STANDARD OPERATING PROCEDURE (SOP)
INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN SAMPLES FROM THE PRIMARY PRODUCTION STAGE
organised by EURL-*Salmonella*
STUDY XVIII - 2015

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 samples from the primary production stage. The matrix used in this study is pork faeces. 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 faeces and in environmental samples from the primary production stage.

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 biochemical and/or serological 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
MSRV	Modified semi-solid Rappaport Vassiliadis medium
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) laboratories can report their PCR results.

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-agar) (ISO6579 Annex D)

6.3 Solid selective media for first and second isolation

- Xylose-Lysine-Deoxycholate (XLD) agar (ISO6579 Annex B.4)
- Second isolation medium of 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;
- pH-meter; having an accuracy of calibration of ± 0.1 pH unit at 25 °C.

7.2 Glassware

- 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 eighteenth interlaboratory comparison study on detection of *Salmonella* in samples from the primary production stage (pork faeces) 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 normally use 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 pork faeces, C1 and C3 are empty).
We ask you to perform the following controls:

- C1: BPW (225 ml) only
- C2: pork faeces (to which 225 ml BPW need to be added)
- C3: positive control as routinely performed in your laboratory (e.g. 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 16 March 2015)

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

Take the numbered plastic bags (‘Whirl-pak’) and leave at room temperature to defrost.
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 pork faeces is at the bottom of the plastic ‘Whirl-pak’ bag immersed completely in the BPW. If necessary, knead the bag to immerse the faeces completely.
Gently mix the samples: shake carefully or knead shortly. (The use of a stomacher is not advisable as the 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.

8.2 Selective enrichment (day 2, Tuesday 17 March 2015)

Allow the MSRV-agar plates to equilibrate to room temperature, if they were stored at a lower temperature. Dry the surface of the MSRV-agar in a Laminair Air Flow cabinet if necessary. Record the requested data of the MSRV.
Label 21 MSRV-agar plates as follow:

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

After equilibration of the plates:
Inoculate the MSRV-agar 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;
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 18 March and Thursday 19 March 2015)

Record the requested data of the isolation media used.

First isolation after 24 h

Inoculation:
Inoculate from suspected MSRV-agar, 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 have to 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 (record the requested data).
2) Second isolation medium. Follow the instructions of the manufacturer (record the requested data).

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

Second isolation after 48 h

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

8.4 Confirmation of colonies from first and second isolation (day 4 and day 5, Thursday 19 March and Friday 20 March 2015)

For confirmation, take from each Petri dish of each isolation medium at least 1 colony considered to be suspect or typical for *Salmonella* (use only well isolated colonies). Store the plates at 5 °C ± 3 °C.
Before confirmation (see below), optionally, streak the suspect or 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 suspect or typical colonies from the original isolation media (stored at 5 °C). Record the results for each dish in Table 1 of the web based test report.
For the results of detection of Salmonella when using PCR, complete Table 2.

<u>Confirmation of identity</u>
The identity from the colonies 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. Record which media and tests have been used for confirmation.

<u>Store one positive isolate (*Salmonella* strain) from each sample.</u>
After the interlaboratory comparison study, it may be necessary to perform some additional testing (in case of deviating results). Therefore, it is requested to store at least one *Salmonella* confirmed colony from one of the used isolation media of each suspect sample B1-B18 and C1-C3.

9 Reporting of the results

All data have to be reported through an electronic result 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 additional information.

Overview of Interlaboratory Comparison Study
Primary Production Stage, PPS XVIII (2015)
Detection of *Salmonella* spp. in pork faeces

Day	Topic	Description
1	**Pre-enrichment**	Allow the BPW to equilibrate to at least room temperature Defrost the faeces samples Add 225 ml BPW to each plastic bag ('Whirl-pak') Mix gently Incubate at 37 °C ± 1 °C for 18 h ± 2 h
2	**Selective enrichment**	0.1 ml BPW culture on MSRV-agar plate, incubate at 41.5 °C ± 1 °C for 24 ± 3 h
3	**First isolation after 24 h**	Inoculate from suspect MSRV-agar (24h) plates: - XLD agar, incubate at 37 °C ± 1 °C for 24 h ± 3 h - Second isolation medium* (obligatory) * Incubate for specified time at the specified temperature
3	**Continue selective enrichment**	Incubate negative MSRV-agar 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-agar (48h) plates: - XLD agar, incubate at 37 °C ± 1 °C for 24 h ± 3 h - Second isolation medium* * 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
Primary Production Stage, PPS XVIII (2015)
Detection of *Salmonella* spp. in pork faeces