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Detection of *hblA* and *bal* Genes in *Bacillus cereus* Isolates From Cheese Samples Using the Polymerase Chain Reaction

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Abstract

Background: *Bacillus cereus* is a Gram-positive spore-forming bacterium, which causes food poisoning. Spores enable the persistence of *B. cereus* in the environment, and *B. cereus* strains can tolerate adverse environmental conditions, such as temperature and insufficient nutrients. *B. cereus* causes food poisoning via the production of two enterotoxins. Most isolates produce toxins leading to diarrhea (enterotoxins) and vomiting (emetic forms). Diarrhea is caused by the production of three different heat-labile enterotoxins: HBL, NHE, and cytotoxin K. A heat-stable toxin, cereulide, is responsible for emesis.

Objectives: This study aimed to detect enterotoxigenic *B. cereus* isolates in cheese samples using the polymerase chain reaction (PCR).

Materials and Methods: Two-hundred pasteurized (n = 100) and nonpasteurized (n = 100) cheese samples were collected. The initial isolation was performed on PEMBA specific medium. Antibiotic susceptibility testing was performed using several antibiotic disks, according to the guidelines of the Clinical Laboratory and Standards Institute. Specific primers amplifying the *hblA* enterotoxin-encoding gene and *bal* hemolysin-encoding gene were used for the molecular detection of the toxins.

Results: Ten samples were positive for the presence of *B. cereus*, with both Gram staining and biochemical reactions. All the isolates were resistant to penicillin and ampicillin but susceptible to vancomycin, erythromycin, and ciprofloxacin. Six and three isolates were resistant to tetracycline and trimethoprim-sulfamethoxazole, respectively. The *hblA* and *bal* genes were amplified in all the *B. cereus* isolates.

Conclusions: The prevalence of *B. cereus* among the cheese samples was low. All the isolates were positive for genes encoding the *hblA* enterotoxin and *bal* toxin.

Keywords: Hemolysin, Enterotoxin Gene, PCR, *Bacillus cereus*

1. Background

Bacillus cereus is a Gram-positive spore forming motile bacterium, which contaminates food products, such as dairy, and industry foods, similar to several other agents (1, 2). *B. cereus* strains encode two distinct toxins (3) and cause different syndromes, depending on the type of toxin produced (4). Emetic syndrome is caused by ingestion of a preformed heat-stable toxin called cereulide, encoded by the cer gene. Diarrheic syndrome is caused by heat-labile enterotoxins, encoded by *hblA*, *entFM*, and *bceT* genes, that are released by vegetative isolates present in the small intestine (5). Although symptoms are often mild and self-limiting, they can occasionally lead to life-threatening complications (6, 7). To prevent *B. cereus* proliferation, proper heat-preservation strategies are required (8). Foods with a pH of $\geq$ 4.8 provide a sufficient medium for the growth of isolates (9). Many strains are also psychrotolerant and thus can proliferate at low temperatures when nutrients are available (10). A prominent feature of *B. cereus* is the production of spores that are very resistant to heat treatment and harsh conditions, resulting in the survival of strains for long periods in foods and on food-contact surfaces (11, 12). *B. cereus* contamination of dairy products occurs via biofilm production by isolates. Spores can also enter through instruments because of inappropriate heating and storage and next, spores can germinate and spoilage may occur (13). Furthermore, isolates can contaminate dairy environment from various sources, including during production, handling, and processing mainly from insufficient cleaned and sanitized equipment (14). The characterization of the toxigenic potential of *Bacillus* isolates and safety requirements for toxin detection in food products

are dependent upon the availability of reliable assays (15). Usually, toxin genes are detected in rapid state in contaminated foods.

2. Objectives

The aim of this study was to investigate the contamination of cheese products by enterotoxigenic *B. cereus* using the polymerase chain reaction (PCR) assay.

3. Materials and Methods

3.1. Cheese Samples

One-hundred pasteurized and nonpasteurized cheese samples were collected. The amount of each separated sample was 10 g. Each sample was dissolved in 90 mL of autoclaved sterile water, followed by shaking for 2 - 3 minutes. After sinking the cheese, 10 mL of the supernatant was aspirated and inoculated in a tube. The tubes were placed in 75°C water and then diluted to 101, 102, and 103 concentrations.

