Detection of *Bacillus cereus* genes responsible for diarrheal and emetic toxins

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**Abstract.** *Bacillus cereus* isolated from different food sources. The diarrheal toxin genes such as cytK, hemolytic enterotoxin (hbla, hblC and hblD), non-hemolytic enterotoxin (nheA, nheB and nheC), bceT and entFM in addition to emetic toxin gene were detected by PCR. The cytK gene was observed in 94.87% of the isolates. entFM and emetic toxin gene were found very rare in all food samples at the percentage 2.56% and 7.69% respectively. Uncooked rice which has a highest number of bacterial isolation, also showed relatively high percentage of the cytK and bceT genes (90%). These two genes present in 100% of *Bacillus cereus* isolates in most food samples. Bacteria isolated from burger meat contain all investigated genes.

**Keywords.** *Bacillus cereus*, diarrheal toxin, emetic toxin.

1. Introduction

*Bacillus cereus* is a Gram-positive, facultative anaerobic, spore-producing, motile, bacterium in the form of a rod. Enterotoxins produced by bacteria in intestine can cause diarrhea. \(10^5\)–\(10^7\) cells or spores if ingested with food, heat labile toxins are produced. Incubation period of this bacterium is range between 8-16 hours. The sign of toxicity of *B. cereus* toxins appears as nausea, vomiting, diarrhea and abdominal pain [1, 2]. Three toxins produced during the vegetative cell growth of *B. cereus* in the intestine that caused diarrheal syndrome, Cyt K, NHE and HBL [4, 5, 6]. The first enterotoxin-hemolysin BL (HBL) was discovered by [7]. NHE toxin on three molecular types A, B, and C. Bacteria need these proteins for cytotoxicity of multi-component enterotoxins [5]. The HBL complex is encoded by genes on a single operon. The B, L1 and L2 proteins are encoded by the hbla, hblD and hblC genes [8, 9]. NHE toxin is a major cause of cytotoxicity in *B. cereus* [10]. The emetic disease is caused by a toxin produced by *B. cereus* called cereulide. This toxin showed stability against heat, acid, and trypsin. Several studies have been done previously for bacterial food contamination [11, 12 13, 14, 15]. This study was done to investigate the *B. cereus* that responsible for food poisoning by detecting the presence of diarrheal and emetic toxin genes.
2. Materials and Methods

2.1. Bacterial strains

*Bacillus cereus* isolates from different food sources were previously described [16]. The DNA of all isolates was extracted by genomic DNA kit (company and origin). The identification of the isolates was done by DNA sequencing using 16S rRNA gene [16].

2.2. Detection of diarrheal and emetic toxin genes by polymerase chain reaction

The primers used in this study for the detection of diarrheal and emetic toxin genes with the anticipated size of the amplified product were listed in Table 1.

