National Institute for Public Health
and the Environment
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EU Interlaboratory comparison study food VII (2015)

Detection of *Salmonella* in whole liquid chicken egg

RIVM Report 2016-0042

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Colophon

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Synopsis

EU Interlaboratory comparison study food VII (2015)
Detection of *Salmonella* in whole liquid chicken egg

In 2015, it was shown that all 36 National Reference Laboratories (NRLs) in the European Union were able to detect high and low levels of *Salmonella* in whole liquid chicken egg. One NRL reported positive results for two blank whole liquid egg samples, a possible explanation may be cross-contamination. In a follow-up study this laboratory scored all samples correctly and achieved the level of good performance. The laboratories detected *Salmonella* in all contaminated samples. In this report we present some of the conclusions of the 7th EU Interlaboratory Comparison Study of Food Samples, which was organised by the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*).

Interlaboratory comparison study obligatory for EU Member States
The study was conducted in September 2015, with a follow-up study in January 2016. Participation was obligatory for all EU Member State NRLs that are responsible for the detection of *Salmonella* in food samples. EURL-*Salmonella* is part of the Dutch National Institute for Public Health and the Environment (RIVM).

The laboratories used internationally accepted analysis methods to detect the presence of *Salmonella* in whole liquid chicken egg samples. Each laboratory received a package with whole liquid egg samples contaminated with two different concentrations of *Salmonella* Enteritidis or containing no *Salmonella* at all. The laboratories were required to analyse the samples for the presence of *Salmonella* in accordance with the study protocol.

Preparation of samples
In this study, for the first time, liquid egg samples were used that had been artificially contaminated with a diluted culture of *Salmonella* at the laboratory of EURL-*Salmonella*. The results showed that this method of contamination, which had been used and tested in earlier studies involving food matrices (meat), animal feed and products of the primary production stage, could also be used for whole liquid egg.

Keywords: *Salmonella*, EURL, NRL, interlaboratory comparison study, *Salmonella* detection method, whole liquid chicken egg

Publiekssamenvatting

EU Ringonderzoek voedsel VII (2015)
Detectie van *Salmonella* in vloeibaar heel kippenei

In 2015 waren alle 36 Nationale Referentie Laboratoria (NRL's) in de Europese Unie in staat om concentraties *Salmonella* in vloeibaar kippenei aan te tonen, zowel bij hoge als lage concentraties. Eén NRL slaagde er niet in om alles de eerste keer goed uit te voeren, maar wel tijdens de herkansing. Dit blijkt uit het zevende voedselringonderzoek dat is georganiseerd door het referentielaboratorium van de Europese Unie voor *Salmonella* (EURL-*Salmonella*).

Ringonderzoek verplicht voor Europese lidstaten
Het onderzoek is in september 2015 gehouden, de herkansing was in januari 2016. Alle NRL's van de Europese lidstaten die verantwoordelijk zijn voor de opsporing van *Salmonella* in voedsel, zijn verplicht om aan het onderzoek deel te nemen. Het EURL-*Salmonella* is gevestigd bij het Nederlandse Rijksinstituut voor Volksgezondheid en Milieu (RIVM).

De laboratoria toonden de *Salmonella*-bacterie in kippenei aan met behulp van de internationaal erkende analysemethoden. Elk laboratorium kreeg een pakket toegestuurd met vloeibaar kippenei dat ofwel besmet was met *Salmonella* Enteritidis in twee verschillende concentraties, of geen *Salmonella* bevatte. De laboratoria dienden volgens een protocol te onderzoeken of de monsters *Salmonella* bevatten.

Monsterbereiding
In eerdere studies zijn voedsel (gehakt), dierlijke mest en dierenvoer op het laboratorium van het EURL-*Salmonella* kunstmatig besmet met een verdunde cultuur van *Salmonella.* In deze studie is voor het eerst vloeibaar kippenei kunstmatig besmet en is bewezen dat ook deze werkwijze geschikt is.

Kernwoorden: *Salmonella,* EURL, NRL, ringonderzoek, vloeibaar heel kippenei, *Salmonella*-detectiemethode

Contents

Summary

In September 2015 the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*) organized the 7th Interlaboratory Comparison Study on the detection of *Salmonella* in samples from food. The matrix of concern was whole liquid chicken egg. The participants were 36 National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*): 30 NRLs from the 28 EU Member States (EU-MS), 5 NRLs from third countries within Europe (EU candidate MS, potential EU candidate MS or members of the European Free Trade Association (EFTA)) and 1 NRL from a non-European country.

The most important objective of the study was to test the performance of the participating laboratories for the detection of *Salmonella* at different contamination levels in a food matrix. For this purpose, 25 g samples of whole liquid chicken egg, artificially contaminated with *Salmonella* Enteritidis (SE) at various contamination levels, were analysed. The performance of the laboratories was compared with the criteria for good performance.

The participants were not sent a Standard Operating Procedure (SOP) but were asked to follow the normal procedure for detection of *Salmonella* in official samples, following ISO/FDIS 6579-1 (Anonymous, 2015). According to this document, Mueller Kauffmann Tetrathionate novobiocin broth (MKTTn) is prescribed as the first selective enrichment medium; for the second selective enrichment medium either Rappaport Vassiliadis Soya broth (RVS) or Modified Semi-solid Rappaport-Vassiliadis (MSRV) can be used.

The participants were asked to report the results as they would for routine samples, so that the indication 'positive' (1) or 'negative' (0) for each sample (after confirmation) was sufficient (irrespective of the combination of selective enrichment medium and isolation medium). Hence, the results per medium are not visible to EURL-*Salmonella*, as they were in previous studies of this type.

The samples consisted of whole liquid chicken egg artificially contaminated with a diluted culture of *Salmonella* Enteritidis (SE) at a low level (approximately 15–20 CFU/25 g liquid egg) and at a high level (approximately 50–100 CFU/25 g liquid egg) and whole liquid chicken egg with no *Salmonella* at all (blank samples). The samples were artificially contaminated in the laboratory of the EURL for *Salmonella*. Before the start of the study, several experiments were carried out to make sure that the samples were fit for use in an interlaboratory comparison study (e.g. tests on alternative *Salmonella* serovars, assessment of stability at different storage temperatures and influence of background flora).

Eighteen individually numbered blind whole liquid egg samples were to be tested by the participants for the presence or absence of *Salmonella*. These samples consisted of six blank samples, six samples with a low level of SE (inoculum 21 CFU/sample) and six samples with a high level

of SE (inoculum 101 CFU/sample). Additionally, one blank control sample (procedure control (BPW only)) and one own (NRL) positive control sample (with *Salmonella*) were to be tested.
The laboratories found *Salmonella* in all (contaminated) samples, resulting in a sensitivity rate of 100%.

PCR was used as an own method by 11 participants, all of which obtained the same results as with the bacteriological culture method; most of them (10) used a real-time PCR.

Most participants (27) used all three selective enrichment media (MKTTn, MSRV and RVS) and 9 laboratories used either RVS or MSRV in addition to MKTTn.

The *Salmonella* serovars most frequently used for the positive control sample were *S*. Enteritidis (16) and *S*. Typhimurium (8). The majority of participants (21) used a diluted culture of *Salmonella*. The concentration of the positive control varied between 5 and 10^7 CFU/sample. For the positive control it is advisable to use a concentration close to the detection limit of the method and a *Salmonella* serovar not often isolated from routine samples.

The egg samples needed to be stored at 5 °C after receipt at the participating laboratory. Unfortunately, this was not always the case and temperatures up to 10 °C were detected, which can make the samples less stable and make it more difficult to detect *Salmonella*.

In this study for the first time, liquid egg samples were used that had been artificially contaminated with a diluted culture of *Salmonella* at the laboratory of EURL-*Salmonella*. The results showed that this method of contamination, which had been used and tested in earlier studies involving food matrices (meat), animal feed and products of the primary production stage, could also be used for whole liquid egg.

This study shows not only that each matrix and *Salmonella* serovar combination may behave differently but also that different batches of the same matrix may contain different levels of background flora, which may influence the growth of *Salmonella*. For each new study the challenge is therefore to produce stable samples, especially when a new matrix is used.

For the experiments prior to the interlaboratory study and for the study itself, a total of four batches of whole liquid chicken egg were tested. The level of background flora in the batch used in the main study was much lower than that in the batches used in the pre-tests. This made it easier to detect *Salmonella* in the samples used in the interlaboratory study than in the samples used in the pre-tests.

Thirty-five out of the 36 laboratories achieved the level of good performance in the main study. One NRL reported two positive results for a blank whole liquid egg sample; a possible explanation is cross-contamination. In a follow-up study this laboratory scored all samples correctly and also achieved the level of good performance.

1 Introduction

An important task of the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*), as laid down in Commission Regulation EC No. 882/2004 (EC, 2004), is the organization of interlaboratory comparison studies to test the performance of the National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*). The history of the interlaboratory comparison studies on the detection of *Salmonella*, as organized by EURL-*Salmonella* (formerly called CRL-*Salmonella*) since 1995, is summarized on the EURL-*Salmonella* website (EURL-*Salmonella*, 2016).
The objective of the current study, organised by EURL-*Salmonella* in October 2015, was to see whether the participating laboratories could detect *Salmonella* in whole liquid chicken egg at different contamination levels. This information is important in order to know whether the examination of samples is being carried out uniformly in all EU Member States (MS) and whether comparable results are obtained by all NRLs-*Salmonella*.

The participants were asked to follow the normal procedure for detection of *Salmonella* in official samples, by using ISO/FDIS 6579-1 (Anonymous, 2015). According to this document, Mueller Kauffmann Tetrathionate novobiocin broth (MKTTn) is prescribed as the first selective enrichment medium; as the second selective enrichment medium either Rappaport Vassiliadis Soya broth (RVS) or Modified Semi-solid Rappaport-Vassiliadis (MSRV) can be used.

