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Direct Detection of Bacillus ceruse Enterotoxin Gene by Multiplex PCR Kit in Dairy Products

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Abstract

Due to the heat resistance of *Bacillus cereus*, its potential pathogenic character, the capability to grow in milk and reported diseases upon consumption of dairy products, the organism should be considered as hazardous in pasteurized milk. In this study *hblA*, *hblC* and *nheB* genes was investigated. Enterotoxin producing ability of 25 raw milk and 5 pasteurization milk samples was judged by Multiplex PCR reactions. Results showed that *hblA*, *hblC* and *nheB* genes were found to be in 66.36, 23.33 and 66.56 percent of milk samples, respectively. Also, 16.66 % and 13.33 % of samples were contained *hblA+hblC* and all three genes, respectively. Our findings indicated that *hblA*, *hblC* and *nheB* genes specific Multiplex PCR can be employed for differentiation of enterotoxigenic *B. cereus* isolates.

Key words : Foodborne Disease, Milk Products, Food Contamination Detection.

INTRODUCTION

Bacillus cereus is a widespread foodborne disease-causing agent which is commonly correlated with two kinds of illness: emesis and diarrhea [1]. Milk and rice are possibly the two most frequently contaminated foodstuffs [2]. *B. cereus* comprises 90% of the paddy soil bacteria and exposure of udders to soil and grass induces contamination of milk and milk products [3] In the U.S, *B. cereus* is culpable for 1-2% of the total epidemics resulting from bacteria. The temperature range for the growth of *B. cereus* has been reported to be from 4 to 50^{0}C with the optimum standing in the range of 30-40^{0}C [4].

Although there are accounts of isolation and characterization of this pathogen from various food stuffs all around the world, there are no reports on the levels, toxin producing ability, or growth characteristics from Iranian dairy products. The Researcher reported that *B. cereus* exists in both raw and pasteurized milk with a prevalence of 2-37% [5]. *B. cereus* spores are a further complication in dried products [6]. *B. cereus* amounts are capable of attaining dangerous levels during the soaking of dried legumes prior to cooking [7]. Some isolates of *B. cereus* can grow at refrigerated temperatures and their spore manage to endure high temperatures [8].

The two types of gastrointestinal diseases caused by *B. cereus* are derived from two considerably distinct kinds of toxins [9]. Three different heat labile diarrheal enterotoxins are produced by *B. cereus* during its vegetative growth in the small intestine [10]. *nhe* and *hbl* genes are structurally pertaining to illness and incorporating three proteins [11]. The multi-component enterotoxins need all three protein constituents for their maximum activities [12, 13,14]. Even though it has been reported that cytotoxicity is chiefly because of nhe, it is not evident whether the other two toxins contribute to diarrhea [15]. Customarily, cultural methods applying selective media are adopted for detection and identification of *B. cereus* cells from environmental samples including fermented foods [16]. Nevertheless, selective media such as polymyxin-egg yolk-mannitol-bromothymol agar (PEMBA) or mannitol-egg yolk-polymyxin (MYP) regularly lead to misidentifications among closely related species [17]. Commercial assay kits are available for semi-quantitive detection of *nhe* and *hbl* enterotoxins. Currently, PCR methods are usually employed for identification purposes [18, 19]. Parts of toxin gene sequences have been utilized as primers for the detection of *B. cereus*. Despite the development of diverse procedures, any discrete method is not even close to sufficient for the accurate identification of *B. cereus* strains from foods.

Consequently, *B. cereus* contamination is a crucial issue for dairy products. In this article, we report a Multiplex-PCR protocol that can be used for the rapid detection of *B. cereus* from dairy products and probably other fermented foods.

MARTIALAND METHODS

Samples and reference bacterial strains

25 samples of raw milk and 5 of pasteurized milk were procured from Mashhad, Iran. The milk was purchased from points of sale and was bought without sharing any knowledge that it was for research purposes, so as not to affect the result of the experiment. The samples were transported to the laboratory at 4^{0}C. The Bacillus cereus was kindly provided by the faculty of veterinary (Ferdowsi University of Mashhad, Iran) on Luria-Bertani agar medium and was used as the positive control.