| Genes | Primers 5’-3’ | Program cycling | Product (bp) | References |
|-------|---------------|-----------------|--------------|------------|
| entFM | F: ATGAAAAAA GTA ATT TGC AGG<br>R: CGT GCA TCT GTT TCA TGAAA | Initial denaturation 94°C 5min, denaturation 94°C 30sec, annealing 50°C 45 sec, extension 72°C 45 sec , final extension 72°C 5 min , 35 cycles | 1269 | [17] |
| hblA  | F: AAG CAA TGG AAT ACA ATG GG<br>R: AGA ATC TAA ATCATGCCA CGT C | Initial denaturation 95°C 15min, denaturation 95°C 30sec, annealing 60°C 30 sec, extension 72°C 1min, final extension 72°C 5 min , 40 cycles | 1154 | [18] |
| hblC  | F: GAT AC(T,C) AAT GTG GCA ACT GC<br>R: TTG AGA CTG CTC G(T,C)T AGT TG | Initial denaturation 94°C 2min, denaturation 94°C 15sec, annealing 55°C 45 sec, extension 72°C 2min, final extension 72°C 5 min , 35 cycles | 740 | [18] |
| hblD  | F: ACC GGT AAC ACT CAT GC<br>R: GAG TCC ATA TGC TTA GATGC | Initial denaturation 94°C 2min, denaturation 94°C 15sec, annealing 55°C 45 sec, extension 72°C 2min, final extension 72°C 5 min , 35 cycles | 829 | [18] |
| nheA  | F: TAC GCT AAG GAG GGG CA<br>R: GTT TTT ATT GCT TCA TCG GCT | Initial denaturation 94°C 2min, denaturation 94°C 15sec, annealing 55°C 45 sec, extension 72°C 2min, final extension 72°C 5 min , 35 cycles | 499 | [19] |
| nheB  | F: CTA TCA GCA CTT ATG GCA G<br>R: ACT CCT ACG GGT GTT CC | Initial denaturation 94°C 2min, denaturation 94°C 15sec, annealing 55°C 45 sec, extension 72°C 2min, final extension 72°C 5 min , 35 cycles | 769 | [19] |
| nheC  | F: CGG TAG TGA TTG CTG GG<br>R: CAG CAT TCG TAC TTG CCA A | Initial denaturation 94°C 2min, denaturation 94°C 15sec, annealing 55°C 45 sec, extension 72°C 2min, final extension 72°C 5 min , 35 cycles | 581 | [19] |
| bceT  | F: CGT ATC GGT CGT TCA CTC GG<br>R: TTT CTT TCC CGC TTG CCT TT | Initial denaturation 94°C 2min, denaturation 94°C 15sec, annealing 56°C 45 sec, extension 72°C 2min, final extension 72°C 5 min , 35 cycles | 924 | [19] |
| cytK  | F: CGA CGT CAC AAG TTG TAA CA<br>R: CGT GTG TAA ATA CCC CAG TT | Initial denaturation 94°C 1min, denaturation 94°C 45sec, annealing 54°C 1 min, extension 72°C 2min, final extension 72°C 5 min , 35 cycles | 565 | [20] |
| Emetic Toxin gene | F: GAC AAG AGA AAT TTC TAC GAG CAA GTA CAA T<br>R: GCA GCC TTC CAA TTA CTC CTT CGC CCA CAG T | Initial denaturation 95°C 15min, denaturation 95°C 30sec, annealing 60°C 30 sec, extension 72°C 1min, final extension 72°C 5 min , 40 cycles. | 635 | [21] |
3. Results

3.1. Detection of diarrheal toxin genes

3.1.1. Detection of cyt K gene

cyt K gene was found in 94.87% of the isolated bacteria. No significant differences (P > 0.05) in rate of detection of this gene in the investigated samples (Table 2, Figure 1).

Figure 1. PCR amplification of cytK gene of Bacillus cereus isolates. M1 = Ladder; 1-4 & 6 = cyt K gene approximately 565 bp.; 5 = negative for cyt K gene; 7 = control negative.

3.1.2. Detection of (hbl and nhe) genes by multiplex PCR

Bacillus cereus isolated from different food were tested for presence of genes code for enterotoxin such as hblA, hblC, hblD, nheA, nheB, and nheC by multiplex PCR (Table 2, Figure 2). In cream isolates, the genes were detected in 6 isolates. hblC gene had the highest percentage 100.00 % followed by hblA, hblD, nheA, nheB and nheC genes were found in 50.00, 16.66, 50.00, 66.66 and 50.00%, respectively. In beef isolates, the genes were detected in 6 isolates. hblC gene had the highest percentage 66.66 % followed by nheB 50% and hblD, nheA and nheC genes were found as 33.33% for each one. The gene hblA was not found. In frozen beef isolates, the genes were detected in 5 isolates. hblC and nheB genes had the highest percentage 60.00 % followed by nheA and nheC genes were found in 40.00 %. The lowest percentage was found in hblA gene. The hblD gene was not found in all of the isolates. In burger isolates, the genes were detected in 7 isolates. hblC and nheB genes had the highest percentage 57.14 % followed by hblD, nheC genes were found in 42.85%. The lowest percentage was found in hblA and nheA in 28.57%. In cooked rice isolates, the genes were detected in 5 isolates. hblC, hblD and nheC genes had the highest percentage 40.00 % followed by hblA, nheA and nheB genes were found in 20.00 % for each one. In uncooked rice isolates, the genes was detected in 10 isolates. hblC, hblD and nheC genes had the highest percentage 40.00 % followed by hblA, nheA and nheB genes were found in 20.00 % for each one.
3.1.3. Detection of bce T gene

