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

Microbiologie des aliments — Méthode horizontale pour la recherche de Shigella *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 21567 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

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Introduction

Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products. In this case, different methods which are specific to these products may be used if absolutely necessary for justified technical reasons. Nevertheless, every attempt should be made to apply this horizontal method as far as possible.

When this International Standard is next reviewed, account will be taken of all information then available regarding the extent to which this horizontal method has been followed and the reasons for deviations from this method in the case of particular products.

The harmonization of test methods cannot be immediate and, for certain groups of products, International Standards and/or national standards may already exist that do not comply with this horizontal method. It is hoped that when such standards are reviewed they will be changed to comply with this International Standard so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

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

WARNING — In order to safeguard the health of laboratory personnel, it is essential that the whole of this method is only carried out by skilled personnel using good laboratory practices and preferably working in a containment facility. Relevant national Health and Safety Regulations relating to this organism shall be adhered to. Care shall be taken in the disposal of all infectious materials.

1 Scope

This International Standard specifies a horizontal method for the detection of *Shigella* species.

Subject to the limitations discussed in the Introduction, this International Standard is applicable to

- products intended for human consumption and the feeding of animals, and
- environmental samples in the area of food production and food handling.

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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.

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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 6887-2, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 2: Specific rules for the preparation of meat and meat products*

ISO 6887-3, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 3: Specific rules for the preparation of fish and fishery products*

ISO 6887-4, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 4: Specific rules for the preparation of products other than milk and milk products, meat and meat products, and fish and fishery products*

ISO 7218:1996, *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations*

ISO 8261, *Milk and milk products — General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination*

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 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*

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3 Terms and definitions

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

3.1
Shigella
microorganisms which form colonies fitting the description of these species on the solid selective media used, and which display the biochemical and serological characteristics described when tests are carried out in accordance with this International Standard

3.2
detection of *Shigella* spp.
determination of the presence or absence of these microorganisms in a particular mass of product, when tests are carried out in accordance with this International Standard

4 Principle

4.1 General

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

4.2 Enrichment in selective liquid medium

A test portion is inoculated into *Shigella* broth containing 0,5 µg/ml of novobiocin, then incubated anaerobically at (41,5 ± 1) °C for 16 h to 20 h.

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4.3 Plating out and identification of colonies

From the enrichment culture obtained, three selective differential media are inoculated: MacConkey agar with low selectivity; XLD agar with moderate selectivity; and Hektoen enteric agar with the greatest selectivity. All are incubated at 37 °C for 20 h to 24 h.

4.4 Biochemical and serological confirmation

Typical and suspect colonies are selected from each of the three selective agars. The colonies are purified on nutrient agar, then biochemical and serological characterizations are carried out using the tests described.

5 Culture media, reagents and antisera

For current laboratory practices, see ISO 7218, ISO/TS 11133-1 and ISO/TS 11133-2 for the preparation, production and performance testing of culture media.

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

See Annex B for descriptions of all media, reagents and antisera.

Commercially available dehydrated media should give more consistent results than media prepared from their component parts in the laboratory. Follow the manufacturer's instructions exactly, as small changes in preparation can significantly change the performance of selective media. Excessive heating of the selective agars used in this International Standard by autoclaving, storage and then re-heating for use may result in loss of selectivity.

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6 Apparatus and glassware

Disposable equipment is an acceptable alternative to re-usable 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) and **wet sterilization (autoclave)** of equipment.

See ISO 7218.

6.2 Drying cabinet or **oven**, ventilated by convection, and capable of operating at a set temperature between 37 °C and 55 °C.

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

6.4 Modified atmosphere jars or **anaerobic incubation cabinets**, and related apparatus to achieve anaerobic conditions, with a gas composition of < 1 % O_2 and 9 % to 13 % CO_2. See ISO 7218.

6.5 Water baths, operating at a set temperature of (47 ± 3) °C, for the cooling of molten media prior to plate pouring, and another set at (50 ± 1) °C (see B 6.3.2 and B 10.2.2).

