INTERNATIONAL STANDARD

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Microbiology of the food chain — Horizontal method for the detection and enumeration of *Enterobacteriaceae* —

Part 1: Detection of *Enterobacteriaceae*

Microbiologie de la chaîne alimentaire — Méthode horizontale par la recherche et le dénombrement des Enterobacteriaceae —

Partie 1: Recherche des Enterobacteriaceae

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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.

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).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

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 ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*, in accordance with the agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This second edition cancels and replaces the first edition (ISO 21528-1:2004), which has been technically revised with the following main changes:

— the MPN method has become an informative Annex A;

— the pre-enrichment step in BPW followed by enrichment in EE broth has been changed to enrichment in BPW[7] and confirmation now takes place in Glucose OF medium instead of using glucose agar;

— performance testing for the quality assurance of the culture media has been added;

— performance characteristics for this method have been added to Annex C.

A list of all the parts in the ISO 21528 series can be found on the ISO website.

Introduction

This document is intended to provide general guidance for the examination of products not dealt with by existing International Standards and to be taken into account by organizations preparing microbiological test methods for application to foods or animal feeding stuffs. Because of the large variety of products within this field of application, these guidelines may not be appropriate in every detail for certain products, and for some other products it may be necessary to use different methods. Nevertheless, it is hoped that in all cases, every attempt will be made to apply the guidelines provided as far as possible and that deviations from them will only be made if absolutely necessary for technical reasons.

The main changes, listed in the foreword, introduced in this document compared to ISO 21528-1:2004 are considered as major (see ISO 17468).

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 document so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

Microbiology of the food chain — Horizontal method for the detection and enumeration of *Enterobacteriaceae* —

Part 1: Detection of *Enterobacteriaceae*

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *Enterobacteriaceae* 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.

1 Scope

This document specifies a method, with enrichment, for the detection of *Enterobacteriaceae*. It is applicable to

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

— environmental samples in the area of primary production, food production and food handling.

This method is applicable

— when the microorganisms sought are expected to need resuscitation by enrichment, and

— when the number sought is expected to be below 100 per millilitre or per gram of test sample.

A limitation on the applicability of this document is imposed by the susceptibility of the method to a large degree of variability (see Clause 11).

NOTE Enumeration can be carried out by calculation of the most probable number (MPN) after incubation in liquid medium. See Annex A.

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 the food chain — 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, *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
Enterobacteriaceae
microorganism that forms characteristic colonies on violet red bile glucose agar and that ferment glucose and show a negative oxidase reaction when the tests are carried out in accordance with the methods specified in this document

3.2
detection of *Enterobacteriaceae*
determination of *Enterobacteriaceae* (3.1), in a particular mass or volume of product or surface area, when tests are carried out in accordance with this document

4 Principle

4.1 Enrichment in non-selective medium

Buffered peptone water (BPW) is inoculated with the test portion, and then incubated at 37 °C (or 30 °C) for 18 h.

NOTE The incubation temperature of 37 °C for enrichment and isolation/enumeration on plating medium is generally used when the detection and enumeration of *Enterobacteriaceae* is for a hygiene indicator. Alternatively, a temperature of 30 °C can be chosen when the detection or enumeration of *Enterobacteriaceae* is conducted for technological purposes and includes psychrotrophic *Enterobacteriaceae*. In this document, 37 °C will be used throughout the text.

4.2 Isolation and selection for confirmation

Violet red bile glucose (VRBG) agar is inoculated with the culture obtained after enrichment in BPW, then incubated at 37 °C. It is examined after 24 h to detect the presence of typical colonies of presumptive *Enterobacteriaceae.*

4.3 Confirmation

Typical colonies of presumptive *Enterobacteriaceae* are subcultured onto non-selective medium, and confirmed by means of tests for the fermentation of glucose and the presence of oxidase.

5 Diluent, culture media and reagent

For current laboratory practice, see ISO 7218.

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

For performance testing of culture media, see ISO 11133 and 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 Incubator, capable of operating at 37 °C ± 1 °C (or 30 °C ± 1 °C).

6.3 Drying cabinet (ventilated by convection) or **incubator**, capable of operating between 25 °C and 50 °C.

6.4 Water bath, or similar apparatus, capable of being maintained between 47 °C to 50 °C.

6.5 Containers (e.g. bottles, tubes, flasks), suitable for the sterilization and storage of culture media.

6.6 Test tubes or **flasks** of appropriate capacity.

6.7 Petri dishes, made of glass or plastics, of diameter 90 mm to 100 mm.

6.8 Loops (of diameter approximately 3 mm) and **wires**, made of platinum/iridium or nickel/chromium, or **glass rods**, or equivalent sterile disposable loops or inoculating needles.

6.9 Graduated pipettes or **automatic pipettes**, of nominal capacities 10 ml, 1 ml and 0,1 ml.

6.10 pH-meter, accurate to within ±0,1 pH unit at 25 °C.

