



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

# Combined **EURL-Salmonella** Proficiency Test Primary Production Stage and Food, 2022

Detection of *Salmonella* in hygiene swab samples



**Combined EURL-*Salmonella* Proficiency Test**  
**Primary Production Stage and Food, 2022**  
Detection of *Salmonella* in hygiene swab samples

RIVM report 2022-0108

## Colophon

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## Synopsis

### **Combined EURL-*Salmonella* Proficiency Test for Primary Production Stage and Food, 2022**

#### Detection of *Salmonella* in hygiene swab samples

In the annual European Reference Laboratory (EURL)-*Salmonella* Proficiency Test (PT) performed in 2022, the National Reference Laboratories (NRLs) of the European Member States (EU-MS) were able to detect *Salmonella* in hygiene swab samples. All laboratories were successful in detecting *Salmonella* in high and low concentrations in the contaminated hygiene swab samples. All laboratories achieved good results. The Proficiency Test (PT) also included a number of negative samples which contained no *Salmonella*, but did contain two different mixtures of background flora. Forty-three laboratories did not detect *Salmonella* in these four negative hygiene swab samples. However, 25 laboratories detected *Salmonella* in one or two out of four negative samples. Since 10% of the total number of negative samples tested positive for *Salmonella*, the EURL-*Salmonella* has decided not to evaluate the results of the negative samples.

This was the outcome of the combined Proficiency Test for detection of *Salmonella* in hygiene swab samples organised by the coordinating EURL-*Salmonella* in September 2022.

Since 1992, all NRLs from EU-MS are required to participate in the annual quality control PTs for *Salmonella*. Each EU MS has to appoint a NRL which is responsible for analysing *Salmonella* in food samples or samples taken from the animal primary production stage (PPS). This year's PT was compulsory for both NRLs PPS as well as NRLs Food. In total, 68 NRLs participated in this study: 34 NRLs PPS and 34 NRLs Food originating from 27 EU-MS, 11 NRLs were based in other countries in Europe and one NRL was based in a non-European country.

The EURL-*Salmonella* is based at the Dutch National Institute for Public Health and the Environment (RIVM). An important task of the EURL-*Salmonella* is to monitor and improve the performance of the NRLs for *Salmonella* in Europe.

Keywords: *Salmonella*, EURL, NRL, Proficiency Test, Hygiene swab samples, *Salmonella*-detection method



## Publiekssamenvatting

### **Het gecombineerde EURL-*Salmonella* ringonderzoek productiedieren en Voedsel (2022)**

#### Detectie van *Salmonella* in hygiënesponsjes

De Nationale Referentie Laboratoria (NRL's) van de Europese lidstaten waren in 2022 in staat om *Salmonella* aan te tonen in hygiënesponsjes. Alle deelnemers konden hoge en lage concentraties *Salmonella* aantonen. In deze PT zijn ook *Salmonella* negatieve monsters verstuurd die alleen twee verschillende mengsel van stoorflora bevatten. Drieënveertig laboratoria hebben geen *Salmonella* aangetoond in deze 4 negatieve hygiënesponsjes. In totaal hebben 25 laboratoria toch *Salmonella* gevonden in een of twee van de 4 negatieve monsters. Omdat in ongeveer 10% van het totale aantal negatieve monsters toch *Salmonella* is gevonden, heeft het EURL-*Salmonella* besloten om deze negatieve monsters buiten beschouwing te laten. Dit blijkt uit het ringonderzoek dat het overkoepelende laboratorium in oktober 2020 organiseerde.

Sinds 1992 zijn de NRL's van de Europese lidstaten verplicht om elk jaar mee te doen aan kwaliteitstoetsen. Dit zijn de zogeheten ringonderzoeken voor *Salmonella*. Elke lidstaat wijst hiervoor een laboratorium aan, het NRL. Dit keer was het ringonderzoek verplicht voor de laboratoria die verantwoordelijk zijn voor testen van monsters uit de leefomgeving van dieren alsook voor laboratoria die verantwoordelijk zijn voor testen van monsters uit voedselproductie.

In totaal hebben 68 NRL's aan dit ringonderzoek deelgenomen: 34 NRL's leefomgeving van dieren voor voedselproductie en 34 NRL's voedsel, afkomstig uit 28 EU-lidstaten, 11 NRL's uit andere Europese landen en een NRL uit een niet-Europees land.

Het Europese Referentielaboratorium (EURL) *Salmonella* is gevestigd bij het Nederlandse Rijksinstituut voor Volksgezondheid en Milieu (RIVM). Een belangrijke taak van het EURL-*Salmonella* is toezien op de kwaliteit van de nationale referentielaboratoria voor deze bacterie in Europa.

Kernwoorden: *Salmonella*, EURL, NRL, ringonderzoek, hygiënesponsjes, *Salmonella*-detectiemethode



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## Summary

In September 2022, the combined European Reference Laboratory (EURL)-*Salmonella* Proficiency Test on the detection of *Salmonella* in samples from the primary production stage (PPS) and Food was held. Participation was mandatory for the National Reference Laboratories (NRLs) for *Salmonella* of all European Union (EU) Member States (MSs) that are responsible for the detection of *Salmonella* in PPS samples and for all NRLs responsible for detection of *Salmonella* in Food samples. A total of 68 NRLs-*Salmonella* participated in this study: 34 NRLs PPS and 34 NRLs Food from the 27 EU MSs, 11 NRLs from third European countries (EU candidate MS or potential EU candidate MSs and members of the European Free Trade Association (EFTA)), and one NRL from a non-European country

### Samples

In this study, the samples under analysis were hygiene swab samples, artificially contaminated at the EURL-*Salmonella* laboratory with two different mixtures of background flora, and a diluted culture of *Salmonella* Enteritidis and/or *Salmonella* Infantis.

Each NRL-*Salmonella* had to analyse the following set of blindly coded samples:

- 4 hygiene swab samples with a high level of *S. Infantis* (30 cfu/sample) and *S. Enteritidis* (8 cfu/sample), in combination with a mixture of *E. cloacae* and *C. freundii* ( $10^6$  cfu/sample).
- 6 hygiene swab samples with a low level of *S. Infantis* (8 cfu/sample), in combination with a mixture of *E. cloacae* and *C. freundii* ( $10^6$  cfu/sample).
- 4 negative hygiene swab samples (no *Salmonella* added)
  - o 2 samples: *E. cloacae* and *C. freundii* ( $10^6$  cfu/sample);
  - o 2 samples: *E. cloacae* and *C. youngae* ( $10^6$  cfu/sample).
- 1 procedure control (hygiene swab samples with sterile peptone saline solution (PS) only)
- 1 positive control sample (laboratories' own *Salmonella* control strain)

The samples were prepared at the laboratory of the EURL-*Salmonella* and stored at 5 °C for approximately one week until the day of transport. On Monday, 26 September 2022, the hygiene swab samples were packaged and sent to the NRLs-*Salmonella*. The NRLs were asked to store the samples at 5 °C on arrival until the start of the analysis on Monday, 3 October 2022.

### Method

All laboratories used the prescribed method EN ISO 6579-1:2017(/A1:2020) to test the samples. Only two participating laboratories were not (yet) accredited for this method. Two laboratories reported to be NRL-*Salmonella* for samples from the primary production stage but used Mueller Kaufmann tetrathionate novobiocin broth (MKTn) and Rappaport-Vassiliadis soya broth (RVS) broth instead of

modified semi-solid Rappaport-Vassiliadis (MSRV) agar for the selective enrichment. The use of these broths is not in line with the prescribed method in EN ISO 6579-1:2017 for analysing PPS samples.

Twenty laboratories also reported results for a second method. All these laboratories found identical results using the alternative method compared to the results found with EN ISO 6579-1:2017(/A1:2020).

## Results

All 68 participating laboratories analysed both the procedure control as well as their own positive control sample correctly.

Almost all laboratories detected *Salmonella* in the hygiene swab samples contaminated with a low level of *Salmonella* Infantis (8 cfu/sample). One laboratory (lab code 1) tested one of the six samples negative for *Salmonella*. These results are still within the criteria for good performance, which permit three negative samples. The sensitivity rate was 99,8% for these samples.

All laboratories detected *Salmonella* in all four high-level samples contaminated with a combination of *Salmonella* Infantis (30 cfu/sample) and *Salmonella* Enteritidis (8 cfu/sample). The sensitivity rate was 100% for these samples.

All 4 negative samples were scored correctly as negative by 43 laboratories. However, 25 laboratories detected *Salmonella* in one or two of the four negative samples. Serotyping of these 'false-positive' isolates showed that this strain was *Salmonella* Enteritidis. Additional subtyping using Whole Genome Sequencing WGS revealed that the 'false-positive' isolate had an identical WGS pattern to the *Salmonella* Enteritidis strain used in this PT to artificially contaminate the positive samples. Since almost 10% of the total number of negative samples were tested as positive for *Salmonella*, the EURL-*Salmonella* decided not to evaluate the results of the negative samples.

Overall, the laboratories scored well in this Proficiency Test analysing the positive samples, with an accuracy of 99,9%. All 68 laboratories fulfilled the criteria of good performance.

# 1 Introduction

An important task of the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*), as laid out in Commission Regulation No 625/2017 (EC, 2017), is the organisation of Proficiency Tests (PTs) to evaluate the performance of the National Reference Laboratories (NRLs) for *Salmonella*. The history of the PTs held by EURL-*Salmonella* from 1995 onwards is summarised on the EURL-*Salmonella* website (EURL-*Salmonella*, 2023).

In September 2022, the EURL-*Salmonella* held a PT to evaluate whether the NRLs responsible for the detection of *Salmonella* in samples from the Primary Production stage (PPS) and Food could detect *Salmonella* at different contamination levels in hygiene swab samples. The results from PTs like this show whether the examination of samples in the EU Member States (EU-MS) is carried out uniformly and whether comparable results can be obtained by all NRLs-*Salmonella*.

The method prescribed for the detection of *Salmonella* species (spp.) is set out in EN ISO 6579-1:2017(/A1:2020).

The design of this study was comparable to previous PTs held by EURL-*Salmonella* (Diddens & Mooijman, 2021; Pol-Hofstad & Mooijman, 2020 and Pol-Hofstad & Mooijman, 2021). For the current study, hygiene swabs were artificially contaminated with either a combination of *Enterobacter cloacae* and *Citrobacter freundii* ATCC 8090 or *Enterobacter cloacae* and *Citrobacter youngae* to mimic background flora in natural samples. In addition, the hygiene swabs were contaminated with a diluted culture of *Salmonella* Enteritidis (SE) and/or *Salmonella* Infantis (SI) at the laboratory of the EURL-*Salmonella*.

