ACTA UNIVERSITATIS AGRICULTURAE ET SILVICULTURAE MENDELIANAE BRUNENSIS

Volume LVIII 50 Number 5, 2010

DETECTION OF CODING GENES FOR ENTEROTOXINS IN *Bacillus cereus* BY PCR AND THEIR PRODUCTS BY BCET-RPLA AND ELISA ASSAY

M. Vyletělová, J. Banykó

Received: June 3, 2010

Abstract

VYLETĚLOVÁ, M., BANYKÓ, J.: *Detection of coding genes for enterotoxins in Bacillus cereus by PCR and their products by BCET-RPLA and ELISA Assay.* Acta univ. agric. et silvic. Mendel. Brun., 2010, LVIII, No. 5, pp. 417–426

Determination of enterotoxin production, diarrhoeal and emetic gene identification was studied in 41 *Bacillus cereus* strains isolated from raw cows' and raw goats' milk, pasteurized milk, dairy products during technological processing and from dairy plant equipment. Presence of enterotoxins was detected by BCET-RPLA (HBL) and ELISA immunoassay (NHE). Gene identification (*nheA*, *nheB*, *nheC*, *hblA*, *hblC*, *hblD*, *bceT*, *cytK-1*, *cytK-2*, *entFM* and *ces*) was achieved by means of PCR. Enterotoxin HBL was detected in 32 strains, enterotoxin NHE in all 41 strains. Presence of all three genes *nheA*, *nheB* and *nheC* was confirmed in 40 strains and genes *hblA*, *hblC* and *hblD* in 29 strains. Comparison of used methods was as follow: 1) BCET-RPLA (which detects L2 component) and PCR (positive or negative all three *hblA*, *hblC* and *hblD* gene detection) were identical in 30 (73%); 2) ELISA (NheA) and PCR (all three *nheC*, *nheB* and *nheA* gene expression) were identical in 40 (98%) cases isolated strains.

milk, *Bacillus cereus*, genes, enterotoxins, PCR, BCET-RPLA, ELISA assay

Spore-forming microorganisms are a serious problem in contamination both of raw milk or dairy products. Their ability to survive pasteurization temperatures or ultrahigh-temperature treatment (UHT) facilitates their infiltration into the food-chain, resulting in the potential incidence of alimentary disorders. *Bacillus cereus* is the primary cause of alimentary intoxication due to dairy product consumption in both the Czech Republic and other countries (Vyletělová *et al.*, 2001, 2002; Páčová *et al.*, 2003; Stenfors *et al.*, 2008; Bartoszewicz, 2008).

Alimentary intoxications by *Bacillus cereus* enterotoxins in food are divided into diarrhoeal and emetic poisoning, according to toxin type. The diarrhoeal type enterotoxin is formed by heat-labile proteins which can be inactivated by heat treatment at 56 °C for 5–30 min (Murray *et al.*, 1999). *Bacillus cereus* produces the following different diarrhoeal enterotoxins: protein complex (haemolytic HBL and non-haemolytic NHE enterotoxins), enterotoxic proteins (enterotoxin T), cytotoxin K and enterotoxin FM (Yang *et al.*, 2005; Lindbäck *et al.*, 2004). Sergeev *et al.* (2006) classified enterotoxin FM as a haemolytic enterotoxin and as cytotoxin.

HBL toxins are formed from three proteins: two lytic components (L2 and L1) and a binding component B. These are encoded by genes *hblC*, *hblD* and *hblA* (Beecher *et al.*, 1995). All three components are required for toxic activity (Yang *et al.*, 2005). NHE consists of three proteins: NheA, NheB and NheC which are encoded by genes *nheA*, *nheB and nheC*, respectively. All three components are also needed for maximum cytotoxic activity (Lindbäck *et al.*, 2004; Yang *et al.*, 2005). The nucleotide sequence denoted *bceT* has been reported to encode a single component toxin T (BceT, 41-kDa protein) which exhibits Vero cell cytotoxicity and has also been attributed to having a role in the diarrheal syndrome (Agata *et al.*, 1995a). An additional study has indicated that the BceT enterotoxin was most likely an experimental artifact that probably could not contribute to food poisoning (Choma and Granum, 2002). CytK toxin is a pore-forming cytotoxin linked to human necrotic enteritis cases (Hardy *et al.*, 2001). Lund *et al.*

(2000) isolated CytK from *Bacillus cereus* strains that caused a severe food poisoning outbreak of enteritis which killed three people and they found a highly cytotoxic, necrotic and haemolytic protein of 34kDa. CytK forms two variants: CytK-1 and Cyt K-2 which are encoded by *cytK-1* and *cytK-2* genes, respectively (Fagerlund *et al.* 2004). CytK-2 is highly toxic to human intestinal cells but not as toxic as CytK-1 (Fagerlund *et al.*, 2004; Guinebretiere *et al.*, 2006). Enterotoxic protein FM (entFM) is a relatively unknown haemolytic enterotoxin that is encoded by the *entFM* gene (Asano *et al.*, 1997).

In contrast to the diarrhoeal type, the emetic toxin (cereulide) is a small, heat-stable cyclic dodecadepsipeptide (Agata *et al.*, 1995b) which can survive a temperature of 120 °C for 90 min (Lund, 1990). This enterotoxin is synthesized by a non-ribosomal peptide synthetase, encoded by the *ces* gene (Ehling-Schulz *et al.*, 2004). The primary symptom of emetic toxin is vomiting but in animal models it can cause cellular damage (Shinagawa *et al.*, 1995) and rarely liver failure in humans (Mahler *et al.*, 1997).

