
**Microbiology of food and animal feed —
Horizontal method for the detection,
enumeration and serotyping of
Salmonella —**

Part 2:

**Enumeration by a miniaturized most
probable number technique**

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*Microbiologie des aliments — Méthode horizontale pour la recherche, le
dénombrement et le sérotypage des Salmonella —*

ISO/TS 6579-2:2012

*Partie 2: Dénombrement par une technique miniaturisée du nombre le
plus probable*

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of document:

- an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;
- an ISO Technical Specification (ISO/TS) represents an agreement between the members of a technical committee and is accepted for publication if it is approved by 2/3 of the members of the committee casting a vote.

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An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

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Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO/TS 6579-2 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

ISO 6579 consists of the following part, under the general title *Microbiology of food and animal feed — Horizontal method for the detection, enumeration and serotyping of Salmonella*:

- *Part 2: Enumeration by a miniaturized most-probable-number technique* [Technical Specification]

Additional parts, dealing with a detection method and guidance for serotyping are planned. ISO 6579:2002 is to be withdrawn at a later date.

Introduction

The procedure described is based on the method reported in Reference [1].

The enumeration procedure described here concerns a miniaturized most probable number (MPN) technique. For this mini-MSRV (modified semi-solid Rappaport–Vassiliadis) MPN technique, the volume of primary dilution tested is less than the volume in the detection method specified in ISO 6579:2002 + Cor.1:2004 + Amd.1:2007.^[5] For this reason, the sensitivity of the mini-MSRV technique is lower than in these detection methods (Reference [1]). The detection limit of the mini-MSRV method is approximately 1 cfu/g, but can vary according to *Salmonella* serovar and per matrix. For the previously mentioned detection methods, this is typically 1 cfu/25 g (0,04 cfu/g). For samples with (very) low numbers of *Salmonella* spp. (<1 cfu/g), it is possible that the mini-MSRV procedure is not sufficiently sensitive. If a quantitative result is requested for samples likely to contain such low numbers (and tested negative with this mini-MSRV technique, for example), it is advisable to enumerate with a “conventional” MPN technique (not miniaturized). For other samples, the mini-MSRV method can have an advantage over the conventional MPN technique because the performance of the miniaturized MPN technique can take less time and need fewer resources (due to small amounts).

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Microbiology of food and animal feed — Horizontal method for the detection, enumeration and serotyping of *Salmonella* —

Part 2: Enumeration by a miniaturized most probable number technique

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *Salmonella*, are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist, and that great care is taken in the disposal of all incubated materials.

Persons using this International Standard should be familiar with normal laboratory practice. This standard does not purport to address all of the safety aspects, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

1 Scope

This part of ISO 6579 gives a method for the enumeration of *Salmonella* spp. present in:

- products intended for human consumption and for the feeding of animals;
- environmental samples in the area of food production and food handling;
- animal faeces;
- environmental samples from the primary production stage;

by calculation of the most probable number (MPN).

The method is based on miniaturization of the dilution, pre-enrichment and selective enrichment steps. The selective enrichment medium, modified semi-solid Rappaport–Vassiliadis (MSRV), is intended for the detection of motile salmonellae and is not appropriate for the detection of non-motile salmonellae.

It is possible that the method is less appropriate to enumerate *Salmonella* ser. Typhi and *Salmonella* ser. Paratyphi.

The method is not appropriate for the enumeration of *Salmonella* spp. in (very) low contaminated samples (<1 cfu/g).

NOTE The number of non-motile salmonellae is generally low in most of the matrices relevant for this method. In this note, examples are given for samples from primary production. The non-motile *Salmonella* biovars of *Salmonella* Gallinarum (*Salmonella* Gallinarum biovar gallinarum and *Salmonella* Gallinarum biovar pullorum) do not seem to survive long in environmental samples and are therefore rarely detected in faecal or environmental (such as dust) samples (regardless of the method). The number of other non-motile *Salmonella* serovars in faecal samples seems to be generally low. For example, in Reference [4] in which approximately 1 000 faecal samples of poultry layer flocks and approximately 900 faecal samples of broiler flocks were analysed, less than 1 % of the total number of samples were positive in a selective broth and at the same time negative on MSRV medium (and likely to be non-motile). Similar results were found in a Dutch study with ca 3 200 faecal samples of pigs (unpublished data). On the other hand, in the case of the study reported in Reference [4], up to almost 40 % of positive samples would not have been detected (i.e. false negatives) if only a selective broth (in this case Rappaport–Vassiliadis) had been used instead of a semi-solid medium.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 6887 (all parts), *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO/TS 11133-1, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 1: General guidelines on quality assurance for the preparation of culture media in the laboratory*

ISO/TS 11133-2, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 2: Practical guidelines on performance testing of culture media*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
Salmonella
microorganism which forms typical or less typical colonies on solid selective media and which displays specific biochemical and serological characteristics

NOTE Suitable tests for the specific biochemical and serological characteristics are specified in this part of ISO 6579.

