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Microbiology of the food chain — Horizontal method for the detection and enumeration of *Clostridium* spp. —

Part 3: Detection of *Clostridium perfringens*

Microbiologie de la chaîne alimentaire — Méthode horizontale pour la recherche et le dénombrement de Clostridium *spp.* —

Partie 3: Recherche de Clostridium perfringens

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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 document 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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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).

A list of all parts in the ISO 15213 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

Clostridium (C.) perfringens is a gram-positive, anaerobic, spore-forming bacterium. As a ubiquitous bacterium, *C. perfringens* is predominantly found in soil, but also in the intestinal tract of humans and animals. Therefore, the presence of *C. perfringens* in high numbers can be an indication of inadequate preparation or handling of food.

High numbers of *C. perfringens* in ready-to-eat-food can cause human illness, mainly diarrhoea. The strains are classified into toxin types, depending on the ability to produce different so called "major" and "minor" toxins. Food poisonings related to *C. perfringens* are mostly caused by *C. perfringens* isolates with the ability to produce *C. perfringens* enterotoxin (CPE). A characteristic feature is the heat resistance of the spores; they have the ability to germinate and multiply in ready-to-eat food after the cooking process. Ingestion of contaminated food is followed by gastrointestinal disease, when enzyme-resistant *C. perfringens* enterotoxins are set free during sporulation in the small intestine. The strains are classified into different types.

This document describes the horizontal method for the detection of *C. perfringens* in food, feed, environmental samples and samples from the primary production stage. The method for the enumeration of sulfite-reducing *Clostridium* spp. is described in ISO 15213-1 and ISO 15213-2 describes the method for the enumeration of *C. perfringens*. These three parts are published as a series of International Standards because the methods are closely linked to each other. These methods are often conducted in association with each other in a laboratory.

Microbiology of the food chain — Horizontal method for the detection and enumeration of *Clostridium* spp. —

Part 3: Detection of *Clostridium perfringens*

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

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 and handling;

— samples from the primary production stage.

This horizontal method was originally developed for the examination of all samples belonging to the food chain. Based on the information available at the time of publication of this document, this method is considered to be fully suited to the examination of all samples belonging to the food chain. However, 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.

NOTE Interlaboratory studies with a small number of participating laboratories (<10) were conducted for the following food categories:

— ready-to-eat, ready-to-reheat meat products;

— eggs and egg products (derivates);

— ready-to-eat, ready-to-reheat fishery products;

— processed fruits and vegetables;

— infant formula and infant cereals (with probiotics);

— multi-component foods or meal components.

It has also been validated with a small number of participating laboratories for the following other category:

— environmental samples (food or feed production).

Since the method is not commonly used for samples in the primary production stage, this category was not included in the interlaboratory study. Therefore, no performance characteristics were obtained for this category. The method has not been validated for the category 'pet food and animal feed', as the test samples used for the interlaboratory study were already naturally contaminated with *C. perfringens*. Given the limited number of participating laboratories in the interlaboratory studies, the calculated performance characteristics can be used as indicative values of the method performance. For detailed information on the validation, see Clause 11 and Annex C to F.

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 the food chain — 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 terminology databases for use in standardization at the following addresses:

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

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

3.1
presumptive *C. perfringens*
presumptive *Clostridium perfringens*
spore-forming bacteria forming typical colonies on a specific selective medium under obligate anaerobic conditions

Note 1 to entry: Presumptive *C. perfringens* are spore-forming bacteria that are able to produce typical colonies under the conditions specified in this document.

3.2
confirmed *C. perfringens*
confirmed *Clostridium perfringens*
bacteria that produce characteristic colonies on the specified selective medium under obligate anaerobic conditions and either possess the enzyme acid phosphatase, or are able to produce sulfite, are not able to produce indole and are not motile (SIM agar)

3.3
human pathogenic *C. perfringens*
human pathogenic *Clostridium perfringens*
confirmed *C. perfringens* (3.2) strains which possess the ability to produce *C. perfringens* enteroxin (CPE), encoded by the *cpe* gene

Note 1 to entry: The *cpe* gene can be located either chromosomally or on large plasmids. These isolates are able to produce CPE in the small intestine on sporulation and cause human illness.

