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Toxigenic Profiling of Enterotoxin-Producing *Bacillus cereus* Isolated from Marketed Raw Chicken Meat and Human Subjects by Triplex and Multiplex PCR

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ABSTRACT

Bacillus cereus incorporates the most important group of endospore-forming micro organism and can cause emetic and diarrheal food poisoning. A total of 42 *B. cereus* strains isolated from marketed raw chicken meat and human subjects swab samples were assessed by a triplex and multiplex PCR for the presence of enterotoxin genes. The detection rate of *nheB*, *hblA*, *hblD*, *cytK, nheA,CER*, *hblC* and *entFM* enterotoxin genes among all *B. cereus* strains was 83.33%, 80.95%, 69.04%, 21.42%, 47.61%, 0%, 61.90%, and 92.85% respectively. Enterotoxigenic profiles were determined in enterotoxin-producing strains showed 19 different patterns. The results offer essential information on toxin genes prevalence and toxigenic profiles of *B. cereus* from sources of origin. The present study was taken into consideration about extreme fitness danger for public health and insuring extra ability in difficulty to food safety amongst all *B. cereus* group members. Also, there may be need for extensive and continuous tracking of food products embracing both emetic toxin and enterotoxin genes.

Keywords: *Bacillus cereus*, enterotoxigenic profiling, PCR, enterotoxin, marketed, Chicken, Meat

Members of the genus *Bacillus* are well known for their great diversity and widespread distribution in nature (Oh *et al.,* 2012) and these bacteria *Bacillus cereus* group (*B. cereus sensulato*) consisting of the genetically closely related species *B. cereus*, *B. thuringiensis*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides*, and *B. weihenstephanensis* is widely recognized as the causative agent of food borne illness (Helgason *et al.,* 2000; Kim *et al.,* 2012; Jimenez *et al.,* 2013). Due to its wide distribution in the environment and ability to produce spores, the Gram-positive, rod-shaped, opportunistic human pathogen grow well in wide adverse environmental condition, the pH ranges from 4.5 to 9.3, with high water activity 0.92 and the temperature ranges for growth from 4ºC to 50ºC (Kramer and Gilbert, 1989). Food borne illness resulting from consumption of *B. cereus* contaminated food may result in diarrheal and emetic type syndromes (Kim *et al.,* 2010; Sandra *et al.,* 2012).

The diarrheal food poisoning is caused by heat-labile protein enterotoxins produced during favorable vegetative growth of *B. cereus* in the small intestine with non-haemolytic enterotoxin (*nhe*), enterotoxin FM (*entFM*), Haemolysin BL (*hbl*) and cytotoxin K (*cytK*) are of the highest importance and therefore often used for the detection of enterotoxic strains (Kim *et al.,* 2012; Park *et al*., 2009).

The emetic food poisoning occur due to emetic toxin (cereulide) is a small cyclic peptide (dodecadepsipeptide), which is heat and acid stable, induces swelling of mitochondria in Hep-2 cells, respiratory distress, and occasional loss of consciousness possibly leading to coma and ultimately death of individual (Ladeuze *et al.,* 2011).

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The heat stable emetic toxin can withstand temperatures of up to 121°C for 90 min. The symptoms usually include nausea, vomiting and stomach pain, which occur 1-5 hrs after food ingestion and can easily be misdiagnosed with *Staphylococcus aureus* food poisoning (Kim *et al.,* 2011; Kumar *et al*., 2017; Kumar *et al*., 2018).

