PCR assay a new approach for detection of enterotoxins

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ABSTRACT

B. cereus group is considered as a potential problem, since it can contaminate many dairy products. In this study, three primer sets were selected to simultaneously detect two different species of the *B. cereus* group by using triple – primer PCR. The triple–primer PCR in this study were synthesized using the CER, CES and groEL genes for the detection of emetic toxin producing strains and another specific primer for the detection of diarrheal toxin (groEL gene only). Results indicated that all the diarrheal enterotoxin producing *B. cereus* strains showed a presence of groEL gene, while CER and CES genes were completely absent. Out of the six *B. cereus* strains tested for the production of diarrheal and emetic enterotoxins by using triple–primer PCR technique, three diarrheal enterotoxin producing strains were only detected. On the other hand, all six *B. cereus* strains had limited ability to produce emetic toxin. All diarrheal enterotoxin producing *B. mycoides* strains showed presence of groEL gene, but CER and CES genes were not detected in any of the *B. mycoides* tested strains. Consequently, four *B. mycoides* strains were tested for the production of diarrheal and emetic enterotoxins by using triple–primer PCR technique, only two strains showed diarrheal enterotoxin producing strains. In contrary all *B. mycoides* tested strains had limited ability to produce emetic toxin.

Keywords: Aerobic spore forming bacteria; Milk products; *B. cereus* group; Enterotoxin producing.

INTRODUCTION

The genus *Bacillus* contains 51 species and is divided into three groups based on the morphology of spores and sporangia (Groups 1-3). Group 1 is subdivided into 1A and 1B based on the cell size and the presence of poly – β – hydroxybutyrate in the cytoplasm. The organisms belong to Group 1A (*B. cereus* group) are; *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. psuedomycoides* and *B. weihenstephanensis*. Enterotoxin production is linked to *Bacillus* induced disease and a majority members of Group 1A produce enterotoxin (Bhunia, 2008).

Bacillus cereus is Gram – positive, rod shaped, spore forming bacterium and a human opportunistic pathogen, which can cause diarrheal and emetic types of food poisoning (Arnsen *et al*.,2008). *B. cereus* is commonly found in a wide variety of different foods and environments (Duc *et al*., 2005; Bartoszewicz *et al*., 2008).

The enterotoxins that have been associated with diarrheal type food poisoning are hemolysin BL (HBL), non-hemolytic enterotoxin (NHE), cytotoxin K (CytK) and enterotoxin FM (Lund *et al*., 2000; Sergeev *et al*., 2005). However, enterotoxins have already been comparatively well characterized at the molecular level (Lund *et al*., 2000) and in immunochemical assay (Dietrich *et al*., 2005).

Additionally, emetic type food poisoning is caused by an emetic toxin (cereulide). This type of food poisoning is small, heat and acid stable circular dodecadepsipeptide with the following stereochemistry: [D-O-Leu-D-Ala-L-O-Val-L-Val]. (Ehling-Schulz *et al*., 2005).

However, since the first case of human food poisoning by Bacillus group was detected, *B. cereus* group has been implicated in various outbreaks worldwide (Stenfors *et al*., 2008; Bennett *et al*., 2013).

Moreover, *B. cereus* group is considered as potential problem since it can contaminate many dairy products in several countries (Sadek *et al*., 2006; Reyes *et al*., 2007; Bartoszewicz *et al*., 2008; Zhou *et al*., 2010; Mohamed *et al*., 2016). This group is able to cause two types of syndroms i.e. diarrheal and emetic. Also, *B. cereus* group is an important spoilage organism to many foods (Tan *et al*., 1997; Fermanian, 1997).

Traditionally, *B. cereus* group have been associated with the spoilage of food products, however, recently they have been linked to potential food poisoning issues (Rodriguez-Lozano *et al*., 2010).

Recently, several investigators used Triple-primer polymerase chain reaction (PCR) assay to detect diarrheal and emetic toxins producing by *B. cereus* group strains (Zhou *et al*., 2010; Kim *et al*., 2013).

Therefore, the aim of the current study was to used a reliable and accuracy method for detection enterotoxic genes by PCR technique in *B. cereus* group strains.

MATERIALS AND METHODS

Gel preparation (1% agarose gel)

Agarose gel 1% is prepared by adding 1g. of agarose to 100 ml. TBE. The solution was boiled to dissolve the agarose in microware oven for 1-3 min., and cooled down to 45°C, 3μL of ethidium bromide (1%) was added and left for solidification at room temperature.

