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

EU Interlaboratory comparison study animal feed II (2012)

Detection of Salmonella in chicken feed

RIVM report 330604029/2013

A.F.A. Kuijpers | J. van de Kassteele |
K.A. Mooijman

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Colophon

Kuijpers A.F.A.
Kassteele van de J.
Mooijman K.A.

Contact:
Angelina Kuijpers
Centre for Zoonoses & Environmental Microbiology (cZ&O)
Angelina.Kuijpers@rivm.nl

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Rapport in het kort

EU Ringonderzoek dierenvoeder studie II (2012)
Detectie van *Salmonella* in kippenvoer

In 2012 waren 30 van de 34 Nationale Referentie Laboratoria (NRL's) in de Europese Unie in staat om hoge en lage concentraties van de *Salmonella*-bacterie in kippenvoer aan te tonen. Van de vier die daar niet in slaagden heeft één NRL de toegestuurde monsters niet ingezet vanwege organisatorische problemen. Drie labs detecteerden onterecht dat er *Salmonella* in een blanco monster zat (vals positief). Een van deze drie behaalde een matig resultaat als gevolg van een foutieve verwerking van ruwe data. De twee overige laboratoria scoorden ook tijdens de herkansing een vals positief resultaat, mogelijk veroorzaakt door een kruisbesmetting tijdens het onderzoek. Vanwege herhaaldelijk slechte prestaties is een van deze NRL bezocht door het overkoepelend orgaan EURL-*Salmonella* en zijn enkele verbeterpunten aangereikt. In totaal hebben de laboratoria, afhankelijk van de gebruikte methoden, tussen de 94 en 97 procent van de besmette monsters *Salmonella* aangetoond.

Ringonderzoek verplicht voor Europese lidstaten
Dit blijkt uit het tweede dierenvoederringonderzoek dat het Referentie-Laboratorium van de Europese Unie (EURL) voor *Salmonella* heeft georganiseerd. Het onderzoek is in september 2012 gehouden, de herkansing was in januari 2013. Deelname aan het onderzoek is verplicht voor alle NRL's van de Europese lidstaten die ervoor verantwoordelijk zijn *Salmonella* op te sporen in diervoeders. Het EURL-*Salmonella* is gevestigd bij het Nederlandse Rijksinstituut voor Volksgezondheid en Milieu (RIVM).

De laboratoria tonen de *Salmonella*bacterie aan met behulp van drie internationaal erkende analysemethodes (RVS, MKTTn en MSRV). Vervolgens moeten zij de studie volgens voorschrift uitvoeren. Elk laboratorium krijgt daarvoor een pakket toegestuurd met kippenvoer (vrij van *Salmonella*) en referentiematerialen, die geen of verschillende besmettingsniveaus van *Salmonella* Enteritidis bevatten. Het kippenvoer en het referentiemateriaal worden vervolgens samengevoegd en onderzocht. Zogeheten *Lenticule discs* zijn als referentiemateriaal gebruikt en gaven in voedsel en veterinaire studies goede resultaten.

MKTTn significant betere analysemethode
De resultaten van dit ringonderzoek onderschrijven het nut om met meerdere analysemethoden te werken. De MKTTn bleek namelijk significant betere resultaten te tonen ten opzichte van RVS en MSRV om *Salmonella* aan te tonen in het kippenvoer. Dit in tegenstelling tot eerdere ringonderzoeken waarbij andere 'producten' werden onderzocht, zoals gehakt of een andere soort kippenvoer.

Trefwoorden: *Salmonella*; EURL; NRL; ringonderzoek; kippenvoer; MKTTn

Abstract

EURL Interlaboratory comparison study on animal feed II (2012)
Detection of *Salmonella* in chicken feed

In 2012, it was shown that 30 of the 34 National Reference Laboratories (NRLs) for *Salmonella* in the European Union were able to detect the presence of *Salmonella* in chicken feed, at both low and high levels. Organizational problems meant that one of the remaining four laboratories was unable to process the samples that it had been sent. The other three laboratories reported false positive results (they detected *Salmonella* in blank samples). One of these three laboratories made an initial transcription error when processing the raw data, which led to it being rated as 'moderate'. The remaining two laboratories in this group also reported false positive results during the follow-up study. This was probably caused by cross-contamination during the proficiency test. Due to its consistently poor performance, one NRL was visited by EURL-*Salmonella* (a central coordinating body), which was able to identify various points for improvement. Depending on the method used, the laboratories detected *Salmonella* in 94 to 97 percent of the contaminated samples tested.

Interlaboratory comparison study obligatory for European Member States
These were the results obtained in the second interlaboratory comparison study on animal feed, which was organized by the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*). The study was conducted in September 2012, with a follow-up study in January 2013. Within the European Member States, all NRLs responsible for the detection of *Salmonella* in animal feed samples were required to participate in this study. EURL-*Salmonella* is part of the Dutch National Institute for Public Health and the Environment (RIVM).

The laboratories used three internationally accepted analytical methods (RVS, MKTTn and MSRV) to test for the presence of *Salmonella* in samples. They were required to proceed in accordance with the study protocol. To this end, each laboratory received a package containing chicken feed (free from *Salmonella*) and a range of reference materials which were either *Salmonella*-free or which contained different levels of this bacterium. The laboratories were instructed to spike the chicken feed with the reference materials before taking samples for testing. The use of *Lenticule discs* as reference material gave good results in both the food and veterinary studies.

MKTTn analytical method significantly superior
This study's results underscore the benefits of using more than just one analytical method, as MKTTn delivered significantly better results than RVS and MSRV in terms of detecting *Salmonella* in chicken feed. This is in contrast to earlier studies in which other "products" were analysed, such as minced meat or another type of chicken feed.

Keywords: *Salmonella*; EURL; NRL; proficiency test; chicken feed; MKTTn

Contents

Summary

In September 2012, the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*) organized the second interlaboratory comparison study on detection of *Salmonella* in an animal feed matrix: poultry feed, mixed meal for laying hens. Participants were 34 National Reference Laboratories for *Salmonella* (NRLs-Salmonella): 29 NRLs from 27 EU Member States (MS), 2 candidate EU MSs and 2 NRLs from member countries of the European Free Trade Association (EFTA) and 1 NRL from a third country (non-European).

The most important objective of the study was to test the performance of the participating laboratories for the detection of *Salmonella* at different contamination levels in an animal feed matrix. To do so, chicken feed samples of 25 grams each were analysed in the presence of reference materials (being lenticule discs) containing *Salmonella* at various contamination levels. The performance of the laboratories was compared to criteria of good performance. In addition, a comparison was made between the prescribed methods (ISO 6579: Anonymous, 2002) and the requested method (Annex D of ISO 6579: Anonymous, 2007). For the prescribed method, the selective enrichment media were Rappaport Vassiliadis Soya broth (RVS) and Mueller Kauffmann Tetrathionate novobiocin broth (MKTTn). For the requested method, the selective enrichment was Modified Semi-solid Rappaport Vassiliadis (MSRV) agar. Optionally, a laboratory could also use a PCR as an additional (own) method for the detection of *Salmonella*.

In comparison with former EURL-Salmonella interlaboratory comparison studies, a lower number of samples were tested containing only one *Salmonella* serovar. For the number of samples and their contamination levels, CEN ISO /TS 22117 (Anonymous, 2010) was followed.

Twenty-three individually numbered lenticule discs had to be tested by the participants for the presence or absence of *Salmonella*. Eighteen lenticule discs had to be examined in combination with each 25 grams of *Salmonella* negative chicken feed: six lenticule discs contained approximately eight colony-forming units (CFU) of *Salmonella* Enteritidis (SE8), six lenticule discs contained approximately 50 CFU of *S*. Enteritidis (SE50) and six lenticule discs contained no *Salmonella* at all (blank lenticule discs). The other five lenticule discs, to which no chicken feed had to be added, were control samples, comprising two lenticule discs SE8, one lenticule disc SE50 and one blank lenticule disc.

The laboratories found *Salmonella* in 94-97% of the (contaminated) samples, depending on the used selective enrichment medium. The accuracy rates for the prescribed selective enrichment media for food, MKTTn and RVS, were respectively 98% and 96%. For the requested method (MSRV), the accuracy rate was 97%. A comparison between the different media did show a significant higher score for the low-level SE contaminated chicken feed samples when analysed with selective enrichment medium MKTTn.
Longer incubation (additional 24 hours) of MSRV resulted in more positive results, which was most clear for the low-level SE contaminated chicken feed samples (8% more positive results).

PCR was used as an own method by five participants. The laboratories scored all tested samples correctly with the PCR method used. One NRL found better results with the PCR than with the bacteriological culture methods.

Thirty out of 34 laboratories achieved the level of good performance at once. One NRL (EU-MS) did not perform the study due to organizational problems and this was considered as an incident. One NRL reported a positive result for a blank sample, which was indicated as a transcription error after the reporting deadline. The performance of this NRL was indicated as moderate. Two laboratories, one EU-MS and one candidate EU-MS reported false positive, blank control samples. For these two NRLs, a follow-up study was organized in January 2013. One NRL (EU-MS) repeatedly showed deviating results in ring trials with animal feed as a matrix and the EURL-*Salmonella* visited this laboratory while they performed the follow-up study. Both poorly performing laboratories again found false positive, blank control samples and did not reach the desired performance level. The EC, DG Sanco was informed about the deviations and the underperformance of both NRLs.

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*, 2013). The objective of the current study, organized by the EURL for *Salmonella* in September 2012, was to see whether the participating laboratories could detect *Salmonella* at different contamination levels in chicken feed. This information is important in order to know whether the examination of samples in the EU Member States (MS) is carried out uniformly and whether comparable results can be obtained by NRLs-*Salmonella*. Additionally, the different methods for the detection of *Salmonella* in chicken feed were compared.

The prescribed method for detection of *Salmonella* in a feed matrix is ISO 6579 (Anonymous, 2002). However, as good experiences have been gained 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 requested to use MSRV for testing the chicken feed.

The set-up of this study was comparable to earlier interlaboratory comparison studies on the detection of *Salmonella* spp. in veterinary, food and feed samples. Animal feed was artificially contaminated with reference materials, consisting of lenticule discs containing a *Salmonella* serovar at low level, high level, or no *Salmonella* at all. Like in the earlier studies, the contamination level of the low-level lenticule discs was close to the detection limit of the method and the level of the high-level samples was approximately 5-10 times above the detection limit. The number of samples tested was lower than in earlier studies.In total, 18 contaminated chicken feed samples were tested: 6 negative samples, 6 low level samples and 6 high level samples from only one *Salmonella* serovar (*Salmonella* Enteritidis). Additional, five control samples (lenticule discs without feed) and two procedure control samples were tested. The number and level of samples tested were in accordance with CEN ISO /TS 22117 (Anonymous, 2010).

