National Institute for Public Health
and the Environment
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Detection of *Salmonella* in chicken faeces

This report contains an erratum d.d. 17-7-2018 after page 34

RIVM Report 2017-0083

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Colophon

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Synopsis

The 20th EU Interlaboratory comparison study in primary production (2017)
Detection of *Salmonella* in chicken faeces

In March 2017, the twentieth EURL-*Salmonella* interlaboratory comparison study on the detection of *Salmonella* in samples taken from the primary production stage was organised. Participation was obligatory for all EU Member State National Reference Laboratories (NRLs) responsible for the detection of *Salmonella* in these types of samples.

Results
All laboratories were able to detect *Salmonella* in all the contaminated chicken faeces samples. Both the blank control sample and the positive control sample were analysed correctly by all laboratories. One laboratory made a labelling mistake and switched the process control for the positive control and therefore scored a 'moderate performance'.
Blank samples containing chicken faeces not contaminated with *Salmonella* were correctly analysed as negative by almost all laboratories. One laboratory found *Salmonella* present in 3 of the 6 blank samples; this was indicated as a 'poor performance'.

Participants
In total, 36 NRLs participated in this study: 29 NRLs from 28 EU Member States, 6 NRLs from other countries in Europe (EU candidate or potential EU candidate Member States and countries of the European Free Trade Association (EFTA)) and, on request of the European Commission, one NRL from a non-European country. EURL-*Salmonella* is situated at the Dutch National Institute for Public Health and the Environment (RIVM). The main task of EURL-*Salmonella* is to monitor and improve the performance of the national reference laboratories in Europe.

Design of study
Each laboratory received a package of chicken faeces samples. The samples were contaminated by the EURL-*Salmonella* at two different *Salmonella* concentrations: high and low. The package also included blank samples without *Salmonella*. The laboratories were asked to analyse the samples according to the internationally prescribed method for the detection of *Salmonella*.

Keywords: *Salmonella*, EURL, NRL, interlaboratory comparison study, *Salmonella* detection method, chicken faeces

Publiekssamenvatting

Het 20e EURL-*Salmonella* ringonderzoek primaire productie (2017)

Detectie van *Salmonella* in kippenmest

In maart 2017 vond het twintigste EURL-*Salmonella* ringonderzoek naar *Salmonella* plaats. Deze jaarlijkse kwaliteitstoets is verplicht voor alle Nationale Referentie Laboratoria (NRL's) van de Europese lidstaten die verantwoordelijk zijn voor het aantonen van *Salmonella* in dierlijke mest.

Resultaten

Alle deelnemers waren in staat om *Salmonella* op te sporen in de kunstmatig besmette kippenmestmonsters. Ook hebben de laboratoria de meegestuurde controlemonsters correct geanalyseerd. Eén laboratorium heeft een fout gemaakt met het labelen van de monsters en heeft daardoor de twee controlemonsters verwisseld. Hiervoor kreeg dit laboratorium een matige score. Bijna alle laboratoria konden de monsters waar geen *Salmonella* aan was toegevoegd (blanco) als zodanig opsporen. Eén laboratorium vond echter *Salmonella* in drie van de zes blanco monsters en scoorde daardoor een onvoldoende.

Deelnemers

In totaal hebben 36 NRL's deelgenomen: 29 NRL's van 28 lidstaten in de EU, zes NRL's uit kandidaat-landen voor het EU-lidmaatschap of landen van de European Free Trade Association (EFTA) en één niet-Europees NRL dat op verzoek van de Europese Commissie is toegevoegd (Israël). Het Europese Referentie Laboratorium (EURL) *Salmonella* is gevestigd bij het Nederlandse Rijksinstituut voor Volksgezondheid en Milieu (RIVM). De hoofdtaak van het EURL-*Salmonella* is toezien op de kwaliteit van de nationale referentielaboratoria voor deze bacterie in Europa.

Werkwijze

Elk laboratorium kreeg een pakket toegestuurd met daarin de monsters met kippenmest. De kippenmest is op het EURL-laboratorium besmet met de *Salmonella*-bacterie in twee concentraties (hoog en laag). Ook zijn er onbesmette blanco monsters meegestuurd. De laboratoria dienden de monsters te analyseren volgens de internationaal voorgeschreven methode op de aanwezigheid van *Salmonella*.

Kernwoorden: *Salmonella*, EURL, NRL, ringonderzoek, kippenmest, *Salmonella*-detectiemethode.

Contents

Summary

In March 2017, the twentieth EURL-*Salmonella* interlaboratory comparison study on the detection of *Salmonella* in samples from the primary production stage was organised. A total of 36 NRLs participated in this study: 29 NRLs from 28 EU Member States, 6 NRLs from third countries within Europe (EU candidate or potential EU candidate Member States) and countries of the European Free Trade Association (EFTA)), and on request of DG-SANTE, one NRL from a non-European country (Israel).

In this study, *Salmonella* free chicken faeces originating from a specific pathogen free (SPF) laying hen farm was used. The chicken faeces samples were artificially contaminated with a diluted culture of *Salmonella* Infantis at the EURL laboratory.

Each NRL received a total of 20 blindly coded samples consisting of 12 chicken faeces samples artificially contaminated with two different levels of *Salmonella* Infantis (6x low (17 cfu/25 g faeces) and 6x high (55 cfu/25 g faeces)), 6 blanc chicken faeces samples and 2 control samples consisting of a procedure control blank and a control sample to be inoculated by the participants using their own positive control strain. The samples were stored at 5 °C until the day of transport. On Monday 13 March 2017, the contaminated chicken faeces samples were packed and sent to the NRLs. On arrival, the NRLs were asked to store the samples at 5 °C until the start of the analysis.