The set-up of this food study was comparable to the interlaboratory comparison studies organised in 2013 and 2014 on the detection of *Salmonella* in minced chicken meat (Kuijpers et al., 2014) and animal feed (Kuijpers et al., 2015). For the current study, the (food) samples were artificially contaminated with a diluted culture of *Salmonella* Enteritidis (SE) at the laboratory of EURL-*Salmonella.*

As in earlier studies, the contamination level of the low-level samples was close to the detection limit of the method and the level of the high-level samples was approximately 5–10 times the detection limit. In total, 18 liquid chicken egg samples were tested: 6 samples per contamination level (blank, low-level and high-level) containing one *Salmonella* serovar (*Salmonella* Enteritidis) or no *Salmonella* (blank). Additionally, two control samples (one blank control sample and one positive control sample) were tested. The number and level of samples tested were in accordance with ISO/TS 22117 (Anonymous, 2010).

2 Participants

Country	City	Institute / NRL-*Salmonella*
Austria	Graz	Austrian Agency for Health and Food Safety (AGES),Institute for Medical Microbiology and Hygiene (IMED)
Belgium	Brussels	Institute of Public Health Lab of Food Pathogens (WIV-ISP)
Bulgaria	Sophia	National Diagnostic Research Veterinary Institute (NDRVMI), National Reference Centre of Food Safety
Croatia	Zagreb	Croatian Veterinary Institute, Lab for Food Microbiology
Cyprus	Nicosia	Ministry of Agriculture, Natural Resources and Environment Veterinary Services Laboratory for the Control of Foods of Animal Origin (LCFAO)
Czech Republic	Prague	State Veterinary Institute (SVI)
Denmark	Ringsted	Danish Veterinary and Food Administration (DVFA-1), Microbiology Ringsted
Estonia	Tartu	Estonian Veterinary and Food Laboratory
Finland	Helsinki	Finnish Food Safety Authority Evira Research Department, Microbiology Unit
France	Ploufragan	Anses Laboratoire de Ploufragan-Plouzané, Unité Hygiène et Qualité des Produits Avicoles et Porcins (HQPAP)
Germany	Berlin	Federal Institute for Risk Assessment (BFR)
Greece	Halkis	Veterinary Laboratory of Chalkis, Hellenic Republic, Ministry of Reconstruction of Production, Environment and Energy
Hungary	Budapest	National Food Chain Safety Office, Food and Feed Safety Directorate
Iceland	Reykjavik	Matis ohf, Icelandic Food and Biotech R&D
Ireland	Kildare	Central Veterinary Research Laboratory CVRL/DAFM Backweston, Department of Agriculture, Food and Marine
Israel	Kiryat Malachi	Southern Laboratory for Poultry Health, Laboratory Egg and Poultry
Italy	Legnaro PD	Istituto Zooprofilattico Sperimentale delle Venezie, OIE
Latvia	Riga	Institute of Food Safety, Animal Health and Environment, BIOR Animal Disease Diagnostic Laboratory

Country	City	Institute / NRL-*Salmonella*
Lithuania	Vilnius	National Food and Veterinary Risk Assessment Institute, Food Microbiology Section
Luxembourg	Dudelange	Laboratoire National de Santé, Département des Laboratoires officiels d'analyses de contrôle
Macedonia, FYR of	Skopje	Faculty of Veterinary Medicine UKIM, Food Institute
Malta	Valletta	Public Health Laboratory (PHL), Microbiology Evans Building
Netherlands	Bilthoven	National Institute for Public Health and the Environment (RIVM/CIb) Infectious Disease Control, Centre for Zoonoses and Environmental Microbiology (cZ&O)
Netherlands	Wageningen	Netherlands Food and Consumer Product Safety Authority (nVWA) Consumer and Safety Division, Microbiology
Norway	Oslo	Norwegian Veterinary Institute, Bacteriology Section
Poland	Pulawy	National Veterinary Research Institute (NVRI), Department of Hygiene of Food of Animal Origin
Portugal	Vairao	Instituto National de Investigação Agrária e Veterinária Unidade de Tecnologia e Segurança Alimentar (INIAV)
Romania	Bucharest	Hygiene and Veterinary Public Health Institute (IISPV)
Serbia	Belgrade	Institute of Veterinary Medicine of Serbia, Department of Food Hygiene
Slovak Republic	Bratislava	State Veterinary and Food Institute
Slovenia	Ljubljana	National Veterinary Institute, Veterinary Faculty (UL)
Spain	Madrid, Majadahonda	Ministerio de Sanidad, Servicios Sociales e Igualdad Servicio de Microbiologia Alimentaria CNA – AECOSAN Food Microbiology
Sweden	Uppsala	National Veterinary Institute (SVA), Department of Bacteriology
Turkey	Ankara	Veterinary Control Central Research Institute, Bacteriological Diagnosis Laboratory
United Kingdom	Birmingham	Public Health England (PHE) Food Water and Environmental Microbiology, Birmingham Laboratory
	Belfast	Agri-Food and Bioscience Institute (AFBI) Veterinary Sciences, Division Bacteriology

3 Materials and methods

3.1 Whole liquid chicken egg

3.1.1 General

The matrix in this interlaboratory comparison study was pasteurised whole liquid chicken egg. The whole liquid egg was obtained from the retail sector. It was produced by the Global Food Group in Ospel in the Netherlands. For the pre-tests, two whole liquid egg batches (each 10 kg) were tested. For the interlaboratory comparison study, a batch of 30 kg (3 buckets of 10 l) pasteurised whole liquid chicken egg was used. This last batch arrived at EURL-*Salmonella* on 8 September 2015. For the follow-up study a fourth batch of 10 kg whole liquid egg was used, arriving at EURL-*Salmonella* on 11 January 2016.

Immediately after receipt of the liquid egg, 5 samples (for the pre-test) or 10 samples (for the interlaboratory comparison study) of 25 g each were checked for the absence of *Salmonella* in accordance with ISO 6579 (Anonymous, 2002) and Annex D of ISO 6579 (Anonymous, 2007). For this purpose, 225 ml of Buffered Peptone Water (BPW) was added to each of the 25 g samples. After pre-enrichment at 37 (± 1) °C for 16 to 18 hours, selective enrichment was carried out in Rappaport-Vassiliadis Soya broth (RVS) and Mueller Kaufmann Tetrathionate novobiocin broth (MKTTn) and on Modified Semi-solid Rappaport-Vassiliadis (MSRV) agar. Next, the MKTTn and RVS tubes and the suspect growth on MSRV plates were plated out on Brilliance *Salmonella* Agar (BSA) and confirmed biochemically.

After verifying the absence of *Salmonella*, the liquid egg was repacked in portions of 25 g in Whirl-Pak plastic bags and stored at 5 °C (see Section3.3.1).

3.1.2 Total bacterial count in whole liquid egg

The total number of Aerobic bacteria in the liquid egg was investigated by following ISO 4833 (Anonymous, 2003a). A 20 g portion of liquid egg was homogenized in 180 ml of peptone saline solution in a plastic bag. The content was mixed by using a Stomacher (for 60 seconds). Next, tenfold dilutions were prepared in peptone saline solution and 1 ml of each dilution was placed in each of two empty Petri dishes (9 cm diameter). To each dish, 15 ml of molten Plate Count Agar (PCA) was added. After the PCA had solidified, an additional 5 ml of PCA was added to the dishes. The plates were incubated at (30 ± 1) °C for (72 ± 3) hours and the total number of Aerobic bacteria was counted after incubation.

3.1.3 Number of Enterobacteriaceae *in whole liquid egg*

In addition to the total number of Aerobic bacteria, the *Enterobacteriaceae* count was determined by following ISO 21528-2 (Anonymous, 2004). A 20 g portion of the liquid egg was homogenized in 180 ml of peptone saline solution in a plastic bag. The contents were mixed using a Stomacher (for 60 seconds). Next, tenfold dilutions were prepared in peptone saline solution and 1 ml of each dilution was placed

in each of two empty Petri dishes (9 cm diameter). To each dish, 10 ml of molten Violet Red Bile Glucose agar (VRBG) was added. After the VRBG had solidified, an additional 15 ml of VRBG was added to the dishes. These plates were incubated at (37 ± 1) °C for (24 ± 2) hours and the number of typical violet-red colonies was counted after incubation. Five typical colonies were tested for the fermentation of glucose and for a negative oxidase reaction. After this confirmation, the number of *Enterobacteriaceae* was calculated.

3.2 Artificial contamination of liquid egg samples

3.2.1 Pre-tests for the preparation of contaminated liquid egg samples

The liquid egg samples were artificially contaminated at the laboratory of EURL-*Salmonella* with a diluted culture of *Salmonella*. Some experiments were performed prior to the start of the interlaboratory comparison study, especially in relation to the stability of *Salmonella* in the artificially contaminated liquid egg samples when stored at different temperatures.

For the contamination, two different *Salmonella* Enteritidis (SE) strains were tested: SEa from chicken product and SEb from chicken egg. Each strain was inoculated in Buffered Peptone Water (BPW) and incubated at (37 ± 1) °C overnight. Next, each culture was diluted in peptone saline solution to be able to inoculate the liquid egg samples with approximately 5-10 CFU/sample and 50–100 CFU/sample. For the enumeration of the contamination level (CFU/ml), 0.1 ml of the diluted culture was spread over an XLD plate and incubated at 37 °C for 20–24 hours.

Samples of 25 g liquid egg were artificially contaminated with a dilution of a *Salmonella* culture (different levels of SE). Some control samples were also prepared without the addition of *Salmonella* (blank samples). All liquid egg samples were stored at 5 °C and at 10 °C for a period of 0, 7, 14 and 21 days. After each storage time at the different temperatures, the artificially contaminated SE and blank liquid egg samples were tested for the presence of *Salmonella* following Annex D of ISO 6579 (Anonymous, 2007), with selective enrichment on MSRV agar and, for some samples, also with selective enrichment in RVS and MKTTn broth following ISO 6579 (Anonymous, 2002).