2 Participants

Country	City	Institute / NRL *Salmonella*
Austria	Linz	Austrian Agency for Health and Food Safety (AGES) Institut für Tierernährung und Futtermittel, Mikrobiology
Belgium	Brussels	Veterinary and Agrochemical Research Centre (VAR)
Bulgaria	Sophia	National Diagnostic Research Veterinary Institute NDRVMI
Croatia	Zagreb	Croatian Veterinary Institute, Laboratory Microbiology Feed
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	Esjberg	Danish Veterinary and Food Administration Region South Laboratory, Microbiology
Estonia	Tartu	Estonian Veterinary and Food Laboratory
Finland	Helsinki	Finnish Food Safety Authority Evira Research Department, Microbiology Unit
France	Ploufragan	Anses Laboratoire de Ploufragan, Laboratoire d'Etudes et de Recherches Avicoles, Porcines et Piscicoles Unite 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	University of Iceland, Keldur Institute for Experimental Pathology
Ireland	Kildare	Central Veterinary Research Laboratory CVRL/DAF Department of Agriculture
Israel	Kiryat Malachi	Southern Poultry Health Laboratory
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
Luxembourg	Ettelbruck	Laboratoires de controle et d'essais de l'ASTA Service de microbiologie et biochimie
Malta	Valletta	Public Health Laboratory (PHL) Evans Buildings
Netherlands, the	Bilthoven	National Institute for Public Health and the Environment (RIVM/Cib) Centre for Infectious Diseases Control Laboratory for Zoonoses and Environmental Microbiology-LZO
Netherlands, the	Wageningen	Nederlandse Voedsel en Waren Autoriteit (nVWA) Divisie Consument & Veiligheid, Microbiologie Primaire Producten
Norway	Oslo	National Veterinary Institute, Section of Bacteriology

Country	City	Institute
Poland	Pulawy	National Veterinary Research Institute (NVRI) Department of Hygiene of Animal Feeding Stuffs
Portugal*	Lisbon	Instituto Nacional dos Recursos Biológicos Bacteriology Laboratory of the Animal Health Unit in LNIV
Romania	Bucharest	Hygiene and Veterinary Public Health Institute (IISPV)
Serbia	Belgrade	Institute of Veterinary Medicine of Serbia Department of Food Hygiene
Slovak Republic	Bratislava	State Veterinary and Food Institute
Slovenia	Ljubljana	National Veterinary Institute, Veterinary Faculty
Spain	Madrid, Algete	Laboratorio Central de Veterinaria
Sweden	Uppsala	National Veterinary Institute (SVA), Department of Bacteriology
United Kingdom	Addlestone	Animal Health and Veterinary Laboratories Agency(AHVLA) Weybridge
United Kingdom	Belfast	Agri-Food and Bioscience Institute (AFBI) Veterinary Sciences Division Bacteriology

*Laboratory received the samples, but did not perform the study

3 Materials and methods

3.1 Reference materials

3.1.1 Batches of lenticule discs

The reference materials consisted of lenticule discs obtained from the Health Protection Agency (HPA) in Newcastle, United Kingdom. Lenticule discs are microbiological reference materials which are plano-convex discs containing micro-organisms at a defined number in a solid, water soluble matrix (HPA, 2012). They are supplied as a single unit supported on a silica gel insert in a small airtight plastic tube (EURL-*Salmonella*, 2012b). The discs are lens-shaped and coloured, and therefore easily seen on top of the filter insert. The *Salmonella* strains used for the preparation of the lenticule discs originated from the National Collection of Type Cultures (NCTC) of HPA.
Three batches of lenticule discs were prepared by HPA:

- S. Enteritidis (SE) at a level of approximately 8 CFU per lenticule disc: batch NCTC 6676 batch 414-110615B;
- S. Enteritidis (SE) at a level of approximately 50 CFU per lenticule disc: batch NCTC 6676 batch 814-110615R;
- Blank lenticule discs (BL), containing no micro-organisms: batch 000-1229952.

3.1.2 Homogeneity of the lenticule discs

The mean number of organisms of each batch was counted by HPA before the lenticule discs were sent to the EURL-*Salmonella*. For this, the HPA tested 30 lenticules per batch. The data were reported on the insert of the batch of lenticules and were subjected to a homogeneity test at the EURL *Salmonella*. It was tested whether the variation in counts between the lenticule discs was less than two times a Poisson distribution, using the following formula:
$T_2 / (I-1) \leq 2$. Where T_2 is a measure for the variation between lenticule discs of one batch and I is the number of lenticule discs (Heisterkamp, 1993).

3.1.3 Stability and robustness of lenticule discs

In the literature, information can be found on the stability of several types of lenticule discs during storage and transport (Boyd et al., 2006 and Desai et al., 2006). Additional tests of the stability of the lenticule discs at elevated temperatures, as well as tests on the 'robustness' of the lenticule discs (mix with different matrices), were performed on the *Salmonella* lenticule discs at the EURL-*Salmonella* laboratory earlier (Kuijpers and Mooijman, 2011 and 2013; Kuijpers et al., 2012a).
For the current study, the contamination level of each batch of lenticule discs was verified by the EURL by testing 3 lenticule discs per batch (SE8 and SE50) after storage at - 20°C.
For the counting of the lenticule discs, each lenticule disc was placed onto Colombia agar plates with sheep blood (OXOID PB5008A, Germany). After ten minutes of rehydration of the lenticule disc at room temperature, the resultant 'drop' was spread over the plate and incubated at 37°C for 20 to 24 hours. This method is also used by HPA to count the mean number of organisms of each batch of lenticule discs.

3.1.4 Pre-tests for the interlaboratory comparison study

Before organizing the interlaboratory comparison study, it was tested whether *Salmonella* could still be detected after mixing a *Salmonella* lenticule disc with the matrix (chicken feed). For this, lenticules with two different *Salmonella* serovars were tested, i.e. *Salmonella* Enteritidis (SE) and *Salmonella* Typhimurium (STM). The batches of the lenticules used for this pre-test were used in earlier studies and more details can be found in the relevant reports (Kuijpers et al. 2012a and Kuijpers and Mooijman 2013). For the pre-test, 6lenticule discs SE8 (batch 414-110615A) and STM6 (batch 323-101021), 2 lenticule discs SE51 (batch 814-110615), STM61 (batch 523-100927) and 2 Blanc lenticule discs were each added to 25 g chicken feed (free from *Salmonella*) in 225 ml BPW. Additionally, 2 control samples (lenticule discs without matrix) of each batch of lenticule discs were tested.
All samples were tested for the presence of *Salmonella* according the SOP of the study (EURL Salmonella, 2012b), ISO 6579 (Anonymous, 2002) and Annex D of ISO 6579 (Anonymous, 2007) with selective enrichment in RVS, MKTTn and on MSRV.

3.2 Chicken feed samples

3.2.1 General

The chicken feed (poultry feed, mixed meal for laying hens) was obtained from the retail sector and was produced by Kasper Fauna Food, Woerden, in the Netherlands. A batch of seven portions of 4 kg arrived at EURL-*Salmonella* on 10 July 2012 and was immediately checked for the absence of *Salmonella* by testing 25 g samples, randomly picked from the seven portions. For the testing for *Salmonella*, ISO 6579 (Anonymous, 2002) and Annex D of ISO 6579 (Anonymous, 2007) were followed. For this purpose, each sample of 25 g was added to 225 ml of Buffered Peptone Water (BPW). After pre-enrichment at 37 (± 1)°C for 16- 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). Next, the suspect plates were plated-out on Xylose Lysine Deoxycholate agar (XLD) and Brilliance *Salmonella* agar (BSA) and confirmed biochemically.
After checking the absence of *Salmonella*, the animal feed was repacked in portions of approximately 550 grams and stored at room temperature.

3.2.2 Total bacterial count in chicken feed

The total number of aerobic bacteria in the chicken feed was investigated. The procedure of ISO 4833 (Anonymous, 2003a) was followed for this purpose. Portions of 20 grams of chicken feed were homogenized into 180 ml of peptone saline solution in a plastic bag. The content was mixed by using a stomacher (for 60 seconds). Next, tenfold dilutions were prepared in a peptone saline solution. Two times 1 ml of each dilution was 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.

3.2.3 Number of Enterobacteriaceae in chicken feed

In addition to the total count of aerobic bacteria, the *Enterobacteriaceae* count was determined. The procedure of ISO 21528-2 (Anonymous, 2004) was used for this purpose. Portions of 20 grams of chicken feed were homogenized into

180 ml of peptone saline solution in a plastic bag. The content was mixed by using a stomacher (for 60 seconds). Next, tenfold dilutions were prepared in peptone saline solution. Two times 1 ml of each dilution was 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 was counted after incubation. Five typical colonies were tested for the fermentation of glucose and for a negative oxidase reaction. After this confirmation, the number of *Enterobacteriaceae* was calculated.

3.3 Design of the interlaboratory comparison study

3.3.1 Samples: lenticule discs and chicken feed

On 17 September 2012 (two weeks before the study) the reference materials (33 individually numbered lenticule discs) and 550 grams of *Salmonella* negative chicken feed were packed with cooling devices as biological substance category B (UN 3373) and sent by door-to-door courier service to each participant. After arrival at the laboratory, the lenticule discs had to be stored at -20°C and the chicken feed had to be stored at +5°C until the start of the study. Details about the mailing and handling of the samples and reporting of test results can be found in the Protocol (EURL-*Salmonella*, 2012a), Standard Operating Procedure (SOP) (EURL-*Salmonella*, 2012b) and the test report (EURL-*Salmonella*, 2012c). The protocol, SOP and the test report that was used during the study can be found on the EURL-*Salmonella* website or can be obtained through the corresponding author of this report.

Five control lenticule discs had to be tested without chicken feed (numbered C1-C5). Eighteen lenticule discs (numbered B1-B18) were each tested in combination with 25 grams of chicken feed (negative for *Salmonella*). Table 1 shows the types and the number of lenticule discs and chicken feed samples which had to be tested. The number and level of samples tested were in accordance with CEN ISO /TS 22117 (Anonymous, 2010).

Table 1 Overview of the types and the number of lenticule discs tested per laboratory in the interlaboratory comparison study

Lenticule discs	Control lenticule discs (n=5) No matrix added	Test samples (n=18) with 25 grams *Salmonella* negative chicken feed
S. Enteritidis 8 (SE8)	2	6
S. Enteritidis 50 (SE50)	1	6
Blank (BL)	2	6

3.3.2 Sample packaging and temperature recording during shipment

The lenticule discs and the chicken feed were packed in two plastic containers that were firmly sealed with screw caps (biopacks). Both biopacks were placed in one large shipping box, together with three frozen (-20°C) cooling devices. Each shipping box was sent as biological substances category B (UN3373) by door-to-door courier services. For the control of exposure to abusive temperatures during shipment and storage, so-called micro-temperature loggers were used to

record the temperature during transport. These loggers are tiny sealed units in a 16 mm diameter and 6 mm deep stainless steel case. Each shipping box contained one logger, packed in the biopack with lenticule discs. The loggers were programmed by the EURL-*Salmonella* to measure the temperature every hour. Each NRL had to return the temperature recorder, immediately after receipt of the parcel, to the EURL. At the EURL-*Salmonella*, the loggers were read by the computer and all temperatures recorded from the start of the shipment until the arrival at the National Reference Laboratories were transferred to an Excel sheet.

3.4 Methods

The NRLs could use the pre-treatment procedures of the samples as normally used in daily routine analyses (e.g. pre-warming of BPW, different ways of mixing the samples in BPW). According to ISO 6887-4 (Anonymous, 2003c), the chicken feed diluted in BPW needs to stand for 20-30 minutes at 18°C to 27°C before mixing. This was described in greater detail in the SOP of this study (EURL-*Salmonella*, 2012b).
The prescribed method of this interlaboratory comparison study was ISO 6579 (Anonymous, 2002) and the requested (additional) method was Annex D of ISO 6579 (Anonymous, 2007). In addition to the prescribed methods, the NRLs were also free to use their own methods. This could be different medium combinations and/or investigation of the samples with alternative methods, such as Polymerase Chain Reaction (PCR)-based methods.

In summary:

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

Selective enrichment in/on:
- Rappaport Vassiliadis Soya broth (RVS) (prescribed);
- Mueller Kaufmann Tetrathionate novobiocin broth (MKTTn) (prescribed);
- Modified semi-solid Rappaport Vassiliadis agar (MSRV) (requested);
- own selective enrichment medium (optional).

Plating-out on:
- Xylose lysine desoxycholate agar (XLD) (prescribed);
- second plating-out medium for choice (obligatory);
- own plating-out medium (optional).

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

3.5 Statistical analysis of the data

The specificity, sensitivity and accuracy rates were calculated for the control samples and the artificially contaminated samples with chicken feed (negative for *Salmonella* spp.). The specificity, sensitivity and accuracy 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 lenticule discs, enrichment media and isolation media, and a random effect part, consisting of the different laboratories. Mutual differences between media and lenticule discs are shown as odds ratios (OR) stratified by medium. The odds of detecting *Salmonella* is the probability of detecting *Salmonella* divided 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 (Gelman and Hill, 2007). This was done by putting vague prior information on the odds of detecting *Salmonella*. A priori, the odds were set to 1 with a 95% confidence interval of 0.025 - 40. 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, 2013).

