STANDARD OPERATING PROCEDURE (SOP)

BACTERIOLOGICAL COLLABORATIVE STUDY VII (2003) 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) are used. 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.
- *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 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 List of abbreviations

BGA	phenol red/Brilliant Green Agar
BPW	Buffered Peptone Water
MKTTn	Muller Kaufmann Tetrathionate novobiocin broth
MSRV	Modified semi-solid Rappaport Vassiliadis medium
RM	Reference Material
RVS	Rappaport Vassiliadis mediium with Soya
SOP	Standard Operating Procedure
TSI	Triple sugar/iron agar
UA	Urea Agar
XLD	Xylose Lysine Deoxycholate agar

6 Culture media

Composition and preparation of the media and reagents are described in Annex B 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.

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

- Rappaport Vassiliadis medium with soya (RVS broth) (Annex B.2)
- Muller Kauffmann tetrathionate-novobiocin broth (MKTTn) (Annex B.3)
- Modified Semi solid Rappaport Vassiliadis (MSRV) (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.
- Selective enrichment medium routinely used in your laboratory (optional)

6.3 Solid selective media for first and second isolation

- Phenol red/brilliant green agar (Annex B.4, ISO 6579: 1993)
  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).
- 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).
- Third medium (optionally) (Paragraph 4.4)

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°C ± 1°C;
- Water bath, capable of operating at 41,5°C ± 1°C or incubator, capable of operating at 41,5°C ± 1°C;
- Water bath, capable of operating at 37°C ± 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 Prewarming BPW and thawing faeces

Take the frozen faeces out of the freezer at the end of the day 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 gr. 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 °C (± 1 °C). Also place some extra non-labelled jars containing 90 ml of BPW at 37 °C in case some jars might have been contaminated. Record in the test report (page 2) the requested data of BPW.

8.2 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 2 (number of parcel) to jars labelled 1-25 and C12,

Add no faeces to jars labelled C1 - C11,

Add 10 grams of faeces from portion 3 (number of parcel) to jars labelled N1-N20.

Do not shake the jars after adding the faeces.

Place all jars in the 37 °C (± 1 °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 21. Results of PCR can be written in the test report Table 5 (page 34).

8.3 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 MSRV plates in a Laminair Air Flow cabinet. Record (page 4-11) the requested data of the selective enrichment broths and MSRV 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°C ± 1°C for 24 h ± 3 h and later on another 24 h ± 3 h;

Transfer 1 ml of homogenised BPW culture to each tube containing 10 ml MKTTn medium. Incubate at 37°C ± 1°C for 24 h ± 3 h and later on another 24 h ± 3 h;

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°C ± 1°C for 24 h ± 3 h and later on another 24 h ± 3 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 4-11).

8.4 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 12-14) 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 MSRV 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) Phenol red/brilliant green agar BGA)
   Place the Petri dishes with the bottom up in the incubator set at 37°C ± 1°C (record the temperature and time at the start and at the end of the incubation and other requested data in test report, page 12-13).

2) Xylose Lysine Desoxycholate agar (XLD)
   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 14-15).

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 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 ± 3h of the selective enrichment media, repeat the procedure described above (**First isolation after 24 h**).

8.5 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°C ± 3°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 18-19) 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 test report page 22-33. For the results of detection of *Salmonella* using PCR fill in Table 4 on test report page 34.

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.

Scheme of bacteriological collaborative study VII (2003) on detection of *Salmonella* spp.

Day	Topic	Description
1	**Prewarming BPW** **Thawing faeces**	Place at the end of the day sufficient jars, each containing 90 ml BPW, at 37 °C ± 1 °C. Place at the end of the day the chicken faeces in the closed containers at 5 °C ± 3 °C
2	**Pre-enrichment**	1 capsule to 90 ml (prewarmed) BPW Do not shake 45 min. at 37 °C ± 1 °C Add 10 g faeces (thawed at 5 °C) to BPW Incubate 16-20 h at 37 °C ± 1 °C
3	**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 Other selective enrichment medi(um)(a)
4	**First isolation after 24 h**	Inoculate from RVS, MKTTn, suspect MSRV plates and other medi(um)(a) - phenol red/brilliant green agar, incubate at (37 ± 1) °C for (24 ± 3) h - Xylose Lysine Desoxycholate agar, incubate at (37 ± 1) °C for (24 ± 3) h - other selective medi(um)(a), incubate for specified time at the specified temperature
4	**Continue selective enrichment**	Incubate RVS, MKTTn and MSRV medium (see day 3) another 24 (± 3) hours at the relevant temperatures
5	**Second isolation after 24 h**	Inoculate from RVS, MKTTn,suspect MSRV plates and Other medi(um)(a) (see day 4) - phenol red/brilliant green agar - Xylose Lysine Desoxycholate agar - other selective medi(um)(a)
5	**Biochemical confirmation**	Inoculate the media from first isolation media (day 4) for biochemical identification and incubate 24 (± 3)h at the specified temperature
6	**Biochemical confirmation**	Inoculate the media from second isolation media (day 5) for biochemical identification and incubate 24 (± 3)h at the specified temperature