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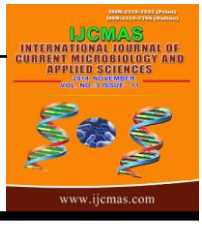


**Original Research Article**

# Detection of *hbl, nhe* and *bceT* Toxin Genes in *Bacillus cereus* Isolates by Multiplex PCR

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**A B S T R A C T**

**Keywords**

*B. cereus*, toxin genes, PCR, milk

Polymerase chain reaction (PCR) was used to detect the presence of hemolytic (*hblA*, *hblC* and *hblD*) and non-hemolytic enterotoxin (*nheA*, *nheB* and *nheC*) genes in *Bcillus cereus* strains isolated from milk and milk product collected from local markets. The study revealed that the percentage of these genes in *B. cereus* isolates were 22.58, 51.61, 9.67, 90.32, 58.06 and 54.83% respectively. Enterotoxin (*bceT*) gene was not detected in the studied isolates.

## Introduction

*Bacillus cereus* is Gram positive, spore-forming, motile, aerobic rod, commonly found in soil, water and food. The organism is isolated from various foods, including cooked rice, dairy products, eggs, meat and spices (Kramer & Gilbert, 1989; Ombui *et al.,* 2008); and from pharmaceutical products (Gracia-Arribas *et al.,* 1988). *B. cereus* occurred ubiquitously in soil and in many raw and processed foods such as rice, milk and dairy products, spices and vegetables (Christiansson *et al.,* 1999; Carlin *et al.,* 2000; Sarrías *et al.,* 2002; Guinebretier *et al.,* 2003). Although it is a Gram-positive bacterium, its Gram property can be changed in a stationary phase of growth. *Bacillus* cultures are Gram-positive when they are young, but may become Gram-negative as they grow old (Anderson - Borge *et al.,* 2001; Shaheen *et al.,* 2009).

The enterotoxins produced by *Bacillus cereus* were: haemolysin BL (HBL), non-haemolytic enterotoxin (NHE), enterotoxin-T and cytotoxin-K (Granum, 1994; Agata *et al.,* 1995; Granum & Lund, 1997; Lund *et al.,* 2000). Three of these (HBL, NHE, and CytK) are related to outbreaks of food borne disease; enterotoxin-T has been classified as enterotoxin on the basis of genetic and structural relationship with bacterial enterotoxins (Agata *et al.,* 1995). Hemolysin BL is a haemolysin consisting of three proteinaceous subunits: B, L1 and L2, a binding factor and two "lytic" factors, respectively. These three subunits have been purified and characterized. The toxin shows dermonecrotic activity as well as activity towards vascular permeability, and causes fluid accumulation in ligated rabbit ileal loops (Granum & Lund, 1997). All three

components are necessary for maximal enterotoxic activity (Beecher *et al.,* 1995b). The genes *hblC* ($L_2$), *hblD* ($L_1$) and *hblA* (B) are arranged in tandem in an operon (Ryan *et al.,* 1997). Granum *et al.,* (1996) concluded that there was at least one enterotoxin in addition to HBL, implicated in the enterotoxin included illness.

Non – Hemolytic Enterotoxin like HBL, consists of three proteinaceous subunits as well: *nheA*, *nheB* and *nheC*, two lytic factors and a binding factor, respectively (Lund & Granum, 1996). Although binary combinations of the subunits show some biological effect, maximal activity is achieved when all three components are present (Lund & Granum, 1997). A single gene codes for each subunit, and the genes of all three subunits are grouped in one operon (Granum *et al.,* 1999). There is substantial similarity between the proteins of the haemolytic and the non-haemolytic enterotoxin (Granum *et al.,* 1999). The *hbl* operon is located in the variable region, and the *nhe* operon is in the constant portion of the *B. cereus* chromosome (Carlson *et al.,* 1996). Polymerase chain reaction assay, as the first nucleic-based assay for detection pathogens, is sensitive, specific and can detect minimum amounts of bacterial DNA in a sample (Leonard *et al.,* 2003; Palchetti & Mascini, 2008). The study was designated for detection the *hbl*, *nhe* and *bceT* toxin genes in *B. cereus* isolates by polymerase chain reaction (PCR).