Detection methods for the diarrhoeal toxin are well-known and commercially available. They include BCET-RPLA test which is specific to the L2 component, and ELISA-(BDE VIA) test which detects mainly the NheA protein (45-kDa) – (Granum and Lund, 1997). The emetic enterotoxins can be detected by bioassay test, based on loss of sperm motility (Andersson *et al.*, 1998; Svensson *et al.*, 2006) or HPLC-MS chemical assay (Häggblom *et al.*, 2002).

HBL and NHE enterotoxins, in order to be active, need all three of their components (L1, L2, B and NheA, NheB, NheC, respectively). However, methods used in practice can detect only one of three enterotoxin components (BCET-RPLA – L2; ELISA BDE – NheA). The results of these methods give us information about possible entorotoxin presence but not whether the toxin is active or not. PCR methods used in this study are able to detect all genes involved but also not their activity.

This study is thus focused on comparison of the BCET-RPLA and ELISA BDE immunoassays with PCR results for genes (*hblC*, *hblD*, *hblA* and *nheA*, *nheB*, *nheC*) identification which encode three enterotoxin components. The aim of this study was to find out, if the commonly used methods (BCET-RPLA, ELISA BDE) are sufficient enough to determine presence and activity of enterotoxins or using some additional methods (as PCR) is necessary.

MATERIAL AND METHODS

Bacillus cereus origin and cultivation

Bacillus cereus strains were isolated from raw cows' and raw goats' milk, pasteurized milk, dairy products during technological processing and from dairy industry equipment (Table I). Samples 1–29 were derived from different samples, whereas samples 30–41 were isolated from one raw cows' milk sample. *Bacillus* strains were isolated on modified MYP Agar supplemented with egg yolk emulsion and Polymyxin B sulphate (HiMedia, Mumbai, India) and cultured at 30 ± 1 °C for 48 hours (CSN EN ISO 7932, 2005). Species identification was provided according to biochemical and physiological characteristics and phenotypic tests (Vyletělová *et al.*, 2001).

Enterotoxin detection

Enterotoxins were detected using two immunoassay kits: HBL enterotoxins using commercial kits BCET-RPLA (*B. cereus* Enterotoxin Reverse Passive Latex Agglutination – Oxoid, Denka SEIKEN Ltd., Japan) and NHE using the ELISA test (BDE-VIA – *Bacillus* Diarrhoeal Enterotoxin Visual Immunoassay) from Tecra International Pty, Ltd., Frenchs Forest, Australia. Both kits were used according to the manufacturer's instructions.

DNA isolation

Colonies obtained from the blood-agar plates, incubated at 37 °C for 24 h, was picked and transferred to the tubes containing 500 µL water for resuspension and centrifuged at 10 000 g for 5 min. Supernatant was discarded, the pellet resuspended in 1 ml of washing solution (10mM Tris-HCl, pH 7.8, Serva, Heidelberg, Germany; 5mM EDTA, pH 8.0, Duchefa, Haarlem, The Netherlands), stirred and spin at 10 000 g/5 min. To the sediment 500 µL of lysis buffer (10mM Tris-HCl, pH 7.8, Serva; 5mM EDTA, pH 8.0, Duchefa; 3 mg/ml lyzozyme, Serva) was added, resuspended properly and incubated at 37 °C for 1 hour. Than 12.5 µL of 20% SDS (Promega, Madison, USA) and 15 µL proteinase K (Machery-Nagel, Düren, Germany) was added, mixed and incubated over night at 55 °C.

The next day equal volume of water saturated, Tris–buffered phenol (Serva) was added, mixed gently and spin at 15 000 g/3 min. The water phase was transferred into the clean eppendorf tubes, than 700 µL of chloroform: isoamyl alcohol 24:1 (Amresco, Solon, USA) was added, mixed again and centrifuged at 15 000 g/3 min. The water phase was transferred into the clean eppendorf tubes, 1/10 volume of 3 M sodium acetate and 1 ml of ethanol were added for precipitation of DNA at –20 °C, then rotated at 15 000 g for 15 min. The supernatant was decanted. After evaporation of ethanol from the sediment the DNA was dissolved in TE buffer.

Primer sequences and multiplex PCR

Amplification was completed using a Tpersonal Combi 48/18 thermal cycler (Whatman Biometra, Baltimore, USA) with a reaction volume of 50 µl. The PCR reaction mixture contained 2.5 µl buffer, 2.5 µl $MgCl_2$ (25mM); 2 µl dNTPs (10mM, Top-Bio, CR); 1 µl forward primer and 1 µl reverse primer; 2 µl Taq 1.1 polymerase enzyme (Top-Bio, CR) and 38 µl PCR water. 1 µl of sample DNA as template was added to the mixture.

Primer sequences used for the detection of the various genes were specified in the work of Yang *et al.* (2005) – Table II. Two primer pairs were used to detect the same region of the *cytK* gene, for there was variation in the sequences (*cytK*1 and *cytK*2) of the region among different strains. The PCR products of this two primer pairs were the same size. Last, one primer pair was to be specific for emetic toxin producing strains of *B. cereus,* was used (Ehling-Schulz *et al.*, 2005b) – Table II.

Amplification consisted of initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s. A final 7-min 72 °C extension followed. The PCR products were analyzed on a 2% (wt/vol) agarose gel, and electrophoresis was performed for 40 min at 100 V. The sizes of DNA fragments were estimated using a 100 bp+ DNA ladder (Promega). Gel patterns were visualized by ethidium bromide staining and photo taken under UV transilluminator (Vilbert Lourmat, Marne-la-Vallée, France).