6.6 Peristaltic homogenizer (stomacher) or **rotary blender**.

See ISO 7218.

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6.7 Inoculation needles and **loops**, made of platinum/iridium or nickel/chrome, of diameter approximately 3 mm, or plastic disposable loops and needles of suitable specifications.

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

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6.9 Flasks and **bottles**, with closures, of suitable capacities for use in the preparation of enrichment broths and agars and their storage.

6.10 Measuring cylinders.

6.11 Tubes, 18 mm in diameter and 160 mm in length (plugged or with screw caps), or **culture bottles**, of nominal capacity 30 ml and 10 ml, with non-toxic metallic caps with liners or plastic disposable caps.

6.12 Petri dishes, of diameter between 90 mm to 100 mm and diameter 140 mm.

6.13 Glass slides or **plates,** suitable for use in agglutination tests.

7 Sampling

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

Sampling is not part of the method specified in this International Standard. 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.

8 Preparation of test sample

Prepare the test sample in accordance with the appropriate part of ISO 6887 and/or ISO 8261.

Analysis of samples should begin as quickly as possible, as survival of *Shigella* is poor.

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9 Procedure (see diagram in Annex A)

9.1 Test portion

See the appropriate part of ISO 6887 and/or ISO 8261 dealing with the procedures for the different types of products concerned.

9.2 Enrichment

In general add x g or x ml of test portion to 9x ml of *Shigella* broth containing 0,5 µg/ml of novobiocin (B.1.2) to make a 1 in 10 dilution of the test sample. Homogenize the test portion in the broth using a peristaltic homogenizer or rotary blender (6.6). Aseptically adjust the pH to 7,0 ± 0,2, if necessary.

Incubate (6.4) the *Shigella* broth under anaerobic conditions with caps and closures loose, or with equipment giving an equivalent effect, so that gas exchange can readily occur without contamination at (41,5 ± 1) °C (6.3) for 16 h to 20 h.

9.3 Plating out and colony selection

9.3.1 Using the cultures obtained in 9.2, gently mix the contents by hand and allow the larger particles to settle.

Inoculate, by means of a loop (6.7), the surface of the following selective agars to obtain well-isolated colonies: MacConkey agar (B.2.1) with low selectivity; XLD agar (B.2.2) with moderate selectivity; and Hektoen enteric agar (B.2.3) with a greater selectivity.

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9.3.2 Incubate (6.3) the plates at (37 ± 1) °C for between 20 h and 24 h.

9.3.3 The appearance of different *Shigella* species can vary on the same selective agar. See Annex C for a description of *Shigella* colonies on the different selective agars used.

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Shigella species can form a minority proportion of the total microbial flora when contaminating a food sample or after enrichment. In these circumstances, the direct streaking of the enrichment broth onto one plate per selective agar may fail to allow the detection of *Shigella* colonies. It may therefore be appropriate in some circumstances (e.g. the investigation of foods implicated in illness) to consider the inoculation of either two 90 mm dishes or one large (140 mm) Petri dish (6.12) to increase the possibility of detection.

The colonies of some Enterobacteriaceae strains are very similar in appearance to those of *Shigella*. Any typical or suspect colonies shall be confirmed (see 9.4) as *Shigella* species or not. Also in some circumstances (e.g. foods implicated in food poisoning), it may be appropriate to investigate more than five colonies from a plate to increase confidence in the absence of *Shigella* in the food sample tested.

Mark any typical or suspect colonies found on each plate.

If no typical colonies are seen and the growth of other microorganisms is weak (particularly on the more selective agar), re-incubate the plates for a further 24 h. Examine them again for typical *Shigella* colonies.

Carry out the confirmation procedure described in 9.4.

9.4 Confirmation of colonies

9.4.1 General

Identification kits (currently commercially available) that have been shown by the user to be reliable for the identification of the different species of *Shigella* may be used. Follow the manufacturer's instructions precisely.

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For confirmation, sub-culture from each dish of each selective medium (see 9.3) five marked typical or suspect colonies.

If on one dish there are fewer than five typical or suspect colonies, take all the marked colonies for confirmation.