PCR master mix

GoTaq Green Master Mix, is a premixed ready-to-use solution containing bacterially drived Taq DNA polymerase, dNTPs, Mg Cl_2 and reaction buffers at optimal concentration for efficient amplification of DNA templates by PCR.

Genomic DNA isolation Kit

Total genomic DNA was extracted from ten isolates according to manufacture instructions using Zymo Research. Fungal/ Bacterial DNA Mini Prep™ Kit (catalog No. D6005 ZR CORP, India). The Kits were purchased from Sigma Company, Egypt. PCR primers and annealing temperature used for the detection of emetic and diarrheal toxins:

The three primer sets which used in the present study are illustrated in Table 1.

Table1. PCR primers and annealing temperature for the detection of emetic and diarrheal toxin producing *B. cereus* group.

Primer name	Primer sequence (5'-3')	AT	Amplicon size (bp)	Reference
CER F-5 CER R-5	CAAGTCAAGATAAGAGGCTTC AAAGCTCTTGCCAAATAACC	54°C	370	Kim *et al*. (2013).
CES F-10 CES R-10	GCATTTCGTGAAGCAGAGGT CCCTTTATCCCCTTCGATGT	54°C	699	Kim *et al*. (2013).
groEL F-1 groELR-1	AGCTATGATTCGTGAAGGT AAGTAATAACGCCGTCGT	54°C	236	Kim *et al*. (2013).

AT= Annealing Temperature.

Amplified fragments visualization

Agarose gel electrophoresis 1.5% (wt/vol) was used for migrating the amplified DNA fragments. Gel was stained with ethidium bromide (0.5 μL/mL) and directly loaded on the gel, DNA ladder was also loaded on the gel for fragment size comparison and visualized under UV light using electrophoresis machine (Mupid-exU, Mupid, Tokyo, Japan). Also, the concentration of the extracted DNA was measured by using UV- spectrophotometer (Model UV-1700, Shimadzu, Tokyo, Japan).

Tested Bacillus group strains

Ten identified Bacillus cultures were used in the present study, six isolates belonging to *Bacillus cereus*, while the rest four cultures were belonging to *Bacillus mycoides*. However, all the tested cultures were isolated from different dairy products.

DNA extraction from Bacillus group strains

The DNA was extracted according to manufacture instructions by using DNA extraction kit (Wizard Genomic DNA purification kit, Promega, Madison, WI, USA).

PCR amplification and PCR reaction condition

The PCR amplification reactions were prepared in a total volume of 20 μL contained 30 pM of each primer, 20 ng of DNA template, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 30 mM KCl, 250 μM dNTP mixture and 1 U Top DNA polymerase (Bioneer, Daejeon, Korea).

The PCR conditions were as follows

Per-denaturation at 94 °C for 10 min., followed by 35 cycles of 94°C for 1 min., annealing temperature was tested at 54 °C and 72 °C for 1 min. and a final elongation cycle at 72 °C for 5 min.

RESULTS AND DISCUSSION

Nowadays, molecular techniques have been increased and successfully applied to detect diarrheal and emetic toxins producing by Bacillus cereus group strains (Kim *et al*. 2013). Thus, polymerase chain reaction (PCR) assay using *B. cereus* group specific triple – primer CER, CES and groEL primers was carried out.

In this respect, a total genomic DNA was extracted from the ten tested isolates by using Zymo Research Fungal / Bacterial DNA Prep™ Kit. Also three species specific primer pairs were used. Results from Table 1 showed that after testing each of the three primer set, groEL-F-1 / groEL-R-1 was highly specific for detection of gene groEl.

Results of amplified PCR fragments for ten tested Bacillus strains are shown in Figure 1. It is very clear from the results obtained that product of *Bacillus cereus* Dc 8-1 which isolated from Domiati cheese scored 236 bp in lan 1 by using primer pair (groEL F-1/groEL R-1). In addition, strains Bacillus cereus Rc 5-1 and Rc6-1 that were isolated from Ras cheese yielded amplified fragment of 236 bp in lan (3 and 4) by using primer (groEL F-1/groEL R-1).

Moreover, it is of interest to notice that both strains belonging to *B. mycoides*, named Rc 2-1 and Dc 7-1, isolated from Ras and Domiati cheese, respectively, showed amplified product of 236 bp in lan (5 and 6) by using the same primer (groEL F-1/groEL R-1).

