STANDARD OPERATING PROCEDURE (SOP)
INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN ANIMAL FEED SAMPLES organised by EURL-*Salmonella*
STUDY III - 2014

1 Scope and field of application
This standard operating procedure (SOP) describes the procedure for the detection of *Salmonella* in the presence of competitive micro-organisms in animal feed sample. The matrix is chicken feed. The application of this SOP is limited to the interlaboratory comparison study for *Salmonella* described in this SOP.

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

ISO 6579:2002/Amd 1 2007. Amendment 1 Annex D: Detection of *Salmonella* spp. in animal faces and in environmental samples from the primary production stage.

International Standard – ISO 6887-4: 2003 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.

3 Definitions
For the purpose of this SOP, the following definition applies:

Salmonella: micro-organisms which form typical colonies on isolation media for *Salmonella* and which display the serological and/or biochemical reactions described when tests are carried out in accordance with this SOP.

4 Principle
The detection of *Salmonella* involves the following stages:
1) Pre-enrichment
2) Selective enrichment
3) Isolation
4) Confirmation of typical colonies as *Salmonella*.

5 List of abbreviations

BPW	Buffered Peptone Water
MKTTn	Muller Kauffmann Tetrathionate novobiocin broth
MSRV	Modified semi-solid Rappaport Vassiliadis medium
RVS	Rappaport Vassiliadis medium with Soya
SOP	Standard Operating Procedure
XLD	Xylose Lysine Deoxycholate agar

6 Culture media

For this study the prescribed method is ISO 6579, additional it is requested also to apply Annex D of ISO 6579. The following media are needed.

Non selective pre-enrichment medium	BPW
Selective enrichment media	MKTTn & RVS (prescribed) MSRV (requested)
Selective plating out media for first and second isolation	XLD and a second medium for choice (obligatory!)

Composition and preparation of the media and reagents are described in Annex B, and in Annex D of ISO 6579: 2002. In the list of media given in 6.1 up to 6.4, reference is made to the relevant parts of ISO 6579. Complete ready-to-use media or dehydrated media are also allowed to be used, as long as the composition is in accordance with the information given below. Check the quality of the media before use.
In addition to the prescribed and requested method (ISO 6579 and Annex D of ISO 6579) laboratories can report their PCR results (optional).

6.1 Non selective pre-enrichment medium

- Buffered Peptone water (BPW) (ISO6579 Annex B.1)

6.2 Selective enrichment media

- Rappaport Vassiliadis medium with soya broth (RVS) (ISO6579 Annex B.2)
- Muller Kauffmann tetrathionate-novobiocin broth (MKTTn) (ISO6579 Annex B.3)
- Modified Semi solid Rappaport Vassiliadis (MSRV) (requested) (ISO6579 Annex D)

6.3 Solid selective media for first and second isolation

- Xylose-Lysine-Deoxycholate (XLD) agar (90 mm plates) (ISO6579 Annex B.4)
- Second isolation medium for choice (obligatory)

6.4 Confirmation media

- Biochemical and/or serological confirmation as described in ISO 6579 Annex B.6-B.11 or by reliable, commercially available identification kits.
- Nutrient agar (optional) (ISO6579 Annex B.5)

7 Apparatus and glassware

The usual microbiological laboratory equipment. Note specifications of the apparatus and glassware.

7.1 Apparatus

- Oven (for dry sterilisation) or autoclave (for wet sterilisation);
- Water bath or incubator, capable of operating at 37 °C ± 1 °C;
- Water bath or incubator, capable of operating at 41.5 °C ± 1 °C;
- Sterile loops of 1 μl and of 10 μl;
- pH-meter; having an accuracy of calibration of ± 0.1 pH unit at 25 °C.

7.2 Glassware

- Sterile micro-pipettes; nominal capacity 0.1 ml and 1 ml;
- Sterile petri dishes; standard size (diameter 90 mm to 100 mm) and/or large size (diameter 140 mm).

8 Procedure

Below the prescribed and requested method of the third interlaboratory comparison study in animal feed samples (chicken feed) of EURL-*Salmonella* is described. The different steps in the procedure are also summarized in Annex A of this SOP. Please record all relevant data. Details of the method can be found in ISO 6579 and Annex D of ISO 6579. For testing the samples, use as much as possible the materials you are normally using for your routine samples.

