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Validation of EN ISO 6579-1 - Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of *Salmonella* - Part 1 detection of *Salmonella* spp.

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ABSTRACT

The European and International Standard method for the detection of *Salmonella* spp. in samples from the primary production stage, EN ISO 6579:2002/Amd.1:2007, was validated by an interlaboratory study in the frame of Mandate M/381, ordered by the European Commission and accepted by the European Standardisation Organisation (CEN). In addition to this study, results from two interlaboratory studies organised earlier by the European Union Reference Laboratory (EURL) for *Salmonella* were used for determination of the performance characteristics. Parallel to the performance evaluation for the Mandate, the revision of EN ISO 6579:2002 started. Part of this revision was the incorporation of the standardised method for detection of *Salmonella* in samples from the primary production stage (EN ISO 6579:2002/Amd.1:2007) and its performance characteristics in the new part 1 of EN ISO 6579. The 2002 version of EN ISO 6579 already contained performance characteristics for the detection of *Salmonella* in food samples, but LOD_{50} values (contamination level at which 50% of the samples are found positive) were not yet included. To be in line with the performance characteristics determined for detection of *Salmonella* spp. in samples from the primary production stage, LOD_{50} values for detection of *Salmonella* in food samples were calculated from the raw data of the validation studies performed in 2000. In this paper, the performance characteristics of EN ISO 6579-1:2017 are determined based not only on the results of the interlaboratory study carried out in 2013 under the Mandate, but also on several other interlaboratory studies. These performance characteristics consist of specificity, sensitivity and LOD_{50}.

1. Introduction

At International and at regional level in Europe, respectively ISO and CEN are active in developing harmonised methods. For microbiology of the food chain several harmonised (EN ISO) standard reference methods exist, which are important tools for the internationally uniform analysis of (food) samples. The majority of the microbiological EN ISO methods concern 'traditional' culture methods, which are considered as the reference methods. Any new/alternative method has to be validated against the reference method to test if this new method performs at least equally well as the reference method. To know how a reference or alternative method performs, it is important to have information on the performance characteristics. However, up to 2010, the majority of microbiological EN ISO methods have been published without performance characteristics. To overcome this omission, the European Commission (EC) issued Mandate M/381 in 2010, which was accepted by the European Standardisation Organisation (CEN), to determine performance characteristics of 15 microbiological standard reference methods related to food hygiene legislation. For this a harmonised experimental design was drafted, based on EN ISO 16140 (Anonymous, 2003a) and its revised version, EN ISO 16140-2 (Anonymous, 2016b), which was still a draft in 2010. For the determination of the performance characteristics of each of the 15 reference methods, one or more interlaboratory studies (ILS) had to be organised testing one (for so called 'vertical methods') to five (for 'horizontal' methods) different matrices. To obtain sufficient data for statistical analysis, at least 10 (for quantitative methods) or 13 (for qualitative methods) collaborators were needed for each ILS.

Part of Mandate M/381 was the determination of the performance characteristics for EN ISO 6579:2002/Amd.1: 2007 for 'Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage' (Anonymous, 2007). As this concerned a so-

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called 'vertical' method, only one matrix (samples from the primary production stage) needed to be analysed in an interlaboratory study.

To judge whether the performance characteristics determined from the results of the ILS performed under the Mandate were representative for samples from the primary production stage, they were compared to the performance characteristics derived from interlaboratory studies organised earlier. This concerned two interlaboratory studies organised by the European Union Reference Laboratory (EURL) for *Salmonella* in 2008 and 2012 (Kuijpers et al., 2008; Kuijpers and Mooijman, 2013). The set-up of these earlier studies deviated from the set-up of the ILS as agreed upon for the Mandate with respect to the number of samples tested. For the study under the Mandate, each participant had to test 8 samples for each contamination level (blank, low and high). In the earlier studies each participant tested (only) 5 samples for each contamination level. On the other hand, in these earlier studies two sets of low and high contaminated samples were included, artificially contaminated with two different *Salmonella* serovars and thus resulting in 10 low and 10 high contaminated samples analysed by each participant. Additionally, the total number of participants in the earlier EURL studies was (much) larger than the prescribed number for the study of the Mandate (approximately 30 participants in the earlier EURL studies, against 13 participants prescribed for the ILS of the Mandate), resulting in a sufficiently large dataset to determine the performance characteristics.

Parallel to the performance evaluation for the Mandate, the revision of EN ISO 6579:2002 started. Part of this revision was the incorporation of the standardised method for detection of *Salmonella* in samples from the primary production stage (EN ISO 6579:2002/Amd.1:2007) and its performance characteristics in the new part 1 of EN ISO 6579 (Anonymous, 2017; Mooijman, 2018). The 2002 version of EN ISO 6579 (Anonymous, 2002) already contained performance characteristics for the detection of *Salmonella* in food samples. However, these consisted of specificity, sensitivity, accordance and concordance, while under the Mandate M/381 accordance and concordance were replaced by LOD_{50} (contamination level at which 50% of the samples are found positive; Anonymous, 2016a). Although not part of the Mandate, but still part of the revision of EN ISO 6579, an attempt was made to calculate the LOD_{50} values for detection of *Salmonella* in food samples from the raw data of the original validation studies performed in 2000 for determining the performance characteristics of the 2002 version of EN ISO 6579 (Feldsine et al., 2003).

In this paper, the performance characteristics of EN ISO 6579-1:2017 are determined based not only on the results of the interlaboratory study carried out in 2013 under the Mandate, but also on several other interlaboratory studies. These performance characteristics consist of specificity, sensitivity and LOD_{50}.

2. Materials and methods

2.1. Design of the studies

Additional to the interlaboratory study (ILS) organised under the Mandate in 2013, data from interlaboratory studies (ILSs) organised by the EURL-*Salmonella* in 2008 and 2012 were used. In all studies, National Reference Laboratories (NRLs) for *Salmonella* of the (by then) 27 different EU Member States participated, as well as some additional NRLs from non-EU Member States. The total number of participants per study is shown in Table 1. All laboratories were accredited or in the process of being accredited according to EN ISO/IEC 17025 (Anonymous, 2005).