In contrast, none of rest tested strains either *B. cereus* or *B. mycoides* yielded any amplified product in this PCR reaction as shown in Figure 1 and Table 1.

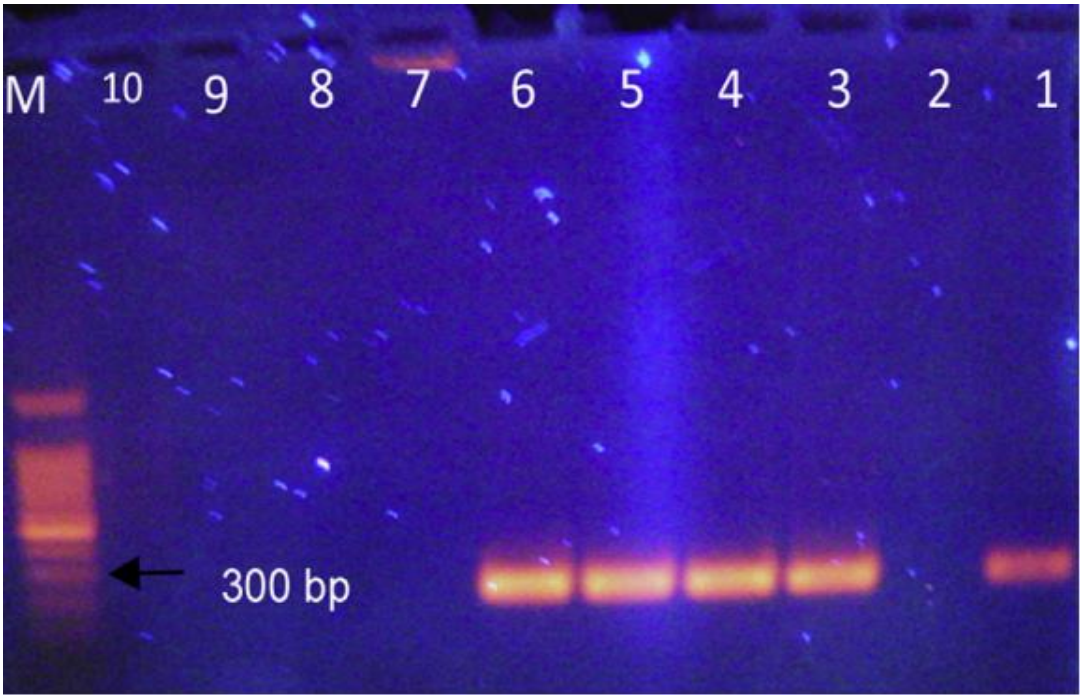

Figure 1. Gel electrophoresis of PCR products amplified with triple-primer on emetic and diarrheal toxin producing *B. cereus* and *B. mycoides* strains. M: DNA ladder 100 bp from promega. Lane 1: *B. cereus* Dc8-1, Lane 2: *B. mycoides* Dc1-1, Lane 3: *B. cereus* Rc5-1, Lane 4: *B. cereus* Rc6-1, Lane 5: *B. mycoides* Rc2-1, Lane 6: *B. mycoides* Dc7-1, Lane 7: *B. mycoides* Rm5-2, Lane 8: *B. cereus* Rc5-2, Lane 9: *B. cereus* Dc10-1, Lane 10: *B. cereus* Dc10-2.

Results of Table (2) showed that all the diarrheal enterotoxin producing *B. cereus* strains showed the presence of groEL gene but CER and CES genes were not present in any of the *B. cereus* strains tested in this study. Results also, clearly indicated that the 3 *B. cereus* strains out of 6 strains from Domiati cheese (one strain), namely *B. cereus* Dc8-1 and Ras cheese (2 strains), namely *B. cereus* Rc5-1 and *B. cereus* Rc6-1 gave positive with groEL gene only and gave negative with CER and CES genes.

Table 2. PCR assays of emetic and diarrheal enterotoxins producing by *B. cereus* strains.

