STANDARD OPERATING PROCEDURE (SOP)
INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN ANIMAL FEED
organised by EURL-*Salmonella*
FEED STUDY II- 2012

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 an animal feed matrix. For this purpose Reference Materials (RMs) containing *Salmonella* spp. as prepared by the Health Protection Agency (HPA, United Kingdom), are used. As matrix, animal feed (negative for *Salmonella*) is used. 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(E) Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.

International Standard – 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.

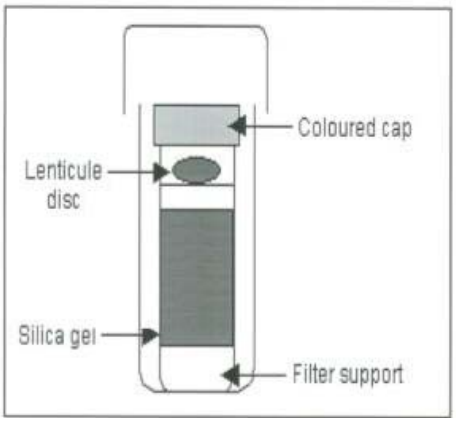
Lenticule Disc Handling Information. HPA Culture Collection, Salisbury, United Kingdom.
More information on the reference materials (lenticule discs) as produced by the HPA can be found on: http://www.hpacultures.org.uk/products/lenticulediscs/index.jsp

3 Definitions
For the purpose of this SOP, the following definitions apply:

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.

-Reference Material: a lenticule disc containing microorganism at a defined number in a water soluble matrix.

Note: Each lenticule is individually packed in small vials as indicated in the picture below.



HPA, UK

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
RM	Reference Material
RVS	Rappaport Vassiliadis medium with Soya
SOP	Standard Operating Procedure
XLD	Xylose Lysine Deoxycholate agar

6 Methods and culture media

For this study the prescribed method is ISO 6579.
Additional to ISO 6579, it is requested also to apply Annex D of ISO 6579.

Non selective pre-enrichment medium	BPW (6.1)
Selective enrichment medium	MKTTn & RVS (prescribed)(6.2) MSRV (requested) (6.2)
Selective plating medium for first and second isolation	XLD and a second medium for choice (obligatory!)(6.3)
Confirmation media	see 6.4

Composition and preparation of the media and reagents are described in Annex B, and in Annex D of the ISO 6579: 2002. In the list of media given in 6.1 up to 6.4, reference is made

to the relevant part 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 method (ISO 6579) and requested method (Annex D of ISO 6579) it is possible to use other methods, e.g. the one(s) routinely used in your laboratory ['Own' method(s)]. This can vary from another culture method to 'a PCR technique'. If necessary prepare media for the 'own' method(s) according to the relevant instructions. Record all relevant information in the test report.

6.1 Non selective pre-enrichment medium

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

6.2 Selective enrichment medium

- Rappaport Vassiliadis medium with soya (RVS broth) (ISO6579 Annex B.2)
- Muller Kauffmann tetrathionate-novobiocin broth (MKTTn) (ISO6579 Annex B.3)
- Modified Semi solid Rappaport Vassiliadis (MSRV) (requested) (ISO6579 Annex D)
- Own selective enrichment medium used in your laboratory (optional)

6.3 Solid selective media for first and second isolation

- Xylose-Lysine-Desoxycholate (ISO6579 Annex B.4)
- Second isolation medium for choice (obligatory)
- Own medium used in your laboratory (optional)

6.4 Confirmation media

- Biochemical confirmation as described in ISO 6579 Annex B.6-B.11 or by reliable, commercially available identification kits.
- Nutrient agar (optional) (ISO6579Annex B.5)

7 Apparatus and glassware

The usual used microbiological laboratory equipment. If requested, note specifications of the apparatus and glassware on the test report.

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 culture containers (bottles, jars or plastic bags) with nominal capacity of approximately 400 ml;
- 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 the requested method of the interlaboratory comparison study in an animal feed matrix of EURL-*Salmonella* is described. The different steps in the procedure are also summarized in Annex A of this SOP. In addition to these methods it is also allowed to use one or more own methods. Please record all relevant data in the test report. Details of the prescribed method can be found in ISO 6579. Details of the requested method can be found in Annex D of ISO 6579 (2007).
For testing the samples use as much as possible the materials you are normally using for your routine samples. For example, use either bags or jars for the pre-enrichment in BPW depending on what you routinely use. Bottles, bags or jars for the pre-enrichment in BPW are further mentioned as containers.