The design of each ILS was described in a detailed protocol, a Standard Operating Procedure (SOP) and a test report in which results were recorded and returned to the organiser of the study for analysis. In the test report, participants were requested to detail all supplementary information that could have influenced their results. The documents of the three interlaboratory studies described here, are available at the EURL-*Salmonella* website (EURL-*Salmonella*, 2017).

The number and type of samples, as well as the matrices varied per ILS. Details per study are given in Table 1. In each study 'real' matrix samples were used, artificially contaminated with two different levels of a *Salmonella* serovar: low and high. Additionally, blank matrix samples were used. For the low level samples, the contamination level should be at or slightly above the detection limit of the method. The objective was to obtain 25% to 75% positive results when testing the samples with the low inoculation level (where the negative results would be mostly being due to the absence of *Salmonella* in these samples). The contamination level of the high level samples was aimed to be 5–10 times above the detection limit of the method. For the ILS under the Mandate, the matrix samples were artificially contaminated with a diluted culture, while for the earlier ILSs reference materials were used to artificially contaminate the samples (for more details on the test materials, see Section 2.3). For the two earlier interlaboratory studies, the participating laboratories received the matrix (chicken faeces or pig faeces) and reference materials separately packed. At the day of analyses, the participants had to prepare the samples by combining 10 g chicken faeces (ILS 2008) or 25 g pig faeces (ILS 2012) with one reference material according to a detailed protocol.

For all studies, all samples (or reference materials) were individually packed and randomly labelled and shipped to the participants by courier service. The samples were packed in double containers with ice packs, and sent in accordance to the International Air Transport Association Regulations as 'Biological Substance category B', classified as UN 3373 (Anonymous, 2013). To obtain more information on the temperature during transport, each parcel contained a small electronic temperature device which measured and registered the temperature every hour. After receipt of the samples, the electronic device had to be returned to the EURL-*Salmonella* after which it was read with the computer. The samples had to be stored at 5 °C (boot socks samples and faeces) or at −20 °C (reference materials), until the day of analysis. Participants used their own media and reagents for each ILS, following the prescriptions of EN ISO 6579:2002/Amd.1:2007 (Anonymous, 2007). The examination of the test materials was initiated on the same day by all participants and started one week (ILS 2013 and 2012) or two weeks (ILS 2008) after dispatch of the samples.

2.2. Method under collaborative trial

In all studies, EN ISO 6579:2002/Amd.1:2007 (Anonymous, 2007) was followed for the detection of *Salmonella* in samples from the primary production stage. The method required the following successive stages:

a) Preparation of the initial suspension, by adding each test portion to a quantity of non-selective broth (Buffered Peptone Water; BPW) to yield a tenfold dilution.
b) Pre-enrichment of the (non-selective) initial suspension a) at 37 °C ± 1 °C for 18 h ± 2 h.
c) Selective enrichment of a culture obtained from b) on a selective semi-solid agar medium, Modified semi-solid Rappaport-Vassiliadis (MSRV) agar, at 41.5 °C ± 1 °C for 24 h ± 3 h and if negative for an additional 24 h ± 3 h.
d) Plating-out of suspect cultures on MSRV agar plates obtained from c) on two selective isolation agar media: Xylose Lysine Deoxycholate (XLD) agar and a second isolation medium for choice. The XLD agar plates were incubated at 37 °C ± 1 °C for 24 h ± 3 h, the second agar plates according to the manufacturer instructions.
e) Confirmation of at least 1 suspect colony obtained from d) by biochemical and serological testing. If negative, 4 additionally suspect colonies had to be confirmed.

For calculation of the LOD_{50} values for detection of *Salmonella* spp. in food samples, raw data were used of two validation studies

Table 1
Number and type of samples analysed in three interlaboratory comparison studies (ILS) for detection of *Salmonella* in samples from the primary production stage.

Year ILS	No. of participants	Matrix		No. of samples with matrix and *Salmonella*[a] at different contamination levels			No. of control samples[b] with *Salmonella*[a] at different contamination levels, no matrix			Inoculation of samples with
		Type	Amount per sample	Blank	Low	High	Blank	Low	High	
2008	32	Chicken faeces	10 g	5	5 STM 5 SE	5 STM 5 SE	2	3 STM 2 SE 2 SPan	1 SE	Reference materials (capsules)
2012	33	Pig faeces	25 g	5	5 STM 5 SD	5 STM 5 SD	2	2 STM 2 SD	1 SD	Reference materials (lenticules)
2013	36	Boot socks + chicken faeces	10 g/pair of boot socks	8	8 STM	8 STM	2	2 STM	2 STM	Diluted culture

[a] STM: *Salmonella* Typhimurium; SE: *Salmonella* Enteritidis; SD: *Salmonella* Derby; SPan: *Salmonella* Panama.
[b] Control samples consisted in the studies of 2008 and 2012 of reference materials only (no matrix added) and in the study of 2013 of moistened boot socks inoculated with a diluted culture of STM (no matrix added) or without *Salmonella* (blank).

performed in the frame of another EU project in 2000 (Feldsine et al., 2003). In these studies, EN ISO 6579:2002 was followed which comprises of the same successive culture steps as described above, but with the use of two selective enrichment broths instead of MSRV agar for step c): Rappaport Vassiliadis broth with soya (RVS), incubated at 41.5 °C ± 1 °C for 24 h ± 3 h, and Muller-Kauffmann tetrathionate-novobiocin (MKTTn) broth incubated at 37 °C ± 1 °C for 24 h ± 3 h.

