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AMENDMENT 1
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Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of presumptive Bacillus cereus — Colony-count technique at 30 degrees C

AMENDMENT 1: Inclusion of optional tests

Microbiologie des aliments — Méthode horizontale pour le dénombrement de Bacillus cereus présomptifs — Technique par comptage des colonies à 30 degrés C

AMENDEMENT 1: Ajout de tests optionnels

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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Fax: +41 22 749 09 47
Email: copyright@iso.org
Website: www.iso.org

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This document was prepared by ISO Technical Committee 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).

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Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of presumptive Bacillus cereus — Colony-count technique at 30 degrees C

AMENDMENT 1: Inclusion of optional tests

In the Scope

Designate the existing NOTE as NOTE 1 and add the following new NOTE:

NOTE 2 The diversity within the *Bacillus cereus* group is large with 7 phylogenetic groups[21][22] and a growing number of species.

After 9.4

Add the following new subclause 9.5:

9.5 Optional tests

9.5.1 General

All the tests mentioned below are optional and intended for complementary investigations (i.e. epidemiological) on isolated *Bacillus cereus* group strains obtained in 9.4.1, following the procedures described in Annexes C to F.

In this amendment, the term "*B. cereus* group" is used instead of "presumptive *B. cereus*", as it is scientifically more precise, as explained in the EFSA scientific opinion published in 2016[28].

9.5.2 Detection of *cytK-1* or *cytK-2* gene variants of the gene encoding Cytotoxin K

Some strains within the *B. cereus* group bacteria carry one of the two variants found for the gene encoding Cytotoxin K, *cytK-1* and *cytK-2.* The *cytK-1* gene is specific to *Bacillus cytotoxicus*[17][22] and thus constitutes the possibility to rapidly identify *B. cytotoxicus*[20]. The procedure in Annex C describes a validated PCR method that targets both *cytK* gene variants and, if present, indicates which of the two forms is present. It also allows confirmation of isolates as *B. cytotoxicus*.

9.5.3 Detection of *Bacillus cereus* group strains able to produce cereulide

Some strains within the *B. cereus* group bacteria are able to produce a heat-stable dodecadepsipeptide, named cereulide. This cereulide, when produced in food, can cause an emetic food poisoning syndrome.

NOTE The method for cereulide quantification is described in ISO 18465[10].

A cereulide peptide synthetase (*ces*) gene is involved in the non-ribosomal synthesis of cereulide[16]. The procedure in Annex D describes a rapid and validated PCR method that targets the *ces* gene.

9.5.4 Motility test for *B. anthracis* screening

The motility test described in Annex E allows for screening for presumptive *B. anthracis* among isolated *B. cereus* group bacteria.

NOTE This test has nevertheless strong limitations as indicated in Annex E (see E.1 and Table E.1).

9.5.5 Microscopic examination of the parasporal crystal from *Bacillus thuringiensis*

B. thuringiensis, one of the *B. cereus* group species, can be distinguished from the other species of this group by the microscopic examination of the parasporal crystal formation.

The procedure for the examination of the parasporal crystal formation is described in Annex F.

After Annex B

Add the following as Annexes C, D, E and F.

Annex A
(informative)

Polymerase chain reaction for the detection of *cytK-1* or *cytK-2* gene variants of cytotoxin K in isolated strains of *Bacillus cereus* group and identification of *Bacillus cytotoxicus*

A.1 General

The chromosomally located *cytK-2* gene encodes cytotoxin K, an enterotoxin that is present in *B. cereus sensu* stricto and *B. thuringiensis* strains[22].

Presence of *cytK-2* genes are also mentioned in strains of other *B. cereus* group species[29]. *CytK-1* gene is a variant of *cytK-2* gene due to a marked polymorphism and encodes to a more cytotoxic form of cytotoxin K that is present only in *B. cytotoxicus*[18].

This method is applicable to well-isolated colonies of *B. cereus* group strains, after appropriate preparation of the DNA.