RESULTS AND DISCUSSION

Detection of diarrheal enterotoxin genes

Results, concerning the occurrence of diarrheal enterotoxin genes in the 41 *Bacillus cereus* strains are presented in Table I. The presence of all three genes *nheA, nheB* and *nheC* was confirmed in 40 strains (98%). Only one strain (2.4%) was negative for *nheA* gene (Table I). Compared to this, detection of all haemolytic genes *hblA, hblC* and *hblD* was confirmed in only 29 strains (71%), two strains (4.9%) lacked the *hblC* gene, 12 strains (29.3%) the *hblA* gene and 4 strains (9.8%) the *hblD* gene. There were 3 strains (7.3%) with no detected *hblA* and *hblD* and one strain (2.4%) with no *hblA* and *hblC* genes, respectively. Only one strain (2.4%) lacked all three haemolytic genes (Table I). Yang *et al.* (2005) reported similar results. They discovered that all *B. cereus* strains carried the *nheA, nheB* and *nheC* genes, while *hbl* genes were carried by 46.8% of strains. Al-Khatib *et al.* (2007) also found a higher frequency of nonhaemolytic enterotoxins in patients with diarrhoea, detected in 71% (*nheA*), 84% (*nheB*) and 90% (*nheC*), respectively. The genes for the haemolytic enterotoxin were detected in 58% (*hblA*), 58% (*hblC*) and 68% (*hblD*). Guinebretiere *et al.* (2002) described *nhe* genes in almost all strains of *B. cereus* coming from food-poisoning and food-associated ecosystems.

In this study, the *bceT* gene was detected only in 8 out of 41 observed strains (20%). In contrast, Hansen and Hendriksen (2001) showed that *bceT* is widely distributed in *B. cereus* (55%) and its sequence is different among different strains. Rowan *et al.* (2003) reported the detection of enterotoxins HBL and *bceT* in a number of veterinary isolates of *Bacillus* spp. from nongastrointestinal infections.

The results presented here for *cytK-1* and *cytK-2* genes are similar. These were detected in 21 (51%) and in 20 (49%) strains, respectively. Twelve (29%) *B. cereus* strains possessed both genes (strains coming from one sample). Fagerlund *et al.* (2004) who first described gene *cytK-2* also detected *cytK-1* and *cytK-2* genes in five *B. cereus* strains (17%).

The *entFM* gene detection was positive in all 41 observed strains. The wide distribution of *entFM* gene was also confirmed by Hsieh *et al.* (1999) who found the gene in 78 out of 84 *B. cereus* strains (93%).

The isolated strains didn't produce all observed enterotoxins. There is interesting that 29 (71%) strains carried the genes encoding diarrheal enterotoxins (non-haemolytic NHE and haemolytic HBL and entFM). Considering the emetic toxin occurrence (1 positive gene detection) dairy products can represent potential source of diseases mainly in the case of diarrheal enterotoxins.

Distribution of diarrheal enterotoxin genes

We analysed samples from different areas of the dairy production chain (Table I). Negative occurrences were determined in the case of *cytK-1* and *bceT* genes in strains originating from heat treated samples (dry and pasteurized milk, yoghurts, thermized milk) with the exception of thermized dessert, and *hblA, bceT* and *cytK-2* were detected in strains isolated from dairy equipment. The genes *nheB* and *nheC* were confirmed in all samples. It is interesting that the results of identified genes were identical in samples 30–41 which were isolated from one raw cows' milk sample (Table I; Figure 1, 2). These results suggest the same origin of the *B. cereus* strains which most likely came from one primary strain. Prüß *et al.* (1999) detected the haemolytic enterotoxin HBL in all species of the *B. cereus* group regardless of environment or geographic origin.

Detection and distribution of emetic gene

The *ces* gene encoding emetic toxin was confirmed in only one case (Table I). This strain came from pasteurized cows' milk. The occurrence of this toxin is very sporadic and mostly strains do not produce this type of enterotoxin (Altayar and Sutherland, 2006). These authors describe production in 3 mesophilic *B. cereus* strains of 177 (1.7%) isolated strains from soil, vegetables and animal faeces. They were the first to confirm emetic toxin production within psychrotrophic strains and its production at lower temperatures. Svensson *et al.* (2006) also identified only 0.05% emetic strains among 3401 isolates obtained from dairy operations. However, Ehling-Schulz *et al.* (2005a) detected *ces* gene in 27% of 90 observed strains.

Detection of enterotoxins using immunoassay kits BCET-RPLA and ELISA

The HBL enterotoxin was detected in 32 (78%) of *B. cereus* strains using commercial kit BCET-RPLA (Table I). The strains from heat treatment samples (dry and pasteurized milk, yoghurts with the exception of thermized milk and dessert) produced no haemolytic enterotoxin. The NHE enterotoxin occurrence