2.3. Preparation of test materials

2.3.1. Test materials for interlaboratory study organised under the Mandate in 2013

For the interlaboratory study organised under the Mandate, boot sock samples were used. Boot socks are frequently used for sampling of chicken stables. The socks are worn over the boots and by walking through the stable, environmental material from the stable is collected. To make sure that the environmental material sticks well, the socks are moistened before use. Instead of walking through stables, the samples for the interlaboratory study were prepared in a more controlled way to make sure that each sample contained the same amount of 'environmental material'.

Each sample consisted of one pair of boot socks in a plastic bag (Sodibox, Nevez, France), moistened with 15 ml peptone saline solution (1.0 g enzymatic digest of casein, 8.5 g sodium chloride, in 1 l distilled water), to which 10 g chicken faeces from a pathogen-free laying hen flock was added. The absence of *Salmonella* in the chicken faeces was previously checked following EN ISO 6579:2002/Amd.1:2007 (Anonymous, 2007). The amount of (natural) background flora in the chicken faeces was determined after receipt of the faeces, as well as after storage at 5 °C, at the start of the interlaboratory study. For this, the total bacterial count as well as the number of *Enterobacteriaceae* were determined, by following respectively EN ISO 4833 (Anonymous, 2003b) and EN ISO 21528-2 (Anonymous, 2004).

The boot socks (with faeces) were artificially contaminated at the laboratory of the EURL-*Salmonella*. For this, *Salmonella* Typhimurium (STM) ATCC 14028 (American Type Culture Collection, USA) was cultured in BPW at 37 °C overnight. Next, tenfold dilutions were made in peptone saline solution, and the number of colony forming units (cfu) per dilution were counted by plating on XLD-agar and incubating the plates at 37 °C for 20–24 h. Meanwhile, the dilutions were stored at 5 °C. Based on the plate counts, a sufficient amount of two (final) dilutions were prepared from the stored dilutions and used to inoculate individual boot sock samples with approximately 0.1 ml, resulting in samples with 5–10 cfu/pair of boot socks (low level samples), or with 50–100 cfu/pair of boot socks (high level samples). For each level, the boot sock samples were inoculated with the same volume from the same (mixed) dilution of *Salmonella* Typhimurium. In this way, the variation in the contamination level between the samples of one batch was kept as low as possible. The control boot sock samples (without faeces), were inoculated in the same way. The concentration of the inoculums used to contaminate the boot sock samples was confirmed by plating the relevant dilution on XLD agar plates. A sub-set of the boot sock samples were not inoculated with *Salmonella*, to become the blank samples.

A few days before dispatch of the samples to the participants of the interlaboratory study, a total of 1200 boot sock samples were prepared and stored at 5 °C until the day of mailing.

2.3.2. Test materials for interlaboratory study organised by EURL-Salmonella in 2008

Five batches of reference materials were prepared, a few weeks before the interlaboratory study. For this purpose milk, artificially contaminated with a *Salmonella* strain from the National Institute for Public Health and the Environment (RIVM) culture collection, was spray-dried (In 't Veld et al., 1996). The obtained highly contaminated milk powder was mixed with sterile (γ-irradiated) milk powder (Carnation, Nestlé, the Netherlands) to obtain the desired contamination level. The mixed powder was filled in gelatine capsules resulting in the final reference materials (RMs), with the following mean contamination levels (Kuijpers et al., 2008):

- 5 and 44 cfu/capsule for *Salmonella* Typhimurium (STM5 and STM44): ALM40 (RIVM Collection, the Netherlands);
- 7 and 91 cfu/capsule for *Salmonella* Enteritidis (SE7 and SE91): LBA88-8993 (RIVM Collection, the Netherlands);
- Blank capsules containing no microorganisms.

The contamination levels of the capsules were determined following the procedure as described by Schulten et al. (2000). In short the procedure is as follows:

- reconstitution of each capsule in 5 ml peptone saline solution in a Petri dish at 38.5 °C ± 1 °C for 45 min ± 5 min;
- repair of *Salmonella* by the addition of 5 ml molten double concentrated plate count agar to the reconstituted capsule solution, and after solidification incubation at 37 °C ± 1 °C for 4 h ± 0.5 h;
- after incubation, 10 ml of molten double concentrated Violet Red Bile Glucose agar was added as an over layer and after solidification the plates were incubated at 37 °C ± 1 °C for 20 h ± 2 h.

For the interlaboratory study, each participant received a batch of pathogen-free chicken faeces separately packed from the reference materials. At the day of analyses, the participants had to prepare the samples by combining 10 g chicken faeces with one reference material according to a detailed protocol.

2.3.3. Test materials for interlaboratory study organised by EURL-Salmonella in 2012

Five batches of reference materials were prepared by the Health Protection Agency (HPA, currently named Public Health England), Newcastle, United Kingdom, 1–4 months before the interlaboratory study. These reference materials consisted of so-called lenticule discs, being plano-convex (lens-shaped) discs containing microorganisms at a defined number in a solid water-soluble matrix (Boyd et al., 2006; Desai et al., 2006). Lenticules with the following mean contamination levels were used for the ILS of 2012:

- *Salmonella* Typhimurium at a level of 10 cfu/lenticule disc (STM10): NCTC 12023 (National Collection of Type Cultures, UK) batch 323–111,025;
- *Salmonella* Typhimurium at a level of 58 cfu/lenticule disc (STM58): NCTC 12023 batch 523-100927R;
- *Salmonella* Derby at a level of 6 cfu/lenticule disc (SD6): NCTC 5722 batch 624–111,215;
- *Salmonella* Derby at a level of 37 cfu/lenticule disc (SD37): NCTC 5722 batch 634–111,214;
- Blank lenticule discs containing no microorganisms: batch 000–110,825.

