	Number of samples tested positive for *Salmonella* per laboratory		
	High level SE n = 4	Low level SE n = 6	Negative n = 4
Criteria of good performance	**≥3**	**≥3**	**0**
24	4	5	0
All other NRLs-*Salmonella*	4	6	0

High-level contaminated Salmonella *liquid whole egg samples*

All laboratories detected *Salmonella* in all four liquid whole egg samples contaminated with a high level of *Salmonella* Enteritidis. The results are shown in figure 4.5.

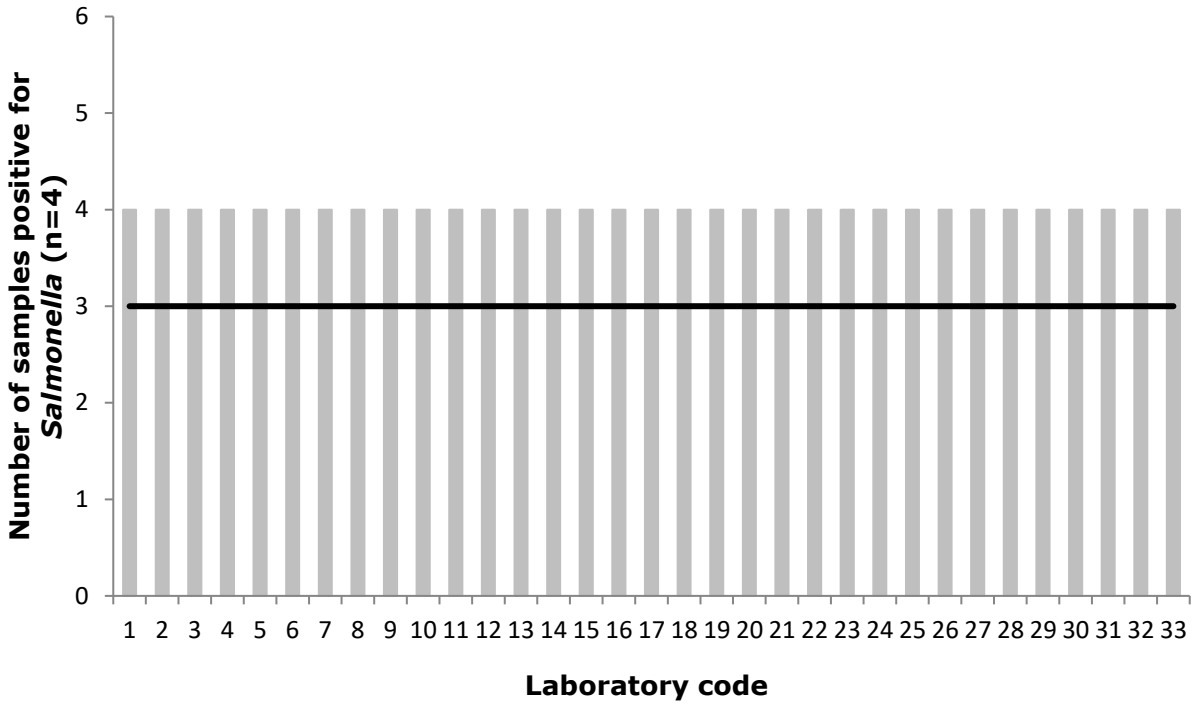

Figure 4.5 Number of liquid whole egg samples artificially contaminated with a high level of Salmonella *Enteritidis (n=4) that tested positive per laboratory*

Low-level contaminated Salmonella *liquid whole egg samples*
Thirty-two laboratories detected *Salmonella* in all six liquid whole egg samples contaminated with a low level of *Salmonella* Enteritidis. One laboratory (laboratory 24) detected *Salmonella* in five of six PT samples contaminated with a low level of *Salmonella* Enteritidis, well above the level of good performance. The level of good performance for the low-level samples for this PT was set at the detection of *Salmonella* in at least three of the six samples.

Figure 4.6 shows the number of samples in which *Salmonella* was detected per laboratory.

