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

RIVM Report 2015-0080

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Colophon

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EU Ringonderzoek diervoeder III (2014)

Detectie van *Salmonella* in kippenvoer

In 2014 waren 32 van de 34 Nationale Referentie Laboratoria (NRL's) in de Europese Unie in staat om hoge en lage concentraties *Salmonella* in kippenvoer aan te tonen. Twee NRL's behaalden een matig resultaat als gevolg van een rapportagefout. Vanwege herhaaldelijk slechte prestaties is een van deze NRL tijdens een herkansing bezocht door het overkoepelende orgaan EURL-*Salmonella* Daarbij zijn enkele verbeterpunten aangereikt, waarna er een goed resultaat is bereikt. In totaal hebben de laboratoria in 97 tot 100 procent van de besmette monsters *Salmonella* aangetoond. Dit blijkt uit het derde diervoederringonderzoek dat is georganiseerd door het referentielaboratorium van de Europese Unie voor *Salmonella* (EURL-*Salmonella*).

Ringonderzoek verplicht voor Europese lidstaten

Het onderzoek is in september 2014 gehouden, de herkansing was in februari 2015. Alle NRL's van de Europese lidstaten die verantwoordelijk zijn voor de opsporing van *Salmonella* in diervoeder, zijn verplicht om aan het onderzoek deel te nemen. Het EURL-*Salmonella* is gevestigd bij het Nederlandse Rijksinstituut voor Volksgezondheid en Milieu (RIVM).

De laboratoria toonden de *Salmonella*-bacterie in kippenvoer aan met behulp van de drie internationaal erkende analysemethoden (RVS, MKTTn en MSRV). Elk laboratorium kreeg een pakket toegestuurd met kippenvoer dat ofwel besmet was met *Salmonella* Senftenberg in twee verschillende concentraties, of geen *Salmonella* bevatte. De laboratoria dienden volgens een protocol te onderzoeken of de monsters *Salmonella* bevatten. Uit de studie blijkt dat het gebruik van meerdere analysemethodes zijn nut heeft, aangezien het aantal monsters waarin *Salmonella* is aangetroffen per methode significant verschilt.

Monsterbereiding

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

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

Synopsis

EU Interlaboratory comparison study animal feed III (2014)
Detection of *Salmonella* in chicken feed

In 2014, it was shown that 32 out of 34 National Reference Laboratories (NRLs) in the European Union were able to detect high and low levels of *Salmonella* in chicken feed. Two laboratories made a reporting error, which led to their performance being rated as 'moderate'. Due to its consistently poor performance, one NRL was visited by the EURL-*Salmonella* (a central coordinating body) during a follow-up study. Some points of improvement were given, after which the NRL reached the level of good performance. The laboratories detected *Salmonella* in 97% to 100% of the contaminated samples. These are some of the conclusions of the third EU Interlaboratory Comparison Study of Animal Feed Samples, which was organised by the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*).

Interlaboratory comparison study obligatory for EU Member States
The study was conducted in September 2014, with a follow-up study in February 2015. Participation was obligatory for all EU Member State NRLs that are responsible for the detection of *Salmonella* in animal feed samples. EURL-*Salmonella* is part of the Dutch National Institute for Public Health and the Environment (RIVM).

The laboratories used internationally accepted analysis methods (RVS, MKTTn and MSRV) to detect the presence of *Salmonella* in chicken feed. Each laboratory received a package of chicken feed contaminated with two different concentrations of *Salmonella* Senftenberg or containing no *Salmonella* at all. The laboratories were required to analyse the samples for the presence of *Salmonella* in accordance with the study protocol. This study underscores the benefits of using different analysis methods, as a significant difference was observed in the number of positive results between the methods.

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

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

Contents

Summary

In September 2014, the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*) organised the third interlaboratory comparison study on the detection of *Salmonella* in animal feed. The matrix of concern was mixed meal for laying hens.
The participants were 34 National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*): 30 NRLs from the 28 EU Member States (EU-MS), 4 NRLs from third countries within Europe (EU candidate MS or potential EU candidate MS and members of the European Free Trade Associations (EFTA)) and one NRL from a non-European country.

The main objective of the study was to test the performance of the participating laboratories with respect to detecting *Salmonella* at different contamination levels in animal feed. For this purpose, chicken feed samples of 25 grams that had been artificially contaminated with *Salmonella* Senftenberg (SSE) at various contamination levels were analysed. The performance of the laboratories was compared with criteria of good performance. In addition, a comparison was made between the prescribed method (ISO 6579: Anonymous, 2002), using selective enrichment in Rappaport Vassiliadis Soya broth (RVS) and Mueller Kauffmann Tetrathionate novobiocin broth (MKTTn), and the requested method (Annex D of ISO 6579: Anonymous, 2007), using selective enrichment on Modified Semi-solid Rappaport Vassiliadis (MSRV) agar.

The samples consisted of chicken feed artificially contaminated with a diluted culture of *Salmonella* Senftenberg (SSE) at a low level (approximately 15-20 CFU/25 g of feed), at a high level (approximately 50-100 CFU/25 g of feed) and with no *Salmonella* at all (blank samples). The samples were artificially contaminated at the laboratory of the EURL-*Salmonella*, which was a new procedure for an animal feed study. Before the start of the study, several experiments were carried out to make sure that the samples were fit for use in an interlaboratory comparison study (e.g. choice of *Salmonella* serovar, stability at different storage temperatures, influence of background flora).

Eighteen individually numbered blind samples of chicken feed had to be tested by the participants for the presence or absence of *Salmonella*. These samples consisted of six blank samples, six samples with a low level of SSE (inoculum 20 CFU/sample) and six samples with a high level of SSE (inoculum 61 CFU/sample). Additionally, three control samples had to be tested: two blank control samples (procedure control (BPW) and matrix control sample (chicken feed)) and one own (NRL) positive control sample (with *Salmonella*).

The laboratories found *Salmonella* in almost all the (contaminated) samples, resulting in a sensitivity rate of 97-100%. A comparison between the different media was made. Isolation on Xylose Lysine Deoxycholate agar (XLD) gave a significantly higher chance of finding *Salmonella* Senftenberg in chicken feed than did isolation on other isolation media (most often Brilliant Green Agar – BGA or Rambach). The difference was 3-7% and was independent of the selective enrichment medium used (RVS,

MKTTn or MSRV). There was a slightly higher chance of finding *Salmonella* after selective enrichment on MSRV compared with RVS and MKTTn, but this was not significant (difference only 1%). Longer incubation (two times 24 h) of MSRV gave 2-3% more positive results.

For the positive control, the majority of the participants (20 laboratories) used a diluted culture of *Salmonella*. The *Salmonella* serovars most frequently used for the positive control sample were *S*. Enteritidis (15) and *S*. Typhimurium (9). The concentration of the positive control varied from an 8 to 10^9 CFU/sample. For the positive control, it is advisable to use a concentration close to the detection limit and to use a *Salmonella* serovar not often isolated from routine samples.

PCR was used as their own method by seven participants, five of which found the same results that were produced using the bacteriological culture method. Most of them used a Real-Time PCR. Two NRLs used a PCR method based on the presence of an InvA gene but, as this gene was absent in the strain used in this study, those PCR results were negative in contrast to the prescribed culture method (RVS, MKTTn and MSRV).

Thirty-two out of 34 laboratories achieved the level of good performance. One NRL reported a false positive result for a blank procedure control sample and another NRL reported a false negative result for their own positive control sample. Both laboratories showed correct results for the samples with animal feed contaminated with *Salmonella*. Those latter results, however, are not reliable when deviations are found in the positive or negative control samples at the same time. The results of these two laboratories were therefore indicated as ‘moderate performance’. One of them showed repeated moderate performance in food and animal feed studies. The EURL staff visited this NRL during a follow-up study organised in February 2015. The laboratory scored all samples correctly in this follow-up study and achieved a good performance. The EC, DG-Sanco, was informed accordingly.

This study, in which animal feed samples artificially contaminated with a diluted culture of *Salmonella* were used for the first time at the laboratory of the EURL-*Salmonella,* was successful. It showed that this method of preparing interlaboratory comparison samples – used and tested in earlier studies with food matrices and products of the primary production stage (chicken faeces) – is also possible for relatively dry samples such as animal feed.

1 Introduction

An important task of the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*), as laid down in the Commission Regulation EC No 882/2004 (EC, 2004), is the organization of interlaboratory comparison studies to test the performances of the National Reference Laboratories (NRLs) for *Salmonella*. The history of the interlaboratory comparison studies on the detection of *Salmonella*, as organized by EURL-*Salmonella* (formerly called CRL-*Salmonella*) since 1995, is summarized on the EURL-*Salmonella* website (EURL-*Salmonella*, 2015).
The objective of the current study, organised by the EURL for *Salmonella* in October 2014, was to see whether the participating laboratories could detect *Salmonella* in chicken feed at different contamination levels. This information is important in order to know whether the examination of samples in the EU Member States (MS) is being carried out uniformly and whether comparable results can be obtained by NRLs-*Salmonella*. Additionally, the different methods used for the detection of *Salmonella* in chicken feed were compared.

The prescribed method used to detect *Salmonella* in a feed matrix is ISO 6579 (Anonymous, 2002). Yet, because there have been good experiences with selective enrichment on Modified Semi-solid Rappaport-Vassiliadis (MSRV) for the detection of *Salmonella* spp. in animal faeces (Annex D of ISO 6579: Anonymous, 2007), as well as for the detection of *Salmonella* in food and animal feed samples, participating laboratories were also asked to use MSRV for testing the chicken feed.

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

Like in earlier studies, the contamination level of the low-level contaminated samples was close to the detection limit of the method used and the level of the high-level samples was approximately 5-10 times above the detection limit. In total, 18 chicken feed samples were tested, 6 samples per contamination level (blank, low level and high level) containing one *Salmonella* serovar (*Salmonella* Senftenberg). Additionally, three control samples (two blank control samples and one positive control sample) were tested. The number and level of samples tested were in accordance with ISO/TS 22117 (Anonymous, 2010).

2 Participants

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

Country	City	Institute / NRL *Salmonella*
Netherlands, the	Bilthoven	National Institute for Public Health and the Environment (RIVM/CIb) Infectious Disease Control, Centre for Zoonoses and Environmental Microbiology (cZ&O)
Netherlands, the	Wageningen	Netherlands Food and Consumer Product Safety Authority (nVWA) Consumer and Safety Division, Microbiology
Norway	Oslo	Norwegian Veterinary Institute, Section of Bacteriology
Poland	Pulawy	National Veterinary Research Institute (NVRI) Department of Hygiene of Animal Feeding Stuffs
Portugal	Lisbon	Instituto National de Investigação Agrária e Veterinária Unidade de Tecnologia e Segurança Alimentar (INIAV)
Romania	Bucharest	Hygiene and Veterinary Public Health Institute (IISPV)
Serbia	Belgrade	Institute of Veterinary Medicine of Serbia Department of Nutrition and Feed Safety
Slovak Republic	Bratislava	State Veterinary and Food Institute
Slovenia	Ljubljana	National Veterinary Institute, Veterinary Faculty (UL)
Spain	Madrid, Algete	Laboratorio Central de Veterinaria
Sweden	Uppsala	National Veterinary Institute (SVA), Department of Bacteriology
United Kingdom	Addlestone	Animal and Plant Health Agency (APHA), Department of Bacteriology
United Kingdom	Belfast	Agri-Food and Bioscience Institute (AFBINI) Veterinary Sciences Division Bacteriology

3 Materials and methods

3.1 Chicken feed

3.1.1 General

The matrix in this interlaboratory comparison study was chicken feed: mixed meal for laying hens (free-range). The chicken feed was obtained from the retail sector and was produced by De Heus Voeders, Ede in the Netherlands. For the pre-test, two different chicken feed meals were tested: chicken breeding meal (flour No2) and mixed meal for laying hens, both obtained from Kasper Faunafood, Woerden, the Netherlands. For the interlaboratory comparison study, a batch of 25 kg of mixed meal for laying hens (De Heus Voeders) was obtained. This latter batch arrived at the EURL-*Salmonella* on 25 August 2014.
Immediately after receipt of the chicken feed, 5 samples (for the pre-test) or 10 samples (for the interlaboratory comparison study) of 25 g each were checked for the absence of *Salmonella* in accordance with ISO 6579 (Anonymous, 2002) and Annex D of ISO 6579 (Anonymous, 2007). For this purpose, the 25-gram samples were each added to 225 ml of Buffered Peptone Water (BPW). After pre-enrichment at 37 (± 1)°C for 16 to 18 hours, selective enrichment was carried out in Rappaport-Vassiliadis Soya broth (RVS), Mueller Kaufmann Tetrathionate novobiocin broth (MKTTn) and on Modified Semi-solid Rappaport-Vassiliadis (MSRV) agar. Next, the MKTTn and RVS tubes and the suspect growth on MSRV plates were plated-out on Brilliance *Salmonella* agar (BSA) and confirmed biochemically.
After checking the absence of *Salmonella*, the chicken feed was repacked in portions of 25 g in Whirl-pak plastic bags and stored (see 3.3.1).

