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

Part 1: Detection of *Salmonella* spp.

Microbiologie de la chaîne alimentaire — Méthode horizontale pour la recherche, le dénombrement et le sérotypage des Salmonella *—*

Partie 1: Recherche des Salmonella *spp.*

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html

This document was prepared by the European Committee for Standardization (CEN), Technical Committee CEN/TC 275, *Food analysis — Horizontal methods*, in collaboration with ISO Technical Committee TC 34, *Food products*, Subcommittee SC 9, *Microbiology*, in accordance with the agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This first edition of ISO 6579-1 cancels and replaces ISO 6579:2002 and ISO 6785:2001, which have been technically revised. It also incorporates ISO 6579:2002/Amd 1:2007 and ISO 6579:2002/Cor 1:2004.

The main changes, compared to ISO 6579:2002, are the following.

— ISO 6785 has been incorporated in this document.

— Samples from the primary production stage have been added to the scope.

— Detection of *Salmonella* Typhi and *Salmonella* Paratyphi is described in Annex D.

— Descriptions of preparations of initial suspensions have been removed and references made to relevant parts of ISO 6887, whenever possible.

— The temperature range for incubation of non-selective media has been extended from 37 °C ± 1 °C to 34 °C to 38 °C without further tolerance.

— For selective enrichment, there is a choice between using the broth or the semi-solid agar of Rappaport Vassiliadis medium (RVS or MSRV) for food, animal feed samples, and for environmental samples from the food production area.

— The inoculation of the isolation medium has become less prescriptive; the objective is to obtain well-isolated colonies after incubation.

— For confirmation, it is acceptable to perform the tests on only one suspect colony (instead of one suspect colony of each medium combination). If this isolate tests negative for *Salmonella,* four more suspect isolates from different media combinations shall be tested.

— It is permitted to perform the biochemical confirmation directly on a suspect, well-isolated colony from the selective plating medium. The purity check on the non-selective agar medium can then be performed in parallel.

— Two confirmation tests have become optional (*ß*-galactosidase test and indole reaction) and one confirmation test has been deleted (Voges-Proskauer reaction).

— In this document, serological confirmation (to serogroup level) is described. For guidance on serotyping (to serovar level), reference is made to ISO/TR 6579-3.

— Table 1 has been improved.

— Performance testing for the quality assurance of the culture media has been added to Annex B.

— Performance characteristics of MSRV have been added to Annex C.

A list of all parts in the ISO 6579 series can be found on the ISO website.

Introduction

This document describes a horizontal method for the detection of *Salmonella* spp. in food (including milk and milk products, originally described in ISO 6785), in animal feed, in animal faeces, and in environmental samples from the primary production stage (the latter two were originally described in ISO 6579:2002/Amd 1:2007).

The main changes, listed in the foreword, introduced in this document compared to ISO 6579:2002, are considered as minor (see ISO 17468[37]).

A procedure for the enumeration of *Salmonella* spp. is described in ISO/TS 6579-2.[3]

Guidance for serotyping of *Salmonella* spp. is described in ISO/TR 6579-3.[24]

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

Part 1: Detection of *Salmonella* spp.

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 document should be familiar with normal laboratory practice. This document 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 document specifies a horizontal method for the detection of *Salmonella*. It is applicable to the following:

— products intended for human consumption and the feeding of animals;

— environmental samples in the area of food production and food handling;

— samples from the primary production stage such as animal faeces, dust, and swabs.

With this horizontal method, most of the *Salmonella* serovars are intended to be detected. For the detection of some specific serovars, additional culture steps may be needed. For *Salmonella* Typhi and *Salmonella* Paratyphi, the procedure is described in Annex D.

The selective enrichment medium modified semi-solid Rappaport-Vassiliadis (MSRV) agar is intended for the detection of motile *Salmonella* and is not appropriate for the detection of non-motile *Salmonella* strains.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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 feed — 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 11133:2014, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at http://www.iso.org/obp

3.1
Salmonella
microorganism which forms typical or less typical colonies on solid selective media and which displays the characteristics described when confirmation tests are carried out in accordance with this document

3.2
detection of *Salmonella*
determination of *Salmonella* (3.1), in a particular mass or volume of product or surface area or object (e.g. boot socks), when tests are carried out in accordance with this document

4 Principle

4.1 General

The detection of *Salmonella* requires four successive stages as specified in Annex A.

NOTE *Salmonella* can be present in small numbers and is often accompanied by considerably larger numbers of other *Enterobacteriaceae* or bacteria of other families. Pre-enrichment is used to permit the detection of low numbers of *Salmonella* or injured *Salmonella*.