Method
All laboratories used the prescribed method (Annex D of ISO 6579:2007 or ISO 6579-1:2017) with selective enrichment on MSRV.

Results control samples
All laboratories scored well analysing both the procedure control as well as their own positive control samples. One laboratory made a labelling mistake and switched the process control for the positive control. This laboratory (lab code 16) scored a moderate performance.

Results artificially contaminated chicken faeces samples
Almost all laboratories detected *Salmonella* in all the chicken faeces samples contaminated with a high level of *Salmonella*. Two laboratories (lab codes 3 and 21) scored 1 of the 6 high level samples negative. This is still within the criteria for good performance which allows for 1 negative sample.

In addition, almost all laboratories detected *Salmonella* in all 6 low level samples. Three laboratories (lab codes 9, 34 and 36) scored 1 of the 6 low level samples negative for *Salmonella.* This is well above the criteria for good performance which allows for three negative samples out of 6. The sensitivity score was 99% for these samples.

The specificity of the study is given by the correctly scored blank samples; this was 99% for this study. Only 1 laboratory did not score all 6 blank samples negative (lab code 18). This laboratory reported 3 of the 6 blank samples positive for *Salmonella* and scored a poor performance.

Overall, the laboratories scored well in this interlaboratory study. The accuracy was 99%. Thirty-four laboratories fulfilled the criteria of good performance, one laboratory scored moderate performance, and one laboratory scored poor performance. An offer for a follow-up study received no reaction.

1 Introduction

An important task of the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*), as laid down in Commission Regulation No 882/2004 (EC, 2004), is the organisation of interlaboratory comparison studies to test the performance of the National Reference Laboratories (NRLs) for *Salmonella*. The history of the interlaboratory comparison studies as organised by EURL-*Salmonella* (formerly called CRL-*Salmonella*) from 1995 onwards is summarised on the EURL-*Salmonella* website (http://www.eurlsalomonella.eu).

In March 2017, the EURL-*Salmonella* organised an interlaboratory study to test whether the NRLs for *Salmonella* could detect *Salmonella* at different contamination levels in chicken faeces samples. The results from interlaboratory studies like this show whether the examination of samples in the EU Member States (EU-MS) is carried out uniformly and whether comparable results can be obtained by all NRLs-*Salmonella*.

The method prescribed for the detection of *Salmonella* spp. in animal faeces, with selective enrichment on Modified Semi-solid Rappaport-Vassiliadis (MSRV), is set out in ISO 6579:2002/Amd 1:2007 recently replaced by ISO 6579-1:2017.

The study design of this study was comparable to previous interlaboratory comparison studies. For this study, the chicken faeces was artificially contaminated with a diluted culture of *Salmonella* Infantis (SI) at the EURL-*Salmonella* laboratory.
In total, 18 samples with chicken faeces were tested: six samples per contamination level (blank, low and high concentrations of *Salmonella* Infantis). Additionally, two control samples were tested: one procedure control and one positive control. The number and contamination levels of the samples were in accordance with ISO/TS 22117:2010.

2 Participants

Country	City	Institute
Austria	Graz	Austrian Agency for Health and Food Safety (AGES IMED/VEMI)
Belgium	Brussels	Scientific Institute of Public Health (WIV-ISP)
Bosnia-Herzegovina	Sarajevo	Veterinary Faculty of Sarajevo Laboratory for bacterial disease in poultry
Bulgaria	Sofia	National Diagnostic and Research Veterinary Institute (NDRVMI), National Reference Centre of Food Safety
Croatia	Zagreb	Croatian Veterinary Institute Poultry Centre, Laboratory for General Bacteriology and Microbiology
Cyprus	Nicosia	Cyprus Veterinary Services Pathology, Bacteriology, Parasitology Laboratory
Czech Republic	Prague	State Veterinary Institute
Denmark	Ringsted	Danish Veterinary and Food Administration
Estonia	Tartu	Estonia Veterinary and Food Laboratory, Bacteriology-Pathology Department
Finland	Kuopio	Finnish Food Safety Authority Evira Research Department, Veterinary Bacteriology
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) National Veterinary Reference Laboratory for *Salmonella*
Greece	Chalkida	Veterinary Laboratory of Chalkida
Hungary	Budapest	National Food Chain Safety Office, Food and Feed Safety Directorate
Iceland	Reykjavik	Matís ohf, Icelandic Food and Biotech R&D
Ireland, Republic of	Kildare	Central Veterinary Research Laboratory (CVRL/DAFFM) Laboratories Backweston, Department of Agriculture, Food and the Marine, Bacteriology
Israel	Kiryat Malachi	Southern Poultry Health Laboratory (Beer Tuvia)
Italy	Padova Legnaro	Istituto Zooprofilattico Sperimentale delle Venezie, OIE National Reference Laboratory for *Salmonella*
Latvia	Riga	Institute of Food Safety, Animal Health and Environment BIOR Animal Disease Diagnostic Laboratory
Lithuania	Vilnius	National Food and Veterinary Risk Assessment Institute