3.1.2 Total bacterial count in chicken feed

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

3.1.3 Number of Enterobacteriaceae *in chicken feed*

In addition to the total number of aerobic bacteria, the *Enterobacteriaceae* count was determined by following ISO 21528-2 (Anonymous, 2004). A 20-gram portion of the chicken feed was homogenized in 180 ml of peptone saline solution in a plastic bag. The contents were mixed using a stomacher (for 60 sec). Next, tenfold dilutions were prepared in peptone saline solution. Two times 1 ml of each dilution were placed in two empty Petri dishes (diameter 9 cm). To each dish, 10 ml of molten Violet Red Bile Glucose agar (VRBG) was added. After the VRBG was solidified, an additional 15 ml of VRBG was added to the agar. These plates were incubated at (37 ± 1) °C for (24 ± 2) hours and the number of typical

violet-red colonies were counted after incubation. Five typical colonies were tested for the fermentation of glucose and for a negative oxidase reaction. After this confirmation, the number of *Enterobacteriaceae* was calculated.

3.2 Artificial contamination of chicken feed samples

3.2.1 Pre-tests for the preparation of contaminated chicken feed samples

The chicken feed samples were artificially contaminated at the laboratory of the EURL-*Salmonella* with a diluted culture of *Salmonella*. Some experiments were performed prior to the start of the interlaboratory comparison study, especially in relation to the stability of *Salmonella* in the artificially contaminated chicken feed samples when stored at different temperatures. For the contamination, two different *Salmonella* serovars were tested: *Salmonella* Typhimurium (STM) ATCC 14028 and *Salmonella* Senftenberg (SSE), isolated from cacao in 2008. The ATTC strain was obtained from the American Type Culture Collection (ATCC, Manassas, USA).
Each strain was inoculated in Buffered Peptone Water (BPW) and incubated at (37 ± 1) °C overnight. Next, each culture was diluted in peptone saline solution to be able to inoculate the chicken feed samples with approximately 5-10 CFU/sample and 50-100 CFU/sample. For the enumeration of the contamination level (CFU/ml), 0.1 ml of the diluted culture was spread over an XLD plate and incubated at 37 °C for 20-24 hours.
Samples of 25 g of chicken feed were artificially contaminated with a dilution of a *Salmonella* culture (different levels of STM or SSE). Some control samples were also prepared without the addition of *Salmonella* (blank chicken feed samples). All chicken feed samples were stored at -20 °C, 5 °C and 10 °C for a period of 0, 7, 14, 21 and 35 days. After each storage time at the different temperatures, the artificially contaminated SSE, STM and blank chicken feed samples were tested for the presence of *Salmonella* following Annex D of ISO 6579 (Anonymous, 2007), with selective enrichment on Modified Semi-solid Rappaport-Vassiliadis (MSRV) and, for some samples, also following ISO 6579 (Anonymous, 2002) with selective enrichment in Rappaport Vassiliadis Soya broth (RVS) and/or Mueller Kauffmann Tetrathionate novobiocin Broth (MKTTn).
To obtain an indication of the amount of the background flora in the samples after storage at different temperatures, the blank chicken feed samples (without the addition of *Salmonella*) were tested for the number of aerobic bacteria and *Enterobacteriaceae* (see 3.1.2 and 3.1.3).

3.2.2 Determination of contamination level in chicken feed samples by MPN

The level of contamination in the final chicken feed samples, as used at the time of the study, was determined by using a five-tube, most probable number (MPN) technique. For this purpose, tenfold dilutions of five chicken feed samples of each contamination level were tested representing 25 g, 2.5 g and 0.25 g of the original sample. The presence of *Salmonella* was determined in each dilution by following Annex D of ISO 6579 (Anonymous, 2007) and ISO 6579 (Anonymous, 2002). From the number of confirmed positive dilutions, the MPN of *Salmonella* in the original sample was calculated by using an MPN software program in Excel, freely available on the Internet (Jarvis et al., 2010).

3.3 Design of the interlaboratory comparison study

3.3.1 Chicken feed samples

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

- labelling of each plastic bag;
- adding 25 g of chicken feed to each plastic bag;
- adding approximately 0.1 ml of a diluted culture of S. Senftenberg (SSE) to the chicken feed sample. The contamination levels aimed at were 15–20 CFU/25 of chicken feed, 50–100 CFU/25 g of chicken feed and blank;
- storage of samples at 5 °C until transport to the NRLs on 29 September 2014.

On 29 September 2014 (one week before the study), the chicken feed samples were prepared for shipment (see 3.3.2) and sent to the participants by door-to-door courier service. After arrival at the laboratories, the chicken feed samples had to be stored at 5 °C until the start of the study.
Further details about the shipping and handling of the samples and the reporting of the test results can be found in the protocol (EURL-*Salmonella*, 2014a), in the Standard Operation Procedure (SOP, EURL-*Salmonella*, 2014b) and in a print-out from the web-based test report (EURL-*Salmonella*, 2014c).

Eighteen chicken feed samples (numbered B1–B18) and three control samples (numbered C1-C3) had to be tested by each participant. Table 1 gives an overview of the number and type of samples to be tested by the participants.
For the control samples, the laboratories were asked to use their own positive *Salmonella* control, which they normally use when analysing routine samples for the detection of *Salmonella*. In addition to this, controls of the BPW and of the matrix had to be analysed (both blank controls).

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

Contamination level	Test samples with chicken feed (n=18)
S. Senftenberg low level (SSE)	6
S. Senftenberg high level (SSE)	6
Blank (BL)	6
	Control samples (n=3)
Own positive control with *Salmonella*	1
Chicken feed (blank matrix control)	1
BPW (blank procedure control)	1

3.3.2 Shipment of parcels and temperature recording during shipment

To each NRL, 21 plastic bags were sent containing the chicken feed samples artificially contaminated with *Salmonella*, blank chicken feed samples or no chicken feed at all (controls). The 21 bags were packed in

one plastic safety bag. The safety bag was placed in one large shipping box, together with three frozen (-20 °C) cooling devices. Each shipping box was sent to the participants as 'biological substances category B (UN3373)' using a door-to-door courier service. To monitor exposure to abusive temperatures during shipment and storage, micro temperature loggers were used to record the temperature during transport. These loggers are tiny units sealed in a stainless steel case that is 16 mm in diameter and 6 mm deep. Each shipping box contained one logger packed in one of the safety bags. The loggers were programmed by the EURL-*Salmonella* to measure the temperature every hour. Each NRL had to return the temperature recorder to the EURL-*Salmonella* on the day the laboratory started the study. At the EURL-*Salmonella,* the loggers were read using a special computer program and all recorded temperatures from the start of the shipment until the start of the study were transferred to an Excel sheet.

3.4 Methods

The NRLs could follow the procedures for as they are normally used in daily routine analyses (e.g. pre-warming of BPW, different ways of mixing the samples in BPW). For the pre-treatment of the chicken feed samples, reference was made to ISO 6887-4 (Anonymous, 2003c). According to this ISO, the chicken feed diluted in BPW needs to stand for 20 to 30 minutes at 18 °C to 27 °C before mixing. This was described in more detail in the SOP of this study (EURL-*Salmonella*, 2014b).
The prescribed method of this interlaboratory comparison study for detection of *Salmonella* in the chicken feed samples was ISO 6579 (Anonymous, 2002) and the requested (additional) method was Annex D of ISO 6579 (Anonymous, 2007). In addition, the NRLs could use their own method, such as a Polymerase Chain Reaction (PCR) procedure.

The prescribed (and requested) method in summary:

Pre-enrichment in:
- Buffered Peptone Water (BPW)

Selective enrichment in/on:
- Rappaport Vassiliadis Soya broth (RVS);
- Mueller Kaufmann Tetrathionate novobiocin broth (MKTTn);
- Modified Semi-solid Rappaport-Vassiliadis medium (MSRV) (requested);

Plating-out on the following isolation media:
- Xylose Lysine Desoxycholate agar (XLD);
- second plating-out medium of choice (obligatory);

Confirmation:

- Confirmation by means of appropriate biochemical tests (ISO 6579, Anonymous, 2002) or by reliable, commercially available identification kits and/or serological tests.

3.5 Statistical analysis of the data

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

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

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

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

Mixed effect logistic regression (Gelman and Hill, 2007) was used for modelling the binary outcomes as a function of a fixed effect part, consisting of the level of contamination (CFU), enrichment media and isolation media, and a random effect part, consisting of the different laboratories. Mutual differences between media and contamination level are shown as odds ratios (OR) stratified by medium. The odds of detecting *Salmonella* is calculated by dividing the probability of detecting *Salmonella* by the probability of not detecting it. An odds ratio is the ratio of the odds of detecting *Salmonella* in one group to the odds of detecting it in another group and can be interpreted as an effect size. Groups are, for instance, two different media.

A Bayesian approach was adopted to prevent spurious odds ratios, i.e. zero or infinite odds ratios. This was done by putting a uniform prior on the probability of detecting *Salmonella*. As a result, the eventual odds and odds ratios will be 'shrunken' towards one and values equal to zero or infinity are made impossible.

Results were analysed using the statistical software R (R Development Core Team, 2014).

3.6 Good performance

For the determination of good performance, the criteria indicated in Table 2 were used. For the determination of 'good performance' per laboratory, the results found with all combinations of the prescribed and requested selective enrichment media and isolation media used by the laboratory were taken into account. For example, if a laboratory found 5/6 low-level contaminated samples positive with RVS/XLD, but no positives with any other selective enrichment medium or isolation medium, this was still

considered as a good result. The opposite was used for the blank samples. Here also, all combinations of media used per laboratory were taken into account. If, for example, a laboratory found 2/6 blank samples positive with MKTTn/BGA but no positives with the other media, this was still considered a 'no-good' result.
The results will therefore be presented for selective enrichment in RVS, MKTTn or on MSRV in combination with the isolation medium (XLD or non-XLD) that gave the highest number of *Salmonella* isolations (e.g. RVS/x).

Table 2. Criteria for testing good performance in the Animal feed III study (2014)

Minimum result		
Contamination level	Percentage positive	No. of positive samples/ total no. of samples
Samples Chicken feed artificially contaminated		
S. Senftenberg high level (SSE high)	80 %	5/6
S. Senftenberg low level (SSE low)	50 %	3/6
Blank (BL)[1]	20 % at max[1]	1/6 at max[1]
Control samples		
Positive control (Own control with *Salmonella)*	100 %	1 /1
Procedure control (BPW)	0 %	0 /1
Matrix control (Chicken feed)	0 %	0 /1

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

4 Results

4.1 Chicken feed, mixed meal for laying hens

All batches of chicken feed tested negative for *Salmonella.*
For the interlaboratory comparison study, the artificially contaminated chicken feed samples were sent to the NRLs-*Salmonella* on Monday, 29 September 2014. After receiving them, the NRLs had to store the samples at 5 °C.
The number of aerobic bacteria and the number of *Enterobacteriaceae* were tested twice at the laboratory of the EURL-*Salmonella*; first on the day the chicken feed arrived at the EURL (27/09/2014) and, a second time, after storage at 5 °C for one week (6/10/2014). Table 3 summarizes the results, showing that the amount of background flora remained stable during storage of a few weeks.