The bce T gene was detected in 37 isolates (94.87%) out of 39 isolates. The high rate (100%) found in beef, burger, frozen beef, and cooked rice. The lowest percentage was found in cream 83.33%. Results showed differ significantly (P < 0.05) in the detection of this gene in tested samples (Table 2, Figure 3).
3.1.4. Detection of enterotoxin (ent FM) gene

The *entFM* gene was detected in 1 isolate (2.5%) out of 39 isolates, it was found in burger 16.66%. The difference is non-significant (P > 0.05) in the detection of this gene in the tested samples (Table 2, Figure 4).

![Figure 4. Detection of enterotoxin ent FM gene. M1, M2=Ladder; Lane 2=positive for ent (FM) gene approximately 1269 bp.](image)

3.1.5. Detection of emetic toxin gene in Bacillus cereus

The emetic gene was detected in 3 isolates (7.6 %), distributed in cream and frozen beef and burger in 16.66, 20 and 14.28 %, respectively but was not detected in beef, cooked rice, and uncooked rice. The differences is non-significant found in the detection of emetic gene in the tested samples at (P > 0.05), (Table 2, Figure 5).

![Figure 5. PCR Amplification of emetic toxin gene. M= Ladder; 2 positive for emetic gene approximately 635 bp.; 1 negative for emetic toxin gene; 3 control negative](image)
3.2. The occurrence of diarrheal and emetic toxins genes in Bacillus cereus isolates

The presence of diarrheal and emetic toxins genes in B. cereus isolated from food sources are shown in (Table 2).

Table 2. The occurrence of diarrheal enterotoxin and emetic toxins genes in Bacillus cereus isolates.

| Sample          | hblA No. (%) | hblC No. (%) | hblD No. (%) | nheA No. (%) | nheB No. (%) | nheC No. (%) | cytK No. (%) | bceT No. (%) | entFM No. (%) | Emetic toxin No. (%) |
|-----------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|----------------------|----------------------|
| Cream(6)        | 3 (50)        | 6 (100)       | 1 (16.66)     | 3 (66.66)     | 4 (50)        | 3 (83.33)     | 5 (83.33)     | 5 (83.33)     | 0 (0)               | 1 (16.66)             |
| Beef (6)        | 0 (0)         | 4 (66.66)     | 2 (33.33)     | 2 (33.33)     | 3 (50)        | 3 (100)       | 2 (100)       | 6 (100)       | 0 (0)               | 0 (0)                |
| Frozen beef (5) | 1 (20)        | 3 (60)        | 0 (0)         | 2 (40)        | 3 (60)        | 2 (100)       | 5 (100)       | 5 (0)         | 0 (0)               | (20)                 |
| Burger (7)      | 2 (20.87)     | 4 (57.14)     | 3 (42.85)     | 2 (57.14)     | 4 (42.85)     | 3 (100)       | 7 (100)       | 7 (14.28)     | 1 (14.28)           | 1 (14.28)            |
| Cooked rice (5) | 1 (20)        | 2 (40)        | 2 (40)        | 1 (20)        | 2 (40)        | 1 (100)       | 5 (100)       | 5 (0)         | 0 (0)               | (0)                  |
| Uncooked rice (10) | 2 (20)    | 7 (70)        | 5 (50)        | 2 (70)        | 6 (50)        | 5 (90)        | 9 (90)        | 9 (0)         | 0 (0)               | (5)                  |
| Total (39)      | 9 (23.07)     | 26 (66.6)     | 13 (33.3)     | 12 (30.7)     | 21 (53.8)     | 17 (43.5)     | 37 (94.87)    | 37 (94.87)    | 2 (2.5)             | 3 (7.6)              |
| (%)            | (23.07)       | (66.6)        | (33.3)        | (30.7)        | (53.8)        | (43.5)        | (94.87)       | (94.87)       | (2.5)               | (7.6)                |

