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Water quality — Detection of *Salmonella* spp.

Qualité de l'eau — Recherche de Salmonella *spp.*

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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.

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 19250 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 4, *Microbiological methods*.

This edition cancels and replaces ISO 6340:1995, which has been technically revised.

Introduction

Salmonella species are bacteria which are widely distributed all over the world. They are usually classified as pathogens, although their virulence and pathogenesis vary widely. The natural hosts of *Salmonella* include humans, agricultural and domestic livestock, and wild animals including birds. Humans and animals can excrete these bacteria while carrying them asymptomatically as well as during disease. It is therefore impossible to eliminate them from the environment. Following the infection of humans, the transmission of *Salmonella* can cause severe disease.

Since water is a recognized vehicle of infection, the presence or absence of *Salmonella* is monitored in water where there is perceived to be a risk of infection. *Salmonella* can be present in all types of domestic and agricultural waste water, freshwaters, including ground and drinking waters, as well as sea water.

The detection of *Salmonella* in water usually requires a concentration step. Since *Salmonella* cells can be present in low numbers and injured in the aqueous environment, their detection in water usually requires a pre-enrichment step.

Water quality — Detection of *Salmonella* spp.

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *Salmonella*, and especially *S. enterica* subsp. *enterica* ser. Typhi (*Salmonella* ser. Typhi) and *S. enterica* subsp. *enterica* ser. Paratyphi (*Salmonella* ser. Paratyphi), be undertaken only in properly equipped laboratories, under the control of a skilled microbiologist, and that great care be 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 problems, 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.

IMPORTANT — It is absolutely essential that tests conducted according to this International Standard be carried out by suitably trained staff.

1 Scope

This International Standard specifies a method for the detection of *Salmonella* spp. (presumptive or confirmed) in water samples. It is possible that, for epidemiological purposes or during outbreak investigations, other media are also required.

WARNING — It is possible that the method does not recover all *Salmonella* ser. Typhi and ser. Paratyphi.

NOTE For a semi-quantitative approach, most probable number (MPN) tests can be performed using appropriate sample volumes. For these cases, the volume of the buffered peptone water is adjusted accordingly.

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 6579, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection of* Salmonella *spp.*

ISO 6887-1, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions*

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

ISO 7704, *Water quality — Evaluation of membrane filters used for microbiological analyses*

ISO 8199, *Water quality — General guidance on the enumeration of micro-organisms by culture*

ISO 19458, *Water quality — Sampling for microbiological analysis*

3 Terms and definitions

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

3.1
presumptive *Salmonella* spp.
bacteria which grow in the selective enrichment medium specified, and form typical or atypical colonies on the solid selective media

3.2
confirmed *Salmonella* spp.
bacteria which grow in the selective enrichment medium specified, and form typical and suspicious colonies on the solid selective media, and which display specfic biochemical and serological characteristics

NOTE The specific biochemical and serological characteristics are determined by tests specified in this International Standard.

3.3
***Salmonella* detection**
determination of the presence or absence of ***Salmonella*** (3.4)

3.4
***Salmonella* spp.**
Salmonella
microorganisms which form typical or atypical colonies on solid selective media and which display specific biochemical and serological characteristics

4 Principle

4.1 General

The detection of *Salmonella* necessitates four successive stages (see also Annex A).

Pre-enrichment is often necessary to permit detection of low numbers of *Salmonella* or injured *Salmonella*. Some *Salmonella* and those which are sublethally injured may require additional incubation time (4.3). Furthermore, *Salmonella* can be present in small numbers and are often accompanied by considerably larger numbers of other members of Enterobacteriaceae or of other families. Therefore, selective enrichment is necessary.

4.2 Pre-enrichment in non-selective liquid medium

Buffered peptone water (B.1) is inoculated at ambient temperature with a known volume of the sample or its dilutions, then incubated at (36 ± 2) °C for (18 ± 2) h. Larger volumes can be concentrated using membrane filtration and the membrane filter is then added to buffered peptone water.

NOTE For waste water it has been shown that shorter incubation times or direct inoculation of the sample in selective medium (4.3) produce better results.

For a semi-quantitative approach, MPN tests can be performed using appropriate sample volumes. In these cases, adjust the volumes of the buffered peptone water accordingly.

4.3 Enrichment in selective liquid media

Rappaport-Vassiliadis medium with soya (RVS broth) and Muller-Kauffmann tetrathionate-novobiocin broth (MKTTn) are inoculated with the culture obtained in 4.2.

The RVS broth is incubated at (41,5 ± 1) °C for (24 ± 3) h and the MKTTn broth at (37 ± 1) °C for (24 ± 3) h.

To detect slow-growing *Salmonella* spp., incubate the enrichment broth for a further (24 ± 3) h to a total of (48 ± 4) h at (41,5 ± 1,0) °C.