Table 3. Number of aerobic bacteria and number of Enterobacteriaceae per gram of chicken feed

Date	*Enterobacteriaceae* CFU / g	Aerobic bacteria CFU / g
25 August 2014	$9*10^2$	$5*10^4$
6 October 2014 (stored at room temperature until 29 September and placed at 5 °C until 6 October)	$2*10^2$	$5*10^4$

4.2 Artificial contamination of chicken feed samples

4.2.1 Pre-tests for the preparation of chicken feed samples

Three sets of experiments were performed. For each set of experiments, the stability of *Salmonella* in the chicken feed samples was tested during storage of the samples at different temperatures for up to five weeks. During each set of experiments, different variables were tested in different combinations (see Section 3.1.1). Table 4 and Figure 1a and 1b show the results of all tested samples.

Table 4. Stability tests of chicken feed artificially contaminated with Salmonella Typhimurium (STM) and S. Senftenberg (SSE)

Days of storage	Storage at -20 °C					Storage at +5 °C					Storage at +10 °C
	STM13	**STM77**	**SSE14**	**SSE20 SSE67**	**SSE47**	**STM13**	**STM77**	**SSE14**	**SSE20 SSE67**	**SSE47**	**SSE14 SSE47**
number of positive samples/number of tested samples											
0	4/4	4/4	6/6	6/6	6/6	4/4	4/4	6/6	6/6	6/6	6/6
7	**3**/4	4/4		6/6		**3**/4	4/4		6/6		6/6
14	**2**/4	4/4	**3**/6	6/6	6/6	**1**/4	4/4	**1**/6		6/6	
35			**3**/6		6/6						

All samples were analysed by using selective enrichment media RVS, MKTTn and MSRV and the highest number of positive samples tested by any of the media is given. Indicated are the serovars and contamination levels in the chicken feed. For example, SSE20 indicates *Salmonella* Senftenberg at a level of 20 CFU/25 g of chicken feed.

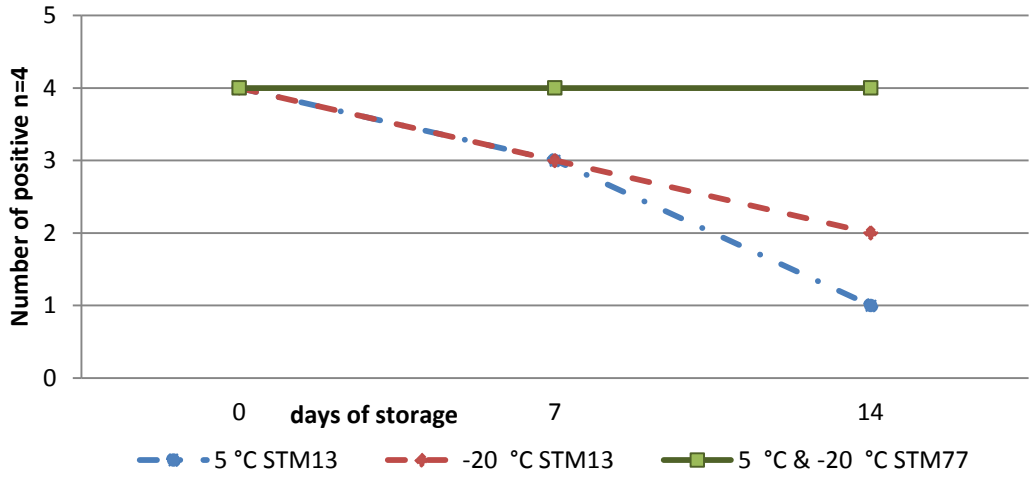


Figure 1a. Stability test of chicken feed samples artificially contaminated with Salmonella *Typhimurium (STM)*

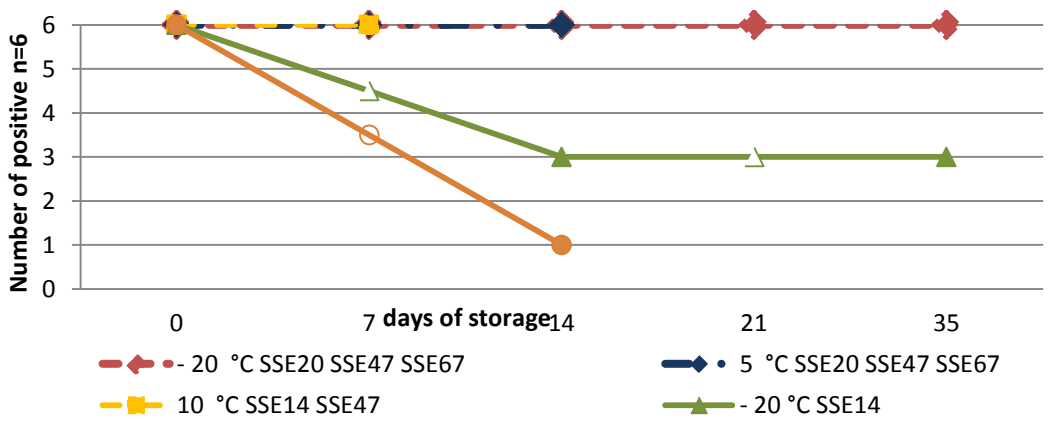


Figure 1b. Stability test of chicken feed samples artificially contaminated with Salmonella *Senftenberg (SSE)*

The major findings are summarized below:

Samples artificially contaminated with low levels of *Salmonella* Typhimurium (chicken breeding meal (flour No2) contaminated with STM 13 CFU) and *Salmonella* Senftenberg (mixed meal for laying hens contaminated with SSE 14 CFU) showed a decrease in the number of Salmonella positive samples after storage at –20 ºC and 5 ºC for 1-2 weeks.

- The number of positive samples was reduced by approx. 50 % after 1 week of storage at 5 ºC.
- Only one sample from both serotypes (STM and SSE) tested positive after storage at -20 ºC for 2 weeks.
- During the different experiments, the number of Enterobacteriaceae in the chicken breeding meal was < 10 CFU/g, while the mixed meal for laying hens gave 10^4 CFU/g.
- The background flora in the chicken breeding meal was not stable at a storage temperature of 5 ºC and -20 ºC. The number of aerobic bacteria showed a decrease at both storage temperatures, from 10^4 CFU/g to
- 10^2 CFU/g within 1 week.

All subsequent experiments were performed with mixed meal for laying hens contaminated with *S.* Senftenberg (SSE). To mimic abuse temperatures during transport, the samples were additionally stored at 10 ºC.

- Samples artificially contaminated with Salmonella Senftenberg at a level of 20 CFU or higher were shown to be stable in chicken feed samples during storage at 10 ºC or lower for at least 1-2 weeks.

The background flora in the samples of mixed meal for laying hens was stable during storage at 5 ^{0}C and –20 ^{0}C, but increased during storage at 10 ^{0}C.

- Storage for one week at 10 ^{0}C showed an increase of approximately one $\log_{10}$ CFU/g in the number of Enterobacteriacea, as well as in the total number of aerobic bacteria.
- The number of aerobic bacteria (initially 10^5 CFU/g) and Enterobacteriaceae (initially 10^4 CFU/g) in the mixed meal for laying hens remained the same after storage at -20 ºC and 5 ºC, even after 5 weeks.

From the results of the experiments, a decision was taken to use the following samples for the interlaboratory comparison study:

- For each participant, 18 x 25 g of chicken feed (ISO/TS 22117; Anonymous, 2010);
- Each sample individually inoculated with a diluted culture of Salmonella:
  - low-level SSE: 15-20 CFU/25 g of chicken feed
  - high-level SSE: 50-100 CFU/25 g of chicken feed
  - blank: 0 CFU/25 g of chicken feed

Table 5. Number of Salmonella Senftenberg (SSE) in the inoculum and in the chicken feed samples

Date of testing	**Low-level SSE CFU / 25 g chicken feed** (95% confidence limit)	**High-level SSE CFU / 25 g chicken feed** (95% confidence limit)
26 September 2014 (inoculum of chicken feed)	20	61
6 October 2014 after storage at 5 °C for 1 week MPN of chicken feed inoculated with SSE (95 % confidence limit)	2* (0.5-4.75)	5* (0.6-30)
20 October 2014 after storage at 5 °C for 3 weeks MPN of chicken feed inoculated with SSE (95 % confidence limit)	2 (0.8-7)	11 (3.75-30)

*In the MPN of 6 October, one dilution (1/100) was missing, so the MPN is at least the calculated number

4.2.2 Contamination level of the artificially contaminated chicken feed samples

Table 5 shows the contamination levels of the low-level and high-level contaminated chicken feed samples. The inoculum level of the diluted SSE culture (tested on XLD), as well as the contamination level of the chicken feed samples after the inoculation with the diluted culture, were tested. The latter was tested using a five-tube MPN test (see Section 3.1.2). The number of positive chicken feed samples tested on 20 October for 25 g, 2.5 g and 0.25 g were, respectively, for the low-level SSE 5/5, 0/5 and 0/5 and for high-level SSE 5/5, 3/5 and 1/5. The calculated MPN/25 g of chicken feed is given in Table 5.

4.3 Technical data: interlaboratory comparison study

4.3.1 General

Thirty-four NRLs for *Salmonella* participated in this study: 30 NRLs from 28 EU-Member States (MS) and 4 NRLs from non-EU MS. The non-EU MS consisted of EU candidate MS or potential EU candidate MS, members of the European Free Trade Association (EFTA) and a non-European country.
All laboratories performed the study on the planned date (week 41, starting on 5 or 6 October 2015).

4.3.2 Accreditation/certification

Thirty-two laboratories are accredited for their quality system according to ISO/IEC 17025 (Anonymous, 2005) and two EU-MS laboratories (12 and 25) are still in the process of accreditation. Twenty-nine laboratories are accredited for ISO 6579 (detection of *Salmonella* in food and animal feeding stuffs), 24 of them are also accredited for Annex D of ISO 6579. Three laboratories (4, 20 and 26) are accredited only for the detection of *Salmonella* in animal faeces and veterinary samples by using MSRV (Annex D of ISO 6579).

4.3.3 *Transport of samples*

Twenty-one participants received the samples within one day after dispatch, six participants within two days and five participants after three days of transport due to a strike at the airport. For two parcels (non-EU-MS), it was not possible to arrange door-to-door transport. The parcels for laboratories 13 and 20 were retained by the customs and arrived only after 4 and 6 days of transport, respectively, at the participating laboratory
The NRLs returned the temperature recorders to the EURL-*Salmonella* at the time they started the study, as requested. One temperature logger was lost and did not arrive at the EURL-*Salmonella* (lab code 8). For the majority of the parcels, the temperature did not exceed 5 °C during transport, with the exception of laboratories 4, 20 and 31. The temperature in the parcels of these laboratories increased to a maximum of 16 °C for 2 to 3 days. During storage at the NRL, the temperature was generally between 0 °C and 5 °C. At eight laboratories (lab codes 1, 11, 13, 15, 17, 19, 29 and 33), the samples were stored between 5 °C and 10 °C.

4.3.4 *Media*

Each laboratory was asked to test the samples using the prescribed method (ISO 6579; Anonymous, 2002) and the requested method (Annex D of ISO 6579; Anonymous, 2007). All laboratories except one used the selective enrichment media RVS, MKTTn and MSRV in combination with XLD and a second plating-out medium of their own choice. Laboratory 18 (EU-MS) did use the prescribed selective enrichment media RVS and MKTTn, but did not use the requested medium MSRV.

Table 6 provides information on the reported pH, the concentration of Novobiocin, the incubation time and temperature that deviated from the prescribed method. The table lists only the reported deviations from the method.
Three laboratories (1, 11 and 18) reported a longer incubation time for the pre-enrichment in BPW. Two laboratories (5 and 16) reported a pH of 7.3 instead of the prescribed maximum pH of 7.2 for BPW.