In total, 16 samples had to be tested:

- 4 hygiene swab samples with a high level of SI (30 cfu/sample) and SE (8 cfu/sample), in combination with a mixture of *E. cloacae* and *C. freundii* ( $10^6$  cfu/sample);
- 6 hygiene swab samples with a low level of SI (8 cfu/sample), in combination with a mixture of *E. cloacae* and *C. freundii* ( $10^6$  cfu/sample);
- 4 negative hygiene swab samples (no *Salmonella* added):
  - o 2 samples: *E. cloacae* and *C. freundii* ( $10^6$  cfu/sample);
  - o 2 samples: *E. cloacae* and *C. youngae* ( $10^6$  cfu/sample).
- 1 procedure control (hygiene swab samples with sterile peptone saline solution (PS) PS only);
- 1 positive control sample (laboratories' own *Salmonella* control strain).

The number of samples and the contamination levels were based on information described in EN ISO 22117:2019.



## 2 Participants

In tables 2.1 and 2.2, the country, city and the name of the institute of the participating NRLs are displayed.

*Table 2.1 List of participants NRLs Primary Production Stage*

Country	City	Institute
<b>Austria</b>	Graz	Austrian Agency for Health and Food Safety (AGES/VEMI)
<b>Belgium</b>	Brussels	Sciensano
<b>Bulgaria</b>	Sofia	National Diagnostic and Research Veterinary Institute (NDRVMI), National Reference Centre of Food Safety
<b>Croatia</b>	Zagreb	Croatian Veterinary Institute, Poultry Centre, Laboratory for General Bacteriology and Microbiology
<b>Cyprus</b>	Nicosia	Cyprus Veterinary Services Pathology, Bacteriology, Parasitology Laboratory
<b>Czech Republic</b>	Praha	State Veterinary Institute
<b>Denmark</b>	Ringsted	Danish Veterinary and Food administration
<b>Estonia</b>	Tartu	Estonian Veterinary and Food Laboratory, Bacteriology-Pathology Department
<b>Finland</b>	Kuopio	Finnish Food Authority, Laboratory and Research Division
<b>France</b>	Ploufragan	Anses, Laboratoire de Ploufragan-Plouzané Unité Hygiène et Qualité des Produits Avicoles et Porcins (HQPAP)
<b>Germany</b>	Berlin	German Federal Institute for Risk Assessment (BfR) Biological Safety Department
<b>Greece</b>	Chalkida	Veterinary Laboratory of Chalkis
<b>Hungary</b>	Budapest	National Food Chain Safety Office, Food and Feed Safety Directorate, Microbiological NRL
<b>Iceland</b>	Reykjavik	Mátis ohf, Food Safety and Analytical services
<b>Ireland, Republic of</b>	Kildare	Central Veterinary Research Laboratory (CVRL/DAFFM) Laboratories Backweston, Department of Bacteriology
<b>Israel</b>	Kiryat Malachi	Laboratory of the Israel Poultry and Egg Board

Country	City	Institute
<b>Italy</b>	Padova Legnaro	Istituto Zooprofilattico Sperimentale delle Venezie, OIE
<b>Kosovo</b>	Pristina	Food and Veterinary Laboratory
<b>Latvia</b>	Riga	Institute of Food Safety, Animal Health and Environment BIOR Bacteriology and Parasitology Division
<b>Lithuania</b>	Vilnius	National Food and Veterinary Risk Assessment Institute, Laboratory of Microbiology and Pathology, Bacteriology Group
<b>Luxembourg, Grand-Duchy of</b>	Diddeléng	Laboratoire de Médecine Vétérinaire de l'Etat, Bacteriologie
<b>Malta</b>	Valletta	Malta Public Health Laboratory (PHL), Evans Building
<b>Netherlands, the</b>	Bilthoven	National Institute for Public Health and the Environment (RIVM), Centre for Zoonosis and Environmental Microbiology (Z&O)
<b>Northern Ireland</b>	NRL tasks PPS are carried out by NRL Ireland	
<b>Norway</b>	Ås	Norwegian Veterinary Institute, Section of Microbiology
<b>Poland</b>	Pulawy	National Veterinary Research Institute, department of microbiology
<b>Portugal</b>	Vairão	Instituto Nacional de Investigação Agrária e Veterinária , Food Microbiology Laboratory
<b>Republic of North Macedonia</b>	Skopje	Food Institute, Faculty of Veterinary Medicine, Laboratory for food and feed microbiology
<b>Romania</b>	Bucharest	Institute for Diagnosis and Animal Health
<b>Slovak Republic</b>	Dolny Kubin	State Veterinary and Food Institute
<b>Slovenia</b>	Ljubljana	National Veterinary Institute, Veterinary Faculty (UL, NVI)
<b>Spain</b>	Madrid Algete	Laboratorio Central de Veterinaria
<b>Spain</b>	Lugo	Laboratorio Nacional de Sanidad Vegetal
<b>Sweden</b>	Uppsala	National Veterinary Institute
<b>Switzerland</b>	Zurich	National Reference Centre for Poultry and Rabbit Diseases (NRGK), Institute of Food Safety and Hygiene, University of Zurich

Table 2.2 List of participants NRL Food

Country	City	Institute / NRL-Salmonella
<b>Austria</b>	Graz	Austrian Agency for Health and Food Safety (AGES/VEMI)
<b>Belgium</b>	Brussels	Sciensano
<b>Bulgaria</b>	Sophia	National Diagnostic and Research Veterinary Institute (NDRVMI), National Reference Centre of Food Safety
<b>Croatia</b>	Zagreb	Croatian Veterinary Institute, Laboratory for Food Microbiology (CVI)
<b>Cyprus</b>	Nicosia	Cyprus Veterinary Services Laboratory for the control of food of animal origin
<b>Czech Republic</b>	Prague	State Veterinary Institute (SVI)
<b>Denmark</b>	Ringsted	Danish Veterinary and Food Administration, Department of Microbiology
<b>Estonia</b>	Tartu	Estonian Veterinary and Food Laboratory, Department of Food Microbiology
<b>Finland</b>	Helsinki	Finnish Food Authority, Laboratory and Research Division
<b>France</b>	Ploufragan	ANSES Laboratoire de Ploufragan-Plouzané, Unité Hygiène et Qualité des Produits Avicoles et Porcins (HQPAP)
<b>Germany</b>	Berlin	German Federal Institute for Risk Assessment (BfR)
<b>Greece</b>	Chalkida	Veterinary Laboratory of Chalkida
<b>Hungary</b>	Budapest	National Food Chain Safety Office, Food Chain Safety Laboratory Directorate, Microbiological NRL
<b>Ireland</b>	Kildare	Central Veterinary Research Laboratory CVRL/DAFM Backweston, Department of Bacteriology
<b>Italy</b>	Legnaro PD	Istituto Zooprofilattico Sperimentale delle Venezie, OIE
<b>Latvia</b>	Riga	Institute of Food Safety, Animal Health and Environment, BIOR, Microbiology and Pathology Laboratory
<b>Lithuania</b>	Vilnius	National Food and Veterinary Risk Assessment Institute, Bacteriology Unit
<b>Luxembourg</b>	Dudelange	Laboratoire National de Santé, surveillance alimentaire
<b>Kosovo</b>	Pristina	Food and Veterinary Laboratory
<b>Malta</b>	Valletta	Malta Public Health Laboratory (PHL), Evans Building

Country	City	Institute / NRL-Salmonella
<b>Netherlands, the</b>	Bilthoven	National Institute for Public Health and the Environment (RIVM), Centre for Zoonoses and Environmental Microbiology (cZ&O)
<b>Netherlands, the</b>	Wageningen	Wageningen Food Safety Research (WFSR)
<b>Norway</b>	Ås	Norwegian Veterinary Institute, Bacteriology Section
<b>Northern Ireland</b>	NRL tasks carried out by NRL Belgium (NRL food)	
<b>Poland</b>	Pulawy	National Veterinary Research Institute (NVRI), Department of Hygiene of Food of Animal Origin
<b>Portugal</b>	Vairão	Instituto Nacional de Investigação Agrária e Veterinária, Food Microbiology
<b>Republic of North Macedonia</b>	Skopje	Food Institute, Faculty of Veterinary Medicine Laboratory for Food and Feed Microbiology
<b>Romania</b>	Bucharest	Hygiene and Veterinary Public Health Institute (IISPV)
<b>Serbia</b>	Belgrade	Institute of Veterinary Medicine of Serbia.
<b>Slovak Republic</b>	Dolny Kubin	State Veterinary and Food Institute
<b>Slovenia</b>	Ljubljana	Institute of Microbiology and Parasitology, Veterinary Faculty (UL, NVI)
<b>Spain</b>	Majadahonda	Centro Nacional de Alimentacion
<b>Sweden</b>	Uppsala	National Veterinary Institute (SVA), Department of Microbiology
<b>Switzerland</b>	Zürich	Institute for Food Safety and Hygiene, University of Zurich
<b>United Kingdom</b>	Wiltshire	UK Health Security Agency

### 3 Materials and Methods

#### 3.1 Preparation of artificially contaminated hygiene swab samples

##### 3.1.1 General

The matrix used for this PT was hygiene swabs. Hygiene swabs are suitable to be used as control samples for the Food production area, as well as for the (animal) primary production stage (PPS). The hygiene swabs were artificially contaminated with background flora, consisting of two different mixtures of two bacteria, and with a diluted culture of SE and/or SI at the EURL-*Salmonella* laboratory.

##### 3.1.2 Pre-tests for the preparation of hygiene swab samples

Hygiene swab samples were ordered from supplier VWR (no: vwrc710-1020; size dry sponges: 7,5 cm by 3,8 cm). The hygiene swabs were pre-moisturised by adding 10 ml of peptone saline solution (PS) and left at room temperature until totally soaked (approx. 30 minutes). The moisturised hygiene swabs were artificially contaminated with background flora by adding 1 ml of an even mixture of *Enterobacter cloacae* and *Citrobacter youngae* (approx.  $10^6$  cfu/swab) or with different concentrations of SE and SI, see Table 3.1. The *C. youngae* strain in the background mixture is H<sub>2</sub>S positive so that it can easily be mixed up with *Salmonella*. The interference of the background flora in the *Salmonella* detection was tested in this pre-test.

