AMENDMENT NO. 1 JANUARY 2024

TO

IS 5887 (PART 6) : 2012/ISO 7932 : 2004

MICROBIOLOGY OF FOOD AND ANIMAL FEEDING STUFFS — HORIZONTAL METHOD FOR THE ENUMERATION OF PRESUMPTIVE *Bacillus cereus*

PART 6 COLONY-COUNT TECHNIQUE AT 30 °C

(First Revision)

This Amendment No. 1 is identical with Amendment No. 1 'Inclusion of optional tests' of ISO 7932 : 2004 'Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of presumptive *Bacillus cereus* — Colony-count technique at 30 °C' issued in March 2020 by International Organization for Standardization (ISO).

Price Group 10

Indian Standard

MICROBIOLOGY OF FOOD AND ANIMAL FEEDING STUFFS — HORZONTAL METHOD FOR THE ENUMERATION OF PRESUMPTIVE *Bacillus cereus*

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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 C
(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*

C.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.

C.2 Principles

C.2.1 General

The method comprises the following consecutive steps:

a) nucleic acid extraction;

b) amplification of target gene and interpretation.

C.2.2 Nucleic acid extraction

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

C.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.

C.3 Reagents

C.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].

C.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.

C.3.3 Reagents for PCR

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

C.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

C.4 Equipment and consumables

C.4.1 General

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

C.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.

C.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.

C.4.4 Equipment for the detection of PCR products

Refer to ISO 20838[12].

C.5 Procedure

C.5.1 General

See Figure C.1.

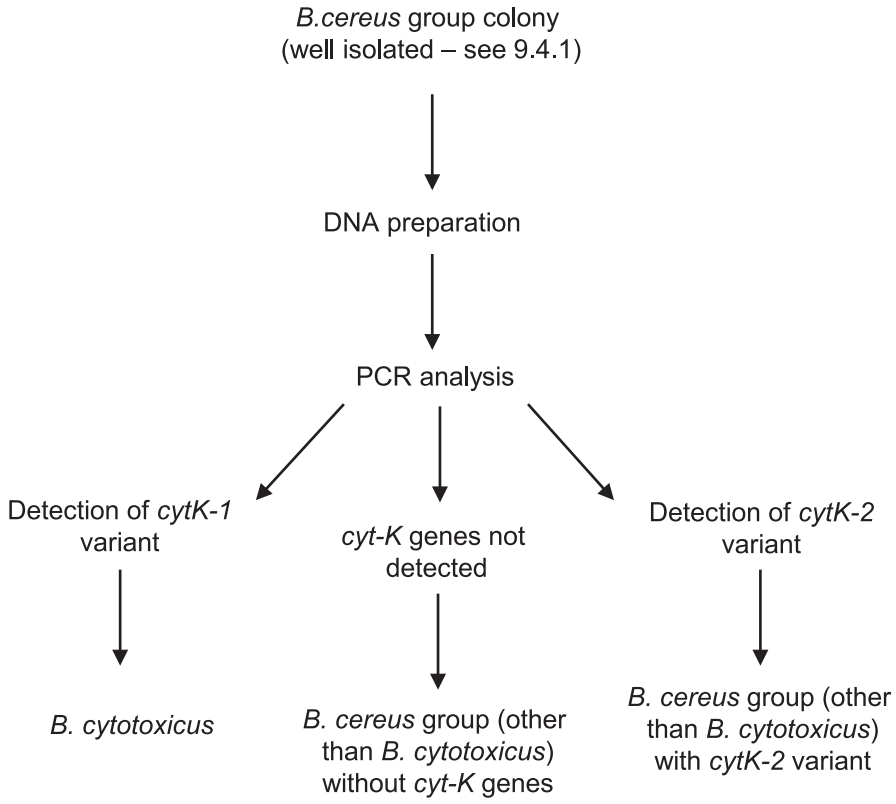


Figure C.1 — Flow diagram for PCR detection of cytotoxin K gene (*cytK-1* or *cytK-2* variants) in *B. cereus* group strains and identification of *B. cytotoxicus*

C.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.

C.5.3 PCR amplification

C.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.

C.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.

C.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

C.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]).

C.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.

C.5.5 Confirmation of the PCR product

Refer to ISO 22174[14].

C.6 Performance characteristics

C.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).

C.6.2 Selectivity

C.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.

C.6.2.2 Inclusivity

Inclusivity of the PCR assay for *cytK-2* fragment was tested on 66 target strains. A 100 % inclusivity was obtained. Inclusivity of the PCR assay for *cytK-1* fragment was tested on 5 target strains. A 100 % inclusivity was obtained. The tested strains for *cytK-2* variant were *B. cereus sensu* stricto and *B. thuringiensis* strains. Target strains for *cytK-1* correspond to all 5 *B. cytotoxicus* strains that were available at the time of evaluation. They were enough distant from the remaining species of the group to exhibit a particular polymorphism resulting in *cytK-1* variant for *cytK* gene.

C.6.2.3 Exclusivity

Exclusivity of the assay for *cytK-1* fragment was tested on 157 non-target strains. A 100 % exclusivity was obtained. Exclusivity of the assay for *cytK-2* fragment was tested on 94 non-target strains. A 100 % exclusivity was obtained.

Strains tested were from all species of the *B. cereus* group and eight diverse food-related species.