4. Discussion

The Bacillus cereus spoilage depends on two main factors: bacterial concentration in dairy products and cytotoxicity of isolates [22]. Several studies reported isolates produced cytotoxins and caused spoilage of milk, while others reported that cytotoxin production was not required for spoilage. As noted earlier, several factors affected toxin production, including environment and temperature signals [22]. The cyt K gene was found in 37 isolates (94.87 %) out of 39 isolates. In cream sample, hblC gene had the highest percentage 100% while in beef samples, hblC gene had the highest percentage (66.66). On the other hand, the gene hblA was not found in beef. In frozen beef samples, hblC and nheB genes have the highest percentage of 60% (for each) followed by nheA and nheC genes which were found in 40% for each. In burger samples, each hblC and nheB genes had the highest percentage (57.14%) followed by hblD, nheC genes were found in 42.85% for each. The lowest percentage (28.57%) was found in each of hblA and nheA. In cooked rice samples, each of hblC, hblD and nheC genes had the highest percentage (40%) followed by each of hblA, nheA and nheB genes which were found in 20% of samples isolates. In uncooked rice samples, hblC, hblD and nheC genes had the highest percentage 40% for each, followed by hblA, nheA and nheB genes which were found in 20% for each one. [20, 23, 24, 25] detected the gene cytK in 50, 88, 70.40 and 65.98% of their isolates. [26] found the cytK gene in 27 (87.09%) out of 31 isolates. nhe, hbl, ceses, ctyK and cry genes also reported in the 92 isolates of B. cereus which were isolated in the previous study [16]. The rate is higher than the presence of nhe and hbl in B. cereus from Korea’s food [27]. The positive rates for nhe and hbl in twenty raw milk samples were higher than those found for fifty four milk samples in China. In the study of [28], the positive rates of nhe and hbl were 62.0% and 37.0%, respectively.[29] found that the hemolysin gene hblA, hblC and hblD found in in most isolates. Haemolytic enterotoxin gene was isolated from vegetables [30, 31]. While haemolytic enterotoxin gene of B. cereus not reported from milk [30, 32]. Enterotoxin gene bceT gene was detected in 37 isolates (94.87%). This agreed with other studies [33, 34]. Whereas [31] found that all isolates were negative for this gene. entFM gene was found in one bacterial isolate (2.5%) out of 39 isolates, it was found in burger. Other studies were indicated that this gene was specific for entotoxigenic B. cereus [17, 35, 36]. Whereas other workers noticed that the entFM gene was found in 27 (93 %) B. cereus isolates [37]. In this study, 3 isolates (7.6%) detected the emetic toxin gene. While [38] found the emetic gene at percentage of
41.6%. Analysis of PCR is quick and easy to identify foods suspected of causing enterotoxigenic Bacillus cereus food poisoning.

5. Conclusion

It can be concluded that most investigated food have Bacillus cereus bacteria and these bacteria harboring different types of genes that can be harmful for man when ingested with food. The genes are relatively highly occurred in studied samples especially cytK and beeT.