Table 3.1 Overview of the strains and contamination levels of the hygiene swabs in stability pre-test

Additions	Strains	Test for	Test on (days)
<b>a. Background flora</b>	<i>E. cloacae</i> + <i>C. youngae</i>	<i>Enterobacteriaceae</i> /total aerobic count	0, 6, 13, 22
<b>b. Low SI + High SE</b>	7 cfu SI/17 cfu SE	<i>Salmonella</i> , O:7, O:9	0, 6, 13, 22
<b>c. Medium SI + Medium SE</b>	14 cfu SI/14 cfu SE	<i>Salmonella</i> , O:7, O:9	0, 6, 13, 22
<b>d. High SI + Low SE</b>	17 cfu SI/7 cfu SE	<i>Salmonella</i> , O:7, O:9	0, 6, 13, 22

To test the stability of the artificially contaminated hygiene swab samples during transport and storage conditions, the pre-test samples were stored at 5 °C for a period of up to three weeks. After 0, 6, 13 and 22 days of storage, five samples were tested for the presence of *Salmonella* at each time interval and according to EN ISO 6579-1:2017. In addition, one hygiene swab sample was tested at each time interval for the concentration of background flora according to EN ISO 21528-2:2017 and EN ISO 4833-1:2013. Finally, the ratio between SI and SE compared to the initial ratio on the day of contamination was evaluated by testing 12 single colonies per plate using antisera to distinguish between *Salmonella* O groups O:7 and O:9 (see 3.1.6).

### 3.1.3 *Preparation of hygiene swab samples for the Proficiency Test*

Pre-moisturised hygiene swab samples were artificially contaminated with a suspension of background flora, consisting of either an even mixture of *E. cloacae* and *C. freundii* (approx.  $10^6$  cfu/ml) or an even mixture of *E. cloacae* and *C. youngae* (approx.  $10^6$  cfu/ml), followed by artificial contamination with a low concentration of SI or a high concentration of both SI and SE according to the following scheme:

- 4 hygiene swab samples with a high level of SI (30 cfu/sample) and SE (8 cfu/sample), in combination with a mixture of *E. cloacae* and *C. freundii* ( $10^6$  cfu/sample);
- 6 hygiene swab samples with a low level of SI (8 cfu/sample), in combination with a mixture of *E. cloacae* and *C. freundii* ( $10^6$  cfu/sample);
- 4 negative hygiene swab samples (no *Salmonella* added)
  - o 2 samples: *E. cloacae* and *C. freundii* ( $10^6$  cfu/sample);
  - o 2 samples: *E. cloacae* and *C. youngae* ( $10^6$  cfu/sample).
- 1 procedure control (hygiene swab samples with sterile peptone saline solution (PS) only);
- 1 positive control sample (laboratories' own *Salmonella* control strain).

The concentration of the inoculum used to contaminate the hygiene swabs was confirmed by streaking the inoculum on xylose lysine deoxycholate (XLD) agar plates (or TSA plate in case of background flora). Immediately after artificial contamination, the high, low and negative samples were stored at 5 °C until transportation to the participating laboratories on Monday, 26 September 2022.

### 3.1.4 *Determination of the level of background flora in hygiene swab samples*

Moisturised hygiene swab samples were artificially contaminated with a mixture of *E. cloacae* and *C. freundii* (ATCC 8090) or with a mixture of *E. cloacae* and *C. youngae* to mimic the presence of background flora, aiming for an end concentration of  $10^6$  cfu/swab. The total number aerobic bacteria and the number of *Enterobacteriaceae* in hygiene swabs was assessed by following EN ISO 4833-1:2013 and EN ISO 21528-2:2017 respectively. The hygiene swab samples were homogenised (kneaded) in peptone saline solution and 10-fold dilutions were analysed on plate count agar (PCA) and violet red bile glucose (VRBG) agar.

### 3.1.5 *Determination of the number of Salmonella in hygiene swab samples by MPN*

The contamination level of *Salmonella* in the artificially contaminated hygiene swab samples was determined using a five-tube most probable number (MPN) technique. For this, 10-fold dilutions of five artificially contaminated hygiene swab samples at 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 following EN ISO 6579-1:2017. The MPN of *Salmonella* in the original sample was calculated from the number of confirmed positive dilutions, using freely available Excel-Based MPN software (Jarvis et al., 2010).

### 3.1.6 *Determination of the Salmonella serotype by antisera*

To distinguish between SI (antigenic formula: 6,7,14:r:1,5) and SE (antigenic formula 1,9,12:g,m:-), antisera to identify group O:7 and

group O:9 were used (SSI Diagnostica, Denmark). Serotyping was performed on a fresh, pure culture of *Salmonella* isolated on a non-selective agar according to instructions of the producer. Twelve single colonies of *Salmonella* (from XLD plates) were grown overnight at 35-37 °C on tryptone soya agar (TSA). Each single colony was transferred to a glass slide containing a small drop of antiserum (approx. 20 µl) and mixed well using an inoculation loop. The slide was tilted for 5-10 seconds. Visible agglutination within 10 seconds is reported as a positive reaction to that O-group. A late or weak agglutination was reported as negative.

### 3.2 Design of the Proficiency Test

#### 3.2.1 Number and type of samples

Each participant received 14 artificially contaminated hygiene swab samples, numbered B1 to B14. In addition, the laboratories had to test two control samples (C1 and C2). Table 3.2 gives an overview of the number and type of samples tested by the participants.

For the control samples, the laboratories were asked to use their own positive *Salmonella* control strain, which they normally use when analysing routine samples for the detection of *Salmonella*. In addition to this positive control (C2), a procedure control (C1) consisting of only Buffered Peptone Water (BPW) had to be analysed. The protocol and result form can be found on the EURL-*Salmonella* website (EURL-*Salmonella* 2022a, 2022b).

Table 3.2 Overview of the number and type of samples tested per laboratory in the Proficiency Test PPS-Food 2022

Strain and contamination level	Hygiene swab samples (n=14)
<b>S. Infantis low level</b>	6
<b>S. Infantis + S. Enteritidis high level</b>	4
<b>Negative (no <i>Salmonella</i> added)</b>	4

Strain and contamination level	Control samples (n=2)
<b>C1: Blank procedure control (BPW only)</b>	1
<b>C2: Positive control (own control with <i>Salmonella</i>)</b>	1

#### 3.2.2

##### *Shipment of parcels and temperature recording during shipment*

The 16 blindly coded samples containing the contaminated and the negative hygiene swab samples plus the two sterile bags with hygiene swabs for the control samples, were packed in two safety bags. These were placed in one large shipping box, together with four frozen (-20 °C) cooling elements. The shipping boxes were sent to the participants as 'biological substances category B' (UN3373) via a door-to-door courier service. The participants were asked to store the samples at 5 °C upon receipt. To monitor exposure to abusive temperatures during shipment and storage, a micro temperature logger was placed between the samples to record the temperature.

### 3.3 Methods

The method for detection of *Salmonella* prescribed for this PT was EN ISO 6579-1:2017 including A1:2020. Hygiene swabs can be considered as control samples for the (animal) PPS as well as control samples for the food production area. NRLs should use the appropriate method for the chosen matrix approach (Food or PPS).

The method starts with pre-enrichment in BPW, followed by a selective enrichment in Mueller Kaufmann tetrathionate novobiocin broth (MKTn) and in Rappaport-Vassiliadis soya broth (RVS) and/or modified semi-solid Rappaport-Vassiliadis (MSRV) agar when considering the hygiene swabs as food samples. When the hygiene swabs are considered as PPS samples, selective enrichment is carried out on modified semi-solid Rappaport-Vassiliadis (MSRV) agar only. Plating-out is carried out on XLD and a second isolation medium of choice. Confirmation is performed using the appropriate biochemical and serological tests as prescribed in EN ISO 6579-1:2017 or using reliable, validated identification kits. The hygiene swabs were moisturised with 10 ml peptone saline solution. In this case 90 ml of BPW had to be added to prepare the initial suspension.

In addition to the EN ISO method, the NRLs were free to use their own method, such as a polymerase chain reaction (PCR) procedure. Only the results obtained with the prescribed method, EN ISO 6579-1:2017(/A1:2020), were used to assess the performance of each participant. Results had to be reported using the EURL-*Salmonella* result form (EURL *Salmonella*, 2022b). Participants received their individual laboratory performance results in a performance report (See example in Annex I), in addition to the interim summary report (EURL *Salmonella*, 2022c).

### 3.4 Statistical analysis of the data

The specificity, sensitivity and accuracy rates were calculated for the artificially contaminated hygiene swab samples. For the control samples, only the accuracy rates were calculated. The rates were calculated with 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

For the determination of 'good performance', the criteria indicated in Table 3.3 were used.

Table 3.3 Criteria for testing good performance in the combined EURL-Salmonella PT PPS-Food 2022

Contamination level	% positive	# positive samples/ total # samples
<b>Hygiene swab samples</b>		
<b>S. Infantis + S. Enteritidis high-level</b>	Min. 80 %	Min. 3/4
<b>S. Infantis low-level</b>	Min. 50 %	Min. 3/6
<b>Negative (no <i>Salmonella</i> added)</b>	not evaluated <sup>1</sup>	Not evaluated <sup>1</sup>

Contamination level	% positive	# positive samples/ total # samples
<b>Control samples</b>		
<b>Procedure control (BPW only)</b>	0 %	0 / 1
<b>Positive control with <i>Salmonella</i></b>	100 %	1 / 1

<sup>1</sup> not evaluated due to unexpected high number of *Salmonella* positive results found by participants



## 4 Results and Discussion

### 4.1 Preparation of artificially contaminated hygiene swab samples

#### 4.1.1 Pre-tests for the preparation of hygiene swab samples

The study design was based on the tests performed for the combined PT PPS-Food organised in 2020 by the *EURL-Salmonella* (Pol-Hofstad and Mooijman, 2021). Background flora was selected based on previous experiences (*C. freundii*) and on suggestions of NRLs during workshop 2022 to use *E. cloacae*. *C. youngae* was included because of similar appearance as *Salmonella* on selective media (H<sub>2</sub>S positive). Since previous experience showed that *C. freundii* survived well on hygiene swabs for up to three weeks, in this pre-test only the survival of the combination of *E. cloacae* and *C. youngae* was investigated. The pre-test samples were stored at 5 °C to mimic storage and transport conditions for up to three weeks and analysed for the presence of *Enterobacteriaceae*, total aerobic count and *Salmonella* using EN ISO 4833-1:2013, EN ISO 21528-2:2017 and EN ISO 6579:1-2017 respectively. The results are presented in Figure 4.1 and Figure 4.2. In addition, tests were performed to determine the survival of both SI and SE in the hygiene swab samples (see 3.1.6).