3.6 Good performance

The criteria used for testing good performance in this study are given in Table 2. For determining good performance per laboratory, all combinations of selective enrichment media and isolation media used by the laboratory were taken into account. For example, if a laboratory found for the SE8 lenticule discs with matrix 4/6 a positive with RVS/XLD but no positives with MKTTn or any other selective enrichment or isolation medium, this was still considered a good result. For the blank lenticule discs, all combinations of media used per laboratory were also taken into account. If, for example, a laboratory found 2/6 blank lenticule discs positive with MKTTn/BGA but no positives with the other media, this was still considered a 'not good' result.

Table 2 Criteria for testing good performance in the Animal Feed II study (2012)

Control samples (lenticules, no matrix)	Minimum result	
	Percentage positive	No. of positive samples/ total No. of samples
SE50	100%	1/1
SE8	50%	1/2
Blank control lenticules	0%	0/2

Samples: artificially contaminated chicken feed (lenticules with matrix)	Minimum result	
	Percentage positive	No. of positive samples/ total No. of samples
Blank[1]	20% at max[1]	1/6 at max[1]
SE50	80%	5/6
SE8	50%	3/6

1: All should be negative. However, as no 100% guarantees about the *Salmonella* negativity of the matrix can be given, one positive out of six blank samples (20% pos.) will still be considered as acceptable.

4 Results

4.1 Reference materials

4.1.1 Contamination level, homogeneity and stability of the lenticule discs

Table 3 summarises the information on the contamination level of each batch of lenticule discs as tested by HPA and by the EURL-*Salmonella*. The mean levels, as well as the lowest and highest counts (in CFU) found per batch, are indicated. Additionally, the results of the homogeneity test of each batch as performed by the EURL *Salmonella* are indicated. The results of the homogeneity test show that each batch fulfilled the pre-set criteria (variation less than two times Poisson distribution).
The verification of the contamination levels of both batches of SE lenticule discs after arrival at the EURL-*Salmonella* showed values between the minimum and maximum CFU counted by HPA after preparation.

Table 3 Level of contamination and homogeneity of SE lenticule discs

	SE8	SE50
Batch number	414-100515B	814-110615R
Date testing lenticules*	01.07.2011	29.06.2012
Number of lenticules tested	30	30
Mean CFU per lenticule	8	50
Min-max CFU per lenticule	3-13	34-65
T_2 / (I-1)**	0.73	1.33
Date testing lenticules**	04.09.12	04.09.12
Number of lenticules tested	3	3
Mean CFU per lenticule disc	7	48
Min-max CFU per lenticule	4-10	44-50

CFU = colony forming units

min-max = enumerated minimum and maximum CFU

* Tested by HPA

** Tested or Calculated by EURL-*Salmonella*

formula T_2: I is the number of lenticule discs; Demand for homogeneity $T_2/(I-1) \leq 2$ (Heisterkamp, 1993)

4.1.2 Pre-test for the interlaboratory comparison study

The pre-test of the full procedure of the interlaboratory comparison study performed at the EURL-*Salmonella* showed good results. All samples of artificially contaminated chicken feed (with lenticules of SE8, STM6, SE51, STM61 and BL) and control samples with lenticule discs (without matrix) were scored correctly with MKTTn, RVS and MSRV. Eventually, a decision was taken to organize the study with the SE lenticules.

4.2 Chicken feed samples

The batch of chicken feed tested negative for *Salmonella* and was stored at room temperature on 10 July 2012. On Monday 17 September 2012, the chicken feed was posted to the NRLs. After receipt, the NRLs had to store the chicken feed at 5°C. The number of aerobic bacteria and the number of *Enterobacteriaceae* were tested twice; firstly, on the day the chicken feed arrived at the EURL (10/07/2012) and, secondly, after storage at room temperature since 10 July 2012 and at 5°C for two weeks, close to the planned

date of the interlaboratory comparison study (28 September 2012). Table 4 shows the results.

Table 4 Number of aerobic bacteria and the number of Enterobacteriaceae per gram of chicken feed

Date	*Enterobacteriaceae* CFU/g	Aerobic bacteria CFU/g
10 July 2012	$1.4*10^4$	$4.7*10^4$
28 September 2012 (stored at room temperature until 17 September and placed at 5°C until 28 September)	$9.1*10^2$	$5.3*10^4$

4.3 Technical data from interlaboratory comparison study

4.3.1 General

In this study, 34 NRLs participated: 29 NRLs from 27 EU-MS, two NRLs from members of the EFTA countries, two NRLs of EU candidate MSs and one NRL from a third country. Twenty-nine laboratories performed the study on the planned date (week 40 starting on 1 October 2012). Three laboratories performed the study one week earlier. Laboratory 31 made a mistake with the treatment of four tubes containing reference materials. A second parcel with the relevant samples was sent to the laboratory on 1 October 2012. At last, the laboratory performed the study in three different weeks. One laboratory, of an EU-MS (lab code 28), did not perform the study.

4.3.2 Accreditation/certification

All laboratories, with the exception of laboratory 31 (EU-MS), indicated that they were accredited according ISO/IEC 17025 (Anonymous, 2005). Twenty-four laboratories are accredited for ISO 6579 for the detection of *Salmonella* in food and animal feedstuffs, 20 of them are also accredited for Annex D of ISO 6579 for different matrices and four of them also mentioned that they were accredited for other methods (e.g. VIDAS). Six laboratories (lab codes 2, 10, 12, 24, 27 and 32) are accredited only for the detection of *Salmonella* in animal faeces and veterinary samples by using MSRV (Annex D of ISO 6579). One laboratory (lab code 29, non EU-MS) is accredited for the detection of *Salmonella* in food and faeces by using RVS and MSRV. One laboratory (lab code 6) did not mention the method for which they are accredited. Laboratory 31 is planning to become accredited in 2013.

4.3.3 Transport of samples

Twenty-three participants received the materials within one day of transport. For seven participants (lab codes 1, 3, 11, 13, 14, 19 and 33) the parcel arrived within two days of transport. The transport to both laboratories 7 and 29 took three days and four days to laboratory 10, which is a non EU-MS. The parcel of laboratory 21 (non-EU-MS) was delivered at the airport within one day and placed at -20°C until it arrived at the institute, after eight days of transport, on 25 September 2012.

For the majority of the parcels, the transport temperature did not exceed 5°C. The parcel of laboratory 3 had a transport time of two days, for which the temperature was 22°C for 20 hours. For two laboratories (lab codes 7 and 11) the parcels were exposed to a temperature of 9-10°C for 16-20 hours. For two NRLs (lab codes 5 and 26) the date of arrival of the parcel recorded on the test report did not correspond with the date reported by the courier (difference of one day).

Table 5 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	19 :10	7.1	5.2	8.1	40	5.2	**50**
2	17 :00	6.9	5.2	8.1	40	**5.5**	10
4	**20:40**	7.2	5.2	8.0	**10**	5.2	10
5	18:40	7.0	5.2	8.0	40	5.1	**20**
6	19:50	7.0	5.2	8.2	40	5.2	**20**
7	18:30	7.0	5.2	**7.2**	39	ND	ND
10	19:50	7.1	5.3	7.8	40	5.1	10
11	**22:00**	7.0	5.2	8.0	40	5.2	10
12	19:20	7.1	-	-	39	5.2	10
13	19:00	7.1	5.2	8.0	40	**5.6**	**0**
15	20:00	7.1	5.2	8.2	40	5.2	**50**
16	18:00	**5.2**	5.2	8.0	40	5.2	10
17	19:45	7.0	5.1	7.8	40	**-**	10
18	17:30	7.2	**5.5**	**-**	40	**5.5**	10
21	19:25	7.0	5.4	8.0	40	5.3	**20**
25	18:00	7.2	5.2	8	40	5.2	**20**
26	**23:45**	**7±0.2**	**5±0.2**	**8±0.2**	40	**5.4±0.2**	10
27	**20:20**	**7.3**	5.4	8.2	40	5.1	10
29	**21:15**	7.2	**5.9**	ND	ND	5.3	10
32	17:45	**-**	**-**	**-**	**50**	**-**	10
33	19:00	7.2	5.2	8.2	**20**	5.2	10
34	18:30	**7.3**	5.45	8.0	40	5.4	10

Bold numbers/ grey cells: Deviating from ISO 6579 and/or ISO 6579 Annex D
ND (Not Done): Laboratory 7 did not perform MSRV and laboratory 29 did not perform MKTTn
-= No information

4.3.4 Media

Each laboratory was asked to test the samples using the prescribed method (ISO 6579) and the requested (Annex D of ISO 6579) method. Thirty-one participants used the selective enrichment media RVS, MKTTn and MSRV in combination with XLD and a second plating-out medium of their own choice.

Laboratory 29 (non-EU) did not use the prescribed medium MKTTn and laboratory 7 did not use the requested medium MSRV. Six laboratories (lab codes 1, 4, 19, 20, 22, 23 and 33) used more than two isolation media.

Table 5 gives information on the pH and the concentration of Novobiocin of the media that were prescribed and requested and on the incubation times. The table only indicates the laboratories who reported deviations.
Five laboratories (lab codes 4, 11, 26, 27and 29) reported a longer incubation time of the pre-enrichment in BPW. Three laboratories (lab codes 16, 27 and 34) reported a deviation of the pH value of BPW.
Two laboratories (lab codes 18 and 29) used a higher pH for the RVS than the prescribed maximum pH of 5.4
Three laboratories (lab codes 7, 10 and 17) used a low pH of 7.2-7.8, the prescribed pH for MKTTn is 7.8-8.2. Two laboratories (lab codes 4 and 33) used MKTTn with a lower concentration of novobiocin and laboratory 32 reported a higher concentration than the prescribed 0.04 g/L of novobiocin.
Six laboratories (lab codes 1, 5, 6, 15, 21 and 25) used MSRV with a higher concentration of novobiocin than the prescribed 0.01 g/L. Laboratory 13 used MSRV without novobiocin. Three laboratories (lab codes 2, 13 and 18) reported a higher pH for the MSRV than the prescribed maximum pH of 5.4.
The laboratories 12, 17, 18 and 32 did not always mention the pH of the media.

A second plating-out medium of choice was obligatory. Table 6 shows the second plating media used by the participants. Most laboratories used BGA modified (Anonymous, 1993) as a second plating-out medium followed by Rambach agar.

Table 6 Media used as second plating-out medium.

Media	Number of users	Lab code
BGA^{mod} (ISO 6579, 1993) (=BPLS= BGPA)	15	1, 5, 9, 13, 14, 15, 17, 18, 19, 22, 23, 26, 27, 29, 33
Rambach	7	2, 4, 6, 7, 8, 19, 21
BGA	4	10, 11, 20, 24
BSA (=OSCM)	4	16, 20, 28, 30
RS	4	1, 3, 32, 34
SM(ID)2	3	22, 23, 31
ASAP	1	12
BPLSA (Kristensen-Kauffmann)	1	25
HE	1	4
MAC	1	33
MLCB	1	22

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

The use of an extra plating agar between the 'isolation' and the 'confirmation' steps was optional. A total of 21 laboratories performed this extra culture step on many different media (e.g. Nutrient agar: ISO 6579, 2002).

All participating laboratories performed confirmation tests for *Salmonella*: biochemically, serologically or both. Tables 7 and 8 summarize the confirmation

media and tests. Two laboratories (lab codes 2 and 14) performed serological tests only and four laboratories (lab codes16, 23, 26 and 31) performed only a biochemical test.

Table 7 Biochemical and other confirmation tests of Salmonella.

