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Microbiology of the food chain — Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species) —

Part 1: Method using Baird-Parker agar medium

Microbiologie de la chaîne alimentaire — Méthode horizontale pour le dénombrement des staphylocoques à coagulase positive (Staphylococcus aureus et autres espèces) —

Partie 1: Méthode utilisant le milieu gélosé de Baird-Parker

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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 of the voluntary nature of standards, 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 www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology* in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 463, *Microbiology of the food chain*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This second edition cancels and replaces the first edition (ISO 6888-1:1999), which has been technically revised. It also incorporates the amendments ISO 6888-1:1999/Amd 1:2003 and ISO 6888-1:1999/Amd 2:2018. The main changes compared with the previous edition are as follows:

— the title has been changed to relate to the “Food chain”;

— the status of this document and ISO 6888-2 has been clarified;

— the document has been aligned with ISO 7218:2007, i.e. pour molten agar medium at 44 °C to 47 °C;

— all occurrences, when appropriate, have been changed from “35 °C or 37 °C” to “34 °C to 38 °C”;

— all occurrences of incubation time, when appropriate, have been changed from “18 h to 24 h” to “24 h ± 2 h”;

— requirements have been added to use ISO 11133;

— all available standards related to sampling techniques have been updated;

— a description of typical and atypical colonies on Baird-Parker agar (BPA) medium has been updated;

— the rabbit plasma fibrinogen agar (RPFA) medium has been added as alternative to the coagulase test for confirmation;

— the flow diagram procedure in Annex A has been updated;

— the culture media and reagent with performance testing in Annex B has been added;

— results of the interlaboratory study (from ISO 6888-1:1999/Amendement1:2003, Precision data) has been updated;

— the Bibliography has been updated.

A list of all parts in the ISO 6888 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

This document, ISO 6888-2 and ISO 6888-3 describes three horizontal methods for the detection and enumeration of coagulase-positive staphylococci among which enterotoxinogenic strains are encountered. It is mainly concerned with *Staphylococcus aureus*, but also with *S. intermedius* and certain strains of *S. hyicus*.

For the purposes of this document, the confirmation of typical and atypical colonies is based on a positive coagulase reaction, but it is recognized that some strains of *Staphylococcus aureus* give weakly positive coagulase reactions. These latter strains can be confused with other bacteria but they can be distinguished from such other bacteria by the use of additional tests not included in this document, such as the sensitivity to lysostaphin, the production of haemolysin, thermostable nuclease and acid from mannitol (see ISO 7218 and Reference [15]).

The main technical changes listed in the Foreword, introduced in this document compared with the previous edition are considered as minor (see ISO 17468). They have a minor impact on the performance characteristics of this method.

Results of the interlaboratory study and tested samples are described in Annex C.

Microbiology of the food chain — Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species) —

Part 1: Method using Baird-Parker agar medium

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detecting staphylococci 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 horizontal method for the enumeration of coagulase-positive staphylococci by counting the colonies obtained on a solid medium (Baird-Parker medium)[10] after aerobic incubation at 34 °C to 38 °C and coagulase confirmation.

This document is applicable to:

— products intended for human consumption;

— products intended for animal feeding;

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

— samples from the primary production stage.

This horizontal method was originally developed for the examination of all samples belonging to the food chain.

Because of the large variety of products in the food chain, it is possible that this horizontal method is not appropriate in every detail for all products. Nevertheless, it is expected that the required modifications are minimized so that they do not result in a significant deviation from this horizontal method.

Based on the information available at the time of publication of this document, this method is not considered to be (fully) suited to the examination of fermented products or other products containing technological flora based on *Staphylococcus* spp (e.g. *S. xylosus*) (such as cheeses made from raw milk and certain raw meat products) likely to be contaminated by:

— staphylococci forming atypical colonies on a Baird-Parker agar medium;

— background flora that can obscure the colonies being sought.

Nevertheless, both this document and ISO 6888-2 are given equivalent status.