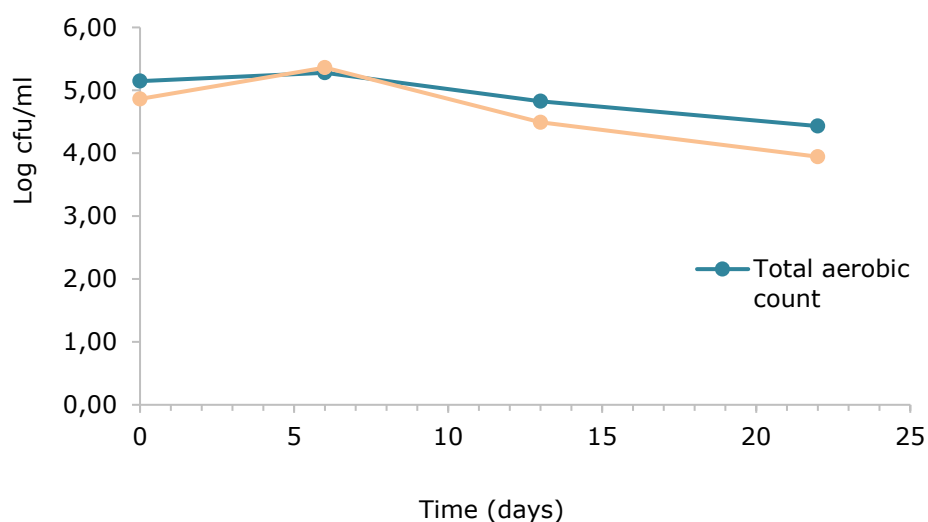


Figure 4.1 The effect of storage time on the number of *Enterobacteriaceae* and total aerobic count in hygiene swab samples

Results in Figure 4.1 show that the number of background flora remains stable in the hygiene swab samples at approx. log 5, when stored at 5 °C for three weeks. The stability of SI in the presence of SE was studied in order to determine if both strains equally survive storage at 5 °C for three weeks or if one of the two strains will become dominant over time. Three combinations of SI and SE were tested: a) SE in surplus over SI, b) both strains in equal concentrations and c) SI in surplus over SE. Table 4.1 shows the aimed concentrations and the actual concentrations used in the 3 mixtures to contaminate the hygiene swabs.

The hygiene swab samples (n=5) were analysed for the presence of *Salmonella* after 0, 6, 13 and 22 days of storage at 5 °C. Twelve single colonies (from XLD plate) per sample were tested serologically, using antisera to identify group O:7 or group O:9 according to 3.1.6. In the hygiene swab samples contaminated with combinations a) and b), almost all colonies belonged to group O:9 already on day 0. This showed that SE almost immediately became dominant over SI. Therefore, only combination c) was analysed for the two O groups on day 6, 13 and 22. Results are shown in Figure 4.2.

*Table 4.1 Concentration of Salmonella Infantis and Salmonella Enteritidis in the inoculum to artificially contaminate the test samples*

<b><i>Salmonella</i> strain + aimed cfu</b>	<b>Actual cfu</b>	
	<b>XLD</b>	<b>TSA</b>
SI 7 cfu	2	15
SI 14 cfu	13	16
SI 17 cfu	7	25
SE 7 cfu	13	7
SE 14 cfu	24	19
SE 17 cfu	25	19

<b><i>Salmonella</i> mixtures used</b>
a 7 cfu SI + 17 cfu SE
b 14 cfu SI + 14 cfu SE
c 17 cfu SI + 7 cfu SE

Combination c) aimed for a surplus of SI over SE, however serotyping 12 colonies on day 0 showed that SI and SE were present in almost equal concentrations. On day 6 there were 4 samples showing higher survival of SE over SI, and only 1 sample showing dominant presence of SI over SE. On day 13 and day 22, SE was detected in almost all sample as the sole *Salmonella* serovar.

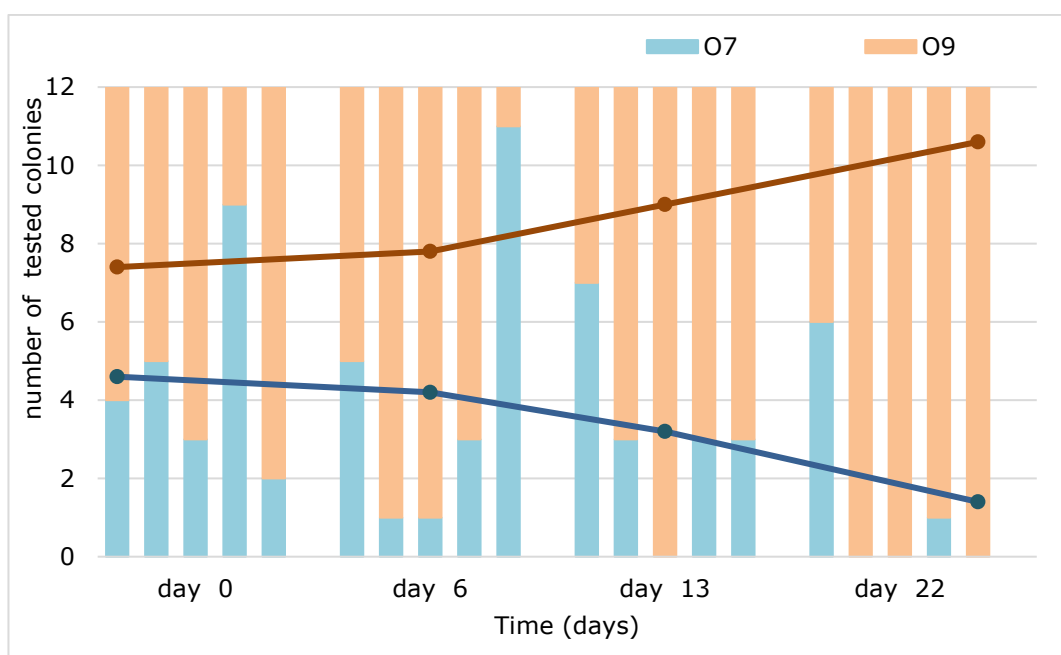


Figure 4.2 Stability tests of hygiene swab samples artificially contaminated with a mixture of 17 cfu *Salmonella Infantis* (group O:7) and 7 cfu *Salmonella Enteritidis* (group O:9) after storage at 5 °C for three weeks. Each bar indicates the number of colonies serologically confirmed as O:7 or O:9. The dark lines represent the average number of colonies SI (O:7) or SE (O:9) present in 5 hygiene swab samples per time frame.

Figure 4.2 shows that SE has better survival capacity in the hygiene swab samples or outgrows SI in the pre-enrichment step of the detection method. To guarantee that both strains are still present in the hygiene swab samples when the participants start their analysis, it was decided to use a mixture of two *Salmonella* strains only in the high contaminated samples and to add SI in a higher concentration than SE (anticipated concentrations: 40 cfu SI and 10 cfu SE).

**4.1.2 Preparation of hygiene swab samples for the Proficiency Test**  
Samples for the PT were prepared as described in 3.1.3. Samples were artificially contaminated with approx. 15 cfu SI/sample and approx. 40 cfu SI +10 cfu SE/sample, representing low and high levels of contamination in the hygiene swab samples.

**4.1.3 Background flora in the hygiene swab samples**  
The hygiene swab samples were contaminated by adding 1 ml of an even mixture of *E. cloacae* and *C. freundii* with a concentration of  $8,0 \times 10^5$  cfu/swab (see Table 4.2). Two of the four negative samples were inoculated with a different mixture of background flora: *E. cloacae* and *C. youngae* with a concentration of  $9,7 \times 10^5$  cfu/swab.

Table 4.2 Number of background flora in the cell suspensions used to artificially contaminate the hygiene swabs (cfu per ml)

Date	<i>E. cloacae</i> and <i>C. freundii</i> (cfu/ml)	<i>E. cloacae</i> and <i>C. youngae</i> (cfu/ml)
<b>21 Sept 2022</b>	8,0 × 10 <sup>5</sup>	9,7 × 10 <sup>5</sup>

#### 4.1.4 Number of *Salmonella* in the Hygiene swab samples

The hygiene swab samples were artificially contaminated at the EURL-*Salmonella* laboratory by adding the appropriate volume of a diluted SI culture or a mixture of SI and SE. Table 4.3 shows the contamination levels of the diluted cultures of *Salmonella* used as inoculum to contaminate the hygiene swab samples.

Table 4.3 Number of *Salmonella* Infantis (SI) and the mixture of *Salmonella* Infantis (SI) and *Salmonella* Enteritidis (SE) in the inoculums and in the hygiene swab samples

Date of testing	Low level SI (cfu/sample)	High level SI + SE (cfu/sample)
<b>21 Sept 2022</b> (inoculum level diluted culture)	8	30 + 8
<b>3 Oct 2022<sup>a</sup></b> MPN contaminated hygiene swab samples (95 % confidence limit)	2,2 (0,9-5,5)	17,35 (6,5-45)

<sup>a</sup> After storage at 5°C for approx. 1,5 week

After inoculation, the samples were stored at 5 °C for 1,5 week until being transported to the participants on 26 September 2022. The final contamination level of *Salmonella* in the hygiene swab samples was determined by performing a five-tube Most Probable Number (MPN) test in the week of the PT study (see Table 4.3).

## 4.2 Technical data of the Proficiency Test

### 4.2.1 General

A total of 68 NRLs-*Salmonella* subscribed to this study: 34 NRLs PPS and 34 NRLs Food originating from 35 countries. The participants originated from 27 EU-MS, 11 NRLs from third European countries (EU candidate or potential EU candidate MS and members of the EFTA countries), and one NRL was based in a non-European country. In total, 67 NRLs-*Salmonella* reported their results, one NRL Food did not return their results.

### 4.2.2 Accreditation and Methods used

Sixty-eight laboratories were accredited according to EN ISO/IEC 17025:2017 for EN ISO 6579-1:2017 (/A1:2020) and used this method for the detection of *Salmonella*.

Three laboratories reporting results as NRL PPS (lab code 36, 52 and 60), used Mueller Kaufmann tetrathionate novobiocin broth (MKTTn) and Rappaport-Vassiliadis soya broth (RVS) broth instead of modified semi-solid Rappaport-Vassiliadis (MSRV) agar for the selective enrichment.