C.6.3 Sensitivity

The limits for PCR amplification ranged from 15 ng to 75 ng of genomic DNA per 15 µl of final reaction.

C.7 Limitations of the PCR assay

This PCR assay allows to a) detect cytotoxin K genes (and particularly one of its two variants), and b) identify *B. cytotoxicus* strains, two characteristics that are both associated with food poisoning. Even if that indicates a potential risk, the sole presence of the gene or species identification does not allow to confirm *cytK* gene expression.

Annex D
(informative)

Polymerase chain reaction for the detection of *ces* gene encoding cereulide peptide synthetase in strains of *Bacillus cereus* group

D.1 Gel-based PCR assay for detection of *ces* gene encoding cereulide peptide synthetase in strains of *Bacillus cereus* group

D.1.1 General

This annex describes a method for the amplification and detection of the *ces* gene specific for the production of cereulide in *Bacillus cereus* group strains using agarose gel electrophoresis.

D.1.2 Principle

Specific DNA fragment of the *ces* gene is amplified by PCR using two primers. The detection of the PCR product is done using agarose gel-electrophoresis.

D.1.3 Reagents

D.1.3.1 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].

D.1.3.2 Reagents for nucleic acid extraction

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

D.1.3.3 Reagents for PCR

D.1.3.3.1 PCR buffer solution.

The 10x 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 Table D.2. Commercially available ready-to-use reagents may be used. The manufacturer's instructions for use should be considered.

D.1.3.3.2 $MgCl_2$ solution, c ($MgCl_2$) = 25 mmol/l.

D.1.3.3.3 Thermostable DNA polymerase (for hot-start PCR), 5 IU/µl.

D.1.3.3.4 dNTP solution, c (dNTP) = 10 mmol/l, for the mix.

D.1.3.3.5 Oligonucleotides.

Sequences of the oligonucleotides are listed in Table D.1.

Table D.1 — Sequences of oligonucleotides

Gene	Primer	Sequence (5′ – 3′)	Amplicon size (bp)
ces	EM1F	GAC AAG AGA AAT TTC TAC GAG CAA GTA CAA T	635
	EM1R	GCA GCC TTC CAA TTAC TCC TTC TGC CAC AGT	

D.1.3.4 Reagents for gel electrophoresis

Refer to ISO 20838[12].

D.1.4 Equipment and consumables

D.1.4.1 General

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

D.1.4.2 Equipment used for nucleic acid extraction

D.1.4.2.1 Micro-centrifuge tubes, with capacities of 1,5 ml or 2,0 ml.

D.1.4.2.2 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*.

D.1.4.2.3 Thermoblock, with heating capacity of up to 100 °C.

D.1.4.2.4 Graduated pipettes and pipette filter tips, for volumes between 1 µl to 1 000 µl.

D.1.4.2.5 Mixer.

D.1.4.3 Equipment used for PCR

D.1.4.3.1 Pipettes and pipette filter tips, having a capacity between 1 µl and 1 000 µl.

D.1.4.3.2 Microcentrifuge tubes, having a capacity of 1,5 ml or 2,0 ml.

D.1.4.3.3 Thin walled PCR microtubes, 0,2 ml or 0,5 ml reaction tubes, multi-well PCR microplates or other suitable equipment.

D.1.4.3.4 Thermal cycler.

D.1.4.4 Equipment used for the detection of the PCR product

Refer to ISO 20838[12].

D.1.5 Procedure

D.1.5.1 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. The protocol described in C.5.2 can be used as well as any nucleic acid extraction procedure appropriate for Gram-positive bacteria and suitable for this purpose (e.g. Reference [15]).

D.1.5.2 PCR set-up

The method is described for a total PCR volume of 50 µl per reaction with the reagents as listed in Table D.2. The PCR can also be carried out in a smaller volume if the solutions are adjusted accordingly. The final concentrations of reagents as outlined in Table D.2 have proven to be suitable.

Table D.2 — PCR reaction reagents

Reagent (concentration)	Final concentration	Volume per reaction (µl)
DNA polymerase buffer without $MgCl_2$[a] (10X)		5
$MgCl_2$ (25 mmol/l)	150 µmol/l	0,3
dNTP mix (10 mmol/l each)	800 µmol/l	4
EM1F (5 µmol/l)	0,5 µmol/l	5
EM1R (5 µmol/l)	0,5 µmol/l	5
Template DNA	/	1
Thermostable DNA polymerase, 5 IU/µl	0,5 IU/µl	5
Adjust the volume to 50 µl using nuclease-free water		

[a] If the DNA polymerase buffer already contains $MgCl_2$, the final concentration of $MgCl_2$ in the reaction mixture is adjusted to 0,15 mmol/l.

D.1.5.3 PCR controls

All appropriate controls as given in ISO 22174[14] shall be performed.

The following strain can be used as positive controls.

Bacillus cereus, emetic strain WDCM 00219.

D.1.5.4 Temperature-time-programme

The temperature-time programme as outlined in Table D.3 and the MasterMix according to Table D.2 were used in the validation study[16]. The use of other thermal cyclers might make an adaptation necessary. The time for activation/initial denaturation depends on the polymerase used. The recommendation of the DNA polymerase manufacturer shall be followed.