To obtain an indication of the amount of background flora in the samples after storage at different temperatures, the blank liquid egg samples (without the addition of *Salmonella*) were tested for the number of Aerobic bacteria and *Enterobacteriaceae* (see Sections 3.1.2 and 3.1.3).

3.2.2 Determination of contamination level in liquid egg samples by MPN

The level of contamination in the final liquid egg samples, as used at the time of the study, was determined by using a five-tube, most probable number (MPN) technique. For this purpose, tenfold dilutions of five liquid 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 Annex D of ISO 6579 (Anonymous, 2007) and ISO 6579 (Anonymous, 2002). From the number of confirmed positive dilutions, the MPN of *Salmonella* in the

original sample was calculated by using an MPN software program in Excel, freely available on the internet (Jarvis et al., 2010).

3.3 Design of the interlaboratory comparison study

3.3.1 Whole liquid chicken egg samples

Approximately two weeks before the study, a total of 810 liquid egg samples were prepared. For this purpose, the following steps were performed:

- labelling of each plastic bag;
- adding 25 g of whole liquid chicken egg to each plastic bag;
- adding approximately 0.1 ml of a diluted culture of *S*. Enteritidis (SE) to the egg sample. The contamination levels aimed at were 10–15 CFU/25 g liquid egg, 50–100 CFU/25 g liquid egg and blank;
- storage of samples at 5 °C until transport to the NRLs on 21 September 2015.

On 14 September 2015 (one week before the study), the egg samples were prepared for shipment (see Section 3.3.2) and sent to the participants by door-to-door courier service. After arrival at the laboratories, the egg samples were to be stored at 5 °C until the start of the study.

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*, 2015a) and in a print-out from the web-based test report (EURL-*Salmonella*, 2015b).

Eighteen egg samples (numbered B1–B18) and two control samples (numbered C1 and C2) had to be tested by each participant. Table 1 gives an overview of the number and type of samples to be tested by the participants.

For the control samples, the laboratories were asked to use their own positive *Salmonella* control, which they normally use when analysing samples for the detection of *Salmonella*. In addition to this, one blank control of the BPW had to be analysed.

Table 1. Overview of the number and type of samples tested per laboratory in the interlaboratory comparison study

Contamination level	**Test samples with whole liquid egg (n=18)**
S. Enteritidis low level (SE low)	6
S. Enteritidis high level (SE high)	6
Blank (BL)	6
	Control samples (n=2)
Positive control (own control with *Salmonella*)	1
Blank procedure control (BPW only)	1

3.3.2 Shipment of parcels and temperature recording during shipment

Twenty plastic bags were sent to each NRL, containing the egg samples artificially contaminated with *Salmonella*, blank liquid egg samples and controls (no egg at all). The 20 bags were packed in one plastic safety bag. The safety bag was placed in one large shipping box, together with three frozen (-20 °C) cooling devices. Each shipping box was sent to the participants as 'biological substances category B (UN3373)' using a door-to-door courier service. To monitor exposure to excessive temperatures during shipment and storage, micro temperature loggers were used to record the temperature during transport. These loggers are tiny units sealed in a stainless steel case 16 mm in diameter and 6 mm deep. Each shipping box contained one logger packed in the safety bag. The loggers were programmed by EURL-*Salmonella* to measure the temperature every hour. Each NRL had to return the temperature recorder to EURL-*Salmonella* on the day the laboratory started the study. At EURL-*Salmonella,* the loggers were read using a computer program and all recorded temperatures from the start of the shipment until the start of the study were transferred to an Excel sheet.

3.4 Methods

The NRLs were to follow the procedures used for routine analyses (e.g. pre-warming of BPW, different ways of mixing the samples in BPW). For the pre-treatment of the liquid egg samples, reference was made to ISO 6887-4 (Anonymous, 2003b). According to this ISO, the liquid egg diluted in BPW needs to be homogenized by manual shaking.
The NRLs were asked to follow ISO/FDIS 6579-1 (Anonymous, 2015) for the detection (and confirmation) of *Salmonella*, and the underlying EN ISO documents, e.g. the EN ISO 6887 series, for preparation of test samples. ISO/FDIS 6579-1 describes the (final) updated technical steps for the detection of *Salmonella* in food, animal feed and samples from the primary production stage.

An important change in this document from the current version of ISO 6579 (Anonymous, 2002) is the option to choose between RVS and MSRV for the selective enrichment of *Salmonella* from food and animal feed samples. This option was also available in the current study, meaning that, in addition to MKTTn, either RVS or MSRV could be used for selective enrichment. The NRLs were also permitted to use all three selective enrichment media. In addition, the NRLs could use their own method, such as a Polymerase Chain Reaction (PCR) procedure. The method in summary:

- Pre-enrichment in:
  - Buffered Peptone Water (BPW);
- Selective enrichment in/on:
  - Mueller Kaufmann Tetrathionate novobiocin broth (MKTTn);
  - Rappaport Vassiliadis Soya broth (RVS) and/or
  - Modified Semi-solid Rappaport-Vassiliadis medium (MSRV);
- Plating-out on the following isolation media:
  - first plating-out: Xylose Lysine Desoxycholate agar (XLD);
  - second plating-out (obligatory): medium of choice;
- Confirmation by means of:

- appropriate biochemical tests (ISO/FDIS 6579-1, Anonymous, 2015) or reliable, commercially available identification kits and/or serological tests.

3.5 Statistical analysis of the data

The specificity, sensitivity and accuracy rates were calculated for the artificially contaminated liquid egg samples. For the control samples, only the accuracy rates were calculated. The rates were calculated according to the following formulae:

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

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

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

3.6 Good performance

For the determination of 'good performance', the criteria indicated in Table 2 were used. For the determination of good performance per laboratory, the results obtained with all combinations of selective enrichment media and isolation media used by the laboratory were taken into account.

Table 2. Criteria for testing good performance in the food VII study (2015)

Minimum result		
Contamination level	Percentage positive	No. of positive samples/ total no. of samples
Samples Whole liquid egg artificially contaminated		
S. Enteritidis high level (SE high)	80%	5/6
S. Enteritidis low level (SE low)	50%	3/6
Blank (BL)[1]	20% max.[1]	1/6 max.[1]
Control samples		
Positive control (own control with *Salmonella*)	100%	1/1
Procedure control (BPW only)	0%	0/1

1. All should be negative. However, as no 100% guarantee of the *Salmonella* negativity of the matrix can be given, 1 positive out of 6 blank samples (20% pos.) is considered acceptable.

4 Results

4.1 Whole liquid chicken egg

All batches of liquid egg were tested negative for *Salmonella.* The number of Aerobic bacteria and the number of *Enterobacteriaceae* were tested twice at the laboratory of EURL-*Salmonella*: first on the day the liquid egg arrived (08/09/2015), the second time after storage at 5 °C for two weeks (22/09/2015). Table 3 summarizes the results of these tests, showing that the amount of background flora remained stable (or increased) during storage at 5 °C.

Table 3. Number of Aerobic bacteria and number of Enterobacteriaceae *per gram of whole liquid chicken egg*

Date	*Enterobacteriaceae* CFU/g	Aerobic bacteria CFU/g
8 September 2015	< 10	$5*10^1$
22 September 2015 (stored at 5 °C)	< 10	$5*10^3$

4.2 Artificial contamination of liquid egg samples

4.2.1 Pre-tests for the preparation of liquid egg samples

Two sets of experiments were performed. For each set of experiments, the stability of *Salmonella* in the egg samples was tested during storage of the samples at different temperatures for up to three weeks. During each set of experiments, different variables were tested in different combinations (see Section 3.2.1). Table 4 shows the results of the tests on all samples. Figure 1 shows the results for the selective enrichment medium giving the lowest number of positive samples (RVS) of low contaminated egg samples.

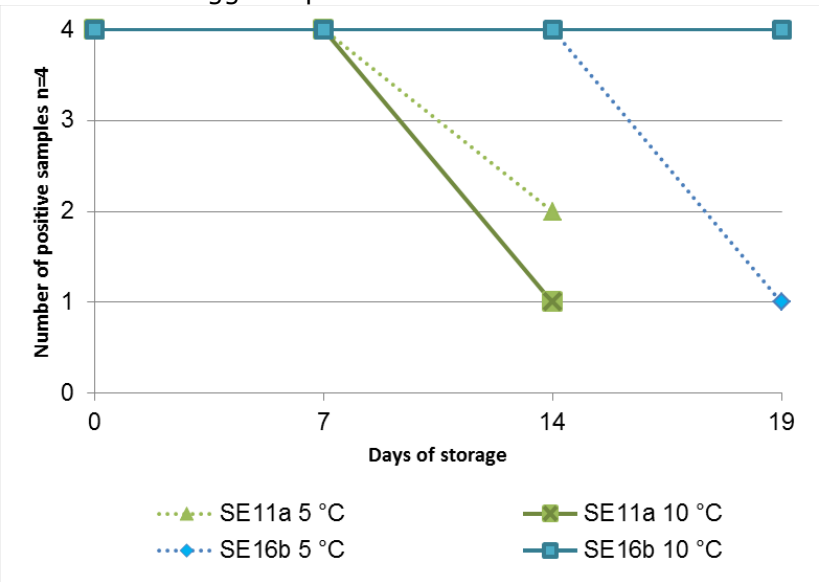


Figure 1. Stability test of liquid egg samples (n=4) artificially contaminated with two different strains of Salmonella Enteritidis (SEa and SEb) at low levels of 11 and 16 CFU/25g

Table 4. Stability tests of whole liquid chicken egg artificially contaminated with Salmonella *Enteritidis (SE)*

Days of storage	**Storage at 5 ºC**				**Storage at 10 ºC**			
	Batch 1 whole liquid egg							
	SE11a n=4	**SE16b** n=4	**SE182a** n=4	**SE74b** n=4	**SE11a** n=4	**SE16b** n=4	**SE182a** n=4	**SE74b** n=4
number of positive samples								
0	4	4	4	4	4	4	4	4
7	4	4	4	4	4	4	4	4
14	4/3/2	4	4	4	3/3/1	4	4	4
19 or 21		2/1/1		4/4/3		4		4
	Batch 2 whole liquid egg							
	SE16b n=6		**SE80**b n=6		**SE16**b n=6		**SE80**b n=6	
number of positive samples								
0	6		6		6		6	
7	6		6		6		6	
14	6		6		6		6	
19 or 21	6		6		6		6	

The number of positive samples tested is given as the results obtained after selective enrichment in MKTTn/MSRV/RVS. When only one figure is given, all three media gave the same result. Indicated are the strains and contamination levels in the liquid egg. For example, SE80 indicates *Salmonella* Enteritidis at a level of 80 CFU/25 g of liquid egg. Two different strains of *Salmonella* Enteritidis were used, marked as SEa (chicken product) and SEb (chicken egg).