We ask you to perform the controls (especially the positive control) your laboratory is normally using for analysing routine samples for the detection of *Salmonella*. Three plastic bags numbered C1 to C3 are included in your parcel (C2 contains 25 g of chicken feed, C1 and C3 are empty).
We ask you to perform the following controls:

- C1: BPW (225 ml) only
- C2 :chicken feed (to which BPW need to be added)
- C3 : positive control as routinely performed in your laboratory (e.g.a reference material or a (diluted) culture containing *Salmonella*) added to 225 ml BPW.

Record the requested data of the three control samples through the web based reporting form.

8.1 Pre-enrichment (day 1, Monday 6 October 2014)

Use BPW equilibrated to at least room temperature (follow your routine procedure).
Record the requested data of BPW.

Take the numbered plastic bags (‘Whirl-pak’).
Add 225 ml BPW to each plastic ‘Whirl-pak’ bag (B1- B18 and C1-C3).

Add your own positive control to plastic 'Whirl-pak' bag C3 or mark your positive control as C3.

Before proceeding, ensure that the chicken feed is at the bottom of the plastic 'Whirl-pak' bag immersed completely in the BPW. If necessary, knead the bag to immerse the chicken feed completely.

Leave the samples to stand for 20 to 30 minutes at 18 °C to 27 °C (room temperature) (according ISO 6887-4: 2003)

Mix the samples by using a pulsifier, stomacher (rotary blender) or shake the samples carefully.
NOTE: Hard materials (e.g. grains) may puncture bags for a peristaltic homogenizer; double bagging may prevent this.

Incubate all samples at 37 °C ± 1 °C for 18 h ± 2 h. Record the temperature and time at the start and at the end of the incubation period and other requested data.

8.2 Selective enrichment (day 2, Tuesday 7 October 2014)

Allow the selective enrichment broths RVS and MKTTn (prescribed method) to equilibrate to room temperature, if they were stored at a lower temperature. Dry the surface of the MSRV plates (requested method), if necessary. Record the requested data of the selective enrichment broths (RVS and MKTTn) and MSRV plates.

Label 21 RVS tubes, MKTTn tubes and MSRV plates as follow:

- 18 tubes/plates from B1 to B18
- 3 tubes/plates from C1 to C3 (control)

After equilibration:
Prescribed methods:

- Transfer 0.1 ml of each BPW culture to each tube containing 10 ml RVS medium. Incubate at 41.5 °C ± 1 °C for 24 h ± 3 h.
- Transfer 1 ml of each BPW culture to each tube containing 10 ml MKTTn medium. Incubate at 37 °C ± 1 °C for 24 h ± 3 h.

Requested method:

- Inoculate the MSRV plates with 0.1 ml of each BPW culture as 1-3 equally spaced spots on the surface of the medium. Incubate **(not upside down)** at 41.5 °C ± 1 °C for 24 h ± 3 h and if negative for another 24 h ± 3 h.

Record the temperature and time at the start and at the end of the incubation period and other requested data.

8.3 Isolation media (first and second isolation, day 3 and 4, Wednesday 8 October and Thursday 9 October 2014)

Record the requested data of the isolation media.

First isolation after 24 h

Inoculation:

Inoculate, by means of a 10 µl loop, from MKTTn and RVS cultures the surface of isolation media in large size Petri dishes (or two standard size Petri dishes) with the corresponding label numbers. Inoculate, by means of a 1 µl loop from suspect MSRV plates, the surface of isolation media in one standard size Petri dish with the corresponding label numbers. Inoculate the isolation media in such a way that isolated colonies will be obtained.
The following isolation media will be used:

1) Xylose Lysine Deoxycholate agar (XLD)
   Place the Petri dishes with the bottom up in the incubator set at 37 °C ± 1 °C (for 24 h ± 3 h).
2) Second isolation medium. Follow the instructions of the manufacturer.

Record temperature and time and other requested data of the isolation media.

After incubation for 24 h ± 3 h, examine the isolation media for the presence of typical or suspect colonies of *Salmonella*.

Second isolation after 48 h

If the MSRV plates do not show suspect growth after 24 h incubation, return them to the incubator (41,5 °C) for another 24 h.
After a total incubation time of 48 h ± 3 h of the MSRV plates repeat the procedure described above (**First isolation after 24 h**). Repeat the full procedure only when the First isolation after 24 h of MSRV is negative.