This is not in line with the prescribed method in EN ISO 6579-1:2017 for analysing PPS samples. There was also 1 laboratory (lab code 66) that did not use the prescribed XLD agar as plating out medium but used Rambach and Endo agar instead.

#### 4.2.3 *Transport of samples*

The samples were transported using a door-to-door courier service on Monday, 26 September 2022. Two laboratories received the parcel on the day of dispatch. Forty-nine parcels were delivered after one day, seven parcels after two days, five parcels after three days and four parcels arrived after four days after dispatch. One parcel arrived somewhat delayed after eight days due to hold-up at the border (lab code 23).

The samples had to be stored at 5 °C upon arrival at the laboratory. The temperature during transport and storage was registered using a temperature probe. The temperature of the parcels during transport was predominantly between -3 °C and 4 °C. The temperature of the parcel arriving late was checked in greater detail. The parcel of laboratory 23 arrived at the laboratory on 4 October 2022 with a temperature of 10 °C (see Figure 4.3). The samples were stored at 5 °C before the analyses started on the next day.

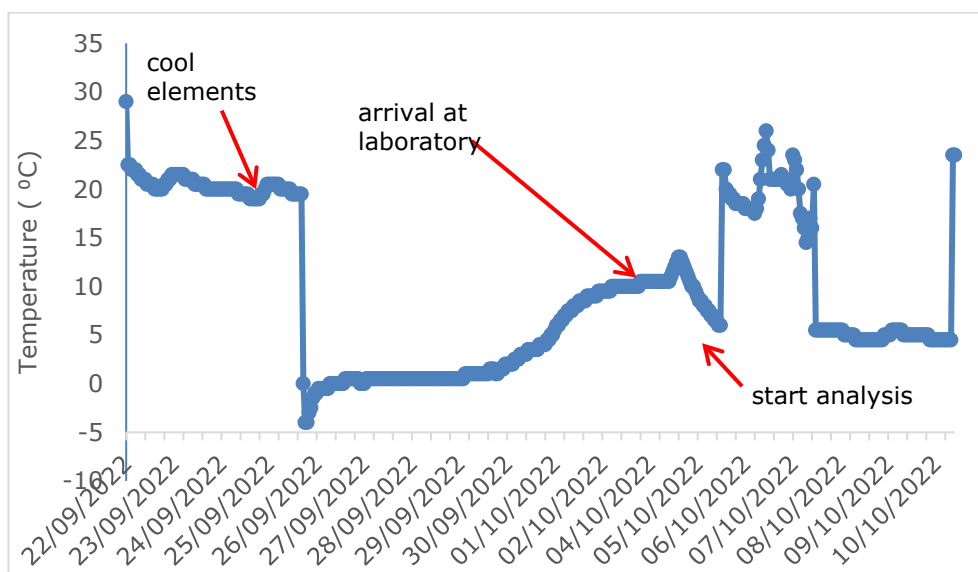


Figure 4.3 Temperature profile of parcel of Laboratory 23

The parcel of laboratory 21 showed a deviating temperature profile. The temperature rose quickly from just above 0 °C (29-9-2022) to 11 °C in 2 days. The sample arrived at the laboratory shortly after and samples were stored cool at 3 °C until the analyses started on 3 October (see Figure 4.4).

The participants were asked to store the parcel at 5 °C upon arrival at their laboratories. The storage temperature at the receiving laboratories ranged from 0 – 9 °C. The majority of laboratories started the analyses on 3 October 2022. However, five laboratories started the analysis one day later (lab codes 12, 40, 41, 64 and 65) and one laboratory started

two days later, after the late arrival of their parcel (lab code 23). Lab codes 29 and 46 started early on 29 and 30 September respectively (two and three days after arrival of their parcel). Laboratory 50 started at the day the parcel arrived (28 September 2022).

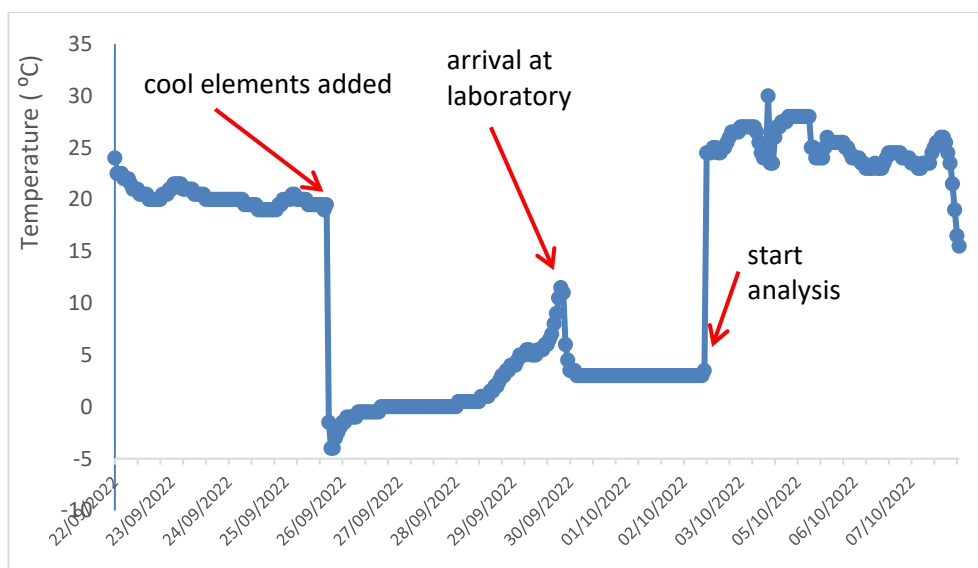


Figure 4.4 Temperature profile of parcel of Laboratory 21

#### 4.2.4

##### Methods

The prescribed method was EN ISO 6579-1:2017. MKTTn, RVS and/or MSRV agar had to be used as selective enrichment media, and XLD agar and a second medium of choice for plating out. Table 4.4 shows which second plating-out media were chosen by the participants. Although the use of at least XLD is prescribed, one laboratory did not use XLD but used Rambach and Endo agar instead (lab code 66).

Table 4.4 Second plating-out media used by the NRLs

Media	No. of users
<b>ASAP</b>	2
<b>BGA</b>	14
<b>BGA mod</b>	8
<b>BSA</b>	5
<b>BxLH</b>	1
<b>SM2</b>	3
<b>Rambach</b>	17
<b>Chromo</b>	2
<b>Compass Salmonella agar</b>	2
<b>Rapid Salmonella</b>	7
<b>RSAL</b>	3
<b>Endo agar</b>	1
<b>BPLS</b>	6
<b>XLD</b>	67

Explanations of the abbreviations used are given in the list of abbreviations.

Technical details on the method that deviated from the prescribed EN ISO method (EN ISO 6579-1:2017) are listed in Table 4.5 (grey-shaded cells);

13 laboratories reported details of deviations. One laboratory (lab codes 13) incubated their BPW solution for too many hours. One laboratory (lab code 52) incubated RVS at 37 °C instead of the prescribed 41,5 °C. Two laboratories used RVS with an incorrect pH (lab codes 2 and 31). Four laboratories used MKTTn with a too low concentration novobiocin (lab codes 13, 27, 28 and 31). Two laboratories used MKTTn with an incorrect pH (lab codes 19 and 51). One laboratory did not report details of the pH at all (lab code 2). One laboratory (lab code 31) used MSRV with a novobiocin concentration higher than prescribed 10 mg/l and one laboratory (lab code 56) did not use MSRV at the correct pH (slightly too high).

Table 4.5 Reported technical deviations from the prescribed EN ISO 6579-1:2017

Lab code	BPW		RVS		MKTTn			MSRV		
	Incu-bation time	T (°C)	pH	T (°C)	T (°C)	pH	Novo-biocin	T (°C)	pH	Novo-biocin
EN ISO 6579-1	16–20 h	37	5,0-5,4	41,5	37	7,0–8,2	40 mg/l	41,5	5,1–5,4	10 mg/l
2	19,5	37	0 <sup>1</sup>	41.5	37	? <sup>1</sup>	40 mg/L			
13	24 <sup>1</sup>	37	5.1	41.5	37	7.9	4 mg/L <sup>1</sup>			
19	19 h 45	37			37	0 <sup>1</sup>	40 mg/L	41.5	5.28	10 mg/L
27	20	37	5.43	41.5	37	8.02	10 mg/L <sup>1</sup>	41.5	5.42	10 mg/L
28	20	37	5.43	41.5	37	8.02	10 mg/L <sup>1</sup>	41.5	5.42	10 mg/L
31	20	37	7.2 <sup>1</sup>	41.5	37	8.2	20 mg/L <sup>1</sup>	41.5	5.2	20 mg/L <sup>1</sup>
36	20	37	5.31	41.5	37	7.42	39.02 mg/L			
51	20	36	5.2	41.5	36	6.6 <sup>1</sup>	40mg/l			
52	20	37	5.36	37 <sup>1</sup>						
56	20	37						41.5	5.6 <sup>1</sup>	10 mg/L

<sup>1</sup> Deviations from EN ISO 6579-1:2017 are indicated in grey

All participating laboratories performed one or several confirmation tests for *Salmonella*. Table 4.6 summarises all reported combinations. Fifty laboratories performed a biochemical test. Thirteen laboratories used only one confirmation test. The majority of participants used a combination of two or more confirmation methods, namely a biochemical test in combination with a serological test, serotyping or a PCR test. Other methods used were: Maltitof, Minividas and Chromogenic agar method.

Table 4.6 Number of laboratories using the different confirmation methods

Number of labs	Bio-chemical	Sero-logical	Sero-typing	PCR	Other
4	x				
20	x	x			
4	x		x		
1	x			x	
5	x				Maltitof
7	x	x	x		
2	x	x	x	x	
1	x	x			Maltitof
1	x		x	x	
2	x		x		chromogenic Media
1	x		x		Maltitof
1	x		x		Minividas
2	x		x	x	Maltitof
1		x			
1		x	x		
1			x		
1			x		Maltitof
7					Maltitof
2				x	
1				x	Maltitof
3		x			Maltitof

## 4.3 Control samples

### 4.3.1

#### General

Two sterile bags with hygiene swabs for the control samples were sent to the laboratories. One was used for the procedure control (C1). The other was used for the positive control to which the laboratories had to add their own positive control strain (C2) normally used in their routine analysis for *Salmonella* detection.

