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Molecular Detection of *Bacillus cereus* in Milk by Polymerase Chain Reaction

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ABSTRACT

It is a valuable and good food source with the ingredients it contains in milk, but if hygienic conditions are not ensured during milking, storage and processing, diseases related to milk and dairy products are likely to occur. More than 90% of these diseases are of bacterial origin. *B. cereus*, found in the *Bacillaceae* family, is a significant source of contamination in milk and dairy products due to the presence of psychrotrophic strains. In recent years, it has been reported that milk and milk products contaminated with *B. cereus* have adverse effects on human health, and toxic substances are formed during the storage of contaminated milk and milk products.

This study, it is aimed to molecularly identify the *B. cereus* bacteria species that cause adverse effects such as food poisoning and loss of quality in milk. In this study, the DNA of *Bacillus cereus*, one of the microorganisms found in raw milk, was isolated and toxic gene regions were determined at the molecular level by PCR method using specific primers to the gene regions *Bacillus cereus*. Six primers specific for nheA, nheB, nheC, hblA, hblC, hblD and 16S rRNA gene regions were used for molecular detection of *B. cereus* in milk samples. At the end of the study, *Bacillus cereus* bacteria were detected in 8 of the 13 milk samples. It has been revealed that *B. cereus* can be detected quickly and reliably in milk and dairy products by molecular species identification using specific primers.

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Introduction

Foodborne pathogenic microorganisms cause severe losses such as disease and death, leading to public health problems that can reach epidemics by contaminating the food at any stage of the process from food production to consumption [1]. It is known that raw milk and its products are one of the main causes of foodborne diseases associated with *Listeria monocytogenes, Staphylococcus aureus, Escherichia coli O157: H7, Bacillus cereus, Salmonella spp., Campylobacter spp., Clostridium botulinum* [2,3]. Since milk is a nutrient-rich food, it has a great place in human nutrition. Although the milk in healthy breast cells is reported to be sterile, high water activity and neutral pH conditions may

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cause contamination of the milk by microorganisms if the production processes and storage conditions are not under hygienic conditions. This situation poses a threat to human health [4,5]. The enzyme production of psychrophilic, lipolytic and proteolytic bacteria and changes in milk are the results of long-term storage of raw milk before pasteurization. Therefore, problems are expected to occur during milk processing and in maintaining the quality of dairy products [6,7].

Bacillus cereus bacteria cause foodborne illnesses due to their enterotoxin and emetic toxin content [8]. Non-hemolytic enterotoxin (*Nhe*), hemolysis BL (*Hbl)* and Cytotoxin K (*CytK*), which are enterotoxins originating from *B. cereus*, have caused food-borne poisoning [9,10]. The emetic toxin is a peptide cereulide, a thermostable and acid-resistant dipeptide. *Nhe* complex consists of 3 subunits, namely *nheA*, *nheB* and *nheC*. The subunits of the *Hbl* complex are *hblA*, *hblC* and *hblD* [11].

Protease enzymes secreted by *B. cereus*, which is one of the psychrophilic bacteria, can maintain their stability during the processing of raw milk and causes clotting in processed milk products in a short time depending on the amount and activity of the enzyme. *B.* cereus is a rod-shaped, gram-positive, facultative anaerobe, a spore bacterium [12,13] and causes many different health problems, especially food poisoning, in terms of food safety. Its spores show high resistance to other environmental conditions such as humidity, pH and temperature. *B. cereus* has hydrophobic properties. They can adhere to surfaces (tool-equipment) very quickly [14]. Diseases from *B. cereus* can be classified as non-gastrointestinal and gastrointestinal diseases. Gastrointestinal diseases can be seen in two different ways. One is diarrhea, and the other is the emetic form [15]. Different enterotoxins from the diarrhea form, while the emetic form is formed by the heat-resistant toxin (Cereulide).

Determination of foodborne pathogens is important for accurate and standardized clinical diagnosis in the food industry and monitoring food hygiene [16]. Protocols for existing traditional biochemical studies are routinely used to detect *B. cereus* require labour and time consuming [17].