8.1 Pre-enrichment (day 1)

Use BPW equilibrated to at least room temperature (follow your routine procedure).
Record in the test report (page 2) the requested data on BPW.

Take the numbered vials with the *Salmonella* lenticules out of the freezer, 10-15 minutes before they are added to the BPW, to allow them to equilibrate to room temperature.

-Label 25 containers as follow:

- 18 containers from B1 to B18
- 7 containers from C1 to C7 (control)

Add 25 g of chicken feed to each container labelled B1 – B18 and C7.
Add 225 ml BPW to each container (B1- B18 and C1-C7).
When your containers are already pre filled with 225 ml BPW, add 25 g of chicken feed to the BPW.
Add no matrix/ feed to the containers labelled C1 – C6.
One container is a procedure control to which no lenticule or feed is added (= C6).
One container is the negative feed control to which only 25 g animal feed is added (= C7).
These control conainers should be handled in the same way as the other containers.

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

Add to the 23 labelled containers (containing BPW with or without animal feed) a lenticule disc from the vial with the corresponding label number (B1- B18 and C1 – C5).
No lenticules are added to C6 and C7.

Leave all containers for an additional 10 – 15 minutes at room temperature to re-hydrate the lenticule. As the disc is coloured, it may be visible when it is re-hydrated. Even when it is not

visible whether the lenticule is re-hydrated, proceed with the next steps of the procedure after at maximum 15 minutes additional standing at room temperature.

Mix the samples by using a pulsifier or a stomacher (rotary blender). When this is not possible (e.g. when the samples are contained in a jar), shake the samples carefully.
NOTE: Hard materials (e.g. grains) will 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 on page 2 of the test report.

If PCR is performed, record all requested data on pages 9 & 13 of the test report.

8.2 Selective enrichment (day 2)

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 (page 3 -5) the requested data of the selective enrichment broths (RVS and MKTTn), MSRV plates and own selective enrichment media (if used) in the test report.
-Label 25 RVS tubes, MKTTn tubes and MSRV plates as follow:

- 18 tubes/plates from B1 to B18
- 7 tubes/plates from C1 to C7 (control)

If other selective enrichment media are used, label them in the same way as described above.

After equilibration:
Prescribed methods:

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

Requested method:

- Inoculate each MSRV plate with three drops of each BPW culture with a corresponding label. Inoculate a MSRV plate with a total volume of 0.1 ml. Incubate **(not upside down)** at 41.5 °C ± 1 °C for 24 h ± 3 h and if negative for another 24 h ± 3 h.

Optional method:

- Inoculate the routinely used selective medium/media (other than those mentioned above), with the corresponding BPW culture (note the inoculation volume of BPW used and the volume of the selective medium/media on the test report). Incubate at the temperature routinely used.
- Place the jars/tubes/plates in the appropriate incubator(s)/water bath(s) and record the temperature and time for the different enrichment media at the start and at the end of the incubation period and other requested data in the test report (pages 3-5).

8.3 Isolation media (first and second isolation) (day 3 and 4)

Record in the test report (pages 6-7) the requested data of the isolation media used.
Allow the isolation media to equilibrate to room temperature, if they were stored at a lower temperature. Dry the surface of the plates, if necessary.

Label three times 25 Petri dishes of each isolation medium from B1 to B18 and C1 to C7.

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 Desoxycholate agar (XLD)
   Incubate the inoculated plates (with the bottom up) at 37 °C ± 1 °C. Record temperature and time and other requested data in the test report, page 6.
2) Second isolation medium. Follow the instructions of the manufacturer. Record temperature and time and other requested data in the test report, page 7.
3) Optionally: additionally selective isolation medium/media routinely used in your laboratory. Incubate the medium/media at the temperature routinely used and record temperature and time and other requested data in the test report, page 7.

After incubation for 24 h ± 3 h, examine the Petri dishes for the presence of typical colonies of *Salmonella*.