I: *Characteristics of studied Bacillus cereus strains*

Strain no/ gene	hblA	hblC	hblD	nheA	nheB	nheC	entFM	bceT	cytK-1	cytK-2	ces	BCET-RPLA ng/ml	ELISA-BDE	Origin of strains
	237	386	436	475	328	557	290	701	800	800	635			
protein	B	L2	L1	NheA	NheB	NheC	entFM	BceT	CytK	CytK	emetic			
1	+	+	+	+	+	+	+	-	+	-	-	64	++++	bulk milk sample (cow´s milk)
2	+	+	+	+	+	+	+	+	+	-	-	128	++	cow (mastitis milk)
3	+	+	+	+	+	+	+	-	+	-	-	64	+++	raw goat milk
4	-	+	-	+	+	+	+	-	-	-	-	< 2	+	dairy equipment
5	-	+	+	+	+	+	+	-	+	-	-	16	+++	dairy equipment
6	-	+	-	+	+	+	+	-	-	-	-	< 2	+	dry milk
7	-	-	+	+	+	+	+	-	-	-	-	< 2	+	bulk milk sample (cow´s milk)
8	+	+	+	+	+	+	+	+	+	-	-	128	+	bulk milk sample (cow´s milk)
9	+	+	+	+	+	+	+	+	+	-	-	128	++	raw skim milk
10	-	+	+	+	+	+	+	-	-	-	-	< 2	+++	raw skim milk
11	-	+	+	+	+	+	+	-	-	+	-	64	+	raw cow´s milk
12	+	+	+	+	+	+	+	-	+	-	-	128	+	raw skim milk
13	+	+	+	+	+	+	+	-	-	-	-	128	++	raw skim milk
14	+	+	+	-	+	+	+	-	-	-	-	128	++	termized milk
15	+	+	+	+	+	+	+	+	+	-	-	128	++	termized dessert
16	+	+	+	+	+	+	+	-	-	-	-	128	+	raw skim milk
17	+	+	+	+	+	+	+	-	-	+	-	32	+	bulk milk sample (cow´s milk)
18	-	+	+	+	+	+	+	-	-	+	-	32	++	bulk milk sample (cow´s milk)
19	+	+	+	+	+	+	+	-	-	+	-	64	+	bulk milk sample (cow´s milk)
20	+	+	+	+	+	+	+	-	-	+	-	32	+	bulk milk sample cow´s milk)
21	-	+	+	+	+	+	+	-	-	-	+	< 2	+	pasteurized cows´ milk
22	+	+	+	+	+	+	+	+	-	+	-	128	++	raw cow´s milk
23	-	+	+	+	+	+	+	-	-	+	-	< 2	+	yoghurt
24	-	+	-	+	+	+	+	+	+	-	-	< 2	+	raw cow´s milk
25	-	-	-	+	+	+	+	-	-	+	-	< 2	+	yoghurt
26	+	+	+	+	+	+	+	+	-	-	-	16	+	raw cow´s milk
27	+	+	+	+	+	+	+	-	-	-	-	16	+	raw cow´s milk
28	+	+	+	+	+	+	+	+	-	-	-	16	+	raw cow´s milk
29	-	+	+	+	+	+	+	-	-	-	-	< 2	++	pasteurized cow´s milk
30	+	+	+	+	+	+	+	-	+	+	-	64	++	bulk milk sample (cow´s milk)

Strain no/ gene	hblA	hblC	hblD	nheA	nheB	nheC	entFM	bceT	cytK-1	cytK-2	ces	BCET-RPLA ng/ml	ELISA-BDE	Origin of strains
protein	B	L2	L1	NheA	NheB	NheC	entFM	BceT	CytK	CytK	emetic			
31	+	+	+	+	+	+	+	-	+	+	-	128	++	bulk milk sample (cow´s milk)
32	+	+	+	+	+	+	+	-	+	+	-	128	++	bulk milk sample (cow´s milk)
33	+	+	+	+	+	+	+	-	+	+	-	128	++	bulk milk sample (cow´s milk)
34	+	+	+	+	+	+	+	-	+	+	-	64	++	bulk milk sample (cow´s milk)
35	+	+	+	+	+	+	+	-	+	+	-	64	++	bulk milk sample (cow´s milk)
36	+	+	+	+	+	+	+	-	+	+	-	128	++	bulk milk sample (cow´s milk)
37	+	+	+	+	+	+	+	-	+	+	-	128	++	bulk milk sample (cow´s milk)
38	+	+	+	+	+	+	+	-	+	+	-	128	++	bulk milk sample (cow´s milk)
39	+	+	+	+	+	+	+	-	+	+	-	64	++	bulk milk sample (cow´s milk)
40	+	+	+	+	+	+	+	-	+	+	-	128	++	bulk milk sample (cow´s milk)
41	+	+	+	+	+	+	+	-	+	+	-	128	++	bulk milk sample (cow´s milk)

BCET-RPLA: < 2 – negative detection of L2 component; *, **, *** - positive detection of L2 component

was positive in all cases using the ELISA-BDE visual immunoassay. Both enterotoxins were produced by 32 (78%) of all strains. Similar results were described by Ombui and Nduhiu (2005) who investigated 96 milk samples and analyzed 47 *B. cereus* strains. They found 81% (38 from 47 isolates) of *B. cereus* strains produced non-haemolytic enterotoxins and 53% (25 strains) produced haemolysin BL. Both haemolysin BL and non-haemolytic enterotoxins were produced by 18 (38%) of all 47 strains. Beattie and Williams (1999) reported 85% positive *B. cereus* strains using the ELISA BDE assay and 51% using BCET-RPLA, respectively.

Comparison of PCR and two commercial immunoassay kits

Comparing the results obtained by PCR method (detection of *hblC* gene encoding the L2 component) and the results of the BCET-RPLA method (L2 detection) the results were consistent for 32 strains, and in 2 strains with negative gene and negative enterotoxin occurrence, as well – 83% (Table I). Different results were found in 7 strains (17%). These *B. cereus* strains possessed the *hblC* gene but the presence of enterotoxin using BCET-RPLA was not confirmed. This difference can be caused by sensitivity of the method or by inactive gene. Guinebretiere *et al.* (2002) found also a high correlation between the presence of this gene and enterotoxin production BCET-RPLA test.

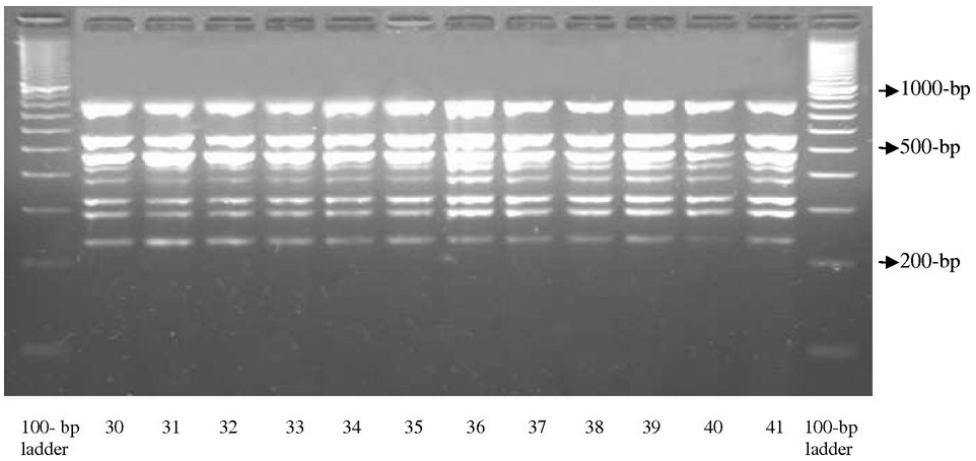
In comparing BCET-RPLA positive results with PCR positive determination for the presence of all three *hbl* genes, we confirmed, that the methods used gave identical results in 30 isolated strains out of 41 (73%), including one strain lacking all three *hbl* genes along with a negative BCET-RPLA result.