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:

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

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

3.1
coagulase-positive staphylococci
bacteria that form either typical or atypical colonies, or both, on the surface of a selective culture medium (Baird-Parker agar medium) and that show a positive coagulase reaction in a tube test or on rabbit plasma fibrinogen agar

Note 1 to entry: The typical and atypical colonies are described in 9.3.1.

3.2
enumeration of coagulase-positive staphylococci
determination of the number of *coagulase-positive staphylococci* (3.1) per gram, per millilitre, per square centimetre or per sampling device/sampled area

Note 1 to entry: A sampled area is an area not defined by a numerical size, for example, a hot tap, a door handle.

4 Principle

4.1 General

Inoculation of the surface of a solid selective culture medium, with a specified quantity of the test sample if the product is liquid, or with a specified quantity of the initial suspension in the case of other products.

Inoculation, under the same conditions, using decimal dilutions of the test sample.

4.2 Incubation

Aerobic incubation of the plates at 34 °C to 38 °C and examination after both 24 h and 48 h.

4.3 Enumeration and confirmation

Calculation of the number of coagulase-positive staphylococci per gram, per millilitre, per square centimetre or per sampling device of sample from the number of either typical or atypical colonies, or both, obtained on plates at dilution levels chosen to give a significant result, and confirmed by a positive coagulase test result, within the counting limits of the method and in accordance with ISO 7218.

NOTE See Annex A for a flow diagram.

5 Culture media and reagents

Follow current laboratory practices in accordance with ISO 7218.

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

For performance testing of culture media and reagents, follow the procedures in accordance with either Annex B or ISO 11133, or both.

For the diluent(s), see the relevant part of the ISO 6887 series.

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) and wet sterilization (autoclave).

See ISO 7218.

6.2 Incubator, capable for maintaining the inoculated media within the temperature range 34 °C to 38 °C.

NOTE The range 34 °C to 38 °C for incubation of media includes the use of incubators set at 35 °C ± 1 °C, 36 °C ± 2 °C or 37 °C ± 1 °C.

6.3 Water bath, or similar apparatus, capable of being maintained at 44 °C to 47 °C.

6.4 Sterile tubes, bottles or flasks with caps, of appropriate capacity. Bottles or flasks with non-toxic metallic or plastic screw-caps may be used.

6.5 Sterile Petri dishes, with a diameter of approximately 90 mm and (optional) large size (diameter approximately 140 mm), made of glass or plastic.

6.6 Straight wire (see ISO 7218) or **Pasteur pipette**.

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.

6.7 Sterile graduated pipettes or automatic pipettes of nominal capacities of 1 ml, 2 ml and 10 ml, graduated in 0,1 ml, 0,1 ml and 0,5 ml divisions, respectively. See ISO 7218.

Graduated pipettes and pipettor tips should be fitted with a non-absorbent cotton wool plug to prevent contamination when used to manipulate microbial cultures.

6.8 Spreaders, sterile, made of glass or plastic.

6.9 pH-meter, it shall be capable of being read to the nearest 0,01 pH unit, enabling measurements to be made with a tolerance of ±0,1 pH unit. The pH meter shall be equipped with either manual or automatic temperature compensation. See ISO 7218.

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

6.11 Membranes, with a 0,2 µm pore size.

7 Sampling

Sampling is not part of the method specified in this document. Follow the specific International Standard dealing with the product concerned. If there is no specific International Standard dealing with the sampling of the product concerned, the parties concerned should come to an agreement on this subject.

Recommended sampling techniques are given in the following documents:

— ISO/TS 17728 for food and animal feed;

— ISO 707 for milk and milk products;

— ISO 6887-3 for fish and fishery products;

— ISO 13307 for the primary production stage;

— ISO 17604 for carcasses;

— ISO 18593 for surfaces.

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

8 Preparation of the test sample

Prepare the test sample from the laboratory sample in accordance with the specific International Standard dealing with the product concerned; follow the procedures specified in the ISO 6887 series and, if necessary, ISO 18593. If there is no specific International Standard available, the parties concerned should come to an agreement on this subject.