4.2 Pre-enrichment in non-selective liquid medium

Buffered peptone water at ambient temperature is inoculated with the test portion, then incubated between 34 °C and 38 °C for 18 h.

For large quantities (e.g. 1 l or more), it is recommended to pre-warm the BPW to 34 °C to 38 °C before mixing it with the test portion.

4.3 Enrichment in/on selective media

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

The RVS broth or the MSRV agar is incubated at 41,5 °C for 24 h and the MKTTn broth at 37 °C for 24 h.

For some products, it may be necessary to incubate the selective enrichment medium/media for an additional 24 h.

NOTE MSRV agar is intended for the detection of motile *Salmonella* strains and is not appropriate for the detection of non-motile *Salmonella* strains.

4.4 Plating out on selective solid media

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

— Xylose Lysine Deoxycholate agar (XLD agar);

— any other solid selective medium complementary to XLD agar (for examples, see Annex E).

The XLD agar is incubated at 37 °C and examined after 24 h. The second selective agar is incubated according to the manufacturer's instructions.

4.5 Confirmation

Colonies of presumptive *Salmonella* are subcultured and their identity is confirmed by means of appropriate biochemical and serological tests.

5 Culture media, reagents, and antisera

For current laboratory practice, see ISO 7218 and ISO 11133.

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

6 Equipment and consumables

Disposable equipment is an acceptable alternative to reusable glassware if it has suitable specifications.

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

6.1 Apparatus for dry sterilization (oven) or **wet sterilization (autoclave)**.

As specified in ISO 7218.

6.2 Drying cabinet or **oven**, capable of operating between 25 °C and 50 °C.

6.3 Incubator(s), capable of operating in the range 34 °C to 38 °C and at 37 °C ± 1 °C.

6.4 Incubator, capable of operating at 41,5 °C ± 1 °C or **water bath** capable of operating at 41,5 °C ± 1 °C.

6.5 Water bath, capable of operating at 47 °C to 50 °C.

6.6 Water bath, capable of operating at 37 °C ± 1 °C.

6.7 Water bath, capable of operating at 45 °C ± 1 °C.

It is recommended to use a water bath (6.4 to 6.7) containing an antibacterial agent because of the low infective dose of *Salmonella*.

6.8 Refrigerator, capable of operating at 5 °C ± 3 °C.

6.9 Freezer, capable of operating at -20 °C ± 5 °C.

6.10 Sterile loops, of approximate diameter, 3 mm (10 µl volume), and of 1 µl volume and **inoculation needle** or **wire**.

6.11 pH-meter, having an accuracy of calibration of ±0,1 pH unit at 20 °C to 25 °C.

6.12 Sterile tubes, **bottles**, or **flasks** with caps of appropriate capacity.

6.13 Sterile graduated pipettes or **automatic pipettes**, of nominal capacities of 25 ml, 10 ml, 1 ml, and 0,1 ml.

6.14 Sterile Petri dishes, with a diameter of approximately 90 mm and (optional) large size (diameter approximately 140 mm).

7 Sampling

Sampling is not part of the method specified in this document (see the specific International Standard dealing with the product concerned). If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

A recommended sampling method is given in ISO/TS 17728[26] for food and animal feed, in ISO 707[27] for milk and milk products, in ISO 13307[28] for sampling at the primary production stage, in ISO 17604[29] for sampling of carcasses, and in ISO 18593[25] for sampling of surfaces.

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

8 Preparation of test sample

Prepare the test sample from the laboratory sample in accordance with the specific International Standard dealing with the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure (see diagrams in Annex A)

9.1 Test portion and initial suspension

For preparation of the initial suspension, in the general case, use as diluent the pre-enrichment medium specified in B.2 (buffered peptone water). Pre-warm the BPW to room temperature before use.

In general, an amount of test portion (mass or volume) is added to a quantity of BPW (mass or volume) to yield a tenfold dilution. For this, a 25 g test portion is mixed with 225 ml of BPW. However, for some type of samples (e.g. boot socks, dust), it may be necessary to use another ratio.

For specific products, follow the procedures specified in ISO 6887 (all parts).

This document has been validated for test portions of 25 g. A smaller test portion may be used without the need for additional validation/verification provided that the same ratio between (pre-)enrichment broth and test portion is maintained. A larger test portion than that initially validated may be used if a validation/verification study has shown that there are no negative effects on the detection of *Salmonella* spp.