Recently, polymerase chain reaction (PCR), ELISA, commercial kits and tests based on different cell cultures are used to diagnose and identification *B. cereus* toxins in foods [18]. The proposed RAPD-PCR procedure for detecting large-scale Bacillus cereus was reported by Nilsson et al. (1998) [19]. Hansen et al. (2001) reported a PCR analysis using

16S rRNA as a target to identify the *B. cereus* group. Specific primers were selected for *16S rRNA* detection of *B. cereus* group bacteria. It was used with consensus primers for *16S rRNA* as a control in PCR [20].

The specificity and sensitivity of its identification, such as *B. cereus*, using the PCR test were evaluated with different food samples types. As a result, the proposed multiplex PCR is stated as a reliable and facile method for the simultaneous identification of the *B. cereus* group in food samples in one tube [21].

Razei et al. (2017) used the multiplex PCR technique for the simultaneous detection of *Campylobacter jejuni*, *Bacillus cereus* and *Listeria monocytogens* bacteria, which are foodborne microorganisms that produce toxins in food (milk). As a result, the new multiplex PCR method is introduced to detect simultaneous *B. cereus, L. monocytogens* and *C. jejuni*, and it has been reported that these results can be used for the detection of other toxin-producing bacteria in food [22].

Banykó et al. (2009) made species identification in raw and pasteurized milk for Bacillus licheniformis and Bacillus cereus. It has been determined that *B. cereus* and *B. licheniformis* both contaminate with the same strains in raw and pasteurized milk and cause contamination with different strains (raw milk and yoghurt/pasteurized milk and yoghurt). As a result, PCR analysis has been reported to be a useful method for characterizing *Bacillus* populations in the dairy environment [23].

In another study, the presence of *B. cereus* toxins was determined by PCR in raw and pasteurized dairy products. For that reason, PCR conditions were optimized for molecular identification of *Listeria monocytogenes* and *Brucella abortus* strains in bovine milk. Lesley et al. (2017) investigated the presence of *B. cereus* in formula and UHT milk samples; As a result, *B. cereus* was detected in 41.7% formula milk and 30% UHT milk samples, respectively [24].

Meena et al. (2019) identified *B. cereus* in dairy products and milk. In the molecular analyzes, while the *gyrB* gene was detected in all isolates, the presence of *cytK* and *hblA* was noted in 60% and 40% of the isolates, respectively. According to the study results, it was emphasized that the presence of enterotoxigenic genes (*cytK, hblA*) in the isolates should cause a potential health threat so that more samples should be studied from different regions [25].

A study conducted in 2019 that obtained the toxigenic profile of *B. cereus* strains in cheeses was molecularly detected [26]. PCR technique was used to investigate *Hbl, Nhe* and *cytotoxin K* toxic genes in the samples. According to study results, at least one gene associated with enterotoxins has been identified in most strains (21/23), highlighting the high toxigenic potential of examined cheeses.

This study aims to molecularly determine *Bacillus cereus'* presence in milk by using the PCR method and primers specific to *Bacillus cereus* gene regions.

Materials and Methods

Production of bacteria and culture conditions

As a positive control, *B. cereus* (ATCC 11778 strain) was used. The spreading plate method was applied using the MYP (Mannitol Egg Yolk Polymyxin) agar for bacterial cultivation of *Bacillus cereus*. The bacterial culture was placed in an incubator at 37 °C and left for two days of incubation. Bacterial colonies observed breeding was detected morphologically. The morphologically selected bacterial colony was taken into the LB broth medium and incubated at 37 °C for 150 rev/min overnight. DNA isolation was made from colonies that multiplied after one night.

DNA extraction

The sample containing the bacterial colony was incubated the night before collected by centrifugation. DNeasy Blood & Tissue Kit (Qiagen, Shanghai, China) was applied according to the manufacturer's instructions for bacterial colonies' DNA isolation. After DNA isolation, purified DNA samples were stored at -20 ° C in the elution buffer for reuse.