## Materials and Methods

**Bacterial strains:** Fifty three *B. cereus* isolates were isolated previously from 227 milk and milk products samples (milk 32.7 %, soft cheese 16.66 %, curls cheese 18.00 % and yogurt 26.00 %). Out of fifty three *B. cereus* isolates thirty one isolates were tested for PCR.

**DNA extraction:** Genomic DNA extraction kit (Geneiad / Korea) was used for bacterial DNA extraction. Bacterial cells were cultured in brain heart infusion broth (oxoid). DNA samples were stored at -20°C until used. The purified DNA was detected by electrophoresis in 0.8 % agarose gel with addition of ethedium bromide. Bromophenol blue stain were added to the DNA sample and visualized by U. V. light.

### Detection of *hbl* , *nhe* and *bceT* toxin gene using PCR

Hemolysine, non-hemolytic enterotoxin and enterotoxin T genes (Table 1) were studied according to protocol of (Guinebretière *et al.*,2002; Hansen and Hendriksen, 2001). The PCR amplification mixture (20 µl) which was used for the detection included AccuPower mix, 5 µl of template DNA, 1 µl for forward and reverse of each oligonucleotide primers.

The volume was completed by sterile free water (BioNeer) to 20 µl, and the vortex was used to mix them well. The PCR tubes were transferred to the thermalcycler to start the amplification reaction according to specific program. The cycling conditions for multiplex PCR for detection *hbl, nhe* and *bceT* toxin genes were initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation step at 94°C for 15 min, annealing step at 60°C for 45 min, extension step at 72°C for 2 min, and final extension step at 72°C for 5 min. PCR products were detected in 1.5 % agarose gel stained with ethidium bromide, viewed by U.V. light.

## Results and Discussion

### Detection of ( *hbl, nhe* and *bce T* ) genes by multiplex PCR

*B. cereus* isolated from milk, yogurt, soft

cheese and curls cheese were tested for various enterotoxin genes, *hblA, hblC, hblD, nheA, nheB, nheC* and *bceT* by multiplex PCR (Table 2-5, Figure 1). The genes were investigated in 11 isolates. *nheA gene* had the highest percentage 100.00 % followed by *nheB gene* in 63.63 %, then *nheC* and *hblC* genes were found in 54.54 % for each one. *hblA* and *hblD* had 9.09 % for each one (Table 2).

**Detection of *hbl, nhe,* and *bceT* genes in *Bacillus cereus* isolates from soft cheese**

The genes were investigated in 5 isolates. *nheA gene* had the highest percentage 80.00 % followed by *nheB, nheC, hblC* genes were found in 60, 40 and 20% respectively (Table 3).

**Detection of *hbl, nhe,* and *bceT* genes in *Bacillus cereus* isolates from curls cheese**

The genes were investigated in 5 isolates. *nheA gene* had the highest percentage followed by *hblC* in 100% and 60%, respectively. The genes *nheC* and *hblA* were found in 40% for each one ( Table 4).

**Detection of *hbl, nhe,* and *bceT* genes in *Bacillus cereus* isolates from yogurt**

The genes were investigated in 10 isolates. *nheA gene* had the highest percentage 80% followed by *nheB* and *nheC* genes 70% both ( Table 5 ).

**The occurrence of enterotoxins genes in *Bacillus cereus* isolated from milk and milk products**

The occurrence of the individual enterotoxin and emetic toxin genes in *B. cereus* isolated from milk, yogurt, soft cheese and curls cheese is shown in (Table.6). High prevalence of both *nheA* was detected in 90.32 %. Whereas low prevalence ( 22.58 and 9.67), respectively were observed for *hblA* and *hblD*. Where as the gene *bceT* was not detected.

Polymerase chain reaction assay has been reported recently used for rapid detection and discrimination of enterotoxins genes in *Bacillus cereus* (Guinebretiere *et al.,* 2006 and Nagmwongatit *et al.,* 2008); and for direct detection of food contamination with enterotoxigenic *B. cereus* as well (Ombui *et al.,* 2008). In this study, seven *B. cereus* enterotoxin genes were detected by developing multiplex PCR.

