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Microbiology of the food chain — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Detection of botulinum type A, B, E and F neurotoxin-producing clostridia

Microbiologie de la chaîne alimentaire — Réaction de polymérisation en chaîne (PCR) pour la détection de micro-organismes pathogènes dans les aliments — Détection des clostridies productrices de neurotoxine botulique de type A, B, E et F

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Foreword

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ISO/TS 17919 was prepared by the European Committee for Standardization (CEN) Technical Committee CEN/TC 275 *Food analysis — Horizontal methods*, in collaboration with 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).

Introduction

Botulinum neurotoxin-producing clostridia are ubiquitous in the environment. Botulism is a severe neuroparalytic disease resulting from the action of botulinum neurotoxins (BoNTs). Seven different serotypes of BoNTs (type A to G) and a number of subtypes have been identified to date.

BoNT type A (BoNT/A), type B (BoNT/B), type E (BoNT/E) and type F (BoNT/F) are mainly responsible for botulism in humans and the genes encoding these toxins are the targets of this Technical Specification. BoNT type A, B, E, and F-producing clostridia exist in four physiologically distinct groups (Group I *Clostridium botulinum*, Group II *C. botulinum*, *C. baratii*, *C. butyricum*).

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Microbiology of the food chain — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Detection of botulinum type A, B, E and F neurotoxin-producing clostridia

1 Scope

This Technical Specification specifies a horizontal method for the molecular detection of clostridia carrying botulinum neurotoxin A, B, E, and F genes by a PCR method. This method detects the genes and not the toxins, therefore a positive result does not necessarily mean the presence of these toxins in the sample investigated. This Technical Specification is applicable to products for human consumption, animal feed, and environmental samples.

The PCR assays for detection of genetic sequences encoding specific toxin types are described in Annexes B and C.

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.

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 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*

ISO 20837, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Requirements for sample preparation for qualitative detection*

ISO 20838:2006, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Requirements for amplification and detection for qualitative methods*

ISO 22174, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — General requirements and definitions*

3 Terms and definitions

For the purpose of this document, the terms and definitions given in ISO 22174 apply.

4 Symbols and abbreviated terms

4.1 Symbols

c substance concentration

ρ mass concentration

φ volume fraction

w mass fraction

4.2 Abbreviated terms

BoNT botulinum neurotoxin

5 Principle

5.1 General

The method comprises the following consecutive steps:

a) microbial enrichment (see 5.2);

b) nucleic acid extraction (see 5.3);

c) amplification (see 5.4);

d) detection of PCR products (see 5.5);

e) confirmation (see 5.6).

NOTE Real-time-PCR combines steps c) to e).

5.2 Microbial enrichment

The number of BoNT-producing clostridia (spores or vegetative cells) to be detected is increased by encouraging their germination and growth in non-selective liquid nutrient medium tryptone–peptose–glucose–yeast extract broth under anaerobic conditions.

5.3 Nucleic acid extraction

Bacterial cells are separated from the nutrient medium, lysed and the nucleic acids are extracted for use in the PCR reaction.

5.4 Amplification by PCR

The extracted nucleic acid is transferred to the PCR mix and the amplification is carried out in a thermal cycler.

5.5 Detection of PCR products

PCR products are detected by gel electrophoresis or an appropriate alternative.

5.6 Confirmation

The identity of the PCR products shall be confirmed by any appropriate method, e.g. sequencing, hybridization or restriction analysis.

6 Reagents

6.1 General

For all stages 5.1 b) to e), use only reagents of recognized analytical grade and consumables suitable for molecular biology applications as specified in ISO 20837 and ISO 20838.

Reagent requirements specified in ISO 20838:2006, Clause 5, apply.

6.2 Culture media

6.2.1 General

Follow ISO 11133 for the preparation, production and performance testing of culture media.

6.2.2 Diluent

Follow ISO 6887-1 and the relevant part of ISO 6887[9]–[13] dealing with the product to be examined.

6.2.3 Non–selective enrichment culture medium, tryptone–peptone–glucose–yeast extract broth (TPGY broth) (Reference [7])

6.2.3.1 General

Other approved non-selective enrichment culture media can be used provided equivalent performance is shown.

6.2.3.2 Composition and pH

Tryptone	50 g
Peptone	5 g
Yeast extract	20 g
D-Glucose	4 g
Sodium thioglycolate, $HSCH_2COONa$	1 g
Water	to 1 000 ml

pH 7,0 ± 0,2

6.2.3.3 Preparation

Dissolve the components in the water by boiling. After sterilization, adjust to pH 7,0 ± 0,2 at 25 °C. Dispense the base into flasks or bottles of appropriate capacity. Sterilize for 15 min at 121 °C. Store in a refrigerator at 5 °C ± 3 °C. Discard unused medium 4 weeks after preparation.