The *B.cereus* is present in starch rich foods such as rice, nutrient enriched foods of animal origin like meat, milk, dairy products and chicken meat and desserts (Jay, 2005). In India, prevalence of *B.cereus* has been reported from foods like milk (Garg *et al.,* 1977; Chopra *et al.,* 1980), meat (Bacchil and Negi, 1984; Bacchil and Jaiswal, 1988), chicken (Tahmasebi *et al.,* 2014; Aklilu *et al.,* 2016) and various other foods (Kamat *et al.,* 1989; Meena *et al.,* 2000). Therefore, it is of significant quality food safety issues, medical and economic importance and several methods have been developed for its detection. These methods include a wide range of approaches including but not limited to conventional culture methods (Fricker *et al.,* 2008; Kumar *et al*., 2011), biochemical and morphological tests (Fernandez-No *et al.,* 2011; Kumar *et al*., 2009), and a variety of molecular approaches using toxin or other appropriate genes of *B. cereus* group species as target (Kim *et al.,* 2012). Recently, molecular diagnostic tools mainly focus on the toxin genes since they are broadly distributed within the *B. cereus* group (Wehrle *et al.,* 2010) and the toxin gene profiles might be better than the exact species for the outbreak investigations (Ehling-schulz and Messelhausser, 2013) and assessment of toxin genes prevalence in wild *B. cereus* isolates would help controlling these potential pathogens in food industry (McKillip, 2000).

Further more, such assessments would be valuable to determine the genetic diversity of the species leading to a broader ecological view (Oh *et al.,* 2012; Kumar *et al*., 2014; Kumar *et al.,* 2018). Therefore, there have been some studies investigating the toxigenic diversity of *B. cereus* isolated from different sources. There is restricted information available in comparison to the enterotoxic strains. Accordingly, the reasons of this study became to estimate the virulence pathogenic capacity of *B. cereus* through determining the toxigenic profiles of line strains and investigate the relationship between isolation source and toxigenic profiles, compare toxigenic pattern and compare the results with previous studies to have a better insight on the topic studied.

MATERIALS AND METHODS

Bacterial strains

The toxigenic profiling of enterotoxin-producing *B. cereus* strains used in this study were isolated from marketed raw chicken, swabs of human handlers, slaughtering equipments during eleven months (from June-2017 to March-2018) period. A total of 42 strains were isolated from 280 processed samples.

DNA extraction

All *B. cereus* strains were grown on nutrient agar (HiMedia Pvt. Ltd.) slants at 37 °C for 24 hrs. A single colony was inoculated in Luria-Bertani broth (HiMedia) and incubated at 37 °C for 24 hrs. After recommended incubation, the total genomic DNA of individual isolates was extracted by using *mericon* DNA Bacteria plus Kit (Qiagen) according to the manufacturer's instructions with some modification. The quality, purity and concentration of isolated DNA were determined by NanoDrop™2000/2000c Spectrophotometers as per methods described by Sambrook and Russel (2001).

Thermal cycling condition and primers

Table 1: Triplex PCR primers pairs used for the toxigenic profiling of *B. cereus*

Sl. No.	Target Genes	Primer sequence (5'→3')	Product Size	Reference
1	NheB	**F:** GTG CAG CAG CTG TAG GCG GT **R:** ATG TTT TTC CAG CTA TCT TTCGCA AT	328 bp	(Yang *et al*., 2005)
2	HblA	**F:** ATT AAT ACA GGG GAT GGA GAAACT T **R:** TGA TCC TAA TAC TTC TTC TAGACG CTT	237 bp	
3	HblD	**F:** AGA TGC TAC AAG ACT TCA AAGGGA AAC TAT **R:** TGA TTA GCA CGA TCT GCT TTCATA CTT	436 bp	

The primers show in Table 1 was used in triplex PCR study. Triplex PCR was conducted using a thermal cycler (Bio-

Table 2: Multiplex PCR primers pairs used for the toxigenic profiling of *B. cereus*

Sl. No.	Target Genes	Primer sequence (5'→3')	Product Size	Reference
1	CytK	**F:** TGCTAGTAGTGCTGT AACTC **R:** CGTTGTTTCCAAC CCAGT	881 bp	(Forghani *et al*., 2014)
2	NheA	**F:** GGAGGGGCAAACAGAA GTGAA **R:** CGAAGAGCTGCTTC TCTCGT	750 bp	
3	CER	**F:** GCGTACCAAATCA CCCGTTC **R:** TGCAGGTGGCACAC TTGTTA	546 bp	
4	HblC	**F:** CGCAACGACAAATC AATGAA **R:** ATTGCTTCACGAGC TGCTTT	421 bp	
5	EntFM	**F:** AGGCCCAGCTACATA CAACG **R:** CCACTGCAGTCAAAA CCAGC	327 bp	