Use pure cultures for biochemical and serological confirmation.

9.4.2 Purification of colonies

Streak the selected colonies onto the surface of nutrient agar plates (B.3) so as to gain well-isolated colonies.

Incubate (6.3) the plates at (37 ± 1) °C for 18 h to 24 h.

If the cultures on nutrient agar are mixed, sub-culture the suspect colony onto a further plate of nutrient agar and incubate at (37 ± 1) °C for 18 h to 24 h to obtain the pure culture.

Shigella sonnei can give two colony types on the same agar plate: a smooth round domed colony (phase 1), and a flat irregular colony with a mat surface (phase 2).

NOTE It is possible to first test the most characteristic colony from each selective agar plate. If positive, it is not necessary to test other colonies. If negative, progress through the other selected colonies until either all are negative or a positive is found.

9.4.3 Biochemical confirmation

9.4.3.1 General

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By means of an inoculation needle (6.7), inoculate the media specified in 9.4.3.2 to 9.4.3.9 respectively with each of the cultures selected in 9.4.1 and record all the results.

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9.4.3.2 Triple sugar iron agar (TSI slopes) (B.4)

Stab the butt and streak the agar slope.

Incubate (6.3) at (37 ± 1) °C for (24 ± 3) h.

Interpret the changes in the medium as follows:

Area of slope	Appearance	Indication
Butt	Yellow	Glucose fermented: positive
	Red or unchanged	Glucose not fermented: negative
	Black	Formation of hydrogen sulfide: positive
	Bubbles or cracks	Gas formation
Slant surface	Yellow	Lactose and/or sucrose utilized: positive
	Red or unchanged	Lactose and sucrose not utilized: negative

Typical *Shigella* cultures show a yellow butt (acid formation) and no gas bubbles, there is no change in the colour of the slant (no utilization of lactose or sucrose) and no hydrogen sulfide production (see Table 1).

9.4.3.3 Semi-solid nutrient agar for motility tests (B.5)

Stab the semi-solid nutrient agar with a colony using an inoculation needle (6.7).

Incubate (6.3) tubes at (37 ± 1) °C for 18 h to 24 h.

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Examine the line of inoculation for spreading growth. Non-motile microorganisms will give a discrete line; motile strains will give diffuse growth away from the inoculum line.

All *Shigella* species are non-motile.

9.4.3.4 Urea agar (B.6)

Streak the agar surface.

Incubate (6.3) at (37 ± 1) °C for (24 ± 3) h and examine at intervals.

If urea is hydrolysed, a rose-pink to deep cerise colour develops from the release of ammonia by the decomposition of the urea with a change in the colour of the pH indicator. There is no change in colour of the agar with a negative reaction.

Shigella species do not hydrolyse urea.

9.4.3.5 L-Lysine decarboxylase medium (B.7)

Inoculate below the surface of the liquid broth.

Incubate (6.3) at (37 ± 1) °C for (24 ± 3) h.

Turbidity and a purple colour after incubation indicate a positive reaction; yellow indicates a negative result.

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Shigella species do not decarboxylate lysine.

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NOTE The use of a paraffin overlay in the tubes can help to ensure anaerobic conditions.

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9.4.3.6 L-Ornithine decarboxylase medium (B.8)

Inoculate below the surface of the liquid broth.

Incubate (6.3) at (37 ± 1) °C for (24 ± 3) h.

If a purple colour develops, the test is positive; a yellow colour means a negative result.

Shigella sonnei decarboxylates ornithine, but other *Shigella* species do not (see Table 1).

9.4.3.7 Detection of indole formation (B.9)

Inoculate a tube containing 5 ml of tryptone/tryptophan medium (B.9.1) with the pure culture.

Incubate (6.3) at (37 ± 1) °C for (24 ± 3) h.

After incubation, add 1 ml of Kovac's reagent (B.9.2).

The formation of a red ring within 10 min indicates indole formation, and a yellow/brown colour indicates a negative reaction.

Shigella sonnei is negative whilst other strains give variable reactions (see Table 1).

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