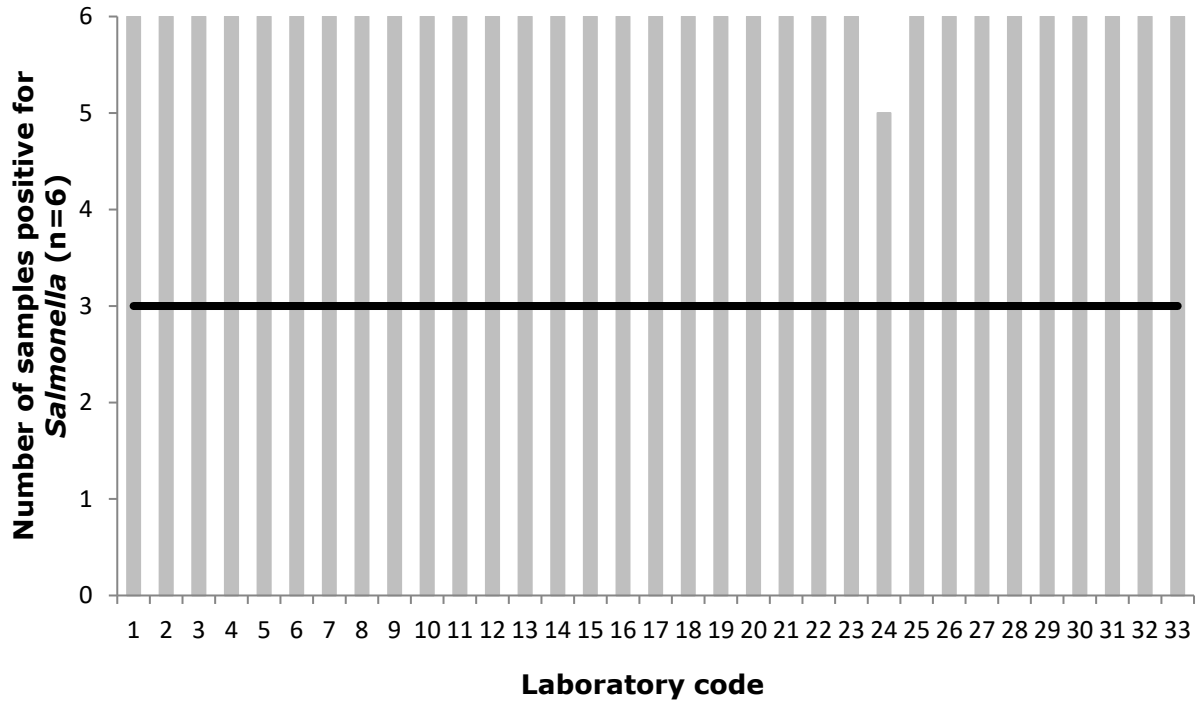

Figure 4.6 Number of liquid whole egg samples artificially contaminated with a low level of Salmonella *Enteritidis (n=6) that tested positive per laboratory*

Negative samples
All laboratories scored the negative liquid whole egg samples correctly negative for *Salmonella*.

4.4.2 *Specificity, sensitivity and accuracy rates of the (artificially contaminated) liquid whole egg samples*

Table 4.10 shows the specificity, sensitivity, and accuracy rates of the liquid whole egg samples tested in this PT. The calculations were performed on the results of all participants and on those of the EU MS participants only.

Table 4.10 Specificity, sensitivity, and accuracy rates calculated from the results found by all participants ('All') and by the laboratories of the EU Member States ('EU MS') only, with the artificially contaminated liquid whole egg samples

Liquid whole egg samples		All n = 33	EU MS n = 28
High level SE (n = 4)	No. of samples	132	112
	No. of positive samples	132	112
	Sensitivity in %	**100**	**100**
Low level SE (n = 6)	No. of samples	198	168
	No. of positive samples	197	168
	Sensitivity in %	**99,5**	**100**
Negative (n = 4)	No. of samples	132	112
	No. of negative samples	132	112
	Specificity in %	**100**	**100**
All whole egg artificially contaminated with *Salmonella*	No. of samples	330	280
	No. of positive samples	329	280
	Sensitivity in %	**99,7**	**100**
All liquid whole egg samples	No. of samples	462	392
	No. of correct samples	461	392
	Accuracy in %	**99,8**	**100**

4.5 Second detection method

Twelve laboratories also used a second method for the detection of *Salmonella* in the PT samples. An overview of the methods used per laboratory is given in Table 4.11. Most laboratories used a PCR or a real-time PCR as second detection method. Eight laboratories use this second detection method routinely for sample analysis. The results of the second detection methods were equal to those obtained with EN ISO 6579-1:2017(/A1:2020).