Rad S1000^{TM} thermal cycler, Sweden), and amplification conditions were as follows: initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 45sec and final extension at 72°C for 7 min. Each reaction tube (25 μL) contained 20 pM of *nheB, hblA and hblD* primers, and 6.3 ml nuclease free distilled water and 5 ml of DNA template. Amplified products were electrophoresed on 2 % agarose gel and viewed under UV light and documented by gel documentation system (Bio-Rad Gel Doc^{TM} XR+ Gel Documentation System, Sweden), using Lab image computer software. A 2 ml DNA molecular weight marker (Gene Ruler^{TM}, 100 bp DNA ladder and O GeneRuler100bp Plus DNA Ladder, Thermo Scientific) was used as molecular weight marker.

The primers used in multiplex PCR mention in Table 2. Multiplex PCR was conducted using a thermal cycler (Bio-Rad S1000^{TM} thermal cycler, Sweden) and amplification conditions were as follows: initial denaturation at 95°C for 10 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 5 min. Each reaction tube (25 μL) contained 20 pM of *cytK, nheA, CER, hblC, entFM* primers, and 5.5 ml nuclease free distilled water and 5 ml of DNA template and amplified products were electrophoresed same as triplex PCR.

Statistical analysis

The data obtained was analyzed and expressed as means ± standard deviations (SD) and compared using chi-square test in IBM®SPSS® software (version 20.0 for Windows, SPSS Inc.) for distribution of toxin genes in *B. cereus* isolated from various sources and their significance of difference was defined at $p \leq 0.05$.

RESULTS AND DISCUSSION

The *B. cereus* strains investigated in this study isolated from different sources did not reveal the emetic toxin-producing strains. These results were in good agreement with the previous studies (Altayar and Suther, 2006). High prevalence of emetic toxin-producing strains in starchy foods such as rice and rice derived products was reported compared to other food sources (Schoeni and Wong, 2005) and that the emetic toxin-producing strains carry enterotoxin genes (Kim *et al.,* 2009; Kim *et al.,* 2010). The *entFM and nheB* were the 1st and 2nd major toxin genes in enterotoxin strains. This result was in good agreement with the reports of Tewari *et al.* (2015) and Forghani *et al.* (2014).

All isolates produced *B. cereus* specific PCR product of 400bp for *groEL* gene and 475bp for *gyrB* gene on Agarose gel (Fig. 1). The isolates were screened by multiplex and triplex PCR for the presence of different enterotoxin genes having the predicted size of 237bp, 436bp, 328bp (by triplex PCR), 750bp, 421bp, 327bp and 881bp (by multiplex PCR) for *hblA*, *hblD*, *nheB*, *nheA*, *hblC*, *entFM* and *cytK*, respectively (Fig. 2 and 3).

The three *nhe* (*nheA, nheB, nheC*) and *hbl* (*hblA, hblC, hblD*) genes exist in 2 separate operons ensuing the synthesis of the *nhe* and *hbl* toxin protein complexes,