DNA Extraction

DNA was extracted from raw and pasteurized milk with some modifications in the kit (#K0721, Thermo). To put it briefly, 10 ml of milk was centrifuged at 10000 rpm for 30 min. The supernatant was discarded and the pellets were washed twice in PBS, pH 7.2 and used for DNA extraction. DNA concentration was assessed by Nanodrop ND2000 (Thermo, USA).

Polymerase chain reaction Assay

For screening of enterotoxigenic genes viz. *hbla, hblc* and *nhe* in all 30 samples of milk, primers specific for those genes were employed in PCR assays. Three sets of primers were used in the

PCR reactions (Table I). 1µl of template was amplified in 24 µl of reaction mixture consisting of PCR buffer 10X 2.5µl, dNTP mixture (20mM) 2 µl, MgCl2 (50 mM) 1.3 µl, primer (20 µM of each) 2µl and 2 U of Taq DNA polymerase. Oligonucleotide primers were procured from Integrated Bionner (South Korea). PCRs were performed using a T-Personal thermocycler (Germany). PCR reaction using *nheB* primers consisted of 35 cycles of 94°C for 30 sec (denaturation), 61°C for 20 sec (annealing) and 72°C for 20 sec (extension). PCR reaction conditions for the primers *hblA* were 35 cycles of 94°C for 30 sec, 59°C for 20 sec and 72°C for 30sec. PCR reaction conditions for the primers *hblC* were 35 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 25 sec. Multiplex PCR reaction conditions for the primers *hblA* were 35 cycles of 94°C for 30 sec, 59°C for 20 sec and 72°C for 30 sec. For the optimization of Multiplex PCR, 2µl of template was amplified in 23 µl of reaction mixture consisting of PCR buffer 10X 2.5µl, dNTP mixture (20mM) 2 µl, MgCl2 (50 mM) 1.3 µl, primer (20 µM of each) 3µl and 2 U of Taq DNA polymerase. Multiplex PCR reaction conditions for the three primers were 35 cycles of 94°C for 40 sec, 62°C for 45 sec and 72°C for 50 sec. In all PCR reactions, an initial denaturation was performed at 95^0 C for 3 min and after completion of 35 cycles, final extension was carried out at 72^0C for 10 min. The PCR product were analyzed in 2 percent agarose gel containing Gel Red (0.3µg/ml). The one positive PCR products for each gene were sent for sequencing.

RESULTS

Identification of Enterotoxin Genes

A total of 30 Milk samples, raw milk and pasteurized milk samples obtained from local markets and farms of Mashhad were screened for the presence of *B. cereus*. Agarose gel electrophoresis of DNA extraction of milk is shown un Figure 1. 24 samples (80%) were discovered to be positive for one of the three genes of enterotoxin (*hblA*, *hblC, nheB*).

hblA gene specific PCR using the primer pair HblA1/HblA2, yielded an amplified product of 306bp only in 11 samples (36.66 %) (Figure 2). *hblC* gene specific PCR applying the primer pair HblC1/HblC2, produced an amplified product of 492bp only in 7 samples (23.3 %) (Figure 3). The primer pair *nhe*B1 /*nhe*B2, which is specific for *nheB* gene, showed 183 bp amplified products in 17 samples (56.66%) (Figure 4). Also, 33.13% of the milk samples had all three genes. 40% of pasteurized milk samples were contaminated with Bacillus cereus. In addition to that, 80% of raw milk samples were contaminated with Bacillus cereus.

Table 1: Primer used in this study

Gene	Primers	Primer lenght	Product size
nheB	5' ATTCATTAGGACCAGAAGGCTTG 3'	23	183
	5' CATTTCCTCTAGCTGTATCTTGGTG 3'	25	
hblC	5' CAATACTCTCGCAACACCAATCG 3'	23	492
	5' CCATCTGTACCTAATATGCCTTGC 3'	24	
hblA	5' ATTACAGATTTGCGAGGTGA 3'	20	306
	5' ATCCCTTGCTACTCCCACTA 3'	20	