Table 4.11 Details of the second detection methods used by twelve laboratories during the Proficiency Test on detection of Salmonella *in liquid whole egg samples*

Laboratory code	Second detection method	Validated	Validated by	Routinely used number of tests/year
3	PCR	Yes	In-house validation	Yes, but not for official control samples
4	qPCR	Yes	AFNOR	8000
5	BAX System, standard PCR assay for *Salmonella* (a commercial end time PCR-system)	Yes	Nordval	3244
6	SureTect Real-time PCR	Yes	Thermo Fisher Scientific	2000
9	PCR	Yes	AFNOR	250
14	PCR	Yes	AFNOR	1600
16	PCR	Yes	AFNOR	NA
18	qPCR	No	NA	-
21	Real-time PCR (primers according to Josefsen et al, 2007)	Yes	In-house validation	151
25	Incubation of MKTTn at 41,5 °C. (Procedure described in EN ISO 6579-1:2017 (/A1:2020) for PPS samples as supplement to incubation of MSRV)	No	NA	NA
28	MINI VIDAS SLM TEST	Yes	AFNOR; AOAC	NA
33	Real-Time PCR	No	NA	NA

- : no information reported

NA: Not Applicable

4.6 Performance of the NRLs

4.6.1 General

All 33 laboratories fulfilled the criteria of good performance for the EURL-*Salmonella* Proficiency Test Food 2021. The participants were informed of their results in an individual laboratory Performance report (Annex I) and an interim summary report containing the results of all participants (Diddens and Mooijman, 2021) within 2 months of performing the PT.

5 Conclusions

All 33 participating laboratories fulfilled the criteria of good performance of the EURL-*Salmonella* Proficiency Test for the detection of *Salmonella* in liquid whole egg samples.

The accuracy rate of the control samples was 100%.

The specificity rate of the negative liquid whole egg samples was 100%.

The sensitivity rates of the liquid whole egg samples artificially contaminated with *Salmonella* Enteritidis was 99,7%.

The accuracy rate of all liquid whole egg samples for all participating laboratories was 99,8%.

Twelve laboratories used a second method for detecting *Salmonella* in the liquid whole egg samples. The results of the second detection methods were equal to those obtained with EN ISO 6579-1:2017(/A1:2020).

List of abbreviations

AFNOR	Association Française de Normalisation (French Standardization Association)
AOAC	Association of Analytical Communities
BGA	Brilliant Green Agar
BGA(mod)	Brilliant Green Agar (modified)
BHI	Brain Heart Infusion broth
BPLS	Brilliant green Phenol-red Lactose Sucrose
BPW	Buffered Peptone Water
BSA	Brilliance *Salmonella* Agar
CEN	European Committee for Standardization
cfu	colony-forming units
DG-SANTE	Directorate-General for Health and Consumer Protection
EC	European Commission
EFTA	European Free Trade Association
EU	European Union
EURL	European Union Reference Laboratory
ISO	International Organization for Standardization
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization – Time Of Flight
MKTTn	Muller-Kauffmann tetrathionate-novobiocin broth
MPN	most probable number
MS	Member State
MSRV	Modified semi-solid Rappaport-Vassiliadis
NMKL	Nordic Committee on Food Analysis
NRL	National Reference Laboratory
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PPS	Primary Production Stage
PT	Proficiency Test
qPCR	quantitative Polymerase Chain Reaction
RIVM	Rijksinstituut voor Volksgezondheid en het Milieu (National Institute for Public Health and the Environment)
RVS	Rappaport-Vassiliadis Soya broth
SE	*Salmonella* Enteritidis
SM (ID)2	*Salmonella* Detection and Identification-2
VRBG	Violet Red Bile Glucose agar
XLD	Xylose Lysine Deoxycholate agar

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Annex I. Example of an individual laboratory Performance report of the EURL-*Salmonella* PT Food 2021

Performance

EURL-*Salmonella* PT Food 2021

Detection of *Salmonella* in liquid whole egg

Number of positive samples / Total number of samples per level

	Liquid whole egg samples			control samples	
Labcode	High	Low	Negative	BPW	Pos control
#	4/4	6/6	0/4	0/1	1/1

Evaluation: Good performance

Number	Level	Your result	Media choices
A1	Negative	Not detected	Selective enrichment:
A2	Low	Detected	MKTTn and MSRV
A3	High	Detected	
A4	Negative	Not detected	Selective isolation media:
A5	Low	Detected	Rambach and XLD
A6	Low	Detected	
A7	High	Detected	
A8	Low	Detected	
A9	Negative	Not detected	
A10	High	Detected	
A11	Negative	Not detected	
A12	Low	Detected	
A13	High	Detected	
A14	Low	Detected	
CTRL1	BPW	Not detected	
CTRL2	Pos control	Detected	

High = High concentration *S.* Enteritidis (Inoculation level: 69 cfu / sample)
Low = Low concentration *S.* Enteritidis (Inoculation level: 10 cfu / sample)
Negative = Negative liquid whole egg sample (no *Salmonella* added)
BPW = Buffered Peptone Water (procedure control)
Pos control = own positive control with *Salmonella*

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