Table 3: Different toxigenic gene profiling of total 42 *B. cereus* isolates

Pattern	*entFM*	*nheA*	*cytK*	*CER*	*hblC*	*nheB*	*hblA*	*hblD*	Nr (%) of *B. cereus* Isolates
I	+	+	-	-	+	+	+	+	9 (21.42)
II	+	+	+	-	+	+	+	+	4 (9.52)
III	+	-	-	-	+	+	+	+	4 (9.52)
IV	+	-	-	-	-	+	+	+	3 (7.14)
V	+	-	+	-	+	+	+	+	3 (7.14)
VI	+	-	+	-	-	+	+	+	2 (4.76)
VII	+	+	-	-	-	-	-	-	2 (4.76)
VIII	+	-	-	-	-	-	-	-	2 (4.76)
IX	+	-	-	-	-	+	-	-	2 (4.76)
X	+	-	-	-	+	+	+	-	2 (4.76)
XI	-	-	-	-	-	+	+	-	1 (2.38)
XII	-	-	-	-	+	+	+	+	1 (2.38)
XIII	-	+	-	-	+	+	+	+	1 (2.38)
XIV	+	-	-	-	-	+	+	-	1 (2.38)
XV	+	+	-	-	+	+	-	+	1 (2.38)
XVI	+	+	+	-	+	+	+	-	1 (2.38)
XVII	+	-	-	-	-	-	+	-	1 (2.38)
XVIII	+	+	-	-	-	-	-	+	1 (2.38)
XIX	+	+	-	-	-	-	+	-	1 (2.38)

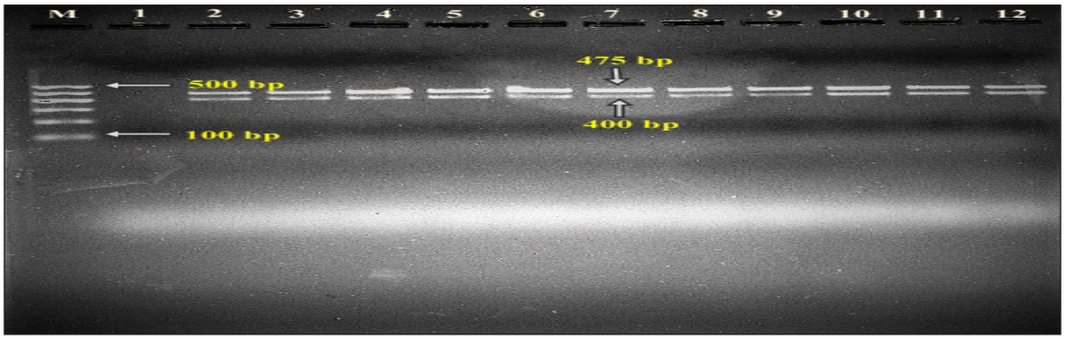

Fig. 1: Agarose gel showing PCR amplified product of 400bp for *groEL* gene in *B. cereus* group and 475bp for *gyrB* gene in *B. cereus* isolates. **Lane M:** 100bp DNA ladder, **Lane 1:** Negative control (Reagent), **Lane 2:** Positive control (MTCC 25061), **Lane 3-12:** Isolates of *B. cereus* from chicken meat and human subjects origin

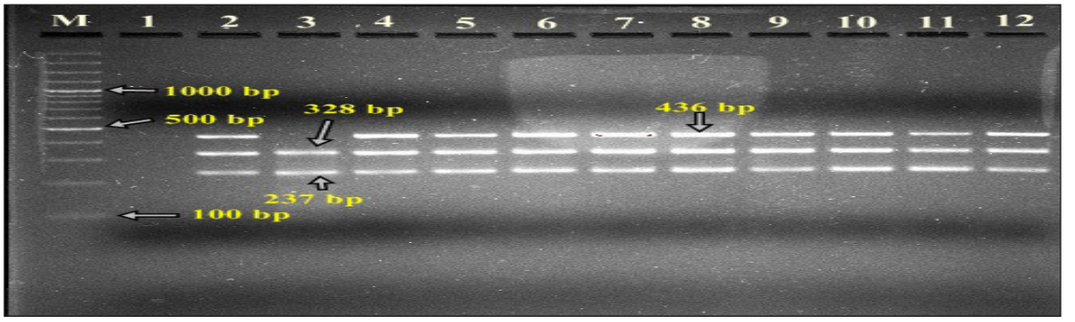

Fig. 2: Agarose gel showing triplex PCR amplified product of 237bp for *hblA*, 436bp for *hblD*, and 328bp for *nheB* gene in *B. cereus* isolates. **Lane M:** 100bp plus DNA ladder, **Lane 1:** Negative control (Reagent), **Lane 2, 4-12:** Samples positive for gene *hblA*, *hblD* and *nheB,* **Lane 3:** Samples positive for *hblA* and *nheB.*