The background flora

For the stability test two separate batches of whole liquid egg were used (referred to in Table 4 and Figure 2 as Batches 1 and 2).

After 7 days of storage at 5 °C and 10 °C the number of Aerobic bacteria had increased from 10^3–10^4 to 10^8–10^9 CFU/g in both batches. The number of *Enterobacteriaceae* in Batch 1 increased over the 7 days to 10^6 CFU/g. In Batch 2 the number of *Enterobacteriaceae* was < 10 CFU/g during the whole test period of 28 days. See Figure 2.

Figure 2 also shows the amount of background flora in Batches 3 (main study) and 4 (follow-up study). The number of *Enterobacteriaceae* also remained at < 10 CFU/g during storage in both batches. The number of Aerobic bacteria in Batch 4 was comparable to that in batches 1 and 2, but in Batch 3 (main study) it was relatively low at day 0 (50 CFU/g) and increased only to 5000 CFU/g after two weeks of storage.

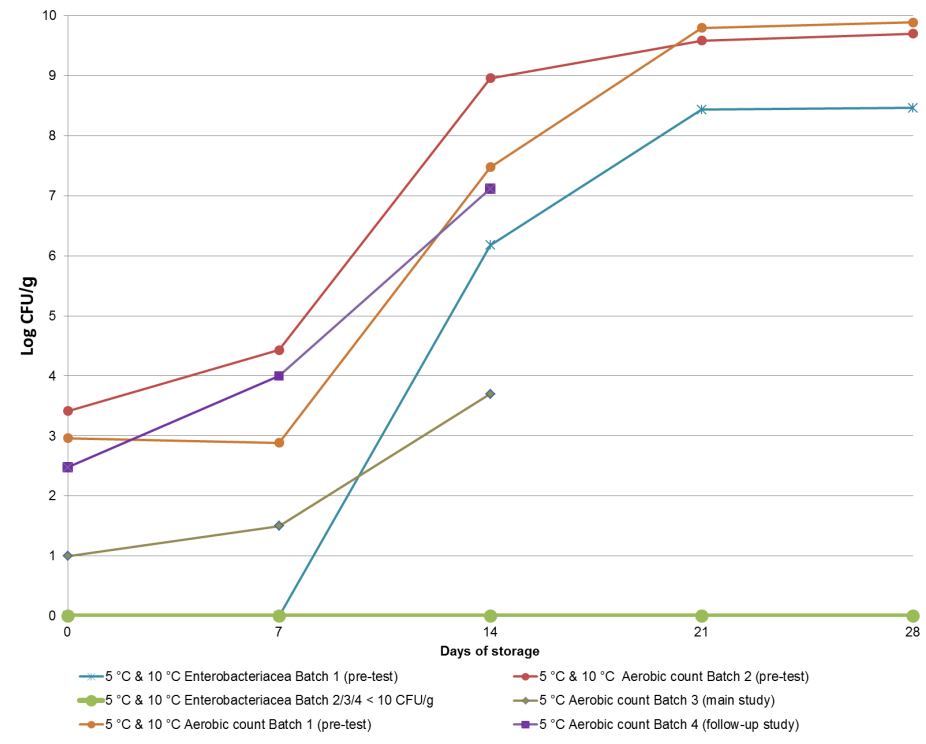


Figure 2. Stability test of background flora in the whole liquid egg samples, showing the Aerobic count and the number of Enterobacteriaceae (CFU/g) in the four batches of whole liquid egg samples

The major findings are summarized on the next page:

- Liquid chicken egg samples artificially contaminated with a low level of *Salmonella* Enteritidis (11–16 CFU/25 g) isolated from chicken product (SEb) were more stable than the samples contaminated with the strain isolated from egg (SEa).
  - The strain isolated from egg showed decreases of 50% and 75% in the number of *Salmonella*-positive samples after storage at 5 °C and 10 °C, respectively, for two weeks.
- All subsequent experiments were performed with the SEb strain isolated from chicken product with liquid whole egg from Batch 2.
  - Liquid whole chicken egg samples artificially contaminated with *Salmonella* Enteritidis at a level of 16 and 80 CFU/25 g were shown to be stable during storage at 5 °C and 10 °C for at least three weeks.
- From the results of these experiments, a decision was taken to use the following samples for the interlaboratory comparison study:
  - for each participant, 18 x 25 g of whole liquid egg (ISO/TS 22117; Anonymous, 2010);
  - each sample individually inoculated with a diluted culture of *Salmonella*:
    - low-level SE: 15–20 CFU/25 g of liquid egg;
    - high-level SE: 50-100 CFU/25 g of liquid egg;
    - blank: 0 CFU/25 g of liquid egg.

4.2.2 Contamination level of the artificially contaminated liquid egg samples

Table 5 shows the contamination levels of the liquid egg samples contaminated with SE at low and high levels. The inoculum level of the diluted SE culture (tested on XLD), as well as the contamination level of the liquid egg samples after inoculation with the diluted culture, were tested. The latter was tested using a five-tube MPN test (see Section 3.2.2). The number of positive whole liquid egg samples tested on 21 September for 25 g, 2.5 g and 0.25 g were, respectively, for low-level SE 5/5, 5/5 and 1/5 and for high-level SE 5/5, 5/5 and 5/5. The calculated MPN/25 g of egg is given in Table 5.

Table 5. Number of Salmonella Enteritidis (SE) in the inoculum and in the whole liquid egg samples

Date of testing	**Low-level SE CFU/25 g liquid egg** (95% confidence limit)	**High-level SE CFU/25 g liquid egg** (95% confidence limit)
10 September 2015 (inoculum of liquid egg)	21	101
21 September 2015 MPN of liquid egg, inoculated with SE (95 % confidence limit) after storage at 5 °C for 3 weeks	35 (11–110)	> 65

4.3 Technical data: interlaboratory comparison study

4.3.1 General

Thirty-six NRLs-*Salmonella* participated in this study: 30 NRLs from the 28 EU Member States (MS) and 6 NRLs from non-EU countries. The non-EU countries consisted of EU candidate MS or potential EU candidate MS, members of the European Free Trade Association (EFTA) and a non-European country.

All laboratories performed the study on the planned date (week 39, starting on 21 September 2015).

4.3.2 Accreditation/certification

All laboratories are accredited for their quality system according to ISO/IEC 17025 (Anonymous, 2005). Thirty-four laboratories are accredited for ISO 6579 (detection of *Salmonella* in food and animal feeding stuffs), 27 of them are also accredited for Annex D of ISO 6579 (Anonymous, 2002). Two laboratories (10 and 28) are accredited only for the detection of *Salmonella* in animal faeces and veterinary samples by using MSRV (Annex D of ISO 6579; Anonymous, 2007).

4.3.3 Transport of samples

The transport of all parcels was delayed by at least one day, due to logistical problems encountered by the courier service. Twenty-seven participants received the samples within two days of dispatch, six participants within three days and three participants after four days of transport. For two parcels (non-EU countries), it was not possible to arrange door-to-door transport. The parcels for laboratories 8 and 33

were retained by customs and arrived only after seven days of transport at the participating laboratory.

All NRLs returned the temperature recorders to EURL-*Salmonella* at the time they started the study, as requested. For the majority of the parcels, the temperature did not exceed 5 °C during transport; the exceptions were laboratories 8, 14, 24 and 27. The temperature in the parcels of laboratories 8, 24 and 27 increased to a maximum of 10 °C for 2 to 3 days. The temperature in the parcel of laboratory 14 increased to a maximum of 18 °C for 10 hours. During storage at the NRL, the temperature was generally between 0 °C and 5 °C. At nine laboratories (lab codes 19, 20, 21, 24, 25, 27, 30, 31 and 35), the samples were stored between 5 °C and 10 °C. An example of the temperature record during transport and storage at a laboratory (lab code 31) is given in Figure 3.

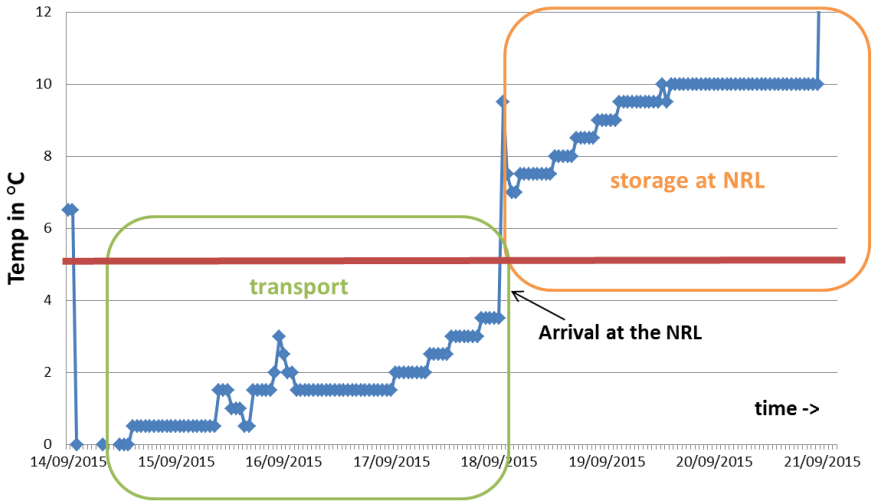


Figure 3 Record of the temperature of a parcel during transport and storage at a laboratory (lab code 31)

4.3.4 *Media*

Each laboratory was asked to follow the final draft version of ISO 6579-1 (Anonymous, 2015). As requested, all laboratories used MKTTn as a selective enrichment medium. Twenty-seven participants used all three selective enrichment media (MKTTn, RVS and MSRV). Six laboratories (3, 5, 9, 22, 23 and 36) used only RVS in combination with MKTTn and three laboratories (21, 31 and 32) used only MSRV in combination with MKTTn. Laboratory 13 used BPLS as a fourth selective enrichment medium.