3.2. Identification Tests

From each dilution, 1 mL was inoculated in brain heart infusion (BHI) medium and incubated at 32°C for 24 hours. Next, one loop of each BHI growth isolate was inoculated on polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA) medium and incubated at 37°C for 48 hours. Then, pink colonies with a lecithinase halo were cultivated on nutrient agar. For these colonies, several tests were performed, including Gram staining, spore formation, biochemical tests such as catalase, VP, nitrate reduction, amylase (starch hydrolysis), motility and beta- hemolysis on sheep blood agar, repeated three times for confirmation.

3.3. Hemolysis

The hemolytic activity of the isolates on 5% sheep blood agar was assessed, as described in previous studies.

3.4. Antimicrobial Susceptibility Testing

Antibiotic susceptibility testing was performed using penicillin, ampicillin, vancomycin, erythromycin, ciprofloxacin, tetracyclin, and trimethoprim-sulfamethoxazole, according to the 2013 guidelines of the Clinical Laboratory and Standards Institute (16).

3.5. DNA Extraction for PCR Assay

DNA from the *B. cereus* isolates were extracted via the freeze-thaw-boiling method. The bacterial isolates were first cultured in nutrient agar at 37°C for 16 hours. One loop of each colony was then removed and dissolved in 150 μL of sterile H_2O and placed at -80°C for 20 minutes and then into 100°C boiling water for 10 minutes. In the next stage, the tubes were centrifuged at 11000 rpm for 10 seconds. Each supernatant was placed in a new tube and stored at -20°C.

3.6. PCR Assay

A PCR assay was performed for the detection of *bal* and *hblA* genes and molecular identification of the *B. cereus* isolates. The PCR master mix was as follows: 10 mM of 10X buffer (50 mM KCl^+ 10 mM Tris- HCl), 50 mM $MgCl_2$, 10 mM dNTP, 10 pM F + R primer, and 5U Taq polymerase and DNA (20 ng/μL), to give a total volume of 25 μL (Sinagen).

The thermal profile of the reaction was 30 cycles of initial denaturation at 95°C for 3 minutes, 94°C for 45 seconds, and an annealing temperature of 55°C (*bal* gene) and 60°C (*hblA* gene) for 45 seconds, followed by extension of 72°C for 1 minute and a final extension of 72°C for 10 minutes. The PCR products were visualized on 1% agarose gel.

4. Results

4.1. Bacterial Isolates

Each pink colony on the PEMBA medium and with lecithinase on specific medium was identified for more confirmation with biochemical tests. The total positive isolates was 5% (n = 10). Three of the pasteurized samples and seven of the nonpasteurized samples were positive. All 10 isolates showed hemolytic activity. Several conditions of the samples and positive isolates are depicted in Table 2. Two isolates were detected in the 10 - 3 dilution, demonstrating high contamination of the samples (Table 2). Five and three isolates were counted in the 102 and 10 -1 concentrations, respectively.

4.2. Antibiotic Susceptibility Assay

All the isolates were resistant to penicillin and ampicillin, but they were susceptible to vancomycin, erythromycin, and ciprofloxacin. Six of the isolates were resistant to tetracyclin, and three were resistant to trimethoprim-sulfamethoxazole.

4.3. PCR assay

The *bal* and *hblA* genes were detected in all 10 isolates. The isolates from both cold (refrigerated) and ambient temperature were positive for these genes (Table 2).