Country	City	Institute
Luxembourg	Luxembourg	Laboratoire de Médecine Vétérinaire de l'Etat, Animal Zoonosis
Macedonia, FYR of	Skopje	Food Institute, Faculty of Veterinary Medicine Laboratory for food and feed microbiology
Malta	Valletta	Public Health Laboratory (PHL) Evans Building
Netherlands, the	Bilthoven	National Institute for Public Health and the Environment (RIVM/Cib) Centre for Infectious Diseases Control Centre for Zoonoses and Environmental Microbiology (Z&O)
Norway	Oslo	Norwegian Veterinary Institute, Section of Bacteriology
Poland	Pulawy	National Veterinary Research Institute (NVRI) Department of Microbiology
Portugal	Oeiras	Instituto Nacional de Investigação Agrária e Veterinária (INIAV) Unidade de Produção e Saúde Animal Laboratorio de Bacteriologia
Romania	Bucharest	Institute for Diagnosis and Animal Health, Bacteriology
Serbia	Belgrade	Institute of Veterinary Medicine of Serbia
Slovak Republic	Bratislava	State Veterinary and Food Institute Reference Laboratory for *Salmonella*
Slovenia	Ljubljana	National Veterinary Institute, Veterinary Faculty
Spain	Madrid Algete	Laboratorio Central de Veterinaria
Sweden	Uppsala	National Veterinary Institute (SVA), Department of Bacteriology
Switzerland	Bern	National Centre for Zoonoses, Bacterial Animal Diseases and Antimicrobial Resistance (ZOBA), Institute of veterinary bacteriology, Vetsuisse faculty Berne
United Kingdom	Addlestone	Animal Health and Veterinary Laboratories Agency (AHVLA) Weybridge, Bacteriology Department
	Belfast	Agri-Food and Bioscience Institute (AFBI) Veterinary Sciences Division Bacteriology

3 Materials and methods

3.1 Preparation of artificially contaminated chicken faeces samples

3.1.1 General

The matrix in this interlaboratory comparison study was chicken faeces from a pathogen-free (SPF) laying hen farm. The batch of faeces was collected by the Animal Health Service (GD) Deventer from a laying hen flock in Deventer (GD, Deventer, NL). The chicken faeces was artificially contaminated with a diluted culture of *Salmonella* at the laboratory of the EURL-*Salmonella*.

3.1.2 Pre-tests for the preparation of chicken faeces samples

The batch of faeces (5 kg) for the pre-test arrived at the EURL on 21 November 2016 and was stored at 5 °C. Immediately on receipt, five samples of 25 grams were taken randomly from the batch and tested for the presence of *Salmonella* according to ISO 6579-1:2017.

The chicken faeces samples (25 g) were then artificially contaminated with a high or a low concentration of a diluted culture of *Salmonella* Infantis culture (own culture collection). To test the stability of the contaminated chicken faeces samples, they were stored at 5 °C and 10 °C for a period of 0, 7, 14 and 21 days, and tested for the presence of *Salmonella* according to ISO 6579-1:2017.

3.1.3 Preparation of the chicken faeces samples for the interlaboratory comparison study

A large batch (15 kg) of chicken faeces from the same flock used for the pre-tests arrived at the EURL-*Salmonella* laboratory on Monday, 6 March 2017. Five samples, 25 gram each, were tested for the presence of *Salmonella* according to ISO 6579:1 (2017). After testing negative, samples of 25 gram chicken faeces were artificially contaminated with *Salmonella* Infantis by adding 0.1 ml of the appropriate dilution of an overnight culture. Three concentration levels were used; blank, low (17 cfu/sample) and high (55 cfu/sample). The concentration of the inoculum used to contaminate the chicken faeces was confirmed by plating the relevant dilution on XLD agar plates. Immediately after artificial contamination, the samples were stored at 5 °C until transport to the participating laboratories on Monday, 13 March 2017.

3.1.4 Determination of amount of background flora in chicken faeces

The total number of aerobic bacteria and the number of *Enterobacteriaceae* in chicken faeces were investigated by following ISO 4833:2003 and ISO 21528-2:2004. The chicken faeces was homogenised in peptone saline solution and ten-fold dilutions were analysed on Plate Count Agar (PCA) and Violet Red Bile Glucose (VRBG) Agar respectively.

3.1.5 Determination of the number of Salmonella *in chicken faeces samples by MPN*

The level of contamination in the artificially contaminated samples was determined using a five-tube most probable number (MPN) technique. For this, ten-fold dilutions of five chicken faeces samples at 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 ISO 6579-1:2017. The MPN of *Salmonella* in the original sample was calculated from the number of confirmed positive dilutions, using an MPN program in Excel (Jarvis et al., 2010).

3.2 Design of the interlaboratory comparison study

3.2.1 Number and type of samples

Each participant received 18 artificially contaminated chicken faeces samples numbered B1 to B18. In addition, the laboratories had to test two control samples (C1 and C2). Table 1 gives an overview of the number and type of samples tested by the participants.

For the control samples, the laboratories were asked to use their own positive *Salmonella* control strain which they normally use when analysing routine samples for the detection of *Salmonella*. In addition to this positive control (C2), a procedure control (C1) consisting of Buffered Peptone Water (BPW) only had to be analysed. The protocol and test report used during the study can be found on the EURL-*Salmonella* website or can be obtained from the author of this report (EURL-*Salmonella* 2017a and 2017b).

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

Contamination level	Test samples chicken faeces (n=18)
***S*. Infantis low-level (SI low)**	6
***S*. Infantis high-level (SI high)**	6
Blank (BL)	6
	Control samples (n=2)
Blank procedure control (BPW)	1
Positive control (own control with *Salmonella*)	1

3.2.2 Sample packaging and temperature recording during shipment

The 20 coded samples containing the contaminated chicken faeces, the blank samples, and the control samples, were placed in two safety bags. The safety bags were placed in one large shipping box together with four frozen (-20 °C) cooling devices. The shipping boxes were sent to the participants as 'biological substances category B (UN3373)' via a door-to-door courier service. The participants were asked to store the samples at 5 °C on receipt. To monitor exposure to abusive temperatures during shipment and storage, a micro-temperature logger was placed in between the samples to record the temperature.