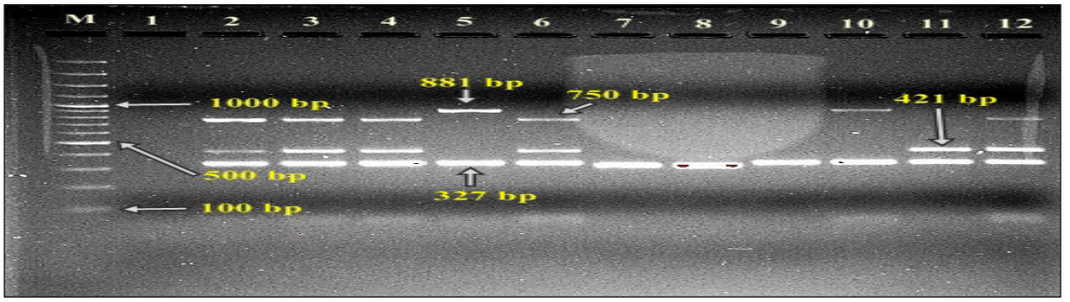

Fig. 3: Agarose gel showing multiplex PCR amplified product of 881bp for *cytK*, 750bp for *nheA*, 421bp for *hblC* and 327bp for *entFM* gene in *B.cereus* isolates. **Lane M:** 100bp plus DNA ladder, **Lane 1:** Negative control (Reagent), **Lane 2, 3, 4, 6, and 12:** Samples positive for *nheA*, *hblC* and *entFM* gene, **Lane 5, 10:** Samples positive for *cytK* and *entFM* gene, **Lane 7, 8, 9:** Samples positive for *entFM* gene, **Lane 11:** Samples positive for *hblC* and *entFM* gene

respectively (Granum *et al.,* 1999). However, the presence of all three genes is not essential for the activity of the toxins, however co-presence of other two genes will bring about maximal virulence (Lindback *et al.,* 2004). In addition, the results obtained in the works of Seong *et al.* (2008), Molva *et al.* (2009) and Kim *et al.* (2010) showed that *nheA, B* and *C* as well as *hblC, D* and *A* genes were always present at the same time in the *B. cereus* strains. In another study Ngamwongsatit *et al.,* 2008 reported co existence of *nhe* and *hbl* in their respective operons. Based on Rosenquist *et al.* (2005), Park *et al.* (2009), Wehrle *et al.* (2010) and others studies, the best used one out of the three genes of every operon for the detection of *nhe* or *hbl* toxin in *B. cereus* strains. As a result, *nheA, nheB, nheC* and *hblA, hblC, hblD* genes had been selected from the three general genes of *nhe* and *hbl* coding operons, respectively, as a way to put extra effort on investigating a higher wide variety of strains and a numerous pool of isolation resources, which might bring about presenting more useful facts to have a higher perception into *B. cereus* food poisoning.

The distribution of toxic genes among the 42 toxin producing strains *B. cereus* isolates from different sources was divided into 19 different patterns of toxigenic profiles. The pattern I (9 strains, 21.42%) was found to be the major pattern containing the 6 toxin genes except *CER* and *cyt*K followed by pattern II (4 strains, 9.52%), which contained the entire 7 toxin gene except *CER* and Pattern III (4 strains, 9.52%), which carried *entFM, hblC, nheB, hblA*, and *hblD* genes. Four patterns showed the presence of four toxin genes *entFM*, *nheB, hblA*, and *hblD.* Pattern V (3 strains, 7.14%) was containing the 6 toxin genes except *nheA* and *CER.* Pattern VI (2 strains, 4.76%), which contained five toxin genes except *nhe*A, CER and *hblC*. Pattern VII (2 strains, 4.76%), which only possessed two toxin genes *entFM* and *nheA* followed by pattern VIII (2 strains, 4.76%), which carried only *entFM* gene; all other pattern contained two or more virulence genes. The analyzed toxic gene profiles of all 42 strains of *B. cereus* isolates were presented in Table 3 and in Fig. 4.