Table 1. Specific Primers Used for the *bal* and *hblA* Genes

Primer	Sequence 5'→3'	Amplicon Size, bp
bal		553
F:	TGCAACTGTATTAGCACAAGCT	
R:	TACCACGAAGTTTGTTCACTACT	
hblA		834
F:	GCTAATGTAGTTTCACCTGTAGCAAC	
R:	AATCATGCCACTGCGTGGACATATAA	

Table 2. The Characteristics of the Cheese Samples and Positive Isolates[a]

Isolate	Pasteurized	Sample Temperature, °C	Spoilage	Count	Hemolysis	*bal*	*hblA*
1	Yes	Ambient	No	10^{-1}	+	+	+
2	Yes	Cold	Yes	10^{-2}	+	+	+
3	Yes	Cold	No	10^{-1}	+	+	+
4	No	Ambient	Yes	10^{-2}	+	+	+
5	No	Cold	No	10^{-1}	+	+	+
6	No	Ambient	No	10^{-3}	+	+	+
7	No	Ambient	No	10^{-2}	+	+	+
8	No	Ambient	Yes	10^{-3}	+	+	+
9	No	Cold	No	10^{-2}	+	+	+
10	No	Ambient	No	10^{-2}	+	+	+

[a] Cold, refrigerated.

5. Discussion

The spoilage potential of *B. cereus* is dependent on two important factors: the bacterial concentration in dairy products (or other foods) and the cytotoxic activity of the isolates. In the present study, the concentration growth of *B. cereus* isolates was ranged from 10 - 103 in cheese products. To our knowledge, there is no previous study regarding this. According to Langeveld (17), these concentrations place in range of spoilage ability, showing a threshold value of *B. cereus*. A previous study showed that isolates from milk were capable of causing spoilage, but no toxin gene was detected among them (18). In this study, few cheese samples from low temperature were in spoilage state and were positive for *B. cereus*, demonstrating that rarely isolates could grow at this temperature (2 - 8°C) (18). Other studies also showed that *B. cereus* strains were mesophiles and that they rarely grew at this temperature as psychrotolerant strains. Ten of the 200 samples in this study were *B. cereus* positive, and the *bal* and *hblA* were amplified in all 10 isolates All the isolates were resistant to penicillin and ampicillin, but they were susceptible to vancomycin, erythromycin, and ciprofloxacin, similar to that reported in another study (19). Six of the isolates were resistant to tetracyclin, and three were resistant to trimethoprim-sulfamethoxazole. In the Savic1 study, 10 of 12 isolates were *hblA* positive (19). Another study of *B. cereus* isolates reported that most were *hblA* positive (20). A study of *B. cereus* isolates from clinical and food samples reported that 90% of the isolates were *hblCDA* positive and that 83.3% were HBL positive (21). Another study reported that the hblC gene was amplified in 59.47% of isolates (22). Therefore, based on the literature, the prevalence of enterotoxin *hblA-C* genes among foodborne *B. cereus* isolates seems to be high.

Several studies reported that isolates produced cytotoxins and caused milk spoilage, whereas other reported that cytotoxin production was not essential for spoilage. However, as noted previously, several factors, including environmental signals and temperatures, affect the production of toxins (Ceuppens 2011). In the present study, two psychrophilic isolates were present in the cheese samples and caused spoilage, similar to that caused by mesophilic strains. It is important to maintain the cheese samples

at lower temperatures because of the possible growth of these isolates. Several previous studies demonstrated that the pathogenicity potential of mesophilic strains was greater than that of psychrophilic strains. Psychrophilic strains of *B.cereus* caused only mild emesis when they contaminated refrigerated foods, and they had a low sanitary risk. Nonemetic strains have been detected in pasteurized egg products. In a study conducted in India, the prevalence of *B. cereus* in cheese, milk powder, ice cream, and milk was high (33% - 55%), and the level of contamination in various dairy products ranged from 10 to 10^8 cfu.g^{-1} or mL^{-1}. A high level of dairy product contamination can lead to the production of heat-stable toxins, with resulting economic losses and health problems. Several studies have demonstrated that isolates from cheese and milk were positive for enzymes, such as amylase, lipase, and protease, and that they produced biofilms.

A limitation of the present study was its focus on the detection of only one type of enterotoxin and one type of hemolysin gene in the cheese samples. Further studies should focus on the detection of a range of emetic toxins and enterotoxins in dairy products.

5.1. Conclusion

The prevalence of *B. cereus* in nonpasteurized and pasteurized cheese samples was low. All the isolates were positive for genes encoding the HBLA enterotoxin and hemolysin. These results show that it is important to detect toxigenic isolates of *B. cereus* in food products.

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