In this study, the *hblA* gene was found in milk and cheese in the percentage 9.09 % for milk and 40.00 % for soft cheese, but this gene did not find in curls cheese. The *hblC* gene was found in milk and cheese in the percentage 54.54 % for milk and 20.00 , 60.00 % for soft cheese and curls cheese, respectively. The gene *hblD* was found in milk at 9.09 % and in soft cheese 20.00 % and did not find in curls cheese. The *nheA* was found in milk at 100.00 % and in soft cheese and curls cheese in 80.00 % and 100.00 % , respectively. The gene *nheB* was found in milk, soft cheese and curls cheese in a percentage 63.63 %, 60.00 % and 20.00 %, respectively. The gene *nheC* was found at the percentage 54.54 % in milk and found in soft cheese and curls cheese at 40.00 % for each one.

Gitahi *et al*., (2009) found the occurrence of *nheA* and *nheC* genes in isolates from both milk and cheese was 3.9 %. The occurrence of *nheB* was 19.5 %, 11.8 % and 59 % in isolates from milk, cheese and rice. The prevalence of *hblA* gene was 9.8 %, 11.8 % and 15.7 % among isolates from milk, cheese and rice respectively, also found that 13.7 %, 7.8 % and 29.4 % of isolates from respective foods had *hblC* gene. The

occurrence of *hblD* gene in both milk and cheese isolates was 11.8 %, while the prevalence in rice isolates was 27.4 %.

In this study, the occurence of the enterotoxins *hblA* and *hblC* were detected in 22.58 %, 51.61 %, respectively. This value is approximately lower than those previously reported from other countries (50 % - 86 %) using similar methods (Mäntynen & Lindstrom, 1998; Hansen & Hendriksen, 2001; Al-Khatib *et al*., 2007). I think the differences in result related to differences in regions, the season of the collection.

The occurrence of *bceT* gene in this study is not detected. The result of present study is similar to the study of Gitahi *et al*., (2009) in Kenya. However we found that the *bceT* gene seems to be relatively rare and could not be found in the enterotoxic strains used in this study. This is contrast to the results of (Agata *et al*., 1995; Ombui *et al*., 1997; Hsieh *et al*., 1999), who were reported that the *bceT* gene was widely distributed among the isolates at 47 % to 41 %. The *bceT* gene sequence has been shown to vary among the isolates (Hansen & Hendriksen, 2001).

**Table.1** Oligonucleotide primers sequences used in this study

| Target gene | Oligonucleotide sequence (5'–3') | Product size (bp) | Reference |
|---|---|---|---|
| ***hblA*** | **F:** AAG CAA TGG AAT ACA ATG GG<br>**R:** AGA ATC TAA ATC ATG CCA CTG C | 1154 | 21 |
| ***hblC*** | **F:** GAT AC(T,C) AAT GTG GCA ACT GC<br>**R:** TTG AGA CTG CTC G(T,C)T AGT TG | 740 | 21 |
| ***hblD*** | **F:** ACC GGT AAC ACT ATT CAT GC<br>**R:** GAG TCC ATA TGC TTA GAT GC | 829 | 21 |
| ***nheA*** | **F:** TAC GCT AAG GAG GGG CA<br>**R:** GTT TTT ATT GCT TCA TCG GCT | 499 | 22 |
| ***nheB*** | **F:** CTA TCA GCA CTT ATG GCA G<br>**R:** ACT CCT AGC GGT GTT CC | 769 | 22 |
| ***nheC*** | **F:** CGG TAG TGA TTG CTG GG<br>**R:** CAG CAT TCG TAC TTG CCA A | 581 | 22 |
| ***bceT*** | **F:** CGT ATC GGT CGT TCA CTC GG<br>**R:** TTT CTT TCC CGC TTG CCT TT | 924 | 22 |

**Table.2** Detection of *hbl, nhe,* and *bceT* genes in *Bacillus cereus* isolates from milk samples

| Gene | | No. of tested isolates = 11 | |
|---|---|---|---|
| | | **PCR positive** | **%** |
| ***Hbl*** | ***hblA*** | 1 | 9.09 |
| | ***hblC*** | 6 | 54.54 |
| | ***hblD*** | 1 | 9.09 |
| ***Nhe*** | ***nheA*** | 11 | 100.00 |
| | ***nheB*** | 7 | 63.63 |
| | ***nheC*** | 6 | 54.54 |
| ***bceT*** | | 0 | 0.00 |

