Interim summary report EURL-*Salmonella*

Interlaboratory Comparison study FEED IV (2018)

Detection of *Salmonella* in chicken feed

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Introduction

In January 2018, the fourth EURL-*Salmonella* interlaboratory comparison study on the detection of *Salmonella* in a feed matrix (Feed IV) was organised for the NRLs for *Salmonella.* In total 35 NRLs participated in this study: 30 NRLs from 28 EU-Member States (MS) and five NRLs from third countries (EU candidate MS or potential EU candidate MS, members of the European Free Trade Association (EFTA) or non-European countries).
This interim summary report consists of two parts. One part contains the individual NRL results; the other part contains the overall results of all NRLs which is described here.

Materials & Methods

Samples

Each NRL analysed in total 20 samples: 18 samples of each 25 g chicken feed artificially contaminated with three different levels (Blank, low and high level) of *Salmonella* Mbandaka (SMb) and 2 control samples.
A batch of 20 kg chicken feed was obtained from, Kasper Faunafood, Woerden in the Netherlands. The chicken feed arrived at EURL-*Salmonella* on 12 January 2018 and was tested negative for *Salmonella*. The chicken feed was packed in portions of 25 gram, after which the test portions were artificially contaminated with three different levels (Blank, low and high level) of *Salmonella* Mbandaka (SMb) and stored at 5 °C. On Monday 19 February, the feed samples were mailed to the NRLs, and were stored at 5 °C after arrival. Table 1 shows the number of aerobic bacteria and *Enterobacteriaceae* in the feed determined by the EURL-*Salmonella* on 15 January and 26 February 2018.

Table 1 Number of aerobic bacteria and Enterobacteriaceae *per gram of chicken feed (negative for* Salmonella*)*

Date	*Enterobacteriaceae* CFU/g	Aerobic bacteria CFU/g
15 January 2018	$9.7*10^4$	$1.7*10^5$
26 February 2018 After storage for 6 weeks at +5 °C	$3.5*10^4$	$5.2*10^5$

Table 2 shows the inoculum levels of the diluted culture with *Salmonella* Mbandaka (SMb) used to artificially contaminate the chicken feed samples. Of the artificially contaminated chicken feed samples with low and high level SMb, also a five tube Most Probable Number (MPN) test was performed. These results are also summarised in Table 2.

Table 2 Number of Salmonella *Mbandaka (SMb) in the inoculum for artificial contamination of the chicken feed, and in the chicken feed samples after storage at 5 °C.*

Date of testing	Low level SMb CFU/per sample	High level SMb CFU/per sample
13 February 2018 (Inoculation of chicken feed)	8	91
26 February 2018 MPN of feed, inoculated with SMb (95% confidence limit) after storage for 13 days at 5 °C	0 (0-0.675)	1.1 (0.4-3)

The NRLs had to analyse the following samples:

6x (25g chicken feed + low level of SMb)
6x (25g chicken feed + high level of SMb)
6x (25g chicken feed)

Furthermore some control samples had to be analysed, being:

1x only BPW (Procedure control Blank)
1x own control sample with *Salmonella* (Own positive control)

The chicken feed samples were individually packed and labelled. The decoding of these samples can be found in the tables of the individual NRL results.

Calculation of specificity, sensitivity and accuracy rates:

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

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

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

Analysis of samples according to ISO 6579-1

The prescribed method was EN ISO 6579-1:2017 (Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of *Salmonella* - Part 1: Detection of *Salmonella* spp.) and the underlying EN ISO documents, e.g. the EN ISO 6887 series for preparation of test samples. It was also allowed to use the former version of EN ISO 6579-1:2017 (EN ISO 6579: 2002). EN ISO 6579-1:2017 describes the updated technical steps for the detection of *Salmonella* in food, animal feed and samples from the primary production stage. An important change in this document compared to the earlier version of EN ISO 6579 (2002), is the possibility to choose between RVS and MSRV for the selective enrichment of *Salmonella* from food and animal feed samples, meaning that additional to MKTTn, either RVS or MSRV could be used for selective enrichment. It was also allowed to use all three selective enrichment media.

For the reporting of the results, the participants were asked to report what would have been reported in case these samples would have been routine samples, meaning that the indication 'positive' (1) or 'negative' (0) per sample (after confirmation) was sufficient (independent of the combination of selective enrichment medium and isolation medium).