Five laboratories (7, 12, 18, 21 and 26) used MKTTn at a pH that deviated from the prescribed pH of 7.8-8.2. Ten laboratories used MKTTn with a lower concentration of novobiocin than the prescribed 0.04 g/L and laboratory 5 used MKTTn without the addition of Novobiocin.
Three laboratories (14, 23 and 27) used MSRV with a higher concentration of novobiocin than the prescribed 0.01 g/L and laboratory 11 used a lower concentration of novobiocin. Five laboratories (7, 15, 26, 30 and 33) reported a deviating pH for MSRV.
Laboratory 9 did not report the pH of any of the used media.

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

Lab code	BPW		RVS	MKTTn		MSRV	
	Incubation time (h:min)	pH	pH	pH	Novo-biocin	pH	Novo-biocin
Prescribed ISO 6579 or ISO 6579 annex D	16-20 h	6.8-7.2	5.0-5.4	7.8-8.2	40 mg/L	5.1-5.4	10 mg/L
1	**22:00**	7	5.2	8	**20**	5.4	10
2	18:05	-	-	-	40	5.11	10
3	17:45	7 + 0.2	5.2 + 0.2	8 + 0.2	**10**	5.2 + 0.2	10
4	19:00	7.1	5.4	8.1	**4**	5.2	10
5	18:15	**7.3**	5.4	7.8	**0**	5.4	10
7	20:00	7.2	5.3	**7.4**	40	**5.5**	10
9	20:00	-	-	-	-	-	-
11	**24:00**	7.0	5.2	8	**0.04**	5.2	**0.05**
12	18:00	7.1	5.2	**6.7**	40	5.2	10
14	19:00	7.2	5.2	8	**10**	5.2	**20**
15	18:40	7	5.4	-	40	**5.5**	10
16	18:00	**7.3**	5.0	8.1	40	5.1	10
18	**22:20**	7	5.3	**7.2**	39	NA	NA
21	18:00	7	5.2	**6.6**	40	5.2	10
22	18:15	7.0	5.3	8.2	40	5.2	**50**
23	20:00	7.2	5.2	8	**10**	5.2	10
26*	18:40	7.0	5.2	**8.5**	40	**5.0**	10
27	17:30	7.1	5.0	8.1	40	5.2	**20**
28	20:00	7.0	5.4	8.0	**10**	5.5	10
29	19:55	7	5.3	8	**20**	5.1	10
30	18:00	7.1	5.3	8.1	**20**	**5.8**	10
33	17:30	7.2	5.2	8.0	40	**5.5**	10

Bold numbers/ grey cells =Deviating from ISO 6579 and/or from ISO 6579 Annex D

\- =No information

NA =Not applicable

* BPW incubation at 35.3 °C instead of at the prescribed 36-38 °C

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

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

Media	Number of users	Lab code
BGA	7	1, 5, 13, 15, 20, 22, 28
Rambach (Merck)	7	8, 18, 19, 23, 26, 33, 34
BGA^{mod} (ISO 6579, 1993)	5	4, 16, 24, 30, 31
BPLS (Merck & Biolife)	4 +1	11, 14, 17, 29 (10*)
RS (Bio-rad)	3	6, 7, 9
SM(ID)2 (Biomerieux)	2	10, 27
BSA (Oxoid)	2	2, 32
Compass *Salmonella* agar (Biokar)	2	12, 21
XLT (Liofilchem Diagnostic)	1	3
ASAP (Biomerieux)	1	25

Explanations of the abbreviations used are given in the 'List of abbreviations'.
*Laboratory 10 used a third plating-out medium

Table 8. Biochemical and other confirmation tests of Salmonella used by the NRLs

Lab code	TSI	UA	LDC	Gal	VP	Indole	Kit	Other
1, 11, 28, 29	+	+	+	-	-	+		
2	+	+	+	-	-	+		oxidase
3, 23	+	+	+	+	+	+		PCR
4, 16, 30	-	-	-	-	-	-		
5	+	-	+	-	-	-		
6, 31	+	+	+	+	+	+		
7, 25	-	-	-	-	-	-	API 2 E	
8, 9	+	-	+	-	-	-		
10	-	-	-	-	-	-		Lysine Iron Agar
12	+	-	-	-	-	-	API 2 E	
13	+	+	+	+	+	+	API 2 E	
14	-	-	-	-	-	-	MICROBACT	PCR
15	+	+	+	-	-	-		
17	+	-	-	-	-	-	Microgen GnA-ID system	
18	+	+	-	-	-	-	API 2 E	
19	+	+	+	+	-	+		semi-solid glucose agar
20	-	-	-	-	-	+		PCR
21	-	-	-	-	-	-	rapid ID32E	
22	-	-	-	-	-	-	VITEK2	
24	+	+	+	+	-	+		
26	-	-	-	-	-	-		
27	-	-	-	-	-	+	BBL	PCR
32	+	+	+	+	+	+		PCR
33	-	-	-	-	-	-	Enterotest 24	MALDI-TOF
34	+	+	+	+	-	+		MALDI-TOF

The use of an extra non-selective plating agar between the 'isolation' and 'confirmation' steps was optional. A total of 26 laboratories performed this extra step (e.g. by using Nutrient agar; Anonymous, 2002).

All participating laboratories performed one or several confirmation tests for *Salmonella, see* Tables 8 and 9. Three laboratories (16, 26 and 30) performed serological tests only and five laboratories (1, 2, 9, 29 and 32) performed only a biochemical test. Two laboratories (33 and 34) used the MALDI-TOF test and seven (3, 13, 14, 20, 23, 27 and 32) used a PCR method for confirmation.

Table 9. Serological confirmation tests of Salmonella used by the NRLs

Lab code	Serological			
	O antigens	**H antigens**	**Vi antigens**	**Other**
1, 2, 4, 9, 10, 29, 32	-	-	-	
3, 5, 6, 11, 14, 15, 16, 18, 19, 21, 22, 23, 26, 28, 31, 33	+	+	-	
7	+	-	+	
8, 13, 20, 24, 25, 30, 34	+	-	-	
17	+	+	+	
27	-	-	+	
10				omnivalent
12				*Salmonella* O Anti-serum Poly A-I & Vi

- = Not done / not mentioned.

4.4 Control samples

4.4.1 General

Table 10 gives the results of all control samples. The results given in the table are the highest number of positive isolations found with all combinations of selective enrichment media and isolation media per laboratory. Annex 1 gives more details on the results for each selective enrichment medium (RVS, MKTTn and MSRV) in combination with the isolation media used per laboratory.
Thirty-two laboratories scored all three control samples correctly with at least one of the used media.

Table 10. Total number of positive results from the control samples per laboratory

Lab code	The highest number of positive isolations found with any used medium combination		
	Own control with *Salmonella* n=1	**BPW n=1**	**Chicken feed n=1**
Good performance	**1**	**0**	**0**
• 1,2, 4-16, 18-34	1	0	0
• 3	**0**	0	0
• 17	1	**1**	0

Bold number = deviating result.
Grey cell = result below level of good performance.

Positive control with Salmonella
Thirty-three laboratories scored good results with their own *Salmonella* positive control sample and detected *Salmonella* with all used media. Laboratory 3 could not detect *Salmonella* in all the used media.

For the positive control samples, the majority of the participants used a diluted culture of *Salmonella* (20 laboratories). Others used a lenticule disc (8), a freeze-dried ampoule (2), kwik-stik (2) or a culti-loop (2) with *Salmonella.* Table 11 shows the *Salmonella* serovars used for the positive control samples. Most often, *Salmonella* Enteritidis (15) and *Salmonella* Typhimurium (9) were used. The concentration of *Salmonella* in the positive control samples used by the different participants varied between 8 and 10^9 CFU/sample.

Table 11. Salmonella serovars used by the participants for the positive control samples

Salmonella serovar	Number of users
S. Enteritidis	15
S. Typhimurium	9
S. Nottingham	3
S. Goldcoast	2
S. Poona, *S.* Bongori, *S.* Kedougou, *S.* Alachua, *S.* Tennessee	1

Procedure control Blank (only BPW)
Thirty-three laboratories correctly analysed the one procedure control sample (no matrix, only BPW) correctly as negative for *Salmonella*. Laboratory 17 reported this sample as positive for *Salmonella* with all selective enrichment media.

Matrix control Blank (chicken feed)
All laboratories correctly analysed the one chicken feed control sample (25 g of matrix) as negative for *Salmonella*.

The results were compared with the definition of ‘good performance’ (see Section 3.6). The Laboratories 3 and 17 did not fulfil these criteria for the control samples.

Table 12. Correct scores found with the control samples by all laboratories ('All') and by the laboratories of the EU member states ('EU')

Control Samples		RVS / X		MKTTn / X		MSRV / X*	
	Laboratories	All n=34	EU n=30	All n=34	EU n=30	All n=33	EU n=29
Positive control (Own *Salmonella*) n=1	No. of samples	34	30	34	30	33	29
	No. of positive samples	33	29	33	29	32	28
	Correct score in %	**97**	**97**	**97**	**97**	**97**	**97**
Procedure control Blank (BPW) n=1	No. of samples	34	30	34	30	33	29
	No. of negative samples	33	29	33	29	32	28
	Correct score in %	**97**	**97**	**97**	**97**	**97**	**97**
Matrix control Blank chicken feed n=1	No. of samples	35	30	35	30	33	29
	No. of positive samples	35	30	35	30	33	29
	Correct score in %	**100**	**100**	**100**	**100**	**100**	**100**
All control samples	No. of samples	102	90	102	90	99	87
	No. of correct samples	100	88	100	88	97	85
	Accuracy in %	**98**	**98**	**98**	**98**	**98**	**98**

X = isolation medium with the highest number of positives

*Result without Laboratory 18 (EU-MS): they did not use MSRV

4.4.2 Correct scores of the control samples

Table 12 shows the correct scores found with the control samples for the different selective enrichment media (RVS, MKTTn and MSRV) in combination with the isolation medium that gave the highest number of positives. The calculations were performed on the results of all participants and on the results of only the EU-MS. Only minor differences were found between these groups.

The laboratories scored a good result for the control samples, with accuracy rates of 98%.

4.5 Results of chicken feed samples artificially contaminated with *Salmonella*

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

General

Table 13 shows the results of the chicken feed samples artificially contaminated with *Salmonella* Senftenberg. The results given in this table are the highest number of positive isolations found with the different selective enrichment media (RVS, MKTTn and MSRV) in combination with 'the best' isolation medium. Annex 2 gives more details on the results for each selective enrichment medium (RVS, MKTTn and MSRV) in combination

with the used isolation media per laboratory. Not all media combinations gave the same results.

Blank samples
Thirty-two laboratories correctly scored all six blank chicken feed samples as negative for *Salmonella* with all used media. Two laboratories (2 and 30) found one blank sample of the six positive for *Salmonella* with the selective enrichment medium RVS, while identifying the same sample correctly as negative with the other selective enrichment media (MKTTn and MSRV) inoculated from the same BPW.
All blanks should test as negative. However, because no 100% guarantee for the *Salmonella* negative status of the chicken feed could be given, one positive out of six blank samples (80% negative) is still considered to be acceptable.

High-level contaminated Salmonella *Senftenberg samples*
All laboratories detected *Salmonella* in all six samples that contained *Salmonella* Senftenberg at an inoculum level of approximately 61 CFU/25 g of chicken feed with at least one of the used selective enrichment media. Laboratory 24 could not detect *Salmonella* in one of the six samples with a high-level of contamination using the selective enrichment medium MKTTn, though they found the same sample to be positive using the other selective enrichment media (RVS and MSRV) inoculated from the same BPW.

Table 13. Number of positive results found with the artificially contaminated chicken feed samples (25g) at each laboratory

Lab code	The highest number of positive isolations found with selective enrichment medium (RVS, MKTTn or MSRV) in combination with 'the best' isolation medium		
	Blank n=6	SSE Low n=6	SSE High n=6
Good performance	**≤1**	**≥3**	**≥5**
1, 3, 5-6, 8–13, 15–29, 31-32, 34	0	6	6
2	**1**	6	6
4	0	**4**	6
7	0	**5**	6
14	0	**5**	6
30	**1**	6	6
33	0	**5**	6

Bold number = deviating result.
Grey cell = result below level of good performance.