Lab code	TSI	UA	LDC	Gal	VP	Indole	Kit	Other
1	+	+	+	+	+	+	Rapid ID 32E	-
2	-	-	-	-	-	-	-	-
3, 19, 24	+	+	+	+	+	+	-	-
4	+	+	+	+	+	+	-	Real time PCR
5	+	+	+	-	-	+	-	Glucose
6, 8, 9	+	+	+	+	-	+	-	-
7	+	+	-	-	-	-	API20E	-
10	-	-	-	-	-	+	Enterotest	Real time PCR
11	+	+	+	+	+	+	Microgen GN-10-A	-
12	-	-	-	-	-	-	API20E	Real time PCR
13	+	+	+	-	-	+	-	Mac Conkey
14	-	-	-	-	-	-	-	Chromogenic agar
15	+	+	+	-	-	+	-	-
16, 18, 27	+	+	+	-	-	-	-	-
17	-	-	-	-	-	-	API20E	Kohns N0 1 medium
20	+	+	+	+	+	+	-	PCR
21	+	-	+	-	-	+	BBL	-
22	-	-	-	-	-	+	BBL	Oxidase
23	-	-	+	-	-	-	API 10S	
25	-	-	-	-	-	-	-	ONPG, Dulcitol, Malonate, Salicin
26	+	+	+	-	+	+	-	-
29	+	+	-	-	-	-	-	Lysine Iron Agar
30	+	-	-	-	-	-	Enterotest	MALDI-TOF
31	-	-	-	-	-	-	API 32E	-
32	+	-	+	-	-	-	-	Sorbitol mobility
33	+	+	+	+	+	+	API32E	PCR
34	-	-	-	-	-	-	Enterotube II	-

- = Not done / not mentioned.

Table 8 Serological confirmation of Salmonella.

Lab code	Serological		
	O antigens	**H antigens**	**Vi antigens**
1, 2, 3, 4, 5, 6, 7, 12, 13, 15, 17, 18, 22, 24, 25, 30	+	+	-
8, 9, 10, 14, 21, 27, 32, 33	+	-	-
11	+	+	+
16, 20, 23, 26, 31	-	-	-
19	-	+	-
23	Polyvalent *Salmonella* Serum		
29	Latex Agglutination Test		
34	Poly A-S + Vi		

- = Not done / not mentioned.

4.4 Control samples

4.4.1 General

Twenty-nine laboratories scored correct results for all the control lenticule discs. Table 9 summarizes the highest number of positive isolations found with all combinations of selective enrichment media and isolation media per laboratory (lenticule discs without chicken feed). In Annex 1 more details per laboratory are given on the results found with the selective enrichment media RVS, MKTTn and MSRV in combination with the used isolation media per laboratory.

Table 9 Total number of positive results from the control samples (lenticule disc without animal feed) per laboratory

Lab code	The highest number of positive isolations found with all combinations of selective enrichment media and isolation media		
	SE8 n=2	**SE50 n=1**	**Blank n=2**
Good Performance	**≥ 1**	**1**	**0**
15, 21 and 31	2	1	**1**
Other laboratories 1 – 14; 16 - 20; 12 – 30; 32 – 34	2	1	0

Bold numbers: deviating results

Grey cell: results are below good performance.

Procedure control without lenticule disc (n=2)
All laboratories correctly analysed the procedure controls as negative: one chicken feed control sample (25 g of chicken feed/no lenticule disc) and one control of BPW only (no animal feed/no lenticule disc).

Blank lenticule discs without addition of chicken feed (n=2)
Thirty laboratories correctly analysed the two blank lenticule discs as negative for *Salmonella* with all used media. Two laboratories (lab codes 15 and 21) found one blank control lenticule disc to be positive on all used media (MKTTn, RVS and MSRV). One laboratory (lab code 31) found one blank control lenticule positive with selective enrichment in RVS on all used isolation media. With the

other selective enrichment media (MKTTn and MSRV) used this laboratory scored this lenticule disc, inoculated from the same BPW, correctly as negative.

SE8 lenticule discs without addition of chicken feed (n=2)
All participating laboratories except one tested both control lenticule discs containing SE8 and found them to be positive. Laboratory 31 could not detect *Salmonella* in one out of two SE8 lenticule discs after selective enrichment on MSRV, but this laboratory scored all samples correctly with the prescribed selective enrichment media RVS and MKTTn.

SE50 lenticule discs without addition of chicken feed (n=1)
All participating laboratories tested the one control lenticule disc containing SE50 and found it to be positive.

The results of all control samples were compared with the definition of 'good performance' (clause 3.6). Three laboratories (lab code 15, 21 and 31) scored below these criteria.

Table 10 Specificity, sensitivity and accuracy rates found with the control samples (lenticule discs without the addition of chicken feed)

Control lenticule disc		RVS/X		MKTTn/X*		MSRV/X**	
	Laboratories	All n=33	EU n=28	All n=32	EU n=28	All n=32	EU n=27
Blank (n=2)	No. of samples	66	56	64	56	64	54
	No. of negative samples	63	54	62	55	62	53
	Specificity in%	**95**	**96**	**97**	**98**	**97**	**98**
SE8 (n=2)	No. of samples	66	56	64	56	64	54
	No. of positive samples	66	56	64	56	63	53
	Sensitivity in%	**100**	**100**	**100**	**100**	**98**	**98**
SE50 (n=1)	No. of samples	33	28	32	28	32	27
	No. of positive samples	33	28	32	28	32	27
	Sensitivity in%	**100**	**100**	**100**	**100**	**100**	**100**
All lenticule discs with *Salmonella*	No. of samples	99	84	96	84	96	81
	No. of positive samples	99	84	96	84	95	80
	Sensitivity in%	**100**	**100**	**100**	**100**	**99**	**99**
All lenticule discs	No. of samples	165	140	160	140	160	135
	No. of correct samples	162	138	158	139	157	133
	Accuracy in%	**98**	**99**	**99**	**99**	**98**	**99**

X = isolation medium with the highest number of positives of all used isolation media.

*Results without Laboratory 29 (non-EU-MS): they did not use MKTTn

**Results without Laboratory 7 (EU-MS): they did not use MSRV

4.4.2 Specificity, sensitivity and accuracy rates of the control samples

Table 10 shows the specificity, sensitivity and accuracy rates for the control lenticule discs without the addition of chicken feed. The rates are calculated 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 (without the results of the EFTA countries, candidate EU-MSs and third countries). No differences were found between these groups.
The maximum possible rates (100%) were found for the SE50 control samples. The sensitivity rate of SE8 was 100% for the prescribed selective enrichment media (MKTTn and RVS). The specificity rate of the blank lenticule discs varied between 95 and 98 per cent.

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

4.5.1 Results per type of lenticule disc and per laboratory

General
Table 11 gives the results of the *Salmonella* negative chicken feed samples artificially contaminated with lenticule discs. This table gives the highest number of positive isolations found with the different selective enrichment media (RVS, MKTTn and MSRV) in combination with any isolation medium per laboratory. In Annex 2, more details per laboratory are given on the results found with the selective enrichment media RVS, MKTTn and MSRV in combination with the isolation media used per laboratory.

Table 11 Number of positive results found with the artificially contaminated chicken feed samples per laboratory

Lab code	The highest number of positive isolations found with all combinations of selective enrichment media and isolation media		
	SE8 n=6	SE50 n=6	Blank n=6
Good Performance	**≥ 3**	**≥ 5**	**≤ 1**
5	6	6	**1**
12	**5**	6	0
Other laboratories 1-4; 6-11; 13 - 34;	6	6	0

Bold numbers: deviating results.

Blank lenticule discs with negative chicken feed (n=6)
Thirty-two laboratories correctly did not isolate *Salmonella* from the blank lenticule discs with the addition of negative chicken feed. One laboratory (lab code 5) found one blank sample added to negative chicken feed positive for *Salmonella* with RVS in combination with both isolation media used. With the other selective enrichment media used (MKTTn and MSRV), this laboratory scored this sample, inoculated from the same BPW, correctly as negative.
In theory, all blanks should test negative**.** However, as no 100% guarantee about the *Salmonella* negativity of chicken feed can be given, one positive out of six blank samples (80% neg.) will still be considered as acceptable.

SE8 lenticule discs with negative chicken feed (n=6)
Thirty-two laboratories were able to isolate *Salmonella* from all the six lenticule discs containing *Salmonella* Enteritidis at a level of approximately 8 CFU/lenticule disc in combination with chicken feed with at least one of the media used. Laboratory 12 could not detect *Salmonella* Enteritidis in three out of six SE8 samples with the prescribed method (RVS and MKTTn). This laboratory scored better results with the requested selective enrichment medium MSRV. Laboratories 17 and 32 could not detect *Salmonella* in four out of six SE8 samples with the requested method MSRV. However, with the prescribed method (RVS and MKTTn) they found all SE8 samples to be positive.

SE50 lenticule discs with negative chicken feed (n=6)
All laboratories isolated *Salmonella* from all the six lenticule discs containing *Salmonella* Enteritidis at a level of approximately 50 CFU/ lenticule disc in combination with chicken feed with at least one of the media used.
Three laboratories (lab codes 3, 10 and 17) could not detect *Salmonella* Enteritidis in two or more out of six SE50 samples with one of the prescribed or requested methods, but they scored all SE50 samples as positive with at least one of the prescribed methods (RVS and/or MKTTn).

The results of all artificially contaminated chicken feed samples were compared with the definition of 'good performance' (clause 3.6). All laboratories fulfilled these criteria for the prescribed media RVS and/or MKTTn.

4.5.2 Results per selective enrichment medium, lenticule disc and per laboratory

Figures 1 and 2 show the number of positive isolations per artificially contaminated chicken feed sample and per laboratory after pre-enrichment in BPW, selective enrichment in RVS, MKTTn and on MSRV, followed by isolation on a selective plating agar. To determine good performance per laboratory, all combinations of selective enrichment media and isolation media used by the laboratory were taken into account. The results of all artificially contaminated chicken feed samples were compared with the definition of 'good performance' (clause 3.6). The black horizontal line in Figures 1 and 2 indicates the border of good performance.

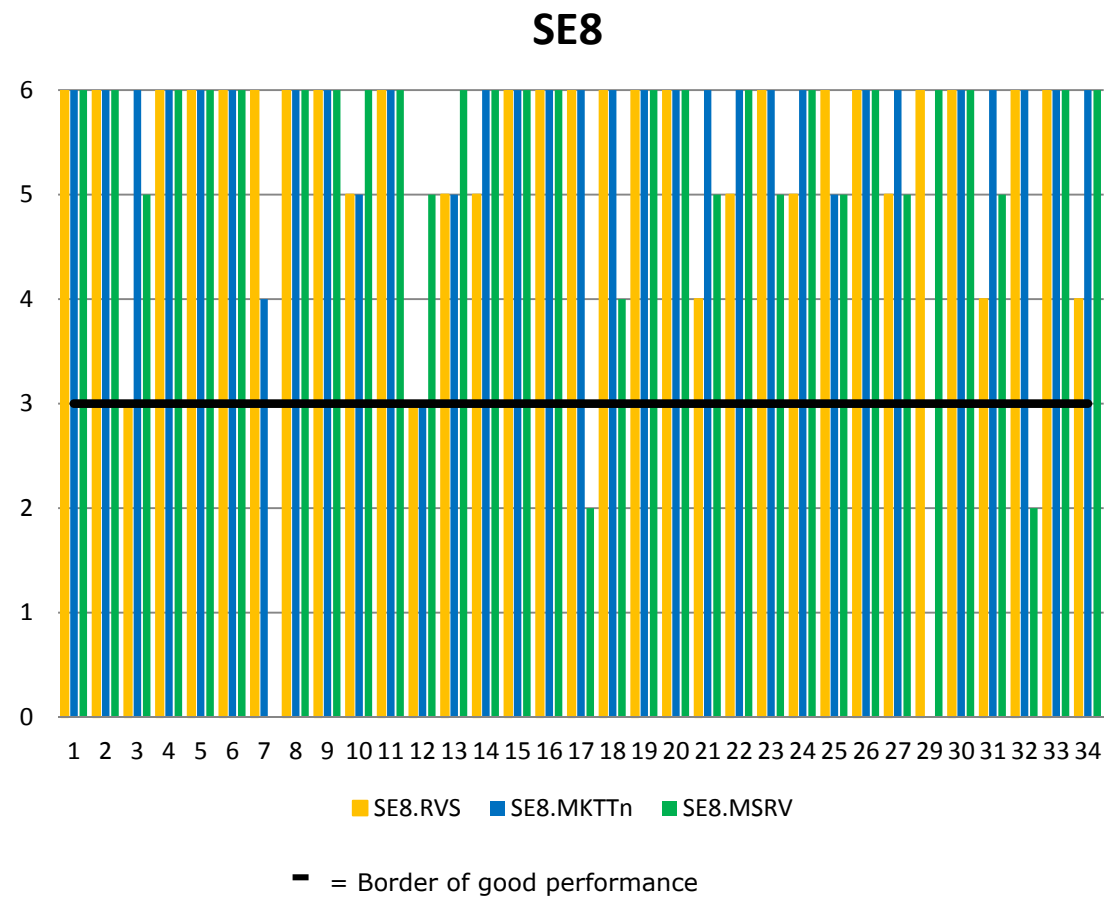

Figure 1 Results per laboratory for the detection of Salmonella in chicken feed samples artificially contaminated with SE8 lenticule discs (n=6) after selective enrichment in RVS, MKTTn and on MSRV followed by isolation on the 'best' selective plating agar.