Table 6 provides information on reported pH, concentration of Novobiocin, incubation time and temperature that deviated from the prescribed method (grey cells). The table lists only those NRLs that reported deviations from the method.

Seven laboratories reported a longer incubation time for the pre-enrichment in BPW.

Two laboratories (16 and 21) reported a pH of 7.3 and 7.4, respectively, instead of the prescribed maximum pH of 7.2 for BPW.
Four laboratories (5, 7, 23 and 27) used MKTTn at a pH that deviated from the prescribed pH of 7.8–8.2.
Six laboratories used MKTTn with a lower concentration of Novobiocin than the prescribed 40 mg/L and laboratory 16 used MKTTn without the addition of Novobiocin.
Four laboratories (4, 12, 24 and 29) used MSRV with a higher concentration of Novobiocin than the prescribed 10 mg/l and laboratory 13 used a lower concentration of Novobiocin.
Four laboratories (1, 7, 21 and 29) reported a deviating pH for MSRV.
Laboratory 21 did not report the pH of RVS. Laboratory 11 used RVS at a pH of 7.1 instead of the prescribed pH of 5.0–5.4.

Table 6. Reported technical deviations from the prescribed/requested procedures

Lab code	BPW		RVS	MKTTn		MSRV	
	Incubation time (h:min)	pH	pH	pH	Novo-biocin	pH	Novo-biocin
ISO 6579	16–20 h	6.8–7.2	5.0–5.4	7.8–8.2	40 mg/l	5.1–5.4	10 mg/l
1	20:00	7.1	5.25	8.1	40	5.5	10
4	20:00	7	5.4	8	40	5.4	20
5	18:00	7	5.2	6.6	40	NO	NO
7	19:30	7	5.2	6.7	40	5	10
11	18:15	7.1	7.1	7.8	40	5.2	10
12	24:00	7.0	5.2	8.0	40	5.2	20
13	20:00	7.0	5.2	8.0	0.040	5.2	0.05
14	20:30	7	5.2	8.2	40	5.2	10
16	17:45	7.27	5.28	7.8	0	5.22	10
17	17:30	7	5.2	8.0	0.04	5.2	10
18	21:00	6.95	5.28	8.05	10	5.41	10
19	20:30	7.1	5.1	8	40	5.2	10
21	20:00	7.4	-	8	40	5.5	10
22	25:55	7.1	5.4	8.0	39	NO	NO
23	19:00	7.2	5.2	6.94	40	NO	NO
24	19:00	6.95	5.2	8.05	40	5.21	20
27	18:30	6.99	5.28	7.08	39	5.11	10
29	19:00	7.08	5.33	8.16	40	5.52	10
30	21:00	7.2	5.2	8.0	10	5.3	10
33	17:45	7	5.2	8	4	5.4	10
34	16:35	7	5.1	8.2	40	5.3	20
36	24:00	7.2	5.2	8.0	10	NO	NO

Grey cells = Deviating from ISO/FDIS 6579-1
- = No information
NO = Did not use MSRV

A second plating-out medium of choice was obligatory. Table 7 shows the second isolation media used by the participants. Most laboratories used BGA (Anonymous, 1993) or a Chromogenic medium (e.g. Rambach) as a second plating-out medium.

Table 7. Second plating-out media used by the NRLs

Media	No. of users	Lab code
BGA^{mod} (ISO 6579, 1993)	9	3, 11, 18, 21, 22, 28, 29, 31, 32
Rambach (Merck)	7	20, 24, 25, 27, 30, 33, 35
BPLS (Merck & Biolife)	5	4, 10, 13, 14, 15
BGA	4	8, 16, 17, 26
RS (Bio-rad)	4	1, 2, 7, 23
SM(ID)2 (Biomerieux)	2	12, 34
CHROMagar Salmonella	2	9, 19
Macconkey (Oxoid)	1	36
Compass *Salmonella* agar (Biokar)	1	5
BSA (Oxoid)	1	6

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

The use of an extra non-selective plating agar between the 'isolation' and 'confirmation' steps was optional. A total of 30 laboratories performed this extra step (e.g. by using Nutrient agar; Anonymous, 2002).

All participating laboratories performed one or several confirmation tests for *Salmonella* (see Tables 8 and 9). Two laboratories (3 and 12) performed serological tests only and eleven laboratories performed only a biochemical test. Two laboratories (10 and 20) used the MALDI-TOF test in addition to other confirmation tests. Eleven participants used a PCR method to confirm *Salmonella* in addition to biochemical and/or serological tests. Laboratory 34 performed only PCR to confirm *Salmonella*.

Table 8. Serological confirmation tests for Salmonella *used by NRLs*

Lab code	Serological			
	O antigens	**H antigens**	**Vi antigens**	**Other**
1	-	+	-	
2, 5, 11, 12, 13, 15, 17, 18, 22, 24, 25, 30, 35	+	-	-	
7, 14, 32	+	+	-	
4				*Salmonella* Poly A-S+Vi antiserum
9				O-antigens, Vi-antigens and H-antigens

- = Not done

Table 9. Biochemical and other confirmation tests for Salmonella *used by NRLs*

Lab code	TSI	UA	LDC	Gal	VP	Indole	Other kit, PCR
1	-	-	-	-	-	-	API 20E
2	+	-	-	-	-	-	API 20E
3	-	-	-	-	-	-	Chromogenic media
4	+	+	+	-	-	-	API 20 E
5	+	+	+	+	-	+	RAPID ID32E
6	+	+	+	-	-	-	
7	+	+	+	+	+	+	VITEK2 GN ID Card
8	+	-	-	-	-	-	API 20E, PCR
9	+	+	+	+	+	+	Cytochrome oxidase, Malonate / phenylalanine, PCR
10	+	+	+	-	-	-	MALDI TOF
11	-	-	-	-	-	-	Kohns No1 Mast
12	-	-	-	-	-	-	
13	+	+	+	-	-	+	
14	+	+	+	+	+	+	Microgen GnA - ID system
15	-	-	-	-	-	-	PCR
16	+	-	-	-	-	-	Wellcolex, Lysine iron agar
17	+	+	+	+	+	+	PCR
18	+	+	+	-	-	+	
19	+	-	-	-	-	+	B&D BBL ENT/NF, PCR
20	+	+	+	+	-	+	MALDI-TOF, PCR
21	+	+	+	-	-	-	
22	-	-	-	-	-	-	API 20E
23	+	-	-	-	-	-	API RAPID ID 32E
24	+	-	-	-	-	-	VITEK2 GN
25	+	+	+	-	-	-	
26	+	+	+	+	+	+	PCR
27	+	+	+	-	-	-	API20E, Brolacin agar
28	-	-	-	-	-	+	Hy-Enterotest, PCR
29	+	+	+	+	+	+	
30	+	+	+	+	+	+	PCR
31	+	+	+	+	-	+	
32	+	+	+	+	+	+	
33	+	+	+	+	+	+	
34	-	-	-	-	-	-	PCR
35	+	+	+	+	-	+	Semi-solid glucose agar, PCR
36	+	+	+	+	+	+	GN (VITEK II)

- = Not done

4.4 Control samples

4.4.1 General

All laboratories scored both control samples (positive and blank) correctly.

Positive control with *Salmonella*

All laboratories obtained good results with their own *Salmonella* positive control sample and detected *Salmonella*.
For the positive control samples, the majority of the participants (21 laboratories) used a diluted culture of *Salmonella*. Others used a lenticule disc (7), a freeze-dried ampoule (3), a culti loop (2), a capsule

(1), a kwik-stik (1) or a pellet (1) with *Salmonella.* Table 10 shows the *Salmonella* serovars used for the positive control samples. Most often, *Salmonella* Enteritidis (16) and *Salmonella* Typhimurium (8) were used. The concentration of *Salmonella* in the positive control samples used by the different participants varied between 5 and 10^7 CFU/sample.

Table 10. Salmonella *serovars used by participants for the positive control samples*

Salmonella serovar	Number of users
S. Enteritidis	16
S. Typhimurium	8
S. Nottingham	4
S. Infantis	2
S. Poona, *S.* Bongori, *S.* Abony, *S.* Alachua, *S.* Tennessee, *S.* Harleystreet	1 (per serovar)

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

The results were compared with the definition of 'good performance' (see Section 3.6). All laboratories fulfilled these criteria for the control samples.

4.4.2 Correct scores of the control samples

Table 11 shows the number of correct scores found with the control samples for the different selective enrichment media in combination with the isolation medium. The calculations were performed on the results of all participants and on the results of only the EU-MS. No difference was found between these two groups. All laboratories obtained correct results for the control samples, with accuracy rates of 100%.