3.3 Methods

The method prescribed for this interlaboratory comparison study was ISO 6579-1:2017 which consists, for samples of the primary production stage, of a pre-enrichment in Buffered Peptone Water (BPW) and selective enrichment on Modified Semi-solid Rappaport-Vassiliadis (MSRV) agar, followed by plating-out on Xylose Lysine Deoxycholate agar (XLD) and a second medium

of choice. Confirmation was performed using the appropriate biochemical tests (ISO 6579-1:2017) or using reliable, commercially available identification kits and/or serological tests. In addition to the ISO method, the NRLs were free to use their own method such as a Polymerase Chain Reaction (PCR) procedure.

3.4 Statistical analysis of the data

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

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

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

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

3.5 Criteria for good performance

The criteria indicated in Table 2 were used for the determination of 'good performance'. 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 interlaboratory comparison study.

Minimum result		
Contamination level	**Percentage positive**	**No. of positive samples/ total No. of samples**
Samples Chicken faeces artificially contaminated		
***S*. Infantis high-level (SI high)**	Min. 80%	Min. 5/6
***S*. Infantis low-level (SI low)**	Min. 50%	Min. 3/6
Blank (BL)[1]	Max. 2%[1]	Max. 1/6[1]
Control samples		
Blank procedure control (BPW)	0%	0 /1
Positive control (own control strain of *Salmonella*)	100%	1 /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% positive) is considered acceptable.

4 Results and discussion

4.1 Preparation of artificially contaminated chicken faeces

4.1.1 Pre-tests for the preparation of chicken faeces samples

The design of the study was based on previous studies organised by the EURL *Salmonella* (Pol-Hofstad and Mooijman, 2016a and 2016b). Chicken faeces originating from an SPF farm was artificially contaminated with different concentrations of two *Salmonella* Infantis strains (strain 1 and 2) to test for stability during storage (see 3.1.2.). Samples were stored at 5 °C to mimic storage conditions and at 10 °C to test the effect of temperature abuse during transport. The pre-test samples were stored for up to three weeks and analysed for presence of *Salmonella* using ISO 6579-1:2017. Results of the different pre-tests are shown in Table 3.

Table 3. Stability tests of chicken faeces samples artificially contaminated with Salmonella *Infantis (SI)*

***Salmonella* Infantis**	**Concentration/ 25 g**	**Temperature (°C)**	**Time (days)**				
			0	**1**	**7**	**14**	**21**
Strain 1	Low (11 cfu)	5°C	5	2	3	1	2
Strain 1	Low (11 cfu)	10°C	5	2	1	0	
Strain 1	High (87 cfu)	5°C	5	5	5	5	5
Strain 1	High (87 cfu)	10°C	5	5	5	3	
Strain 2	Low (8 cfu)	5°C	5	5	3	3	0
Strain 2	Low (8 cfu)	10°C	5	5	1	0	
Strain 2	High (52 cfu)	5°C	5	5	5	5	5
Strain 2	High (52 cfu)	10°C	5	5	5	5	

From Table 3 it is clear that the storage of the pre-test samples at 5 °C or 10 °C for three weeks affected the number of samples positive for *Salmonella*. The high-level contaminated samples were all positive after storage at 5 °C and 10 °C for two to three weeks, except for the *Salmonella* Infantis strain 1 stored for 2 weeks at 10 °C, where two of the 5 samples scored negative. Strain 1 was more sensitive towards storage then strain 2. For the low-level samples artificially contaminated with *Salmonella* Infantis 1 stored at 5 °C, the number of positive samples decreased from 5 to 1 within two weeks, while 3 of the 5 samples contaminated with *Salmonella* Infantis 2 were still positive after the same storage time. When stored at 10 °C, both strains showed a similar poor survival: only 1 of the 5 samples was positive after 1 week of storage.

Salmonella Infantis strain 2 was selected for further tests as this strain showed more stable results. The stability tests were repeated with slightly higher concentrations of *Salmonella* Infantis to retain more positive samples after storage.

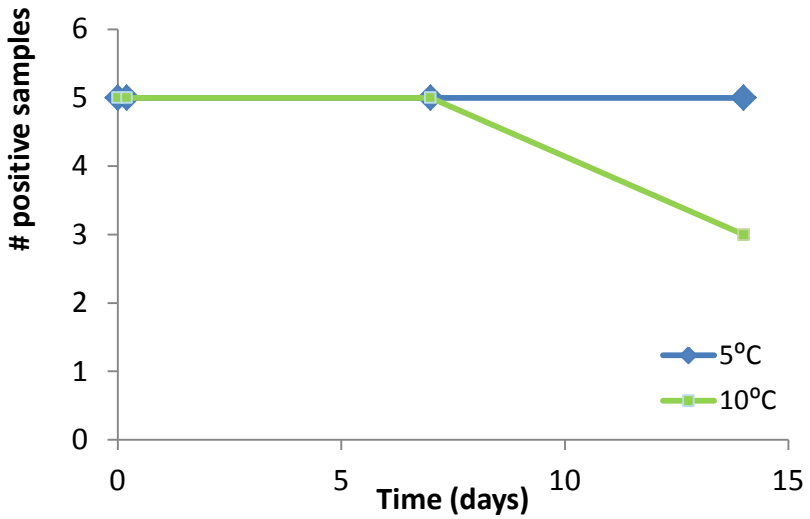

Figure 1. Stability test of chicken faeces samples (n=5) artificially contaminated with Salmonella *Infantis strain 2 with low contamination level (12 cfu/25 g))*

Results are shown in Figure 1. For this test, the chicken faeces was contaminated with 12 cfu/25 g and stored at 5 °C and 10 °C for two weeks. Results show that storage of these samples at 5 °C for two weeks had no effect on the number of positive samples. However, storage at 10 °C had a small effect, resulting in two negative samples out of five. Because of the good results in survival of *Salmonella* at 5 °C, it was decided to use this concentration for the interlaboratory samples. Samples may experience 10 °C during transportation and storage at the laboratory, but generally this will only last a few hours or less than 1 day. It was expected that this would not result in more than 2 negative samples, which is still acceptable.