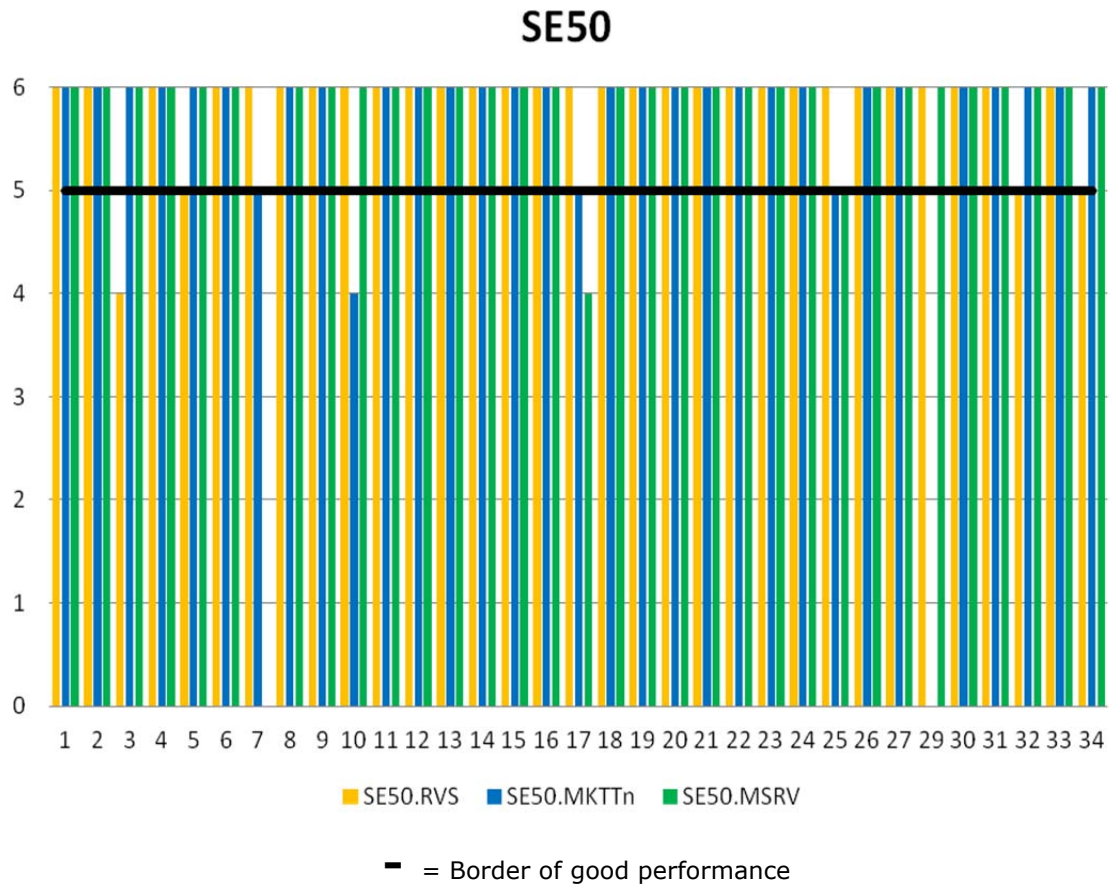

Figure 2 Results per laboratory for the detection of Salmonella in chicken feed samples artificially contaminated with SE50 lenticule discs (n=6) after selective enrichment in RVS, MKTTn and on MSRV followed by isolation on the 'best' selective plating agar.

Table 12 presents the percentages of positive isolations after 24 hours of incubation for RVS, MKTTn and MSRV and after an additional 48 hours of incubation for MSRV. When MKTTn or RVS was used for selective enrichment, XLD gave 3-5% more positive results than other plating-out media. The majority of the laboratories used BGA(modified) as the second plating-out medium (see Table 6). An extra incubation time of 24 h for MSRV gave on average 6% more positive results and no differences were seen between different plating-out media. For SE8 samples, the percentages of positive results were 83% after 24 h and 91% after 48 h of incubation on MSRV. For the SE50 samples, the percentage was 93% and 98% respectively. The majority of positive results after an additional incubation of 48 h came from laboratories 12 and 32, with respectively eight and six more positive results out of the 12 artificially contaminated samples with *Salmonella*.
Laboratory 22 did not mention the results after 24 hours of incubation on MSRV.

Table 12 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 additionally for 48 hours on MSRV, followed by isolation on different plating-out media.

Plating-out medium	Selective enrichment medium		
	RVS	MKTTn	MSRV
	24 h	24 h	24 / 48 h
XLD	91%	96%	88 / 94%
Other (most often BGA)	88%	91%	88 / 94%
Difference XLD/other	3%	5%	0%

Tables 13 and 14 show the differences between selective enrichment media and isolation media per lenticule as odds ratios (OR). In addition, the 95% confidence intervals and p-values are given.

In Table 13, the odds of finding a positive isolation with the different plating-out media are compared, given a selective enrichment medium. For instance, the odds of finding *Salmonella* from the SE8 samples after selective enrichment in MKTTn is a factor of 2.28 higher when XLD is used as an isolation medium compared to an isolation medium other than XLD. In general, if MKTTn is used as a selective enrichment medium, the ORs are greater than the ORs of RVS and MSRV. In other words, when MKTTn is used for selective enrichment it is easier to detect *Salmonella* if XLD is used compared to other isolation media. This is significant for all lenticules. For the other selective enrichment media, RVS and MSRV, there is no significant difference for the detection of *Salmonella* after plating out on XLD or on another isolation media.

The interpretation of Table 14 is similar to that of Table 13, except that selective enrichment media are mutually compared, given XLD as isolation medium. For instance, the odds of finding *Salmonella* from all SE8 samples after selective enrichment in RVS is a factor of 0.33 lower than with MKTTn. In general, if MKTTn is used as selective enrichment medium, the chance of finding *Salmonella* is greater than when RVS is used. This difference is significant. When RVS is used as a selective enrichment medium compared to MSRV, this gives a smaller chance (factor 0.7). However, this difference is not significant.

Table 13 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 positive lenticule discs

Selective enrichment medium	Compared isolation media	Lenticule disc	Odds Ratios	95% lower	95% upper	p-value*
RVS	XLD compared with other than XLD	SE8	1.58	0.89	2.82	0.11
		SE50	1.50	0.63	3.57	0.36
		all SE	1.54	0.92	2.63	0.10
MKTTn	XLD compared with other than XLD	SE8	2.28	1.06	5.21	0.03
		SE51	2.71	1.02	7.71	0.05
		all SE	2.49	1.32	4.89	0.01
MSRV	XLD compared with other than XLD	SE8	0.99	0.49	2.01	0.99
		SE51	1.72	0.48	7.13	0.42
		all SE	1.31	0.62	2.93	0.48
All enrichment media	XLD compared with other than XLD	SE8	1.53	1.03	2.29	0.04
		SE51	1.89	1.01	3.53	0.05
		all SE	1.70	1.17	2.47	0.01

* significant difference in case p < 0.05.

Table 14 Number of positive isolations found with a selective enrichment medium compared with the number of positive isolations found with another selective enrichment medium, given that the isolation is on XLD
Samples: chicken feed, artificially contaminated with Salmonella positive lenticule discs

Compared selective enrichment media	Isolation medium	Lenticule disc	Odds Ratios	95% lower	95% upper	p-value*
RVS compared with MKTTn	XLD	SE8	0.33	0.15	0.70	0.00
		SE50	0.56	0.18	1.60	0.29
		all SE	0.43	0.22	0.82	0.01
RVS compared with MSRV	XLD	SE8	0.70	0.36	1.35	0.29
		SE50	0.37	0.10	1.21	0.10
		all SE	0.51	0.24	1.02	0.06
MKTTn compared with MSRV	XLD	SE8	2.11	0.95	4.97	0.07
		SE50	0.66	0.16	2.53	0.57
		all SE	1.18	0.53	2.64	0.68

* Significant difference in case p < 0.05.

Figure 3 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 lenticules are not used. The mean (OR=1) is defined as the odds of detecting *Salmonella* based on the fixed effects only (lenticule, enrichment medium and isolation medium). Laboratories below the mean have a lower probability of detecting *Salmonella*. In general, the laboratories performed very well. There is only a small difference between the performance of the laboratories just above the mean (e.g. laboratory 1) or just below the mean

(e.g. laboratory 13). Laboratory 1 missed two lenticule discs on only one of the two isolation media and laboratory 13 missed two lenticule discs on both isolation media used. From the laboratories with a score of OR < 1, ten laboratories scored a significantly lower performance ($p < 0.05$). The laboratories 10 and 18 showed a lower performance because they missed samples with their second isolation medium, but mostly scored the same samples correctly with XLD. The laboratories 3, 10, 12, 17 and 32 scored a lower number of positive results with one of the selective-enrichment media, but with another selective enrichment medium they scored better results.
Six laboratories, 4, 8, 9, 16, 26 and 33, scored all samples correctly for all media used.

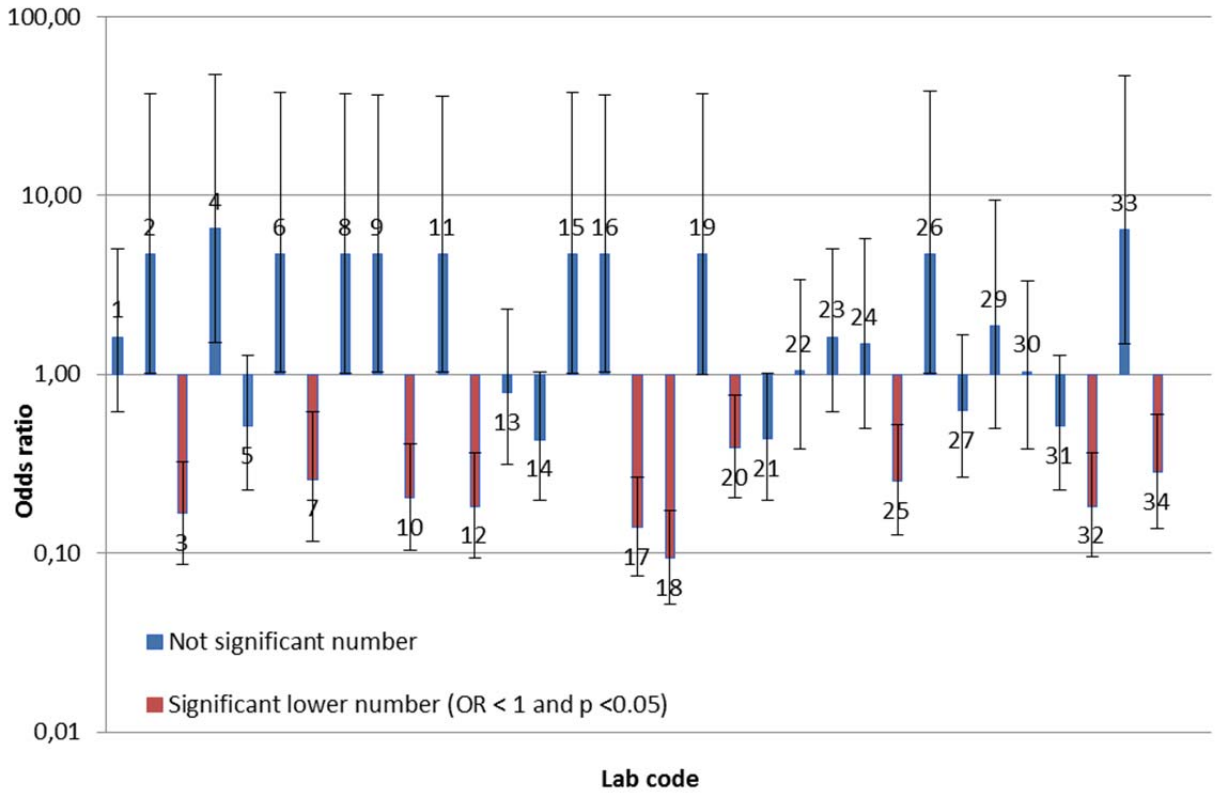

Figure 3 Performance of each laboratory compared to the mean of all laboratories for the artificially contaminated chicken feed samples (without blanks)

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

Table 15 shows the specificity, sensitivity and accuracy rates for all types of lenticule discs added to the chicken feed. The rates are calculated for the different selective enrichment media (RVS, MKTTn and MSRV) with plating-out medium XLD. The calculations were performed on the results of all participants and on the results of only the EU-MS (without the results of the European Free Trade Association countries, candidate EU-MS and third countries (non-EU-MS)). No or only small differences were found between these groups. The specificity rates (of the blank lenticule discs) were 99 -100%. The highest rates were found with MKTTn. This was most clear for the SE8 samples (sensitivity rate 96%). In

general RVS gave the lowest sensitivity rates (94%) followed by MSRV (95%) with the highest sensitivity results for MKTTn (97%).