3.2
count of Salmonella
number of *Salmonella* spp. found per millilitre or per gram of a test sample or per surface area or on an object (e.g. bootsocks)

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4 Principle

4.1 General

The enumeration of *Salmonella* spp. in the MPN format necessitates four successive stages (4.2 to 4.5).
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4.2 Pre-enrichment in non-selective liquid medium

Preparation of a 10⁻¹ dilution of the sample in buffered peptone water (BPW) (initial suspension).

Addition of the initial suspension to the first (empty) row of three wells of a 12-well microtiter plate.

Inoculation of the second row of three wells containing non-selective pre-enrichment broth (BPW) with a specified quantity, from the first row in a 12-well microtiter plate.

Inoculation of the third, fourth and if necessary more rows of three wells containing BPW.

Incubation of the 12-well microtiter plates at 37 °C for 18 h.

4.3 Enrichment on a selective semi-solid medium

Subculturing of each well obtained in 4.2 in a well containing a semi-solid agar (MSRV).

Incubation at 41,5 °C (MSRV) for 24 h. If MSRV is negative after 24 h, the plate is incubated for a further 24 h.

4.4 Selective plating and identification

From the (suspect) cultures (the highest dilutions) obtained in 4.3, a selective solid medium xylose–lysine–deoxycholate (XLD) agar is inoculated and incubated at 37 °C for 24 h.

4.5 Confirmation

Colonies of presumptive *Salmonella* obtained in 4.4 are confirmed by means of appropriate biochemical and serological tests.

4.6 Calculation of most probable number

From the number of confirmed positive wells, the MPN of *Salmonella* spp. per millilitre or per gram of the test sample is calculated.

5 Culture media and sera

5.1 General

For current laboratory practice, see ISO 7218.

For the performance testing of media, follow the recommendations of ISO/TS 11133-1, ISO/TS 11133-2 and the information as given in A.8.

All media and reagents needed are described in Annex A. Alternatively, dehydrated complete media or diluents can be used. In the latter case, follow the manufacturer's instructions.

5.2 Culture media

5.2.1 Non-selective pre-enrichment medium: Buffered peptone water (BPW). See A.1.

5.2.2 Semi-solid selective enrichment agar: Modified semi-solid Rappaport–Vassiliadis medium (MSRV). See A.2.

5.2.3 Xylose–lysine–deoxycholate (XLD) agar. See A.3.

5.2.4 Nutrient agar. See A.4.

5.2.5 Triple sugar–iron agar (TSI) agar. See A.5.

As an alternative, a double sugar–iron agar (Kligler–Hajna) may be used.

5.2.6 Urea agar (Christensen). See A.6.

5.2.7 L-Lysine decarboxylation medium (LDC). See A.7.

5.2.8 Antisera. Several types of agglutinating sera containing antibodies for one or several O-antigens and for one or several H-antigens are commercially available.

Every attempt should be made to ensure that the anti-sera used are adequate to provide for the detection of all *Salmonella* serotypes.

6 Apparatus and glassware

Disposable supplies are an acceptable alternative to reusable glassware if it has similar specifications.

Usual microbiological laboratory equipment and, in particular, the following.

6.1 Apparatus for dry sterilization (oven) or wet sterilization (autoclave). See ISO 7218.

6.2 Drying cabinet or ventilated oven, capable of being maintained at between 25 °C and 50 °C or a **laminar air flow cabinet.**

6.3 Incubators, capable of operating at 37 °C ± 1 °C and 41,5 °C ± 1 °C.

- 6.4 **Water bath**, capable of operating at 47 °C to 50 °C.
- 6.5 **Refrigerator** (for storage of prepared media), capable of operating at 5 °C ± 3 °C.
- 6.6 **pH-meter**, having a resolution of 0,01 pH and accurate to within ± 0,1 pH units at 25 °C. See ISO 7218.
- 6.7 **Sterile test tubes and flasks**, of appropriate capacity. Flasks or bottles and test tubes with non-toxic metallic or plastic (screw) caps may be used.
- 6.8 **Sterile loops** of 1 µl.
- 6.9 **Sterile graduated pipettes or automatic pipettes**, of nominal capacities 10 ml (with 0,5 ml division), 2 ml (with 0,1 ml division), 0,1 ml (with 0,01 ml division). Multi-channel pipettes of 0,5 ml and 0,02 ml nominal capacity for pipetting three wells at a time.
- 6.10 **Sterile Petri dishes**, of approximately 90 mm diameter.
- 6.11 **Sterile 12-well microtiter plates** with wells of approximately 25 mm diameter and 20 mm deep (5 ml) with a flat bottom with lid.
- 6.12 **Homogenizer**. See ISO 7218.