In determinations of non-haemolytic protein by the ELISA immunoassay which detects 45-kDa protein (NheA related to *nheA* gene) and PCR the results were identical in 40 cases (98%). The only exception was the absence of *nheA* gene in thermized milk. Hansen & Hendriksen (2001) described negative *nheA* gene detection and positive ELISA BDE immunoassay in three *B. cereus* strains among 22 isolated strains. This difference can be explained by mutation in sequences complementary to the primers and protein can be functionless. Guinebretiere *et al.* (2002) found a high correlation between gene occurrence and enterotoxin production using ELISA tests.

The presence of all three HBL and NHE components is needed for toxic activity (Lindbäck *et al.*, 2004; Yang *et al.*, 2005). This implies that in the case of positive results using ELISA and BCET-RPLA kits which detect only one HBL or NHE component (L2 or NheA respectively) the enterotoxin need not be toxically active. In practice, it is advised to use both methods for determination of possible presence of enterotoxins (ELISA and PCR or BCET-RPLA and PCR) but to use other method for determination of toxic activity enterotoxins.

II: *PCR primers used for multiplex PCR*

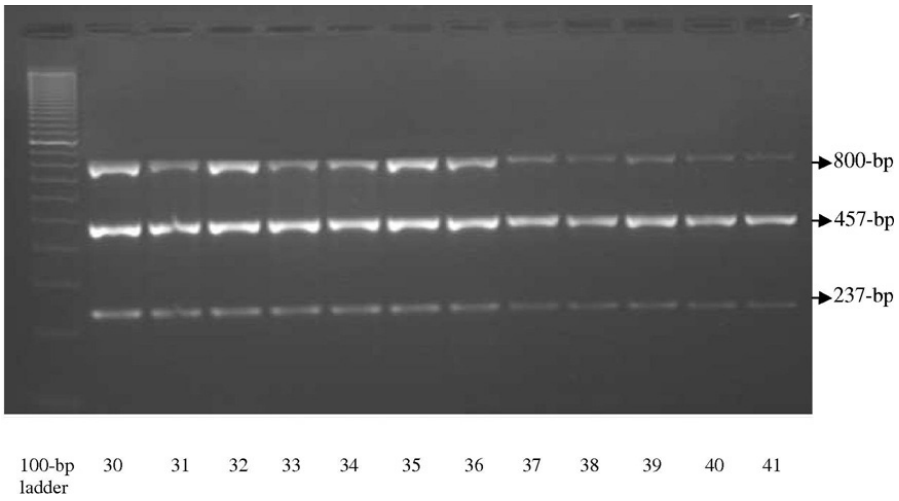
Target gene	Primer	Sequence (5´→3´)
hblA **(237bp)**	HA-F1	ATT AAT ACA GGG GAT GGA GAA ACT T
	HA-R1	TGA TCC TAA TAC TTC TTC TAG ACG CTT
hblC **(386bp)**	HC-F1	CCT ATC AAT ACT CTC GCA ACA CCA AT
	HC-R1	TTT TCT TGA TTC GTC ATA GCC ATT TCT
hblD **(436bp)**	HD-F1	AGA TGC TAC AAG ACT TCA AAG GGA AAC TAT
	HD-R1	TGA TTA GCA CGA TCT GCT TTC ATA CTT
nheA **(457bp)**	NA-F1	ATT ACA GGG TTA TTG GTT ACA GCA GT
	NA-R1	AAT CTT GCT CCA TAC TCT CTT GGA TGC T
nheB **(328bp)**	NB-F1	GTG CAG CAG CTG TAG GCG GT
	NB-R1	ATG TTT TTC CAG CTA TCT TTC GCA AT
nheC **(557bp)**	NC-F1	GCG GAT ATT GTA AAG AAT CAA AAT GAG GT
	NC-R1	TTT CCA GCT ATC TTT CGC TGT ATG TAA AT
bceT **(701bp)**	BceT-R1	AGC TTG GAG CGG AGC AGA CTA TGT
	BceT-F1	GTA TTT CTT TCC CGC TTG CCT TTT
entFM **(290bp)**	FM-R1	CAA AGA CTT CGT AAC AAA AGG TGG T
	FM-F1	TGT TTA CTC CGC CTT TTA CAA ACT T
cyt-K$_1$ **(800bp)**	Cyt-R1	ATC GGG CAA AAT GCA AAA ACA CAT
	Cyt-F1	ACC CAG TTT GCA GTT CCG AAT GT
cyt-K$_2$ **(800bp)**	Cyt-R2	ATC GGT CAA AAT GCA AAA ACA CAT
	Cyt-F2	ACC CAG TTA CCA GTT CCG AAT GT
ces **(635bp)**	CES-F1	GGT GAC ACA TTA TCA TAT AAG GTG
	CES-R2	GTA AGC GAA CCT GTC TGT AAC AAC A