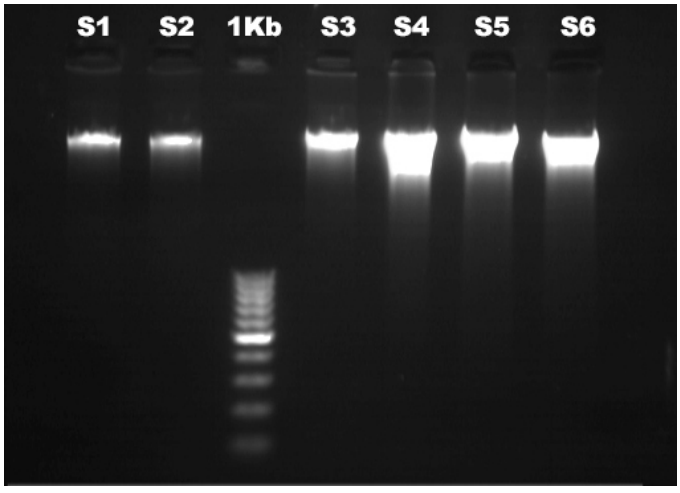

Fig 1: Agarose gel electrophoresis of DNA extraction of milk. Gel Red stained-agarose gel showed good quality of genomic DNA.

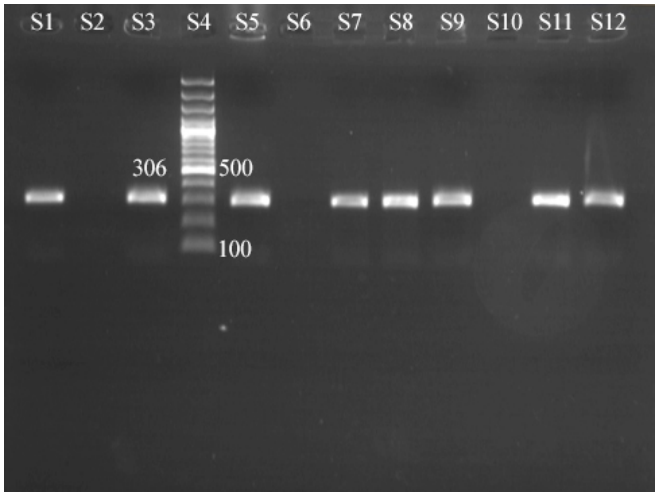

Fig 2: *hblA* gene specific PCR. Lane S1, S3, S5, S7, S8, S9, S11 and S12: Raw milk contaminated by B. cereus, S2: Negative control, S4: M100+ DNA ladder

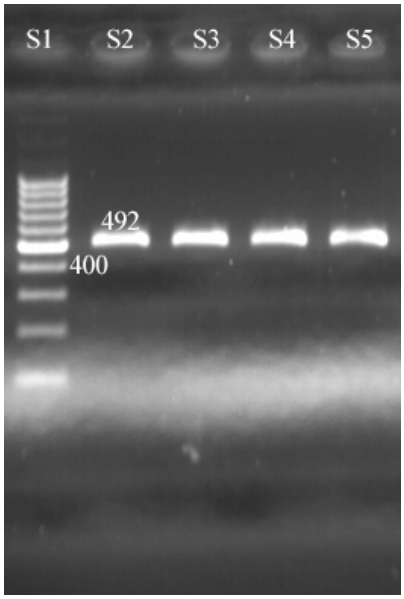

Fig 3: *hblC* gene specific PCR. Lane S1: M100+ DNA ladder, S2-S5: Raw milk contaminated by *B. cereus.*

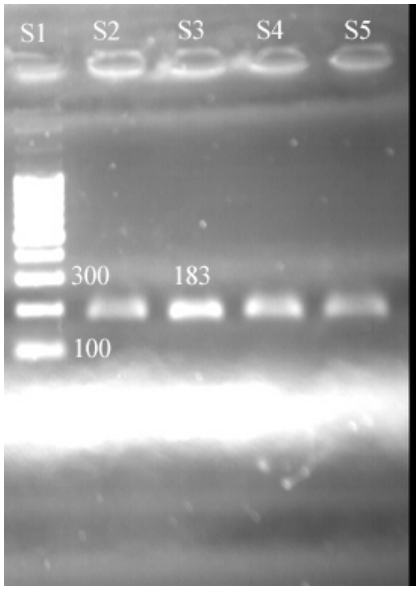

Fig 4: *nheB* gene specific PCR. Lane S1: M100+ DNA ladder, S2-S5: Raw milk contaminated by *B. cereus.*

