

STANDARD OPERATING PROCEDURE (SOP)

INTERLABORATORY COMPARISON STUDY VIII (2004) 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.

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). 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) (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
This medium must be boiled to dissolve (instructions (October, 2004) of
manufacturer). After boiling the medium must be ISO 6579
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 0.02 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 (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) (Annex B.6)

Urea agar (Annex B.7)

L-Lysine decarboxylation medium (Annex B.8)

Nutrient agar (optional) (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 $(37 \pm 1) ^\circ\text{C}$;
Incubator, capable of operating at $(41,5 \pm 1) ^\circ\text{C}$;
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 eighth 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.

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 and thawing faeces

Take the frozen faeces out of the freezer at the end of the day (eg. on Monday) before you start testing and thaw the portions frozen faeces in the closed container **overnight at 5 °C**.

Label 25 jars containing 90 ml of BPW from 1 to 25. For the naturally contaminated samples number 20 jars of BPW from N1 to N20. 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 is added and one jar is a negative faeces control to which only 10 g faeces is added (= C12). These control jars should further be handled in the same way as the other jars. Place all jars **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

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 thawed faeces to the jars according to the following scheme:

- **Add 10 grams of faeces from portion 1 to jars labelled 1-25 and C12,**
- **Add no faeces to jars labelled C1 - C11,**
- **Add 10 grams of faeces from portion 2 to jars labelled N1-N20.**

Do not shake the jars after adding the faeces.

Place all jars in the (37 ± 1) °C incubator for $(4 \pm \frac{1}{2})$ 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. 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) °C.

8.4 Selective enrichment

Allow the MSR-V-plates to equilibrate to room temperature, if they were stored at a lower temperature. Dry the surface of the MSR-V plates in a Laminair Air Flow cabinet. Record (page 4-7) the requested data of the MSR-V plates and if used, the data of the own selective enrichment broth in the test report. Label 25 plates of MSR-V from 1 to 25. Also label 20 MSR-V-plates from N1 to N20 and 12 plates from C1 to C12. All plates are incubated for (24 ± 3) h and later on for another (24 ± 3) h. **Please note that this study is divided into two parts: one with an incubation time of the BPW of $(4 \pm \frac{1}{2})$ h and the other with an incubation time of the BPW of (18 ± 2) h. So, subsequently all media will be inoculated and incubated accordingly.**

After equilibration: inoculate the MSR-V plates with three drops of BPW culture, with a total volume of 0.1 ml. Incubate (**not upside down**) at $(41,5 \pm 1)$ °C for (24 ± 3) h and later on 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).

8.5 Isolation media (first and second isolation)

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 20 large Petri dishes from N1 to N20 and label 12 large Petri dishes from C1 to C12.

First isolation after 24 h

Inoculation:

Inoculate, by means of a loop, from the suspect MSR/V plates, 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 $(37 \pm 1) ^\circ\text{C}$ (note the temperature and time at the start and at the end of the incubation and other requested data on 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 $24 \text{ h} \pm 3 \text{ h}$, 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 MSR/V-plates, repeat the procedure described above (**First isolation after 24 h**).

8.6 Confirmation of colonies from first and second isolation

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

Store the plates at $(5 \pm 3) ^\circ\text{C}$.

Before biochemical confirmation, 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 test report (page 14-15) the requested data of the nutrient agar. Incubate the inoculated plates at $(37 \pm 1) ^\circ\text{C}$ for $(24 \pm 3) \text{ h}$.

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 [incubation time of BPW of (4 ± ½) h and selective enrichment on MSR/V], Table 2 [incubation time of BPW of (18 ± 2) h and selective enrichment on MSR/V] 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 test report page 27.

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 test report (page 20) 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) on page 9).

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

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.