1: *Agarose gel of multiplex PCR amplification of Bacillus cereus strains number 30–41. There are cytK-1 (800bp), nheC (557bp), nheA (457bp), hblD (436bp), hblC (386bp), nheB (328bp), entFM (290bp) and hblA (237bp)*

SUMMARY

This study was focused on a results comparison of the BCET-RPLA and ELISA BDE immunoassay for enterotoxins detection with PCR results for genes identification which encode three enterotoxin components of HBL and NHE enterotoxins (*hblC*, *hblD*, *hblA* and *nheA*, *nheB*, *nheC*). Next to these genes were identified others encoded diarrhoeal and emetic toxins (*bceT*, *cytK-1*, *cytK-2*, *entFM* and *ces*). *Bacillus cereus* strains (n = 41) were isolated from raw cows' and raw goats' milk, pasteurized milk, milk dairy during technological processing and from dairy plant equipment. Enterotoxins were detected using two immunoassay kits: HBL enterotoxins using commercial kits BCET-RPLA and NHE using the ELISA test (BDE-VIA). The DNA amplification was performed with a thermal cycler. The electrophoresis was carried out for 120 min at 80V. DNA in the gel was visualized by exposure to UV light,



2: *Agarose gel of triplex PCR amplification for detection of cytK-2 gene. There are cytK-2 (800-bp) and for positive control nheA (457-bp) and hblA (237-bp)*

photographed with a digital system (DP-001.FDC, Vilber Lourmat) and evaluated using BioLight software. There were used various primer sequences for detection diarrheal enterotoxins.
The HBL enterotoxin was detected in 32 (78%) of *B. cereus* strains using commercial kit BCET-RPLA and the NHE enterotoxin occurrence was positive in all 41 cases using the ELISA-BDE visual immunoassay. Positive gene detection was as follow: *nheA* was detected in 40 strains, *nheB* and *nheC* (41), *hblA* (29), *hblC* (39), *hblD* (37), *bceT* (8), *cytK-1* (21), *cytK-2* (20), *entFM* (41) and *ces* in 1case. The presence of all three HBL (L2, L1 and B) and NHE components (NheA, NheB and NheC) is needed for toxic activity of these enterotoxins. The presence of all three genes *nheA, nheB* and *nheC* was confirmed in 40 strains (98%). Compared to this, detection of all haemolytic genes *hblA, hblC* and *hblD* was confirmed in only 29 strains (71%). Comparing BCET-RPLA positive (or negative in 1 case) results with PCR positive (or 1 negative) determination for the presence of all three *hbl* genes, we confirmed, that the methods used gave identical results in 30 isolated strains (73%). In determinations of non-haemolytic protein by the ELISA immunoassay and PCR the results were identical in 40 cases (98%). In practice, it is advised to use both methods for determination of possible presence of enterotoxins but to use other method for determination of toxic activity enterotoxins.

SOUHRN

Detekce genů kódujících enterotoxiny *Bacillus cereus* pomocí PCR a jejich produktů metodou BCET-RPLA a ELISA assay

Práce byla zaměřena na srovnání výsledků pro detekci přítomnosti enterotoxinů pomocí metod BCET-RPLA a ELISA immunoassay a výsledků identifikace genů odpovědných právě za syntézu složek HBL a NHE enterotoxinů (*hblC, hblD, hblA* a *nheA, nheB, nheC*). Vedle těchto genů byly detekovány i další kódující syntézu dirrhogenních a emetického enterotoxinu (*bceT, cytK-1, cytK-2, entFM* a *ces*). Kmeny *Bacillus cereus* byly izolovány z kravského a kozího mléka, pasterovaného mléka, mléčných produktů během technologického zpracování a z výrobního zařízení. Přítomnost HBL enterotoxinů byla stanovena pomocí metody BCET-RPLA a přítomnost NHE enterotoxinu ELISA (BDE-VIA) testem. Pro amplifikaci DNA byl použit termální cykler. Elektroforéza amplikonů probíhala po dobu 120 min při 80 V, ethidium bromidem barvené gely byly vizualizovány UV světlem, zachyceny digitálním fotosystémem (DP-001.FDC, Vilber Lourmat) a vyhodnoceny pomocí programu BioLight. Pro amplifikaci jednotlivých genů byly použity různé sekvence primerů.
Hemolytický enterotoxin HBL byl identifikován metodu BCET-RPLA u 32 kmenů *B. cereus* (78%). Přítomnost hemolytického enterotoxinu NHE byla potvrzena u všech 41 sledovaných kmenů. Pozitivní detekce genů byla zjištěna následovně: gen *nheA* byl identifikován u 40 kmenů, *nheB* a *nheC* (41), *hblA* (29), *hblC* (39), *hblD* (37), *bceT* (8), *cytK-1* (21), *cytK-2* (20), *entFM* (41) a *ces* pouze v jednom případě. Přítomnost všech tří složek, které tvoří enterotoxin HBL (L2, L1 a B) a všech tří komponent, ze kterých je složen NHE enterotoxin (NheA, NheB a NheC), je nutná pro toxickou aktivitu obou enterotoxinů. Přítomnost všech tří genů *nheA, nheB* a *nheC* byla pozitivní u 40 kmenů (98%) a genů *hblA, hblC* a *hblD* pouze u 29 kmenů (71%). Srovnáme-li identifikaci všech tří *hbl* genů (pozitivní i negativní) s pozitivními či negativními výsledky BCET-RPLA, zjistíme, že jsou shodné ve 30 případech (73%). V pří-

padě srovnání výsledků ELISA immunoassay pro stanovení NHE enterotoxinu s výsledky PCR pro všechny tři *nhe* geny zjistíme, že jsou shodné ve 40 případech (98 %). Pro praxi to znamená použití obou metod pro stanovení možné přítomnosti enterotoxinů, ale použití další jiné metody pro stanovení jejich toxické activity.

mléko, *Bacillus cereus*, enterotoxin, geny, PCR, BCET-RPLA, ELISA assay

Acknowledgements

This work was supported by Ministry of Education project MSM2678846201 and by Ministry of Agriculture project NAZV QF 3162.