The mean number of organisms of each batch was counted by HPA before the lenticule discs were sent to the EURL-*Salmonella*. For this, the HPA tested 30 lenticules per batch. For the current study, the contamination level of each batch of lenticule discs was verified at the EURL-*Salmonella* by testing 2 lenticule discs (containing STM) or 5 lenticule discs (containing SD) per batch after receipt and storage at −20 °C. For the counting of the lenticule discs, each lenticule disc was placed onto Colombia agar plates with sheep blood (Oxoid PB5008A, Germany). After 10 min of rehydration of the lenticule disc, the resultant 'drop' was spread over the plate and incubated at 37 °C for 20–24 h (Kuijpers and Mooijman, 2013).

For the interlaboratory study, each participant received a batch of pathogen-free pig faeces separately packed from the reference materials. At the day of analyses, the participants had to prepare the samples by combining 25 g pig faeces with one reference material according to a detailed protocol.

2.4. Homogeneity and stability of the test materials

2.4.1. Test materials for interlaboratory study organised under the Mandate in 2013

Acceptance of the production batches for use in the interlaboratory study was made on the basis of achieving satisfactory homogeneity and stability according to the laboratories' own criteria.

For an indication on the variation between samples in the number of *Salmonella*, and to gain information on the contamination levels in the final samples, a five-tube most probable number (MPN) technique was used. For this, tenfold dilutions of five boot sock samples of each contamination level were tested, representing 10 g, 1 g and 0.1 g of the original sample. The presence of *Salmonella* was determined in each dilution by following EN ISO 6579:2002/Amd.1:2007 (Anonymous, 2007). From the number of confirmed positive dilutions, the MPN of *Salmonella* in the original sample was calculated, by using an MPN program in Excel, freely available on the Internet (Jarvis et al., 2010).

The stability of the artificially contaminated boot sock samples was tested prior to the interlaboratory study in three experiments with specially prepared batches with different contamination levels. For this, low and high contaminated test samples were stored at +5 °C, the normal storage temperature of the samples, and at elevated temperatures to test the influence of abusive transport and/or storage temperatures (+10 °C and +15 °C). The presence of *Salmonella* was tested in 5 samples at each storage temperature at day 0, 7, 14 and 21, following EN ISO 6579:2002/Amd.1:2007 (Anonymous, 2007). The set-up of the three experiments was as follows:

- Experiment 1: boot sock samples artificially contaminated with *Salmonella* Typhimurium at 7 cfu/sample stored at 5 °C and at 15 °C;
- Experiment 2: boot sock samples artificially contaminated with *Salmonella* Typhimurium at 55 cfu/sample stored at 5 °C and at 15 °C;
- Experiment 3: boot sock samples artificially contaminated with *Salmonella* Typhimurium at 14 cfu/sample stored at 5 °C and at 10 °C.

The stability of the (natural) background flora was tested in blank boot sock samples (with faecal material, but without the addition of *Salmonella*) for the same storage conditions. For this purpose the EN ISO procedures for establishing the total number of aerobic bacteria (EN ISO 4833; Anonymous, 2003b) and for analysing the number of *Enterobacteriaceae* (EN ISO21528-2; Anonymous, 2004) were followed.

2.4.2. Test materials for interlaboratory study organised by EURL-Salmonella in 2008

Before filling all mixed contaminated milk powders into gelatine capsules, test batches of 60 capsules were prepared of each mixture to determine the mean number of cfu per capsule and the homogeneity of the mixture. The remaining mixed powders were stored at −20 °C. If the test batches fulfilled the pre-set criteria for contamination level and homogeneity, the relevant mixed powders were completely filled into gelatine capsules and stored at −20 °C.

Information on (long-term) stability of the capsules was obtained from studies performed earlier (2001–2006) on the same highly contaminated milk powders as used for preparing the batches of capsules for the interlaboratory study of 2008. Additionally, also short term stability of the capsules was tested when stored at elevated (abusive) temperatures. Details on the homogeneity and stability studies can be found in Kuijpers et al., 2008.

2.4.3. Test materials for interlaboratory study organised by EURL-Salmonella in 2012

Each batch of lenticule discs were delivered with an insert, giving information on the contamination level and homogeneity (tested by HPA).

Tests on the long-term stability at storage temperature (−20 °C) and short-term tests for stability at abusive storage temperatures on lenticule discs containing S. Enteritidis (SE) and S. Typhimurium (STM) were performed earlier by the EURL-*Salmonella*, and showed good results (Kuijpers and Mooijman, 2011, 2012).

For the interlaboratory study of 2012, some additional stability tests were performed for storage of the lenticule discs at storage temperature (−20 °C) and at elevated temperatures. Details on the homogeneity and stability studies can be found in Kuijpers and Mooijman, 2013.

2.5. Statistical analysis of the interlaboratory studies data

For the performance of a qualitative method, specificity, sensitivity and LOD_{50} (contamination level at which 50% of the samples are found positive) were calculated. LOD_{50} was calculated with the model described in EN ISO 16140-2, using a program in Excel, freely available on the Internet (Anonymous, 2016b). In this program, the contamination level of each batch of test samples has to be entered in cfu/g or cfu/ml. For this, the inoculum levels of each batch of samples were used for the ILSs of 2008, 2012 and 2013. For the earlier EURL-*Salmonella* studies this concerned the mean level of each batch of reference materials per 10 g faeces (ILS 2008) or 25 g faeces (ILS 2012). For ILS 2013, this concerned the level of *Salmonella* in the inoculum to contaminate the low level and high level samples per 10 g chicken faeces (the weight of the pair of boot socks was not taken into account). Of the food samples used in the validation studies in 2000, information on the inoculum

levels was no longer available. However, MPN results of the artificially contaminated food samples were available and these were used for calculation of the LOD_{50} values.

The calculation of specificity and sensitivity (in %) is described below.

Specificity (*SP*):

$$SP = \frac{N_-}{N} * 100\%$$

where *N*- is the number of negative results at blank level and *N* is the total number of analysed samples at this level.