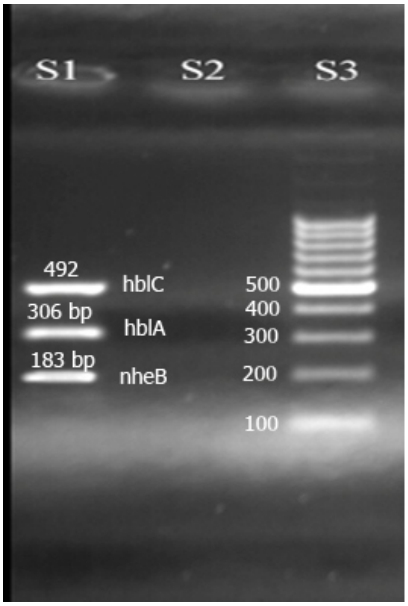

Fig 5: Multiplex PCR electrophoresis for *hblA*, *hblC* and *nheB* genes. Lane: S1: *B. cereus* contain *hblA*, *hblC* and *nheB* genes, S2: Negative control, S3: M100+ DNA ladder.

Most of the positive results were achieved with 1l of extracted DNA in the PCR mixture. At greater and lower amount of DNA template, the number of positive results was lower. There were no differences between the intensity of ethidium bromide- stain bands obtained from the 5 concentrations of the template (from 0.25 to 5 µl) in a PCR mixture of the final volume of 50 µl.

Optimization of Multiplex PCR Kit

The direct multiplex PCR assay technique was applied to examine the level of contamination with *B. cereus* of 30 dairy products. The multiplex PCR results yielded distinctly amplified PCR products, with bands highly conspicuous in gel electrophoresis (Figure 5).

DISCUSSION

The detection limit of *B. cereus* directly from the food samples produced different results, since the matrix of the food influenced the detection effectiveness of the microbial cells using the PCR technique. As well-known, sometimes extracting microbial DNA directly from food, Taq polymerase inhibitory substances present in the sample could decrease the efficiency of the amplification reaction. The high specificity demonstrated by the three couple of primers used in this study towards *B. cereus* (no amplification products were obtained for other microorganisms) allowed us to exploit them for the selective detection of *B. cereus* strains directly in foods. On the basis of different compositions of the food matrices, it was essential to develop several pre-extraction methods for the different foods inspected so as to achieve DNA amplification using the PCR technique. For the DNA extraction from milk it was more effective to clean the pellet applying EDTA solution, and an amplicon was gained down to the level of 20 cells/g of B. cereus (Figure 1).

In this research and others [20] there have been cases of *B. cereus* isolates producing *nhe* in the absence of *nheB*. These results emphasize the polymorphism associated with *nheB*. 6/16 percent of milk samples contained both genes of *hblA* and *hblC*. Das et al (2007) reported that 37 percent of the fish examined contained *Bacillus cereus* and 29 percent of them had the enterotoxin *hblA* [21]. Multiplex PCR results demonstrated the feasibility of employing direct multiplex PCR in detecting foods contaminated with entrotoxigenic *B. cereus*.

CONCLUSION

The Multiplex PCR procedure adopted in order to identify *B. cereus* in milk from cows was examined in contrast to bacterial culture as the standard method. In this study we managed to detect all *B. cereus* strains isolated from infected dairy products and the positive strain by amplification of the *hblA*, *hblC* and *nheB* genes. With the primers used we were not able to amplify and detect DNA from milk samples infected with bacteria apart from *B. cereus*. The significant specificity of the approach was

characterized by several authors. We succeeded in establishing a multiplex PCR for direct detection of three *B. cereus* enterotoxin genes in processed foods by combining both raw milk and pasteurized milk. Nonetheless, a particular threshold bacterial load is necessary for acquiring a positive gene detection. The failure to identify all the PCR products with direct multiplex PCR, at low bacterial counts, could be ascribed to inhibition effects of the *Taq* polymerase by food components. It was perceived that some of the genes would be detected at higher bacterial loads but become undetectable at lower bacterial loads, whereas other genes were detectable at both higher and lower bacterial loads. Genes which were detected at lower bacterial loads were believed to exist in higher copy numbers than those genes that were not detectable at lower bacterial loads; therefore less amplification of some of the genes renders them undetectable in gel electrophoresis.