6.2.4 TPGY broth buffered — for acidic and acidifying foodstuffs only

6.2.4.1 Stock solution (phosphate buffer)

6.2.4.1.1 Solution 1

Sodium dihydrogenphosphate monohydrate [$NaH_2PO_4 \cdot H_2O$]	138 g
Water	to 1 000 ml

6.2.4.1.2 Solution 2

Disodium hydrogenphosphate [Na_2HPO_4]	142 g
Water	to 1 000 ml

6.2.4.1.3 Preparation

Dissolve the components in the water by boiling. To 250 ml solution 1, add solution 2 until the pH reaches 7,2. Store in a refrigerator at 5 °C ± 3 °C.

6.2.4.2 Preparation of the complete medium

Dissolve the components given for the base (6.2.3.2) in 500 ml water by boiling. Add 100 ml phosphate buffer (6.2.4.1) The final phosphate concentration of the complete medium is 0,1 mol/l. Add water up to 1 000 ml. Dispense the complete medium into flasks or bottles of appropriate capacity. Sterilize for 15 min at 121 °C. Store in a refrigerator at 5 °C ± 3 °C. Discard unused medium 4 weeks after preparation.

6.3 Nucleic acid extraction

6.3.1 Chloroform, $CHCl_3$.

6.3.2 Ethanol, $\varphi(C_2H_5OH)$ = 96 %.

6.3.3 Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), $C_{10}H_{14}N_2O_8Na_2$.

6.3.4 Hexadecyl(trimethyl)ammonium bromide [(cetyl(trimethyl)ammonium bromide, **CTAB]**, $C_{19}H_{42}BrN$.

6.3.5 Hydrochloric acid, $\varphi(HCl)$ = 37 %.

6.3.6 Isopropanol, $CH_3CH(OH)CH_3$.

6.3.7 Proteinase-K, approximately 20 units/mg of lyophilizate.

6.3.8 Sodium chloride, NaCl.

6.3.9 Sodium hydroxide, NaOH.

6.3.10 Tris(hydroxymethyl)aminomethane (tris), $C_4H_{11}NO_3$.

6.3.11 CTAB extraction buffer, ρ(CTAB) = 20 g/l, c(NaCl) **=** 1,4 mol/l, c(tris) = 0,1 mol/l, $c(Na_2EDTA)$ = 0,02 mol/l.

Adjust to pH 8,0 with HCl or NaOH.

6.3.12 CTAB-precipitation buffer, ρ(CTAB) = 5 g/l, c(NaCl) = 0,04 mol/l.

6.3.13 Sodium chloride solution, c(NaCl) = 1,2 mol/l.

6.3.14 Ethanol solution, $\varphi(C_2H_5OH) = 70\ \%$.

6.3.15 Proteinase-K solution, $\rho = 20$ mg/ml, dissolved in sterile water.

Do not autoclave. Store at −20 °C, but avoid repeated freezing and thawing.

6.3.16 Tris–EDTA (TE) buffer, $c(\text{tris}) = 0{,}01$ mol/l, $c(Na_2EDTA) = 0{,}001$ mol/l.

Adjust to pH 8,0 with HCl or NaOH.

6.4 Reagents for PCR

6.4.1 Thermostable DNA polymerase, as specified in ISO 20838 and ISO 22174.

6.4.2 Deoxyribonucleoside triphosphates (dNTPs) containing dATP, dCTP, dGTP and dTTP or dUTP, as specified in ISO 20838 and ISO 22174.

6.4.3 PCR buffer solution, as specified in ISO 20838 and ISO 22174.

The PCR buffer solution is usually delivered with the DNA polymerase, which may or may not include $MgCl_2$ in a concentration specified by the manufacturer. The final $MgCl_2$ concentrations are method specific and are therefore listed in the annexes. It is possible that ready-to-use reagents are commercially available. If so, follow the manufacturer's instructions for use.

6.4.4 Primers and probes

Primers and probes for specific detection of the neurotoxin gene sequences are listed in Annexes B and C.

7 Apparatus and equipment

7.1 General

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

7.2 Equipment for sample preparation prior to enrichment

7.2.1 Water bath, capable of being maintained at 50 °C ± 1 °C.

7.2.2 Centrifuge, for 50 ml and 100 ml tubes and with an adjustable acceleration of up to 12 000 × *g*.

7.2.3 Membrane filter, nitrocellulose-filter, pore size 0,45 µm.

7.2.4 Centrifuge tubes, of capacities of 50 ml and 100 ml.

7.3 Equipment for microbial enrichment

7.3.1 Water baths, capable of being maintained at 30 °C ± 1 °C, 65 °C ± 1 °C and 100 °C ± 1 °C.

7.3.2 Anaerobic jar or anaerobic cabinet, capable of being maintained at 30 °C ± 1 °C, according to ISO 7218.

7.3.3 Incubator, capable of operating at 30 °C ± 1 °C.

7.3.4 Flasks or bottles, of appropriate capacity.

7.4 Equipment used for nucleic acid extraction

Appropriate equipment according to ISO 20837 and, in particular, the following.