A.2 Principles

A.2.1 General

The method comprises the following consecutive steps:

a) nucleic acid extraction;

b) amplification of target gene and interpretation.

A.2.2 Nucleic acid extraction

Bacterial cells are harvested from well isolated colonies and the nucleic acid is extracted for use in PCR reaction.

A.2.3 Amplification of target gene and interpretation

The extracted nucleic acid is selectively amplified using PCR. Detection of the PCR products is achieved by electrophoresis on agarose. Interpretation is deduced from presence or absence of the expected band.

A.3 Reagents

A.3.1 General

All reagents needed for this annex are molecular grade reagents and consumables suitable for molecular biology. They shall be used as given in ISO 20837[11] and ISO 20838[12].

A.3.2 Nucleic acid extraction

Nucleic acid extraction procedure and reagents appropriate for Gram-positive bacteria shall be used.

Commercial kits can also be used.

A.3.3 Reagents for PCR

Refer to ISO 22174[14] and ISO 20838[12].

A.3.4 Primers

The primers used for detection of cytotoxin K genes are listed in Table C.1.

Table C.1 — Sequences of oligonucleotides, characteristics and resulting amplicon

Primer		Sequence (5′ – > 3′)	Gene variant	Position on *cytK* gene	Amplicon size (bp)
CK1F	F	CAA TTC CAG GGG CAA GTG TC	*cytK-1*	314–333	426
CK1R	R	CCT CGT GCA TCT GTT TCA TGA G	Accession number[a] DQ885233.1	740–719	
CK2F	F	CAA TCC CTG GCG CTA GTG CA	*cytK-2*	314–333	585
CK2R	R	GTG IAG CCT GGA CGA AGT TGG	Accession number[a] AJ318876.2	899–879	
Key F: forward R: reverse [a] Make reference to the publicly available nucleotide sequences available at http://www.www.ncbi.nlm.nih.gov					

A.4 Equipment and consumables

A.4.1 General

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

A.4.2 Equipment for nucleic acid extraction

C.4.2.1 Micro-centrifuge tubes, with capacities of 1,5 ml or 2,0 ml.

C.4.2.2 Centrifuge, for reaction tubes with a capacity of 1,5 ml or 2,0 ml and capable of achieving an acceleration up to approximately 14 000 *g*.

C.4.2.3 Thermoblock, with heating capacity of up to 100 °C.

C.4.2.4 Graduated pipettes and pipette filter tips, for volumes between 1 µl to 1 000 µl.

C.4.2.5 Mixer.

A.4.3 Equipment for PCR

C.4.3.1 PCR thermal cycler.

C.4.3.2 Thin-walled PCR microtubes, 0,2 ml or 0,5 ml reaction tubes, multi-well PCR microplates or other suitable equipment.

A.4.4 Equipment for the detection of PCR products

Refer to ISO 20838[12].

A.5 Procedure

A.5.1 General

See Figure C.1.

