# STANDARD OPERATING PROCEDURE (SOP)

## INTERLABORATORY COMPARISON STUDY IX (2005) ON THE DETECTION OF *SALMONELLA* spp. organised by CRL-*Salmonella*

## 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. For this purpose Reference Materials (RMs) containing sublethally injured *Salmonella* Typhimurium (STM), *Salmonella* Enteritidis (SE) or *Salmonella* Panama (SPan) as prepared by the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*) are used. Furthermore poultry faeces 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.

Draft Amendment ISO 6579:2002/DAmd 1, 2005. Amendment 1 Annex D: Detection of *Salmonella* spp. in animal faces and in samples from the primary production stage.

Beckers, H.J., Van Leusden, F.M., Meijssen, M.J.M., Kampelmacher, E.H. 1985.
Reference material for the evaluation of a standard method for the detection of *Salmonella* in foods and feeding stuffs. J. Appl. Bacteriol., 59, 507-512.

## 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.
*Detection of Salmonella*: detection of *Salmonella* from reference materials in the presence of competitive organisms, when the test is carried out in accordance with this SOP.
*Reference Material*: a gelatine capsule containing a quantified amount of a test strain 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 |
| MSRV | Modified semi-solid Rappaport Vassiliadis medium |
| RM | Reference Material |
| SOP | Standard Operating Procedure |
| TSI | Triple sugar/iron agar |
| UA | Urea Agar |
| XLD | Xylose Lysine Deoxycholate agar |

## 6 Culture media

For this study the 'prescribed' method (according to the draft Annex D of ISO 6579) is as follows:

| | |
|---|---|
| Non selective pre-enrichment medium | BPW (beside incubation of 18 h also incubation of 4 h) |
| 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 of the 'prescribed' method are described in Annex B, and in draft 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 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. Note all relevant information in the test report.

### 6.1 Non selective pre-enrichment medium

Buffered Peptone water (BPW) (ISO 6579, Annex B.1)

Mind to distribute the BPW in portions of **90 ml** into suitable flasks before sterilisation.

### 6.2 Selective enrichment medium

Modified Semi solid Rappaport Vassiliadis (MSRV) (Draft Annex D 2005 ISO 6579)

This medium must be boiled to dissolve (instructions of manufacturer). After boiling the medium must be transparent blue. After cooling down to 50 °C the supplement or the novobiocin has to be added.

The final concentration of the novobiocin in the medium should be 0.01 g/l. Plates should be poured with a volume of 15 to 20 ml.

Selective enrichment medium routinely used in your laboratory (optionally)

### 6.3 Solid selective media for first and second isolation

Xylose-Lysine-Desoxycholate (ISO 6579, Annex B.4)
This medium must be boiled to dissolve (instructions manufacturer). After boiling the medium must be transparent red. Plates should be poured with a volume of 15-20 ml in 90 mm plates or 30-40 ml in 140 mm-plates.
Second selective plating medium of choice (obligatory)
Own medium (optionally)

### 6.4 Confirmation media

*Biochemical confirmation*

Triple sugar/iron agar (TSI agar) (ISO 6579, Annex B.6)
Urea agar (ISO 6579, Annex B.7)
l-Lysine decarboxylation medium (ISO 6579, Annex B.8)
Nutrient agar (optional) (ISO 6579, 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);
Incubator, capable of operating at (37 ± 1) °C;
Incubator, capable of operating at (41,5 ± 1) °C;
Loops;
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 200 ml;
Culture tubes with approximate sizes: 8 mm in diameter and 160 mm in length;
Micro-pipettes; nominal capacity 0,1 ml;
Petri dishes; small size (diameter 90 mm to 100 mm) and/or large size (diameter 140 mm).

# 8 Procedure

## 8.1 General

Below the 'prescribed' method of the ninth interlaboratory comparison study of CRL-*Salmonella* is described. The different steps in the procedure are also summarised in Annex A of this SOP. Beside the 'prescribed' method 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 draft Annex D of ISO 6579.
**Mind that for the 'prescribed' method two incubation times of BPW is prescribed [(4 ± ½) h and (18 ± 2) h]. Use for your own method(s) the incubation time of BPW which is relevant for the method (note on the test report)**

## 8.2 Prewarming BPW (day 0)

Label 25 jars containing 90 ml of BPW from 1 to 25 (eg. on Monday). For the dust samples number 10 jars of BPW from D1 to D10. Also label 12 jars of BPW from C1 to C12 (control capsules). One jar is a procedure control (= C11) to which no capsule or faeces will be added and one jar is a negative faeces control to which only 10 g faeces will be added (= C12). These control jars should further be handled in the same way as the other jars. Place all jars with BPW **overnight** at (37 ± 1) °C. Also place some extra non-labelled jars containing 90 ml of BPW at 37 °C. These extra jars can be used in case some jars might have been contaminated. Record in the test report (page 2) the requested data of BPW.

