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Prevalence of *ces* and *cytk* Genes of *Bacillus cereus* Isolated From Raw Milk in Tabriz, Iran

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Abstract

Background: *Bacillus cereus* is a gram-positive and spore-forming bacterium which is widespread in nature. It also has been known as a major foodborne pathogen that often plays a role in the contamination of ready-to-eat and dairy products. It causes two different types of food poisoning in human: the diarrheal type and the emetic type.

Objective: The current study was planned to determine the prevalence of *ces* and *cytk* genes of *Bacillus cereus* isolated from raw milk in Tabriz, Iran.

Materials and Methods: In this study, 40 *B. cereus* strains isolated from cow raw milk, that had already been identified phenotypically, were assessed for molecular confirmation by polymerase chain reaction (PCR) method. Then, they were evaluated for presence of *ces* and *cytK* genes by specific primers.

Results: Of 40 *B. cereus* strains, 39 strains were confirmed molecularly. The frequency of *cytK* and *ces* genes was reported 38 (97.43%) and 0 (0%), respectively.

Conclusion: The results of present study showed that *B. cereus* strains isolated from raw milk had high potential in causing diarrhea poisoning. Therefore, using procedures to reduce the bacterial contamination during the processing of dairy product is essential.

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Background

Foodborne diseases are one of the serious problems in developed and developing countries.[1] It has been suggested that *Bacillus cereus* has significant impacts on human health, agricultural crops, and food processing.[2] *B. cereus* commonly results in spoilage of food products.[3] Moreover, it is known as an opportunistic pathogen which could cause two types of food poisoning among humans, characterized by either nausea and vomiting or abdominal pain and diarrhea.[4,5] Although swallowing more than 10^5 bacteria per gram of food is necessary to cause illness, eating too much bacteria does not always produce the disease.[6] The spores of this bacterium remain in the food even after cooking, and if the food is kept under warm and humid conditions, spores germinate and produce a type of enterotoxin that can lead to food poisoning.[7,8] Cytotoxin K (*cytK*) and NHE complex proteins are among the first virulence factors in *B. cereus*, which can cause diarrhea.[9] The virulent character of emetic strains is linked to the production of a heat stable cereulide, which is synthesized by a non-ribosomal peptide synthetase encoded by *ces* genes.[10] The emetic toxin, which is usually pre-made in food, is not inactivated during food processing or gastrointestinal passage since it is highly resistant to heat treatments, extreme pH conditions, and protease activities.[11] Therefore, eating live *B. cereus* is not necessary for this type of disease to occur. In addition, diarrheal food poisoning is not the outcome of pre-made toxins in food; rather it is caused by viable vegetative *B. cereus* cells (not spores) producing enterotoxins in the small intestine. This is due to the fact that spores do not produce enterotoxins. Furthermore, spores can be easily degraded under gastrointestinal conditions by the host's digestive enzymes.[12,13] Recently, there has been an increasing concern in intestinal infections associated with this bacterium. Often these infections are present in immunocompromised patients. Accordingly, the current study aimed to determine the prevalence of *ces* and *cytk* genes of *B. cereus* isolated from raw milk in Tabriz, Iran.

Materials and Methods

Sampling

In this study, 200 samples of raw cow milk were randomly selected from the stores selling dairy products in Tabriz from March to September 2018. The samples were transferred to the laboratory of food hygiene under sterile conditions to isolate and identify *B. cereus* according to the national standard method of Iran. The samples were cultured in MYP agar (Mannitol Egg yolk Polymyxin agar) (Merck, Germany). After the incubation period, large, pink, and haloed colonies were identified as possible *B. cereus*, and hemolysis and gram staining tests were

performed.[14] Finally, 40 strains of *B. cereus* were isolated by biochemical methods.

DNA Extraction

DNA extraction of tested samples was performed by using of kit (Pak gene Yakhteh company, Catalog No. 30535). After DNA extraction, the samples were evaluated in order to determine DNA concentration in ng/L using a Nanodrop device. The priority of reading indices, the ratio of 260/280 nm, and the ratio of 260/230 nm were considered. Moreover, the optical density (OD) was read for performing PCR.

PCR Test to Confirm the Molecular Diagnosis of Bacillus cereus

Specific primers for *B. cereus* (Table 1) were prepared from Nano Zist Fanavaran Company (Iran).

PCR Test for Identification of cytk and ces Genes

The polymerase chain reaction (PCR) was performed in 25 µL volume, which included 15.8 µL of master mix of PCR, extracted DNA containing 8 µL (10 ng), and specific primers (0.6 µL from each of the forward and reverse primers). Table 2 presents PCR conditions. PCR product in 1.5% agarose was electrophoresed and illustrated using gel document. *B. cereus* ATCC 11778 was used as positive control. According to Table 2, amplification of *Bal, cytk* and *ces* genes in thermocycler was performed. *B. cereus* samples harboring *cytK* gene were sent for sequencing (Bioneer Company, South Korea). Clustal Omega program was used for drawing phylogenetic tree.

