STANDARD OPERATING PROCEDURE (SOP)

INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN FOOD organised by CRL-*Salmonella*

FOOD STUDY IV- 2010

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 a food matrix. For this purpose Reference Materials (RMs), containing sublethally injured *Salmonella* spp., as prepared by the Community Reference Laboratory (CRL) for *Salmonella,* are used. As matrix, minced meat (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.

Amendment 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 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 gelatine capsule containing a quantified amount of a test organism in spray dried milk.

4 Principle

The detection of *Salmonella* involves the following stages:
a) Pre-enrichment
b) Selective enrichment
c) Isolation
d) Confirmation of typical colonies as *Salmonella*.

5 List of abbreviations

BPW	Buffered Peptone Water
MKTTn	Muller Kaufmann 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, with an extra incubation step of 24 h of the selective enrichment media. 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(E). 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. Control the quality of the media before use.

Beside the prescribed method (ISO 6579) and requested method (Annex D of ISO 6579) it is allowed to use other methods, e.g. the one(s) routinely used in your laboratory [‘Own’ method(s)]. 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)
  Distribute the BPW in portions of **225 ml** into suitable flasks before sterilisation.

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 (optionally)

6.3 Solid selective media for first and second isolation

- Xylose-Lysine-Desoxycholate (140 mm and 90 mm plates) (ISO6579 Annex B.4)
- Second isolation medium for choice (obligatory)
- Own medium used in your laboratory (optionally)

6.4 Confirmation media

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

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

- Culture bottles or jars with nominal capacity of 300 ml;
- Culture tubes with approximate sizes: 8 mm in diameter and 160 mm in length;
- Micro-pipettes; nominal capacity 0.1 ml and 1 ml;
- 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 a food matrix of CRL-*Salmonella* is described. The different steps in the procedure are also summarized in Annex A of this SOP. Beside 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).

8.1 Prewarming BPW (day 0)

Label 31 jars, each containing 225 ml of BPW as follow:

- 24 jars from 1 to 24
- 7 jars from C1 to C7 (control capsules)

Place all jars (at least) **overnight** at 37 °C (± 1 °C). Also place some extra non-labelled jars containing 225 ml of BPW at 37 °C in case some jars might have been contaminated. Record in the test report (page 2 & 3) the requested data on BPW.

8.2 Pre-enrichment (day 1)

Take the numbered vials with the *Salmonella* capsules and the control capsules out of the freezer one hour before they are added to the BPW, to allow them to equilibrate to room temperature.
Shortly before adding the capsules, take the jars with BPW from the 37 °C incubator and inspect them for visual growth. Discard infected jars.
Add to 29 labelled jars a gelatine capsule from the vial with the corresponding label number. Do not open the gelatine capsule and do not shake the BPW to dissolve the capsule more rapidly. Place the jars with the capsules in the 37 °C incubator for **45 minutes** for dissolving of the capsules. Record the temperature and time at the start and at the end of this period in the test report (page 3).
After 45 minutes add the faeces to the jars according to the following scheme:

- **Add 25 g of meat to each jar labelled 1-24 and C7,**
- **Add no meat to jars labelled C1 – C6,**

Do not shake the jars after adding the meat.

One jar is a procedure control (= C6) to which no capsule or meat is added and one jar is a negative meat control to which only 25 g meat is added (= C7). These control jars should be handled in the same way as the other jars.

Place all jars in the 37 °C (± 1 °C) incubator 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, fill in all requested data on page 20 & 29 of the test report.

8.3 Selective enrichment (day 2)

Allow the selective enrichment broths RVS and MKTTn (prescribes method) to equilibrate to room temperature, if they were stored at a lower temperature. Dry the surface of the MSRV plates (requested method) in a Laminair Air Flow cabinet if necessary. Record (page 4-11) 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 31 RVS tubes, MKTTn tubes and MSRV plates as follow:

- 24 tubes/plates from 1 to 24
- 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 and later on for another 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 and later on for another 24h ± 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 (page 4-11).

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

Record in the test report (page 12-17) the requested data of the isolation media used. Label two times 24 large size Petri dishes and 24 standard size Petri dishes of the isolation media from 1 to 24 and label two times 7 large size Petri dishes and 7 standard size Petri dishes from C1 to C7.

Note:
In case you do not have large dishes (140 mm) at your disposal use two standard size (90 - 100 mm) dishes, one after the other, using the same loop.

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. Use a 1 µl loop to inoculate from suspect MSRV plates, the surface of isolation media in one standard size Petri dish with the corresponding label number. 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)
   Place the inoculated plates with the bottom up in the incubator set at 37 °C (record temperature and time and other requested data in the test report, page 12-13).
2) Second isolation medium. Follow the instructions of the manufacturer (record temperature and time and other requested data in the test report, page 14-15).
3) Optionally: selective isolation medium/media routinely used in your laboratory. Incubate the medium/media at the temperature routinely used (record temperature and time and other requested data in the test report, page 16-17).

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 media, repeat the procedure described above (**First isolation after 24 h**). Repeat the full procedure only when the First isolation after 24 h on selective enrichment media is negative.

8.5 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 18) 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). Report the number of colonies tested and the number of colonies confirmed as *Salmonella* for each dish in Table 1 (isolation using RVS), Table 2 (isolation using MKTTn), Table 3 (isolation using MSRV) and Table 4 (isolation using own enrichment) on the test report pages 21-28. For the results of detection of *Salmonella* using PCR fill in Table 5 on the test report page 29.

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 19) 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 19) 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 1-25 and C1-C7.

9 Test report

The test report will contain all information that might influence the results and is not mentioned in this SOP. Some incidents or deviations from the specified procedures will also be recorded. 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 FOOD IV (2010) On detection of Salmonella spp. in minced meat (see Annex A)		
Day	Topic	Description
0	**Prewarming BPW**	Place at least at the end of the day sufficient jars, each containing 225 ml BPW, at 37 °C ± 1 °C.
1	**Pre-enrichment**	Add 1 capsule to 225 ml (prewarmed) BPW Do not shake Incubate 45 min. at 37 °C ± 1 °C Add 25 g minced meat to BPW 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* • Own selective medi(um)(a)* * incubate for specified time at the specified temperature
3	**Continue selective Enrichment**	Incubate RVS, MKTTn, MSRV and own medium another 24 (± 3) hours at the relevant temperatures
4	**Second isolation after 48 h**	If the first isolation was negative, inoculate from RVS, MKTTn, suspect MSRV plates (48 h) and Own medi(um)(a): • 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 first isolation media (day 4).