Polymerase Chain Reaction

The polymerase Chain Reaction (PCR) process was performed using BIO-RAD C1000 Touch Thermal Cycler. PCR was performed by optimizing reaction conditions for each primer and the PCR reactions were performed in a total volume of 25 µl containing 0,4 (*IAC, 16S rRNA*) or 0,6 (*hblA, hblC, hblD, nheA, nheB/nheC*) µM of each primer, 2.5 µl 10X *Taq* Buffer with KCl, 1,6 (*IAC, 16S rRNA*) or 0,4 (*hblA, hblC, hblD, nheA, nheB/nheC*) mM of each dNTP (Thermo Scientific, USA), DNA Taq polymerase (*IAC, 16S rRNA* 2,5 U; *hblA, hblC, hblD, nheA, nheB/nheC* 1 U; Thermo Scientific, USA), $MgCl_2$ (*IAC, 16S rRNA* 4 mM; *hblA, hblC, hblD, nheA, nheB/nheC* 2 mM) and 2 µl DNA preparation. The PCR cycle reactions are as follows: 10 minutes in the first denaturation

at 95 °C, then 94 °C for 60 seconds, 50-60 °C for 60 seconds (the annealing temperatures changing for each primer), 72 °C for 60 seconds and 72 °C is completed with a final extension step of 10 minutes in. Thermo Scientific EC 1000 XL Power Supply Electrophoresis system was used to execute DNA samples. BIO-RAD ChemiDOC MP Imaging system (UV transilluminator) was used for imaging the gels where DNA and PCR products were operated. PCR fragments were run for 30 min at 100 volts in 0.5 x TBE buffer solution with 2% agarose gel electrophoresis added with ethidium bromide and visualized under UV light. Agarose gels (120 ml of 2%) were prepared to contain 24 ml 5 x TBE, 96 ml ddH2O, 2.4 g Agarose and 5 µl Ethidium Bromide (10 mg/ml).

The sequence of primer sets (IAC-F IAC-R, 16S-F 16S-R, 45c1 45c2, nheBC1 nheBC2, HblA1 HblA2, L1a-F L1a-R, L2a-F L2a-R) used in this study and targeting specific gene regions information, expected band sizes (bp) for *B. cereus*, and target gene regions are given in Table 1.

Table 1 Expected band sizes and sequences information of primers used

Primer Name	**Primer Sequences (5'--- 3')**	**Expected Band Sizes (bp) for *B. cereus* (bp)**	**Target Region**	**Reference**
AC-F AC-R	GCAGCCACTGGTAACAGGAT GCAGAGCGCAGATACCAAAT	118	*IAC*	[27]
IblA1 IblA2	GCTAATGTAGTTTCACCTGTAGCAAC AATCATGCCACTGCGTGGACATATAA	874	*hblA*	[28]
6S-F 6S-R	GCGGCGTGCCTAATACTGC CTCAGGTCGGCTACGCATCG	267	16S rRNA	[29]
5c1 5c2	GAGGGGCAAACAGAAGTGAA TGCGAACTTTTGATGATTCG	186	*nheA*	[27]
heBC1 heBC2	ACATTGCGAAAGATAGCTGGA TGTTCTGCTGCAAAAGGATG	300	*nheB/ nheC*	[30]
L1a-F L1a-R	AGGTCAACAGGCAACGATTC CGAGAGTCCACCAACAACAG	205	*hblD*	[28]
L2a-F L2a-R	CGAAAATTAGGTGCGCAATC TAATATGCCTTGCGCAGTTG	411	*hblC*	[28]

Results and Discussion

In this study, primers specific to IAC, 16S rRNA, nheA, nheB / nheC, hblA, hblC and hblD gene regions were used for molecular screening *Bacillus cereus* in DNA samples

taken from milk samples. For this purpose, *Bacillus cereus* determination was performed on 13 milk samples collected using six primers (16S-F 16S-R, 45c1 45c2, nheBC1 nheBC2, HblA1 HblA2, L1a-F L1a-R, L2a-F L2a-R) specific to different toxin genes. For all primers, 50 bp ladder (L), positive control (PC) and milk samples 1-13 were loaded into the gel, respectively. The internal amplification control (IAC) is a non-targeted DNA sequence used to prevent false-negative PCR results and eliminate the inhibitory effect. It is amplified with the target sequence and takes part in the same reaction as the sample [31]. Although the IAC design may vary, a general guideline for using IAC in the PCR applications of pathogens has been proposed by the European Standardization Committee. For this reason, IAC-F / IAC-R primers [27]. were used in this study. The band results of the gel images of the molecular screening performed with the primers used in the study are given in Table 2.