Criteria for good performance

For determining good performance per laboratory, all combinations of selective enrichment media (MKTTn and/or RVS and/or MSRV) and isolation media used by the laboratory were taken into account.
This study showed an unexpected high number of negative results of the artificially contaminated chicken feed samples. Therefore it was decided not to set criteria for these samples, but only to compare the number of positive samples found per laboratory with the mean number of positive samples found by all participants. The results of the control samples and blank chicken feed samples were judged according the good performance criteria in Table 3

Table 3 Used criteria for good performance and mean number of positive results of the contaminated samples in the Feed IV study (2018)

Minimum result for good performance		
	Percentage positive	No. of positive samples/ total No. of samples
Control samples		
Own control with *Salmonella*	100%	1 /1
BPW	0%	0 /1
Samples: chicken feed		
Blank[1] Feed	20% at max[1]	1/6 at max[1]
Mean number of positive samples found by all participants		
Samples: chicken feed artificially contaminated		
SMb high	50%	3/6
SMb low	5%	0.3/6

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

Results

General

On Monday 19 February 2018 (week 8) the samples were sent to 35 laboratories. The majority of the parcels were delivered at the NRLs within 1- 2 days.
Thirty-four laboratories performed the study as requested in week 9. Most of them started on 26 February 2018, one participants performed the study 1 week later (lab code 34). Thirty-three laboratories used as requested MKTTn as selective enrichment medium. Thirteen participants used additional both selective enrichment media RVS and MSRV. Nine laboratories used only RVS in addition to MKTTn and eleven laboratories used only MSRV in combination with MKTTn. Two laboratories (lab code 2, non-EU and lab-code 27, EU-MS) used only MSRV for selective enrichment and no MKTTn.

Controls

Procedure control Blank (only BPW)
Thirty-four laboratories analysed the one procedure control sample (no matrix, only BPW) correctly negative for *Salmonella*. Laboratory 35 reported this sample as positive for *Salmonella*.

Positive control with Salmonella
Thirty-two laboratories scored good results with their own *Salmonella* positive control sample. Laboratories 1, 2 and 35 reported this sample as negative for *Salmonella*.

For the positive control, the majority of the participants used a diluted culture of *Salmonella* (19 laboratories). Other participants used a lenticule disc (8), capsule (1), freeze dried ampoule, Kwik stik or Culti loop (2) with *Salmonella.* The *Salmonella* serovars most frequently used for the positive control sample were *Salmonella* Enteritidis (16), *Salmonella* Typhimurium (7) and *Salmonella* Nottingham (4).

Table 4 gives the correct scores for the control samples with accuracy rates of 94%.

Artificially contaminated chicken feed samples

Blank samples
Thirty-one laboratories correctly scored all 6 blank feed samples negative for *Salmonella*. Three laboratories (lab code 9, 15 and 30) found one blank sample out of six positive for *Salmonella*. All blanks should be tested negative. However, as no 100% guaranty 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. A false positive result for a blank sample may also been caused by cross-contamination, exchange of samples or by misinterpretation of the results. When the number of background flora in a matrix is relatively high (like for this study) this may cause problems with reading of the isolation media. In combination with a limited confirmation, the *Enterobacteriaceae* present in a matrix can be misinterpreted as *Salmonella*, resulting in a false positive blank result.

High level contaminated Salmonella *Mbandaka samples*
Thirty-three laboratories detected *Salmonella* in at least one of six high contaminated feed samples. Two laboratories (lab codes 2 and 6) could not detect *Salmonella* in any of the six high contaminated samples.

Low level contaminated Salmonella *Mbandaka samples*
Nine laboratories detected one or two of the six low contaminated feed samples positive for *Salmonella*. Most laboratories could not detect *Salmonella* in any of six low contaminated samples.

Laboratory 2 could not detect *Salmonella* in any of the samples (including the positive control). This laboratory indicated a technical problem with the temperature during the incubation of the pre-enrichment (BPW).

Figures 1 and 2 give for all possible combinations of media (MKTTn and RVS or/and MSRV), the highest number of positive samples found per laboratory for respectively the low and high (SMb) contaminated chicken feed samples. The mean number of positive samples found by all participants is also indicated in the figures.

The MPN analysis of the chicken feed samples (Table 2), shows a very low level of *Salmonella* in even the high contaminated samples at the day of performance. The number of positive samples found by all participants was evenly distributed over the different samples of both high and low level contaminated samples. This indicates that the detection of *Salmonella* in the chicken feed was influenced evenly over all samples. This was not expected from the pre-test, for which the same chicken feed and SMb strain were used. The batch chicken feed used in the interlaboratory study contained 1 log CFU/g higher number of *Enterobacteriaceae* compared to the batch chicken feed in the pre-test. This high amount of backgroundflora may influence the detection of *Salmonella* negatively, but is not likely to be the only clarification for the high number of negative feed samples. After the interlaboratory study, the EURL-*Salmonella* repeated the inoculation of animal feed samples using the same batch of chicken feed, the same SMb strain and the same inoculation levels. Similar results were observed as found with the samples of the interlaboratory study (Table 1 and 2). Additionally to the inoculation of the 25 g chicken feed samples with 10 and 100 SMb, feed samples were inoculated with 1000 CFU. These latter samples were all tested positive for *Salmonella*. This 'confirms' that a reduction of almost 2 log CFU of SMb occurred after addition to the chicken feed samples. This reduction explains the high number of negative samples in the interlaboratory study.