8.4 Confirmation of colonies from first and second isolation (day 4 and day 5, Thursday 9 October and Friday 10 October 2014)

For confirmation, take from each Petri dish of each isolation medium at least 1 colony considered to be typical or suspect (use only well isolated colonies). Store the plates at 5 ^{o}C ± 3 ^{o}C.
Before confirmation, optionally, streak the typical colonies onto the surface of nutrient agar plates with the corresponding label numbers, in a manner which allows to develop well isolated colonies. Incubate the inoculated plates at 37 °C ± 1 °C for 24 h ± 3 h.

If the selected colony is not confirmed as *Salmonella*, test a further 4 typical colonies from the original isolation media (stored at 5 °C). Record the results for each dish.
Table 1: isolation from RVS
Table 2: isolation from MKTTn
Table 3: isolation from MSRV
For the results of detection of *Salmonella* when using PCR, complete Table 4.

Confirmation of identity

The identity from the colonies selected above (either direct from the isolation medium, or from nutrient agar) is confirmed by means of appropriate biochemical and serological tests. Follow the instructions of ISO 6579. Record which media and tests have been used for confirmation.

Store one positive isolate (*Salmonella* strain) from each sample.

After the interlaboratory comparison study, it may be necessary to perform some additional tests (in case of deviating results). Therefore it is requested to store at least one *Salmonella* confirmed colony from at least one of the used isolation media of each used selective enrichment media from each suspect sample B1-B18 and C1-C3.

9 Reporting of the results

This is the first animal feed EURL-*Salmonella* detection study for which all data have to be reported through an electronic reporting form. The web based report should contain all information that might influence the results and is not mentioned in this SOP. It is no longer necessary to report results of 'own' culture media. Results of an 'own' PCR method can still be recorded. Incidents or deviations from the specified procedures should also be recorded at the end of the test report (remarks/ comments). Furthermore, the name of the person in charge of the NRL, and the name(s) of the person(s) carrying out the work are requested to be recorded. If the study was carried out by another laboratory than the NRL, please also give the details of this laboratory.

We have made a selection of the most important questions in the web based test report, resulting in a shorter test report than before. As all NRLs are accredited according to ISO 17025, we assume that additional information, not requested in the test report, will be available in your system. In case of deviating results, the EURL-*Salmonella* may ask the NRLs to send this additional information.

Overview of Interlaboratory Comparison Study
Feed III (2014)
Detection of *Salmonella* spp. chicken feed

Day	Topic	Description
1	**Pre-enrichment**	Allow the BPW to equilibrate to at least room temperature Add 225 ml BPW to each plastic bag ('Whirl-pak') Leave 20 to 30 minutes at room temperature Mix (pulsifier or stomacher) sample Incubate at 37 °C ± 1 °C for 18 h ± 2 h
2	**Selective enrichment**	- 0.1 ml BPW culture in 10 ml RVS, incubate at 41.5 °C ± 1 °C for 24 h ± 3 h - 1 ml BPW culture in 10 ml MKTTn, incubate at 37 °C ± 1 °C for 24 h ± 3 h - 0.1 ml BPW culture on MSRV plate, incubate at 41.5 °C ± 1 °C for 24 h ± 3 h
3	**First isolation after 24 h**	Inoculate from RVS, MKTTn, suspect MSRV plates (24h): - XLD agar, incubate at 37 °C ± 1 °C for 24 h ± 3 h - Second isolation medium (obligatory) incubate at the specified temperature for specified time
3	**Continue selective enrichment**	Incubate negative MSRV plates another 24 h ± 3 h at 41.5 °C ± 1 °C
4	**Second isolation after 48 h**	If the first isolation was negative, inoculate from suspect MSRV (48h) plates: - XLD agar, incubate at 37 °C ± 1 °C for 24 h ± 3 h - Second isolation medium incubate at the specified temperature for specified time
4	**Confirmation of identity**	Confirm the identity of the *Salmonella* suspect colonies from the first isolation media (day 3).
5	**Confirmation of identity**	Confirm the identity of the *Salmonella* suspect colonies from the second isolation media (day 4).

Annex A of SOP

Interlaboratory Comparison Study
Feed III (2014)
Detection of *Salmonella* spp. in chicken feed