4.1.2 *Preparation of the chicken faeces samples for interlaboratory comparison study*

Samples for the interlaboratory comparison study were prepared in the same way as the pre-test samples (see 3.1.3). The chicken faeces came from the same flock of laying hens as the batch of faeces used in the pre-test. The faeces was tested for the absence of *Salmonella* immediately on arrival of the new batch of chicken faeces (25 kg).

4.1.3 *Background flora in chicken faeces*

The amount of background flora in the chicken faeces was tested by analysing the number of aerobic bacteria and *Enterobacteriaceae.* Results are shown in Table 4. The amount of background flora in the fresh faeces was 4.2×10^8 cfu/g for aerobic bacteria and 8.7×10^4 cfu/g for *Enterobacteriaceae*. Storage of the faeces at 5 °C for two weeks had hardly any effect on the aerobic count or on the number of *Enterobacteriaceae*.

Table 4. Number of aerobic bacteria and Enterobacteriaceae *per gram of chicken faeces*

Date of testing	Aerobic bacteria cfu/g	*Enterobacteriaceae* cfu/g
7 March 2017	4.2×10^8	8.7×10^4
20 March 2017, after storage at 5 °C	1.0×10^8	5.5×10^4

4.1.4 *Number of* Salmonella *in chicken faeces samples*

Chicken faeces samples (25 g) were artificially contaminated at the EURL laboratory by adding 60 or 300 µl of a diluted *Salmonella* culture. The target concentration was 12 cfu/25g for the low level samples and 50 cfu/25g for the high level samples. In practice, it is very difficult to reach the exact selected levels. Table 5 shows the contamination level of the diluted culture of *Salmonella* Infantis used as inoculum to contaminate the chicken faeces samples. The chicken faeces samples contaminated at a low level were inoculated with 17 cfu, while the samples contaminated at a high level were inoculated with 55 cfu. After inoculation, the samples were stored at 5 °C for almost one week until transport to the participants on the 20th of March 2017. The final contamination level of *Salmonella* in the chicken faeces samples was determined by performing a five-tube Most Probable Number (MPN) test in the week of the interlaboratory comparison study. Results show that the low level samples were contaminated to a higher extent than anticipated, while the high level samples seemed to contain less *Salmonella* than expected (see Table 5).

Table 5. Number of Salmonella *Infantis (SI) in the inoculum and in the contaminated chicken faeces samples*

Date of testing	Low-level SI cfu/25 g chicken faeces	High-level SI cfu/25 g chicken faeces
9 March 2017 (Inoculum level diluted culture)	17	55
20 March 2017, after storage at 5 °C MPN of contaminated chicken faeces (95% confidence limit)	22 (8.5-56)	35 (11 - 110)

4.2 Technical data interlaboratory comparison study

4.2.1 *General*

A total of 36 NRLs for *Salmonella* participated: 29 NRLs from 28 EU-MS (Northern Ireland has its own NRL laboratory) and 6 third countries within Europe (EU candidate or potential EU candidate MS) and countries of the European Free Trade Association (EFTA) and, on request of DG-SANTE, one NRL from a non-European country (Israel).

4.2.2 *Accreditation*

Thirty-five laboratories were accredited for Annex D of ISO 6579, 14 laboratories were also accredited for ISO 6579, and one EU-MS laboratory (lab code 1) is still in the process of accreditation. This laboratory

was asked for its accreditation plans, and measures were taken by the NRL to fulfil the accreditation in future studies.

4.2.3 *Transport of samples*

The samples were transported using a door-to-door courier on Monday 13 March 2017. Thirty laboratories received the parcels within one day of dispatch and three participants within two days. One laboratory received the parcels after three days, one laboratory after four days, and one laboratory after six days, due to problems at the border. Participants were asked to store the parcel at 5 °C on arrival in their laboratories. The temperature during transport and storage was recorded using a temperature recorder placed between the samples in the sample bag. The temperature during transport was predominantly between -4.5 °C and +6 °C. The storage temperature at the receiving laboratories ranged from 0 – 9 °C. Two laboratories had a storage temperature between 13 and 23 °C for several days (lab codes 13 and 18).

4.2.4 *Media*

Each laboratory was asked to test the samples using the prescribed method (ISO 6579-1:2017) using MSRV agar as selective enrichment medium and XLD agar plus a second plating-out medium of their own choice. Table 6 shows which second plating-out media were chosen by the laboratories. Explanations of the abbreviations used are given in the 'List of abbreviations'

Table 6. Second plating-out media used by the NRLs

Media	No. of users
BGA^{mod}	5
Rambach	7
BPLS	4
BGA	9
RS	4
SM(ID)2	3
BxLH	1
ASAP	1
BSA	2

Technical details on the method which deviated from the prescribed ISO method (ISO 6579-1:2017) are listed in Table 7 (darker-shaded cells). Only 6 laboratories reported details of deviations. Three laboratories (lab codes 3, 8 and 18) incubated the BPW for a longer period than prescribed. In addition, 3 laboratories (lab codes 4, 5, and 14) used MSRV with a deviating pH. Two of these (lab codes 4 and 14) used a Novobiocin concentration which was a factor 100 too high or too low. Laboratory 14 did not report the requested details.