Low-level contaminated Salmonella *Senftenberg samples*
Thirty laboratories detected *Salmonella* in all six samples that contained *Salmonella* Senftenberg at an inoculum level of approximately 20 CFU/25 g of chicken feed using all the selective enrichment media in combination with at least one of the used isolation media. Three laboratories (lab codes 7, 14 and 33) could not detect *Salmonella* in one out of six samples with a

low-level of contamination. Laboratory 4 could not detect *Salmonella* in two out of six samples with a low-level of contamination with any of the used media (RVS, MKTTn and MSRV).

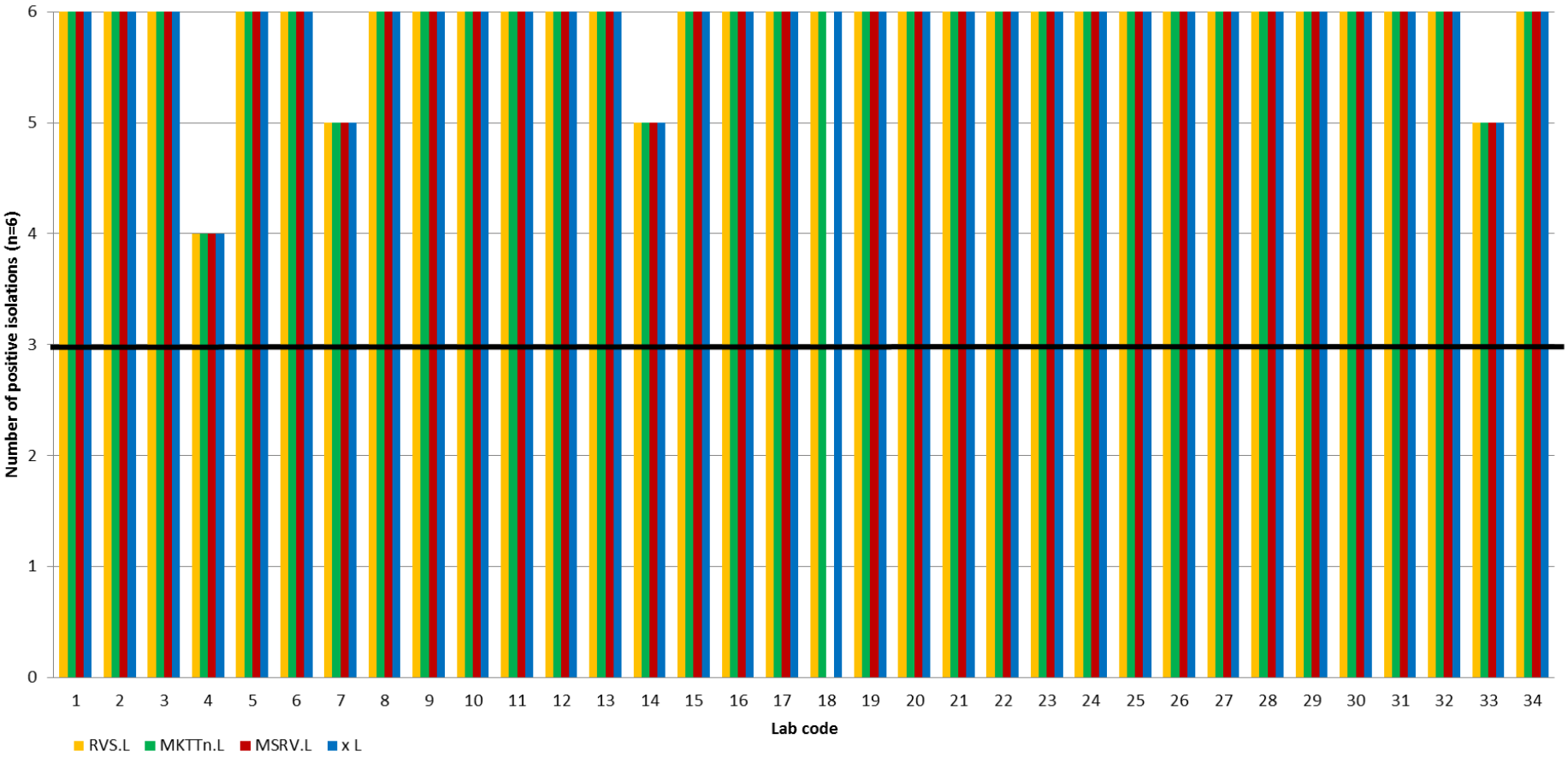
The results of the artificially contaminated chicken feed samples were compared with the definition of 'good performance' (see Section 3.6) and all laboratories fulfilled these criteria.

4.5.2 Results for each selective enrichment medium, each level of contamination and each laboratory

Figures 2 and 3 show the number of positive isolations for each level of artificially contaminated chicken feed sample and for each laboratory after pre-enrichment in BPW and selective enrichment in RVS, MKTTn and on MSRV, followed by isolation on selective plating agar (XLD and other). Furthermore, all possible combinations of media that produced the highest number of positive results (x) are given. The selective enrichment medium and/or isolation medium that gave the highest number of positives varied per laboratory.

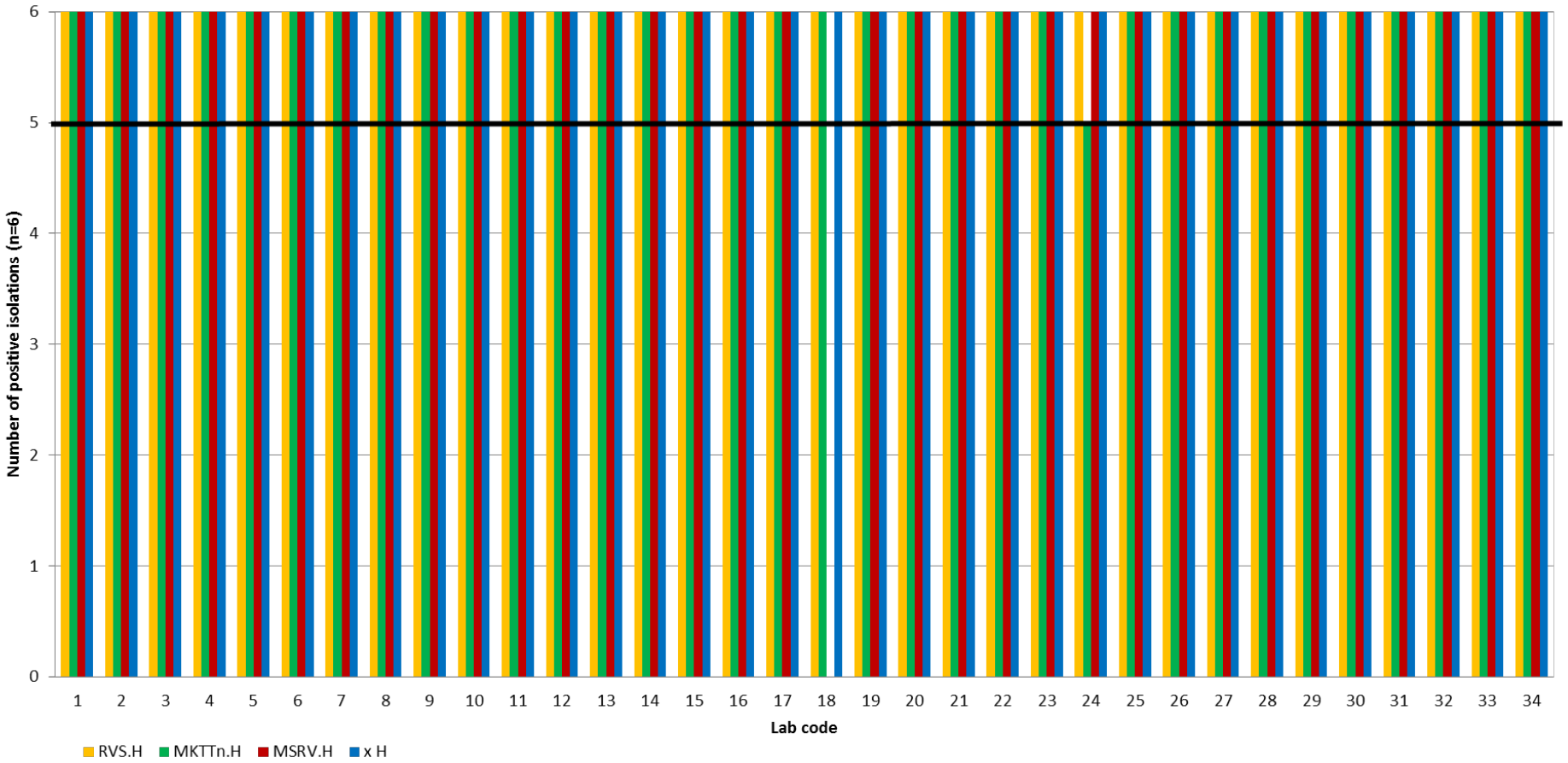
The results found with the artificially contaminated chicken feed samples were compared with the agreed definition of 'good performance' (see Section 3.6). In Figures 2 and 3, the border of good performance is indicated by a black horizontal line.

Table 14 presents the percentages of samples testing positive for *Salmonella* after 24 hours of incubation in RVS, MKTTn and on MSRV and after an additional 24 hours of incubation on MSRV. The majority of the laboratories used BGA (modified) or Rambach as the second plating-out medium (see Table 7).
When MKTTn was used for selective enrichment, XLD produced 7% more positive results than other plating-out media; for RVS and MSRV this was 3-4%.
An extra incubation time of 24 hours for MSRV produced 3-5% more positive results.



▬ = border of good performance

Figure 2. Results per laboratory found with the chicken feed samples artificially contaminated with a low-level of SSE (n=6) after selective enrichment in RVS, MKTTn and on MSRV, followed by isolation on the 'best' selective plating agar and all possible combinations of media producing the highest number of positive results (x).



▬ = border of good performance

Figure 3. Results per laboratory found with the chicken feed samples artificially contaminated with a high-level of SSE(n=6) after selective enrichment in RVS, MKTTn and on MSRV, followed by isolation on the 'best' selective plating agar and all possible combinations of media producing the highest number of positive results (x).

Table 14. Mean percentages of positive results for the detection of Salmonella in the artificially contaminated chicken feed samples after selective enrichment in RVS, MKTTn and on MSRV incubated for 24 hours, and for a total of 48 hours on MSRV, followed by isolation on different plating out media

Plating out medium	Selective enrichment medium					
	RVS 24h		MKTTn 24h		MSRV 24 / 48 h	
Contamination level SSE	Low	High	Low	High	Low	High
XLD	97%	99%	96%	99%	94/97%	96/100%
Other isolation media (most often BGA or Rambach)	93%	96%	89%	92%	88/93%	91/96%
Difference XLD/other	4%	3%	7%	7%	6/4%	5/4%

Tables 15 and 16 show the differences between selective enrichment media and isolation media for each contamination level as odds ratios (OR). In addition, the 95% confidence intervals and p-values are given.

In Table 15, the odds of finding a positive isolation using the different plating-out media are compared, given a selective enrichment medium. For instance, the odds of finding *Salmonella* in the low-level contaminated SSE samples after selective enrichment in MKTTn is a factor of 3.69 higher when XLD is used as the isolation medium, compared with an isolation medium other than XLD. In general, if RVS is used as selective enrichment medium, the Odds Ratios (ORs) are smaller than the ORs for MKTTn and MSRV. In other words, when MKTTn or MSRV is used for selective enrichment, it is easier to detect *Salmonella* if XLD is used compared to other isolation media. The use of XLD produces significantly higher scores for RVS, MKTTn and MSRV. Only for the high-level contaminated samples with selective enrichment in RVS was the higher score using XLD not significant compared with isolation medium other than XLD.