**Table.3** Detection of *hbl, nhe,* and *bceT* genes in *Bacillus cereus* isolates from soft cheese samples

| Genes | | No. of tested isolates = 5 | |
|---|---|---|---|
| | | PCR positive | % |
| *Hbl* | *hblA* | 0 | 0.00 |
| | *hblC* | 1 | 20.00 |
| | *hblD* | 0 | 0.00 |
| *Nhe* | *nheA* | 4 | 80.00 |
| | *nheB* | 3 | 60.00 |
| | *nheC* | 2 | 40.00 |
| *bceT* | | 0 | 0.00 |

**Table.4** Detection of (*hbl, nhe,* and *bceT*) genes in *Bacillus cereus* isolated from curls cheese samples

| Genes | | No. of tested isolates = 5 | |
|---|---|---|---|
| | | PCR positive | % |
| *Hbl* | *hblA* | 2 | 40.00 |
| | *hblC* | 3 | 60.00 |
| | *hblD* | 1 | 20.00 |
| *Nhe* | *nheA* | 5 | 100.00 |
| | *nheB* | 1 | 20.00 |
| | *nheC* | 2 | 40.00 |
| *bceT* | | 0 | 0.00 |

**Table.5** Detection of *hbl, nhe,* and *bceT* genes in *Bacillus cereus* isolates from yogurt samples

| Genes | | No. of tested samples = 10 | |
|---|---|---|---|
| | | PCR positive | % |
| *Hbl* | *hblA* | 4 | 40.00 |
| | *hblC* | 6 | 60.00 |
| | *hblD* | 1 | 10.00 |
| *Nhe* | *nheA* | 8 | 80.00 |
| | *nheB* | 7 | 70.00 |
| | *nheC* | 7 | 70.00 |
| *bceT* | | 0 | 0.00 |

**Table.6** The occurrence of enterotoxin and emetic toxins genes in *B. cereus* isolated from milk and milk products

| Sample (No. of *B. cereus* isolates) | *hblA* No. + (%) | *hblC* No. + (%) | *hblD* No. + (%) | *nheA* No. + (%) | *nheB* No. + (%) | *nheC* No. + (%) | *bceT* No. + (%) |
|---|---|---|---|---|---|---|---|
| **Milk (11)** | 1 (9.09) | 6 (54.54) | 1(9.09) | 11 (100) | 7 (63.63) | 6 (54.54) | 0 (0.00 ) |
| **Yogurt (10)** | 4 (40.00) | 6 (60.00) | 1 (10.00) | 8 (80.00) | 7 (70.00) | 7 (70.00) | 0(0.00 ) |
| **Soft cheese(5)** | 0 (0.00 ) | 1 (20.00) | 0(0.00 ) | 4 (80.00) | 3 (60.00) | 2 (40.00) | 0 (0.00 ) |
| **Curls cheese(5)** | 2 (40.00) | 3 (60.00) | 1 (20.00) | 5 (100.00) | 1 (20.00) | 2 (40.00) | 0 (0.00 ) |
| **Total (31) (%)** | 7 (22.58) | 16 (51.61) | 3 (9.67) | 28 (90.32) | 18 (58.06) | 17 (54.83) | 0 (0.00 ) |