REFERENCE

AGATA, M., OHTA, M., ARAKAWA, Y. and MORI, M., 1995a: The *bceT* gene of *Bacillus cereus* encodes an enterotoxic protein. *Microbiology*, 141: 983–988. ISSN 1350-0872.

AGATA, M., OHTA, M., MORI, M. and ISOBE, M., 1995b: A novel dodecadepsipeptide, cereulide, is an emetic toxin of *Bacillus cereus*. *FEMS Microbiol. Lett*., 129: 17–20. ISSN 0378-1097.

AL-KHATIB, M. S., KHYAMI-HORANI, H., BADRAN, E. and SHEBABI, A. A., 2007: Incidence and characterization of diarrheal enterotoxins of fecal *Bacillus cereus* isolates associated with diarrhoea. *Diagn. Microbiol. Infect. Dis*., 59: 383–387. ISSN 0732-8893.

ALTAYAR, M. and SUTHERLAND, A. D., 2006: *Bacillus cereus* is common in the environment but emetic toxin producing isolates are rare. *J. Appl. Microbiol*., 100: 7–14. ISSN 1364-5072.

ANDERSSON, M. A., MIKKOLA, R., HELIN, J., ANDERSSON, M. C. and SALKINOJA-SALONE, M., 1998: A novel sensitive bioassay for detection of *Bacillus cereus* emetic toxin and related depsipeptide ionophores. *Appl. Environ. Microbiol*., 64: 1338–1343. ISSN 0099-2240.

ASANO, S. I., NUKUMIZU, Y., BANDO, H., IIZUKA, T. and YAMAMOTO, T., 1997: Cloning of novel enterotoxin genes from *Bacillus cereus* and *Bacillus thuringiensis*. *Appl. Environ. Microbiol*., 63: 1054–1057. ISSN 0099-2240.

BARTOSZEWICZ, M., HANSEN, B. M. and SWIECICKA I., 2008: The members of the *Bacillus cereus* group are commonly present contaminants of fresh and heat-treated milk *Food Microbiol*., 25: 588–596. ISSN 0740-0020.

BEATTIE, S. H. and WILLIAMS, A. G., 1999: Detection of toxigenic strains of *Bacillus cereus* and other *Bacillus* spp. with an improved cytotoxicity assay. *Lett. Appl. Microbiol*., 28: 221–225. ISSN 0266-8254.

BEECHER, D. J., SHOENI, J. L. and WONG, A. C. L., 1995: Enterotoxin activity of haemolysin BL from *Bacillus cereus*. *Infect. Immun*., 63: 4423–4428. ISSN 0019-9567.

CHOMA, C. and GRANUM, P. E., 2002: The enterotoxin T (BcET) from *Bacillus cereus* can probably not contribute to food poisoning. *FEMS Microbiol. Lett*., 217: 115–119. ISSN 0378-1097.

CSN EN ISO 7932 (IDT. 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. Czech Standards Institute, August 2005.

EHLING-SCHULZ, M., FRICKER, M. and SCHERER, S., 2004: Identification of emetic toxin producing *Bacillus cereus* strains by a novel molecular assay. *FEMS Microbiol. Lett*., 232: 189–195. ISSN 0378-1097.

EHLING-SCHULZ, M., SVENSSON, B., GUINEBRETIERE, M-H., LINDBÄCK, T., ANDERSSON, M., SCHULZ, A., FRICKER, M., CHRISTIANSSON, A., GRANUM, P. E., MÄRTLBAUER, E., NGUYEN-THE, C., SALKINOJA-SALONE, M. and SCHERER, S., 2005a: Emetic toxin formation of *Bacillus cereus* is restricted to a single evolutionary lineage of closely related strains. *Microbiology*, 151: 183–197. ISSN 1350-0872.

EHLING-SCHULZ, M., VUKOV, N., SCHULZ, A., SHAHEEN, R., ANDERSSON, M., MÄRTLBAUER, E. and SCHERER, S., 2005b: Identification and partial characterization of the nonribosomal peptide synthetase gene responsible for cereulide production in emetic *Bacillus cereus*. *Appl. Environ. Microbiol*., 71: 105–113. ISSN 0099-2240.

FAGERLUND, A., WEEN, O., LUND, T., HARDY, S. P. and GRANUM, P. E., 2004: Genetic and functional analysis of the *cytK* family of genes in *Bacillus cereus*. *Microbiology*, 150: 2689–2697. ISSN 1350-0872.

GRANUM, P. E. and LUND, T., 1997: Mini review. *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiol. Lett*., 157: 223–228. ISSN 0378-1097.

GUINEBRETIERE, M. H., BROUSSOLLE, V. and NGUYE-THE, C., 2002: Enterotoxigenic profiles of food poisoning and food-borne *Bacillus cereus* strains. *J. Clin. Microbiol*., 40: 3053–3056. ISSN 0095-1137.

GUINEBRETIERE, M. H., FAGERLUND, A., GRANUM, P. E. and NGUYEN-THE., C., 2006: Rapid discrimination of *cytK-1* and *cytK-2* genes in *Bacillus cereus* by a novel duplex PCR system. *FEMS Microbiol. Lett*., 259: 74–80. ISSN 0378-1097.

HÄGGBLOM, M. M, APETROAIE, C., ANDERSSON, M. A. and SALKINOJA-SALONE, M. S., 2002: Quantitative analysis of cereulide, the emetic toxin of *Bacillus cereus*, produced under various

conditions. *Appl. Environ. Microbiol.*, 68: 2479–2483. ISSN 0099-2240.