#### Procedure control (BPW only)

All laboratories analysed the procedure control correctly as being negative for *Salmonella* and scored good results for this control sample.

#### Positive control with *Salmonella*

All laboratories correctly scored their own *Salmonella* positive control sample as positive. The *Salmonella* serovars used for the positive control sample are shown in Table 4.7. The majority of the NRLs-*Salmonella* use

SE or *S. Typhimurium* for their positive control samples. However, the use of a less common *Salmonella* serovar as control strain may be advisable in order to make the detection of possible cross-contamination easier.

#### 4.3.2 Correct scores of the control samples

Table 4.8 shows the number of correctly analysed control samples for all participants and for the EU-MS NRLs PPS and NRLs Food separately. No differences were found between these two groups. All laboratories showed correct results, resulting in accuracy rates of 100%.

Table 4.7 *Salmonella* serovars used by participants for the positive control samples

<i>Salmonella</i> serovar	Number of users
<b>S. Enteritidis</b>	18
<b>S. Typhimurium</b>	16
<b>S. Nottingham</b>	9
<b>S. Abaetetuba</b>	5
<b>S. Alachua, S. Blegdam, S. Bongori, S. Harleystreet, S. Tranaroa, S. Infantis, S. Adabraka, S. Agbeni</b>	2 (per serovar)
<b>S. Tennessee, S. Regent, S. Poona, S. Weltevreden</b>	1 (per serovar)

Table 4.8 Correct scores found with the control samples by all participants and by the laboratories of the EU NRLs PPS and EU NRLs Food separately

Control samples		All labs n = 68	EU NRLs only	EU NRLs PPS n = 28	EU NRLs Food n = 28
Procedure control n=1	No. of samples	68	56	28	28
	No. of negative samples	68	56	28	28
	<b>Specificity in %</b>	<b>100%</b>	<b>100%</b>	<b>100%</b>	<b>100%</b>
Positive control (own <i>Salmonella</i> ) n=1	No. of samples	68	56	28	28
	No. of positive samples	68	56	28	28
	<b>Sensitivity in %</b>	<b>100%</b>	<b>100%</b>	<b>100%</b>	<b>100%</b>
All control samples n=2	No. of samples	136	112	56	56
	No. of correct samples	136	112	56	56
	<b>Accuracy in %</b>	<b>100%</b>	<b>100%</b>	<b>100%</b>	<b>100%</b>

## 4.4 Artificially contaminated hygiene swab samples

### 4.4.1 General

Hygiene swab samples artificially contaminated with two different mixtures of background flora (*E. cloacae* and *C. freundii* or *E. cloacae*

and *C. youngae*) and two different concentrations of two *Salmonella* strains: SI and/or SE were analysed for the presence of *Salmonella* by the participants. Table 4.9 shows the overall results found by the participants.

Table 4.9 Number of positive results found with the artificially contaminated hygiene swab samples at each laboratory

	Number of positive samples		
	Negative n=4	SI low n=6	SI + SE high n=4
Criteria good performance	No evaluation	≥3	≥3
All other NRLs (n = 43)	0	6	4
Lab code (4, 5, 9, 14, 15, 18, 19, 23, 25, 35, 36, 42, 49, 51, 53, 54, 55, 56, 57, 62, 63, 64, 65 (n = 23; 12 NRLs PPS + 11 NRLs Food)	1	6	4
Lab code 1 (NRL PPS)	2	5	4
Lab code 31 (NRL PPS)	2	6	4

#### Negative hygiene swab samples

The negative samples were artificially contaminated with background flora in two different combinations. Samples B1 and B8 contained a mixture of *E. cloacae* and *C. freundii* ( $10^6$  cfu/sample), while samples B9 and B14 contained a mixture of *E. cloacae* and *C. youngae* ( $10^6$  cfu/sample). Forty-three laboratories tested all four of these hygiene swab samples negative for *Salmonella* (see Figure 4.5a and 4.5b).

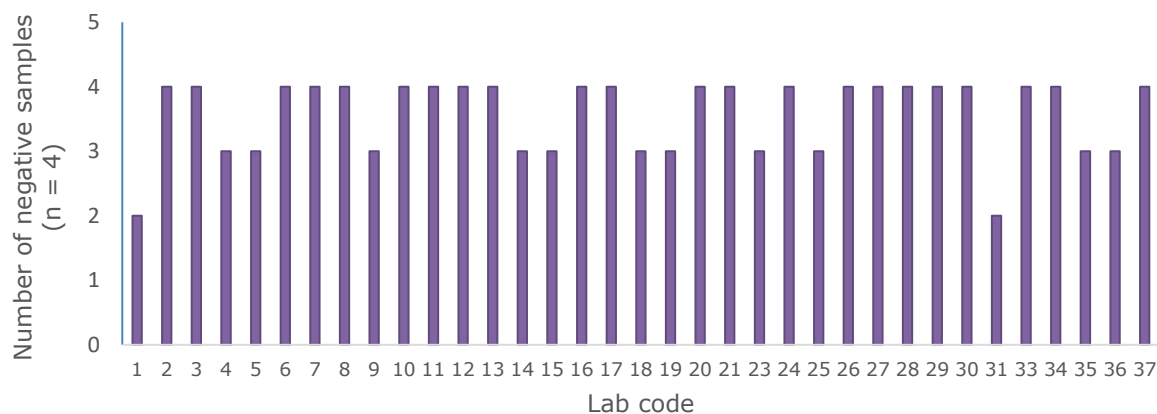


Figure 4.5a Number of negative hygiene swab samples tested negative for *Salmonella*, per laboratory with lab codes 1-37.

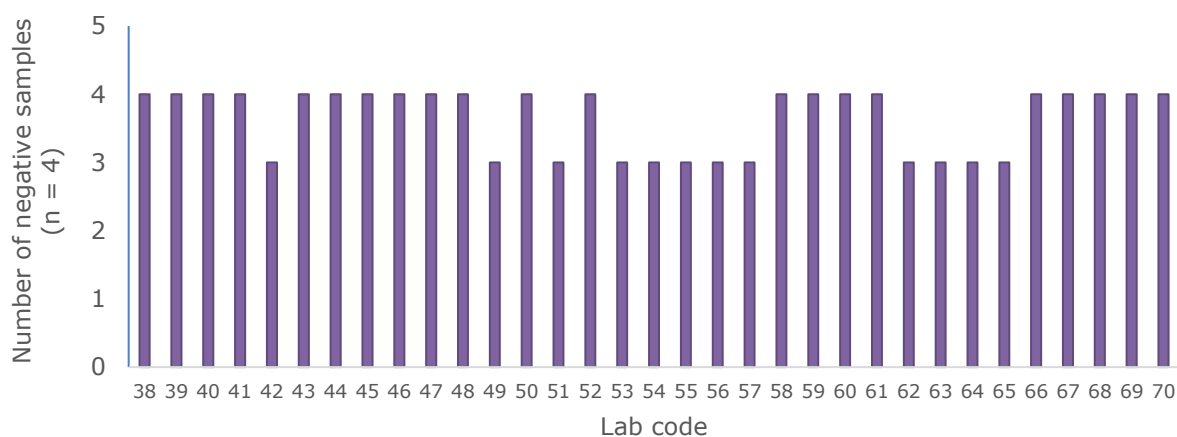


Figure 4.5b Number of negative hygiene swab samples tested negative for *Salmonella*, per laboratory with lab codes 38-70.

However, in total 25 laboratories tested one or two negative samples positive for *Salmonella* (see Table 4.10). Eleven laboratories detected *Salmonella* in sample B1, four laboratories detected *Salmonella* in sample B8, five laboratories detected *Salmonella* in sample B9 and seven 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).

Table 4.10 Number of (in)correct results per hygiene swab sample

Samples	Contamination	# labs Correct	# labs False	Lab codes
<b>B1</b>	Negative (EC/CF)	57	11	4, 5, 18, 19, 23, 31, 51, 56, 57, 64, 65,
<b>B8</b>	Negative (EC/CF)	64	4	25, 31, 49, 54
<b>B9</b>	Negative (EC/CY)	63	5	1, 35, 53, 62, 63
<b>B14</b>	Negative (EC/CY)	61	7	1, 9, 14, 15, 36, 42, 55
<b>B2</b>	Low SI	68		
<b>B4</b>	Low SI	68		
<b>B5</b>	Low SI	68		
<b>B6</b>	Low SI	68		
<b>B7</b>	Low SI	68		
<b>B13</b>	Low SI	67	1	1
<b>B3</b>	High SI/SE	68		
<b>B10</b>	High SI/SE	68		
<b>B11</b>	High SI/SE	68		
<b>B12</b>	High SI/SE	68		
<b>C1</b>	Negative	68		
<b>C2</b>	Positive	68		

E.C = *E. cloacae*; CF = *C. freundii* ; CY = *C. youngae* SI = *S. Infantis*, SE = *S. Enteritidis*

The laboratories which found *Salmonella* in the negative samples were requested to send information and raw data on these samples to investigate the results in more detail.

Information on the second isolation media used by the participants did not reveal any explanation, since the (false) positive results were scored with twelve different media.

Information on the type of *Salmonella* strains used as a positive control showed that nine of the 25 laboratories had used SE as a positive control and sixteen laboratories had used other *Salmonella* serovars. Cross contamination with the positive control strains was therefore not likely.

The EURL-*Salmonella* investigated seven spare samples sets (prepared for the PT, but not used) each containing four negative swab samples, which were kept at 5°C since the preparation of the samples. In all 28 negative samples, *Salmonella* could not be detected.

Some laboratories serotyped their (false) positive samples and reported the presence of SE. In addition, some of these laboratories also sub-typed the strains isolated from the negative samples by using WGS (lab codes 9, 14, 18, 54 and 62). The results were sent to the EURL-*Salmonella*, where the WGS data was compared to WGS data of the *S. Enteritidis* used by the EURL-*Salmonella* to artificially contaminate the hygiene swab samples (Figure 4.7). Results show that the strains isolated from the negative samples by the five participants are identical

to the SE strain used in the high contaminated samples. Laboratory 54 also sent in the WGS data of their SE strain used as a positive control in this PT. The red sphere in the left part of the minimum spanning tree (MST) in Figure 4.6 shows that this strain is not the same as found in the negative samples.