3.4
detection of *C. perfringens*
detection of *Clostridium perfringens*
determination of confirmed *C. perfringens* (3.2) in a particular mass, volume of product, surface area or object, when a specified test is conducted

Note 1 to entry: Specified tests are given in Clause 9.

4 Principle

4.1 General

The detection of *C. perfringens* requires three successive stages as specified in Annex A.

4.2 Enrichment in selective liquid medium

A selective culture medium (at ambient temperature) is inoculated with a specified quantity of the test sample if the initial product is liquid, or a specified quantity of an initial suspension in the case of other products. The inoculated selective medium is incubated at 46 °C for 18 h.

4.3 Isolation on selective solid medium

From the cultures obtained in 4.2, two selective plating media are inoculated. The plates are incubated at 37 °C and at 46 °C for 24 h anaerobically.

4.4 Confirmation

Confirmatory tests are carried out. The result is expressed as *C. perfringens* detected or not detected per sample volume. Additionally, the method mentioned in Annex G can be used for molecular differentiation between non-pathogenic and human pathogenic *C. perfringens* strains.

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, follow the procedures in accordance with ISO 11133 and Annex B.

6 Apparatus and consumables

Disposable equipment is an acceptable alternative to reusable glassware if it has suitable specifications. The usual microbiological laboratory apparatus (see ISO 7218) and, in particular, the following shall be used.

6.1 Appropriate apparatus for achieving an anaerobic atmosphere, a jar that can be hermetically sealed or any other appropriate equipment which enables anaerobic atmosphere conditions to be maintained for the total incubation time of the culture medium. Other systems of equivalent performance, such as anaerobic cabinets, may be used. Follow the manufacturer's instructions for installation and maintenance.

The composition of the atmosphere required can be achieved by means of the addition of a gas mixture (e.g. from a gas cylinder) after evacuation of air from the jar, by displacement of the atmosphere in a cabinet or by any other appropriate means (such as commercially available gas packs). In general, anaerobic incubation requires an atmosphere of less than 1 % volume fraction oxygen, 9 % volume fraction to 13 % volume fraction carbon dioxide.

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

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

6.4 Freezers, capable of operating at -20 °C ± 2 °C and below -70 °C.

6.5 Incubator(s), capable of operating at 37 °C ± 1 °C, 46 °C ± 1 °C.

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

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

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

6.9 Sterile graduated pipettes or **automatic pipettes**, of nominal capacities of 10 ml and 1 ml.

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

6.11 Sterile Petri dishes, with a diameter of approximately 90 mm.

6.12 Thermostatically controlled water bath, capable of operating at 44 °C to 47 °C.

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, it is recommended that the parties concerned 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 raw molluscs, tunicates and echinoderms from primary production areas;

— ISO 13307 for primary production stage;

— ISO 17604 for carcasses;

— ISO 18593 for surfaces.

It is important that the laboratory receives a sample that is representative of the product under consideration. The sample should not have 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. Follow the procedures as specified in the ISO 6887 series.

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

The procedure as given in Annex A shall be followed.

9.2 Test portion and initial suspension

Follow the procedures in accordance with the ISO 6887 series and the specific International Standard dealing with the product concerned.

Prepare the initial suspension in the case the product of concern is not liquid. Add 1 ml (6.9) of the liquid sample or 1 ml (6.9) of the initial suspension (0,1 g product) to 9 ml of Rapid Perfringens Medium (RPM, B.2). Alternatively, 10 ml (6.9) of the liquid sample or of the initial suspension (1 g product) is added to 90 ml of RPM (B.12).

It is possible to composite or pool samples of the same type, to reduce workload when a large number of samples are required to be examined. This may be necessary to reflect microbiological quality of a large batch of product of environmental samples or required by regional legislation.

Similarly, a number of test portions may be pooled and examined together in larger quantities of media, or the (pre)enrichment cultures from the test portions may be pooled and carried out as a single test. These pooling procedures are described in ISO 6887-1:2017, Annex A. Whether it is possible to pool samples of a certain type, this shall be verified according to the protocol described in ISO 6887-1:2017, Annex D.