NOTE 1 Validation can be conducted according to the appropriate parts of ISO 16140. Verification for pooling samples can be conducted according to the protocol described in ISO 6887-1:2017, Annex D[38].

For large quantities (e.g. 1 l or more), it is recommended to pre-warm the BPW to 34 °C to 38 °C before mixing it with the test portion.

NOTE 2 When more than one 25 g test portion from a specified lot of product is to be examined and when evidence is available that combining test portions does not affect the result for that particular food, the test portions can be pooled. More information on pooling of samples as well as a procedure to test the influence of pooling on the sensitivity of the method can be found in ISO 6887-1[38].

9.2 Non-selective pre-enrichment

Incubate the initial suspension (9.1) between 34 °C and 38 °C (6.3) for 18 h ± 2 h.

It is permissible to store the pre-enriched sample after incubation at 5 °C (6.8) for a maximum of 72 h (see References [30] to [34]).

9.3 Selective enrichment

9.3.1 General

Allow the selective enrichment media, RVS broth or MSRV agar (B.3 or B.4), and MKTTn broth (B.5) to equilibrate at room temperature if they were stored at a lower temperature.

Minimize the transfer of particulate material from the pre-enrichment into the selective enrichment media.

After incubation, it is permissible to store the selective enrichment at 5 °C (6.8) for a maximum of 72 h (see References [30] to [34]).

NOTE MSRV agar is intended for the detection of motile *Salmonella* strains and is not appropriate for the detection of non-motile *Salmonella* strains.

9.3.2 Procedure for food, animal feed samples, and environmental samples from the food production area

Transfer 0,1 ml of the culture obtained in 9.2 to a tube containing 10 ml of the RVS broth (B.3) or to the surface of a MSRV agar plate (B.4). Inoculate the MSRV agar with one to three equally spaced spots on the surface of the medium.

Transfer 1 ml of the culture obtained in 9.2 to a tube containing 10 ml of MKTTn broth (B.5).

Incubate the inoculated RVS broth at 41,5 °C (6.4) for 24 h ± 3 h.

Incubate the inoculated MSRV agar plates at 41,5 °C (6.4) for 24 h ± 3 h. **Do not invert the plates**.

Incubate the inoculated MKTTn broth at 37 °C (6.3) for 24 h ± 3 h.

Suspect MSRV plates will show a grey-white, turbid zone extending out from the inoculated drop.

In dried milk products and cheese, *Salmonella* may be sublethally injured. Incubate the selective enrichment media from these products for an additional 24 h ± 3 h (see Reference [35]).

For some other products, e.g. when investigating outbreak samples, this additional incubation time may also be beneficial.

9.3.3 Procedure for samples from the primary production stage

Inoculate the MSRV agar (B.4) with 0,1 ml of the pre-enriched culture (9.2) as one to three equally spaced spots on the surface of the medium.

Incubate the inoculated MSRV plates at 41,5 °C (6.4) for 24 h ± 3 h.

Do not invert the plates.

Suspect MSRV plates will show a grey-white, turbid zone extending out from the inoculated drop.

If the plates are negative after 24 h, re-incubate for a further 24 h ± 3 h.

NOTE Sensitivity can be improved by using a second selective enrichment procedure, e.g. MKTTn broth incubated at 41,5 °C for 24 h.[36]

9.4 Plating out

9.4.1 General

From the selective enriched cultures (9.3), inoculate two selective isolation agar media. The first isolation medium is Xylose Lysine Deoxycholate (XLD) agar. The second isolation medium is chosen by the testing laboratory.

Choose a second selective plating medium which is complementary to XLD agar and is based on different diagnostic characteristics to those of XLD agar to facilitate detection of, for instance, lactose positive or H2S-negative *Salmonella.* For examples of isolation media, see Annex E.

Allow the XLD agar (B.6) plates and the second selective plating medium to equilibrate at room temperature if they were stored at a lower temperature. If necessary, dry the surface of the plates before use (see ISO 11133).

9.4.2 Procedure for food, animal feed samples, and environmental samples from the food production area

From the culture obtained in the RVS broth (9.3.2), inoculate by means of a 10 µl loop (6.10) the surface of an XLD plate (B.6) so that well-isolated colonies will be obtained. Proceed in the same way with the second selective plating-out medium.

From the positive growth obtained on the MSRV agar (9.3.2), determine the furthest point of opaque growth from the inoculation points and dip a 1 µl loop (6.10) just inside the border of the opaque growth. Withdraw the loop ensuring that no large lumps of MSRV agar are extracted. Inoculate the surface of an XLD plate (B.6) so that well-isolated colonies will be obtained. Proceed in the same way with the second selective plating-out medium.