6. References

[1] Granum PE 1994 Bacillus cereus and its toxins J. App. Bacteriol. Symp. Suppl. 76 618.
[2] Arnesen LPS, Fagerlund A and Granum PE 2008 From soil to gut: Bacillus cereus and its food poisoning toxins FEMS Microbiol Rev. 32 579.
[3] Logan NA 2011 Bacillus and relatives in foodborne illness J. App. Microbiol. 112 417.
[4] Heinrichs J, Beecher D, MacMillan J and Zilinskas B 1993 Molecular cloning and characterization of the hblA gene encoding the B component of hemolysin BL from Bacillus cereus J. Bacteriol. 175 6760.
[5] Lund T and Granum PE 1997 Comparison of biological effect of the two different enterotoxin complexes isolated from three different strains of Bacillus cereus Microbiology 143 3329.
[6] Lund T, De Buyser ML and Granum PE 2000 A new cytotoxin from Bacillus cereus that may cause necrotic enteritis Mol. Microbiol. 38 254.
[7] Beecher D and MacMillan J 1991 Characterization of the components of hemolysin BL from Bacillus cereus Infect. Immun. 59 1778.
[8] Ryan PA, Macmillan JD and Zilinskas BA 1997 Molecular cloning and characterization of the genes encoding the L1 and L2 components of hemolysin BL from Bacillus cereus J. Bacteriol. 179 2551.
[9] Granum PE, O’Sullivan K and Lund T 1999 The sequence of the non-hemolytic enterotoxin operon from Bacillus cereus FEMS Microbiol. Lett. 177 225.
[10] Moravek M, Dietrich R, Buerk C, Broussole V, Guinebretiere M, Granum PE, Nguyen C and Maerlbauer E 2006 Determination of the toxin potential of Bacillus cereus isolates by quantitative enterotoxin analysis FEMS Microbiol. Lett. 257 293.
[11] Abbas BA and Jaber GM 2012 Occurrence of Listeria monocytogens in raw milk of ruminants in Basrah province Iraqi J. Vet. Sci. 26 47.
[12] Abbas BA, Ghadban MK and Alghanim AM 2017 Microbial evaluation of milk and milk products during a past two decades, in Basrah southern Iraq: A Review Ann. Rev. Biol. 14 1.
[13] Abbas BA, Khudaier BY and Amaal MK 2017 Studies on mecA gene in methicillin resistant Staphylococcus aureus isolates Jokull 67 58.
[14] Saeed BM, Al-Jadaa SA and Abbas BA 2019 Synthesis of a Novel 4, 4’-[1, 4-phenylenebis (1, 3, 4-thiadiazole-5, 2-diyl)] bis (azaneylylidene) bis (methaneylylidene) diphenol and Determination of Its pharmacological and antimicrobial Activities. In J. Physics: Conference Series (1279 012037). IOP Publishing.
[15] Saeed BM, Al-Jadaa SA and Abbas BA 2019 Pharmacological and Biological Evaluation of 5, 5’[(1, 4-Phenelene) bis (1, 3, 4-thiadiazol-2-amine)]. J. Physics: Conference Series (1279 012038). IOP Publishing.
[16] Saeed BMS, Abbas BA and Al-Jadaa SAN 2018 Molecular Detection of Tetracycline Resistance Genes in Bacillus cereus Isolated from Food Sources. Bas. J. Vet. Res. 17 223.
[17] Martinez-Blanch JF, Sanchez G, Garay F and Aznar R 2009 Development of real-time PCR assay for detection and quantification of enterotoxigenic members of Bacillus cereus group in food samples Int. J. Food Microb. 135 15.
[18] Guinebretiere MH, Broussolle V and Nguyen-The C 2002 Enterotoxigenic profiles of food-poisoning and food-borne Bacillus cereus strains J. Clin. Microbiol. 40 3053.

[19] Hansen BM and Hendriksen NB 2001 Detection of enterotoxin Bacillus cereus and Bacillus thuringiensis strains by PCR analysis Appl. Environ. Microbiol. 67 185.

[20] Ngaamwongsatit P, Buasri W, Pianariyanon P, Pulskirkan C, Ohba M, Assavanig A and Panbangred W 2008 Broad distribution of enterotoxins genes (hblCDA, nheABC, cytK and entFM) among Bacillus thuringiensis and Bacillus cereus as shown by novel primers Int. J. Food Microbiol. 121 352.

[21] Ehling-Schulz M, Fricker M and Scherer S 2004 Bacillus cereus, the causative agent of an emetic type of food-borne illness Mol. Nutr. Food Res. 48 479.

