Pak. J. Agri. Sci., Vol. 57(3), 887-891; 2020
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
DOI: 10.21162/PAKJAS/20.9246
http://www.pakjas.com.pk

MULTIPLEX PCR BASED DETECTION OF TOXIN PRODUCING *Bacillus cereus* FROM DIFFERENT MILK SAMPLES RETAILED IN PAKISTAN

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Bacillus cereus is a gram positive, spore forming bacteria. It produces hemolytic and non-hemolytic enterotoxins, which lead to diarrhea, food poisoning and self-limiting gastrointestinal track (GIT) infections. The organism occurs worldwide, mostly the outbreaks due to *B. cereus* are linked to fried rice and milk products. The present study focused on detection of hemolytic (*hblA, hblC*) and non-hemolytic (*nheA*) toxin encoding gene subunits of *B. cereus* isolated form different milk samples. A total of 100 milk samples (refrigerated pasteurized, raw, heat treated and packed milk) were tested for the presence of *B. cereus*. Isolates were confirmed by biochemical testing. DNA was isolated through phenol chloroform method and confirmed by gel electrophoresis. For the detection of hemolytic and non-hemolytic subunits multiplex polymerase chain reaction (PCR) was performed. Out of 100 samples, 20% were found positive to *B. cereus* by biochemical testing. Among the 20% positive milk samples 50, 40 and 10% were heat treated refrigerated milk, raw milk and packaged milk, respectively. Results of multiplex PCR showed 100, 30 and 9% positive *nheA*, *hblC* and *hblA* genes subunits, respectively in positive isolates of *B. cereus*. Prevalence of *B. cereus* in milk could be a potential threat for milk consumers. Appropriate pasteurization and storage conditions must be followed to evade the*B. cereus* contamination.
Keywords: *Bacillus cereus*, enterotoxin genes, multiplex PCR.

INTRODUCTION

Bacillus cereus belongs to genus Bacillus that is spore forming rods, aerobic and naturally existent in water, dust, soil and contaminated milk, rice and other food products. *B. cereus*is gram positive, motile and rod-shaped bacteria that produce different enzymes: proteinase, lipase and phospholipases. Bacilli are frequently non-pathogenic, but some species harvest numerous toxins that cause self-limiting GIT infections and food poisoning. *B. cereus* produces mainly two major toxins, emetic and diarrhea genictoxins (Bhunia *et al.*, 2018; Mahmood *et al.*, 2018). *B. cereus being* capable to resist different heat treatments and a psycho-tolerant can limit the quality of both refrigerated and pasteurized dairy products. It is Hazard group 2 organism as defined in the European Legislation (European Commission Council Directive 93/88/EEC). Food poisoning caused by *B. cereus* occurs in two types of illness: emetic and diarrheal syndrome (Dogan *et al.*, 2018; Hameed *et al.*, 2018). Usually *B. cereus* is detected as opportunistic pathogen. *Bacillus* spp. are phenotypically similar to each other, difference is only determined on the basis of genetic comparison (Riaz *et al.*, 2018; Zhou *et al.*, 2008).

First time ever pathogenic strain of *B. cereus* was detected by Hauge and pathogenesis was described as diarrhea, nausea, vomiting and abdominal pain (Hauge, 1955; Memon *et al.*, 2018). It causes food curdling, decline the flavor and quality of food products. Mainly two types of diseases are associated with *B. cereus* pathogenesis, diarrheal and emetic syndrome. Diarrheal type is caused by enterotoxins and emetic toxins cause emetic syndrome. Heat labile toxins are produced in small intestine by vegetative growth of *B. cereus* (Chaikhun-Marcou *et al.*, 2018; Hussein *et al.*, 2015). Heat stable toxins cause cereulide, while heat labile toxins causes diarrhea. Both diseases are self-limiting and insignificant, but the recent studies shows that emetic syndrome caused by *B. cereus* can be severe that lead to death (Fricker *et al.*, 2007). Enterotoxins has two subclasses Hemolytic and non-hemolytic enterotoxins, both are possibly related with diarrheal outbreaks. Through different contaminated food products like burger, rice, pasta, they can cause diarrheal syndrome (Soleimani *et al.*, 2018). Hemolytic and non-hemolytic toxins, enterotoxins T and cytotoxin K are produced by *B. cereus*.