Table 15. Number of positive isolations found with XLD compared with the number of positive isolations found with other isolation media, given a selective enrichment medium.
Samples: chicken feed, artificially contaminated with Salmonella Senftenberg

Selective enrichment medium	Compared isolation media	CFU	odds ratios	95% lower	95% upper	p-value*
RVS	XLD compared with media other than XLD	Low	2.89	1.1	8.05	0.03
		High	2.67	0.76	10.66	0.12
		Low & High	2.78	1.25	6.48	0.01
MKTTn	XLD compared with media other than XLD	Low	3.69	1.58	8.99	0
		High	9.38	2.72	44.93	0
		Low & High	5.88	2.74	14.37	0
MSRV	XLD compared with media other than XLD	Low	2.89	1.03	8.51	0.04
		High	17.28	2.08	446.11	0
		Low & High	7.07	2.14	37.1	0
All selective enrichment media	XLD compared with media other than XLD	Low	3.14	1.79	5.54	0
		High	7.51	2.9	25.69	0
		Low & High	4.85	2.76	9.69	0

* significant difference in case $p < 0.05$, indicated in grey.

The interpretation of Table 16 is similar to that of Table 15, except that selective enrichment media are compared with each other, with XLD as the isolation medium. For instance, the odds of finding *Salmonella* in low-level contaminated SSE samples after selective enrichment in RVS is a factor of 1.39 higher than when MKTTn is used. When RVS or MKTTn is used as the selective enrichment medium, compared with MSRV, the odds become smaller (a factor of 0.37). In general, if MSRV is used as the selective enrichment medium, the chance of finding *Salmonella* is higher than when MKTTn or RVS is used. However, these differences are not significant.

Table 16. Number of positive isolations found using a selective enrichment medium compared with the number of positive isolations found when using another selective enrichment medium, given that the isolation is on XLD. Samples: chicken feed artificially contaminated with Salmonella Senftenberg

Compared selective enrichment media	Isolation medium	CFU	Odds Ratios	95% lower	95% upper	p-value*
RVS compared with MKTTn	XLD	Low	1.39	0.48	4.22	0.55
		High	0.69	0.12	3.56	0.68
		Low & High	0.98	0.35	2.57	0.99
RVS compared with MSRV	XLD	Low	0.85	0.26	2.81	0.78
		High	0.16	0.01	1.61	0.13
		Low & High	0.37	0.06	1.35	0.15
MKTTn compared with MSRV	XLD	Low	0.62	0.19	1.88	0.39
		High	0.23	0.01	2.64	0.27
		Low & High	0.37	0.06	1.43	0.17

* Significant difference in case $p < 0.05$.

Figure 4 shows the performance of each laboratory as odds ratios compared with the mean of all laboratories for the artificially contaminated samples. In this calculation, the blank samples are not used. The mean (OR = 1) is defined as the odds of detecting *Salmonella* based on the fixed effects only (SSE low or high, enrichment medium and isolation medium). Laboratories below the mean (OR < 1) have a lower probability of detecting *Salmonella*. The laboratories 4, 7, 14, 15, 16, 20, 25, 29, 30 and 33 scored a significantly lower probability of detecting *Salmonella,* but still scored within the lines of good performance. Yet these laboratories still may have a sensitivity problem with one of their media. For example, the laboratories 4, 7, 14 and 33 (marked yellow in Figure 4) scored one or two low-level contaminated samples as negative when using all selective-enrichment media. The laboratories 15, 16, 25, 29 and 30 (marked pink in Figure 4) scored 5 to 23 fewer positive results with their second isolation medium (other than XLD). Laboratory 20 (marked red in Figure 4) found 4 more positive samples with their second isolation medium (BGA), compared with XLD for both selective enrichment media RVS and MKTTn, while with MSRV they scored all samples correctly, regardless of the isolation medium used.
Figure 4 shows the highest scores for eighteen laboratories (1, 3, 5, 8, 9, 10, 12, 13, 17, 19, 21, 23, 26, 27, 28, 31, 32 and 34). They scored all artificially contaminated samples correctly for all used media.

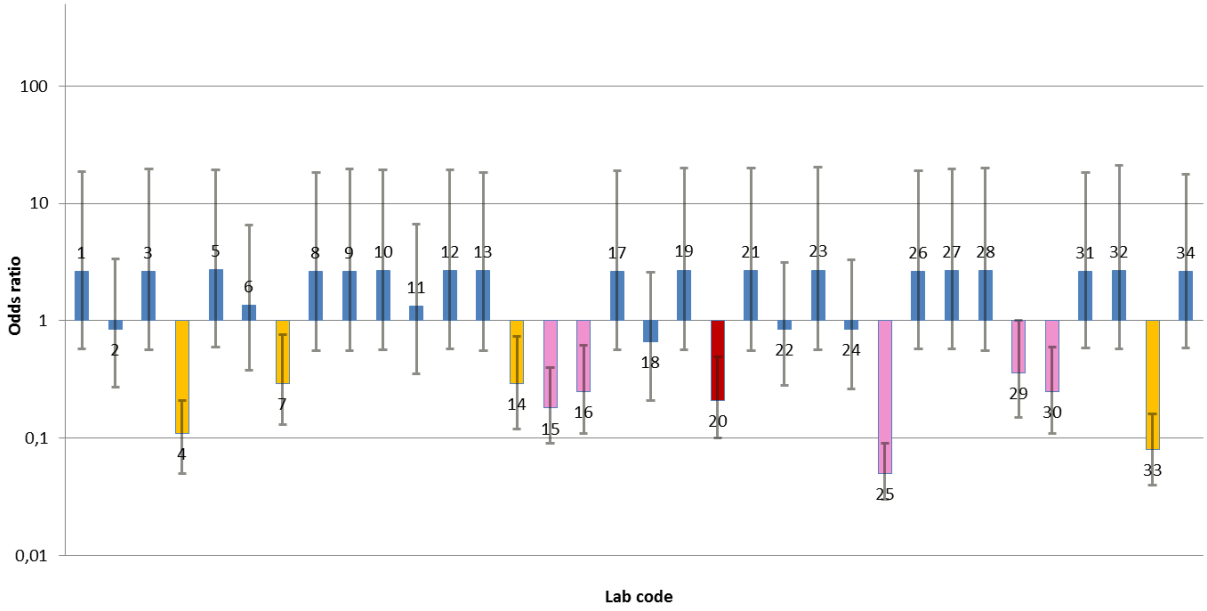


Figure 4 Performance of each laboratory compared with the mean of all laboratories for the artificially contaminated chicken feed samples (without blanks)

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

Table 17 shows the specificity, sensitivity and accuracy rates for all levels of artificially contaminated chicken feed samples. This table gives the results for the different selective enrichment media (RVS, MKTTn and MSRV) and isolation on selective plating agar showing the highest number of positives (x). 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 rates were comparable for the different selective enrichment media: specificity rates 99-100%, sensitivity rates 97-100% and accuracy rates of 99%.

Table 17. Specificity, sensitivity and accuracy rates found by the participating laboratories with the artificially contaminated chicken feed samples after selective enrichment in RVS, MKTTn and on MSRV, in combination with an isolation medium with the highest number of positives

Chicken feed samples		RVS / X		MKTTn / X		MSRV / X*	
	Laboratories	All n=34	EU n=30	All n=34	EU n=30	All n=33	EU n=29
Blank (n=6)	No. of samples	204	180	204	180	198	174
	No. of negative samples	202	178	204	180	198	174
	Specificity in %	**99**	**99**	**100**	**100**	**100**	**100**
SSE low (n=6)	No. of samples	204	180	204	180	198	174
	No. of positive samples	199	177	199	177	193	171
	Sensitivity in %	**98**	**98**	**98**	**98**	**97**	**98**
SSE high (n=6)	No. of samples	204	180	204	180	198	174
	No. of positive samples	204	180	203	179	198	174
	Sensitivity in %	**100**	**100**	**99**	**99**	**100**	**100**
All samples with *Salmonella*	No. of samples	408	360	408	360	396	348
	No. of positive samples	403	357	402	356	391	345
	Sensitivity in %	**99**	**99**	**99**	**99**	**99**	**99**
All samples	No. of samples	612	540	612	540	594	522
	No. of correct samples	605	535	606	536	589	519
	Accuracy in %	**99**	**99**	**99**	**99**	**99**	**99**

X = isolation medium with the highest number of positives

* = results without Laboratory 18 (EU-MS): they did not use MSRV

4.6 PCR (own method)

Seven laboratories (3, 13, 14, 20, 23, 27 and 32) applied a PCR method as an additional detection technique. Table 18 gives further details about the PCR techniques used.
Laboratory 27 initially did not report their PCR results, but discussed their results with the EURL after the summary results of the study were reported.

Table 18. Details of Polymerase Chain Reaction procedures used as their own method during the interlaboratory comparison study

Lab code	PCR method	Vali-dated	Commer-cially available	Routinely used number of tests/year	DNA extraction after enrichment in	Reference
3	Real-time (InvA)	+	-	-	BPW	
13	Three step	-	+ (Qiagen)	100	Confirmation from nutrient agar	Stone et al. (1994)
14	Real-time	+	-	89	BPW	Malorny et al. (2004)
20	Real-time	+	-	850	BPW	Malorny et al. (2004) Lofstrom et al. 2010 and 2012
23	Real-time	+	-	100	BPW	ISO 16140 (Anonymous, 2003b)
27	Real-time(InvA)					
32	Real-time	+	-	16	BPW	

Table 19 gives the results of both the PCR method and the bacteriological culture technique (BAC). Five laboratories (13, 14, 20, 23 and 32) found the same results when using the PCR method and the bacteriological culture method. Two laboratories (3 and 27) did not find any positive results with their Real Time PCR (InvA) method, although all bacteriological culture results were scored correctly.

Table 19. Number of positive results found with the artificially contaminated chicken feed samples by using a PCR technique and the bacteriological culture technique

	Lab 3 & 27		Lab 13, 20, 23, 32		Lab 14	
	BAC	PCR	BAC	PCR	BAC	PCR
SSE low (n=6)	6	**0**	6	6	**5**	**5**
SSE high (n=6)	6	**0**	6	6	6	6
Blank (n=6)	0	**0**	0	0	0	0

BAC = bacteriological culture results (best score of selective enrichment in RVS, MKTTn and on MSRV)
Bold numbers = unexpected results
Grey cells = different results found with the PCR method in comparison with the bacteriological culture technique (BAC)

4.7 Performance of the NRLs

4.7.1 General

Thirty-two NRLs fulfilled the criteria of good performance and two laboratories scored below these criteria. For the determination of good performance, the results of all media were taken into account. Some laboratories did not score well with one medium, but still scored a 'good

performance' overall. Laboratory 3 reported their positive control sample as testing negative for *Salmonella* with all media combinations. Laboratory 17 reported one blank procedure control sample (only BPW) as testing positive for *Salmonella* with all media combinations.

The two deviating laboratories (3 and 17) were contacted by the EURL-*Salmonella* in November 2014 and asked for possible explanations for their deviating results. Both laboratories made a transcription error when reporting the results and this was confirmed by their raw data.

Laboratory 3 made a transcription error while they filled in the electronic form. Their raw data showed that the positive control sample was correctly identified as positive for all used media and also in their PCR method. After they provided the raw data, it was decided that no further actions were considered necessary for this laboratory and their results were designated as being a 'moderate performance'.

Laboratory 17 indicated multiple technical causes for the problem: mistyping, probably because of the digital method of reporting, and problems with saving the digital format due to problems with the Internet connection. After they provided the raw data, it was decided that their results for this study should be designated as being a 'moderate performance'. Unfortunately, this laboratory has made the same kind of reporting mistake with Blank samples for the third time: in studies in 2011 (Food V), in 2013 (Food VI) and in the current study (Feed III). In cases of repeated moderate performance, further actions are needed, namely: a follow-up (e.g. a visit of staff members of the EURL to the NRL) and informing EC DG-Sanco (DG-Sanco, 2007) about the performance. DG-Sanco was informed about the repeated moderate performance in December 2014 and a visit to the NRL was planned during a follow-up study in February 2015.