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7 Sampling

Sampling is not part of the method specified in this part of ISO 6579. See the specific International Standard dealing with the product concerned.

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It is important that the laboratory receive a truly representative sample which has not been damaged or changed during transport or storage.

If there is no specific International Standard dealing with sampling of the product concerned, it is recommended that the parties concerned come to an agreement on the subject.

8 Preparation of test sample

Prepare the test sample in accordance with the specific International Standard appropriate to the product concerned.

If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on the subject.

9 Procedure

9.1 Test portion, initial suspension

See ISO 6887 and any specific International Standard appropriate to the product concerned. Prepare an initial suspension by diluting the test portion 10-fold in BPW (5.2.1). For example, add 25 g of sample to 225 ml of BPW and homogenize, e.g. in a stomacher (6.12), for 1 min.

9.2 Dilution and pre-enrichment in non-selective liquid medium

Take a 12-well microtiter plate (6.11) with empty wells in the first row of three wells and containing 2 ml BPW (5.2.1) in the other wells (second, third and fourth row with each 3 wells; see Annex B).

NOTE 1 As a general case, the described procedure specifies dilutions for one 12-well microtiter plate. When a higher number than 500 cfu/g of *Salmonella* is suspected, it is necessary to proceed with a second 12-well microtitre plate containing 2 ml BPW (5.2.1) in each well. Prepare a sufficient number of plates (dilutions) to ensure that the final well in the 12-well microtiter plate yields a negative result.

Transfer to each well of the first (empty) row of three wells, using a pipette (6.9), 2,5 ml of the initial suspension (9.1).

Transfer 0,5 ml (e.g. by using a multi-channel pipette; 6.9) of each well from the first row into the 2 ml BPW in the successive wells in the second row (first 5⁻¹ dilution).

Transfer 0,5 ml (e.g. by using a multi-channel pipette; 6.9, with new tips) of each well from the second row into the 2 ml BPW in the successive wells in the third row (second 5⁻¹ dilution).

Before transferring the 0,5 ml of the second row into the third row, mix the suspensions in the wells by repeatedly (carefully) sucking up and blowing out of the suspension in the pipette and in the wells.

Proceed in the same way for the other rows.

Incubate the 12-well microtiter plate at 37 °C (6.3) for 18 h ± 2 h.

NOTE 2 As the contamination level of the tested samples is generally unknown and often low, it can prove worthwhile to check for the presence of *Salmonella* spp. in the sample by also culturing the initial suspension. For this purpose, incubate (6.3) the initial suspension (9.1) at 37 °C for 18 h ± 2 h. For the next culture steps, follow the procedures as described in 9.3 to 9.5. In 9.3: inoculate a Petri dish containing MSR/V with 1–3 equally spaced spots with a total volume of 0,1 ml of the incubated BPW culture of the initial suspension (9.1).

9.3 Selective enrichment on a semi-solid medium

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Allow the MSR/V (5.2.2) in the 12-well microtiter plates to equilibrate at room temperature if they were stored at a lower temperature.

Inoculate each well containing 2 ml MSR/V with 20 µl of the BPW culture (9.2), e.g. using a multi-channel pipette (6.9). Use new tips for each row of three wells.

Place the 20 µl BPW culture at the margin of the well and on the surface of the medium (see Annex B).

When taking a subculture from BPW, try not to disturb particulate samples. Therefore, move the microtitre plates carefully. Avoid pipetting particulate matter on to the MSR/V plates.

Incubate the inoculated MSR/V plates at 41,5 °C (6.3) for 24 h ± 3 h.

Do not invert the plates.

Suspect wells show a grey-white, turbid zone extending out from the inoculated drop. The turbid zone is characterized by a white halo with a clearly defined edge.

If the wells are negative after 24 h, reincubate for a further 24 h ± 3 h.

9.4 Selective plating

Allow the XLD agar (5.2.3) plates to equilibrate at room temperature if they were stored at lower temperature. If necessary, dry the surface of the plates before use.

Subculture suspect MSR/V wells (9.3) by dipping a 1 µl loop (6.8) just inside the border of the opaque growth and by inoculating this culture material on the surface of one normal size XLD plate, so that well-isolated colonies are obtained.