MULTIPLE PC BASED DETECTION OF TOXIN PRODUCING Bacillus cereus FROM DIFFERENT MILK SAMPLES RETAILED IN PAKISTAN

Azhar Rafique¹,², M. Luqman², Zeeshan Nawaz³, Asma Ashraf³, Shabab Nasir¹, Shaymaa Fadhel Abbas Albaayit¹,², M. Shahid Mahmood², Rao Zahid Abbas², Farhat Jabeen¹, Tayyaba Sultana¹, Salma Sultana¹, Shabana Naz¹ and Farkhanda Asad¹

¹Department of Zoology, GC University Faisalabad, Pakistan; ²Institute of Microbiology, University of Agriculture, Faisalabad Pakistan; ³Department of Microbiology, GC University Faisalabad, Pakistan; ⁴Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq; ⁵Department of Parasitology, University of Agriculture, Faisalabad, Pakistan

*Corresponding author’s e-mail: azharrafique96@gmail.com; shaymaa_albaayit@yahoo.com

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 (hbl, 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 refrigerate 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. Bacillus 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. Bacillus 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 (Fricke 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). Bacillus cereus is the most important specie related to food poisoning diseases. Its optimum growth conditions range from pH 4.5-9.3, moisture >0.92 and temperature range is very wide from 4 °C -50 °C (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 °C 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.

Table 1. List of Primers used in Multiplex PCR.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Oligonucleotide sequence (5-3)</th>
<th>Product size (bp)</th>
<th>Annealing temperature</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NheA (non-hemolytic subunit)</td>
<td>F: TAC GCT AAG GAG GGG CA R: GTT TTT ATT GCT TCA TCG GCT</td>
<td>499</td>
<td>60</td>
<td>(Abbas et al., 2014)</td>
</tr>
<tr>
<td>HblA (hemolytic subunit)</td>
<td>F: AAG CAA TGG AAT ACA ATG GG R: AGA ATC TAA ATC ATG CCA CTG C</td>
<td>1154</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>HblC (hemolytic subunit)</td>
<td>F: GAT ACT AAT GTG GCA ACT GC R: TTG AGA CTG CTC GTT AGT TG</td>
<td>740</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>
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 milk was 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 isolated in pure culture streaks (Figure 1).

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

Molecular detection of Hemolytic and non-hemolytic subunits: Multiplex PCR was used for the detection of enterotoxin subunits of pathogenic B. cereus elaborated in Figure 2. Non-hemolytic subunit nheA was 100%, hblC hemolytic subunit was 30% and 9% hblA hemolytic subunit gene was detected (Graph 1).

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) and Banyko et al. (2009). In our findings out of 50% samples taken from refrigerated heated milk highest prevalence of 10% was found and all were confirmed with nheA 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 sensulato group which include B. anthracis, B. thuringiensis, B. mycoides, B. pseudomycoideus 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.

**REFERENCES**


Bacillus cereus detection by multiplex PCR