Table D.3 — Temperature-time programme

Activation/initial denaturation	95 °C for 15 min
Amplification	95 °C for 30 s
	59 °C for 30 s
	72 °C for 60 s
Number of cycles (amplification)	30
Final extension	72 °C for 5 min

D.1.5.5 Detection of PCR products

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

The target sequence is considered to be detected if the size of the PCR product corresponds to the expected length of the target DNA sequence (see amplicon size in Table D.1). For the interpretation of the results, see ISO 22174[14].

D.1.5.6 Confirmation of a positive PCR result

Refer to ISO 22174[14].

D.1.6 Performance characteristics

D.1.6.1 General

The method has been validated for DNA extracted from different emetic *Bacillus cereus* group strains[16].

D.1.6.2 Theoretical evaluation of the method

Theoretical evaluation was done by performing a sequence similarity search against the GenBank/EMBL/DDBJ database[27]. The result of the search confirmed a complete identity of the primers only with the expected target sequence.

D.1.6.3 Selectivity

D.1.6.3.1 Inclusivity

The inclusivity of the method was tested with 30 emetic *Bacillus cereus* group strains, see Table D.4.

Table D.4 — Inclusivity of the PCR using target strains

Source	Number of strains	*ces* gene detected
Food (without connection to a foodborne illness/outbreak)	5	5
Food connected to foodborne illness	13	13
Human samples (vomit, faeces)	12	12

D.1.6.3.2 Exclusivity

The exclusivity of the method was tested with 178 non-target organisms, see Table D.5. No cross-reactivity was observed with the non-target bacteria.

Table D.5 — Exclusivity of the PCR using non-target strains

Species	Number of strains	*ces* gene detected
Bacillus cereus non-emetic strains	100	0
Bacillus anthracis	7	0
Bacillus mycoides	6	0
Bacillus thuringiensis	6	0
Bacillus pseudomycoides	3	0
Bacillus weihenstephanensis	7	0
other *Bacillus* spp.	8	0
Staphylococcus aureus	10	0
Staphylococcus equorum	1	0
Clostridium perfringens	3	0
Listeria monocytogenes	6	0
Campylobacter spp.	3	0
Escherichia coli	4	0
Salmonella spp.	6	0
Yersinia enterocolitica	8	0

D.1.6.4 Sensitivity

The detection limit was found to be 2,5 pg template DNA for a 30-cycle PCR protocol, which corresponds to approximately 500 genome equivalents[16].

D.2 Real-time PCR assay for detection of *ces* gene encoding cereulide peptide synthetase in strains of *Bacillus cereus* group

D.2.1 General

This annex describes a probe-based real-time PCR method based on TaqMan technology for the detection of the *ces* gene specific for emetic *Bacillus cereus* group strains.

D.2.2 Principle

Specific DNA fragment of the *ces* gene specific for emetic *Bacillus cereus* group strains is amplified by real-time-PCR. The PCR product is detected by measuring the fluorescence of the probe.

D.2.3 Reagents

D.2.3.1 General

For the quality of reagents used, refer to ISO 22119[13] and ISO 22174[14].

D.2.3.2 Reagents for nucleic acid extraction

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].

D.2.3.3 Ready to use MasterMix for real-time-PCR

A ready-to-use MasterMix contains PCR-buffer solution, $MgCl_2$-solution, dNTP-solution, optional a decontamination System (dUTP included uracil N-glycosylase) and Taq-Polymerase and is mostly adapted to the thermal cycler used. The manufacturer's instructions for use should be considered.

D.2.3.4 pUC 19 plasmid

The plasmid is used as internal amplification control (IAC).

D.2.3.5 Oligonucleotides

Sequences of the oligonucleotides are listed in Table D.6.

Table D.6 — Sequences of oligonucleotides

Gene	Primer/probe	Sequence (5′ – 3′)
ces	ces_TaqMan_for	CGC CGA AAG TGA TTA TAC CAA
	ces_TaqMan_re	TAT GCC CCG TTC TCA AAC TG
	ces_TaqMan_probe	FAM[a]-GGGAAAATAACGAGAAATGCA-TAMRA[b]
Internal amplification control (IAC) (see ISO/TS 17919[9])	IAC_for	TGT GAA ATA CCG CAC AGA TG
	IAC_re	AGC TGG CGT AAT AGC GAA G
	IAC_probe	HEX[c]- GAG AAA ATA CCG CAT CAG GC -TAMRA[b]

[a] FAM: 6-carboxyfluorescein

[b] TAMRA: 6-carboxytetramethylrhodamine

[c] HEX: 5′-Hexachloro-Fluorescein

NOTE The use of other fluorescence labels for the probes has not been tested or validated.

D.2.4 Equipment and consumables

D.2.4.1 General

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

D.2.4.2 Equipment for nucleic acid extraction

D.2.4.2.1 Micro-centrifuge tubes, with capacities of 1,5 ml or 2,0 ml.

D.2.4.2.2 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.*

D.2.4.2.3 Thermoblock, with heating capacity of up to 100 °C.

D.2.4.2.4 Graduated pipettes and pipette filter tips, for volumes between 1 µl to 1 000 µl.

D.2.4.2.5 Mixer.

D.2.4.3 Equipment for PCR

D.2.4.3.1 PCR thermal cycler.

D.2.4.3.2 Thin-walled PCR microtubes, 0,2 ml or 0,5 ml reaction tubes, multi-well PCR microplates or other suitable equipment.