NOTE *Salmonella* Typhi and *Salmonella* Paratyphi A are usually not important in routine water quality monitoring, but can be relevant in epidemiological investigations. MKTTn broth is used for enrichment with incubation at (36 ± 2) °C for up to (24 ± 3) h and recovers most strains of *Salmonella*, including some strains of *Salmonella* Paratyphi, but is not thought to be able to recover strains of *Salmonella* Paratyphi C. MKTTn broth is not used if *Salmonella* Typhi is suspected after the use of selenite cystine broth.

4.4 Plating out and recognition

From the cultures obtained in 4.3, two selective solid media are inoculated:

a) xylose lysine deoxycholate agar (XLD agar);

b) any other solid selective medium complementary to XLD agar and, if applicable, appropriate for the isolation of lactose-positive *Salmonella* and *Salmonella* Typhi and *Salmonella* Paratyphi strains — the laboratory may choose which medium to use.

Incubate the XLD agar at (36 ± 2) °C and examine after (24 ± 3) h to check for the presence of colonies which are considered to be presumptive *Salmonella*. Incubate the second selective agar according to the manufacturer's recommendations.

NOTE For information, brilliant green agar (BGA), bismuth sulfite agar, etc., can be used as the second plating-out medium.

4.5 Confirmation

Subculture colonies of presumptive *Salmonella*, then plate out as described in 4.4 and confirm their identity by means of appropriate biochemical (8.5.3) and serological (8.5.4) tests.

5 Apparatus

Usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following.

5.1 General. Except for disposable glassware which is delivered sterile, sterilize glassware as specified in ISO 8199. Disposable apparatus is an acceptable alternative to reusable glassware if it has suitable specifications.

5.2 Autoclave, capable of being maintained at (121 ± 3) °C and at (115 ± 3) °C.

5.3 Water bath or **incubator**, capable of being maintained at (36 ± 2) °C.

5.4 Water bath or **incubator**, capable of being maintained at (41,5 ± 1,0) °C.

5.5 Water baths, capable of operating at (70 ± 1) °C and at 50 °C to 55 °C.

5.6 Membrane filtration apparatus, as specified in ISO 8199.

5.7 Sterile membrane filters, with a nominal pore size of 0,45 µm.

The quality of membrane filters may vary from brand to brand or even from batch to batch. It is therefore advisable to check the quality on a regular basis, as specified in ISO 7704.

5.8 pH-meter, with an accuracy of calibration of ± 0,1 pH at 20 °C to 25 °C.

5.9 Sterile forceps.

5.10 Sterile loops, approximate diameter 3 mm (10 µl volume), and inoculation needle or wire.

6 Sampling

Sampling is not part of the method specified in this International Standard. Samples should be taken in accordance with ISO 19458.

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

7 Culture media and reagents

NOTE For guidelines on quality assurance and performance testing, see ISO/TS 11133-1[2] and ISO/TS 11133-2[3].

7.1 Basic materials. For uniformity of results, in the preparation of media, either use a dehydrated complete medium or use constituents of uniform quality and reagents of recognized analytical grade.

Other grades of reagents may be used provided they can be shown to produce comparable results.

7.2 Water, ISO 3696[1], grade 3.

7.3 Culture media, prepared in accordance with Annex B.

7.3.1 Buffered peptone water, non-selective pre-enrichment medium buffered peptone water (BPW, B.1).

7.3.2 Rappaport-Vassiliadis broth with soya (RVS broth, B.2), selective enrichment medium.

7.3.3 Xylose lysine deoxycholate agar (XLD agar, B.3).

7.3.4 Second solid selective plating-out medium, whose choice is left to the discretion of the testing laboratory. Follow the manufacturer's instructions precisely regarding its preparation for use.

7.3.5 Nutrient agar (B.4), or other appropriate non-selective agar.

7.3.6 Triple sugar and iron agar (TSI agar, B.5).

As an alternative, iron and two sugar agar may be used.

7.3.7 Urea agar, Christensen (B.6).

7.3.8 L-Lysine decarboxylation medium (B.7).

7.3.9 Selenite cystine broth (B.8).

7.3.10 Muller-Kaufmann tetrathionate-novobiocin broth (MKTTn, B.9).

7.3.11 Filter aid (B.10).

8 Procedure

See Figure A.1.

8.1 Preparation of the sample

For the preparation of the sample, filtration and inoculation on isolation media, follow the instructions as specified in ISO 8199 and ISO 6887-1. Start the examination preferably immediately after taking the samples. If the samples are kept at ambient temperatures, start the examination within 12 h after sampling. Under exceptional circumstances, it is allowable for the samples to be kept at (5 ± 3) °C for up to 24 h prior to examination.

The volume of the sample to be analysed depends on the type of water. Usual volumes for bathing water and drinking water are 1 000 ml to 5 000 ml. For polluted surface waters and waste water, smaller volumes are usually analysed.