There was a high number of laboratories detecting *Salmonella* in one or two of the negative samples (n=25) and a high number of the total amount of negative samples was found positive (11% = 27 samples out of the 272 total number of negative samples). Therefore, cross contamination in those laboratories was excluded as an explanation for these unusual results. More likely, the contamination of the negative samples may have occurred during the preparation of the samples at the laboratory of the EURL-*Salmonella*. As the contamination was seen throughout all four series of negative samples, it seems most probable that the mixture of background flora has become contaminated with *Salmonella*. Most likely, the *E. cloacae* suspension was contaminated since that strain was used in both background flora mixtures to inoculate the hygiene swab samples. Because almost 90% of the these samples were still negative for *Salmonella*, as well as the extra 28 samples from the spare sets kept at the EURL-*Salmonella*, the contamination level must have been very low. Unfortunately, the original inoculum suspensions were no longer available to verify this explanation.

Due to the high number of false-positive results, the EURL-*Salmonella* decided not to evaluate the negative samples in this PT.

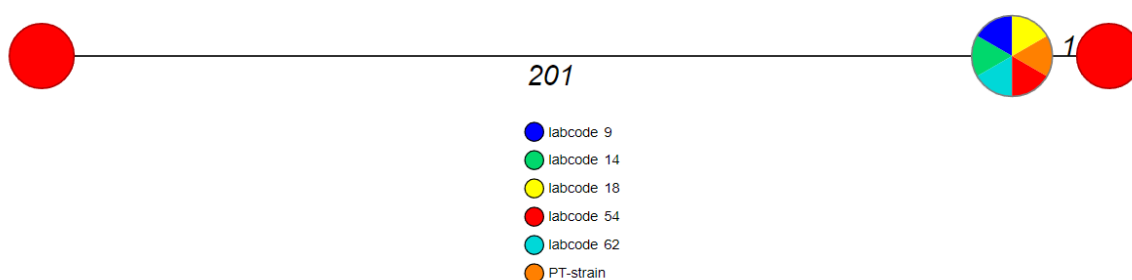


Figure 4.6 Minimum spanning tree (MST) based on the cgMLST analyses of the strains isolated from the negative samples by some of the participants.

#### Hygiene swab samples contaminated with a low level of *Salmonella* Infantis

Almost all participating laboratories were able to detect *Salmonella* in all six hygiene swab samples that were contaminated with a low inoculum level of approximately 8 cfu SI. One laboratory (lab code 1) reported one of the six samples as negative for *Salmonella*. With respect to low-level samples, a negative score for a maximum of three out of six samples is regarded as acceptable. Thus, this laboratory met the criteria

for a good performance score. The results of all participants are shown in Figure 4.7a and Figure 4.7b.

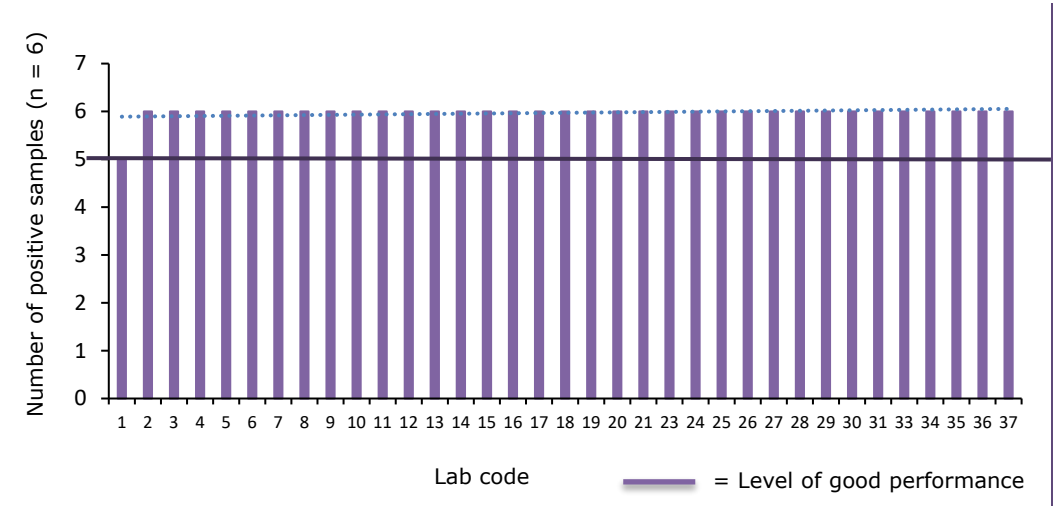


Figure 4.7a Number of positive Salmonella isolations found in the hygiene swab samples contaminated with a low level of Salmonella Infantis (n=6), per laboratory with lab codes 1-37

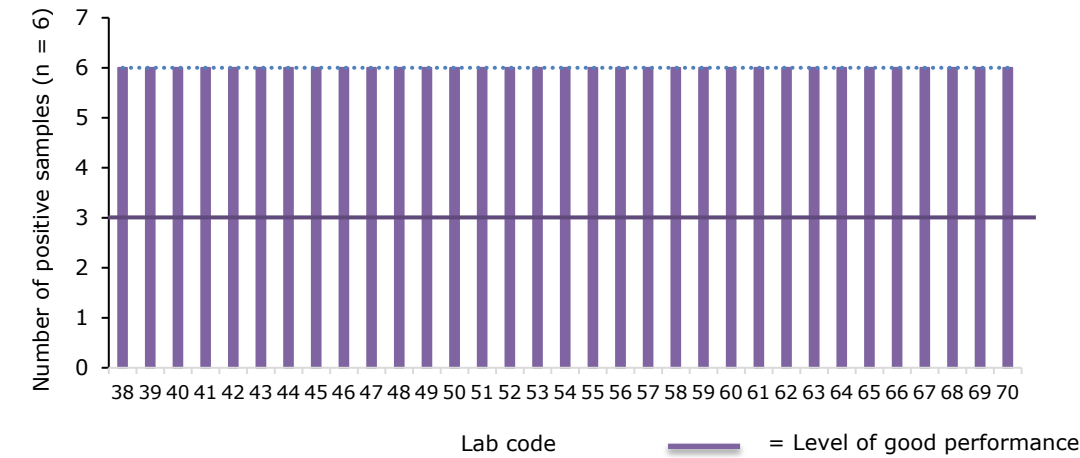


Figure 4.7b Number of positive Salmonella isolations found in the hygiene swab samples contaminated with a low level of Salmonella Infantis (n=6), per laboratory with lab codes 38-70

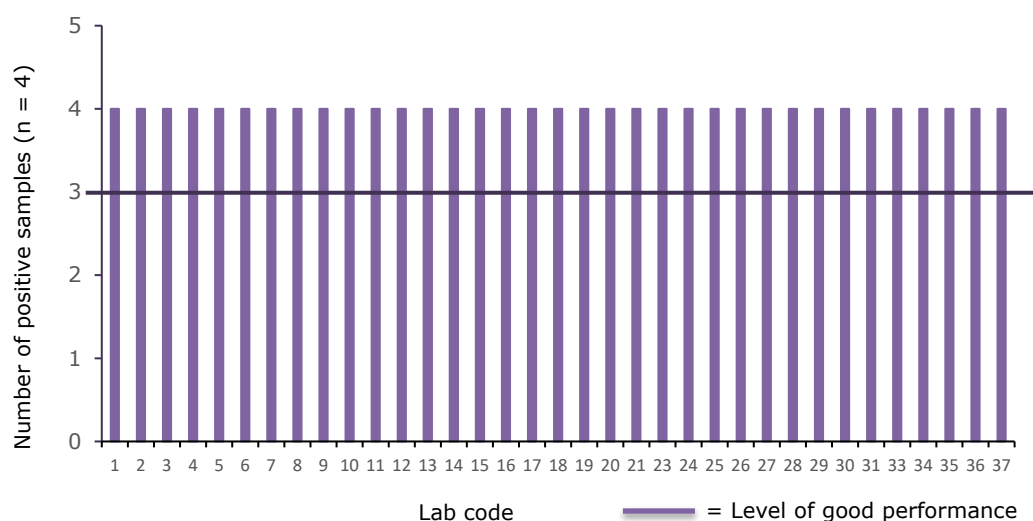


Figure 4.8a Number of positive *Salmonella* isolations found in the hygiene swab samples contaminated with a high level of *Salmonella Infantis* + *Salmonella Enteritidis* (n=4), with lab codes 1-37

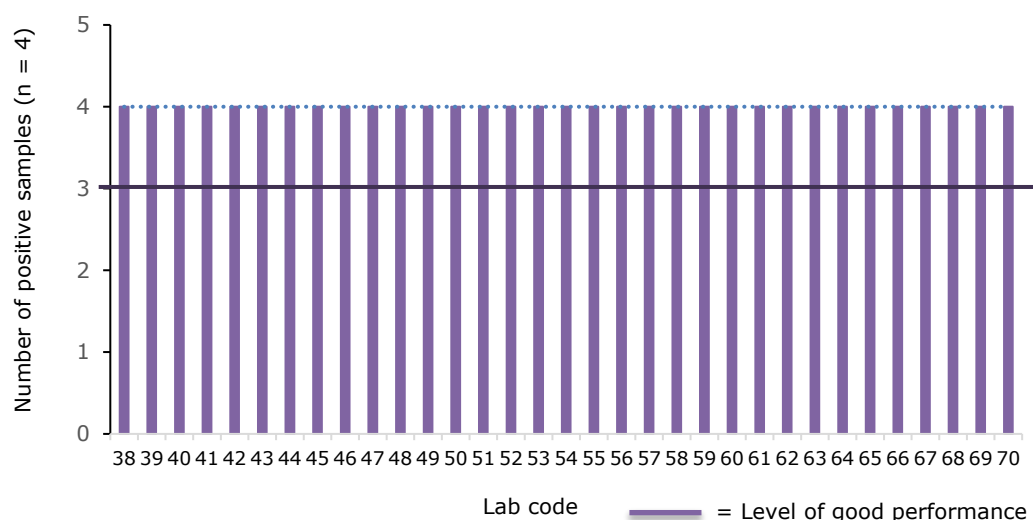


Figure 4.8b Number of positive *Salmonella* isolations found in the hygiene swab samples contaminated with a low level of *Salmonella Infantis* + *Salmonella Enteritidis* (n=4), per laboratory with lab codes 38-70

Hygiene swab samples contaminated with a high level of *Salmonella Infantis* and *Salmonella Enteritidis*

All participating laboratories were able to detect *Salmonella* in all four hygiene swab samples that were contaminated with a high concentration of SI (30 cfu/sample) and SE (8 cfu/sample). The results are shown in Figure 4.8a and Figure 4.8b.

