SLOVENSKI STANDARD
SIST EN ISO 7932:2005/oprA1:2018

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Mikrobiologija živil in krme - Splošno navodilo za štetje domnevno prisotnih Bacillus cereus - Štetje kolonij pri 30 °C - Dopolnilo A1: Vključitev izbirnega preskusa (ISO 7932:2004/DAM 1:2018)

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 (ISO 7932:2004/DAM 1:2018)

Mikrobiologie von Lebensmitteln und Futtermitteln - Horizontales Verfahren zur Zählung von präsumtivem Bacillus cereus - Koloniezählverfahren bei 30 °C - Änderung 1: Aufnahme optionaler Testmethoden (ISO 7932:2004/DAM 1:2018)

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 (ISO 7932:2004/DAM 1:2018)

Ta slovenski standard je istoveten z: **EN ISO 7932:2004/prA1**

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07.100.30	Mikrobiologija živil	Food microbiology

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ISO 7932:2004/DAM 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

ICS: 07.100.30

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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

Page 6 after Subclause 9.4

Add Subclause 9.5

9.5 Optional tests

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

9.5.1 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 *B. cytotoxicus* [17, 22] and thus constitutes a way to rapidly identify *B. cytotoxicus* [20]. The procedure in annex C describes a rapid and validated PCR method that targets both *cytK* gene variants and, if present, indicates which of the two forms is present. It also allows to identify isolates to *B. cytotoxicus*.

9.5.2 Detection of [cereulide-producing] *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, may cause an emetic food poisoning syndrome.

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

9.5.3 Motility and hemolysis tests

These tests allows to screen presumptive *B. anthracis* among isolated *B. cereus* group bacteria.

Note these tests have strong limitations that are presented in annex E (E.1 and Table E.1).

9.5.4 Microscopic examination of the parasporal crystal from *B. thuringiensis*

The *B. thuringiensis* species, one of the B. cereus group species, can be distinguished of the other species of this group by the microscopic examination of the parasporal crystal that its isolated strains are able to produce under the sporulation conditions described in annex F.

Page 12 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 *B. cytotoxicus*

C.1 Introduction

The chromosomally located *cytK-2* gene encodes cytotoxin K, an enterotoxin that is present only in *B. cereus sensu stricto* and *B. thuringiensis* [22], although all strains do not carry *cytK-2* genes. *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 on 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 [10] and ISO 20838 [11].

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 [13] and ISO 20838 [11].

C.3.4 Primers

The primers used for detection of cytotoxin K genes are listed below.

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* Accession number [a] DQ885233.1	314-333	426
CK1R	R	CCT CGT GCA TCT GTT TCA TGA G		740-719	
CK2F	F	CAA TCC CTG GCG CTA GTG CA	*cytK-2* Accession number [a] AJ318876.2	314-333	585
CK2R	R	GTG IAG CCT GGA CGA AGT TGG		899-879	
F : Forward ; R : Reverse [a] Make reference to the publicly available nucleotide sequences available on http://www. www.ncbi.nlm.nih.gov					

C.4 Equipment and consumables

C.4.1 Equipment for nucleic acid extraction

C.4.1.1 Micro-centrifuge tubes, with capacities of 1,5 ml and 2,0 ml.

C.4.1.2 Centrifuge, for reaction tubes with a capacity of 1,5 ml and 2,0 ml and capable of achieving an acceleration up to approximately 14 000 x *g*.

C.4.1.3 Thermoblock, with heating capacity of up to 100 °C.

C.4.1.4 Graduated pipettes and pipette filter tips, for volumes between 1 µl to 1000 µl.

C.4.1.5 Mixer

C.4.2 Equipment for PCR

C.4.2.1 PCR thermal cycler

C.4.2.2 Thin-walled PCR microtubes, 0,2 ml or 0,5 ml reaction tubes, multi-well PCR microplates or other suitable equipment.

C.4.3 Equipment for the detection of PCR products

Refer to ISO 20838 [11]

