STANDARD OPERATING PROCEDURE (SOP)

INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN CHICKEN FAECES organised by EU-RL-*Salmonella*

STUDY XIV - 2011

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 chicken faeces. For this purpose Reference Materials (RMs) containing *Salmonella* Typhimurium (STM) or *Salmonella* Enteritidis (SE) as prepared by the Health Protection Agency (HPA, United Kingdom) are used. As matrix, chicken faeces 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

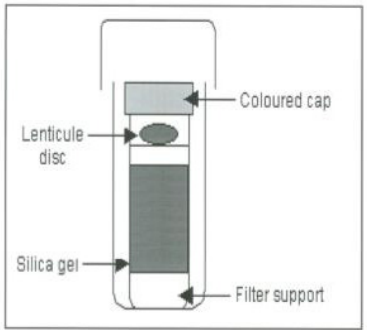
International Standard – ISO 6579: 2002(E) Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.

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.

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 figure below.



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
MSRV	Modified semi-solid Rappaport Vassiliadis medium
RM	Reference Material
SOP	Standard Operating Procedure
XLD	Xylose Lysine Deoxycholate agar

6 Culture media

For this study the prescribed method is the procedure as described in Annex D of ISO 6579, for which the following media are needed.

Non selective pre-enrichment medium	BPW
Selective enrichment medium	MSRV
Selective plating medium 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 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 (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

-Modified Semi solid Rappaport Vassiliadis (MSRV) (ISO6579 Annex D)
-Own selective enrichment medium routinely used in your laboratory (optional)

6.3 Solid selective media for first and second isolation

-Xylose-Lysine-Desoxycholate (XLD) agar (90 mm plates) (ISO6579 Annex B.4)
-Second isolation medium of 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) (ISO6579 Annex B.5)

7 Apparatus and glassware

The usual 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;
-pH-meter; having an accuracy of calibration of ± 0.1 pH unit at 25 °C.

7.2 Glassware

-Culture containers (bottles, jars or plastic bags) with nominal capacity of approximately 400 ml;
-Culture tubes with approximate sizes: 8 mm in diameter and 160 mm in length;
-Micro-pipettes; nominal capacity 0.1 ml;
-Petri dishes; standard size (diameter 90 mm to 100 mm)

8 Procedure

Below the prescribed method of the fourteenth interlaboratory comparison study in chicken faeces of EU-RL-*Salmonella* is described. The different steps in the procedure are also summarized in Annex A of this SOP. In addition to this method it is also allowed to use one or more own methods. Please record all relevant data in the test report. 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. For example, either use 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 (pages 2 & 3) 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 34 containers as follow:

- 25 containers from B1 to B25
- 9 containers from C1 to C9 (control lenticules)

-Add 25 g of faeces to each container labelled B1 – B25 and C9.
-Add 225 ml BPW to each container (B1- B25 and C1-C9).
When your containers are already pre filled with 225 ml BPW, add 25 g of faeces to the BPW.

Add no faeces to the containers labelled C1 – C8.
One container is a procedure control to which no lenticule or faeces is added (= C8).
One container is the negative faeces control to which only 25 g faeces is added (= C9).
These control conainers should be handled in the same way as the other containers.

-Add to the 32 labelled containers (containing BPW with or without faeces) a lenticule disc from the vial with the corresponding label number (B1- B25 and C1 – C7).
No lenticules are added to C8 and C9.

- Leave all the containers for 10 – 15 minutes at room temperature to re- hydrate the lenticule. Before proceeding, ensure that the disc is completely dissolved. 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 15 minutes standing at room temperature.

-Gently mix the samples: shake carefully when your samples are in a jar or knead shortly when the samples are in a plastic bag. (The use of a pulsifier or stomacher is not advisable as the chicken faeces may contain sharp particles).

-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 3 of the test report.

If PCR is performed, record all requested data on pages 16-17 & 22 of the test report.

8.2 Selective enrichment (day 2)

Allow the MSRV plates to equilibrate to room temperature, if they were stored at a lower temperature. Dry the surface of the MSRV plates in a Laminair Air Flow cabinet if necessary. Record (pages 4-7) the requested data of the MSRV and own selective enrichment media (if used) in the test report.

-Label 34 MSRV plates as follow:

- 25 plates from B1 to B25
- 9 plates from C1 to C9 (control)

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

After equilibration of the media:

Prescribed method:

-Inoculate the MSRV plates with three drops of BPW culture, 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 and for the time 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 4-7).

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

Record in the test report (pages 8-13) the requested data of the isolation media used. Label 38 (standard size) Petri dishes of each isolation medium from B1 to B25 and C1 to C9.

First isolation after 24 h

Inoculation:

Inoculate from suspect MSRV plates, the surface of an isolation medium in one standard size Petri dish with the corresponding label number in such a way that well isolated colonies will be obtained. The following isolation media will be used:

1) Xylose Lysine Desoxycholate agar (XLD)
   Place the Petri dishes with the bottom up in the incubator set at 37 °C (record temperature and time and other requested data in the test report, pages 8 & 9).

2) Second isolation medium. Follow the instructions of the manufacturer (record temperature and time and other requested data in the test report, pages 10 & 11).

3) Optional: selective isolation medium/media routinely used in your laboratory. Incubate the medium/media at the temperature and for the time routinely used (record temperature and time and other requested data in the test report, pages 12 &13).

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 MSRV and, if relevant of own selective enrichment media, repeat the procedure described above (**First isolation after 24 h**). Repeat the full procedure only when the First isolation after 24 h of MSRV, and if relevant from own selective enrichment media, 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 isolation 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 14) 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 a further 4 typical colonies from the original isolation medium (stored at 5 °C). Report the number of colonies tested (in the column named 'col') and the number of colonies confirmed as *Salmonella* (in the column 'sal') for each dish in Table 1 (isolation using MSRV) and Table 2 (isolation using own enrichment) on the test report (pages 18-21).

If a PCR method has been used, report the results in Table 3 of the test report (page 22).

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 15) 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 in the test report (page 15) the requested data.

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 medium from the samples B1-B25 and C1-C9.

9 Test report

The test report should contain all information that might influence the results and is not mentioned in this SOP. Incidents or deviations from the specified procedures should also be recorded. The test report should include the name of the person in charge of 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 ANIMAL FAECES XIV (2011) on the detection of *Salmonella* spp. in chicken faeces		
Day	Topic	Description
1	**Pre-enrichment**	Allow the BPW to equilibrate to at least room temperature Add 25 g faeces to container (jar or plastic bag) Add 225 ml BPW to faeces (or add 25 faeces directly to 225 ml BPW) Add 1 lenticule disc to BPW Leave 10- 15 minutes at room temperature Mix or shake gently Incubate (18 h ± 2) h at (37 °C ± 1) °C
2	**Selective enrichment**	0.1 ml BPW culture on MSRV plate, incubate at (41.5 ± 1) °C for (24 ± 3) h Own selective enrichment medium/ media
3	**First isolation after 24 h**	Inoculate from suspect MSRV (24h) plates and from Own selective medium/ media – XLD 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 medium and if necessary Own medium/ media another 24 (± 3) hours at the relevant temperatures
4	**Second isolation after 48 h**	If the first isolation was negative, inoculate from suspect MSRV (48h) plates and if relevant from Own medium/ media – XLD agar, incubate at (37 ± 1) °C for (24 ± 3) h – Second isolation medium* – Own selective medium/ media* *=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 animal faeces XIV
on the detection of *Salmonella* spp.
EU-RL-*Salmonella* 2011