Results

Of 40 *B. cereus* isolates, 39 (97.5%) isolates possessed *Bal* gene, which were identified as *B. cereus* (Figure 1).

Of 39 *B. cereus* isolates, 38 (97.43%) isolates harbored *cytk* gene (Figure 2) and none of the isolates harbored *ces* gene.

According to the gained sequences in online program (NCBI), two *B. cereus* isolates showed more homology to each other and three *B. cereus* isolates showed homology together. The homology of the intended isolates with other strains of *B. cereus* is presented in Figure 3.

Discussion

Many factors affect the microbial quality of raw milk, of which four main sources are considered for microbial contamination as follows: inside the udder tank, the outer parts of the udder, environmental factors, milking equipment and storage of raw milk. In order to produce safe products from milk, good hygienic practice based on control methods HACCP (Hazard Analysis and Critical Control Point) during the production chain to consumption must be observed, and these prerequisites in the raw milk supply chain must be considered.[17] The findings of the present study showed that out of 200 samples of raw cow milk, 39 (19.5%) samples were infected with *B. cereus*. Moradi-Khatoonabadi et al reported that 9% of raw milk samples were contaminated with *B. cereus*.[17] Reyes et al revealed that 24.23% of milk and dairy products marketed in Brazil were contaminated with *B. cereus*.[18] In another study, Heydarzadeh and

Table 1. Sequence of Primers Used for Detection of *B. Cereus* and *cytK* and *ces* Genes

Gene	Primer Sequence (5′→3′)	Amplicon Size (bp)	Reference
Bal	F- TGCAACTGTATTAGCACAAGCT R- TACCACGAAGTTTGTTCACTACT	533	[15]
cytk	F- ACAGATATCGGGCAAAATGC R- TCCAACCCAGTTTGCAGTTC	809	[16]
ces	F- GTGACACATTATCATATAAGGTG R- GAACCTGTCTGTAACAACA	1271	[10]

Table 2. Conditions for Performing PCR of *Bacillus Cereus* Isolates to Amplify the Desired Genes

Stage	Number of Cycles	Gene Time *Bal/cytk/ces*	Temperature (°C)
Primary denaturation	1	3′/3′/4′	94/94/94
Denaturation	35	30"/30"/30"	94/94/94
Annealing	35	45"/45"/45"	54/57/54
Extension	35	60"/60"/70"	72/72/72
Terminal extension	1	5'/5'/5'	72/72/72

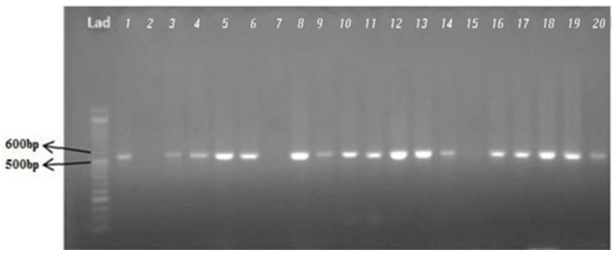

Figure 1. Electrophoresis of the *Bal* Gene PCR Product on 2% Agarose. *Note*. Lad: ladder 50 bp; No. 2: positive control (*B. cereus* ATCC 11778); No. 3: negative control (double-distilled water); No. 4-7 and 9-21: positive *Bal* gene samples (533 bp); No. 8: negative *B. cereus* sample.

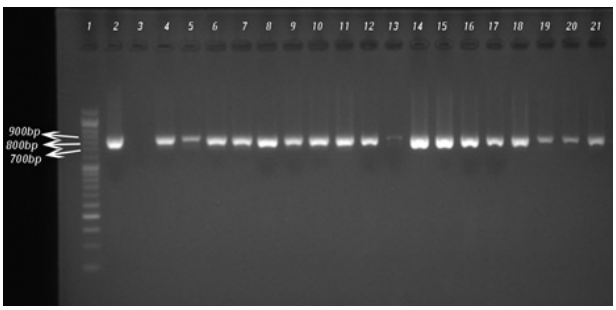

Figure 2. Electrophoresis of the *cytK* Gene PCR Product on 2% Agarose. *Note*. No. 1: ladder 50 bp; No. 2: positive control (*B. cereus* ATCC 11778); No. 3: negative control (double-distilled water); No. 4-21: positive *cytK* gene samples (809 bp).