Second isolation after 48 h

After a total incubation time of 48 h ± 3 h of the selective enrichment medium MSRV, repeat the procedure described above (**First isolation after 24 h**). Repeat the full procedure only when the First isolation after 24 h on MSRV is negative.

8.4 Confirmation of colonies from first and second isolation (day 4 and day 5)

For confirmation, take from each Petri dish of each selective medium, at least 1 colony considered to be typical or suspect (use only well isolated colonies).

Store the plates at 5 °C ± 3 °C.
Before confirmation (see below), 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. Record on the test report (page 8) the requested data of the nutrient agar. Incubate the inoculated plates at 37 °C ± 1 °C for 24 h ± 3 h.
If the selected colony is not confirmed as *Salmonella*, test at maximum another 5 typical colonies from the original isolation medium (stored at 5 °C). You do not need to mention the numbers of colonies tested. Mention the final result of your analyse for each petri-dish: “1” for a positive result (when colonies are confirmed as *Salmonella*) and “0” for a negative result.
Report the results in the Tables 1-3 on page 10-12.
Table 1: isolation from RVS & MKTTn
Table 2: isolation from MSRV
Table 3: isolation from own selective enrichment medium
For the results of detection of *Salmonella* when using PCR, complete Table 4 on the test report at page 13.

Confirmation of identity
The identity from the colony selected above (either directly from the isolation medium, or from nutrient agar) is confirmed by means of appropriate biochemical and serological tests. Follow the instructions of ISO 6579. Note in the test report (page 8) which media/tests have been used for confirmation. The interpretation of the biochemical tests is given in Table 1 of ISO 6579:2002 on page 9. Optionally inoculate other media which are routinely used for confirmation. Record the requested data in the test report (page 8).

Conserve one positive isolate (*Salmonella* strain) from each sample.
After the interlaboratory comparison study it may be necessary to perform some additional testing (in case of deviating results). Therefore it is requested to conserve one *Salmonella* confirmed colony from one of the used isolation media of each of the used selective enrichment media from each suspect sample B1-B18 and C1-C7.

9 Test report

Record in the test report all information that might influence the results and is not mentioned in this SOP. Also record incidents or deviations from the specified procedures. The test report should include the name of the person in charge for the NRL, and the names of the persons who are carrying out the work. If the study was carried out by another laboratory than the NRL, please also give the details of this laboratory in the test report.

Scheme of Bacteriological Interlaboratory Comparison Study FEED II (2012) On detection of *Salmonella* spp. in chicken feed (see Annex A)

Day	Topic	Description
1	**Pre-enrichment**	Allow the BPW to equilibrate to at least room temperature Add 25 g chicken feed to container (jar or plastic bag) Add 225 ml BPW to chicken feed (or add 25 chicken feed to 225 ml BPW) Leave 20 minutes at room temperature Add 1 lenticule disc to BPW Leave 10- 15 minutes at room temperature Mix (pulsifier or stomacher) sample Incubate (18 h ± 2) h at (37 °C ± 1) °C
2	**Selective enrichment**	0.1 ml BPW culture in 10 ml RVS, incubate at (41.5 ± 1) °C for (24 ± 3) h 1 ml BPW culture in 10 ml MKTTn, incubate at (37 ± 1) °C for (24 ± 3) h 0.1 ml BPW culture on MSRV plate, incubate at (41.5 ± 1) °C for (24 ± 3) h Own selective enrichment medi(um)(a)
3	**First isolation after 24 h**	Inoculate from RVS, MKTTn, suspect MSRV plates (24h) and own medi(um)(a): • Xylose Lysine Desoxycholate agar, incubate at (37 ± 1) °C for (24 ± 3) h • Second isolation medium* (obligatory) • Own selective medi(um)(a)* (optional) * incubate for specified time at the specified temperature
3	**Continue selective Enrichment**	Incubate MSRV another 24 (± 3) hours at (41.5 ± 1) °C
4	**Second isolation after 48 h**	If the first isolation was negative, inoculate from suspect MSRV plates (48 h): • Xylose Lysine Desoxycholate agar, incubate at (37 ± 1) °C for (24 ± 3) h • Second isolation medium* • Own selective medi(um)(a)* * incubate for specified time at the specified temperature
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 II
on the detection of *Salmonella* spp. in chicken feed
EURL-*Salmonella* 2012