Table 11. Correct scores found with the control samples by all laboratories ('All') and by the laboratories of the EU Member States ('EU')

Control Samples		MKTTn and RVS or/and MSRV XLD or 2nd plate	
	Laboratories	All n=36	EU n=30
Positive control (own *Salmonella*) n=1	No. of samples	36	30
	No. of positive samples	36	30
	Correct score in %	**100**	**100**
Procedure control blank (BPW) n=1	No. of samples	36	30
	No. of negative samples	36	30
	Correct score in %	**100**	**100**
All control samples	No. of samples	72	60
	No. of correct samples	72	60
	Accuracy in %	**100**	**100**

4.5 Results of whole liquid egg samples artificially contaminated with *Salmonella*

4.5.1 Results for each level of Salmonella *and each laboratory*

General
Table 12 shows the results of the liquid egg samples artificially contaminated with *Salmonella* Enteritidis. The results given in this table are the highest number of positive isolations found with the different selective enrichment media (RVS, MKTTn and MSRV) in combination with the 'best' isolation medium.

Table 12. Number of positive results found with the artificially contaminated whole liquid egg samples (25g) at each laboratory

Lab code	Number of positive isolations		
	Blank n=6	SE low n=6	SE high n=6
Goodperformance	≤1	≥3	≥5
13	2	6	6
All other NRLs	0	6	6

Grey cell = result below level of good performance

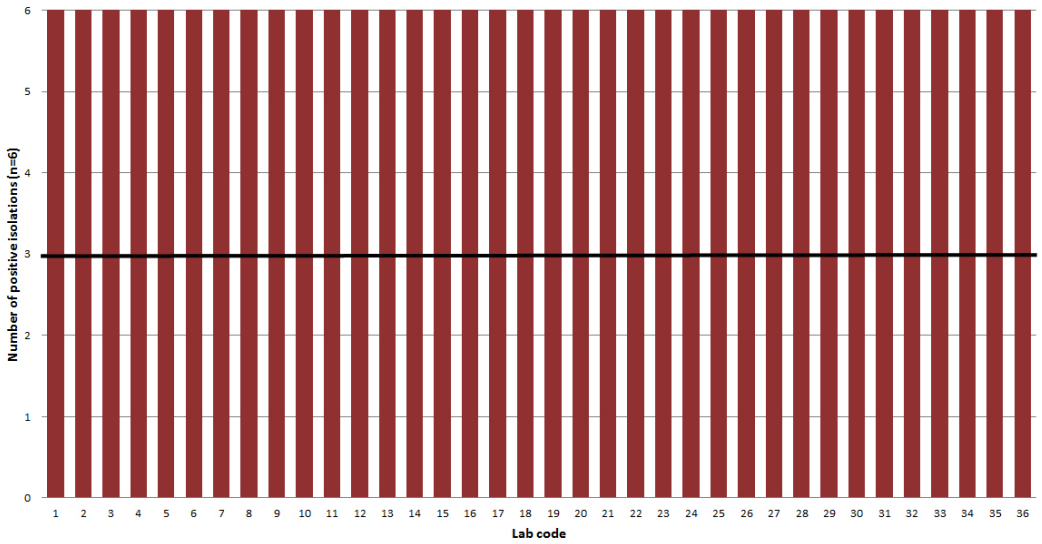
Blank samples
Thirty-five laboratories correctly scored all six blank whole liquid egg samples as negative for *Salmonella.* Laboratory 13 found two blank samples of the six positive for *Salmonella.*
All blank samples should be tested negative. However, because no 100% guarantee of the *Salmonella*-negative status of the egg could be given, one positive out of six blank samples (80% negative) is considered to be acceptable. Finding more than one blank sample positive is not very likely. A false positive result for a blank sample may have been caused by cross-contamination or by misinterpretation of the results.

High-level contaminated *Salmonella* Enteritidis samples
All laboratories detected *Salmonella* in all six samples that contained *Salmonella* Enteritidis at an inoculum level of approximately 100 CFU/25 g whole liquid egg.

Low-level contaminated *Salmonella* Enteritidis samples
All laboratories detected *Salmonella* in all six samples that contained *Salmonella* Enteritidis at an inoculum level of approximately 20 CFU/25 g whole liquid egg.
The results of the artificially contaminated whole liquid egg samples were compared to the definition of 'good performance' (see Section 3.6) and laboratory 13 did not fulfil these criteria.

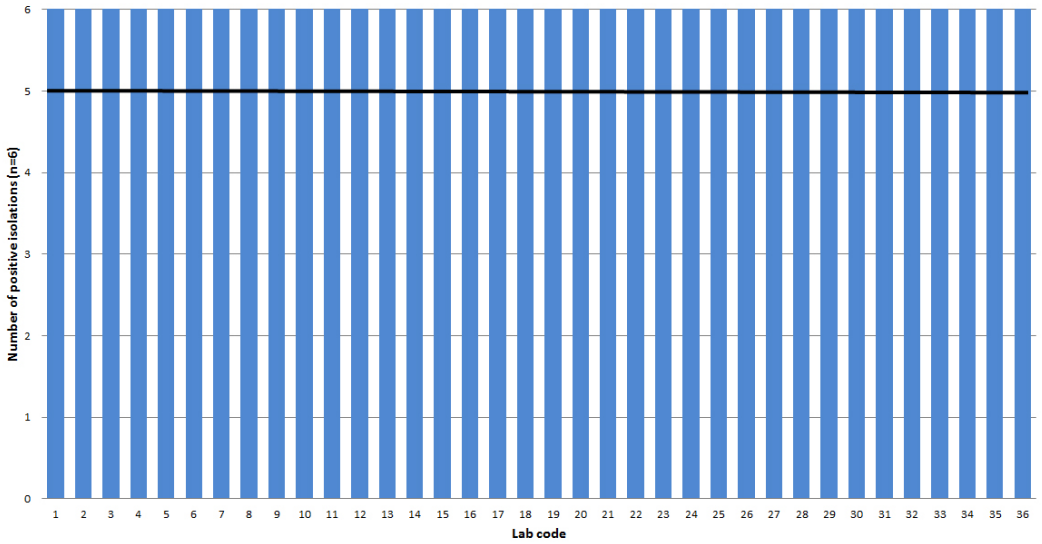
4.5.2 Results for each selective enrichment medium, each level of contamination and each laboratory
Figures 4 and 5 show the number of positive isolations for each level of artificially contaminated egg sample and for each laboratory after pre-enrichment in BPW and all combinations of selective enrichment media (MKTTn and RVS and/or MSRV) and plating-out media (XLD and own choice), giving the highest number of positive samples. These results

were compared to the agreed definition of 'good performance' (see Section 3.6). In Figures 4 and 5, the border of good performance is indicated by the horizontal black line.



▬ = border of good performance

Figure 4. Number of positive isolations per laboratory after analysing six samples of 25 g whole liquid egg artificially contaminated with low-level S. Enteritidis. Results include all combinations of selective enrichment media (MKTTn and RVS and/or MSRV) and plating-out media (XLD and 2nd plate), giving the highest number of positive samples.



▬ = border of good performance

Figure 5. Number of positive isolations per laboratory after analysing 6 samples of 25 g whole liquid egg artificially contaminated with high-level S. Enteritidis. Results include all combinations of selective enrichment media (MKTTn and RVS and/or MSRV) and plating-out media (XLD and own medium), giving the highest number of positive samples.

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

Table 13 shows the specificity, sensitivity and accuracy rates for all three levels of artificially contaminated liquid egg samples. This table gives the results for all possible combinations of media (MKTTn and RVS and/or MSRV), giving the highest number of positive results. The calculations were performed on the results of all participants and on the results of the participants of the EU-MS only. No difference was found between these two groups. The specificity and accuracy rates were 99% and sensitivity rates for both low-level and high-level contaminated egg samples were 100%.

Table 13. Specificity, sensitivity and accuracy rates found by the participating laboratories with the artificially contaminated whole liquid egg samples.

Egg samples		**MKTTn and RVS and/or MSRV XLD and 2nd plate**	
	Laboratories	All n=36	EU n=30
Blank (n=6)	No. of samples	216	178
	No. of negative samples	214	180
	Specificity in %	**99**	**99**
SE low (n=6)	No. of samples	216	180
	No. of positive samples	216	180
	Sensitivity in %	**100**	**100**
SE high (n=6)	No. of samples	216	180
	No. of positive samples	216	180
	Sensitivity in %	**100**	**100**
All samples with *Salmonella*	No. of samples	432	360
	No. of positive samples	432	360
	Sensitivity in %	**100**	**100**
All samples	No. of samples	648	538
	No. of correct samples	646	540
	Accuracy in %	**99**	**99**

4.6 PCR (own method)

Eleven laboratories applied a PCR method as an additional detection technique. Table 14 gives further details about the PCR techniques used.

All laboratories found the same results when using the PCR method and the bacteriological culture method. Laboratory 35 did not report its results for the positive control.

*Table 14. Details of Polymerase Chain Reaction procedures used by NRLs-*Salmonella *as own method during the interlaboratory comparison study*

Lab code	PCR method	Validated (by)	Commer-cially available	Routinely used number of tests/year	DNA extraction after enrichment in	Reference
8	Conven-tional 3-step PCR	No	+	approx. 70	Pure culture from nutrient agar	Stone et al. (1994)
9	Real-time	AFNOR and others	+	91	BPW	
15	Real-time	Yes but no details	-	79	BPW	Malorny et al. (2004)
17	Real-time	AFNOR	+	2,000	BPW	
19	Real-time	AFNOR	+	875	BPW	
20	Real-time	No	-	-	BPW	Daum et al. (2002)
26	Real-time	Intra-laboratory	-	45	BPW	
28	Real-time	Lofstrom et al. (2010); Lofstrom and Hoorfar (2012)	-	1000	BPW	Malorny et al. (2004)
30	Real-time	Yes, but no details	-	-	BPW	
34	Real-time	No	-	> 500	BPW	
35	Real-time	Internally validated	+	-	BPW	

4.7 Performance of the NRLs

4.7.1 General

Thirty-five NRLs fulfilled the criteria of good performance and one laboratory scored below these criteria: laboratory 13 reported two blank egg samples(false) positive for *Salmonella*.