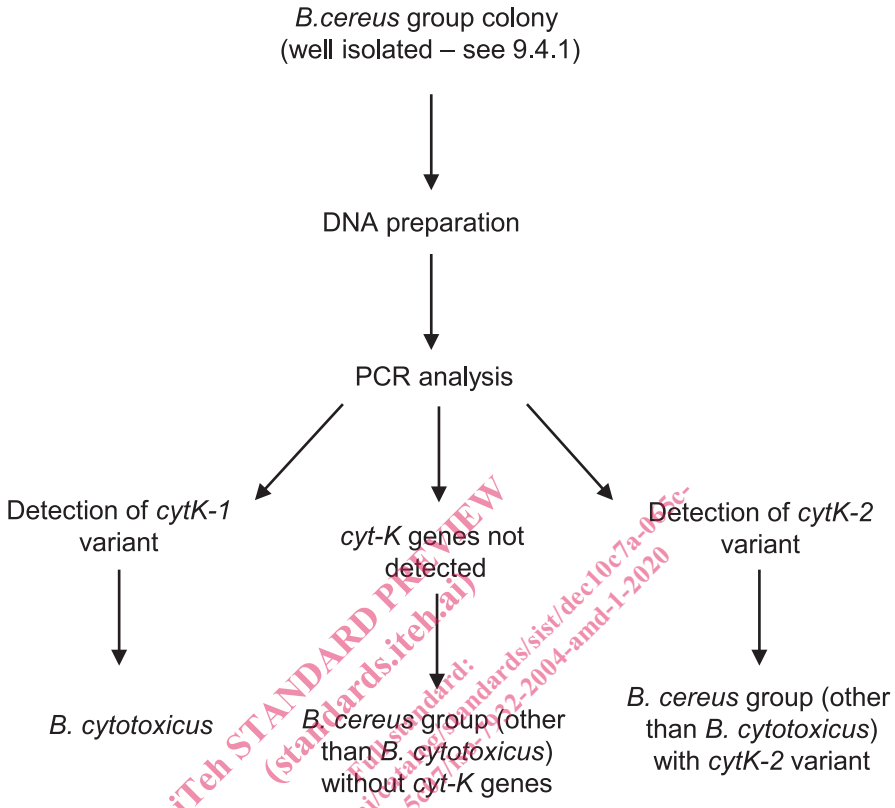

Figure C.1 — Flow diagram for PCR detection of cytotoxin K gene (*cytK-1* or *cytK-2* variants) in *B. cereus* group strains and identification of *B. cytotoxicus*

A.5.2 Nucleic acid extraction

Confirmed *B. cereus* group colonies according to 9.4 should be used for DNA extraction. Prior to DNA extraction, the colonies can be optionally washed by centrifugation in 1 ml of nuclease free water. Any nucleic acid extraction procedure appropriate for Gram-positive bacteria suitable for this purpose can be used (e.g. Reference [15]).

A 10 µl loopfull of colony material is harvested (from MYP or non selective agars) and suspended in 1 ml of nuclease free water, pelleted at 11 000*g* for 15 min. The pellet is resuspended in 500 µl extraction buffer [1,7 g/l sodium dodecylsulfate, 200 mmol/l Tris-HCl (pH 8), 20 mmol/l EDTA, 200 mmol/l NaCl]. The suspension is incubated at 55 °C for 1 h with 25 µl of proteinase K (10 µg/µl). DNA is extracted with one volume of phenol and subsequently with one volume of chloroform. The aqueous phase is precipitated with 2,5 volumes of cold ethanol (100 % volume fraction) and centrifuged at 11 000*g* for 20 min. The supernatant is discarded and the pellet washed once with 800 µl of cold ethanol (70 % volume fraction). After drying, the pellet is dissolved in 50 µl nuclease free water and stored at –20 °C. DNA amount is quantified by absorbance at 260 nm in a spectrophotometer and shall be adjusted to a concentration compatible with the sensitivity of the PCR (see C.6.3).

Other methods or commercial ready-to-use purification kits can be used if controls (see C.5.3.2) are scrupulously used.

A.5.3 PCR amplification

A.5.3.1 General

The total PCR volume is 15 µl per reaction. The reagents are listed in Table C.2. The final concentrations of reagents as outlined in the table have proven to be suitable.

Table C.2 — PCR reaction reagents

Reagent (concentration)	Final concentration	Volume per reaction (µl)
DNA polymerase buffer[a] (10X)	1x	1,5
dNTPs mix (5 mmol/l each)	0,2 mmol/l each	0,6
CK1F (10 µmol/l)	0,25 µmol/l	0,375
CK1R (10 µmol/l)	0,25 µmol/l	0,375
CK2F (10 µmol/l)	0,25 µmol/l	0,375
CK2R (10 µmol/l)	0,25 µmol/l	0,375
$MgCl_2$ (25 mmol/l)	2,5 mmol/l	1,5
DNA polymerase[a]	0,75 U	0,15
Template DNA (Genomic - 25 ng/µl)		2,5
Adjust the volume to 15 µl using nuclease free water		

[a] This protocol has been validated using commercially available AmpliTaq®10x Buffer and AmpliTaq®[1)] polymerase and Master Mix containing the four dNTPs.