D.2.4.4 Equipment for the detection of PCR products

Refer to ISO 20838[12].

D.2.5 Procedure

D.2.5.1 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]).

D.2.5.2 PCR amplification

The method is described for a total PCR volume of 25 µl per reaction with the reagents as listed in Table D.7. The PCR can also be carried out in a larger volume if the solutions are adjusted appropriately. The final concentrations of reagents as outlined in Table D.7 have proven to be suitable.

Table D.7 — PCR reaction reagents

Reagent (concentration)	Final concentration	Volume per reaction (µl)
Brilliant QPCR Multiplex MasterMix (2X)[a]	1 X	12,5
PCR primers: ces_TaqMan_for and ces_TaqMan_re, IAC_for and IAC_re, according to Table D.6 (10 mmol/l each)	500 µmol/l each	1,25
PCR probes: ces_TaqMan_probe and IAC_probe, according to Table D.6 (10 mmol/l each)	200 µmol/l each	0,5
pUC 19-plasmid (1 fg/µl)	0,04 fg/µl	1 µl
Nuclease-free water	/	0,5
Template DNA	maximum 250 ng/reaction	5

[a] Brilliant QPCR Multiplex MasterMix is an example of a suitable product available commercially from Agilent Technologies. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to give the same results.

D.2.5.3 PCR controls

All appropriate controls as given within ISO 22174[14] shall be performed.

The following strains can be used as positive controls: *Bacillus cereus*, emetic strain WDCM 00219.

An IAC can be used. An example for a heterologous IAC is given in D.2.3.4.

D.2.5.4 Temperature-time-programme

The temperature-time programme as outlined in Table D.8 has been used for the validation study (see Reference [19]). The use of other thermal cyclers than those used in the validation study might make an adaptation necessary. The time for activation/initial denaturation depends on the polymerase used.

Table D.8 — Temperature-time programme

Activation/initial denaturation	95 °C for 10 min
Amplification	95 °C for 15 s
	55 °C for 60 s
Number of cycles (amplification)	45

D.2.5.5 Interpretation of the results

The threshold value to determine the cycle of threshold (Ct) shall be defined by the analyst or by the cycler-specific software. A positive sample generates an amplification plot with at least the exponential phase of a typical amplification curve, see ISO 22119[13]. The amplification curve of these samples crosses the defined threshold setting after a certain number of cycles. A sample with a fluorescence signal above the threshold is considered positive, if the controls specified above (see D.2.5.3) give the expected results.

D.2.6 Performance characteristics

D.2.6.1 General

The method has been validated for DNA extracted from different emetic *Bacillus cereus* group strains and from naturally contaminated samples in a single-laboratory validation study.

The method has been published in Reference [19] with a modified IAC as described in Reference [24] and also used in ISO/TS 17919[9]. Messelhäusser et al.[24] demonstrated this IAC does not affect performance characteristics of the PCR detection system initially published.

D.2.6.2 Theoretical evaluation of the method

Theoretical evaluation was done by performing a sequence similarity search against the GenBank/EMBL/DDBJ database[27]. The result of the search confirmed a complete identity of the primers and probe only with the expected target sequence.

D.2.6.3 Selectivity

D.2.6.3.1 Inclusivity

The inclusivity of the method was tested with 23 emetic *Bacillus cereus* group strains (see Reference [19]).

D.2.6.3.2 Exclusivity

The exclusivity of the method was tested with 77 non-target organisms. No cross-reactivity was observed with the non-target bacteria, see Table D.9.

Table D.9 — Exclusivity of the real-time-PCR using non-target strains

Species	Number of strains	*ces* gene detected
Bacillus cereus non-emetic strains	16	0
Bacillus anthracis	4	0
Bacillus mycoides	4	0
Bacillus thuringiensis	4	0
Bacillus weihenstephanensis	4	0
other *Bacillus* spp.	9	0
Staphylococcus aureus	14	0
Clostridium perfringens	3	0
Listeria monocytogenes	4	0
Campylobacter spp.	3	0
Escherichia coli	4	0
Salmonella spp.	4	0
Yersinia enterocolitica	4	0

D.2.6.4 Sensitivity

The detection limit was found to be 0,6 pg template DNA which corresponds to approximately 100 genome equivalents[19].

Annex E
(informative)

Motility as a screening test

NOTE Source: Reference [26].

E.1 General

WARNING — *B. anthracis* (Biosafety level 3 microorganism) is highly pathogenic and causes human and animal serious diseases. In case of suspicion of isolation of potential *B. anthracis* strain, regulations on biosafety shall be taken into account and a reference laboratory shall be warned.

The motility test is intended to assist in differentiating *B. anthracis* from other *B. cereus* group members. Nevertheless, correct identification of *B. anthracis* strains is very complex and needs additional tests, which are outside the scope of this document.

E.2 Culture medium

E.2.1 Composition of BC motility medium

Trypticase Peptone from casein, tryptic digest (CAS N°: 91079-40-2)	10 g
Yeast extract (CAS N°: 8013-01-2)	2,5 g
Dextrose (CAS N°: 5996-10-1)	5 g
Na_2HPO_4 (CAS N°: 7558-79-4)	2,5 g
Agar (CAS N°: 9002-18-0)	3 g
Distilled water	1 l

E.2.2 Preparation of BC motility medium

Heat with agitation to dissolve and homogenize agar. Dispense 2 ml portions into 13 × 100 mm tubes. Autoclave the tubes 15 min at 121 °C. Store the sealed tubes at room temperature two days before use or at 5 °C ± 3 °C for up to six months. Final pH, 7,4 ± 0,2.