Table 15 Specificity, sensitivity and accuracy rates for all participating laboratories of the artificially contaminated chicken feed samples (each lenticule disc added to 25 g of chicken feed) for the selective enrichment in RVS, MKTTn and on MSRV and plating-out medium with highest number of positives.

Lenticule disc with chicken feed		RVS/X		MKTTn/X*		MSRV/X**	
	Laboratories	All n=33	EU n=28	All n=32	EU n=28	All n=32	EU n=27
Blank (n=6)	No. of samples	198	168	192	168	192	162
	No. of negative samples	197	167	192	168	192	162
	Specificity in%	**99**	**99**	**100**	**100**	**100**	**100**
SE8 (n=6)	No. of samples	198	168	192	168	192	162
	No. of positive samples	180	154	184	161	175	147
	Sensitivity in%	**91**	**92**	**96**	**96**	**91**	**91**
SE50 (n=6)	No. of samples	198	168	192	168	192	162
	No. of positive samples	193	163	187	165	189	159
	Sensitivity in%	**97**	**97**	**97**	**98**	**98**	**98**
All lenticule discs with *Salmonella*	No. of samples	396	336	384	336	384	324
	No. of positive samples	373	317	371	326	364	306
	Sensitivity in%	**94**	**94**	**97**	**97**	**95**	**94**
All lenticule discs	No. of samples	594	504	576	504	576	486
	No. of correct samples	570	484	563	494	556	468
	Accuracy in%	**96**	**96**	**98**	**98**	**97**	**96**

X = isolation medium with the highest number of positives of all isolation media used.

* Results without Laboratory 29 (non-EU): they did not use MKTTn

** Results without Laboratory 7 (EU-MS): they did not use MSRV

4.6 PCR (Own method)

Five laboratories (lab codes 4, 10, 12, 20 and 33) applied a Polymerase Chain Reaction (PCR) method as an additional detection technique. All laboratories tested the samples after pre-enrichment in BPW. Two of the four PCR methods were validated. Only laboratory 20 used the PCR routinely. Laboratory 33 did not give any details on the PCR used. Table 16 gives further details on the PCR methods used.
The laboratories scored all tested samples correctly with the PCR method used. One laboratory (lab code 12) did not mention the PCR result for one sample (SE8 with chicken feed). Laboratory 33 tested only 12 of the 25 samples with PCR.

Table 16 Details on Polymerase Chain Reaction procedures, used as their own method during the interlaboratory comparison study by five participants

Lab code	PCR method: Reference
4	Real time PCR, Microval 2011, Commercial
10	Real time PCR, Not validated, Malorny 2004, Non-commercial
12	Real time PCR, Validated*, Hein 2006, Non-commercial
20	PCR no further information, Validated*, Non-commercial
33	PCR no further information

* Participants indicated that the PCR method has been validated. However, it is not clear whether the method has been validated in accordance with ISO 16140 (Anonymous, 2003b) and no information on the certificate number was given.

4.7 Performance of the NRLs

4.7.1 General

Thirty NRLs fulfilled the criteria of good performance and three laboratories scored below these criteria. As for the determination of good performance, the results of all media were taken into account. It may be that some laboratories did not score well with one medium, but overall still scored a 'good performance'. For example, laboratory 20 found, for all samples cultivated on their second isolation medium, one or more negative results independent of the method used (RVS, MKTTn or MSRV), but scored all samples correctly with the isolation medium XLD. One laboratory (lab code 28) did not send in their results. Three laboratories (lab codes 15, 21 and 31) reported one (out of two) positive blank control sample. All blanks should have tested negative.

The four deviating laboratories were contacted by the EURL-*Salmonella* in November 2012 and asked to give possible explanations for their deviating results and some were asked to perform some additional tests (e.g. biochemical tests, serotyping and phage typing). Laboratory 28 was asked for an explanation for not participating.

Laboratory 15 found one blank control sample (lenticule discs without chicken feed) positive on all media used (RVS, MKTTn and MSRV). The laboratory indicated it had made a transcription error, which was proved by their raw data. Hence, no further actions were considered necessary for this laboratory and their results were indicated as a 'moderate performance'.

Laboratory 21 found one blank control sample (lenticule discs without chicken feed) positive on all media used (RVS, MKTTn and MSRV). The laboratory checked their procedures, but did not find any omission or a clarification for the (false) positive blank. The laboratory did some extra biochemical and serological tests on the false positive sample and it turned out to be a *Salmonella* Enteritidis.

Laboratory 28 gave the following explanation for not performing the study: The laboratory in which the Production and Animal Health Unit and the Technology and Food Safety Unit are situated has been integrated in a newly created Institute. This process caused some difficulties in the coordination of

action between units. In addition to this complex process, the institute is under severe budgetary constraints, which also prevented the laboratory from participating in the October 2012 proficiency test. It is expected that these problems will be solved soon, enabling the laboratory to participate in future studies on *Salmonella*.

Laboratory 31 found one blank control sample (lenticule discs without chicken feed) to be positive with RVS. The other selective enrichment media (MKTTn and MSRV), inoculated from the same BPW, were scored correctly as negative. The laboratory was not able to find an explanation for the false positive blank result, although their own negative control also happened to give positive results. Additionally, this laboratory analysed the samples over the course of 3 weeks. When they started the study (24-9-2012), they had some problems with recognising the lenticules and spoiled four samples and analysed some of the other samples. They contacted the EURL-*Salmonella* with this problem and immediately a new parcel containing replacements for the relevant samples was sent. The other samples were analysed on 1-10-2012 and on 8-10-2012 (containing the replacement samples). The laboratory indicated that it split the work up because it was considered to be too much for a limited number of staff to do on one working day.
The problem that laboratory 31 is facing in this study (false positive blank result) is the same problem it faced in the study of 2008, including the follow-up study.

No follow-up study was organized for laboratory 15, its results were indicated as 'moderate performance'. The lack of participation of laboratory 28 is considered as an incident and no further action was taken. It is expected that this laboratory will participate in the next study. If new problems in participation arise, this will be reported to EC, DG-Sanco.

To check whether the actions taken have been successful, laboratories 21 and 31 participated in a follow-up study organized by the EURL-*Salmonella* in January 2013.

4.7.2 Follow-up study

The set-up of the follow-up study was the same as the one used for the full interlaboratory comparison study organized in September 2012, but with a lower number of samples. In this follow-up study, more blank samples were tested, as these samples caused the main problems. Table 17 gives an overview of the samples used in the follow-up study.

Table 17 Overview of the types and the number of lenticule discs tested in the follow-up interlaboratory comparison study

Lenticule discs	Control lenticule discs (n=5) No matrix added	Test samples (n=8) with 25 grams of *Salmonella* negative chicken feed
S. Enteritidis 50 (SE50)	2	4
Blank	3	4

On Monday 21 January 2013, one parcel with two plastic containers was sent to the laboratories 21 and 31 containing: 5 control lenticule discs (numbered C1 – C5), 8 lenticule discs (numbered B1 – B8), 250 grams of chicken feed and one temperature recorder.

On 18 January 2013, the number of aerobic bacteria (3.6 *10^3 CFU/g) and *Enterobacteriaceae* (4.5 *10^2 CFU/g) in the chicken feed was tested after it was stored at 5°C since September 2012. These numbers were 1 log lower than the numbers found in the chicken feed used in the full study (see Table 4).
The performance of this follow-up study started in week 5 (28 January 2013). Each laboratory had to follow the same SOP and protocol as used in the study of September 2012 (EURL-*Salmonella*, 2012a, 2012b). The test report was shorter, but comparable to the September study (EURL-*Salmonella*, 2012c). For the media used, only the differences with the September study needed to be indicated.

For the media compositions, incubation times and temperatures, no or only minor differences were observed in comparison with the full study.

Laboratory 21 performed the follow-up study, but unfortunately the problem with the false positive blank sample was still not solved. They found again one blank control sample (lenticule disc without chicken feed) to be positive for *Salmonella* with all selective enrichment media used, i.e. RVS, MKTTn and MSRV.
The laboratory went through all the procedures and drafted a report of their findings. After 24 h of incubation, the BPW was clear, so there was no growth in the sample that was found to be a false positive (blank lenticule in BPW). But the BPW became turbid after 48 hours of incubation. The cultivation on XLD and Rambach after 24 h of selective enrichment in RVS and MKTTn were positive. It is therefore likely that the cross-contamination took place after the pre-enrichment step in BPW during the inoculation of RVS and MKTTn from the BPW.
The selective enrichment on MSRV was not suspect after 24 h of incubation, but it was so after 48 hours and the cultivations on XLD and Rambach were positive. A positive result after 48 h of selective enrichment on MSRV, while being negative after 24 h, is possible when there is a very low concentration of *Salmonella* inoculated on the MSRV.
The false positive sample was serotyped as *Salmonella* Enteritidis.
A possible explanation may be the contamination of the pipette or other equipment used to transfer the BPW culture to the selective enrichment medium. If there was a low concentration of *Salmonella* present on, for example, the pipette (or other equipment), both the (clear) BPW, as well as the selective enrichment media, were possibly contaminated. This explanation may also explain why the BPW became turbid after 48 hours of incubation, while it was still clear after 24 hours.
The laboratory did take some actions. They checked the sterility of their pipettes and shields and it was proved that they were sterile. The most important action was the introduction of positive and negative controls in their daily analyses. The effect of this latter action still needs to be shown.

Laboratory 31 showed repeatedly deviating results in ring trials with animal feed as a matrix (the full and follow-up study of 2008 and this second animal feed study of 2012). Two staff members of the EURL-*Salmonella* visited this laboratory while they performed a follow-up to the current study (starting on 28 January 2013). During a two-day visit (28 and 29 January 2013), the procedures were checked for possible (technical) problems 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 31.
In the reporting to the EURL, relevant information was regularly missing and the test report or replies were often sent after the deadline.

In the test report of the follow-up study, several deviations were noticed:

- the pre-enrichment in BPW of the control samples was performed at 31°C instead of at the prescribed 37°C. The control samples and the test samples were placed in different incubators and one incubator lost temperature due to an error in turning the temperature limit knob.
- the selective enrichment in MKTTn was performed at 31°C instead of at the prescribed 37°C.
- the pH of the MKTTn medium was 7.4 instead of 8.0 (± 0.2).

Unfortunately, no improvement in the performance of laboratory 31 was seen in the follow-up study, the results were again below the criteria of good performance. The blank control sample (lenticule disc without chicken feed) and both procedure controls (the control sample with only BPW and the control sample with BPW and chicken feed) tested positive for *Salmonella*.