7.4.1 Microcentrifuge tubes, of capacities of 1,5 ml and 2,0 ml.

7.4.2 Thermo block, with a mixing frequency between 300 r/min and 1 400 r/min.

7.4.3 Graduated pipettes and pipette filter tips, for volumes between 1 µl and 1 000 µl.

7.4.4 Centrifuge, for reaction tubes having a capacity of 1,5 ml and 2,0 ml, e.g. microcentrifuge, capable of achieving an acceleration of up to 12 000 × *g*.

7.4.5 Mixer, e.g. vortex type.

7.5 Equipment used for PCR

Appropriate equipment according to the method and, in particular, the following.

7.5.1 Pipettes and pipette filter tips, having a capacity between 1 µl and 1 000 µl.

7.5.2 Microcentrifuge tubes, having a capacity of 1,5 ml and 2,0 ml.

7.5.3 Thin-walled PCR microtubes, 0,2 ml or 0,5 ml reaction tubes, multi-well PCR microplates or other suitable equipment.

7.5.4 Thermal cycler.

7.6 Equipment used for the detection of the PCR product

Appropriate equipment according to the method and, in particular, the following.

7.6.1 Gel-based PCR

7.6.1.1 Horizontal gel system.

7.6.1.2 Power supply.

7.6.1.3 Ultraviolet (UV) transilluminator or UV light box.

7.6.1.4 Gel documentation system.

7.6.2 Real-time PCR

7.6.2.1 Real-time PCR thermal cycler.

7.6.2.2 Appropriate detection and analysis software.

8 Sampling

Sampling is not part of the method specified in this Technical Specification. 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 the subject.

It is important the laboratory receive a truly representative sample which has not been damaged or changed during transport or storage.

9 Procedure

9.1 Sample preparation prior to enrichment

9.1.1 General

See Figure A.1.

It is recommended that at least 25 g be analysed, particularly for honey samples; however, if supply is limited, smaller sample sizes may be used.

9.1.2 Preparation of the sample

Prepare and homogenize the sample according to ISO 6887-1 and the relevant parts of ISO 6887[9]–[13] concerning the relevant matrix.

9.1.3 Preparation of honey samples

Place the vessel of honey in a water bath (7.2.1) at 50 °C ± 1 °C for 30 min to melt the honey. Invert the vessel several times to mix the sample.

Weigh 25 g ± 2 g of honey into a sterile centrifuge tube with a capacity of 100 ml (7.2.4) and add at least 50 ml of sterile distilled or deionized water containing 1 % volume fraction polysorbate 80, preheated to 50 °C ± 1 °C. Mix until the solution is homogeneous. Centrifuge the mixture at 12 000 × *g* (7.2.2) for 30 min. Remove the supernatant carefully and pass it through a 0,45 µm membrane filter (7.2.3). In case of blockage, pass any remaining supernatant through a fresh filter. Use all filters in the subsequent steps. Store the sediment temporarily at 5 ± 3 °C.

9.2 Microbial enrichment

9.2.1 Inoculation

9.2.1.1 General

Remove dissolved oxygen from the enrichment medium (6.2) by boiling for 10 min to 15 min in a water bath (7.3.1).

If the sample is acidic or acidifying, the enrichment broth shall be prepared according to 6.2.4.

9.2.1.2 Inoculation of test portion

9.2.1.2.1 Recovery of vegetative cells and spores

Adjust the temperature of the enrichment medium to 30 °C ± 1 °C in a water bath (7.3.1). Transfer the test portion into the degassed enrichment medium to give a final dilution of 10^{-1}.

9.2.1.2.2 Recovery of spores

Adjust the temperature of the enrichment medium to 65 °C ± 1 °C in a water bath (7.3.1). Transfer the test portion into the preheated enrichment medium to give a final dilution of 10^{-1} at 65 °C ± 1 °C. After inoculation, maintain the flask or bottle at 65 °C ± 1 °C for further 10 min, and then quickly cool to 30 °C ± 1 °C in a water bath (7.3.1).

9.2.1.3 Inoculation of honey test portions

Adjust the temperature of the enrichment broth to 65 °C ± 1 °C (7.3.1). Transfer the sediment (9.1.3) to one flask or bottle (7.3.4) and the filter or filters (9.1.3) into a second flask or bottle (7.3.4) each containing at least 10 ml of the heated enrichment broth, but in all cases ensure sufficient liquid covers the filters. Incubate both flasks or bottles at 65 °C ± 1 °C for 10 min in a water bath (7.3.1).