Sensitivity (SE):

$$SE = \frac{N_+}{N} * 100\%$$

where $N+$ is the number of positive results at a low/high contamination level and N is the total number of analysed samples at this level.

3. Results

3.1. Stability and homogeneity of the test samples of the interlaboratory study organised under the Mandate in 2013 (boot sock samples)

Three batches of boot sock samples were subjected to the stability tests prior to the interlaboratory study. These batches were artificially contaminated with *Salmonella* Typhimurium at two low levels (7 cfu/sample and 14 cfu/sample) and one high level (55 cfu/sample). The results of the stability studies are shown in Fig. 1. The artificially contaminated boot sock samples were relatively stable when stored at 5 °C and at 10 °C. After 14 days, 4 to 5 of the 5 samples (high contaminated as well as low contaminated) were still tested positive for *Salmonella*. A longer storage time (21 days), especially in combination with a higher storage temperature (15 °C) resulted in a lower number of positive samples. This was most clear for the low contaminated samples containing 7 cfu *Salmonella*/sample and stored at 15 °C. An explanation could be that some die off occurred of *Salmonella* in the samples and/or that due to the increase of the background flora during storage at 10–15 °C (see below), the growth of *Salmonella* was suppressed.

The amount of natural background flora in the chicken faeces added to the boot socks showed to be stable when stored at 5 °C for 3 weeks (Fig. 2). When stored at higher temperatures (10 °C and 15 °C) the total aerobic count and the number of *Enterobactericeae* increased with 1 to 1.5 $\log_{10}$ cfu/g (Fig. 2).

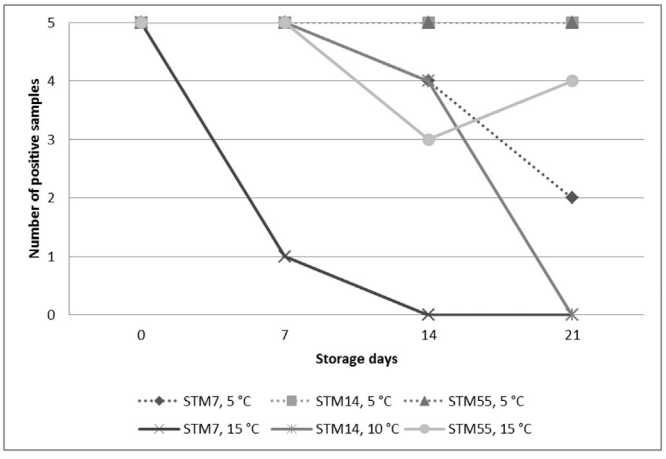

Fig. 1. Stability tests of boot sock samples (5 samples per batch, time and temperature combination) artificially contaminated with *Salmonella* Typhimurium at three different levels, 7 cfu/sample (STM7), 14 cfu/sample (STM14) and 55 cfu/sample (STM55), and stored at three different temperatures (5 °C, 10 °C and 15 °C).

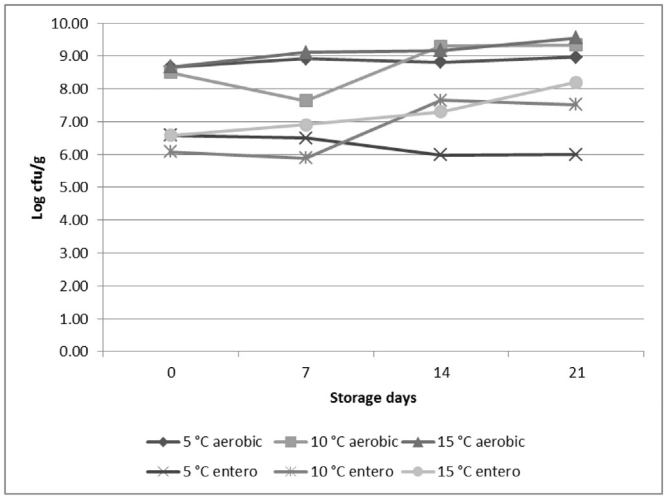

Fig. 2. Stability tests of natural background flora in 'blank' boot sock samples with chicken faeces from a laying hen flock, stored at three different temperatures (5 °C, 10 °C and 15 °C), given in $\log_{10}$ cfu/g total aerobic count ('aerobic') and $\log_{10}$ cfu/g *Enterobacteriaceae* ('entero').

A stability of 14 to 21 days of the artificially contaminated boot sock samples was considered sufficient for the test samples of the interlaboratory study, when taking into account the following: preparation of the test samples shortly before the performance of the interlaboratory study, contamination level of the low contaminated samples between 5 and 10 cfu/sample, contamination level of the high contaminated samples approx. 10 times higher than the low contaminated samples, storage of the samples at 5 °C and short transport time (1–2 days) at 5–10 °C.

The final batches of boot sock samples for the interlaboratory study were inoculated with *Salmonella* Typhimurium at a low level of 9 cfu/sample and at a high level of 81 cfu/sample. After 10 days of storage at 5 °C, the contamination level in the inoculated test samples were enumerated with an MPN technique, showing an MPN of 3.3 *S.* Typhimurium per sample (95% confidence interval 1.1–10) for the low contaminated samples, and an MPN of 160 (53–490) *S.* Typhimurium per sample for the high contaminated samples, which was considered acceptable for use in the interlaboratory study. The amount of background flora at the date of the study was approximately 10^7 cfu/g for the aerobic bacteria and approximately 10^4 cfu/g for the number of *Enterobacteriaceae* (Kuijpers and Mooijman, 2014).

Details on the results of the homogeneity and stability studies of the test materials used in the EURL-*Salmonella* interlaboratory studies of 2008 and 2012 can be found in respectively Kuijpers et al., 2008 and in Kuijpers and Mooijman, 2013. For these latter studies, the matrix samples were artificially contaminated by the participating laboratories by adding a reference material to a prescribed amount of matrix at the day of analysis. For both type of reference materials used in the two studies (capsules and lenticules), it was shown that the homogeneity fulfilled the pre-set criteria and that the reference materials were stable when stored at −20 °C for at least 1 year. Additionally, the reference materials were shown to be stable when stored at +5 °C for approx. 1 week, which was sufficient for mailing of the samples.