Table 2 PCR results of the primers used in the study

Milk Sample Numbers	**Primer Name**						
	IAC-F IAC-R	16S-F 16S-R	45c1 45c2	nheBC1 nheBC2	L1a-F L1a-R	L2a-F L2a-R	HblA1 HblA2
PC	+	+	+	+	+	+	+
1	+	+	+	+	+	+	+
2	+	-	-	-	-	-	-
3	+	+	+	+	+	+	+
4	+	-	-	-	-	-	-
5	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+
7	+	-	-	-	-	-	-
8	+	-	-	-	-	-	-
9	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+
12	+	-	-	-	-	-	-
13	+	+	+	+	+	+	+

PC: Positive Control, + and – represent the positive and negative result, respectively

Differentiation between the subtypes can be determined by PCR and can be concluded quickly without pre-enrichment in clinical samples [31]. Ogawa et al. (2015) developed multiplex PCR that allows the detection of *B. anthracis* virulence plasmids and can distinguish genetically related strains of *B. anthracis* from other *B. cereus* group species [32]. They designed six primer sets targeting similar strains, one chromosome of *B.*

anthracis, two virulent plasmids (pXO1 and pXO2), a bacterial gene (16S rRNA gene) and a mammalian gene (actin-beta gene). These primers have been used to identify genetically relevant clinical strains of B. anthracis and *B. cereus* isolated from outbreaks of nosocomial infections in Japan. That study found that the newly developed primers are an accurate and valuable method for detecting *B. cereus* and *B. anthracis* [32]. In this study, primers designed specifically for the *16S rRNA* gene region were used for the molecular detection of *B. cereus*. The band profiles obtained as a result of the PCR are shown in Fig 1.

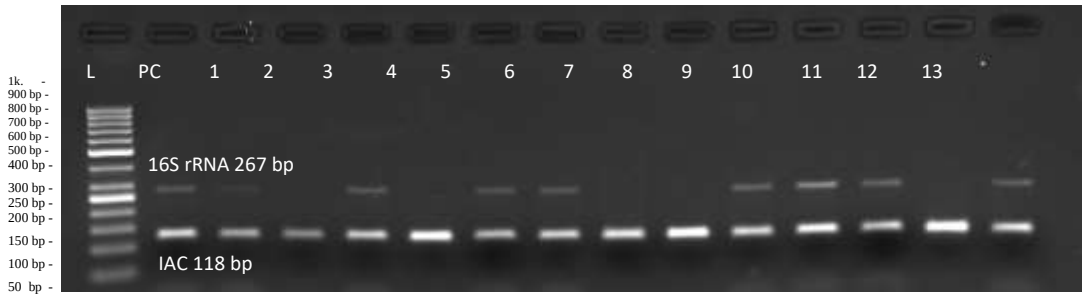

Fig 1 Molecular characterization with 16S-F 16S-R primer (L: 50 bp, PC: Positive Control)

In food microbiology, it is crucial to characterize suspicious isolates in terms of pathogenicity and species identification to identify the toxigenicity of strains isolated by molecular techniques.

In this study, it was determined that 62% of the milk samples resulting from molecular screening performed with primers of 45c1 and 45c2 contained the *nhA* toxic gene region of *Bacillus cereus* Non-haemolytic enterotoxin (nhe) caused food poisoning in an organization in Norway in 1995, causing 152 people to get sick due to diarrhea. The nhe operon system, consisting of 3 protein subunits, was 90% or more in *B. cereus* cultures in molecular biology studies [18, 33].

In a different study, Moravek et al. (2004) observed that the samples isolated from baby food based on diarrhea type food poisonings of *B. cereus* gave a 186 bp size band in the gene region-specific studies of *B. cereus* 45c1 45c2 primers (nheA). According to our research, the expected band size of samples including *B. cereus* was confirmed by using 45c1 45c2 primers in milk samples [28].

Dierick et al. (2005) screened a total of 50 different strains with nheBC1 nheBC12 primers to detect *B. cereus* by PCR method specific to nheB / nheC gene regions. In this

study, according to these gene regions (nheB / nheC), the molecular size screening for nheBC1 nheBC2 primer set was obtained in which 8 of 13 samples in total were used [30].

Hbl is an enterotoxin and was first described in 1984 by isolating *B. cereus* F837 / 76 strain [34]. The enterotoxin complex Hbl with hemolytic effect; consists of three large proteins synthesized by three polycistronic genes consisting of hblA, hblC and hblD. Molecular screening in this study using the L2a-F L2a-R primer revealed that 62% of 13 samples had the *B. cereus* hblC toxic gene. Similarly, due to molecular screenings with the L1a-F L1a-R primer, 8 of the milk samples examined in this study were found to have the HblD toxic gene region of *B. cereus*.