Strains	Sources	Enterotoxin producing *B. cereus* specific PCR primers			Emetic toxin producing	Diarrheal toxin producing
		CER	CES	groEL		
B. cereus Dc8-1	Domiati cheese	-	-	+	-	+
B. cereus Rc5-1	Ras cheese	-	-	+	-	+
B. cereus Rc6-1	Ras cheese	-	-	+	-	+
B. cereus Rc5-2	Ras cheese	-	-	-	-	-
B. cereus Dc10-1	Domiati cheese	-	-	-	-	-
B. cereus Dc10-2	Domiati cheese	-	-	-	-	-
No. of positive producing toxin strains					0	3

It could be extracted from Table (3) that, all the diarrheal enterotoxin producing *B. mycoides* strains showed the presence of groEL gene but CER and CES genes were not present in any of the *B. mycoides* strains tested in this study. Results also, indicated that the 2 *B. mycoides* strains out of 4 strains from Ras cheese (one strain), namely *B. mycoides* Rc2-1 and Domiati cheese (one strain), namely *B. mycoides* Dc7-1 gave positive with groEL gene only and gave negative with CER and CES genes.

Table (4) also, shows that 50% of tested *B. cereus* strains from both Ras cheese and Domiati cheese samples were toxigenic and showed the presence of groEL gene but CER and CES genes were not present in any of the *B. cereus* strains tested. The same behavior was noticed with 50% of tested *B. mycoides* strains which showed the presence of groEL gene but CER and CES genes were not present in any of the *B. mycoides* strains tested

CONCLUSION

The obtained results in this study revealed that the tested strains of B. cereus and B. mycoides showed the presence of groEL genes, while CER and CES genes were completely absent. B. cereus group had an ability to produce diarrheal enterotoxin, but the tested strains of all B. cereus and B. mycoides had a limited ability to produce emetic toxin.

Table 3. PCR assays of emetic and diarrheal enterotoxins producing by *B. mycoides* strains.

Strains	Sources	Enterotoxin producing *B. mycoides* specific PCR primers			Emetic toxin producing	Diarrheal toxin producing
		CER	CES	groEL		
B. mycoides Dc1-1	Domiati cheese	-	-	-	-	-
B. mycoides Rc2-1	Ras cheese	-	-	+	-	+
B. mycoides Dc7-1	Domiati cheese	-	-	+	-	+
B. mycoides Rm5-2	Raw milk	-	-	-	-	-
No. of positive producing toxin strains					0	2

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Table 4. Enterotoxin genes in *B. cereus* and *B. mycoides* strains.

Primers	Genes	Percentage positive in *B. cereus* (No=6)	Percentage positive in *B. mycoides* (No=4)
CER F-5 CER R-5	CER	0	0
CES F-10 CES R-10	CES	0	0
groEL F-1 groEL R-1	groEL	50%	50%

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استخدام تقنية الـ PCR كمقارنة حديثة في الكشف عن التوكسينات

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الملخص العربي

تعتبر بكتيريا مجموعة cereus أحد أهم المشكلات الواردة، لأنها يمكن أن تلوث العديد من منتجات الألبان. في هذه الدراسة، تم اختيار ثلاثة أنواع من B. cereus بإستخدام Triple-primer PCR. تم تخليق Triple-primer PCR في هذه الدراسة بإستخدام primerللكشف عن نوعين مختلفين من مجموعة جينات CER، CES، groEL للكشف عن السلالات المنتجة لسم القيء، primer متخصص واحد للكشف عن سم الإسهال (فقط groEL). دلت النتائج على أن كل سلالات B. cereus المنتجة لسم الإسهال أظهرت وجود جين groEL لكن جينات CER ، CESلم تكن موجودة في سلالات B. cereus المختبرة. قد أظهرت النتائج بوضوح أنه من بين 6 سلالات B. cereus المختبرة لإنتاج سم الإسهال والقيء بإستخدام تقنيةTriple-primer PCR وجد أن 3 سلالات كانت لديها القدرة على إنتاج السم المسبب للإسهال، من ناحية آخرى وجد أن كل 6 سلالات B. cereus لم يكن لديهم القدرة على إنتاج السم المسبب للقيء. أيضًا، لقد أظهرت النتائج أن كل سلالات B. mycoides المنتجة لسم الإسهال أظهرت وجود جين groEL لكن جينات CERو CES لم تكن موجودة في سلالات B. mycoides المختبرة. ونستنتج من هذا أنه من بين 4 سلالات B. mycoides مختبرة لإنتاج سم الإسهال والقيء بإستخدام تقنية-Triple primer PCRوجد أن اثنتين من السلالات كانت لديها القدرة على إنتاج السم المسبب للإسهال، من ناحية أخرى كل أربع سلالات B. mycoides لم يكن لديهم القدرة على إنتاج السم المسبب للقيء.

الكلمات المفتاحية: البكتيريا الهوائية، منتجات الألبان، المجموعة B. cereus، إنتاج السم المعوي.

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