3.2. General results of the interlaboratory study organised under the Mandate in 2013

All participants received the boot sock samples within 48 h after dispatch. For the majority of the parcels, the temperature did not exceed 5 °C during transport and storage at the participating laboratory. Parcels of four laboratories were subjected to temperatures of 5 °C - 10 °C for only a few hours and the parcel of one laboratory was

transported and/or stored at 10 °C - 16 °C for two days. Still, no negative effects were seen on the results of the laboratories concerned as the laboratory with the longest transport time in combination with the highest temperatures still tested all contaminated samples positive for *Salmonella*. All participants used the prescribed method (EN ISO 6579:2002/Amd.1:2007) for analysis of the test samples. No problems were encountered with the analysis of the test samples, neither with the analysis of the control samples.

3.3. Results excluded from further analysis

On forehand it was agreed that results from laboratories would not be excluded unless they reported (major) technical deviations which might have influenced the results. For that reason, data of some laboratories have not been used for calculation of the performance characteristics. The number of datasets which were excluded from further analysis and the reasons for exclusion are given below.

- Interlaboratory study organised under the Mandate in 2013: the results of 3 laboratories were excluded because these laboratories used deviating concentrations of novobiocin in MSRV agar. The prescribed concentration is 0.01 g/l, and the three laboratories used a higher concentration of novobiocin (two laboratories used 0.02 g/l and one laboratory used 0.05 g/l). Earlier studies have shown that the concentration of novobiocin can affect the bacterial motility (Soutourina et al., 2001; Veenman et al., 2007) and as the specific growth of *Salmonella* on MSRV agar is based on motility, it is essential that the prescribed concentration novobiocin in MSRV agar is used.
- Interlaboratory study organised by EURL-*Salmonella* in 2008: the results of 13 laboratories were excluded for various reasons. One laboratory used a longer time to reconstitute the reference materials (60 min instead of 45 min), which may have resulted in possible growth of *Salmonella* during reconstitution and thus in more positive samples than expected. One laboratory incubated the pre-enrichment broth (BPW) for 24 h instead of 16–20 h. A longer incubation time of the BPW may cause overgrowth of background flora and suppression of the growth of *Salmonella* and therefore possibly more negative samples than expected. One laboratory incubated the MSRV agar plates at 37 °C instead at 41.5 °C. Incubation temperature is a selective factor for the growth on MSRV agar. Using a lower incubation temperature than prescribed may result in more growth of the background flora and may negatively affect the growth of *Salmonella*. Ten laboratories used deviating concentrations of novobiocin in MSRV agar of which four did not report the concentration or reported the concentration to be 0 g/l and six laboratories reported a higher concentration than 0.01 g/l.
- Interlaboratory study organised by EURL-*Salmonella* in 2012: the results of 7 laboratories were excluded because of a longer incubation time of the BPW (23 h) than prescribed (one laboratory) and because of using deviating concentrations of novobiocin in MSRV agar (six laboratories).

3.4. Statistical analysis of data

3.4.1. Performance characteristics detection of Salmonella in samples from the primary production stage

The results of the data analysis of the interlaboratory study organised under the Mandate in 2013 are summarised in Table 2. After exclusion of the data of three laboratories (see Section 3.3), a total of 264 data per contamination level retained for the analysis of the performance characteristics. Only one laboratory tested one blank boot sock sample (false) positive for *Salmonella*, resulting in an overall specificity of 99.6%. The sensitivity rates for the low contaminated boot sock samples as well as for the high contaminated samples were 94.7% and 98.1%, respectively. Thirteen laboratories did not detect *Salmonella* in 14 low contaminated samples and 5 laboratories did not detect *Salmonella* in 5 high contaminated samples. The overall LOD_{50} was approximately 4 cfu/sample.

Table 2
Results of data analysis of interlaboratory study 2013, detection of *Salmonella* in boot sock samples.

Parameter	Pair of boot socks + 10 g laying hen faecal material +		
	Blank	STM9[a]	STM81[a]
Number of participating collaborators	36	36	36
Number of samples per collaborator	8	8	8
Number of laboratories retained after evaluation of the data	33	33	33
Number of samples retained after evaluation of the data	264	264	264
Sample size	Boot socks		
Specificity, in %	99.6	–	–
Sensitivity per level, in %	–	94.7	98.1
LOD_{50} (95% confidence interval), in cfu/sample	–	3.8 (3.2 to 4.4)	

–: not applicable.
[a] The boot sock samples were artificially contaminated with a diluted culture of *Salmonella* Typhimurium (STM) at a level of 9 cfu/sample and a level of 81 cfu/sample.

The results of the data analysis of the interlaboratory study organised by EURL-*Salmonella* in 2008 are summarised in Table 3. After exclusion of the data of 13 laboratories (see Section 3.3), a total of 95 data per contamination level retained for the analysis of the performance characteristics. All laboratories tested the blank chicken faeces samples correctly negative for *Salmonella*, resulting in an overall specificity of 100%. For the artificial contamination of the chicken faeces samples, two *Salmonella* serovars were used. The sensitivity rate of the highly contaminated samples with *Salmonella* Typhimurium as well as with *Salmonella* Enteritidis was 100%. The overall sensitivity rate of the low contaminated samples with *Salmonella* Enteritidis was 67.4% (14 laboratories did not detect *Salmonella* in 31 samples), while for the low

Table 3
Results of data analysis of interlaboratory study 2008, detection of *Salmonella* in chicken faeces samples.