This study obtained that milk samples containing hblC and hblD toxin gene regions are also positive for the hblA gene (Fig 2). Another study conducted a PCR study to detect *B. cereus* isolates in cheese samples and used hblA and bal genes for molecular detection [35]. As a result of the study, although the presence of *B. cereus* was low in cheese samples, all isolates were found positive for genes encoding hblA enterotoxin.

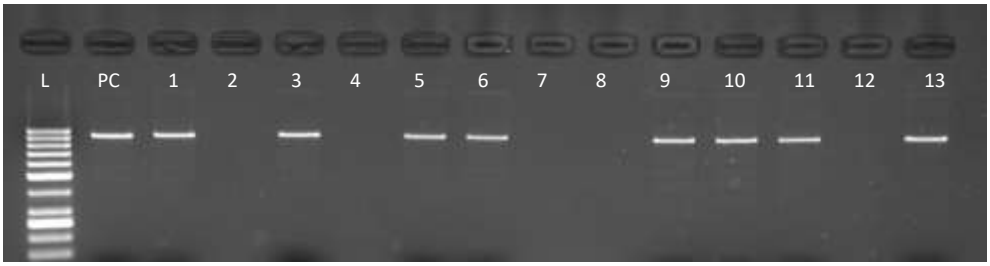

Fig 2 Molecular characterization with HblA1 HblA2 primer (L: 50 bp, PC: Positive Control)

Yang et al. (2005) characterized one emetic and five different enterotoxins *Bacillus cereus* toxin. Polymerase chain reactions were performed with 12 primer pairs to replicate the enterotoxin and emetic specific sequences of the species in the *B. cereus* group. Screening tests with the developed primers have been successfully applied to analyze 162 food poisoning and toxigenic potential of food-related strains. The results contained at least one toxin gene among all *B. cereus* strains, and ten toxigenic patterns were identified for all test strains. The two species are closely related, but the toxin profiles of *B. mycoides* strains were significantly different from *B. cereus* ($P < 0.05$). As a result of the study, the

molecular detection of the species in the *B. cereus* group, along with the importance of detecting toxin genes, was also indicated [36].

In another study, Ngamwongsatit et al. (2008) designed eight new PCR primer pairs specific for eight toxin genes (*nheA, nheB, nheC, hblC, hblD, hblA, entFM* and *cytK*). They used these primers in molecular screening on 205 *B. Thuringiensis* (43 serovars, 152 soil and ten food isolates) and 411 *B. cereus* strains (290 soil and 121 food isolates). With the detection of these eight toxin genes, a total of 616 isolates were divided into four groups [37].

Zhang et al. (2016) used multiplex PCR to investigate enterotoxin distribution in 62 enterotoxin-producing strains in milk powder, noodles, and rice to selectively detect Bacillus cereus, which is responsible for various toxin outbreaks of foodborne diseases. The specificity of the enterotoxin (*cytK*, *nheA* and *hblD*) genes of *B. cereus* was verified using a single PCR [38].

Conclusion

In this study, a molecular determination was made using the PCR method and primers specific to the gene regions of *Bacillus cereus* bacteria that cause negative effects in raw and pasteurized milk samples. For this purpose 6 primers (16S-F 16S-R, 45c1 45c2, nheBC1 nheBC2, HblA1 HblA2, L1a-F L1a-R, L2a-F L2a-R) used that specific to 7 toxin gene regions (16S rRNA, nheA, nheB / nheC, hblA, hblC and hblD) of *Bacillus cereus* bacteria was performed molecular screening. *Bacillus cereus* bacteria were detected molecularly in 13 milk samples examined due to the study.

Microbiological properties in Turkey mainly do determination of microorganisms in milk, but morphologically identified species do not always give accurate results. In this study, *B. cereus* bacteria were detected molecularly, which causes adverse effects such as food poisoning and loss of quality in milk. It is predicted that the results obtained and the methods used will also help future studies for the detection of *B. cereus* bacteria in milk and dairy products.

Abbrevations

PCR: Polymerase Chain Reaction, Nhe: Non-hemolytic enterotoxin, Hbl: hemolysis BL, CytK: Cytotoxin K, MYP: Mannitol Egg Yolk Polymyxin, IAC: internal amplification control

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