Table 7. Reported technical deviations from the prescribed/requested procedure ISO 6579-1:2017

Lab code	BPW		MSRV	
	Incubation time (h:min)	pH	pH	Novobiocin
ISO 6579-1	**16–20 h**	**6.8–7.2**	**5.1–5.4**	**10 mg/l**
3	**21:45**	7.0	**5.4**	10
4	18:35	7.0	**4.6**	**1000**
5	18:40	7.0	**5,5**	10
8	**20:10**	7	5,2	10
14	18:15	**-**	**-**	**0,01**
18	**23:00**	7.0	**5.5**	10

Darker cells = Deviating from ISO 6579-1:2017
- = No information

All participating laboratories performed one or several confirmation tests for *Salmonella*. Table 8 summarises all reported combinations. Other methods were specified as Maldi-tof and automated VITEK-2 method. Ten laboratories used one confirmation test only; most used a combination of two or more confirmation methods.

Table 8: Number of laboratories using the different confirmation methods.

Number of labs	Biochemical	Serological	Other	PCR
1			X	
1			X	X
4		X		
1		X	X	
1	X		X	X
1	X	X	X	
1	X	X	X	X
5	X	X		X
16	X	X		
5	X			

4.3 Control samples

4.3.1 General

Two control samples were sent to the laboratories. One was used as a procedure control (BPW only). The other was used for the positive control to which the laboratories added their own positive control strain normally used in their routine analysis for the detection of *Salmonella*.

Procedure control Blank (BPW only)
All laboratories scored good results for the control samples.

Positive control with *Salmonella*
As positive control, the majority of the participants used a diluted culture of *Salmonella* (24 laboratories). Others used a lenticule disc (6), a freeze-dried ampoule (2) or a capsule (1) with *Salmonella*. The *Salmonella* serovars used for the positive control sample are shown in Table 9.

Table 9. Salmonella *serovars used by participants for the positive control samples.*

***Salmonella* serovar**	**Number of users**
***S.* Enteritidis**	12
***S.* Typhimurium**	9
***S.* Nottingham**	5
***S.* Alachua**	2
***S.* Dublin, *S.* Bongori, *S.* Harleystreet, *S.* Blegdam, *S.* Tennessee, S. Abaetetuba, S. Tranaroa, S. Infantis**	1 (per serovar)

4.3.2 *Correct scores of the control samples*
Table 10 shows the number of correctly analysed control samples for all participants and for the EU Member States. No differences were found between these groups. Results were 100% correct for all laboratories, with accuracy rates of 100%.

One laboratory switched the two control samples, reporting positive results for the sample intended as procedure control and negative results for the sample intended as positive control (lab code 16). The raw data of this laboratory demonstrated that the control samples were switched and that their results were correct. However, as this is a reporting error, the performance of this laboratory was scored as moderate.

Table 10 Correct scores found with the control samples by all participants (All) and by EU laboratories only (EU)

Control samples		**All n=36**	**EU n=29**
Procedure control blank (BPW) n=1	No. of samples	36	30
	No. of positive samples	36	30
	Correct score in %	**100**	**100**
Positive control (own *Salmonella*) n=1	No. of samples	36	30
	No. of negative samples	36	30
	Correct score in %	**100**	**100**
All control samples n=2	No. of samples	72	60
	No. of correct samples	72	60
	Accuracy in %	**100**	**100**

4.4 Artificially contaminated chicken faeces samples

4.4.1 General

Chicken faeces artificially contaminated with two different levels of *Salmonella* Infantis, low (approx. 17 cfu/25 g) and high (approx. 55 cfu/25 g), as well as blank samples, were analysed for the presence of *Salmonella* by the participants. Table 11 shows the overall results obtained by the participants.

Table 11. Number of positive results found with the artificially contaminated chicken faeces samples at each laboratory

	Number of positive isolations		
	Blank n=6	SI low n=6	SI high n=6
Criteria good performance	≤1	≥3	≥5
Lab code 18	**3**	6	6
Lab codes 9, 34 and 36	0	5	6
Lab codes 3 and 21	0	6	5
All other NRLs	0	6	6

Blank chicken faeces samples (n=6)

All but one laboratory correctly analysed the blank samples negative for *Salmonella*. Laboratory 18 found three of the six blank samples positive for *Salmonella* and as a result was scored as 'poor performance'.

Figure 2. Number of chicken faeces samples artificially contaminated with a low level of Salmonella *Infantis (n = 6) that tested positive per laboratory.*

- = Criterion for good performance

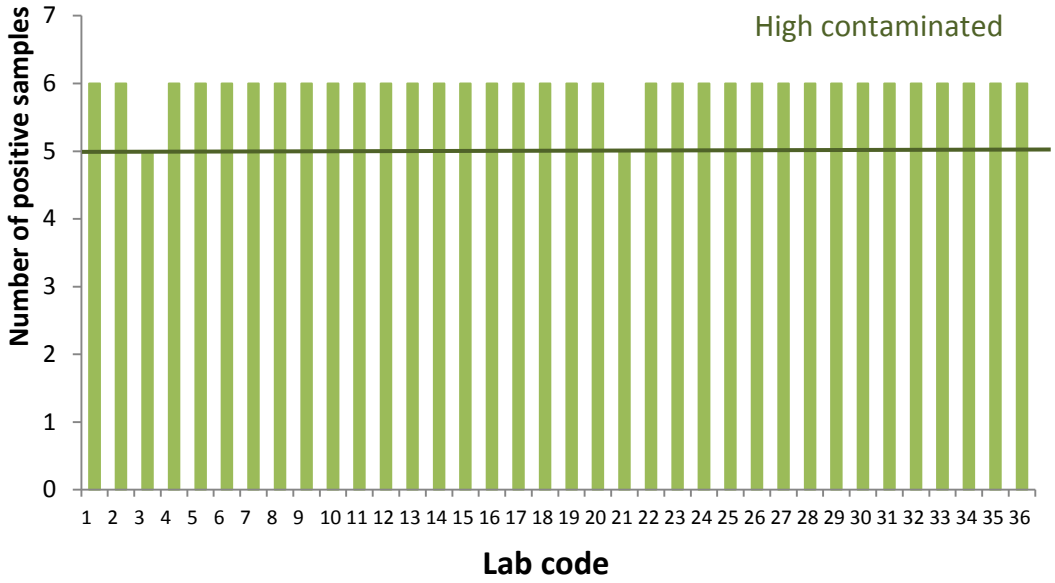

Figure 3. Number of chicken faeces artificially contaminated with high levels of Salmonella *Infantis (n = 6) that tested positive per laboratory*
- = Criterion for good performance