Despite the emetic strains, Pattern I (9 strains, 21.42%) representing the major toxigenic pattern among the enterotoxic strains contained the six enterotoxin genes together except *cytK* (Table 3). This result was in good agreement with the works of Tewari *et al*. (2015) and Forghani *et al.* (2014). The prevalence of single enterotoxin genes was not high among the enterotoxic strains but still more frequent compared to the earlier reported emetic strains (Guinebretiere *et al.,* 2002; Kim *et al.,* 2010; Wehrle *et al.,* 2010).

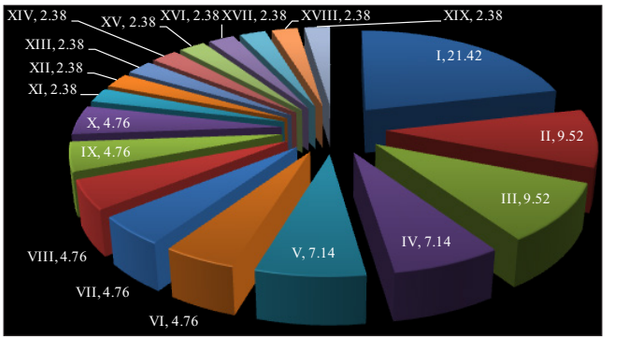

Fig. 4: Pie chart showing toxigenic profiling of amplified potential virulence genes in 42 *B. cereus* isolates

Table 4: Summary of toxin genes distribution of *Bacillus cereus* isolates from raw chicken meat and human subjects

Toxin gene	Organ wise raw chicken meat samples								Swab samples		
	Thigh muscle n=8	Breast muscle n=4	Wing muscle n=4	Rib muscle n=2	Neck muscle n=4	Heart portion n=3	Liver portion n=4	Gizzard portion n=1	Chicken meat handler n=5	Butchers-knife n=3	Chopping board n=4
entFM	7(87.5%)	4(100%)	4(100%)	2(100%)	3(75%)	3(100%)	4(100 %)	1(100%)	4(80%)	3(100%)	4(100%)
nheA	6(75%)	3(75%)	ND	2(100%)	3(75%)	1(33.33%)	1(25%)	ND	ND	2(66.66%)	2(50%)
cytK	4(50%)	ND	1(25%)	1(50%)	ND	2(66.66%)	1(25%)	ND	ND	ND	ND
CER	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
hblC	6(75%)	4(100%)	3(75%)	2(100%)	3(75%)	3(100%)	3(75 %)	ND	2(40%)	ND	1(25%)
nheB	8(100%)	4(100%)	4(100%)	2(100%)	4(100%)	3(100%)	4(100 %)	1(100%)	3(60%)	ND	2(50%)
hblA	8(100%)	3(75)	4(100%)	2(100%)	4(100%)	3(100%)	3(75%)	1(100%)	3(60%)	1(33.33%)	2(50%)
hblD	8(100%)	4(100%)	4(100%)	2(100%)	3(75%)	2(66.66%)	2(50%)	ND	2(40%)	1(33.33%)	1(25%)
χ^2	33.38 (p=1.81)	25.01** (p=0.00)	25.60** (p=0.00)	12.71 (p=0.07)	19.20** (p=0.00)	14.31* (p=0.04)	15.74* (p=0.02)	08.00 (p=0.33)	15.38* (p=0.03)	14.31* (p=0.04)	12.80 (p=0.07)

Note: ** = Highly Significant at p<0.01, * = Significant at p<0.05, ND = Not detected, n = No. of positive sample, χ^2 = Chi-Square, p = Probability value, % = Percentage.