9 Procedure

9.1 Test portion, initial suspension and dilutions

Refer to the relevant part of the ISO 6887 series.

9.2 Inoculation and incubation

Transfer, by means of a sterile pipette (6.7), 0,1 ml of the test sample if liquid, or 0,1 ml of the initial suspension (10^{-1} dilution) in the case of other products, to a Baird-Parker agar (BPA) plate (see B.2). For enumeration techniques in microbiology of the food chain, the number of Petri dishes to be used according tested dilutions is stated into ISO 7218. Repeat the procedure for further decimal dilutions if necessary.

If, for certain products, it is desirable to count low numbers of coagulase-positive staphylococci, the level of detection can be raised by a factor of 10 by inoculating 1,0 ml of the test sample liquid, or 1,0 ml of the initial suspension for other products, either on the surface of one plate (Ø 140 mm) or on the surface of three small agar plates (Ø 90 mm). The number of Petri dishes to be used according to the tested dilutions is stated in ISO 7218.

Carefully spread the inoculum as quickly as possible over the surface of the agar plate, trying not to touch the sides of the Petri dish, using the spreader (6.8). Allow the plates to dry with their lids on for about 15 min at laboratory temperature.

NOTE For inoculation using a spiral plater, see ISO 7218.

Invert the dishes prepared above and place them for 24 h ± 2 h in the incubator (6.2) set at 34 °C to 38 °C. Then re-incubate for a total of 48 h ± 4 h.

NOTE Colonies with typical appearance after 24 h ± 2 h incubation can lose their typical appearance after 48 h ± 4 h incubation, due to overgrowth with enlargement of the clear zone during the second phase of incubation. Counting only at 48h ± 4h can lead to too low counts or no counts.

9.3 Counting of colonies

9.3.1 General description of colonies growing on BPA medium

9.3.1.1 Colonies presumed to be coagulase-positive staphylococci

Typical colonies are black or grey, shining and convex (1 mm to 1,5 mm in diameter after incubation for 24 h ± 2 h, and 1,5 mm to 2,5 mm in diameter after incubation for 48 h ± 4 h) and are surrounded by a clear zone, which can be partially opaque. After incubation for at least 24 h, an opalescent ring immediately in contact with the colonies can appear in this clear zone.

Atypical colonies have the same size as typical colonies and can present one of the following morphologies:

— shining black colonies with or without a narrow white edge; the clear zone is absent or barely visible and the opalescent ring is absent or hardly visible;

— grey colonies free of clear zone.

Atypical colonies are formed mainly by strains of coagulase-positive staphylococci contaminating, for example, dairy products, shrimps and giblets. They are less often formed by strains of coagulase-positive staphylococci contaminating other products.

9.3.1.2 Colonies not presumed to be coagulase-positive staphylococci

Other colonies are all the remaining colonies possibly present on the plates that do not show the typical or atypical appearance described in 9.3.1.1 and are considered as the background flora.

NOTE Bacteria belonging to genera other than staphylococci can give colonies with an appearance similar to staphylococci. Microscopic examination of Gram stain, before confirmation, will enable the distinction of other genera from staphylococci.

9.3.2 Colonies counting procedure

After incubation for 24 h ± 2 h, mark on the bottom of the plates the positions of any typical colonies present.

Re-incubate all plates at 34 °C to 38 °C for a further 24 h ± 2 h and mark any new typical colonies. Also mark any atypical colonies present.

For the enumeration, only retain plates containing a maximum of 300 colonies in total (typical, atypical, background flora), and including a maximum of either 150 typical or atypical colonies, or both, at two successive dilutions.

EXAMPLE 0 typical colony, 150 atypical colonies and 150 of background flora.

150 typical colonies, 0 atypical colony and 150 of background flora

150 typical colonies, 150 atypical colonies and 0 of background flora

One of the plates shall contain at least 10 colonies (of either typical or atypical colonies, or both). Select for confirmation (see 9.4) a given number A (in general five typical colonies if there are only typical colonies, or five atypical colonies if there are only atypical colonies, or five typical and five atypical colonies if both types are present, from each plate).