From the culture obtained in the MKTTn broth (9.3.2), inoculate by means of a 10 µl loop (6.10) the surface of an XLD plate (B.6) so that well-isolated colonies are obtained. Proceed in the same way with the second selective plating-out medium.

NOTE 1 To obtain well-isolated colonies, large size Petri dishes with plating-out media (diameter approximately 140 mm) or two normal size plates (diameter approximately 90 mm) can be used.

Incubate the XLD plates inverted at 37 °C (6.3) for 24 h ± 3 h.

Incubate the second selective plating-out medium in accordance with the manufacturer's instructions.

If the selective enrichment media have been incubated for an additional 24 h, follow the same plating-out procedure as described above.

Typical colonies of *Salmonella* on XLD agar have a black centre and a lightly transparent zone of reddish colour due to the colour change of the indicator.

NOTE 2 *Salmonella* H2S-negative variants grown on XLD agar are pink with a darker pink centre. Lactose-positive *Salmonella* grown on XLD agar are yellow with or without blackening. The occurrence of these phenotypes is summarized in Table 1.

Check the second selective plating medium after the appropriate incubation time for the presence of colonies which, from their characteristics, are considered to be presumptive *Salmonella*.

9.4.3 Procedure for samples from the primary production stage

From the positive growth obtained on the MSRV agar (9.3.3), determine the furthest point of opaque growth from the inoculation points and dip a 1 µl loop (6.10) just inside the border of the opaque growth. Withdraw the loop ensuring that no large lumps of MSRV agar are extracted. Inoculate the surface of an XLD plate so that well-isolated colonies will be obtained. Proceed in the same way with the second selective plating medium.

Incubate the XLD plates inverted at 37 °C (6.3) for 24 h ± 3 h.

Incubate the second selective plating medium in accordance with the manufacturer's instructions.

Return negative MSRV plates to the 41,5 °C incubator and incubate for a further 24 h ± 3 h. Perform the selective plating procedure if, after 48 h of incubation, these MSRV plates become positive.

Typical colonies of *Salmonella* on XLD agar have a black centre and a lightly transparent zone of reddish colour due to the colour change of the indicator.

NOTE *Salmonella* H2S-negative variants grown on XLD agar are pink with a darker pink centre. Lactose-positive *Salmonella* grown on XLD agar are yellow with or without blackening. The occurrence of these phenotypes is summarized in Table 1.

Check the second selective plating medium after the appropriate incubation time for the presence of colonies which, from their characteristics, are considered to be presumptive *Salmonella*.

9.5 Confirmation

9.5.1 General

The combination of biochemical and serological test results indicate whether an isolate belongs to the genus *Salmonella*. For characterization of *Salmonella* strains, full serotyping is needed. Guidance for serotyping is described in ISO/TR 6579-3[24].

For some of the confirmation media as specified in 9.5.3 and in B.8 to B.12, alternative (commercial) formulations exist which may also be applicable for biochemical confirmation of *Salmonella*. These alternative formulations are allowed, provided that the performance for the biochemical confirmation of *Salmonella* is verified before use.

For a clear distinction between positive and negative biochemical reactions, it is helpful to verify the reactions of the media of each biochemical test with well-characterized positive and negative control strains.

NOTE 1 The recognition of colonies of *Salmonella* 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 culture medium used.

If shown to be reliable, miniaturized galleries for the biochemical identification of *Salmonella* may be used (see ISO 7218).

NOTE 2 Alternative procedures can be used to confirm the isolate as *Salmonella* spp. providing the suitability of the alternative procedure is verified (see ISO 7218).

9.5.2 Selection of colonies for confirmation

Mark suspect colonies on each plate (9.4). Select at least one typical or suspect colony for subculture and confirmation. If this is negative, select up to four more suspect colonies ensuring that these colonies are subcultured from different selective enrichment/isolation medium combinations showing suspect growth.

Streak the selected colonies onto the surface of a pre-dried non-selective agar medium (B.7) in a manner which will allow well-isolated colonies to develop. Incubate the inoculated plates between 34 °C and 38 °C (6.3) for 24 h ± 3 h.

Alternatively, if well-isolated colonies (of a pure culture) are available on the selective plating media (9.4), the biochemical confirmation can be performed directly on a suspect, well-isolated colony from the selective plating medium. The culture step on the non-selective agar medium can then be performed in parallel with the biochemical tests for purity check of the colony taken from the selective agar medium.