Tewari *et al.,* 2013). The high variety of toxigenic profiles among strains reconfirmed the progressive emergence of novel toxin gene profiles, confronting food industry and food microbiology laboratories with novel hazards (Thorsen *et al.,* 2006; Chon *et al.,* 2012; Ehling-schulz and Messelhausser, 2013).

The summarized the distribution of enterotoxin genes in *B. cereus* isolated from various sources and their significance of difference in Pearson Chi-Square is (p< 0.05) describe in Table 4. The results indicated that *entFM* was the most prevalent potent enterotoxin gene regardless of the isolation bacterium with a prevalence of 75% to 100%. The second most frequent enterotoxin gene among all bacterium was *nheB,* showing the highest prevalence in all samples wise raw chicken meat isolates (100%) and the lowest in swab sample of chopping board isolated strains (50%). It was also more prevalent than the other enterotoxin genes (*nheA*, *cytK* and *hblC*) in every individual sample wise raw chicken meat isolated bacterium. The *nheA, cytK* and *hblC* genes showed a much higher range of diversity in their prevalence among different isolation as compared to the 3 afore mentioned enterotoxin genes (*nheB, hblA and hblD*). The *nheA* gene showed a similar prevalence of more than 50% in swab samples isolates from chopping board and butchers knife, thigh muscle, breast muscle, rib muscle, neck muscle, while its prevalence was as low as 25% in liver portion isolates. A similar phenomenon was observed for *cytK* gene being present in only 25% of liver portion samples isolates and wing muscle, while it showed a much higher prevalence of 66.66% in heart portion and 50% in thigh muscle and rib muscle bacterium. The *hblC* gene showed more than 75% prevalence in thigh muscle, breast muscle, wing muscle, rib muscle, neck muscle, heart portion and liver portion isolates, while its prevalence was as low as 40% and 25% in human hand swab and swab of chopping board isolated bacterium, respectively. The *CER* emetic gene was not detected in all the isolates.

The *entFM* was always the most prevailing toxin gene among all strains, regardless of isolation source. This result was in good agreement with numerous previous studies (Tewari *et al.,* 2015; Forghani *et al.,* 2014). The *nhe* genes are widely accepted as the most frequent enterotoxin genes and their presence with a lower frequency than any other enterotoxin has rarely been reported (Martınez-Blanch *et al.,* 2009). In present study the *nheB* gene was the 2nd major gene among strains and showed a much higher prevalence among the enterotoxin-producing strains (Table 3). The *hblA* and *hblD* were the 3rd and 4th prevailing genes, respectively.

The presence of *entFM* as the major enterotoxin after *nhe* (Ngamwongsatit *et al.,* 2008; Seong *et al.,* 2008; Kim *et al.,* 2009; Tewari *et al.,* 2013) has previously been reported. However, Park *et al.* (2009) and Kim *et al.* (2011) reported *hbl* as the 2nd major enterotoxin with 84% and

90% prevalence, respectively. The findings in this study as well as a few more recent reports suggest that *entFM*, *Nhe*, *Hbl* and *CytK* which is implicated in adhesion, biofilm formation, and virulence (Tran *et al.,*2010) should be considered as a main enterotoxin in *B. cereus***.**

CONCLUSION

In conclusion, results obtained in the present study revealed that *B. cereus* might be a serious health hazard due to its ubiquity, high prevalence of pathogenic strains and that they may harbor toxin genes regardless of their origin. The *entFM* and *nheB* genes were the major enterotoxin genes found among a high number of *B. cereus* strains. Present study investigated a wider range of *B. cereus* sources leading to a broader insight on the matter. Isolates from different sources occasionally showed different patterns of gene prevalence. Also, future studies may identify strains up to species level followed by toxigenic profiling in order to investigate the relationship between species type and pathogenic potential.

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