E.3 Procedure

Inoculate BC motility medium by stabbing (with an inoculation needle) down the centre of the tube with a 24 h culture of a single well-isolated colony selected and purified as described in 9.4.1. Incubate tubes 18 h to 24 h at 30 °C and examine for type of growth along stab line. Motile organisms produce diffuse growth out into the medium away from the stab. Non-motile organisms produce growth only in and along stab.

Table E.1 — Differential motility characteristics of large-celled Group I *Bacillus* species

Feature	*B. cereus*	*B. thuringiensis*	*B. mycoides*	*B. weihenstephanensis*	*B. anthracis*	*B. cytotoxicus*
Motility[a]	+	+	–	+	–	+
[a] Symbols and abbreviations: +, ≥ 85 % positive; –, 0 % to 15 % positive.						

Annex F
(informative)

Parasporal crystal of *Bacillus thuringiensis* — Protocol for examination by wet mount microscopy

F.1 General

At the time of writing this annex, the delineation of the *B. thuringiensis* species within the *B. cereus* group is phenotypically based, essentially, on the production of protein toxin crystals. These protein crystals are produced along with the spores, hence named parasporal crystals, and can be observed by wet mount microscopy in phase contrast, with an 100X oil immersion objective as described in the current annex.

F.2 Principles

The method comprises the following consecutive steps:

— isolation on sporulation agar (HCT);

— periodic examinations by wet mount microscopy in phase contrast.

F.3 Culture media and reagents

F.3.1 Hydrolysate of casein tryptone (HCT) agar medium

F.3.1.1 Solution A

F.3.1.1.1 Composition

$MgSO_4$, $7H_2O$ (CAS N°: 10034-99-8)	12,3 g
$MnSO_4$, H_2O (CAS N°: 10034-96-5)	0,169 g
$ZnSO_4$, $7H_2O$ (CAS N°: 7446-20-0)	1,44 g
Water	1 000 ml

F.3.1.1.2 Preparation

Dissolve the components in the water by stirring.

Mix and sterilize by filtration through 0,2 µm filter. Store up to six months at 5 °C ± 3 °C.

F.3.1.2 Solution B

F.3.1.2.1 Composition

Ferric ammonium citrate (CAS N°: 1185-57-5)	11,0 g
Water	1 000 ml

F.3.1.2.2 Preparation

Dissolve the component in the water by stirring.

Sterilize by filtration through 0,2 µm filter. Store up to three months at 5 °C ± 3 °C. Protect from light.

F.3.1.3 Solution C

F.3.1.3.1 Composition

$CaCl_2$, $2H_2O$ (CAS N°: 10043-52-4)	14,7 g
Water	1 000 ml

F.3.1.3.2 Preparation

Dissolve the component in the water by stirring.

Sterilize by filtration through 0,2 µm filter or for 15 min at 121 °C. Store up to six months at 5 °C ± 3 °C.

F.3.1.4 Solution D

F.3.1.4.1 Composition

Glucose (CAS N°: 50-99-7)	20,0 g
Water	qsp 100 ml

F.3.1.4.2 Preparation

Dissolve the component in the water by stirring.

Sterilize by filtration through 0,2 µm filter. Store up to 6 months at 5 °C ± 3 °C.

Solutions shall be discarded if any precipitate appears.

F.3.1.5 Base medium

F.3.1.5.1 Composition

Trypton (CAS N°: 91079-40-2)	5,0 g
Bacto casamino Acid (CAS N°: 9000-71-9)	2,0 g
KH_2PO_4 (CAS N°: 7778-77-0)	6,8 g
Solution A (F.3.1.1)	10 ml
Solution B (F.3.1.2)	2 ml
Solution C (F.3.1.3)	10 ml
Agar-Agar (CAS N°: 9002-18-0)	12,0 g to 18,0 g[a]
Water	1 000 ml

[a] Depending on the agar gel strength.

F.3.1.5.2 Preparation

Dissolve the components and solutions A, B and C in the water by stirring and boiling.

Adjust the pH, if necessary, so that the pH of the complete medium, after sterilization, is 7,2 ± 0,2 at 25 °C.

Sterilize for 15 min in an autoclave set at 121 °C.

F.3.1.6 Complete medium

F.3.1.6.1 Composition

Base medium	1 000 ml
Solution D (F.3.1.4)	15 ml

F.3.1.6.2 Preparation

Aseptically mix the base medium, cooled at 44 °C to 47 °C, with the solution D.

Pour 15 ml to 20 ml portions of the complete medium into sterile Petri dishes. Allow it to solidify.

Store up to one month at 5 °C ± 3 °C, protect from light.

The complete medium can also be poured in 250 ml or 500 ml sterile bottles and stored up to three months, protected from light.

F.4 Equipment and consumables

See Clause 6, usual microbiological equipment (see ISO 7218) and, in particular, the following.

F.4.1 **Microscope**, with phase-contrast, and with slides and cover slips.