4.7.2 Follow-up study

The set-up and the number samples in the follow-up study were the same as they were for the full interlaboratory comparison study organized in September 2014. But, since the original problem was due to a false positive blank control, the samples types in the follow-up study were focused on this problem. The study contained 6 blank samples and 12 artificially contaminated chicken feed samples were inoculated with a high level of *S*. Senftenberg (SSE, approximately 68 CFU/sample). And only high-level SSE samples were used to test the possible risk of cross-contamination in the blank samples.

On 16 February 2015, the number of aerobic bacteria ($4.7 * 10^4$ CFU/g) and the number of *Enterobacteriaceae* ($2.8 * 10^2$ CFU/g) in the chicken feed was tested after it had been stored at 5 °C since September 2014. These numbers were still comparable to the numbers found in the chicken feed used in the full study (see Table 5). A duplicate set of the samples used for this follow-up study was tested by the EURL-*Salmonella* for the presence of *Salmonella* and all the samples were scored correctly on all selective enrichment media used (RVS, MKTTn and MSRV).
On Monday, 9 February 2015, one parcel with 21 samples in one plastic safety bag was sent to laboratory 17 containing: 3 control samples

(numbered C1 – C3), 18 (contaminated) chicken feed samples (numbered B1 – B18) and one temperature recorder.
The follow-up study started in week 5 (16 February 2014). The laboratory had to follow the same SOP, protocol and web-based test report as had been used in the study of September 2014 (EURL-*Salmonella*, 2014a, 2014b and 2014c).

Two staff members of the EURL-*Salmonella* visited laboratory 17 while this NRL performed the follow-up study. During a two-day visit (16 and 17 February 2015), the procedures of the NRL were checked for possible (technical) problems in an attempt to explain the deviating results. At the end of the visit, a report containing observations and recommendations for possible improvements was drafted by the staff members of the EURL-*Salmonella* and discussed with the staff members of laboratory 17.

During the follow-up study, laboratory 17 used the same media used in the full study. The reporting of the results is performed differently from routine samples. Not only are the forms for reporting different from the routine samples (special reporting forms of the EURL), but also the person performing the reporting is different. In the main study, the NRL had some problems reporting through the web-based test reports of the EURL-*Salmonella*. They regularly had problems with the Internet, which caused them to lose data several times because the form had not been completed before the Internet stopped working. During the visit of the EURL-*Salmonella,* no problems with the Internet or with completing the web-based test report of the EURL-*Salmonella* were observed.
The NRL had indicated earlier that they thought the reporting had been the main source for the mistakes they had made in the three interlaboratory comparison studies. It is quite likely that this was indeed an important source of the mistakes.

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

A report on laboratory 17 (EU-MS), containing all information about the performance in the food/animal feed studies of 2011, 2013, 2014 and the visit of the EURL-*Salmonella* in 2015, was sent to EC, DG-Sango in April 2015. It was concluded that no further actions were needed.

5 Discussion

Artificial contamination of samples with a diluted culture
After many years of using reference materials (capsule or lenticule discs) to artificially contaminate the matrix in the interlaboratory comparison studies of the EURL-*Salmonella*, it was decided to change to the artificial contamination of the samples with a diluted culture at the laboratory of the EURL. The main reason for this change was to better mimic 'real life' routine samples and to enable easier handling of the study samples by the participants.

The first EURL-*Salmonella* study in which this method of artificial contamination was used successfully were the studies conducted in 2013 for the detection of *Salmonella* in boot socks (Kuijpers and Mooijman, 2014) and for the detection of Salmonella in minced chicken meat (Kuijpers et al. 2014). As each matrix and *Salmonella* serovar combination may behave differently, the samples of the current study, with animal feed used as a matrix for the first time, were tested for their 'long-term' stability at storage temperatures (-20 ºC and 5 ºC) and for 'short-term' stability at temperatures that may occur during the transport of the samples. Experiences from earlier studies had shown that, in general, the transport time of the parcels to the NRLs is 1 to 2 days at temperatures that remain below 10 ºC most of the time. Only occasionally, the temperature of a parcel during transport may be ≥15 ºC for a few hours.

As the number of *Salmonella* in the chicken feed slowly decreased during storage, it was decided to inoculate the low-level contamination samples with 20 CFU of a diluted culture of *Salmonella* Senftenberg to make sure that the level on the date of the study would still be approximately 5-10 CFU. After storage and transport, the contamination level in the samples with a low level of contamination was approximately 11 MPN/25 g (with 95% confidence interval of 0.8-7 MPN/25 g) on the day of the study.

Transport of the samples
To stabilize the level of *Salmonella* Senftenberg in the samples during transport, the materials were packed with frozen cooling elements and transported by courier service. The information provided by the temperature recorders included in the parcels showed that the temperature in the parcels remained below 5 ºC for most of the transport time. It can therefore be assumed that transport did not negatively affect the mean contamination level of the samples. This was confirmed by the fact that the laboratory with the longest transport time and/or in combination with the highest temperatures (lab codes 13 and 31) scored all samples correctly.

Accreditation of laboratories
According to EC regulations 882/2004 (EC, 2004) and 2076/2005 (EC, 2005), each NRL should have been accredited in their relevant field before 31 December 2009. Thirty-two laboratories were accredited. Two participants (EU-MSs, lab codes 11 and 25) were still in the process of being accredited, which is relatively late.

Performance of the laboratories
For the evaluation of the laboratories in terms of 'good performance', the best combination of selective enrichment medium (RVS, MKTTn or MSRV) and isolation medium was taken into account (i.e. the combination with the highest number of positive isolations).

Two laboratories (lab codes 3 and 17) scored an 'underperformance'. Both laboratories found correct results for the samples containing animal feed contaminated with *Salmonella,* as well as for the blank animal feed samples. Yet those results are in fact not reliable when deviations in the positive or negative control sample are found. At both laboratories, the cause of the deviating result was a mistake made in reporting. When reporting the results of routine samples, a transcription error may result in unwanted situations, such as an 'incorrect non-compliance' of an animal feed/food product. To prevent such unwanted situations occurring, it is important to have a system in which the results are checked before reporting to the 'client'. This should be part of the quality system of the laboratory. The results of laboratories 3 and 17 were therefore indicated as a 'moderate performance'. A follow-up study was considered unnecessary for laboratory 3. Laboratory 17 repeatedly showed moderate performances in food and animal feed studies, so a follow-up study was organised in combination with a visit of two staff members of the EURL. The results of the follow-up study fulfilled the criteria for a good performance, indicating that the laboratory is able to perform the analysis for the detection of *Salmonella* in a proper manner. No further actions were considered necessary. The results of the next food/feed study will again be reviewed against the trend analysis of the former studies.

Two laboratories (2 and 30) scored a positive result for *Salmonella* in one blank sample on one selective enrichment medium (RVS), while they correctly found the same sample to be negative using another selective enrichment medium inoculated from the same pre-enriched culture in BPW. This was still considered acceptable because no 100% guarantee of the *Salmonella* negativity of the matrix could be given. An explanation for the false positive sample may be cross-contamination or misinterpretation of the results.

The performance of each laboratory compared with the mean of all laboratories for the artificially contaminated chicken feed samples (Figure 4) is an indication of the performance of a laboratory in general (the blanks are not included in this comparison). A laboratory can show a performance under the mean of all laboratories, yet still score a 'good performance'. This lower score can be caused by a low performance on one of the selective enrichment or isolation media. For the determination of a good performance, the results of the 'best' media are taken into account; while for the analysis as presented in Figure 4, the results of all media combinations are presented. Fifty-three per cent of the participants (18/34) scored all artificially contaminated samples correctly with all used media. Only 12% of the participants (4/34) missed one or two samples from the low-level contaminated samples with all selective enrichment media. These chicken feed samples contained SSE at a low-level (approximately 11 CFU/25 g) close to the detection limit. Due to the chance that one out of two low-level contaminated samples may be negative. This was the case for three participants (9%). However, at this

contamination level, it is less likely that both samples will be identified as negative as this was observed by only one participant (3%). Thirty-five per cent of the participants (12/34) did not find *Salmonella* on one of the isolation media, which may indicate a sensitivity problem with a medium.

According to the criteria used, 32 laboratories scored a 'good performance' and two laboratories scored a 'moderate performance'.

Specificity, sensitivity and accuracy rates
The calculations were performed on the results of all participants and on the results of the EU-MS only. Only minor differences (if any) were found between these groups.
The majority of the blank chicken feed samples tested as negative, resulting in a specificity rate close to 100%. For the samples of chicken feed that were artificially contaminated the rates were comparable for the different selective enrichment media.
The sensitivity rates are influenced not only by the contamination level of the target organism, but also by the level of interfering background flora. For the current chicken feed samples, the growth of *Salmonella* did not seem to be negatively influenced by the background flora, since the sensitivity rates were close to 100 %.

Positive control samples
The participants were asked to use the positive control sample(s) that are routinely used in their laboratory. *S*. Enteritidis and *S*. Typhimurium were the most frequently used serovars and the concentration in the positive control samples varied between 8 – 10^9 CFU/sample. A positive control sample should demonstrate that media are capable of supporting growth of a range of organisms in low numbers. To gain an idea concerning the sensitivity of a method, the concentration of a positive control sample should be just above the detection limit of this method. The majority of the participants used a much higher concentration. Furthermore, it may be advisable to use a serovar rarely isolated from the routine samples analysed in the laboratory. In this way, possible cross-contamination can be detected more easily.

Media and incubation
Some laboratories showed an 'underperformance' for one of the isolation media used, while they scored all samples correctly as positive with another isolation medium, inoculated from the same pre-enriched culture. This may indicate a sensitivity problem with an isolation medium. But it may also indicate that one isolation medium is more suitable for detecting the specific *Salmonella* serovar from the specific matrix. In this study, it was observed that with isolation medium XLD significantly more *Salmonella* isolations were made than were with other isolation media.
During the study, (small) deviations in the prescription of the media (e.g. in pH or concentration of novobiocin) or in incubation times have been reported. The influence of these deviations on the results is not always clear or is hard to trace.

A longer incubation time of 48 hours for MSRV gave only 3-5% more positive results compared with 24 hours of incubation.

PCR
Seven laboratories used a PCR technique in addition to the prescribed method. Five of them found the same results as were obtained using the bacteriological culture technique (BAC). Two laboratories (lab codes 3 and 27) did not find any positive results with the used real time (InvA) PCR. Both participants contacted the EURL-*Salmonella* about their negative PCR results and the laboratories performed additional research. It is possible that the *S*. Senftenberg strain used for this study presented some mutations/deletions on the InvA gene and specifically on the DNA fragments where primers and probes for the used protocol are located. With the conventional PCR method there was an amplicon for the SSE strain used in this study. Other strains of *S*. Senftenberg were tested with the same real time-PCR (InvA) and gave correct positive results. Different strains of *S*. Senftenberg were tested with a PCR on InvE/A gene, according to Stone et al. (1994). The SSE strain used in the study gave a larger PCR product than the other tested *Salmonella* strains. Next Generation Sequencing (NGS) did confirm that the SSE strain used in this study was missing a part of the virulence island SPI-1 gene (this contains InvA), while this gene was present in the other tested strains of *Salmonella,* including other *S*. Senftenberg strains.
It was concluded that the InvA gene was not present in the *S*. Senftenberg strain used in this study. The absence of InvA gene in *S*. Senftenberg strains and in *Salmonella* in general is rare.

Evaluation of this study
The artificial contamination of chicken feed with a diluted culture at the laboratory of the EURL-*Salmonella* was successful. This shows that, besides food matrices (minced meat) and products of the primary production stage (PPS: chicken faeces and boot socks with chicken faeces), also animal feed samples can be artificially contaminated in this way and are applicable in interlaboratory comparison studies.

6 Conclusions

Thirty-two out of 34 NRLs for *Salmonella* scored a good performance for the detection of *Salmonella* in high-level and low-level contaminated chicken feed samples. One laboratory scored a 'moderate performance'. One laboratory achieved the level of good performance in the follow-up study.

High rates for the specificity, sensitivity and accuracy of the artificially contaminated chicken feed were found (blank, low level and high level): 97 - 100%.

The accuracy rate of the chicken feed samples that were artificially contaminated with *S*. Senftenberg was comparable for the different selective enrichment media (97% - 100%).