NOTE Honey samples are only analysed for spores.

9.2.2 Incubation

Incubate under anaerobic conditions (7.3.2) at 30 °C ± 1 °C. After 24 h ± 2 h of incubation, remove 1 ml of the enrichment for the PCR analysis and return immediately to anaerobic conditions.

If the result of the first PCR is negative, continue incubation under the same conditions for a further 48 h ± 2 h, then transfer 1 ml of the enrichment into a flask or bottle (7.3.4) containing 9 ml of fresh enrichment broth (6.2.3). Incubate anaerobically (7.3.2) at 30 °C ± 1 °C for 18 h ± 2 h and perform a second PCR run.

9.2.3 Process controls

Positive and negative process controls shall be included according to ISO 22174.

An example of a method for the preparation of spores is given in Annex D.

9.3 Nucleic acid preparation

9.3.1 General

An appropriate nucleic acid extraction procedure for Gram-positive bacteria shall be used.

An example of a procedure is given in 9.3.2 to 9.3.4. This procedure consists of a lysis step — thermal lysis in the presence of CTAB — followed by several extraction steps in order to remove inhibitors, such as polysaccharides and proteins.

Once the matrix test portion has been prepared, apply the DNA extraction and purification protocol given in 9.3.2 to 9.3.4.

Scale adaptation of masses and buffer volumes is required as a function of the selected size of the test portion.

9.3.2 Sample extraction

Transfer 1 000 µl of the enrichment culture (9.2.2) into a microcentrifuge tube (7.4.1). Centrifuge (7.4.4) for 5 min at approximately 12 000 × *g*. Discard the supernatant (aqueous).

Add 500 µl prewarmed (65 °C) CTAB extraction-buffer (6.3.11) to the pellet and mix gently until the pellet is lysed. Incubate for 30 min at 65 °C, under agitation (7.4.2). Add 20 µl of proteinase-K solution (6.3.7), gently mix the tube and incubate for 30 min at 65 °C, under agitation (7.4.2). Centrifuge for 10 min at approximately 12 000 × *g*. Transfer the supernatant to a new tube, add 0,7 to 1 volume of chloroform (6.3.1) and mix thoroughly.

Centrifuge for 15 min at approximately 12 000 × *g*. Transfer the supernatant (aqueous) to a new tube.

9.3.3 CTAB precipitation

Add 2 volumes of the CTAB precipitation buffer (6.3.12). Incubate for 60 min at room temperature without agitation. Centrifuge for 15 min at 12 000 × *g*. Discard the supernatant. Dissolve the precipitated DNA by adding 350 µl of NaCl solution (6.3.13). Add 350 µl of chloroform (6.3.1) and mix thoroughly. Centrifuge for 10 min at 12 000 × *g*. Transfer the aqueous phase into a new tube.

NOTE CTAB-precipitation is not necessary for all matrices, only for protein- and polysaccharide-rich matrices. Alternatively, a solid-phase purification of the DNA (e.g. by the use of spin columns) is possible assuming the results are equivalent.

9.3.4 DNA precipitation

Add 0,6 volume of isopropanol (6.3.6), mix smoothly by inverting the tube and keep the tube at room temperature for 20 min. Centrifuge for 15 min at 12 000 × *g*. Discard the supernatant. Add 500 µl of ethanol solution (6.3.14) to the tube and invert several times. This is the critical step ensuring the complete removal of CTAB. Centrifuge for 10 min at 12 000 × *g*. Discard the supernatant. Dry the DNA pellet and redissolve it into 100 µl of an appropriate buffer, e.g. TE buffer (6.3.16). This is the DNA master stock. The DNA can be stored at −20 °C until use.

9.4 PCR amplification

Different procedures for PCR amplification can be used. The detection of the PCR product can be either gel based or by detection of the fluorescence signal.

All requirements for the PCR amplification are specified in ISO 20838.

Examples of gel-based PCR methods are described in Annex B and of real-time PCR methods in Annex C.

9.4.1 PCR controls

PCR controls shall be in accordance with ISO 22174.

9.4.2 Detection of PCR products

Different procedures for detection of PCR products can be used. Examples of gel-based PCR methods are described in Annex B and of real-time PCR methods in Annex C.

9.5 Confirmation of a positive PCR result

Follow the procedure specified in ISO 20838.

9.5.1 Interpretation of the results

The results obtained, including the controls specified in ISO 22174, should be unambiguous and the controls should yield expected results, otherwise the procedure shall be repeated.

The PCR result is:

a) positive, if a specific PCR product has been detected and confirmed and all the controls give expected results;

b) negative within the limits of detection, if a specific PCR product has not been detected, and all controls give expected results.

A positive PCR result does not automatically indicate the presence of botulinum neurotoxins. To confirm the presence of the neurotoxins suitable methods targeting the toxins directly shall be applied.