These toxins are termed as enterotoxins due to genetic similarities with toxins related to food poisoning. Non-hemolytic toxins comprises of three subunits *nheA, nheB, nheC*. Hemolytic toxins have three subunits: *hblA, hblC, hblD*. Each subunit is encoded for specific single gene (Abbas *et al.*, 2014).

Milk is proper development medium, for microbes, particularly for the bacteria. Defilement with various microscopic organisms may prompt substantial bacterial load in milk. This habitually occurs in dairy industry. Most of the time, *B. cereus* members of the Bacillaceae family contaminate the milk because of its rottenness capacity and imminent to source of human illnesses (Janstova *et al.*, 2006; Khan *et al.*, 2018). In the form of spores *B. cereus* may contaminate milk and milk products. It is a gram-positive bacterium but during its growth cycle at stationary phase it may appear as gram negative sometimes with the passage of time (Shaheen *et al.*, 2010; Naveed and Anwar, 2018). *B. cereus*is the most important specie related to food poisoning diseases. Its optimum growth conditions range from pH 4.5-9.3, moisture level more than 0.92 and temperature range is very wide from 4 ºC -50 ºC (Drean *et al.*, 2015).

B. cereus is already reported worldwide to cause cytotoxicity in dairy products. Most of the strains grows well at 25-32 ºC and showed high diarrheal food poisoning (Arnesen *et al.*, 2007). Dairy products including butter, milk powder, cheese, ice cream can be the source of food poisoning with the presence of different enzymes (protease, lipase, amylase) produced by the *B. cereus.* These enzymes cause health and spoilage risks in dairy environment (Kumari *at el.*, 2014).

Multiplex PCR sensitive technique is broadly used for the detection of different toxigenic and deteriorative bacteria from different dairy products, milk and other foods (Chiang *et al.*, 2012). This study was conducted to isolate and identify *B. cereus* from various kinds of milk collected form Faisalabad and characterization of isolates on the basis of their hemolytic and non-hemolytic gene subunits.

MATERIALS AND METHODS

***Sample collection*:** Total 100 samples comprising of refrigerated pasteurized, raw, heat treated, and packed milk were collected from District Faisalabad, Punjab, Pakistan.All the samples were collected during spring and winter season in sterile open mouth containers and transported to laboratory under controlled conditions 4-6°C.

Figure 1. The purified streaks of the growth of Beta hemolytic form of *B. cereus.*

***Isolates identification*:** Through the conventional culturing technique milk samples were examined. By spread plate method, 0.1 ml of samples spread on mannitol egg yolk polymyxin B agar (HiMedia™ India. (Bartoszewicz *et al.*, 2008) plates and incubated at 37°C for 12-24 hours. Suspected colonies were stained through Gram's staining. To differentiate from other bacillus species further confirmatory biochemical tests including sugar fermentation test, catalase test, oxidase test and voges-proskauer tests were performed.

***Beta- hemolysis on blood agar*:** Pathogenic*B. cereus* produces extracellular enzymes that lysis the RBC's in 5% sheep RBC's blood agar. Suspected 20 isolates were inoculated on blood agar plates with 5% of sheep RBC's for overnight growth at 37 °C.

***DNA extraction*:** The genomic DNA of isolates were extracted from culture broth by phenol-chloroform method. (Sambrook and Russel, 2006) Albaayit *et al.*, 2020) and confirmed through gel electrophoresis. For the Multiplex PCR reactions 50 ng/µl concentration of DNA was used as template.

***Molecular detection of nheA, hblA, hblC subunits*:** Non-hemolytic subunit *nheA* and hemolytic subunit *hblA, hblC*were investigated by previously known primers (Abbas

Table 1.List of Primers used in Multiplex PCR.