Laboratory 13 was contacted by EURL-*Salmonella* in November 2015 and asked for possible explanations for its deviating results. The false positive isolates were from the same serotype as the positive samples from the study. The laboratory checked each step in the *Salmonella* detection procedure for possible contamination.

Laboratory 13 participated in a follow-up study organised by the EURL-*Salmonella* in September 2015.

4.7.2 Follow-up study

The set-up in the follow-up study was the same as the for the main interlaboratory comparison study organised in September 2015. However, since the original problem was due to a false positive blank,

the sample types in the follow-up study focused on this problem. The study contained 7 blank samples (only liquid egg) and 5 artificially contaminated whole liquid egg samples inoculated with a high level of *S*. Enteritidis (approximately 110 CFU/sample). Only high-level SE samples were used in order to test for cross-contamination of the blank samples.

On 25 January 2016, the number of Aerobic bacteria ($1.3 * 10^7$ CFU/g) and the number of *Enterobacteriaceae* (< 10 CFU/g) in the whole liquid egg was tested after it had been stored at 5 °C since 12 January 2016. The number of *Enterobacteriaceae* were comparable to the number found in the liquid egg used in the main study (see Table 3 and Figure 2) but the number of Aerobic bacteria was much higher.

A duplicate set of the artificially contaminated samples used for this follow-up study was tested by EURL-*Salmonella* for the presence of *Salmonella* and all the samples were scored correctly with all selective enrichment media used (RVS, MKTTn and MSRV).

On Monday, 18 January 2016, one parcel with 14 samples in one plastic safety bag was sent to laboratory 13 containing: 2 control samples (numbered C1 and C2), 12 whole liquid egg samples (numbered B1–B12) and one temperature recorder.

The follow-up study started in week 5 (25 January 2015). The laboratory had to follow the same protocol and web-based test report as had been used in the study of September 2015 (EURL-*Salmonella*, 2015a and 2015b).

During the follow-up study, laboratory 13 used the same media as used in the main study.

The results of the follow-up study fulfilled the criteria for good performance.

The most plausible explanation for the false positive blank results in the main study in September 2015 is cross-contamination in the laboratory.

5 Discussion

The NRLs had to follow EN ISO 6579 (and the underlying EN ISO documents, e.g. the EN ISO 6887 series for preparation of test samples) in accordance with their normal procedure for detection (and confirmation) of *Salmonella* in 'official' samples. The Final Draft International Standard (FDIS) version of ISO 6579-1 was published in 2015. This document describes the (final) updated technical steps for the detection of *Salmonella* in food, animal feed and samples from the primary production stage.

An important change in this document from the current version of ISO 6579 is the option to choose between RVS and MSRV for the selective enrichment of *Salmonella* from food and animal feed samples. For that reason, this choice was also available in the current study, so that in addition to MKTTn, either RVS or MSRV could be used for selective enrichment. The NRLs were also permitted to use all three selective enrichment media (MKTTn, MSRV and RVS). Most participants (27) used all three selective enrichment media and 9 laboratories used either RVS or MSRV besides MKTTn.

Artificial contamination of samples with a diluted culture
The use of matrices that mimic routine samples is considered more appropriate than the use of reference materials in interlaboratory studies. In 2013, EURL-*Salmonella* started with artificial contamination of matrices with a diluted culture of a *Salmonella* serovar, which is more challenging concerning stability of the samples than for reference materials. In earlier EURL-*Salmonella* studies, this method of artificial contamination was successfully used for the detection of *Salmonella* in boot socks and chicken faeces (Kuijpers and Mooijman, 2014 and 2015) and for the detection of *Salmonella* in minced chicken meat (Kuijpers et al. 2014) and chicken feed (Kuijpers et al. 2015).

Each matrix and *Salmonella* serovar combination may behave differently with respect to the survival of *Salmonella* during storage and transport. For that reason, the artificially contaminated whole liquid egg samples were tested for their 'long-term' stability at normal storage temperature (5 °C) and at a temperature that may occur during the transport of the samples (10 °C). Experience from earlier studies had shown that, in general, the transport time of the parcels to the NRLs is 1 to 2 days at temperatures that remain below 10 °C for most of the time. Only occasionally, the temperature of a parcel during transport may be ≥10 °C for a few hours.

As the number of *Salmonella* in the whole liquid egg slowly decreased during storage in the pre-test period, it was decided to inoculate the low-level contamination samples with 15–20 CFU of a diluted culture of *Salmonella* Enteritidis to make sure that the level at the date of the study would still be approximately 5–10 CFU. After storage and transport, the contamination level in the samples with a low level of contamination was approximately 35 MPN/25 g (with a 95% confidence

interval of 11–110 MPN/25 g) on the day of the study, which was somewhat higher than expected.
Additionally, the amount of background flora in the whole liquid egg samples used for the interlaboratory study was much lower than in the samples used for the pre-tests. Hence, the growth of *Salmonella* was hardly influenced by the background flora, making it relatively easy to detect *Salmonella* in the samples used in this study.

Transport of samples
To stabilize the level of *Salmonella* Enteritidis in the samples during transport, the materials were packed with frozen cooling elements and transported by courier service. The information provided by the temperature recorders included in the parcels showed that the temperature in the parcels remained below 5 °C for most of the transport time. It can therefore be assumed that transport did not negatively affect the mean contamination level of the samples. This was confirmed by the fact that the laboratories with the longest transport time and/or the highest temperatures (lab codes 8, 14 and 33) scored all samples correctly.

The liquid egg samples needed to be stored at 5 °C after receipt at the participating laboratory. Unfortunately, this was not always the case and temperatures of up to 10 °C were detected, which could result in die-off of *Salmonella* and greater difficulty in detecting *Salmonella*.

Accreditation of laboratories
According to EC regulations 882/2004 (EC, 2004) and 2076/2005 (EC, 2005), each NRL has to be accredited in its relevant work field. Two participants (lab codes 10 and 28, from non-EU countries) were not accredited for the detection of *Salmonella* in food samples but only for the detection of *Salmonella* in animal faeces and veterinary samples by using MSRV (Annex D of ISO 6579; Anonymous, 2007).

Performance of the laboratories
For the evaluation of the performance of the laboratories, all combinations of selective enrichment media (MKTTn and RVS and/or MSRV) and isolation media were taken into account.

One laboratory (13) found *Salmonella* in two blank samples. All blanks should test negative. However, because no 100% guarantee of the *Salmonella*-negative status of the liquid egg could be given, one positive out of six blank samples (80% negative) was considered acceptable. Finding more than one blank sample positive is not very likely. An explanation for the false positive sample may be cross-contamination. This laboratory scored all samples correctly in a follow-up study and thereby fulfilled the criteria of good performance.

According to the criteria used, all laboratories achieved ‘good performance’.

Specificity, sensitivity and accuracy rates
The calculations were performed on the results of all participants and on the results of the EU-MS only. No differences were found between these two groups. The specificity and accuracy rates were 99% and sensitivity

rates for both low- and high-level contaminated egg samples were 100%.

Positive control samples
Participants were asked to use the positive control sample(s) that are routinely used in their laboratory. *S*. Enteritidis and *S*. Typhimurium were the most frequently used serovars and the concentration in the positive control samples varied between 5 and 10^7 CFU/sample. A positive control sample should demonstrate that media are capable of supporting the growth of a range of organisms in low numbers. To gain insight into the sensitivity of a method, the concentration of a positive control sample should be just above the detection limit of this method. The majority of the participants used a much higher concentration. Furthermore, it may be advisable to use a serovar rarely isolated from the routine samples analysed in the laboratory. In this way, possible cross-contamination can be detected more easily.

Media and incubation
During the study, (small) deviations in the prescription of the media (e.g. in pH or concentration of Novobiocin) or in incubation times were reported. No influence of these deviations could be seen on the results. All samples were scored correctly.

PCR
Eleven laboratories used a PCR technique in addition to the prescribed culture method. All of them obtained the same results as with the bacteriological culture technique. This indicates that the PCR methods used are well suited to the detection of *Salmonella* in whole liquid egg samples. Almost all participants used a validated real time PCR with DNA isolation from BPW. Approximately 50% used a commercially available PCR method.

In comparison with previous EURL-*Salmonella* studies on the detection of *Salmonella* in food samples, an increase was seen in the number of NRLs using a PCR technique as their own method. In food studies organized in 2011 and 2013, nine and three laboratories, respectively, used a PCR technique in addition to the prescribed method, while in the current study eleven laboratories performed PCR (Kuijpers et al., 2012 and 2014). However, from this information no conclusions can be drawn concerning the use of PCR techniques in the NRLs, since the use of an own method is voluntary and not prescribed in the interlaboratory comparison studies.

Evaluation of this study
The set-up of this study was comparable to the interlaboratory comparison studies on the detection of *Salmonella* in food, animal feed and samples from the primary production stage organised since 2013. However, some important changes were introduced:

- A Standard Operating Procedure (SOP) specifying in detail the steps of the procedure for the *Salmonella* detection was for the first time not sent to the participants. The NRLs were asked to follow EN ISO 6579 (and the underlying EN ISO documents, e.g. the EN ISO 6887 series for preparation of test samples) according to their normal procedure for detection (and confirmation) of *Salmonella* in 'official' samples.

- An additional blank matrix control sample was not sent, as the set of samples already included several blank matrix samples.
- Participants were asked to report the results as they would for routine samples, meaning that the indication 'positive' (1) or 'negative' (0) per sample (after confirmation) was sufficient, irrespective of the combination of selective enrichment medium and isolation medium.
- As in earlier EURL-*Salmonella* interlaboratory comparison studies, all results were reported electronically using a standard form. The questions in the web-based report were updated resulting in a shorter test report than before (e.g. only the incubation time of BPW was asked for instead of that of all the media used). As all NRLs have to be accredited according to ISO 17025, additional information not requested in the test report should be available in their system.