Chicken faeces samples containing a low concentration of *Salmonella* Infantis (n=6)
Almost all laboratories were able to detect *Salmonella* in all six chicken faeces samples contaminated with a low inoculum level of approximately 17 cfu/25 g. Only three laboratories (labcodes 9, 34 and 36) reported one of the six samples negative for *Salmonella*. With regards to low level samples, it is acceptable to score a maximum of three of the six samples as negative, so these three laboratories scored well above the criteria for good performance. The results are shown in Figure 2.

Chicken faeces samples containing a high concentration of *Salmonella* Infantis (n=6)
Almost all laboratories were able to detect *Salmonella* in all six samples inoculated with approximately 55 cfu/25 g. Two laboratories (lab codes 3 and 21) reported 1 sample negative for *Salmonella*. As it is permitted to score one of the 6 high level samples as negative, this is still within the criteria of good performance. The results are shown in Figure 3.

4.4.2 Specificity, sensitivity and accuracy rates for the artificially contaminated samples

Table 12 shows the specificity, sensitivity and accuracy rates for all artificially contaminated chicken faeces samples. The calculations were performed on the results of all participants and on the results of the participants of the EU-MS only. Only minor differences were found between these groups. The specificity rate (99.5%) and the sensitivity rates (low level: 99%; high level 99%) were high for both groups of participants.

Table 12. Specificity, sensitivity and accuracy rates found by the participating laboratories with the artificially contaminated chicken faeces samples.

Chicken faeces		All participants n=36	EU-MS n=29
Blank n=6	No. of samples	216	174
	No. of negative samples	213	174
	Specificity in %	**99**	**100**
Low level SI n=6	No. of samples	216	174
	No. of positive samples	213	171
	Sensitivity in %	**99**	**98**
High level SI n=6	No. of samples	216	174
	No. of positive samples	214	172
	Sensitivity in %	**99**	**99**
All chicken faeces samples with SI	No. of samples	432	348
	No. of positive samples	427	343
	Sensitivity in %	**99**	**99**
All chicken faeces samples (pos. and neg.)	No. of samples	648	522
	No. of correct samples	640	517
	Accuracy in %	**99**	**99**

4.5 PCR (own method)

This year, eight laboratories (lab codes 7, 13, 14, 18, 21, 22, 25, and 33) also performed a PCR method on the chicken faeces samples as an additional detection technique (see Table 13). Most laboratories tested the samples after pre-enrichment in BPW; laboratory 7 started the DNA extraction before pre-enrichment in BPW; and laboratory 18 started after selective enrichment on MSRV. All laboratories used a real-time PCR except for laboratory 18 which used a commercially available three-step PCR with reference to Malorny et al. (undated). Six laboratories used a validated PCR method. Of these, four used the PCR method for their routine testing. Two used a non-validated PCR method of which 1 routinely used the PCR method.

The majority of NRLs found identical results with their PCR method and the bacteriological culture method, including the false positive results for the three blank samples (lab code 18). One laboratory (lab code 21) using the PCR method found two high level samples and two low level samples negative, while with the bacteriological culture method, they only found one high level sample negative. Laboratory 14 found one low level sample negative with the PCR method, while this was tested positive with the bacteriological culture method.

Table 13. Details of Polymerase Chain Reaction (PCR) procedures used by NRLs Salmonella as own method

Lab code	PCR method	Validated (by)	Commercially available	Routinely used number of test/2016	Reference
7	Real Time	Nationally	-	68	Malorny *et al.* 2004
13	Real Time	Lofstrom et al.	-	1000	Malorny *et al.* 2004
14	Real-time	AFNOR	+	267	Commercial kit
18	Conventional: Three step	No	+	200	Malorny et al, PDF file
21	Real-time	AFNOR	+	-	-
22	Real-time	Nationally	-	98	Josefsen *et al*. 2007; Malorny *et al*. 2004
25	Real-time	NF validation: AOAC-RI	+	-	Lauer at al. 2009
33	Real-time	No	+	-	Mericon Pathogen Detection Handbook

4.6 Performance of NRLs

4.6.1 General

All laboratories were able to detect *Salmonella* in high and low concentrations in chicken faeces samples. Of the 36 laboratories, 34 fulfilled the criteria for good performance for the prescribed MSRV method. One laboratory (non-EU-MS, lab code 18) scored a 'poor performance' for falsely detecting *Salmonella* in three blank chicken faeces samples. This laboratory did not provide any possible explanation for their problems, and was not able to participate in the follow-up study for unknown reasons. One laboratory scored a 'moderate performance' for making an error in labelling the control samples (lab code 16).

5 Conclusion

All NRLs for *Salmonella* were able to detect high and low levels of *Salmonella* in chicken faeces using the prescribed MSRV method.

Thirty-four NRLs scored a 'good performance'; one laboratory scored a 'moderate performance' due to a labelling mistake in the control samples. One laboratory scored a 'poor performance' for falsely detecting *Salmonella* in three of the six blank chicken faeces samples.

The accuracy, specificity and sensitivity rates of the NRLs with respect to the control samples (without chicken faeces) after selective enrichment on MSRV agar were all 100%.

