

BACTERIOLOGICAL COLLABORATIVE STUDY VI (2002)
ORGANISED BY CRL *SALMONELLA*

**STANDARD OPERATING PROCEDURE FOR THE DETECTION OF
SALMONELLA IN THE PRESENCE OF COMPETITIVE MICRO-ORGANISMS.**

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 or *Salmonella* Enteritidis as prepared by the Community Reference Laboratory for *Salmonella* (CRL). Furthermore poultry faeces is used. The application of this SOP is limited to the bacteriological collaborative 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.

- ① *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.
- ① *Reference Material*: a gelatine capsule containing a quantified amount artificially contaminated 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 Culture media

Composition and preparation of the media and reagents are described in Annex B of the ISO 6579:2002(E).

5.1 Non selective pre-enrichment medium

- ① Buffered Peptone water (Annex B.1)

5.2 Selective enrichment medium

- ① Rappaport Vassiliadis medium with soya (RVS broth) (Annex B.2)
- ① Muller Kauffmann tetrathionate-novobiocin broth (MKTTn) (Annex B.3)
- ① Modified Semi solid Rappaport Vassiliadis (Newsletter, Vol.5, No.2, June 1999)
This medium must be boiled to dissolve (instructions manufacturer). After boiling the medium must be transparent blue. After cooling down to 50°C the supplement or the novobiocine has to be added.
The final concentration of the novobiocine in the medium should be 0.01 g/l. Plates should be poured with a volume of 15 to 20 ml.
- ① Selective enrichment medium routinely used in your laboratory

5.3 Solid selective media for first and second isolation

- ① Phenol red/brilliant green agar (Annex B.4, ISO 6579993)
The medium must be boiled to dissolve (instructions manufacturer). After boiling the medium must be transparent red.
Plates should be poured with a volume of 30-40 ml (140 mm-plates).
- ① 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 30-40 ml (140 mm-plates).
- ① Third medium (optionally) (Paragraph 4.4)

5.4 Confirmation media

Biochemical confirmation

- ① Triple sugar/iron agar (TSI agar) (Annex B.6)
- ① Urea agar (Annex B.7)
- ① l-Lysine decarboxylation medium (Annex B.8)
- ① Nutrient agar (optionally) (Annex B.5)

6 Apparatus and glassware

The usual microbiological laboratory equipment. If requested, note specifications of the apparatus and glassware on the test report.

Apparatus

- ① Oven (for dry sterilisation) or autoclave (for wet sterilisation);
- ① Incubator, capable of operating at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$;
- ① Water bath, capable of operating at $41,5^{\circ}\text{C} \pm 1^{\circ}\text{C}$ or incubator, capable of operating at $41,5^{\circ}\text{C} \pm 1^{\circ}\text{C}$;
- ① Water bath, capable of operating at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$;
- ① Loops;
- ① pH-meter; having an accuracy of calibration of ± 0.1 pH unit at 25°C .

Glassware

- ① Culture bottles or jars with nominal capacity of 250 ml;
- ① Culture tubes; 8 mm in diameter and 160 mm in length;
- ① Micro-pipettes; nominal capacity 0,1 ml;
- ① Petri dishes; small size (diameter 90 mm to 100 mm) and/or large size (diameter 140 mm).

7 Procedure

Take the frozen faeces out of the freezer at the end of the working day and thaw the portions frozen faeces **overnight** at 5 °C.

7.1 Pre-enrichment

Allow the BPW to equilibrate to 37°C for better dissolving of the capsules.

Record in test report (page 3) the requested data of the BPW. 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. Label 25 jars containing 225 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 gr. faeces is added (= C12). These control jars should further be handled in the same way as the other jars.

After equilibration 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 **30 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 30 minutes add the thawed faeces to the jars according to the following scheme:

Add 10 grams of faeces from portion A to jars labelled 1-25 and C12,

Add no faeces to jars labelled C1 - C11,

Add 25 grams of faeces from portion B to jars labelled N1-N20.

Do not shake the jars after adding the faeces.

Place all jars in the 37 °C incubator for 16 h to 20 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 in the test report page 13. Results of PCR can be written in the test report Table 5 (page 26).

7.2 Selective enrichment

Allow the selective enrichment broths 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 selective enrichment broths and MSR/V plates in the test report. Label 25 jars/tubes/plates of each selective enrichment broth from 1 to 25. Also label 20 selective enrichment jars/tubes/plates from N1 to N20 and 12 jars/tubes/plates from C1 to C12. All selective media are incubated for 24 h and later on for another 24 h.