Target Gene	Oligonucleotide sequence (5-3)	Product size (bp)	Annealing temperature	Reference
NheA (non-hemolytic subunit	F: TAC GCT AAG GAG GGG CA R: GTT TTT ATT GCT TCA TCG GCT	499	60	(Abbas *et al.*, 2014)
HbIA (hemolytic subunit)	F: AAG CAA TGG AAT ACA ATG GG R: AGA ATC TAA ATC ATG CCA CTG C	1154	60	
HbIC (hemolytic subunit)	F: GAT ACT AAT GTG GCA ACT GC R: TTG AGA CTG CTC GTT AGT TG	740	60	

et al., 2014) and PCR condition
/s. Pathogenic species of *B. cereus* were confirmed through amplification of hemolytic and non-hemolytic subunits that encodes for specific genes. Primer sequences, annealing temperature, product size and target genes are given in Table 1.

Multiplex PCR master mix was prepared by adding 3µl of 50 ng/µl concentration of template DNA from each sample. For each sample 25µl master mix was used for multiplex PCR reaction. Amplified products were confirmed on 1.5% agarose gel according to the base pairs of the targeted gene.

RESULTS

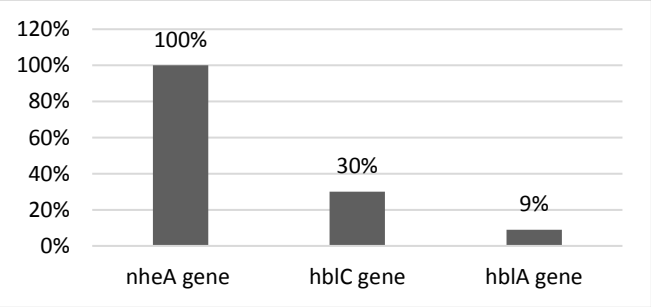
***Prevalence of pathogenic B. cereus associated with hemolytic and non-hemolytic subunits*:**
100 samples encompassed 50% refrigerated pasteurized milk samples, 20% fresh raw milk samples, 10% packed milk of different companies, 20%heat treated milk from tea stalls and milk shops. Highest prevalence of *B. cereus* observed in refrigerated pasteurized milkwas 10% in total positive samples. Table 2 shows prevalence of *B. cereus* in different kind of milk.

In Bacillus genus other species are physiologically related to *B. cereus* through gram staining 30 isolates are confirmed gram positive bacilli.

Further confirmatory biochemical tests were performed that suspects the 20 different milk samples contaminated with pathogenic *B. cereus*.

***Beta-hemolysis of suspected pathogenic B. cereus species*:** All the isolates showed positive Beta- hemolysis on blood agar.

***DNA isolation and confirmation*:** DNA isolated by using phenol chloroform technique and confirmed with gel electrophoresis.



Graph 1. Percentage of hemolytic (*hblC, hblA*) and non-hemolytic (*nheA*) subunit genes in the isolated *Bacillus cereus* from the study samples.

***Molecular detection of Hemolytic and non-hemolytic subunits*:** Multiplex PCR was used for the detection of enterotoxin subunits of pathogenic *B. cereus*. Non- hemolytic subunit *nheA*was100%,*hblC*hemolytic subunit was 30% and 9% *hblA*hemolytic subunit gene was detected.