C.5 Procedure

C.5.1 General

See Figure C.1

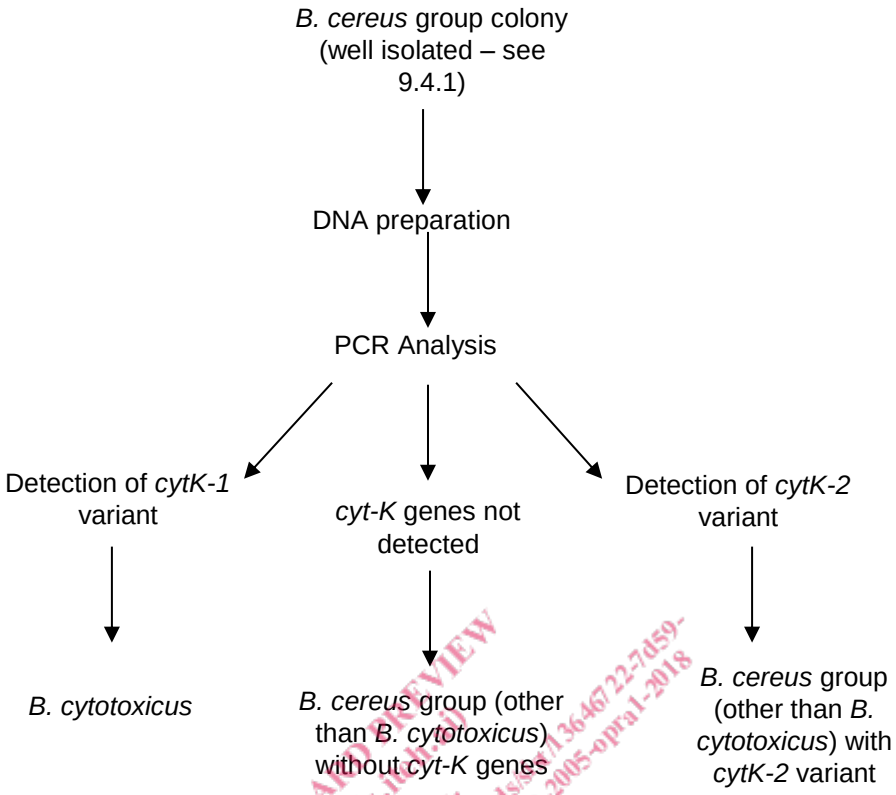

Figure 1: Flow diagram for PCR detection of cytotoxin K gene (*cytK-1* or *cytK-2* variants) in *B. cereus* group strains and identification of *B. cytotoxicus*

C.5.2 Nucleic acid extraction

Confirmed *B. cereus* group colonies according to subclause 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 [23]).

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 x *g* for 15 min. The pellet is resuspended in 500 µl extraction buffer (1,7 % 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 was precipitated with 2,5 volumes of cold 100 % ethanol and centrifuged at 11 000 x *g* for 20 min. The supernatant was discarded and the pellet washed once with 800 µl of cold 70 % ethanol. After drying, the pellet was 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 must be adjusted to a concentration compatible with the sensitivity of the PCR (see C.6.3).

Also 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 (Stock conc.)	Final concentration	Volume per sample (µl)
10x DNA Polymerase Buffer[a]	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
Genomic DNA (25 ng/µl)		2,5
Adjust the volume to 15 µl using nuclease free water		
[a] This protocol has been validated using commercial available AmpliTaq®10x Buffer and AmpliTaq® polymerase (Applied Biosystems, Forster City, CA, USA) and Master Mix containing the four dNTPs (Eurogentec)[1]		

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

C.5.3.2 PCR controls

All appropriate controls as given in ISO 22174 [13] must 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, need to be included in the PCR assay to check the conditions of amplification.

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

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

WDCM number [a]	*cytK-1*	*cytK-2*
WDCM xxxx ~~(ATCC14579^T)~~	Negative control	Positive control
WDCM xxxx ~~DSM 22905^T~~	Positive control	Positive control
WDCM xxxx ~~DSM 11821~~	Negative control	Negative control
[a] Make reference to the reference strain catalogue available on http://www.wfcc.info for information on culture collection strain numbers and contact details.		

C.5.3.3 Temperature-time program

The temperature-time program as outlined below has been used in the evaluation study.

Table C.4 – Temperature-time program

Initial denaturation		94°C for 5 min
Amplification	Denaturation	94°C for 15 s
	Hybridisation	57°C for 30 s

[1] This information is given for the convenience of the user of this standard and does not constitute an endorsement of this product. Equivalent products can be used if they can be shown to give the same results.

	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 product

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

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 either :
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

The specificity of this PCR method for *cytK-1* and *cytK-2* variant was 100 % under the PCR conditions described (see C.6) and with the recommended controls (see C.5.3.2). Nevertheless, as prescribed in ISO 22174 [13], the presence of the PCR product and its specificity shall be demonstrated by a suitable confirmation reaction.

C.6 Performance characteristics

C.6.1 General

This method was evaluated 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

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.6.2.1 Inclusivity test

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 (the two species that may carry *cytK-2*gene [22]). 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.6.2.2 Exclusivity test

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 8 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 i) detect cytotoxin K genes (and particularly one of its two variants) and ii) 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.