In Table 5 the specificity, sensitivity and accuracy rates are given for the artificially contaminated feed samples. The specificity and accuracy rates were respectively 99% and 52% and the sensitivity rates for low and high contaminated animal feed samples were respectively 5% and 52%.

Performance of the participants

Laboratories 1, 2 and 35 reported the absence of *Salmonella* in their own positive control sample. Laboratory 35 also reported a positive blank result with their procedure control sample (only BPW).
Two laboratories (lab codes 2 and 27) did not follow the prescribed method EN ISO 6579-1:2017 for the analysis of animal feed samples by using only MSRV as selective enrichment medium instead of two selective enrichment media (MKTTn and either RVS or MSRV).
The EURL-*Salmonella* will contact these laboratories for further explanations.

Because of the unexpected low level of *Salmonella* in the final chicken feed samples it is not possible to evaluate the performance of the laboratories for the detection of *Salmonella* in the 'positive' chicken feed samples.

The sensitivity rates also showed very low percentages, especially for the low contaminated samples (only 5%, Table 5). The high contaminated samples could have been evaluated as the low contaminated samples as the sensitivity rate was approx. 50% (Table 5), indicating a final level in the feed samples close to the detection limit.
Due to these low contamination levels, it was decided not to set criteria for the analysis of the feed samples artificially contaminated with *Salmonella*, but only to compare the number of positive samples found per participant with the mean number of positive samples found by all participants. Keeping in mind that the change of finding six high level samples negative is still 1.2%.

Table 4 Correct scores of the control samples

Control samples	n=35	**MKTTn and RVS or/and MSRV/ XLD or 2nd plate**
Procedure control Blank (BPW) n=1	No. of samples No. of negative samples **Correct score in %**	35 34 **97**
Positive control (Own *Salmonella*) n=1	No. of samples No. of positive samples **Correct score in %**	35 32 **91**
All Control samples	No. of samples No. of correct samples **Accuracy in %**	70 66 **94**

Table 5 Specificity, sensitivity and accuracy rates of the artificially contaminated chicken feed samples

Chicken feed	n=35	**MKTTn and RVS or/and MSRV/ XLD or 2nd plate**
Blank n=6	No. of samples No. of negative samples **Specificity in %**	210 207 **99**
Low level n=6	No. of samples No. of positive samples **Sensitivity in %**	210 11 **5**
High level n=6	No. of samples No. of positive samples **Sensitivity in %**	210 109 **52**
All chicken feed samples with *Salmonella*	No. of samples No. of positive samples **Sensitivity in %**	420 120 **29**
All chicken feed samples	No. of samples No. of correct samples **Accuracy in %**	630 327 **52**

List of abbreviations

BL	Blank-No colony forming units
BPW	Buffered Peptone Water
CFU	colony forming units
EFTA	European Free Trade Associations
EURL	European Union Reference Laboratory
ISO	International Standardisation Organisation
MKTTn	Mueller Kauffmann Tetrathionate novobiocin broth
MPN	Most Probable Number
MS	Member State
MSRV	Modified Semi-solid Rappaport Vassiliadis
NRL	National Reference Laboratory
RVS	Rappaport Vassiliadis Soya broth
SMb	*Salmonella* Mbandaka
XLD	Xylose Lysine Deoxycholate agar

References

EN ISO 6579-1:2017. Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of *Salmonella* - Part 1: Detection of *Salmonella* spp..

ISO 6887-1 & 4:2017 Microbiology of food and animal feeding stuffs – Preparation of test samples, initial suspension and decimal dilutions for microbiological examination. Part 1: General rules for the preparation of the initial suspension and decimal dilutions. Part 4: Specific rules for the preparation of miscellaneous products International

EN ISO 6579: 2002. Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.