6.11 Homogenizer, as specified in ISO 7218.

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 dealing with the sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject.

Recommended sampling techniques are given in:

— ISO/TS 17728 for food and animal feed;

— ISO 13307 for primary production stage;

— ISO 17604 for carcasses;

— ISO 18593 for environmental samples.

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

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 available, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure

9.1 General

See ISO 7218.

9.2 Test portion and initial suspension

In general, an amount of test portion (mass or volume) is added to a quantity of BPW (mass or volume) to yield a 10-fold dilution. For example, a 10 g test portion is mixed with 90 ml of BPW.

This document has been validated for test portions of 10 g or ml. A smaller test portion may be used, without the need for additional validation/verification, providing that the same ratio between 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 adverse effects on the detection of *Enterobacteriaceae.*

NOTE Validation can be conducted in accordance with the appropriate documents of ISO 16140 (all parts). Verification for pooling samples can be conducted in accordance with the protocol described in ISO 6887-1:2017, Annex D.

9.3 Enrichment

Incubate the initial suspension (9.2) at 37 °C for 18 h ± 2 h.

Continue the procedure with isolation and selection of colonies for confirmation (9.4).

9.4 Isolation and selection for confirmation

9.4.1 Isolation

Using a loop (6.8), streak from the incubated enrichment medium (see 9.3) the surface of a plate containing the selective medium (B.2) and incubate the plate at 37 °C (see note in Clause 4) for 24 h ± 2 h.

9.4.2 Selection of colonies for confirmation

Characteristic colonies are pink to red or purple (with or without precipitation haloes).

Mark suspect colonies from the incubated plates (see 9.4.1). Select at least one typical or suspect colony for subculture (see 9.5) and biochemical confirmation tests (see 9.6). If this is negative, select up to four more suspect colonies.

If more than one morphology is present among the colonies, select one colony of each morphology for subculture.

Certain *Enterobacteriaceae* may cause decolouration of their colonies or of the medium. Therefore, when no characteristic colonies are present, choose whitish colonies for confirmation.

9.5 Subculturing selected colonies

Streak onto non-selective medium (e.g. nutrient agar plates) (B.3) each of the colonies selected for confirmation (see 9.4.2).

Incubate these plates at 37 °C for 24 h ± 2 h.

Select a well-isolated colony from each of the incubated plates for the biochemical confirmation tests (see 9.6).

9.6 Biochemical confirmation tests

9.6.1 Oxidase reaction

Using a platinum/iridium loop, wire or glass rod (6.8), take a portion of each well-isolated colony (see 9.5) and streak onto a filter paper moistened with the oxidase reagent (B.5) or onto a commercially available disc or stick. A nickel/chromium loop or wire shall not be used.

Consider the test to be negative if the colour of the filter paper does not turn dark blue purple within 10 s.

Consult the manufacturer's instructions for ready-to-use discs or sticks.

9.6.2 Fermentation test

Using a wire (6.8), stab the same colonies selected in 9.6 that gave a negative oxidase test into tubes containing Glucose OF medium (B.4). Overlay the surface of the medium with minimal 1 cm of sterile mineral oil (B.6).

Incubate these tubes at 37 °C for 24 h ± 2 h.

If a yellow colour develops throughout the content of the tube, regard the reaction as being positive.

10 Expression of results

If any of the selected typical colonies (see 9.4.2) from a subculture (see 9.4.1) is oxidase-negative and glucose-positive, the sample from which the subculture was derived shall be regarded as being positive for *Enterobacteriaceae*. In accordance with the interpretation of the results, indicate *Enterobacteriaceae* detected or not detected in a test portion of *x* g or *x* ml of product, or on the surface area swabbed, or in entire objects (e.g. boot socks).

11 Precision

11.1 Interlaboratory study

The performance characteristics of the method were determined in an interlaboratory study to determine the specificity, sensitivity and the LOD_{50} of the method. The data are summarized in Annex C. The values derived from the interlaboratory study may not be applicable to food types other than those given in Annex C.

NOTE In this document, the word "type" is combined with "food" to improve the readability of this document. However, the word "food" is interchangeable with "feed" and the other areas of the food chain as mentioned in the scope of this document.

11.2 Sensitivity

The sensitivity is defined as the number of samples found positive divided by the number of samples tested at a given level of contamination. The results are thus dependent on the level of contamination of the sample.

11.3 Specificity

The specificity is defined as the number of samples found negative divided by the number of true negative (or blank) samples tested.

12 Test report

The test report shall specify:

— the test method used, with a reference to this document, i.e. ISO 21528–1;

— the sampling method used, if known;

— the size of the test portion and/or the nature of the objects examined (for detection methods only);

— all operating conditions not specified in this document, or regarded as optional, together with details of any incidents which may have influenced the test result(s);