F.5 Procedure

F.5.1 Isolation on sporulating agar

Streak one well isolated colony obtained in 9.4.1 on HCT agar and incubate at 30 °C ± 1 °C for 24 h ± 3 h and subsequent periods of 24 h ± 3 h, for a maximum of four periods.

Storage of the non-selective agar after incubation in 9.4.1 is allowed for up to 72 h at 5 °C ± 3 °C before isolation on HCT agar.

Also, other sporulation media can be used if it is shown that they produce equivalent results according to F.6.

F.5.2 Microscopic examination

After each incubation period on HCT agar proceed to the wet mount microscopic examination as follows.

— Emulsify a small amount of *Bacillus* culture in approximately 10 µl of water, on a microscope slide, and place over the emulsion a cover slip. Avoid trapping air bubbles. (Place one end of the cover slip on the slide and slowly lower the other end using the end of a toothpick. This will help to prevent air bubbles from getting trapped under the cover slip.) It is important to minimize the thickness of water between the slides in order to reduce bacterial cells movements that may impede the examination.

— Then, examine, in phase contrast, with 100X oil immersion objective.

— Spores are ellipsoidal, do not swell the sporangium and are clearly refringent.

— Parasporal crystals are non refringent inclusion bodies, with oval to tetragonal shape ("diamond-shaped") smaller than the spore. Oval shape is more often observed than the tetragonal one.

— These protein toxin crystals are somewhat difficult to differentiate from other inclusion bodies, especially after being released from the bacterial cells. Thus, observation of these crystals should be performed while still present within the bacterial cell, together with the spore, in other words in a parasporal state.

The parasporal crystal should exhibit the following characteristics:

— be observed within bacterial cells together with a refringent spore (if most of bacterial cells harbour a spore without inclusion and/or inclusion without spores, the strain shall be considered as not producing protein toxin crystals);

— have a minimum size (approximately 1/4 to 1/2 of the spore): smaller inclusions or granular shapes shall not be considered as protein toxin crystals, even if present together with the spore.

Figures F.1, F.2 and F.3 show the aspects of different *B. cereus* group strains that produce or do not produce parasporal crystal, as well as an example of a strain that is not possible to conclude.

As soon as sporulation is complete (most of cells exhibiting spores), incubation should be stopped.

It may be helpful to examine in parallel of the tested strains, the two strains given in Table F.1.

F.5.3 Interpretation of the results

According to the microsopic examination, results are interpreted as follows:

— strain producing parasporal crystal: the strain belongs to *B. thuringiensis* species;

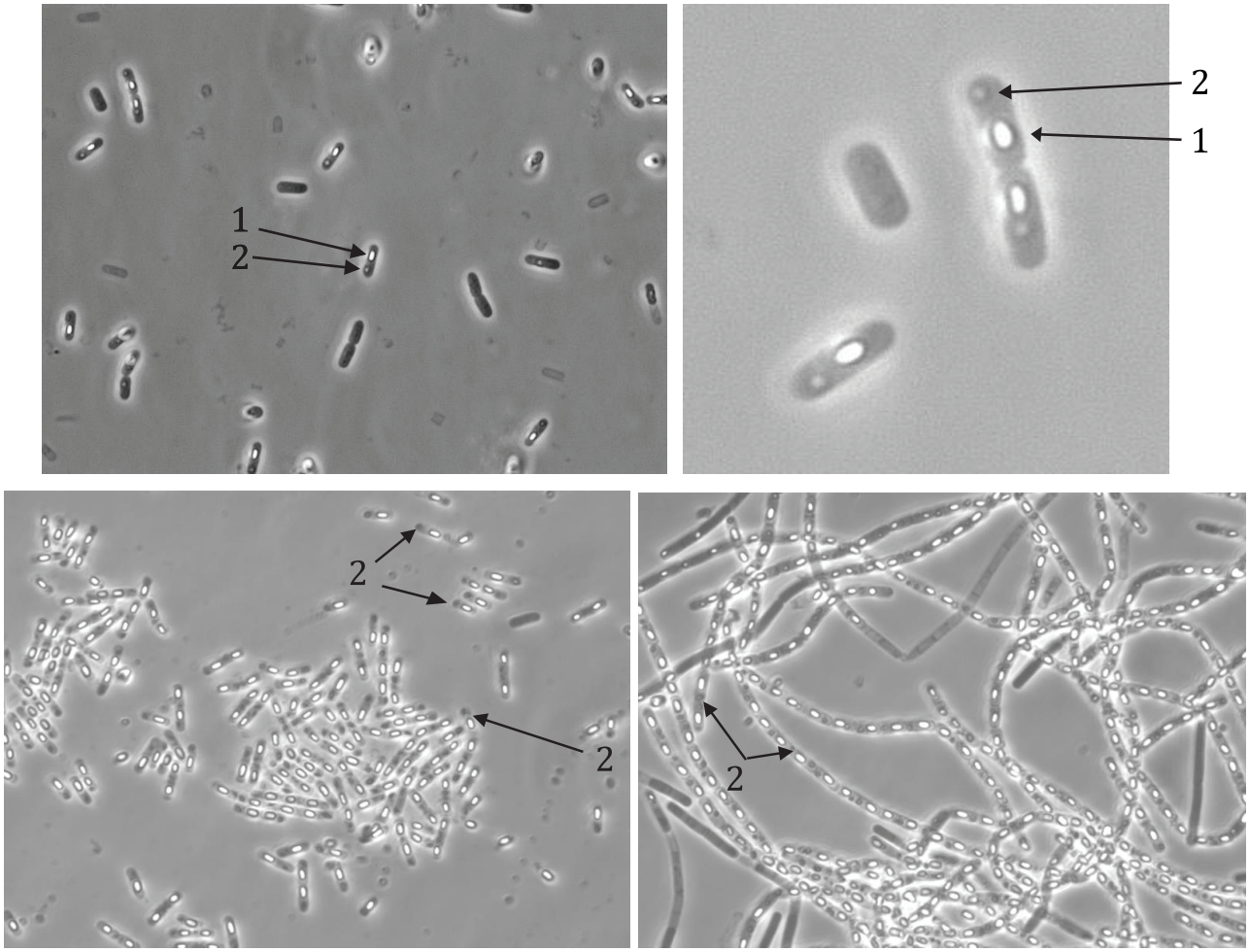
— strain not producing parasporal crystal: *B. cereus* group strain not producing parasporal crystal, very unlikely to be *B. thuringiensis*.