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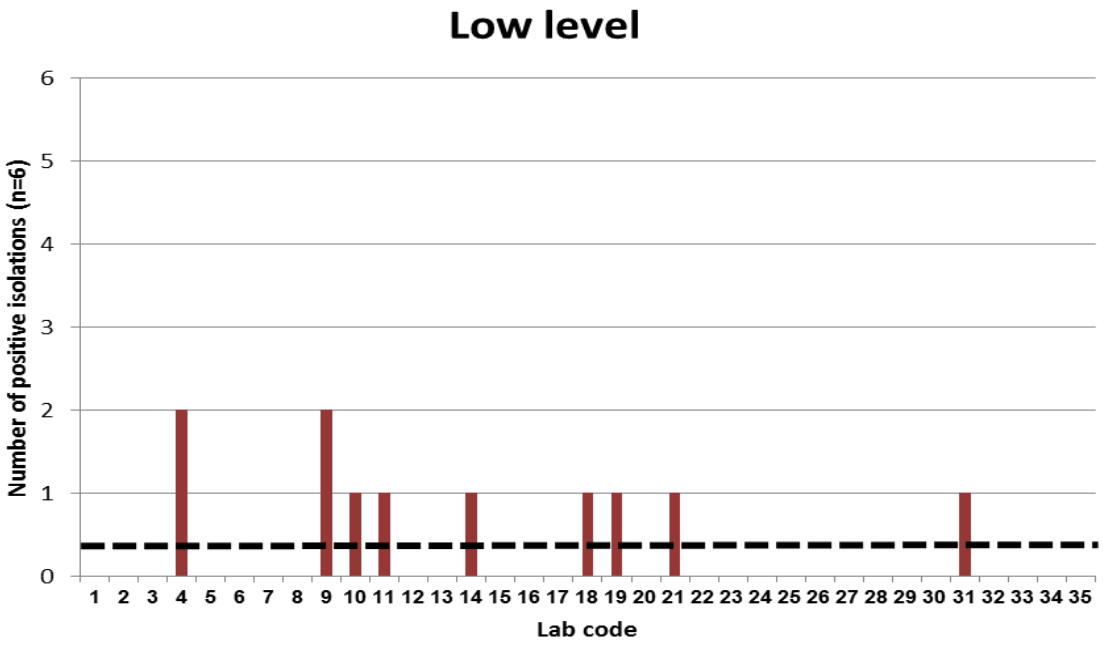
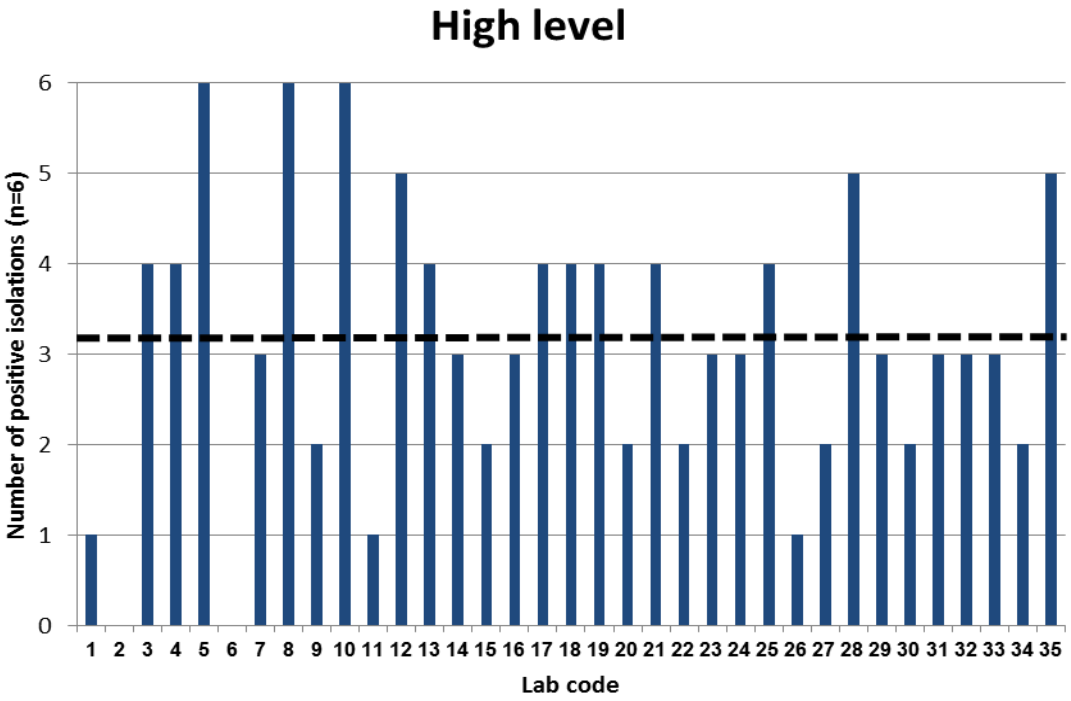


---- *mean number of positive samples found by all participants*

Figure 1 Number of positive isolations per laboratory after analysing 6 samples of each 25 g chicken feed artificially contaminated with low level Salmonella *Mbandaka. Results concern all possible combinations of media (MKTTn and RVS or/and MSRV) giving the highest number of positive samples.*



---- *mean number of positive samples found by all participants*

Figure 2 Number of positive isolations per laboratory after analysing 6 samples of each 25 g chicken feed artificially contaminated with high level Salmonella *Mbandaka. Results concern all possible combinations of media (MKTTn and RVS or/and MSRV) giving the highest number of positive samples.*