The accuracy rates for the control samples after selective enrichment in RVS, MKTTn and MSRV was 98%.

Some participants may need to take the optimization of the positive control sample used in their daily routine analysis into consideration with respect to the choice of the *Salmonella* serovar and/or contamination level.

Isolation on XLD after selective enrichment gave a significantly higher probability of finding *Salmonella* Senftenberg in chicken feed in comparison with other isolation media (e.g. BGA or Rambach).

48 hours of incubation of the selective enrichment medium MSRV resulted in 3-5% more positive results than 24 hours of incubation.

PCR as a laboratory's own method gave the same results as the bacteriological culture technique, except for the PCR based on the presence of an InvA gene. The absence of this gene in the strain used in this study caused negative results in the PCR, in contrast to the results achieved with the prescribed culture method (RVS, MKTTn and MSRV).

The artificial contamination of chicken feed with a diluted culture at the laboratory of the EURL-*Salmonella* was successful. This shows that, besides food matrices (minced meat) and products of the primary production stage (PPS: chicken faeces and boot socks with chicken faeces), also animal feed samples can be artificially contaminated in this way and are applicable in interlaboratory comparison studies.

List of abbreviations

ASAP	AES *Salmonella* Agar Plate
ATCC	American Type Culture Collection
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
CFU	Colony-Forming Units
DG-Sanco	Directorate-General for Health and Consumer Protection
EC	European Commission
EFTA	European Free Trade Association
EU	European Union
EURL	European Union Reference Laboratory
Gal	Galactosidase
ISO	International Organization for Standardization
LDC	Lysine Decarboxylase
MKTTn	Mueller Kauffmann Tetrathionate novobiocin Broth
MPN	Most Probable Number
MS	Member State
MSRV	Modified Semi-solid Rappaport-Vassiliadis
NGS	Next Generation Sequencing
NRL	National Reference Laboratory
OR	Odds Ratio
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PPS	Primary Production Stage
RIVM	Rijksinstituut voor Volksgezondheid en het Milieu (National Institute for Public Health and the Environment)
RS	Rapid *Salmonella*
RVS	Rappaport Vassiliadis Soya broth
SSE	*Salmonella* Senftenberg
SM (ID)2	*Salmonella* Detection and Identification-2
SOP	Standard Operating Procedure
STM	*Salmonella* Typhimurium
TSI	Triple Sugar Iron agar
UA	Urea Agar
VP	Voges-Proskauer
VRBG	Violet Red Bile Glucose agar
XLD	Xylose Lysine Deoxycholate agar
XLT	Xylose Lysine Tergitol

References

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

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

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

Anonymous (2003b). ISO 16140. Microbiology of food and animal feeding stuffs – Protocol for the validation of alternative methods. International Organisation for Standardisation, Geneva, Switzerland.

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

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

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

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

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

DG-Sanco (2007) Protocol for management of underperformance in comparative testing and/or lack of collaboration of National Reference Laboratories (NRLs) with Community Reference Laboratories (CRLs) activities.

EC (2004). Commission Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on the official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules. Official Journal of the European Union L 165 of 30 April 2004. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2004R0882:20060525:EN:PDF(access date December 2015)

EC (2005). Commission Regulation (EC) No 2076/2005 of the European Parliament and of the Council of 5 December 2005 laying down transitional arrangements for the implementation of Regulations (EC) No. 853/2004, (EC) No. 854/2004 and (EC) No 882/2004 of the European Parliament and of the Council and amending Regulations (EC) No. 853/2004 and (EC) No. 854/2004. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2005:338:0083:0088:EN:PDF (access date December 2015)

EURL-*Salmonella* (2014a). Protocol Interlaboratory Comparison Study on the detection of *Salmonella* spp. in animal feed samples organized by EURL-*Salmonella* STUDY III – 2014. http://www.eurlsalmonella.eu/Proficiency_testing/Detection_studies_Feed (access date December 2015)

EURL-*Salmonella* (2014b). Standard operating procedure (SOP) Interlaboratory
Comparison Study on the detection of *Salmonella* spp. in animal feed samples organized by EURL-*Salmonella* STUDY III – 2014. http://www.eurlsalmonella.eu/Proficiency_testing/Detection_studies_Feed (access date December 2015)

EURL-*Salmonella* (2014c). Test report on Interlaboratory Comparison Study on the detection of *Salmonella* spp. in animal feed organized by EURL-*Salmonella* STUDY III – 2014. http://www.eurlsalmonella.eu/Proficiency_testing/Detection_studies_Feed (access date December 2015)

EURL-*Salmonella* (2014). History of EURL-*Salmonella* interlaboratory comparison
studies on the detection of *Salmonella*. http://www.eurlsalmonella.eu/Publications/Interlaboratory_comparison_study_Reports (access date December 2015)

Gelman A and Hill J (2007). Data Analysis Using Regression and Multilevel/Hierarchical Models, Cambridge University Press, New York

Jarvis B, Wilrich C, Wilrich P-T. (2010) Reconsideration of the derivation of most probable numbers, their standard deviations, confidence bounds and rarity values. J. Appl. Microbiol. 109:1660–1667.
Link to MPN calculation programme: http://www.wiwiss.fu-berlin.de/institute/iso/mitarbeiter/wilrich/index.html
(access date December 2015)

Kuijpers AFA and Mooijman KA (2014). EU Interlaboratory comparison study veterinary XVI (2013); Detection of *Salmonella* in chicken faeces adhering to boot socks. RIVM report 330604031, Bilthoven, the Netherlands.
http://www.rivm.nl/bibliotheek/rapporten/330604031.pdf (access date December 2015)

Kuijpers AFA, Kassteele van de J and Mooijman KA (2014). EU Interlaboratory comparison study food VI (2013); Detection of *Salmonella* in minced chicken meat. RIVM report 2014-0010, Bilthoven, the Netherlands.
http://www.rivm.nl/bibliotheek/rapporten/2014-0010.pdf (access date December 2015)

Kuijpers AFA and Mooijman KA (2015). EU Interlaboratory comparison study primary production XVII (2014); Detection of *Salmonella* in

chicken faeces. RIVM report 2014-0011, Bilthoven, the Netherlands.
http://www.rivm.nl/bibliotheek/rapporten/2014-0011.pdf (access date December 2015)

Lofstrom, C, Hansen F and Hoorfar J (2010). Validation of a 20-h real-time PCR method for screening of *Salmonella* in poultry faecal samples. Veterinary Microbiology 144:511-514.

Lofstrom, C.; Hoorfar, J (2012). Validation of an open-formula, diagnostic realtime PCR method for 20-h detection of *Salmonella* in animal feeds.
Veterinary Microbiology 158: 431-435.

Malorny B, Paccassoni E, Fach P, Bunge C, Martin A and Helmuth R (2004). Diagnostic real-time PCR for detection of *Salmonella* in food. Appl. Environ. Microbiol. 70:7046-7052.

R Development Core Team (2014). A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org. (access date December 2015).

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

Annex 1 Number of positive results of the control samples at each laboratory, for each selective enrichment medium and each isolation medium

Lab code	RVS XLD / 2nd *			MKTTn XLD / 2nd *			MSRV XLD / 2nd *		
	Positive control own n=1	Procedure control BPW n=1	Matrix Control feed n=1	Positive control own n=1	Procedure control BPW n=1	Matrix Control feed n=1	Positive control own n=1	Procedure Control BPW n=1	Matrix control feed n=1
Good performance	**1**	**0**	**0**	**1**	**0**	**0**	**1**	**0**	**0**
1	1	0	0	1	0	0	1	0	0
2	1	0	0	1	0	0	1	0	0
3**	**0**	0	0	**0**	0	0	**0**	0	0
4	1	0	0	1	0	0	1	0	0
5	1	0	0	1	0	0	1	0	0
6	1	0	0	1	0	0	1	0	0
7	1	0	0	1	0	0	1	0	0
8	1	0	0	1	0	0	1	0	0
9	1	0	0	1	0	0	1	0	0
10	1	0	0	1	0	0	1	0	0
11	1	0	0	1	0	0	1	0	0
12	1	0	0	1	0	0	1	0	0
13	1	0	0	1	0	0	1	0	0
14	1	0	0	1	0	0	1	0	0
15	1	0	0	1	0	0	1	0	0
16	1	0	0	1	0	0	1	0	0
17**	1	**1**	0	1	**1**	0	1	**1**	0
18	1	0	0	1	0	0	-	-	-
19	1	0	0	1	0	0	1	0	0
20	1	0	0	1	0	0	1	0	0
21	1	0	0	1	0	0	1	0	0
22	1	0	0	1	0	0	1	0	0
23	1	0	0	1	0	0	1	0	0
24	1	0	0	1	0	0	1	0	0
25	1	0	0	1	0	0	1	0	0
26	1	0	0	1	0	0	1	0	0
27	1	0	0	1	0	0	1	0	0
28	1	0	0	1	0	0	1	0	0
29	1	0	0	1	0	0	1	0	0
30	1	0	0	1	0	0	1	0	0
31	1	0	0	1	0	0	1	0	0
32	1	0	0	1	0	0	1	0	0
33	1	0	0	1	0	0	1	0	0
34	1	0	0	1	0	0	1	0	0

* = When only one figure is given, both isolation media gave the same result.

** = deviating result was an error in reporting

\- = not performed

bold numbers = deviating results / grey cells = results are below the criteria of good performance

Annex 2 Number of positive results of the artificially contaminated chicken feed samples at each laboratory, for each selective enrichment medium and each isolation medium

Lab code	RVS XLD / 2nd *			MKTTn XLD / 2nd *			MSRV XLD / 2nd *		
	Blank	SSE Low	SSE High	Blank	SSE Low	SSE High	Blank	SSE Low	SSE High
Good performance	**≤ 1**	**≥ 2**	**≥ 5**	**≤ 1**	**≥ 2**	**≥ 5**	**≤ 1**	**≥ 2**	**≥ 5**
1	0	6	6	0	6	6	0	6	6
2	**1**	6	6	0	6	6	0	6	6
3	0	6	6	0	6	6	0	6	6
4	0	**4 / 3**	6	0	**4 / 3**	6	0	**4**	6
5	0	6	6	0	6	6	0	6	6
6	0	6	6/**5**	0	6	6	0	6	6
7	0	**5**	6	0	**5**	6	0	**5**	6
8	0	6	6	0	6	6	0	6	6
9	0	6	6	0	6	6	0	6	6
10	0	6	6	0	6	6	0	6	6
11	0	6	6	0	6	6/**5**	0	6	6
12	0	6	6	0	6	6	0	6	6
13	0	6	6	0	6	6	0	6	6
14	0	**5**	6	0	**5**	6	0	**5**	6
15	0	6/**4**	6/**5**	0	6/**2**	6/**4**	0	6	6
16	0	6	6	0	6/**3**	6/**2**	0	6	6
17	0	6	6	0	6	6	0	6	6
18	0	6	6	0	6/**4**	6	-	-	-
19	0	6	6	0	6	6	0	6	6
20	0	**5**/6	**3**/6	0	**3**/6	**5**/6	0	6	6
21	0	6	6	0	6	6	0	6	6
22	0	6/**5**	6/**5**	0	6	6	0	6	6
23	0	6	6	0	6	6	0	6	6
24	0	6	6	0	6	**5**	0	6	6
25	0	6/**0**	6/**1**	0	6/**0**	6/**0**	0	6	6
26	0	6	6	0	6	6	0	6	6
27	0	6	6	0	6	6	0	6	6
28	0	6	6	0	6	6	0	6	6
29	0	6	6	0	6/**4**	6/**3**	0	6	6
30	**1**	6	6	0	6	6	0	6/**3**	6/**4**
31	0	6	6	0	6	6	0	6	6
32	0	6	6	0	6	6	0	6	6
33	0	**5**	6	0	**5**	6	0	**5 / 0**	6/**0**
34	0	6	6	0	6	6	0	6	6

* = When only one figure is given, both isolation media gave the same result.

\- = not performed

bold numbers = deviating results

grey cells = results are below the criteria of good performance

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