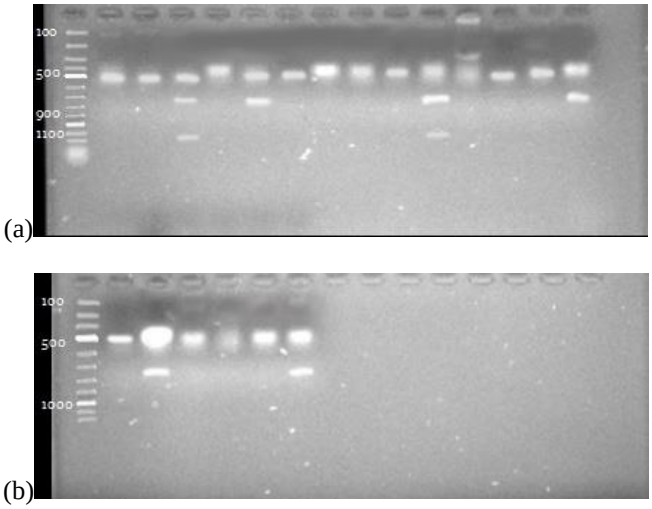


Figure 2. Multiplex PCR results showing different genes amplified in single PCR.
Lane 1= 100+ bp molecular ladder; All the Lanes showing positive results of *nheA* subunit with the band of 499 bp in both (a) and (b) figures. In fig. (a) Lane 4, 6, 11, 15 and in fig. (b) Lane 3 and 7 showing positive result band of *hblC* subunit with 740 bp. Lane 4 and 11 showing the positive result of *hblA* subunit with the 1154 bp band.

DISCUSSION

Refrigerated heated milk and raw milk found to be a source of *B. cereus* with the confirmed enterotoxin producing genes. Multiplex PCR confirmed the primers and PCR conditions reported by Abbas *et al*.(2014). Refrigerated raw and heat-treated milk especially from domestic refrigerators can cause diarrhea and emetic syndrome due to the *B. cereus* spores and it has already been reported by Borge *et al*. (2001) andBanyko *et al*. (2009). In our findings out of 50 % samples taken from refrigerated heated milk highest prevalence of 10% was found

Table 2. Prevalence of suspected pathogenic *B. cereus.*

Type of milk	Sample size	Positive samples	Percentage out of total samples(20/100)
Refrigerated pasteurized milk	50/100	10/50 (20%)	10%
Fresh raw milk	20/100	4/20 (20%)	4%
Heat treated milk	20/100	4/20 (20%)	4%
Packed milk	10/100	2/10 (20%)	4%
	Total sample size =100	Total positive samples = 20	

and all were confirmed with *nhe* hemolytic gene subunit which basically cause diarrheal syndrome. *B. cereus*is one of the most prevalent and food associated strain in Bacillus cereus group mainly isolated from food and dairy products. *B. cereus* was detected in raw cow milk and rice pudding 60% and 55%, respectively by Mohamed *et al.* (2016). Bacillus strains have similarity within their sansulato group which include *B. anthracis*, *B. thuringeinsis*, *B. mycoides*, *B. pseudomycoides* and *B. withenstephanensis,* all these strains have genetic similarities, but *B. cereus* can be differentiated on the basis of enterotoxin genes (Drobniewski, 1993; Senesi *et al.*, 2010) which is done in our study and from suspected 30% samples 20% samples confirmed through multiplex PCR as enterotoxin producing *B. cereus.* As described by Abbas *et al.* (2014) hemolytic and non-hemolytic subunits of enterotoxin gene from milk were *hblA* (9,09%), *hblC* (54.54%), *hblD* (9.09 %), *nheA* (100%), *nheB*(63.63%), *nheC* (54.54%) and in our study all the *B. cereus* strains were positive to non-hemolytic (*nheA)* gene subunit and hemolytic subunit (*hblA* 9% , *hblC* 30%) were detected. As compared to previous findings and our study *B. cereus* present in refrigerated heated milk and raw milk can cause food poisoning and diarrheal syndrome due to presence of enterotoxin genes. Compared to other techniques multiplex PCR proved to be reliable, fast and accurate method for the detection of multiple gene subunits in single reaction.

***Conclusion*:** The present study concludes that presence of *B. cereus* in milk collected from different sources is a potential threat for diarrhea and food poisoning diseases. It is an opportunistic pathogen that harvest hemolytic and non-hemolytic toxins evidenced by multiplex PCR. Appropriate storage conditions and ideal pasteurization must be followed to avoid the contamination of *B. cereus.*

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[Received 25 June 2019: Accepted 19 Dec 2019: Published (online) 08 June 2020]