STANDARD OPERATING PROCEDURE (SOP)

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

STUDY X - 2006

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 pig faeces. 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 CRL for *Salmonella* are used. As matrix, pig faeces 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.

Draft Amendment ISO 6579:2002/amendedDAmd 1 (2006-09-12). Amendment 1 Annex D: Detection of *Salmonella* spp. in animal faces and in 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.
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 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
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 is the procedure as described in draft Annex D of ISO 6579 (2006-09-12).

Non selective pre-enrichment medium	BPW
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 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 (draft 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. Note all relevant information in the test report.

6.1 Non selective pre-enrichment medium

Buffered Peptone water (BPW)	(ISO6579 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)	(ISO6579 Draft Annex D 2006-09-12)
Selective enrichment medium routinely used in your laboratory (optionally)	

6.3 Solid selective media for first and second isolation

Xylose-Lysine-Desoxycholat (90 mm plates)	(ISO6579 Annex B.4)
Second isolation medium for choice (obligatory)	
Own medium (optionally)	

6.4 Confirmation media

Biochemical confirmation

Triple sugar/iron agar (TSI agar)	(ISO6579 Annex B.6)
Urea agar	(ISO6579 Annex B.7)
L-Lysine decarboxylation medium	(ISO6579 Annex B.8)
Nutrient agar (optional)	(ISO6579 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);
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
Loops 1 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 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; standard size (diameter 90 mm to 100 mm)

8 Procedure

8.1 General

Below the requested method of the tenth interlaboratory comparison study in pig faeces of CRL-*Salmonella* is described. The different steps in the procedure are also summarized in Annex A of this SOP. Beside these method it is also allowed to use one or more own methods. Please record all relevant data in the test report. Details of the method can be found in ISO 6579 and draft Annex D of ISO 6579 (version 120906).

8.1 Prewarming BPW (day 0)

Label 25 jars containing 90 ml of BPW from 1 to 25. 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 (at least) **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 & 3) the requested data of 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 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 to the jars according to the following scheme:

- **Add 10 g 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.

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 16 & 21 of the test report.

8.3 Selective enrichment (day 2)

Allow the selective enrichment media 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 if necessary. Record (page 4-7) the requested data of the MSRV and own selective enrichment media (if used) in the test report. Label 25 MSRV plates from 1 to 25. Also label 12 MSRV plates from C1 to C12. Incubate the MSRV plates for 24 h and later on for another 24 h. If own selective enrichment media are used, label them in the same way as described for MSRV and also incubate for two times 24 h.

After equilibration:
Prescribed method:

- 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 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 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 4-7).

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

Record in the test report (page 8-13) the requested data of the isolation media used. Label 25 (standard size) Petri dishes of each isolation medium from 1 to 25 and label 12 (standard size) Petri dishes from C1 to C12.

First isolation after 24 h

Inoculation:

Inoculate from suspect MSRV plates, the surface of an isolation medium in one standard 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 °C (record temperature and time and other requested data in test report, page 8 & 9).
2) Second isolation medium. Follow the instructions of the manufacturer (record temperature and time and other requested data in test report, page 10 & 11).
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 test report, page 12 &13).

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^{o} C ± 3^{o} C.

Before biochemical 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 test report (page 14) 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 MSRV) and Table 2 (isolation using own enrichment) on the test report pages 17-20. For the results of detection of *Salmonella* using PCR fill in Table 3 on the test report page 21.

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 15) 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 on page 9.

TSI agar:

Butt: -yellow by fermentation of glucose; (+)
-black by formation of hydrogen sulphide; (+)
-bubbles or cracks due to gas formation from glucose (+)

Slant: -red or unchanged: lactose and sucrose are not used (-)

Urea agar: yellow: no splitting of ammonia (-)

L-Lysine decarboxylation medium: turbidity and purple colour (+)

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 name of the NRL, the person in charge for the NRL, and the names of the persons who are carrying out the work and will be signed by these persons. 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
ANIMAL FAECES X (2006)
on the detection of *Salmonella* spp. in pig faeces

Day	Topic	Description
0	**Prewarming BPW**	Place at least at the end of the day sufficient jars, each containing 90 ml BPW, at 37 °C ± 1 °C.
1	**Pre-enrichment**	Add 1 capsule to 90 ml (prewarmed) BPW Do not shake Incubate 45 min. at 37 °C ± 1 °C Add 10 g faeces to BPW Incubate 18 h ± 2 h at 37 °C ± 1 °C
2	**Selective enrichment**	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 suspect MSRV (24h) plates 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 MSRV medium another 24 (± 3) hours at the relevant temperatures
4	**Second isolation after 48 h**	Inoculate from suspect MSRV (48h) plates if first isolation was negative) and own medi(um)(a) Xylose Lysine Desoxycholate agar Second isolation medium Own selective medi(um)(a)
4	**Biochemical confirmation**	Inoculate the media from first isolation media (day 3) for biochemical identification and incubate 24 (± 3)h at the specified temperature
5	**Biochemical confirmation**	Inoculate the media from second isolation media (day 4) for biochemical identification and incubate 24 (± 3)h at the specified temperature