If the laboratory is not able to clearly establish production of parasporal crystal, it should be stated that the strain belongs to an undefined *B. cereus* group species.

If the strain is not sporulating (after four incubation periods) the procedure (see F.5.1 to F.5.3) can be repeated. If no sporulation is observed, it should be stated that the strain belongs to an undefined *B. cereus* group species. The absence of sporulation should be noted in the test results.

Table F.1 — Performance testing of HCT agar

Function	Incubation	Control strains	Criteria
Inclusivity	30 °C ± 1 °C for 24 h ± 3 h (1 or 2 times)	*B. thuringiensis* WDCM 00221	Presence of parasporal crystal after 1 or 2 incubation periods
Exclusivity	30 °C ± 1 °C for 24 h ± 3 h (1 or 2 times)	*B. cereus* WDCM 00001	Presence of spores/absence of parasporal crystal after 1 or 2 incubation periods



Key

1 spore

2 parasporal crystal

Figure F.1 — Microsocopic examination of 4 different strains (incubation time = 48 h)

Non refringent bodies and spores are present in most of the cells:

— Interpretation: strains producing parasporal crystal = *Bacillus thuringiensis*

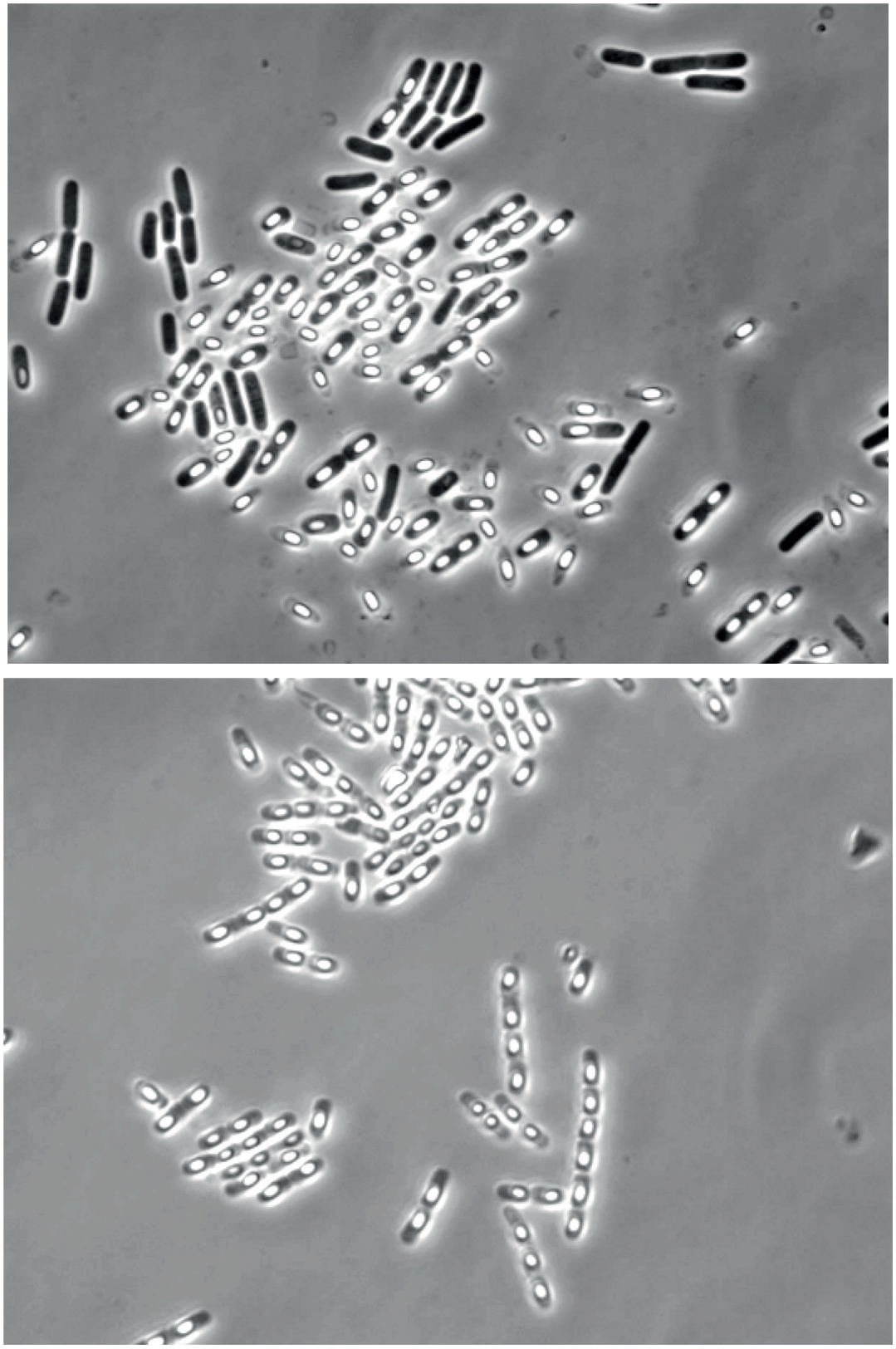


Figure F.2 — Microscopic examination of 2 different strains (incubation time = 48 h)

Only spores are present in most of the cells:

— Interpretation: *B. cereus* group strains not producing parasporal crystal

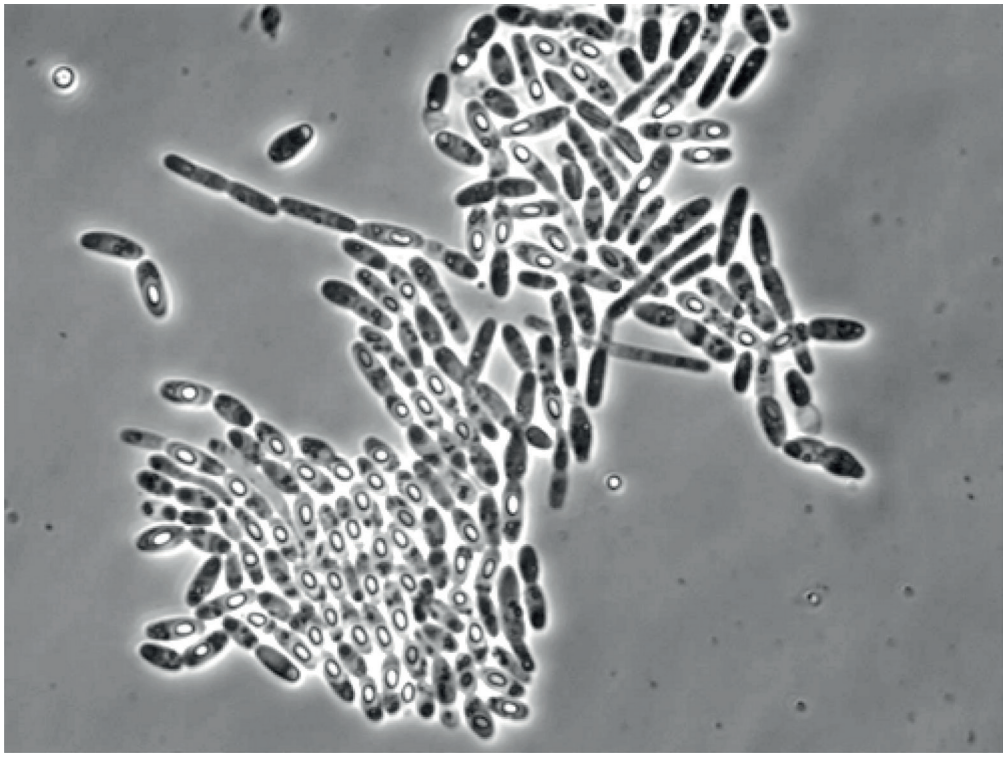


Figure F.3 — Microsocopic examination of a difficult to interpret strain (incubation time = 48 h)

Heterogeneous sporulation, presence of inclusion/granular bodies, sometimes with spores:

— Interpretation: undefined *B. cereus* group strain

F.6 Performance characteristics