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

9.3 Selective enrichment

Incubate the selective enrichment broth RPM in closed tubes or bottles (9.2) at 46 °C (6.5) for 18 h ± 2 h.

9.4 Isolation

Allow the selective plating media (B.3 and B.4) to equilibrate at room temperature if they were stored at a lower temperature. If necessary, dry the surfaces of the plates (see ISO 11133) in a drying cabinet or oven (6.3) before use.

From the selective enrichment obtained at 9.3, inoculate by means of a 10 µl loop (6.10) the surface of a Petri dish (6.11) containing the selective medium tryptose sulfite cycloserine agar (TSC agar, B.3) and a Petri dish (6.11) containing the selective medium Lactose egg-yolk neomycin agar (LENA, B.4).

Incubate the TSC agar plates anaerobically (6.1) in an incubator (6.5) at 37 °C for 24 h ± 2 h. Incubate the LENA plates anaerobically (6.1) in an incubator (6.5) at 46 °C for 24 h ± 2 h.

NOTE After inoculation of the TSC agar plates an overlay of TSC agar can be used to prevent the development of spreading colonies on the surface of the medium. Pour about 5 ml of the TSC medium (see Clause B.3) as overlay and allow to solidify by leaving the Petri dishes standing on a cool horizontal surface.

9.5 Confirmation of *C. perfringens*

9.5.1 Selection of colonies for confirmation

9.5.1.1 Typical colonies on TSC agar are black or grey to yellow-brown staining, even if the colour is faint.

Typical colonies on LENA show yellow colour (acid fermentation from lactose) and precipitation (lecithinase reaction).

Upon removal of the TSC agar plates from the anaerobic atmosphere, plates shall be read within 30 min as the colour of the colonies can rapidly fade and disappear upon exposure to oxygen. If anaerobic jars are used, the plates should be checked jar by jar or in small portions if the incubation was performed in an anaerobic incubator (6.1, 6.5).

For confirmation, take five presumptive *C. perfringens* colonies from each dish containing typical colonies (see 9.4). If more than one morphology is present among the colonies, select one of each morphology for subculture and confirmation.

9.5.1.2 Allow the plates 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).

Streak each of the selected colonies with a sterile loop (6.10) onto one non-selective blood agar plate, e.g. Columbia blood agar (B.5). If blood is not available, Columbia agar base or another nutrient-rich medium (e.g. Tryptone soya agar or Brain heart infusion agar) can be used with or without blood. Several isolates can be streaked onto identified sectors of a non-selective agar plates. Streaks should obtain well-isolated colonies.

Incubate the plates in an anaerobic atmosphere (6.1) at 37 °C (6.5) for 20 h ± 2 h. Right after incubation, select well-isolated freshly grown colonies for confirmation. Confirmation may be done either by the acid phosphatase test (9.5.2) or by the sulfite indole motility (SIM) agar test (9.5.3).

NOTE Alternative procedures (see ISO 7218) can be used to confirm whether the typical colonies are *C. perfringens,* provided that the suitability of the alternative procedure has been validated (see ISO 16140-4 or ISO 16140-6).

After incubation, these plates can be refrigerated at 5 °C (6.7) for a maximum of 48 h before reading. For plates which were incubated anaerobically, maintain the anaerobic atmosphere.

9.5.2 Acid phosphatase test

9.5.2.1 It is known that, beside *C. perfringens*, some other *Clostridium* strains (e.g. some strains of *C. baratii*) can produce acid phosphatase, but this ability is very limited. Therefore, only a very low percentage of false positives is expected.

9.5.2.2 Colonies grown anaerobically on blood or nutrient agar plates are spread on filter paper and 2 to 3 drops of the acid phosphatase reagent (B.6) are placed onto the colonies. If a commercially available test kit is used, follow the manufacturer's instructions.

NOTE It is possible to drip acid phosphatise reagent on colonies, if no further investigation of the colonies is needed.

9.5.2.3 A purplish colour developed within 3 min to 4 min is considered as a positive reaction.