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. pseudomycoideoides 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 microwave 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 driven Taq DNA polymerase, dNTPs, MgCl₂ 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.

**Table 1.** PCR primers and annealing temperature for the detection of emetic and diarrheal toxin producing *B. cereus* group.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5'-3')</th>
<th>AT</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CER F-5</td>
<td>CAAGTCAAGATAAGAGGCTTC</td>
<td>54°C</td>
<td>370</td>
<td>Kim et al. (2013).</td>
</tr>
<tr>
<td>CER R-5</td>
<td>AAAGCTCTTGCCAAAATAACC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CES F-10</td>
<td>GCATTTCGTGAAGCAGAGGT</td>
<td>54°C</td>
<td>699</td>
<td>Kim et al. (2013).</td>
</tr>
<tr>
<td>CES R-10</td>
<td>CCCITTATCCCCCTCGATGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>groEL F-1</td>
<td>AGCTATGATTCGTGAAGGTT</td>
<td>54°C</td>
<td>236</td>
<td>Kim et al. (2013).</td>
</tr>
<tr>
<td>groELR-1</td>
<td>AAGTAATAACGCCGGTCGT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 MgCl₂, 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.
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.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Sources</th>
<th>Enterotoxin producing <em>B. cereus</em> specific PCR primers</th>
<th>Emetic toxin producing</th>
<th>Diarrheal toxin producing</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. cereus</em> Dc8-1</td>
<td>Domiati cheese</td>
<td>- - +</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>B. cereus</em> Rc5-1</td>
<td>Ras cheese</td>
<td>- - +</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>B. cereus</em> Rc6-1</td>
<td>Ras cheese</td>
<td>- - +</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>B. cereus</em> Rc5-2</td>
<td>Ras cheese</td>
<td>- - -</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>B. cereus</em> Dc10-1</td>
<td>Domiati cheese</td>
<td>- - -</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>B. cereus</em> Dc10-2</td>
<td>Domiati cheese</td>
<td>- - -</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>No. of positive producing toxin strains</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>3</strong></td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Sources</th>
<th>Enterotoxin producing <em>B. mycoides</em> specific PCR primers</th>
<th>Emetic toxin producing</th>
<th>Diarrheal toxin producing</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. mycoides</em> Dc1-1</td>
<td>Domiati cheese</td>
<td>- - -</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>B. mycoides</em> Rc2-1</td>
<td>Ras cheese</td>
<td>- - +</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>B. mycoides</em> Dc7-1</td>
<td>Domiati cheese</td>
<td>- - +</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>B. mycoides</em> Rm5-2</td>
<td>Raw milk</td>
<td>- - -</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>No. of positive producing toxin strains</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>2</strong></td>
</tr>
<tr>
<td>Primers</td>
<td>Genes</td>
<td>Percentage positive in B. cereus (No=6)</td>
<td>Percentage positive in B. mycoides (No=4)</td>
<td></td>
</tr>
<tr>
<td>---------</td>
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<td>----------------------------------------</td>
<td>------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>CER F-5</td>
<td>CER</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>CER R-5</td>
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</tr>
<tr>
<td>CES F-10</td>
<td>CES</td>
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<td>0</td>
<td></td>
</tr>
<tr>
<td>CES R-10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>groEL F-1</td>
<td>groEL</td>
<td>50%</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>groEL R-1</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

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

REFERENCES


استخدام تقنية PCR كتقنية حديثة في الكشف عن التوكسينات

خاطر عبد الفتاح أحمد خاطر، شريف عادل سعد
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الملخص العربي
تعتبر بكتيريا مجموعة B. cereus أحد أهم المشكلات الراهنة، لأنها يمكن أن تلوث العديد من منتجات الألبان. في هذه الدراسة، تم اختيار ثلاثة أنواع من primer للكشف عن نوعين مختلفين من مجموعة B. cereus باستخدام Triple-primer PCR. تم تخليق primer متخصص واحد للكشف عن سم السم القيء (groEL فقط). نجح primer المتخصص في الكشف عن السلالت المنتجة لسم السم القيء، ولكن primer المتخصص لم يكن قادرًا على الكشف عن سلالت B. mycoides المنتجة لسم السم القيء.

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