The laboratory additionally performed serotyping and molecular typing (MLVA) on all positive samples of the follow-up study (including the false positive blank samples) and they were all typed as *Salmonella* Enteritidis, having all the same MLVA pattern. This MLVA pattern also corresponded to the pattern found earlier in the (false positive) isolates in the full study of September 2012.

With these results, the laboratories 21 and 31 again showed an 'underperformance' (see clause 3.6) in this follow-up study.
A report on laboratory 31 (an EU-MS), with all information about the performance in the studies of 2008 and 2012 and the visit of the EURL-*Salmonella* in 2013, was sent to EC, DG-Sanco in March 2013. DG-Sanco will contact the responsible authority in the country of this NRL and will take further actions.
A report containing an overview of results from the studies in which laboratory 21 (candidate EU-MS) participated was also sent to EC, DG-Sanco in April 2013.

5 Discussion

Reference materials
In this interlaboratory comparison study, lenticule discs containing *S*. Enteritidis at low and high levels were tested.
The homogeneity tests performed by HPA and by EURL-*Salmonella* on the original data of the HPA showed good homogeneity in the batches of lenticules. The variation in counts between lenticule discs of each batch (SE8 and SE50) were less than two times the Poisson distribution.
The verification of the mean contamination levels of the batches of lenticules performed at the EURL-*Salmonella* showed sufficient stability for both batches of lenticules when stored at -20 ºC.

To prevent the batches of lenticule discs from a decrease in the mean level during transport, the materials were packed with frozen cooling elements and transported by courier service. The information provided by the temperature recorders, which were included in the parcels, showed that the temperature remained below 5 ºC during transport of the majority of the parcels. Therefore it is assumed that transport did not negatively affect the mean contamination level of the samples. This was confirmed by the fact that the laboratories with the longest transport times and/or deviating transport temperatures (lab codes 3, 7, 10, 11, 21 and 29) still found good results.

Performance of the laboratories
According to EC Regulations 882/2004 (EC, 2004) and 2076/2005 (EC, 2005), each NRL should be accredited for their relevant work field since 31 December 2009. All laboratories reported that they were accredited with the exception of one participant from a EU-MS (lab code 31). This latter laboratory indicated it was in the process of becoming accredited in 2013, which is relatively late.

For determining 'good performance' per laboratory, the best performing isolation medium after selective enrichment in RVS, MKTTn or MSRV was taken into account (being the medium with the highest number of positive isolations). Thirty out of a total of 33 laboratories scored 'good performance'. Three laboratories (lab codes 15, 21 and 31) scored an 'underperformance' in the blank control samples. The problem of laboratory 15 concerned a mistake in reporting and their results were indicated as 'moderate performance'. A follow-up study was considered unnecessary for this laboratory. The other two laboratories participated in a follow-up study. Unfortunately, they both made the same mistake made in the full study and again scored an 'underperformance' with false positive blank samples.
False positive blank results can have different causes:
- cross-contamination, which can occur at different stages of the procedure for the detection of *Salmonella;*
- limited confirmation or misinterpretation of the test results.

A possible explanation for the false positive blank results (without matrix) found with all selective enrichment media used at laboratory 21 may have been (cross-contamination in the first step of the procedure: the pre-enrichment in BPW. Laboratory 21 is a non-EU country and was participating for the second time in a EURL detection study and for the first time in an animal feed study. Possible

inexperience with the type of material used in this study may have influenced their performance. In earlier studies it has been observed that laboratories participating for the first time often show an 'underperformance', but improve during the course of the studies. (Kuijpers and Mooijman, 2012b).

Laboratory 31 (EU-MS) is not accredited and was participating for the second time in a EURL study. They showed the same kind of problems with false positive control samples in the first animal feed study in 2008 (Kuijpers et al., 2009).
Furthermore, the laboratory mentioned that it split the work up over the course of three weeks because it was too much work to accomplish on one working day. None of the other participating laboratories mentioned this problem. Analysing the samples of one study over 3 different weeks is not the intention of the interlaboratory study. All samples should be analysed in 'one run', including the control samples.
In addition to the problems in the full study, the laboratory found a blank control sample (lenticule disk without animal feed) and both procedure controls to be false positive for *Salmonella* in the follow-up study.
If growth is found in the blank control of BPW, it is not possible to trust the result of any other sample analysed with the same batch of BPW. Growth in BPW, which is supposed to be sterile, can be caused by insufficient sterilisation of the BPW or by cross-contamination during the process of analysing. However, false positive blank results can also be caused by the mixing up of samples and/or isolates or by misinterpreting the isolation plates and/or of the confirmation tests. As the routine samples of the laboratory are rarely positive for *Salmonella* and as no positive controls are used during the analyses, this latter explanation may also be quite likely due to a lack of experience with positive samples.
It is most likely that the cross-contamination/mixing up of samples was done during the analyses of the samples in the interlaboratory comparison studies. Cross-contamination from routine samples is not very likely as the routine samples are rarely positive for *Salmonella*.
Although, during the visit of the EURL-*Salmonella* staff members, no major source could be found which may have caused the false positive blank results, it cannot be excluded that a series of personal mistakes may have resulted in poor performance in the full study as well as in the follow-up study.

Only one participant (Laboratory 5) scored a positive in one blank sample with chicken feed with selective enrichment in RVS. This was still considered as acceptable as no 100% guarantee concerning the *Salmonella* negativity of chicken feed can be given. This laboratory correctly scored the same blank sample as negative after selective enrichment in MKTTn and MSRV, inoculated from the same BPW. An explanation for this one false positive sample may be cross-contamination or misinterpretation of the results. The number of background flora (*Enterobacteriaceae*) in the matrix was relatively high and this may have caused problems with reading the isolation media. In combination with a limited confirmation, the *Enterobacteriaceae* present in the matrix can be misinterpreted as *Salmonella*, resulting in a false positive blank result.

The performance of each laboratory compared with the mean of all laboratories for the artificially contaminated chicken feed samples (Figure 3) is an indication of the performance of the laboratory in general (the blanks are not included in this comparison). A laboratory can show a performance under the mean of all laboratories but still score a 'good performance'. However, when this is the case, it would be advisable for this laboratory to check the reasons for the lower

number of positive *Salmonella* results compared with the other laboratories. The laboratories 3, 10, 12, 17 and 32 scored a lower number of positive results but still scored within the lines of good performance. However, these laboratories still may have a sensitivity problem with one of their methods and/or media.

Laboratory 12 showed that it had problems with the sensitivity of its prescribed food method: selective enrichment in RVS and MKTTn. This laboratory could not detect *Salmonella* in 50% of the low-level contaminated (SE8) chicken feed samples with MKTTn and RVS. However, with MSRV they only missed one SE8 sample and with their own method (PCR) they correctly found all samples to be positive. The SE8 lenticule discs contained SE at a low levels (approximately 8 CFU/lenticule). Due to change, one out of six lenticule discs containing SE8 may be negative. Finding more than one SE8 lenticule disc to be negative is not likely due to change.
The laboratory scored the minimum results to fulfil the criteria of good performance for the prescribed food method.

Specificity, sensitivity and accuracy rates
The calculations were performed on the results of all participants and on the results of only the EU Member States (without the results of participants from the EFTA, candidate EU-MS countries and third countries). Only minor differences (if any) were found between these groups.
The specificity, sensitivity and accuracy rates were high. Even for the chicken feed samples, artificially contaminated with SE8 lenticule discs, a sensitivity rate of at least 91% was found. As this low contamination level is close to the detection limit of the method a sensitivity rate close to 50% would have been expected.

Media and incubation
Four laboratories (lab codes 3, 10, 17 and 32) showed an 'underperformance' for one of the selective enrichment media used, while they correctly scored all samples as positive with another method inoculated from the same pre-enrichment medium (see Annex 2). This suggests that those laboratories may have a sensitivity problem with that one selective enrichment medium which scored a lower number of positive results.
Explanations for these deviations are hard to find most of the time. Depending on the type of sample analysed, (small) deviations in the prescription of the media (e.g. in pH or concentration novobiocin) or incubation time may stimulate the growth of the background flora, resulting in a suppression of the growth of *Salmonella*. For instance, laboratory 7 reported a lower pH of MKTTn as prescribed and scored a lower number of feed samples as positive. However, whether these low scores were caused by the low pH of MKTTn is hard to trace.

Many participants showed some variation in results between the different selective enrichment media (RVS, MKTTn and MSRV) in combination with the different isolation media (XLD or other). In contrast to earlier studies (other matrices), more samples were found to be positive with MKTTn than with RVS or MSRV. An explanation for this result may be that the type of background flora in the chicken feed was better suppressed in MKTTn.
A few participants did mention that the contaminating organisms in the animal feed were able to grow effectively on MSRV plates and spread like an expected *Salmonella* isolate. Some of the control samples (only chicken feed and BPW) also showed this growth pattern, though it was confirmed to be negative for *Salmonella*. When culturing from RVS, often an overgrowth of mucous, swarming bacteria (probably *Enterobacter sakazaki*) on XLD was seen, which

made it difficult or impossible to detect *Salmonella*. It was easier to detect *Salmonella* on a chromogene agar (e.g. Brilliance Salmonella Agar (BSA)) when inoculated from RVS, while isolations from MSRV did not show differences between XLD and BSA.
When MKTTn or RVS was used for selective enrichment, followed by isolation on XLD, this gave 2-5% more positive results than other plating-out media. After selective enrichment on MSRV, the difference between XLD and another plating-out medium was nil.
When MKTTn was used for analysing the animal feed samples, there was a significant higher chance of detecting *Salmonella* in combination with XLD in comparison with other isolation media. For example, laboratories 10 and 18 showed a lower performance in comparison with the mean of all laboratories (Figure 3) when analysing the samples with the combination MKTTn and their own isolation medium (BGA). However, they scored the same samples correctly with the combination MKTTn and XLD.
When RVS or MSRV was used as selective enrichment medium, no significant differences were observed between isolation on XLD or other isolation media. Still, it was observed that some isolation media (e.g. Rambach, RS and BSA) scored better than XLD, e.g. because of better suppression of disturbing background flora or easier recognition of *Salmonella* on the plate.
Differences were observed between the 11 different isolation media used by the different participants (see Annex 2) as a second plating-out medium, but the number of users per isolation medium (see table 6) was too small to make a comparison.

A longer incubation time of 48 hrs for RVS and MKTTn was not requested in this study as ISO 6579 prescribes only 24 h of incubation (Anonymous, 2002). For selective enrichment on MSRV, this additional incubation of 24 h was still requested, as this is prescribed in Annex D of ISO 6579 (Anonymous, 2007). Depending on the level of contamination in the samples, the additional 24 h of incubation resulted in 5-8% more positive results.

PCR
Five laboratories used a PCR technique in addition to the prescribed method and all of them found at least the same results as were found using the bacteriological detection methods. This indicates that the PCR methods used are well suited for the detection of *Salmonella* in chicken (animal) feed samples.

6 Conclusions

Thirty out of 34 NRLs achieved the level of 'good performance' for the detection of *Salmonella* in chicken feed. Two laboratories scored an 'underperformance' in the full study and in the follow-up study. One laboratory scored a 'moderate performance'. One NRL did not perform the study.

The sensitivity rates for the control samples (without matrix) after selective enrichment in RVS, MKTTn and on MSRV were at least 99%.

The specificity rates for the control samples (blank lenticule discs without matrix) after selective enrichment in RVS, MKTTn and on MSRV were at least 95%.

The specificity rates of the chicken feed samples artificially 'contaminated' with blank lenticule discs was 99% for RVS and 100% for MKTTn and MSRV.

For all chicken feed samples artificially contaminated with *Salmonella*, the sensitivity rates after selective enrichment in MKTTn (97%) were higher than the rates after selective enrichment in RVS (94%) and MSRV (95%).

The accuracy rates of the artificially contaminated chicken feed samples were 96% for RVS, 97% for MSRV and 98% for MKTTn.