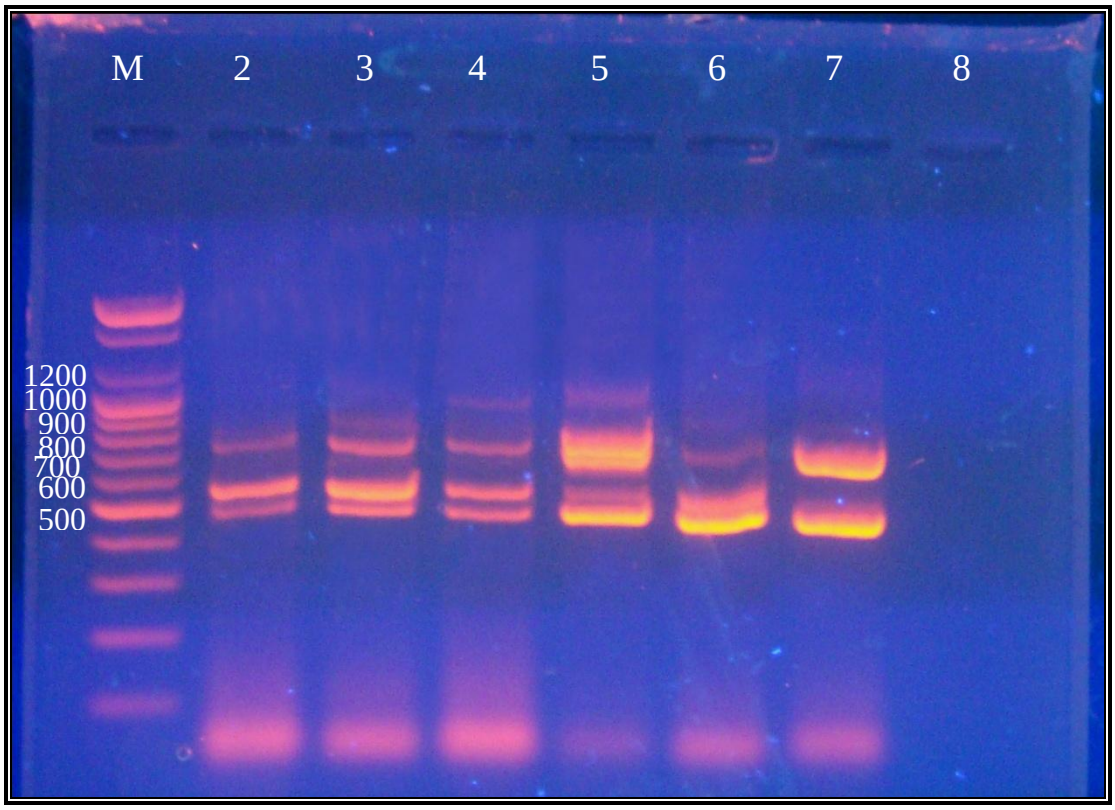


**Figure.1** PCR amplification of *hblACD* and *nheABC* genes of *Bacillus cereus* isolates isolated from milk and milk products

Lane 1 (M)= Molecular size marker.
Lane 2 = *nheA*, *nheC* and *hblC* ( 499, 581 and 740) bp, respectively.
Lane 3 = *nheA*, *nheC*, *nheB* and *hblD* (499, 581, 769 and 829) bp, respectively.
Lane 4 = *nheA*, *nheC*, *hblC* and *hblA* (499, 581, 740, 1154) bp, respectively.
Lane 5 = *nheA*, *nheC*, *nheB*, *hblC* and *hblA* (499, 581, 740, 769, 1154) bp, respectively .
Lane 6 = *nheA*, *nheC* and *nheB* (499, 581, 769) bp, respectively.
Lane 7 = *nheA* and *hblC* (499 and 740) bp, respectively.
Lane 8 = control negative.

In this study, *nheA* gene was the most common gene, it's detected in 90.32 % of the isolates. The *hblD* gene was the least common gene detected in 9.67 % of the isolates. The *nheB* and *nheC* gene were detected in 58.06 % and 54.83 %, respectively.

The results were approximately similar to Guinebretiere *et al*., (2002), who found that 63 % and 36 % of food borne *B. cereus* isolates to lack one or two of the *nhe* and *hbl* genes, respectively, when amplified by PCR, through all the genes were demonstrated by Southern blotting and most of the isolates tested positive for enterotoxins using immunoassay, Oxoid kit for the *hbl* genes and Tecra kit for *nhe* genes (Guinebretiere *et al*., 2002). Granum, (2001) found the *nhe* operon is present in 100 % of *B. cereus* isolates, while the *hbl* operon was present in 50 % of *B. cereus* isolates. However, (Cardazzo *et al*., 2008) reported that 3 and 4 out of 47 new *B. cereus* isolated from industrial dairy products, egg product and farm dairy

product without one or two of the *nhe* and *hbl* gene complex, respectively.

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