[1)] AmpliTaq®10x Buffer and AmpliTaq® polymerase are products supplied by Applied Biosystems, Forster City, CA, USA. Master Mix is a product supplied by Eurogentec. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the products named. Equivalent products can be used if they can be shown to lead to the same results.

Different protocols for PCR amplification can be used, depending on the DNA polymerase and DNA preparation that is used. However, the PCR reaction shall be stringent, using the primers described in Table C.1 with appropriate hybridization temperature (see Table C.4) and appropriate controls (see C.5.3.2), with the reliability of primers being validated with a specific hybridization temperature. The control strains are listed in Table C.3.

Table C.3 — Control strains to be included in PCR assays

WDCM number[a] (species)	*cytK-1*	*cytK-2*
WDCM 00218 (*Bacillus cereus*)	Negative control	Positive control
WDCM 00220 (*Bacillus cytotoxicus*)	Positive control	Negative control
WDCM 00222 (*Bacillus weihenstephanensis*)	Negative control	Negative control

[a] Refer to the reference strain catalogue available on http://www.wfcc.info for information on culture collection strain numbers and contact details.

A.5.3.2 PCR controls

All appropriate controls as given in ISO 22174[14] shall be performed. At least a positive and a negative control, represented for each gene variant by a known positive and a known negative bacterial strain DNA respectively, shall be included in the PCR assay to check the conditions of amplification.

DNA positive controls (including process controls as given in ISO 22174[14]) should be obtained by the same DNA extraction protocol as used for test isolates.

A.5.3.3 Temperature-time programme

The temperature-time programme, as outlined in Table C.4, has been used in the evaluation study.

Table C.4 — Temperature-time programme

Initial denaturation		94 °C for 5 min
Amplification	Denaturation	94 °C for 15 s
	Hybridization	57 °C for 30 s
	Elongation	72 °C for 30 s
Number of cycles		30
Final extension		72 °C for 7 min

A.5.3.4 Detection of the PCR products

The PCR products are detected after electrophoresis on agarose gel (1,5 %) with an appropriate molecular weight marker (refer to ISO 20838[12]).

A.5.4 Interpretation of the PCR result

The result obtained, including the controls specified above (see C.5.3.2), should be as follows. Otherwise, the PCR shall be repeated.

The PCR result will be one of the following:

a) positive for *cytK-1* variant, if a specific PCR product of 426 bp has been detected and all the controls give expected results, or

b) positive for *cytK-2* variant, if a specific PCR product of 585 bp has been detected and all the controls give expected results, or

c) negative for cytotoxin K genes, if a specific PCR product has not been detected, and all controls give expected results.

A.5.5 Confirmation of the PCR product

Refer to ISO 22174[14].

A.6 Performance characteristics

A.6.1 General

This method was evaluated in a single-laboratory validation study. It was tested on a total of 160 *B. cereus* group strains and 10 outgroup species, including Southern blotting or PCR product sequencing[23], and then applied on 391 strains[22]. The assay turned out to be highly reliable with 0 % false-negative reactions and 0 % false-positive reactions. BLASTN[25] analysis also showed that there were no targets in the bacterial organisms other than *cytK* gene for the four primers included in the PCR reaction. The specificity for *cytK-1* and *cytK-2* variant was 100 % under the PCR conditions described[23] and with the recommended controls (see C.5.3.2).

A.6.2 Selectivity

A.6.2.1 General

Selectivity was performed in duplex PCR using the primers CK1F, CK1R, CK2F, CK2R, working by couple (CK1F/CK1R and CK2F/CK2R). It was checked that the respective primer pairs run exclusively on *cytK-1* variant or *cytK-2* variant and target low conserved regions between the two variants.