As a result of these changes, the results per medium were no longer visible to EURL-*Salmonella*, so that no wider conclusions could be drawn on the performance of the various media and on the effect of the prolongation of the incubation time from 24 h to 48 h. However, the changes meant that the routine analysis of samples and reporting of results were more reflecting the daily laboratory practice than in earlier studies. Additional, the reporting of results was easier and faster in the current study.

The artificial contamination of whole liquid egg with a diluted culture at the laboratory of EURL-*Salmonella* was successful, showing it to be applicable in interlaboratory comparison studies.

Not only may each matrix and *Salmonella* serovar combination behave differently, as we see from this study, but also different batches of matrix may contain different levels of background flora, which may influence the growth of *Salmonella.* For each new study the challenge is to prepare stable samples, especially when a new matrix is used.

6 Conclusions

Thirty-five out of the 36 NRLs-*Salmonella* achieved good performance in the detection of *Salmonella* in high-level and low-level contaminated whole liquid chicken egg samples. One laboratory achieved the level of good performance only after a follow-up study.

High rates of specificity, sensitivity and accuracy for the detection of *Salmonella* in artificially contaminated whole liquid egg samples (blank, low-level and high-level) were found: 99–100%.

The accuracy rate for the control samples after selective enrichment in MKTTn and RVS and/or MSRV was 100%.

Some participants may consider the optimisation of the positive control sample used in their routine analysis with respect to the choice of *Salmonella* serovar and/or contamination level.

An increase was seen in the number of NRLs using a PCR technique in the food study (mostly real-time). PCR as a laboratory's own method gave the same results as the bacteriological culture technique.

The background flora in the whole liquid egg batch used for the main study was lower than that in the batch used in the pre-tests. This made it easier to detect *Salmonella* in the samples used in the study itself.

Not only may each matrix and *Salmonella* serovar combination behave differently, but also different batches of matrix may contain different levels of background flora, which may influence the growth of *Salmonella.* For each new study the challenge is to prepare stable samples, especially when a new matrix is used.

The artificial contamination of whole liquid egg with a diluted culture of *Salmonella* at the laboratory of EURL-*Salmonella* was successful. This shows that, in addition to minced meat, chicken feed and samples from the primary production stage (chicken faeces), liquid chicken egg samples can be used in interlaboratory comparison studies.

List of abbreviations

AFNOR	Association Française de Normalisation (French Standardization Association)
BGA(mod)	Brilliant Green Agar (modified)
BL	blank (no colony-forming units)
BPLS	brilliant green phenol-red lactose sucrose
BPW	Buffered Peptone Water
BSA	Brilliance *Salmonella* Agar
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
FDIS	Final Draft International Standard (ISO)
Gal	Galactosidase
ISO	International Organization for Standardization
LDC	Lysine Decarboxylase
MKTTn	Mueller-Kauffmann Tetrathionate-Novobiocin broth
MPN	most probable number
MS	Member State
MSRV	Modified Semi-solid Rappaport-Vassiliadis
NRL	National Reference Laboratory
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
RIVM	Rijksinstituut voor Volksgezondheid en het Milieu (National Institute for Public Health and the Environment)
RS	Rapid *Salmonella*
RVS	Rappaport Vassiliadis Soya broth
SE	*Salmonella* Enteritidis
SM (ID)2	*Salmonella* Detection and Identification-2
SOP	Standard Operating Procedure
TSI	Triple Sugar Iron agar
UA	Urea Agar
VP	Voges-Proskauer
VRBG	Violet Red Bile Glucose agar
XLD	Xylose Lysine Deoxycholate agar

References

Anonymous (1993). ISO 6579. Microbiology – General guidance on methods for the detection of *Salmonella*. International Organization for Standardization, Geneva, Switzerland.

Anonymous (2002). ISO 6579. Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. International Organization for Standardization, Geneva, Switzerland.

Anonymous (2003a). ISO 4833. Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of microorganisms – Colony-count technique at 30 degrees C. International Organization for Standardization, Geneva, Switzerland.

Anonymous (2003b). ISO 6887-4. Microbiology of food and animal feeding stuffs – Preparation of test samples, initial suspension and decimal dilutions for microbiological examination – Part 4: Specific rules for the preparation of products other than milk and milk products, meat and meat products, and fish and fishery products. International Organisation for Standardisation, Geneva, Switzerland.

Anonymous (2004). ISO 21528-2. Microbiology of food and animal feeding stuffs – Horizontal methods for the detection and enumeration of Enterobacteriaceae – Part 2: Colony-count method. International Organization for Standardization, Geneva, Switzerland.

Anonymous (2005). ISO/IEC 17025. General requirements for the competence of testing and calibration laboratories. International Organization for Standardization, Geneva, Switzerland.

Anonymous (2007). Amendment of ISO 6579:2002/Amd1 Annex D: Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage. International Organization for Standardization, Geneva, Switzerland.

Anonymous (2010). ISO/TS 22117. Microbiology of food & animal feeding stuffs - Specific requirements & guidance for Proficiency Testing (PT) by interlaboratory comparison. International Organization for Standardization, Geneva, Switzerland.

Anonymous (2015). ISO/FDIS 6579-1: Microbiology of the food chain – Horizontal method for the detection, enumeration and serotyping of *Salmonella* – Part 1: Horizontal method for the detection of *Salmonella* spp. International Organization for Standardization, Geneva, Switzerland.

Daum LT et al. (2002). Real-time PCR detection of *Salmonella* in suspect foods from a gastrointestinal outbreak in Kerr county, Texas. J Clin Microbiol. 2002 Aug;40(8):3050–2.

EC (2004). Commission Regulation (EC) No. 882/2004 of the European Parliament and of the Council of 29 April 2004 on the official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules. Official Journal of the European Union L 165 of 30 April 2004. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2004R0882:20060525:EN:PDF (access date December 2016).

EC (2005). Commission Regulation (EC) No. 2076/2005 of the European Parliament and of the Council of 5 December 2005 laying down transitional arrangements for the implementation of Regulations (EC) No. 853/2004, (EC) No. 854/2004 and (EC) No. 882/2004 of

the European Parliament and of the Council and amending Regulations (EC) No. 853/2004 and (EC) No. 854/2004. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2005:338:0083:0088:EN:PDF (access date December 2016).

EURL-*Salmonella* (2015a). Protocol Interlaboratory Comparison Study on the detection of *Salmonella* spp. in food samples organized by EURL-*Salmonella* STUDY VII – 2015. http://www.eurlsalmonella.eu/dsresource?type=pdf&disposition=inline&objectid=rivmp:289171&versionid=&subobjectname (access date December 2016).

EURL-*Salmonella* (2015b). Test report on Interlaboratory Comparison Study on the detection of *Salmonella* spp. in food organized by EURL-*Salmonella* STUDY VII – 2015. http://www.eurlsalmonella.eu/dsresource?type=pdf&disposition=inline&objectid=rivmp:289172&versionid=&subobjectname (access date December 2016).

EURL-*Salmonella* (2016). History of EURL-*Salmonella* interlaboratory comparison studies on the detection of *Salmonella*. http://www.eurlsalmonella.eu/Publications/Interlaboratory_comparison_study_Reports (access date December 2016).

Jarvis B, Wilrich C, Wilrich P-T (2010). Reconsideration of the derivation of most probable numbers, their standard deviations, confidence bounds and rarity values. J. Appl Microbiol. 109:1660–7. Link to MPN calculation programme: http://www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/wilrich/index.html (access date December 2016).

Kuijpers AFA, Kassteele van de J, Mooijman KA (2012). EU Interlaboratory comparison study food VI (2011); Detection of *Salmonella* in minced meat. RIVM report 330604025/2012, Bilthoven, the Netherlands. http://www.rivm.nl/bibliotheek/rapporten/330604025.pdf (access date December 2016).

Kuijpers AFA, Mooijman KA (2014). EU Interlaboratory comparison study veterinary XVI (2013); Detection of *Salmonella* in chicken faeces adhering to boot socks. RIVM report 330604031, Bilthoven, the Netherlands. http://www.rivm.nl/bibliotheek/rapporten/330604031.pdf (access date December 2016).

Kuijpers AFA, Kassteele van de J, Mooijman KA (2014). EU Interlaboratory comparison study food VI (2013); Detection of *Salmonella* in minced chicken meat. RIVM report 2014-0010, Bilthoven, the Netherlands. http://www.rivm.nl/bibliotheek/rapporten/2014-0010.pdf (access date December 2016).

Kuijpers AFA, Mooijman KA (2015). EU Interlaboratory comparison study primary production XVII (2014); Detection of *Salmonella* in chicken faeces. RIVM report 2014-0011, Bilthoven, the Netherlands. http://www.rivm.nl/bibliotheek/rapporten/2014-0011.pdf (access date December 2016).

Kuijpers AFA, Kassteele van de J, Mooijman KA (2015). EU Interlaboratory comparison study animal feed VI (2014); Detection of *Salmonella* in chicken feed. RIVM report 2015-0080, Bilthoven, the Netherlands. http://www.rivm.nl/bibliotheek/rapporten/2015-0080.pdf (access date December 2016).

Lofstrom C, Hansen F, Hoorfar J (2010). Validation of a 20-h real-time PCR method for screening of *Salmonella* in poultry faecal samples. Veterinary Microbiology 144:511–14.

Lofstrom C, Hoorfar, J (2012). Validation of an open-formula, diagnostic real-time PCR method for 20-h detection of *Salmonella* in animal feeds. Veterinary Microbiology 158:431–5.

Malorny B, Paccassoni E, Fach P, Bunge C, Martin A, Helmuth R (2004). Diagnostic real-time PCR for detection of *Salmonella* in food. Appl Environ Microbiol. 70:7046–52.

Stone GG, Oberst RD, Hays MP, McVey S, Chengappa MM (1994). Detection of *Salmonella* serovars from clinical samples by enrichment broth cultivation PCR procedure J Clin., Mikrobio. 32(7):1742-9.

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