Parameter	Chicken faeces +				
	Blank	STM5[a]	STM44[a]	SE7[a]	SE91[a]
Number of participating collaborators	32	32	32	32	32
Number of samples per collaborator	5	5	5	5	5
Number of collaborators retained after evaluation of the data	19	19	19	19	19
Number of samples retained after evaluation of the data	95	95	95	95	95
Test portion size, in g	10	10	10	10	10
Specificity, in %	100	–	–	–	–
Sensitivity per serovar and level, in %	–	96.8	100	67.4	100
LOD_{50} per serovar (95% confidence interval), in cfu/test portion	–	1.0 (0.7 to 1.4)		4.3 (3.3 to 5.6)	
LOD_{50} overall (95% confidence interval), in cfu/test portion	–	2.5 (2.1 to 3.0)			

[a]Chicken faeces samples were artificially contaminated with reference materials with the following strains and levels:
Salmonella Typhimurium (STM) at a level of 5 cfu/test portion and a level of 44 cfu/test portion;
Salmonella Enteritidis (SE) at a level of 7 cfu/test portion and a level of 91 cfu/test portion.
–: not applicable.

Table 4
Results of data analysis of interlaboratory study 2012, detection of *Salmonella* in pig faeces samples.

Parameter	Pig faeces +				
	Blank	SD6[a]	SD37[a]	STM10[a]	STM58[a]
Number of participating collaborators	33	33	33	33	33
Number of samples per collaborator	5	5	5	5	5
Number of collaborators retained after evaluation of the data	26	26	26	26	26
Number of samples retained after evaluation of the data	130	130	130	130	130
Test portion size, in g	25	25	25	25	25
Specificity, in %	99.2	–	–	–	–
Sensitivity per serovar and level, in %	–	88.5	97.7	91.5	98.5
LOD_{50} per serovar (95% confidence interval), in cfu/test portion	–	2.8 (2.2 to 3.5)		3.8 (3.0 to 4.7)	
LOD_{50} overall (95% confidence interval), in cfu/test portion	–	3.2 (2.8 to 3.8)			

[a]Pig faeces samples were artificially contaminated with reference materials with the following strains and levels:
Salmonella Derby (SD) at a level of 6 cfu/test portion and a level of 37 cfu/test portion;
Salmonella Typhimurium (STM) at a level of 10 cfu/test portion and a level of 58 cfu/test portion.
–: not applicable.

Table 5
LOD_{50} values and sensitivity rates for detection of *Salmonella* in food samples, calculated from data of two interlaboratory studies (ILS-I and ILS-II) organised in 2000 (Anonymous, 2002; Feldsine et al., 2003).

	Fresh cheese curd[a]		Egg powder[b]			Raw poultry meat[c]			
	ILS-I		ILS-I		ILS-II	ILS-I		ILS-II	
	Low level	High level	Low level	High level	Low level	Low level	High level	Low level	High level
Contamination level of artificially contaminated samples, MPN per 25 g (95% confidence interval)[d]	0.7 (0.2–2.4)	37.2 (7.5–95)	9.6 (2.2–26)	115 (22.5–495)	0.7 (0.2–2.3)	3.7 (1.0–9.5)	5.8 (1.0–25)	0.2 (0.04–0.9)	1.0 (2.2–4.5)
Number of participating collaborators	23	23	26	26	9	25	25	13	13
Number of samples per collaborator	5	5	5	5	5	5	5	6	6
Number of collaborators retained after evaluation of the data	21	21	21	21	8	20	20	13	13
Number of samples retained after evaluation of the data	105	105	105	104	40	99	100	78	78
Test portion size, in g	25	25	25	25	25	25	25	25	25
Sensitivity, in %	74.3	83.8	98.1	99	nd	98	100	nd	nd
LOD_{50} (95% confidence interval), in cfu/test portion	5.7 (4.0–8.1)		6.0 (4.7–7.7)			nd	nd	2.2 (1.5–3.2)	

nd: not determined.
[a] Cheese samples were artificially contaminated with *Salmonella* Montevideo (lactose positive strain).
[b] Egg powder samples were artificially contaminated with *Salmonella* Panama.
[c] Poultry meat samples were artificially contaminated with *Salmonella* Typhimurium in ILS-I and were naturally contaminated with *Salmonella* spp. in ILS-II.
[d] Contamination levels determined using a Most Probable Number (MPN) technique.

contaminated samples with *Salmonella* Typhimurium this was 96.8% (3 laboratories did not detect *Salmonella* in 3 samples). As a result, the LOD_{50} values also differed per serovar: approximately 1 cfu/test portion for *Salmonella* Typhimurium and approximately 4 cfu/test portion for *Salmonella* Enteritidis.

The results of the data analysis of the interlaboratory study organised by EURL-*Salmonella* in 2012 are summarised in Table 4. After exclusion of the data of 7 laboratories (see Section 3.3), a total of 130 data per contamination level retained for the analysis of the performance characteristics. Only one laboratory tested one blank pig faeces sample (false) positive for *Salmonella*, resulting in an overall specificity of 99.2%. For the artificial contamination of the pig faeces samples also two *Salmonella* serovars were used, but the difference in sensitivity rates was less pronounced than for the study of 2008. The overall sensitivity rates of the highly contaminated samples was 98.5% for *Salmonella* Typhimurium (one laboratory did not detect *Salmonella* in two samples) and 97.7% for *Salmonella* Derby (two laboratories did not detect *Salmonella* in three samples). With the low contaminated samples with *Salmonella* Typhimurium a sensitivity rate of 91.5% was found (five laboratories did not detect *Salmonella* in 11 samples) and with the low contaminated samples with *Salmonella* Derby a sensitivity rate of 88.5% was found (six laboratories did not detect *Salmonella* in 15 samples). The overall LOD_{50} for all samples was approximately 3 cfu/test portion.

Tables 2–4 are also published in Annex C of EN ISO 6579-1 (Anonymous, 2017).