REFERENCE S

1. Schoeni JL, Wong AC. *Bacillus cereus* food poisoning and its toxins. J. Food. Prot. 2005: 68:636-48.

2. Rahimi E, Abdos F, Momtaz H, Baghbadorani ZT, Jalali M. Bacillus cereus in Infant Foods: Prevalence Study and Distribution of Enterotoxigenic Virulence Factors in Isfahan Province, Iran. Sci. World J. 2013:5:1-5

3. Kramer J, Gilbert R. *Bacillus cereus* and other Bacillus species. In: Doyle, (Ed.) Foodborne Bacterial Pathogens. Marcel Dekker, New York, 1989. P.21-70.

4. Carlin F, Fricker M, Pielaat A, Heisterkamp S, Shaheen R, Salkinoja-Salonen M. Emetic toxin-producing strains of Bacillus cereus show distinct characteristics within the *Bacillus cereus* group. Int J. Food Microbiol. 2006: 109:132138.

5. Banykó J, Vyletelová M. Determining the source of *Bacillus cereus* and *Bacillus licheniformis* isolated from raw milk, pasteurized milk and yoghurt. Lett Appl Microbiol. 2009: 48(3): 318-23.

6. Ne□mečková I, Solichová K, Roubal P, Uhrová B, Šviráková E. Methods for Detection of Bacillus sp., *B. cereus*, and *B. licheniformis* in Raw Milk. Czech J Food Sci. 2011: 55-60.

7. Vijaylakshmi G, Dwarkanath CT, Sreenivasa MV. Studies on *Bacillus cereus* contamination of rice and rice preparation. J. Food Sci. Technol. 1981: 18: 231-234.

8. Valero M, Fernández PS, Salmerón MC. Influence of pH and temperature on growth of *Bacillus cereus* in vegetable substrates. Int J Food Microbiol. 2003: 82(1):71-9.

9. Granum PE, Lund T. *Bacillus cereus* enterotoxins. FEMS Microbiol. Lett. 1997: 157: 223-228.

10. Lund T, De Buyser ML, Granum PE. A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. Mol. Microbiol. 2000: 38: 254-261.

11. Kyei-Poku G, Gauthier D, Pang A, Frankenhuyzen K. Detection of *Bacillus cereus* virulence factors in commercial products of *Bacillus thuringiensis* and expression of diarrheal enterotoxins in a target insect. Canadian J. Microbiol. 2007: 53: 1283-1290.

12. Beecher D, MacMillan J. Characterization of the components of hemolysin BL from *Bacillus cereus*. Infect Immun. 1991: 59: 17781784.

13. Lindback T, Fagerlund A, Rodland MS, Granum PE. Characterization of the Bacillus cereus Nhe enterotoxin. Microbiol. 2004: 150: 3959-3967.

14. Granum PE, Doyle MP, Beuchat LR. Food Microbiology: Fundamentals and Frontiers, ASM press, Washington, D.C. 2007.

15. Moravek M, Dietrich R, Buerk C, Broussole V, Guinebretiere M, Granum PE, Hguyen C, Maertlbauer E. Determination of the toxin potential of *Bacillus cereus* isolates by quantitative enterotoxin analysis. FEMS Microbiol. Lett. 2006: 257: 293-298.

16. Fricker M, Reissbrodt R, Ehling-Schulz M. Evaluation of standard and new chromogenic selective plating media for isolation and identification of *Bacillus cereus*. Int. J. Food Microbiol. 2008: 121: 27 -34.

17. Luna VA, Gulledge J, Cannons AC, Amuso PT. Improvement of a selective media for the isolation of B. anthracis from soils. J. Microbiol. Methods. 2009: 79: 301-306.

18. Ankolekar C, Rahmati T, Labbe RG. Detection of toxigenic *Bacillus cereus* and *Bacillus thuringensis* spores in US rice. Int. J. Food Microbiol. 2009: 128, 460-466.

19. Kwon GH, Lee HA, Park JY, Kim JS. Development of a RAPD-PCR method for identification of Bacillus species isolated from cheonggukjang. Int. J. Food Microbiol. 2009: 129: 282-287.

20. Hansen B, Hendriksen N. Detection of enterotoxic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. Appl. Environ. Microbiol. 2001: 67: 185-189.

21. S. Das, P. K. Surendran, N.Thampuran, PCR-based detection of enterotoxigenic isolates of *Bacillus cereus* from tropical seafood. Indian J Med Res. 316-320 (2009).