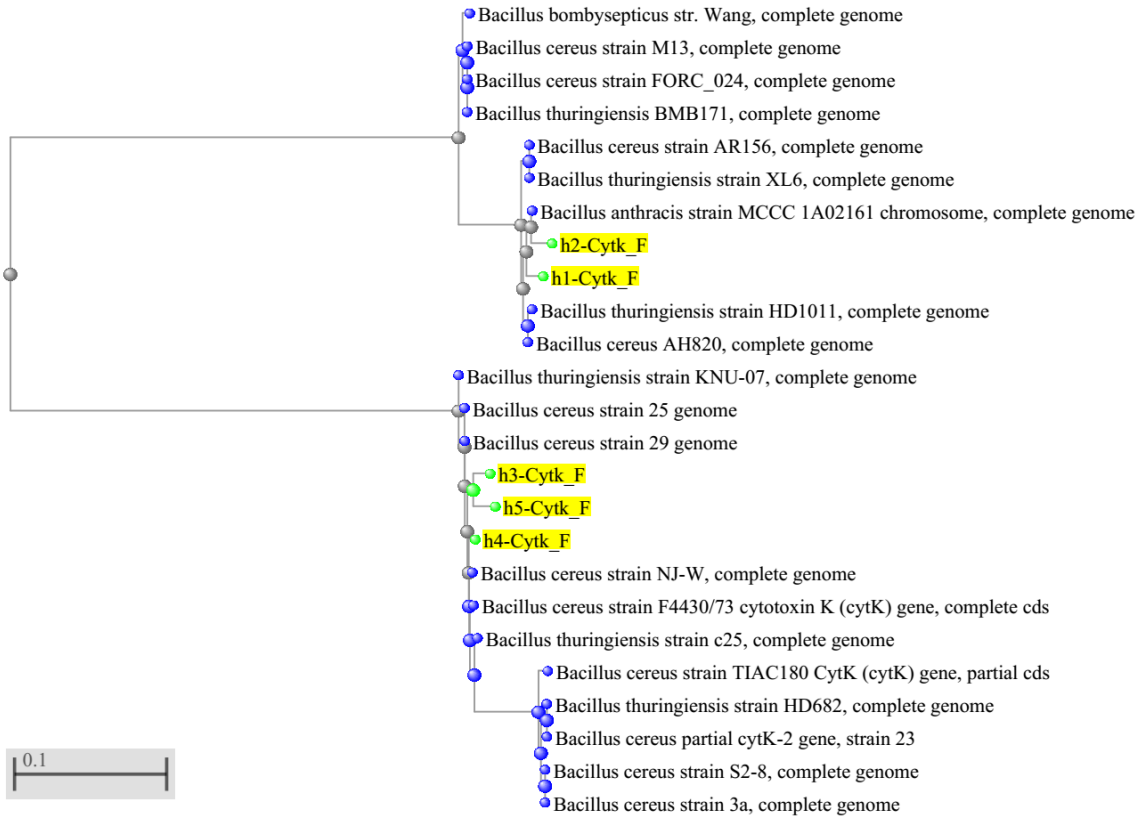

Figure 3. The Phylogenetic Tree on the Base of Nucleotide Sequence of *cytK* Gene in *Bacillus Cereus*. *Note*. The criterion indicates 0.1 change in nucleic acid in *cytK* gene between tested strains.

Javadi showed that 10.83% of raw milk samples were contaminated with *B. cereus*.[19] The results of this research showed the frequency of *cytk* and *ces* genes was 97.43% and 0%, respectively. The findings of the study by Owusu-Kwarteng et al showed that the frequency of *cytk* and *ces* genes in *B. cereus* isolated from dairy farms and traditional dairy products was 75% and 9%, respectively.[20] Kim et al were not able to produce amplicons for the emetic gene, *ces*, in both reference and commercial strains of *B. cereus*.[21] Emetic genes which produce toxin have already been identified at different low rates (1.5% to 17.2%) in isolated *B. cereus* strains isolated from different food sources.[22,23] Hence, these findings suggest that emetic toxin genes are not highly common or are rare among *B. cereus* isolates. Horii et al showed that 13% of *B. cereus* isolates from blood cultures contained the *cytK* gene.[24] Heydarzadeh and Javadi reported that 92.3% of *Bacillus cereus* isolates from raw milk contained *ces* gene.[19]

Conclusion

The findings of the present research revealed the difference in dispersion of *cytk* and *ces* genes in *B. cereus*; this difference probably derives from geographical diversities and different ecological origin of the isolated strains (milk, human and different animals). In this study, from 40 isolates of *B. cereus* previously identified by phenotypic and biochemical tests, 39 (97.5%) isolates were approved by RCR. This indicates a higher accuracy of PCR method than the culture and biochemical methods. The rapid method for detecting the presence of enterotoxigenic *B. cereus* in food is very important to ensure the health of foodstuff.

Authors' contributions

SM supervised the project and wrote the manuscript. All authors read and approved the final manuscript.

Ethical Approval

Not applicable.

Conflict of Interest Disclosures

The authors declare no conflict of interests.

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