HANSEN, B. M. and HENDRIKSEN, N. B., 2001: Detection of enterotoxic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. *Appl. Environ. Microbiol.*, 67: 185–189. ISSN 0099-2240.

HARDY, S. P., LUND, T. and GRANUM, P. E., 2001: CytK toxin of *Bacillus cereus* forms pores in planar lipid bilayers and is cytotoxic to intestinal epithelia. *FEMS Microbiol. Lett*., 197: 47–51. ISSN 0378-1097.

HSIEH, Y. M., SHEU, S. J., CHEN, Y. L. and TSEN, H. Y., 1999: Enterotoxigenic profiles and polymerase chain reaction detection of *Bacillus cereus* group cells and *B. cereus* strains from food and food-borne outbreaks. *J. Appl. Microbiol*., 87: 481–490. ISSN 1364-5072.

LINDBÄCK, T., FAGERLUND, A., RØDLAND, M. S. and GRANUM, P. E., 2004: Characterization of the *Bacillus cereus* Nhe enterotoxin. *Microbiology*, 150: 3959–3967. ISSN 1350-0872.

LUND, B. M., 1990: Food-borne disease due to *Bacillus* and *Clostridium* species. *Lancet*, 336: 982–986. ISSN 0140-6736.

LUND, T, DEBUYSER, M. L. and GRANUM, P. E., 2000: A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. *Mol.. Microbiol*., 38: 254–261. ISSN 0950-382X.

MAHLER, H., PASI, A., KRAMER, J. M., SCHULTE, P., SCOGING, A. C., BÄR, W. and KRÄHENBÜHL, S., 1997: Fulminant liver failure in association with the emetic toxin of *Bacillus cereus*. *New Eng. J. Med*., 336: 1142–1148. ISSN 0028-4793.

MURRAY, P. R., BARON, E. J., PFALLER, M. A., TENOVER, F. C. AND YOLKEN, R. H., 1999: *Manual of clinical microbiology.* 7th edition ASM, Washington D. C., pp. 1776. ISBN 1-55581-126-4.

OMBUI, J. N. and NDUHIU, J. G., 2005: Prevalence of enterotoxigenic *Bacillus cereus* and its enterotoxins in milk and milk products in and around Nairobi. *East Afr. Med. J*., 82: 280–284. ISSN 0012-835x.

PÁČOVÁ, Z., ŠVEC, P., STENTORE, L. P., VYLETĚLOVÁ, M. and SEDLÁČEK, I., 2003: Isolation of the psychrotolerant species *Bacillus weihenstephanensis* from raw cow's milk. *Czech J. Anim. Sci*., 48: 93–96. ISSN 1212-1819.

PRÜSS, B. M., DIETRICH, R., NIBLER, B., MÄRTLBAUER, E. and SCHERER, S., 1999: The haemolytic enterotoxin HBL is broadly distributed among species of the *Bacillus cereus* group. *Appl. Environ. Microbiol.*, 65: 5436–5442. ISSN 0099-2240.

ROWAN, N. J., CALDOW, G., GEMMEL, C. G. and HUNTER, I. S., 2003: Production of diarrheal enterotoxins and other potential virulence factors by veterinary isolates of *Bacillus* species associated with nongastrointestinal infections. *Appl. Environ. Microbiol.*, 69: 2372–2376. ISSN 0099-2240.

SERGEEV, N., DISTLER, M., VARGAS, M., CHIZHIKOV, V., HEROLD, K. E. and RASOOLY, A., 2006: Microarray analysis of *Bacillus cereus* group virulence factors. *J. Microbiol. Meth*., 65: 488–502. ISSN 0167-7012.

SHINAGAWA, K., KONUMA, H., SEKITA, H. and SUGII, S., 1995: Emesis of rhesus monkeys induced by intragastric administration with the HEp-2 vacuolation factor (cereulide) produced by *Bacillus cereus*. *FEMS Microbiol. Lett*., 130: 87–90. ISSN 0378-1097.

STENFORS, ARNESEN, L. P, FAGERLUND, A. and GRANUM, P. E., 2008: From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiol. Rev.*, 32: 579–606. ISSN 0168-6445.

SVENSSON, B., MONTHAN, A., SHAHEEN, R., ANDERSSON, M. A., SALKINOJA-SALONE, M. and CHRISTIANSSON, A., 2006: Occurrence of emetic toxin producing *Bacillus cereus* in the dairy production chain. *Int. Dairy J*., 16: 740–749. ISSN 0958-6946.

VYLETĚLOVÁ, M., HANUŠ, O., PÁČOVÁ, Z., ROUBAL, P. and KOPUNECZ, P., 2001: Frequency of *Bacillus* bacteria in raw cow's milk and its relation to other hygienic parameters. *Czech J. Anim. Sci*., 46: 260–267. ISSN 1212-1819.

VYLETĚLOVÁ, M., ŠVEC, P., PÁČOVÁ, Z., SEDLÁČEK, I. and ROUBAL, P., 2002: Occurrence of *Bacillus cereus* and *Bacillus licheniformis* strains in the course of UHT milk production. *Czech J. Anim. Sci*., 47: 200–205. ISSN 1212-1819.

YANG, I. C., SHIH, D. Y. C., HUANG, T. P., HUANG, Y. P., WANG, J. Y. and PAN, T. M., 2005: Establishment of a novel multiplex PCR assay and detection of toxigenic strains of the species in the *Bacillus cereus* group. *J. Food Prot*., 68: 2123–2130. ISSN 0362-028X.

Adresa

RNDr. Marcela Vyletělová, Ph.D., Agrovýzkum Rapotín, Výzkumníků 267, 788 13 Vikýřovice, Česká republika, RNDr. Juraj Banykó, Výzkumný ústav pro chov skotu Rapotín, Výzkumníků 267, 788 13 Vikýřovice, Česká republika