3.4.2. LOD_{50} values for detection of Salmonella in food samples

In EN ISO 6579 of 2002, performance characteristics were published for the detection of *Salmonella* in three different food samples: fresh cheese curd, dried egg powder and raw poultry meat. These performance characteristics were derived from interlaboratory studies organised with these matrices in 2000 in the frame of the European project SMT CT 96 2098 (AFSSA, 2001; Feldsine et al., 2003) and consisted of specificity, sensitivity, accordance and concordance. The latter two performance characteristics are no longer used because of their limited use, and therefore an attempt was made to calculate the LOD_{50} values instead. For this, the raw data of the interlaboratory studies of spring 2000 could be used as they were still available at the French Agency for Food, Environmental and Occupational Health & Safety (ANSES, formerly called AFSSA). Additional to the interlaboratory study performed to determine the performance characteristics published in EN ISO 6579:2002 (Anonymous, 2002), a second study was organised in fall 2000 to obtain fractional recovery for egg powder samples and raw poultry meat. The results of this second study were published by Feldsine et al. in 2003 and were used, additional to the results of the first study, to calculate LOD_{50} values for the three matrices mentioned above. The specificity and sensitivity rates were already published in EN ISO 6579:2002 (Anonymous, 2002) and were copied unchanged

into the updated tables with performance characteristics of EN ISO 6579-1:2017 (Anonymous, 2017). In Table 5 the LOD_{50} values are given for the three matrices, fresh cheese curd, dried egg powder and raw poultry meat. Table 5 also shows information on the contamination levels of the samples and on the number of data used for calculating the LOD_{50} values. For completeness, also the sensitivity rates are given, copied from the tables in Annex C of EN ISO 6579:2002. The specificity rate was 100% for all three matrices. The updated performance characteristics have also been published in Annex C of EN ISO 6579-1 (Anonymous, 2017).

4. Discussion

In the past, only a few European and International standard methods have been published with information on the performance characteristics of the method. These characteristics are important, e.g. to verify the performance of a laboratory when the method is newly introduced, or to validate an alternative method. To enhance the number of Standard methods with performance characteristics, the European Commission issued a Mandate for the validation of 15 microbiological standard methods. Although EN ISO 6579 (Anonymous, 2002) did contain performance characteristics for the detection of *Salmonella* in food samples, its amendment published in 2007 for the detection of *Salmonella* in samples from the primary production stage (Anonymous, 2007) did not. For that reason, validation of Amd.1 of EN ISO 6579 was part of Mandate M/381. For this so-called 'vertical' method only one matrix needed to be analysed in an interlaboratory study. Boot sock samples with (artificially contaminated) chicken faeces were chosen as this type of samples is often analysed in laboratories and was considered representative for samples from the primary production stage.

The performance characteristics to be determined for qualitative methods under the Mandate differed slightly from the ones already published in EN ISO 6579:2002. Instead of accordance and concordance, LOD_{50} needed to be determined. To be able to calculate LOD_{50} values, samples with a low level should be used so that fractional recovery is obtained in the ILS (ideally, 50% of the samples should be positive, with a range of 25%–75%). The preparation of such samples for an interlaboratory study is quite a challenge. The contamination level should not be too low as die off may occur during storage and mailing of the samples, which may result in too many negative samples. Furthermore, when real matrices with (natural) background flora are used, the growth of the target organism may be disturbed by the background flora present in the matrix, although the amount of disturbance is difficult to predict. Taking all these possible effects into account, the contamination level of *Salmonella* in the low contaminated boot sock samples was aimed to be 5–10 cfu/pair of boot sock samples, which was well achieved with 9 cfu S. Typhimurium/sample at the day of preparation. These low contaminated samples showed to be stable for 2–3 weeks when stored at 5 °C, and for approximately 1 week when stored at 10–15 °C, which was considered sufficient for the purpose of the study. After 10 days of storage at 5 °C, the number of *Salmonella* in the samples was approx. 3 cfu (with a 95% confidence interval of 1–10 cfu), confirming that the initial inoculum of 9 cfu was needed to retain sufficient *Salmonella* in the samples at the day of analysis. The total amount of background flora in the chicken faeces added to the boot socks was relatively high (approx. 10^7 cfu/g), so that some disturbance in the growth of *Salmonella* was expected, probably resulting in fractional recovery. However, the final results showed that the sensitivity rate of the low contaminated samples was almost 95%, meaning that only a (very) small amount of fractional recovery was obtained. This is a good result for the performance of the method, showing that this method is able to detect low levels of *Salmonella* in the presence of high amounts of background flora, but less ideal for the calculation of LOD_{50}. As still some fractional recovery was found, it was possible to give an indication on the LOD_{50} value for the boot sock samples.

To see if the performance characteristics found in the interlaboratory study with boot sock samples were representative for (other) samples from the primary production stage, they were compared to data obtained from interlaboratory studies organised earlier. The performance characteristics derived from these earlier studies showed in general comparable values to the ones of the study with boot socks, especially for the LOD_{50} values.

Thanks to the fact that the raw data of the validation studies performed in 2000 were still available, the LOD_{50} values could relatively easily be determined from these data for the detection of *Salmonella* in food samples. This shows the importance of storage of raw data of validation studies, so that the data remain available for future use. The LOD_{50} values for the food samples were close, or slightly higher than the values found for the samples from the primary production stage: 2–6 cfu/test portion for the food samples against 2–4 cfu/test portion for the samples from the primary production stage.

5. Conclusions

Thanks to the activities performed under, and in line with, Mandate M/381, the revised version of the European and International standard for detection of *Salmonella* in samples of the food chain (EN ISO 6579-1:2017) now contains (updated) performance characteristics not only for detection of *Salmonella* in food samples, but also for detection of *Salmonella* in samples from the primary production stage.

The outcome of the validation studies showed that EN ISO 6579-1 for detection of *Salmonella* works equally well for food samples as for samples from the primary production stage.

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Conflict of interest

No conflict of interest declared.

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