## 8.3 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 35 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 and dust samples to the jars according to the following scheme:

- **Add 10 grams of faeces to jars labelled 1-25 and C12,**
- **Add no faeces to jars labelled C1 - C11,**

***! Do not shake the jars after adding the faeces.***

- **Add 10 grams of dust to jars labelled D1-D10.**

***Shake the jars containing dust to make sure the dust isn't floating on the BPW.***

Place all jars in the (37 ± 1) °C incubator for (4 ± ½ ) 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. After incubation and transferring the requested amount of material to the selective enrichment medium, incubate the jars further for a total of (18 ± 2) h at (37 ± 1) $^{o}$C.

## 8.4 Selective enrichment (day 1 and day 2)

**Please note that in this study two incubation times of the BPW are used: (4 ± ½ ) h and (18 ± 2) h. Therefore MSRV plates should be inoculated on day 1 and on day 2.**

Day 1

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 Laminar Air Flow cabinet if necessary. Record (page 4-7) the requested data of the MSRV plates and if used, the data of the own selective enrichment medium in the test report. Label 25 plates of MSRV from 1 to 25. Also label 10 MSRV-plates from D1 to D10 and 12 plates from C1 to C12. If necessary also indicate on the plates that they are inoculated with the 4h BPW cultures. If you have other means to distinguish between the plates inoculated with 4h BPW cultures from 18h BPW cultures it is not necessary to label each individual plate.
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 ± 1) $^{o}$C for (24 ± 3) h and if negative for another (24 ± 3) h. Record the temperature and time at the start and at the end of the incubation period and other requested data in the test report (page 4-7).

Day 2

Repeat the procedure of day 1 for inoculation of the MSRV plates with the 18h BPW cultures.

## 8.5 Isolation media (first and second isolation;day 2, 3 and 4)

*Note:*
In the case that you do not have large dishes (140 mm) at your disposal use two small (90-100 mm) dishes, one after the other, using the same loop.
Record in the test report (page 8-13) the requested data of the isolation media used. Label 25 large Petri dishes of the isolation media from 1 to 25, label 10 large Petri dishes from D1 to D10 and label 12 large Petri dishes from C1 to C12. If necessary also note the relevant incubation time of BPW on the plates. If you have other means to distinguish between the plates inoculated with 4h BPW cultures from 18h BPW cultures it is not necessary to label each individual plate.

**First isolation after 24 h**
Inoculate, by means of a loop, from the suspect MSRV plates (see draft Annex D of ISO 6579), the surface of an isolation medium in a large size Petri dish with the corresponding label number. 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 ± 1) °C (note the temperature and time at the start and at the end of the incubation and other requested data on the test report, page 8-9).
2) Second isolation agar plate for choice (obligatory !). Please note all relevant details on incubation temperature and time in the test report (page 10-11).

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 two times (24 ± 3) h of the MSRV-plates, repeat the procedure described above at first isolation after 24 h.

## 8.6 Confirmation of colonies from first and second isolation (day 3, 4 and 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 isolation plates at (5 ± 3) $^{o}$C.
Before biochemical confirmation (see below), optionally, streak each typical colony onto the surface of a nutrient agar plate with the corresponding label number, in a manner which allows to develop well isolated colonies. Record on the test report (page 14-15) the requested data of the nutrient agar. Incubate the inoculated plates at (37 ± 1) °C for (24 ± 3) h.

**Biochemical confirmation**

By means of a loop, inoculate the media specified below with the colony selected as described above (either directly from the isolation medium, or from nutrient agar). For each of the mentioned media follow the instructions in 9.5 of ISO 6579 (2002). Optionally inoculate other media which are routinely used for biochemical confirmation. Record in the test report (page 16) the requested data of the media.

TSI agar
Urea agar
l-Lysine decarboxylation medium

**Interpretation of the biochemical tests**

*Salmonella* generally show the reactions given in Table 1 of ISO 6579:2002(E).

TSI agar:
*Butt:* -yellow by fermentation of glucose;
-black by formation of hydrogen sulfide; and
-bubbles or cracks due to gas formation from glucose
*Slant:* -red or unchanged
Urea agar: red to rose pink and later to deep cerise
l-Lysine decarboxylation medium: coloured purple

If the initial 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 [incubation time of BPW of (4 ± ½) h and selective enrichment on MSRV], Table 2 [incubation time of BPW of (18 ± 2) h and selective enrichment on MSRV] and Table 3 (isolation using own enrichment) on test report page 18-26. For the results of detection of *Salmonella* using PCR fill in Table 4 on the test report page 27.

## 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 will include the names of the persons, who are carrying out the work and will be signed by these persons.