The sensitivity rate for the chicken faeces samples artificially contaminated with a high level of *S*. Infantis was 99% for the prescribed MSRV method.

The sensitivity rate for the chicken faeces samples artificially contaminated with a low level of *S*. Infantis was 99%.

The accuracy rate of the NRLs in detecting *Salmonella* in the artificially contaminated chicken faeces samples was 99%.

The majority of the NRLs-*Salmonella* use *S.* Enteritidis or *S*. Typhimurium for their positive control samples. But the use of a less common *Salmonella* serovar in routine samples may be advisable in order to make the detection of possible cross contamination easier.

Eight participants used a PCR technique in addition to the prescribed MSRV method. Six laboratories reported identical results for both methods. Two laboratories found more samples negative for *Salmonella*.

List of abbreviations

AFNOR	Association Française de Normalisation (French Standardization Association)
AOAC	Association of Analytical Communities
ASAP	AES *Salmonella* Agar Plate
BAC	Bacteriological Culture technique
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
BxLH	Brilliant green, Xylose, Lysine, Sulphonamide
cfu	Colony-Forming Units
DG-SANTE	Directorate-General for Health and Consumer Protection
EC	European Commission
EFTA	European Free Trade Association
EU	European Union
EURL	European Union Reference Laboratory
ISO	International Organization for Standardization
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*
SI	*Salmonella* Infantis
SM(ID)2	*Salmonella* Detection and Identification-2
SPF	Specific Pathogen Free
VRBG	Violet Red Bile Glucose agar
XLD	Xylose Lysine Deoxycholate agar

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Erratum Report 2017-083

Date: 13-07-2018
Report title: The 20th EURL-*Salmonella* interlaboratory comparison study primary production (2017)
Detection of *Salmonella* in chicken faeces

In the participants table in Chapter 2, the wrong institute for Switzerland was taken up as participant.

The correct participants list is given below:

For approval:
Kirsten Mooijman
Coordinator EURL *Salmonella*

2 Participants

Country	City	Institute
Austria	Graz	Austrian Agency for Health and Food Safety (AGES IMED/VEMI)
Belgium	Brussels	Scientific Institute of Public Health (WIV-ISP)
Bosnia-Herzegovina	Sarajevo	Veterinary Faculty of Sarajevo Laboratory for bacterial disease in poultry
Bulgaria	Sofia	National Diagnostic and Research Veterinary Institute (NDRVMI), National Reference Centre of Food Safety
Croatia	Zagreb	Croatian Veterinary Institute Poultry Centre, Laboratory for General Bacteriology and Microbiology
Cyprus	Nicosia	Cyprus Veterinary Services Pathology, Bacteriology, Parasitology Laboratory
Czech Republic	Prague	State Veterinary Institute
Denmark	Ringsted	Danish Veterinary and Food Administration
Estonia	Tartu	Estonia Veterinary and Food Laboratory, Bacteriology-Pathology Department
Finland	Kuopio	Finnish Food Safety Authority Evira Research Department, Veterinary Bacteriology
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) National Veterinary Reference Laboratory for *Salmonella*
Greece	Chalkida	Veterinary Laboratory of Chalkida
Hungary	Budapest	National Food Chain Safety Office, Food and Feed Safety Directorate
Iceland	Reykjavik	Matís ohf, Icelandic Food and Biotech R&D
Ireland, Republic of	Kildare	Central Veterinary Research Laboratory (CVRL/DAFFM) Laboratories Backweston, Department of Agriculture, Food and the Marine, Bacteriology

Country	City	Institute
Israel	Kiryat Malachi	Southern Poultry Health Laboratory (Beer Tuvia)
Italy	Padova Legnaro	Istituto Zooprofilattico Sperimentale delle Venezie, OIE National Reference Laboratory for *Salmonella*
Latvia	Riga	Institute of Food Safety, Animal Health and Environment BIOR Animal Disease Diagnostic Laboratory
Lithuania	Vilnius	National Food and Veterinary Risk Assessment Institute
Luxembourg	Luxembourg	Laboratoire de Médecine Vétérinaire de l'Etat, Animal Zoonosis
Macedonia, FYR of	Skopje	Food Institute, Faculty of Veterinary Medicine Laboratory for food and feed microbiology
Malta	Valletta	Public Health Laboratory (PHL) Evans Building
Netherlands, the	Bilthoven	National Institute for Public Health and the Environment (RIVM/Cib) Centre for Infectious Diseases Control Centre for Zoonoses and Environmental Microbiology (Z&O)
Norway	Oslo	Norwegian Veterinary Institute, Section of Bacteriology
Poland	Pulawy	National Veterinary Research Institute (NVRI) Department of Microbiology
Portugal	Oeiras	Instituto Nacional de Investigação Agrária e Veterinária (INIAV) Unidade de Produção e Saúde Animal Laboratorio de Bacteriologia
Romania	Bucharest	Institute for Diagnosis and Animal Health, Bacteriology
Serbia	Belgrade	Institute of Veterinary Medicine of Serbia
Slovak Republic	Bratislava	State Veterinary and Food Institute Reference Laboratory for *Salmonella*
Slovenia	Ljubljana	National Veterinary Institute, Veterinary Faculty
Spain	Madrid Algete	Laboratorio Central de Veterinaria
Sweden	Uppsala	National Veterinary Institute (SVA), Department of Bacteriology
Switzerland	Zurich	National Reference Centre for Poultry and Rabbit diseases (NRGK), Institute of Veterinary Bacteriology, University of Zurich,
United Kingdom	Addlestone	Animal Health and Veterinary Laboratories Agency (AHVLA) Weybridge, Bacteriology Department
	Belfast	Agri-Food and Bioscience Institute (AFBI) Veterinary Sciences Division Bacteriology

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