This method was evaluated through an interlaboratory study involving 12 independent laboratories. It was carried out on a total of 39 *B. cereus* group strains, among which 20 belonged to a *B. cereus* group species other than *B. thuringiensis* (mainly *B. cereus*), and 19 belonged to *B. thuringiensis* species.

The 39 strains were tested by the 12 laboratories, leading to 468 independent results.

— Among 19 *B. thuringiensis* strains, 201 correct identifications over 228 results (88,2 %) were observed.

— Among 20 other *B. cereus* group strains, 219 correct identifications over 240 results (91,3 %) were observed. But among the 21 incorrect results, only 13 (5,4 %) corresponded to real misidentifications (*B. thuringiensis* instead of other *B. cereus* species) whereas 8 were not assigned to a species and were reported as "undefined *B. cereus* group strain".

— Thus, the overall ratio of correct identification was 89,7 % (420 correct identifications over 468 results) or 91,5 % when taking into account the 8 "undefined *B. cereus* group" results.

Those results were considered satisfactory for such a method based on wet mount microscopic examination.

F.7 Limitations

B. thuringiensis strains isolated in food can be wild strains or originate from biocides or phytosanitary products. The test described here does not allow to discriminate between these two origins.

Moreover, *B. cereus* and *B. thuringiensis* strains are usually not discriminated in clinical diagnostics or food microbiology. Thus, the actual contribution of the two species to gastrointestinal and non-gastrointestinal diseases is currently unknown.

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(FAD 31)

Publication, BIS, New Delhi