If sample dilutions are necessary (e.g. for waste water samples), prepare these dilutions as specified in ISO 8199.

8.2 Non-selective pre-enrichment

8.2.1 Non-selective pre-enrichment for volumes less than 10 ml

Inoculate 50 ml of BPW (B.1) at room temperature with the sample or dilutions thereof and incubate at (36 ± 2) °C for (18 ± 2) h.

8.2.2 Non-selective pre-enrichment for volumes greater than 10 ml

Filter a volume of water appropriate for the water being examined.

Immerse the membrane filter in 50 ml of BPW (B.1).

Alternatively, add the sample to the same volume of double strength BPW.

Note that the latter procedure is not suitable for mineral waters with high salt content or sea water.

Incubate the cultures at (36 ± 2) °C for (18 ± 2) h.

8.2.3 Recommendation for turbid or polluted water

For turbid or polluted waters, sterile filter aid (B.10) can be added and the sample filtered through a sterile absorbent pad acting as a supporting base instead of using the membrane.

In this case, filter an aliquot of filter aid, typically 15 ml, to form an initial layer on the absorbent pad. Mix a second aliquot, typically 15 ml, with the volume of sample and filter. For turbid or dirty waters, additional aliquots may be filtered. When filtration is complete, remove the funnel and carefully transfer the absorbent pad and filter aid to BPW (B.1). If necessary, retain a small volume of BPW to rinse the funnel so that the final volume of BPW is 100 ml. Incubate for presence or absence, or dispense as an MPN series for a semi-quantitative count.

8.3 Selective enrichment

Allow the enrichment broth(s) to equilibrate to room temperature if they were stored at a lower temperature. Transfer 0,1 ml of the culture obtained in 8.2 to a tube containing 10 ml of the RVS broth (B.2). When MKTTn (B.9) is also used, transfer 1 ml of the culture obtained in 8.2 to a tube containing 10 ml of the MKTTn broth.

Incubate the inoculated RVS broth at (41,5 ± 1,0) °C for (24 ± 3) h and, if necessary (see 4.3), for (48 ± 4) h. Care should be taken that the maximum allowed incubation temperature (42,5 °C) is not exceeded. Incubate the inoculated MKTTn broth at (36 ± 2) °C for (24 ± 3) h.

NOTE For RVS broth, the magnesium chloride concentration and incubation temperature have been optimized to yield good recovery without losing selectivity according to Reference [5].

8.4 Plating out

8.4.1 General

Allow the XLD agar plates and the second selective plating-out medium (see ISO 6579:2002, 5.2.4.2) to equilibrate at room temperature if they were stored at a lower temperature. If necessary, dry the surface of the plates before use.

8.4.2 Plating from RVS broth

Using the culture obtained in the RVS broth, inoculate, after incubation for (24 ± 3) h and, if necessary (see 4.3), for (48 ± 4) h, by means of a sterile loop (5.10), the surface of the following enrichment media so that well-isolated colonies are obtained:

a) XLD agar (B.3);

b) an additional selective medium (7.3.4).

Invert the dishes so that the bottom is uppermost, and place them in the incubator (5.3) set at (36 ± 2) °C for (24 ± 3) h for the XLD agar. The manufacturer's instructions shall be followed for the second selective plating-out medium.

8.4.3 Plating from MKTTn broth

After incubation at (36 ± 2) °C for (24 ± 3) h using the culture obtained, repeat the procedure specified in 8.4.2 with the two selective plating-out media.

8.5 Confirmation

8.5.1 General

If shown to be reliable, commercially available identification kits for the biochemical examination of *Salmonella* may be used. Use these kits according to the manufacturer's instructions.

8.5.2 Selection of colonies for confirmation

For routine monitoring purposes, take, for confirmation, from each Petri dish of each selective medium (8.4), at least one discrete colony considered to be typical or presumptive *Salmonella*. If the first colony is not confirmed as *Salmonella*, then take a further four colonies.

On XLD agar, typical *Salmonella* colonies usually have a black centre and a lightly transparent zone of reddish colour due to the colour change of the indicator. It is recommended that at least five colonies be identified for epidemiological studies. If on one dish there are fewer than five typical or suspect colonies, take all the typical or suspect colonies for confirmation.

NOTE The recognition of *Salmonella* colonies is to a large extent a matter of experience and their appearance can vary somewhat, not only from serovar to serovar, but also from batch to batch of the selective medium used. *Shigella*, *Providencia* and H_2S-negative *Salmonella* spp.(e.g. *Salmonella* Paratyphi A) appear as pink with a darker pink centre; lactose-positive *Salmonella* grown on XLD are yellow with or without blackening; Enterobacteriaceae e.g. *Escherichia coli, Enterobacter*, *Klebsiella*, *Citrobacter*, *Proteus*, and *Serratia* appear as yellow, opaque colonies.