#### 4.4.2 *Specificity, sensitivity and accuracy rates of the artificially contaminated samples*

Table 4.11 shows the specificity, sensitivity and accuracy rates for all artificially contaminated hygiene swab samples. The calculations were performed on the results of all participants and on the results of the EU NRLs PPS and EU NRLs Food separately. All participants performed well in this study: the sensitivity rates (low level: 99,8%; high level 100%) were very high. Hardly any differences were found between all participants and the EU NRLs PPS or EU NRLs Food as shown in Table 4.11. Due to the unexpected high number of 'false' positive samples, the specificity and the accuracy for this PT was somewhat lower than normal. The specificity of the negative samples was 90.1%. The accuracy calculated on basis of the all the hygiene swab samples including the "false" positive samples was 97,1%.

#### 4.4.3 *Second detection method*

In the current PT, 20 laboratories also used a second method to analyse the hygiene swab samples. An overview of the methods used per laboratory can be found in Table 4.12. Almost all laboratories used a PCR method as a second method. Two laboratories used the VIDAS method. Not alle methods were validated or routinely used by the participants. All NRLs found identical results with their second method compared to the prescribed bacteriological culture method.

*Table 4.11 Specificity, sensitivity and accuracy rates found by the participating laboratories (all participants and EU-MS only) with the artificially contaminated Hygiene swab samples*

Hygiene swab samples		All participants n=68	EU NRLs only n=56	EU NRLs PPS n=28	EU NRLs Food n = 28
<b>Negative samples n=4</b>	No. of samples	272	224	112	112
	No. of negative samples	245	204	101	103
	<b>Specificity in %</b>	<b>90,1%</b>	<b>91,1%</b>	<b>90,2%</b>	<b>92,0%</b>
<b>Low level SI n=6</b>	No. of samples	408	336	168	168
	No. of positive samples	407	336	168	168
	<b>Sensitivity in %</b>	<b>99,8%</b>	<b>100%</b>	<b>100%</b>	<b>100%</b>
<b>High level SI + SE n=4</b>	No. of samples	272	224	112	112
	No. of positive samples	272	224	112	112
	<b>Sensitivity in %</b>	<b>100%</b>	<b>100%</b>	<b>100%</b>	<b>100%</b>
<b>All hygiene swab samples with <i>Salmonella</i> n=10</b>	No. of samples	680	560	280	280
	No. of positive samples	679	560	280	280
	<b>Sensitivity in %</b>	<b>99,9%</b>	<b>100%</b>	<b>100%</b>	<b>100%</b>
<b>All hygiene swab samples (pos. and neg.) n=14</b>	No. of samples	952	784	392	392
	No. of correct samples	924	764	381	383
	<b>Accuracy in %</b>	<b>97,1%</b>	<b>97,4%</b>	<b>97,2%</b>	<b>97,7%</b>

Table 4.12 Details on the second detection method used by NRLs-Salmonella during the Proficiency Test PPS-Food 2022

Lab code	Second detection method	Validated (by)	Reference	Routinely # per year
1	Real-Time PCR	National Laboratory Accreditation Authority	ISO 22119:2011(E)	800
2	SureTect Real-Time PCR	Thermo Fisher Scientific	AOAC 051303, AFNOR UNI 03/07-11/13	2000
7	PCR method	No		195
8	National standard NF U 47-100	No		300
13	Real-Time PCR	AFNOR	TRA 02/12-01/09	No
15	qPCR	AFNOR	BRD 07/06 - 07/04	8000
17	qPCR Biorad IQCheck <i>Salmonella</i> II Kit, qPCR Biotecon <i>Salmonella</i> spp + SE +STM LyoKit	AFNOR, NordVal	BRD 07/06 -07/04, NordVal No 055	20
30	VIDAS SLM TEST	AFNOR	BIO 12/10-09/02	No
36	qPCR iQCheck salmonella II BIORAD	NF	BRD 07/06 -07/04	No
37	Real Time PCR (BAX System)	AFNOR	QUA 18/03-11/02	No
40	Real-time PCR	§64 of the National Food and Feed Code	Malorny et al.(2004) AEM 70:7046-7052	252
41	PCR	§64 of the National Food and Feed Code	Malorny et al.(2004) AEM 70:7046-7052	252
43	PCR	No		
47	VIDAS Rapid <i>Salmonella</i>	National Accreditation Board	AFNOR Bio-12/10-09/02	690
51	Real-Time PCR	AFNOR	BRD 07/06-07/04	2000
56	BAX System, standard PCR assay for <i>Salmonella</i> ( a commercial end time PCR-system)	Nordval	Nordval certificate #030	2500
57	BAX System, standard PCR essay for <i>Salmonella</i> ( a commercial end time system).	Nordval	Nordval certificate #030	2500
62	VIDAS UP <i>Salmonella</i> (SPT)	Adria	BIO 12/32 – 10/11	0
66	PCR method	No	Rahn, K. et al (1992): Mol Cell Probes 6 (4), 271-279	No
67	PCR method	No	Rahn, K. et al (1992): Mol Cell Probes 6 (4), 271-279	No

## 4.5 Performance of the NRLs

### 4.5.1 General

All laboratories were able to detect *Salmonella* in high and low concentrations in hygiene swab samples. All 68 laboratories fulfilled the criteria for good performance on the samples containing *Salmonella*. The negative samples were artificially contaminated with two different combinations of background flora. Approx. 11% of the total number of negative samples were scored positive for *Salmonella* by the participants. Investigations showed that the participants found SE in the negative samples, with a WGS profile identical to the strain used in the *Salmonella*-positive PT samples. Although cross contamination cannot be totally excluded, it not realistic in this case considering the large number of laboratories that detected *Salmonella* in the negative samples. Additionally, *Salmonella* positive results were found in all sets of negative samples. Since 90% of the negative samples were negative, as were the 27 spare negative samples tested at the EURL laboratory after the PT, the most likely explanation for these findings is possible contamination of the *E. cloacae* suspension used for both background flora mixtures. Cross contamination of the *E. cloacae* suspension with SE must have been low since only 10% of the negative samples tested positive for *Salmonella*. Given these problems, the results of the negative samples were not evaluated.



## 5 Conclusions

All NRLs for *Salmonella* were able to detect high and low levels of *Salmonella* in hygiene swab samples, and all 68 NRLs scored a 'good performance'.

The results of the negative samples were not evaluated due to possible contamination of the background flora with SE at the EURL laboratory.

All the control samples were scored correctly. The specificity, sensitivity and accuracy rates of the control samples were all 100%.

The sensitivity rate of all laboratories that tested the hygiene swab samples that were artificially contaminated with a low level of SI was 99,8%.

The sensitivity rate of all laboratories that tested the hygiene swab samples that were artificially contaminated with a high level of SI and SE was 100%.

Twenty participants used a second method in addition to the prescribed bacteriological culture method. All laboratories reported identical results for both methods.



## List of abbreviations

AOAC	Association of Official Analytical Chemists
AFNOR	Association Francaise de Normalisation
ASAP	AES <i>Salmonella</i> agar plate
ATCC	American Type Culture Collection
BGA	Brilliant green agar
BGA (mod)	Brilliant green agar (modified)
BPLS	Brilliant green phenol-red lactose sucrose
BPW	Buffered peptone water
BSA	Brilliance <i>Salmonella</i> agar
BxLH	Brilliant green, xylose, lysine, sulphonamide
CF	<i>Citrobacter freundii</i>
cfu	Colony-forming units
cgMLST	core genome MultiLocus Sequence Typing
CY	<i>Citrobacter Youngae</i>
DG-SANTE	Directorate-General for Health and Consumer Protection
EC	<i>Enterobacter cloacae</i>
EFTA	European Free Trade Association
EN	European Standard
EU	European Union
EURL	European Union Reference Laboratory
ISO	International Organization for Standardization
MKTTn	Mueller Kauffmann tetrathionate with novobiocin (broth)
MPN	Most Probable Number
MS	Member State
MSRV	Modified semi-solid Rappaport-Vassiliadis
MST	Minimum spanning tree
NRL	National Reference Laboratory
PCR	Polymerase chain reaction
PPS	Primary Production Stage
PS	Peptone saline solution
PT	Proficiency Test
RIVM	Rijksinstituut voor Volksgezondheid en Milieu (National Institute for Public Health and the Environment)
RSAL	unknown abbreviation
RVS	Rappaport Vassiliadis with soya (broth)
SI	<i>Salmonella</i> Infantis
SE	<i>Salmonella</i> Enteritidis
SM2	<i>Salmonella</i> detection and identification-2
TSA	Tryptone soya agar
UK	United Kingdom
VRBG	Violet red bile glucose
WGS	Whole Genome Sequencing
XLD	Xylose lysine deoxycholate



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## Annex I Example of an individual laboratory Performance Report of the combined EURL-*Salmonella* PT PPS-Food 2022

# Performance



## EURL-*Salmonella* PT PPS-Food 2022

Number of positive samples/Total number of samples per level

Labcode	Hygiene swab samples			control samples	
	High	Low	Negative	BPW	pos control
# NRL PPS	4/4	6/6	0/4	0/1	1/1

### Evaluation: Good performance

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

Number	Level	Your result	Second method	Media choices:
B1	Negative	Not detected	Not Detected	MSRV
B2	Low	Detected	Detected	XLD/ BGA
B3	High	Detected	Detected	
B4	Low	Detected	Detected	
B5	Low	Detected	Detected	
B6	Low	Detected	Detected	
B7	Low	Detected	Detected	
B8	Negative	Not detected	Not Detected	
B9	Negative	Not detected	Not Detected	
B10	High	Detected	Detected	
B11	High	Detected	Detected	
B12	High	Detected	Detected	
B13	Low	Detected	Detected	
B14	Negative	Not detected	Not Detected	
C1	Negative	Not detected	Not Detected	
C2	Positive	Detected	Detected	

Negative = B1 and B8 (*E. cloacae/C. freundii* 10<sup>5</sup> cfu)

B9 and B14 (*E. cloacae/C. youngae* 10<sup>5</sup> cfu)

Low = Low conc. of *S. Infantis* (8 cfu) + *E. cloacae/C. freundii* (10<sup>5</sup> cfu)

High = High conc. of *S. Infantis* (40 cfu) + *S. Enteritidis* (10 cfu) + *E. cloacae/C. freundii* (10<sup>5</sup> cfu)

BPW = Buffered Peptone Water

Pos control = own positive control

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