In contrast to earlier studies (with other matrices), selective enrichment in MKTTn gave a significantly higher chance of isolating *Salmonella* from the chicken feed compared with the other selective enrichment media (RVS and MSRV). This was most clear for the materials contaminated with low levels of *S*. Enteritidis (SE8), which had a sensitivity rate for MKTTn of 96% compared with 91% for RVS and MSRV.

Selective enrichment in MKTTn, in combination with isolation on XLD, scored a significantly higher chance of isolating *Salmonella* out of the matrix used in this study, in comparison with non-XLD isolation media.

An additional incubation time of 24 hours of MSRV gave 5-8% more positive results.

List of abbreviations

ASAP	AES *Salmonella* Agar Plate
BGA(mod)	Brilliant Green Agar (modified)
BGPA	Brilliant Green Phenol Agar
BPLS(A)	Brilliant Green Phenol-red Lactose Sucrose (Agar)
BPW	Buffered Peptone Water
BSA	Brilliance *Salmonella* Agar (OSCM)
CFU	colony forming units
EC	European Commission
EFTA	European Free Trade Association
EU	European Union
EURL	European Union Reference Laboratory (CRL)
Gal	Galactosidase
HE	Hektoen Enteric agar
HPA	Health Protection Agency
ISO	International Standardization Organization
LDC	Lysine Decarboxylase
MAC	MacConkey Agar
MKTTn	Mueller Kauffmann Tetrathionate novobiocin Broth
MLCB	Mannitol Lysine Crystal Violet Brilliant Green Agar
MLVA	Multiple-Locus Variable number tandem repeat Analysis (molecular typing method)
MS	Member State
MSRV	Modified Semi-solid Rappaport Vassiliadis
NCTC	National Collection of Type Cultures (HPA)
NRL	National Reference Laboratory
OR	Odds Ratio
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
RIVM	Rijksinstituut voor Volksgezondheid en het Milieu (National Institute for Public Health and the Environment)
RS	Rapid *Salmonella*
RV(S)	Rappaport Vassiliadis (Soya) broth
SE	*Salmonella* Enteritidis
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

References

Anonymous (1993). ISO 6579 (E). Microbiology – General guidance on methods for the detection of *Salmonell*a. International Organisation for Standardisation, Geneva, Switzerland.

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

Anonymous (2005). ISO 17025. General requirements for the competence of testing and calibration laboratories. International Organisation for Standardisation, 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 Organisation for Standardisation, Geneva, Switzerland.

Anonymous (2010). ISO 22117. Microbiology of food & animal feedingstuffs - Specific requirements & guidance for Proficiency Testing (PT) by interlaboratory comparison. International Organisation for Standardisation, Geneva, Switzerland.

Boyd P, Eaton S and Magee J (2006). The stability of micro-organisms preserved in LENTICULE discs, demonstrated by repeat sample distributions of the EQUAL Scheme for Indicator Organisms (water microbiology). Journal of Applied Microbiology 100, 272–278.

Desai M, Russell JE and Gharbia S (2006). Genetic stability of strains preserved on LENTICULE discs and by freeze-drying: a comparison using fluorescent amplified fragment length polymorphism analysis. FEMS Microbiol Lett 257, 158–162.

EC (2004). Commission Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on 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 (19 June, 2013)

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 (19 June, 2013)

EURL-*Salmonella* (2012a). Protocol Interlaboratory Comparison Study on the detection of Salmonella spp. in animal feed organised by EURL-*Salmonella* FEED STUDY II – 2012. http://www.eurlsalmonella.eu/Proficiency_testing/Detection_studies_Feed (19 June, 2013)

EURL-*Salmonella* (2012b). Standard operating procedure (SOP) Interlaboratory Comparison Study on the detection of Salmonella spp. in animal feed organised by EURL-*Salmonella* FEED STUDY II – 2012. http://www.eurlsalmonella.eu/Proficiency_testing/Detection_studies_Feed (19 June, 2013)

EURL-*Salmonella* (2012c). Testreport Interlaboratory Comparison Study on the detection of Salmonella spp. in animal feed organised by EURL-*Salmonella* FEED STUDY II – 2012. http://www.eurlsalmonella.eu/Proficiency_testing/Detection_studies_Feed (19 June, 2013)

EURL-*Salmonella* (2013). History of EURL-*Salmonella* interlaboratory comparison studies on the detection of *Salmonella*. http://www.eurlsalmonella.eu/Publications/Interlaboratory_comparison_study_Reports (16 July, 2013)

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

Hein I, Flekna G, Krassnig M, Wagner M (2006). Real-time PCR for the detection of *Salmonella* spp. in food: An alternative approach to a conventional PCR system suggested by the FOOD-PCR project. Journal of Microbiological Methods 66 (2006) 538-547.

Heisterkamp SH, Hoekstra JA, Van Strijp-Lockefeer NGWM, Havelaar A, Mooijman KA, In `t Veld PH, Notermans SHW (1993). Statistical analysis of certification trials for microbiological reference materials. Commission of European Communities, Community Bureau of Reference, Brussels, Luxembourg. EUR Report; EUR 15008 EN

HPA Health Protection Agency (2012). Lenticule Disc Handling Information. HPA Culture Collection, Salisbury, United Kingdom. http://www.hpacultures.org.uk/products/lenticulediscs/index.jsp (2 July 2013) http://www.hpacultures.org.uk/media/C4B/10/LENT_Handling_Website_V.pdf

Kuijpers AFA, Veenman C, Kassteele van de J and Mooijman KA (2009). EU Interlaboratory comparison animal feed I (2009); Detection of *Salmonella* in chicken feed. RIVM Report 330604012, Bilthoven, the Netherlands.

Kuijpers AFA and Mooijman KA (2011). EU Interlaboratory comparison study veterinary XIV (2011); Detection of *Salmonella* in chicken faeces. RIVM report 330604023, Bilthoven, the Netherlands.

Kuijpers AFA, Kassteele van de J and Mooijman KA (2012a). EU Interlaboratory comparison study food V (2011); Detection of *Salmonella* in minced meat. RIVM Report 330604025, Bilthoven, the Netherlands.

Kuijpers AFA and Mooijman KA (2012b). Detection of *Salmonella* in food, feed and veterinary samples by EU laboratories. Food Research International (2012) Vol. 45 pp. 885-890.

Kuijpers AFA and Mooijman KA (2013). EU Interlaboratory comparison study veterinary XIV (2012); Detection of *Salmonella* in pig faeces. RIVM report 330604028, Bilthoven, the Netherlands.

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.

Microval (2011). Certificate RQA2008LR22: Biotest MMB *Salmonella* Prep and MMB *Salmonella* V, R, LC Kits to detect *Salmonella* spp in food and feeding stuffs. http://www.microval.org/resources/News-Certificate_2010LR22_en.pdf http://www.microval.org/resources/RQA2008LR22.pdf (19 June, 2013)

R Development Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org. (19 June 2013).

Annex 1 Number of positive results of the control samples (lenticule disc without matrix) per laboratory and per selective enrichment medium

Lab code	RVS			MKTTn			MSRV		
	SE8 n=2	SE50 n=1	Blank n=2	SE8 n=2	SE50 n=1	Blank n=2	SE8 n=2	SE50 n=1	Blank n=2
Good Performance	**≥ 1**	**1**	**0**	**≥ 1**	**1**	**0**	**≥ 1**	**1**	**0**
1	2	1	0	2	1	0	2	1	0
2	2	1	0	2	1	0	2	1	0
3	2	1	0	2	1	0	2	1	0
4	2	1	0	2	1	0	2	1	0
5	2	1	0	2	1	0	2	1	0
6	2	1	0	2	1	0	2	1	0
7	2	1	0	2	1	0	-	-	-
8	2	1	0	2	1	0	2	1	0
9	2	1	0	2	1	0	2	1	0
10	2	1	0	2	1	0	2	1	0
11	2	1	0	2	1	0	2	1	0
12	2	1	0	2	1	0	2	1	0
13	2	1	0	2	1	0	2	1	0
14	2	1	0	2	1	0	2	1	0
15	2	1	**1**	2	1	**1**	2	1	**1**
16	2	1	0	2	1	0	2	1	0
17	2	1	0	2	1	0	2	1	0
18	2	1	0	2	1	0	2	1	0
19	2	1	0	2	1	0	2	1	0
20	2	1	0	2	1	0	2	1	0
21	2	1	**1**	2	1	**1**	2	1	**1**
22	2	1	0	2	1	0	2	1	0
23	2	1	0	2	1	0	2	1	0
24	2	1	0	2	1	0	2	1	0
25	2	1	0	2	1	0	2	1	0
26	2	1	0	2	1	0	2	1	0
27	2	1	0	2	1	0	2	1	0
28	-	-	-	-	-	-	-	-	-
29	2	1	0	-	-	-	2	1	0
30	2	1	0	2	1	0	2	1	0
31	2	1	**1**	2	1	0	2	1	0
32	2	1	0	2	1	0	**1**	1	0
33	2	1	0	2	1	0	2	1	0
34	2	1	0	2	1	0	2	1	0

- : not performed

bold numbers: deviating results

grey cells: results are below the criteria of good performance

Annex 2 Number of positive results for the artificially contaminated chicken feed samples (with lenticule disc) per laboratory and per selective enrichment medium

Lab code	RVS XLD/2nd *			MKTTn XLD/2nd *			MSRV XLD/2nd *		
	SE8 n=6	SE50 n=6	Blank n=6	SE8 n=6	SE50 n=6	Blank n=6	SE8 n=6	SE50 n=6	Blank n=6
Good Performance	**≥ 3**	**≥ 5**	**≤ 1**	**≥ 3**	**≥ 5**	**≤ 1**	**≥ 3**	**≥ 5**	**≤ 1**
1	6/**5**	**5**/6	0	6	6	0	6	6	0
2	6	6	0	6	6	0	6	6	0
3	**3/2**	**4/3**	0	6/**5**	6	0	**5**	6	0
4	6	6	0	6	6	0	6	6	0
5	6	**5/4**	**1**	6	6/5	0	6	6/5	0
6	6	6	0	6	6	0	6	6	0
7	6	**4**/6	0	**4**	**5**	0	-	-	-
8	6	6	0	6	6	0	6	6	0
9	6	6	0	6	6	0	6	6	0
10	**5**	6	0	**5/2**	**4/2**	0	6	6	0
11	6	6	0	6	6	0	6	6	0
12	**3**	6	0	**3**	6	0	**5**	6	0
13	**5**	6	0	**5**	6	0	6	6	0
14	**3/5**	6	0	6	6	0	6/5	6	0
15	6	6	0	6	6	0	6	6	0
16	6	6	0	6	6	0	6	6	0
17	6/**4**	6	0	6/**5**	**5**	0	**2**	**4**	0
18	6/**1**	6/**3**	0	6/**2**	6/**1**	0	**4**	6/**5**	0
19	6	6	0	6	6	0	6	6	0
20	6/**2/5**	6/**4**/6	0	6/**4/5**	6	0	6/**5**/6	6/**5**/6	0
21	**4/3**	6	0	6	6	0	**5**	6	0
22	**4/5**	6	0	6	6	0	6	6	0
23	6	6	0	6	6	0	**5**	6	0
24	**5**	6	0	6	6	0	6	6	0
25	**5**/6	6	0	**5**	**5**	0	**5**	**5**	0
26	6	6	0	6	6	0	6	6	0
27	**5**	6	0	6/**5**	6	0	**5**	6	0
28	-	-	-	-	-	-	-	-	-
29	**5**/6	6	0	-	-	-	6	6	0
30	6/**5**	6	0	**5**/6	6/**5**	0	6	6	0
31	**4**	6	0	6	6	0	**5**	6	0
32	**3**/6	**5**	0	6	6	0	**2**	6	0
33	6	6	0	6	6	0	6	6	0
34	**4/2**	**5/4**	0	6	6	0	**5**/6	6	0

* When only one figure is given, both isolation media give the same result. In case of three figures, three isolation media were used.

\- : not performed

bold numbers: deviating results

grey cells: results are below the criteria of good performance

National Institute for Public Health and the Environment
P.O. Box 1 | 3720 BA Bilthoven
www.rivm.com