After equilibration:

Transfer 0.1 ml of homogenised BPW culture to each tube containing 10 ml RVS medium. Incubate at $41,5^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for $24\text{ h} \pm 3\text{ h}$ and later on another $24\text{ h} \pm 3\text{ h}$;

Transfer 1 ml of homogenised BPW culture to each tube containing 10 ml MKTTn medium. Incubate at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for $24\text{ h} \pm 3\text{ h}$ and later on another $24\text{ h} \pm 3\text{ h}$;

Inoculate the MSR/V plates with three drops of BPW culture, with a total volume of 0.1 ml. Incubate (**not upside down**) at $41,5^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for $24\text{ h} \pm 3\text{ h}$ and later on another $24\text{ h} \pm 3\text{ h}$;

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 test report). Incubate at the temperature routinely used.

Place the jars/tubes in the appropriate incubator(s)/waterbath(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 4-7).

7.3 Isolation on media (first and second isolation)

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

Record in the test report (page 8-10) 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 all selective enrichment cultures and from suspect MSR/V plates, the surface of an isolation medium in a large size petri dish with the corresponding label number (see also *note* at the beginning of section 7.3). The following isolation media will be used:

- 1) Phenol red/brilliant green agar;
Place the petri dishes with the bottom up in the incubator set at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ (record the temperature and time at the start and at the end of the incubation and other requested data in test report, page 8).
- 2) Xylose Lysine Desoxycholate agar
Place the petri dishes with the bottom up in the incubator set at 37°C (note the temperature and time at the start and at the end of the incubation and other requested data on test report, page 9).
- 3) Optionally: selective isolation medium/media routinely used in your laboratory.
Only if media used are different from those mentioned above.
Incubate the medium/media at the temperature routinely used (record temperature and time and other requested data in test report, page 10).

After incubation for $24\text{ h} \pm 1^{\circ}\text{C}$, examine the petri dishes for the presence of typical colonies of *Salmonella*.

Second isolation after 48 h

After a total incubation time of $48\text{ h} \pm 3\text{h}$ of the selective enrichment media, repeat the procedure described above (**First isolation after 24 h**).

7.4 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 $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$. 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 11) the requested data of the nutrient agar. Incubate the inoculated plates at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for $24\text{ h} \pm 3\text{h}$.

If the selected colony is not confirmed as *Salmonella*, test at maximum another 5 typical colonies. 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 MSR V) and Table 4 (isolation using own enrichment) on test report page 14-25. For the results of detection of *Salmonella* using PCR fill in Table 4 on test report page 26.

Biochemical confirmation

By means of a loop, inoculate the media specified below with the colony selected as described above. Optionally inoculate other media which are routinely used for biochemical confirmation. Record in test report (page 11+12) the requested data of the media.

- ⌚ TSI agar
- ⌚ Urea agar
- ⌚ l-Lysine decarboxylation medium
- ⌚ Nutrient agar (optionally)

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: rose pink to cerise
- ⌚ l-Lysine decarboxylation medium: coloured purple

8 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.

Schedule of the adapted ISO 6579: 2002(E) method

Day	Topic	Description
1	Pre-enrichment	1 capsule to 225 ml BPW Do not shake 30 min. at 37 °C Add 10 or 25 gr. faeces to BPW Incubate 16-20 h at 37 °C
2	Selective enrichment	0.1 ml BPW culture in 10 ml RVS 1 ml BPW culture in 10 ml MKTTn 0.1 ml BPW culture on MSR/V plate Other selective enrichment medi(um)(a) Incubate 24 h at 41,5 °C
3	First isolation after 24 h	Inoculate from RVS, MKTTn, suspect MSR/V plates and other medi(um)(a) ⌚ phenol red/brilliant green agar ⌚ Xylose Lysine Desoxycholate agar ⌚ other selective medi(um)(a) Incubate 24 h at the specified temperature
3	Continue selective enrichment	Incubate RVS and MSR/V medium (see day 2) another 24 hours at 41,5 °C
4	Second isolation after 24 h	Inoculate from RVS, MKTTn, suspect MSR/V plates and Other medi(um)(a) ⌚ phenol red/brilliant green agar ⌚ Xylose Lysine Desoxycholate agar ⌚ other selective medi(um)(a) Incubate another 24 h at the specified temperature
4	Biochemical confirmation	Inoculate the media from first isolation media (day 3) for biochemical identification and incubate 24 h at the specified temperature
5	Biochemical confirmation	inoculate the media from second isolation media (day 4) for biochemical identification and incubate 24 h at the specified temperature