[22] Kohneshahr SM, Deilami Khibi Z, Ghasemian A, Reza Shapoury R, Javid Taghinejad J, Majid Eslami M and Heidarzadeh S 2016 Detection of hbla and bal genes in Bacillus cereus isolates from cheese samples using the polymerase chain reaction. Avicenna J. Clin Microb. Ininfec. 3 36033.

[23] Wijnands LM, Dufrenne JB, Rombouts FM, Veld PH and Leusden FM 2006 Prevalence of potentially pathogenic Bacillus cereus in food commodities in The Netherlands J. Food Prot. 69 2587.

[24] Chitov T, Dispan R and Kasinrerk W 2008 Incidence and diarrheagenic potential of Bacillus cereus in pasteurized milk and cereal products in Thailand J. Food Saf. 28 467.

[25] Rather MA, Aulakh RS, Gill JPS and Ghatak S 2012 Enterotoxin gene profile and antibiogram of Bacillus cereus strains isolated from raw meats and meat products J. Food Saf. 32 22.

[26] Khudor MH, Abbas BA and Saeed BMS 2012 Molecular detection of enterotoxin (cyt k) gene and antimicrobial susceptibility of Bacillus cereus isolates from milk and milk products Bas. J. Vet. Res. 11 164.

[27] Forghani F, Kim JB and Oh DH 2014 Enterotoxigenic profiling of emetic toxin and enterotoxin-producing Bacillus cereus, isolated from food, environmental, and clinical samples by multiplex PCR J. Food Sci. 79 M2288.

[28] Zhou GP, Liu, HZ, He J 2008 The occurrence of Bacillus cereus, B. thuringiensis and B. mycoides in Chinese pasteurized full fat milk Int. J. Food Microbiol. 121 195.

[29] Sood B, Sahota PP and Hunjan M 2017 Multidrug Resistant Bacillus cereus in Fresh Vegetables: A Serious Burden to Public Health Int. J. Curr. Microbiol. App. Sci. 6 649.

[30] Banerjee M, Nair GB and Ramamurthy T 2011 Phenotypic and genetic characterization of Bacillus cereus isolated from the acute diarrheal patients Indian J. of Med. Res. 133 88.

[31] Chon JW, Yim JH, Kim HS, Kim DH, Kim H, Oh DH, Kim SK and Seo KH 2015 Quantitative Prevalence and Toxin Gene Profile of Bacillus cereus from Ready-to-Eat Vegetables in South Korea Foodborne Pathog. Dis. 12 795.

[32] Abbas BA, Khudor MH and Saeed BMS 2014 Detection of hbl, nhe and bceT toxin genes in Bacillus cereus isolates by multiplex PCR Int. J. Curr. Microbiol. App. Sci. 3 1009.

[33] Hsieh YM, Sheu SJ, Chen YL and Tsen HY 1999 Enterotoxigenic profiles and polymerase chain reaction detection of Bacillus cereus group cells and B. cereus strains from foods and food-borne outbreaks J. Appl. Microbiol. 87 481.

[34] Ombui JN, Schmiegier H, Kagiko MM and Arimi SM 1997 Bacillus cereus may produce two or more diarrheal enterotoxins FEBS Microbiol. Lett. 149 245.

[35] Nooratiny I and Sahilah AM 2013 Detection of enterotoxin targeted entFM and hblA genes by inoculating Bacillus cereus (Strain BC1) into ready-to-eat food (RTF) and drink samples using polymerase chain reaction (PCR) Int. Food Res. J. 20 1895.

[36] Granum PE, Anderson A, Gayther C, Giffel ML, Lund T and O’Sullivan K 1996 Evidence of further enterotoxin complex produced by Bacillus cereus FEMS Microb. Lett. 141 145.

[37] Tewari A, Singh SP and Singh R 2015 Incidence and enterotoxigenic profile of Bacillus cereus in meat and meat products of Uttarakhand, India J. Food Sci. Technol. 52 1796.
[38] Aubaid AH and Dakel KM 2010 Detection of emetic toxin genes in Bacillus cereus isolated different types of Foods. J. Coll. Education Pure Sci. 2 111.