Original Article

Mahidol University Journal of Pharmaceutical Sciences 2014; 41 (2), 22-30

Detection of Hemolysin BL Gene of *Bacillus cereus* Isolates

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Abstract

Hemolysin BL (HBL) is the multi-component enterotoxin causing diarrheal type of *Bacillus cereus* food poisoning. The *hbl* operon of *B. cereus* was divided into 4 coding sequences (CDS) and identified as *hblC, hblD, hblA* and *hblB* genes consisting of 1,319, 1,221, 1,128 and 1,401 nucleotides encoding proteins of 439, 406, 375 and 466 amino acids with predicted sizes of 49.3, 43.8, 41.7 and 52.3 kDa, respectively. One hundred of *B. cereus* isolates were detected for the presence of each *hbl* gene by multiplex PCR and HBL production by discontinuous hemolysis on HBL sheep blood agar and *Bacillus cereus* Enterotoxin-Reverse Passive Latex Agglutination (BCET-RPLA) test kit. The results showed that 47 of 100 *B. cereus* isolates (47%) were detected by multiplex PCR with all *hbl* genes and gave positive result with RPLA test. Forty-one of 47 isolates (87.2%) produced discontinuous hemolysis on HBL sheep blood agar. However, this pattern was not observed clearly in 6 of 47 isolates (12.8%). In addition, *B. cereus* isolate E82/44 gave multiplex PCR positive with only 3 *hbl* genes, *hblA, hblC* and *hblD*, also showed positive result with RPLA test and produced discontinuous hemolysis.

Keyword: *Bacillus cereus*, Hemolysin BL, Enterotoxin, Food poisoning.

INTRODUCTION

Bacillus cereus is a Gram-positive, facultatively anaerobic, spore-forming rod normally present in soil, air, dust, water and common contaminant in many raw and processed foods including rice, spices, eggs, vegetables, meat and dairy products[1]. Vegetative cells of *B. cereus* are eliminated by heat treatments but their spores can survive and are extremely resistant to different environmental stresses[2]. They should be considered as a critical point of hygienic importance for processes involving heat treatments that may activate the spores and kill the competitive microflora, since *B. cereus* vegetative cells grow well in the absence of competing organisms[3]. Consumption of food contaminated with *B. cereus* or their toxins can lead to food poisoning illnesses. *B. cereus* is the causative agent of two different types of food poisoning, the emetic and diarrheal syndromes[4]. The emetic syndrome is occurred by directly ingestion of heat stable toxin, the emetic toxin (cereulide), produced by growing cells in food resulting in vomiting a few hours after ingestion[5]. The diarrheal syndrome is probably occurred by several heat labile enterotoxins produced during vegetative growth of *B. cereus* in small intestine resulting in enterotoxicosis, abdominal pain and diarrhea after incubation for 8-16 h[6]. At the present, only three enterotoxins have been shown to be involved in food poisoning including hemolysin BL (HBL), non-hemolytic enterotoxin (NHE) and cytotoxin K (CytK). While there are no any report about this symptom for the remaining two, enterotoxin T (BceT) and enterotoxin FM (EntFM)[7].

Hemolysin BL (HBL), a three-component enterotoxin consists of a binding component B and two lytic components L_1 and L_2, encoded by *hblA, hblD* and *hblC* genes, respectively, and is considered as a primary virulence factor in diarrheal type

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for its ability to cause fluid accumulation in rabbit ileal loops. All three components are required for enterotoxic and hemolytic activity. Function of B´ protein encoded by *hblB* gene, which shows high sequence homology to *hblA* gene, is still unclear. HBL toxin exhibits hemolytic and dermonecrotic activities, increases vascular permeability and is toxic to Chinese hamster ovary cells[8]. Beta hemolysis surrounding colonies on blood agar is a diagnostic character of *B. cereus*[9]. On specific medium designated HBL agar, the hemolytic pattern surrounding HBL-producing strains is discontinuous and therefore diagnostic of hemolysin BL[10].

There are two major molecular techniques applied in the detection of HBL-producing *B. cereus* isolates, PCR-based and hybridizaton-based techniques. PCR-based method has been used extensively for all of the HBL-encoding genes. However, the presence of these toxin genes does not necessarily indicate that whether or not this bacterium is able to produce HBL enterotoxin and cause disease[11]. Detection of *B. cereus* with hybridization is mainly performed to confirm the results obtained with specific PCR, as well as, in the detection of HBL enterotoxic *B. cereus*[12]. The entire HBL enterotoxin complex can be demonstrated by HBL sheep blood agar[13]. However, this method is not widely used and a correlation between the results of the HBL plate and the PCR tests could not be observed for the strains that contained *hbl* genes[14]. Furthermore, there is only one available commercial *Bacillus cereus* Enterotoxin-Reverse Passive Latex Agglutination (BCET-RPLA) test kit of Oxoid, which commonly used to detect L_2 part of the HBL toxin[15]. This is noticeable that this method performed based upon the specificity of some part of HBL. Application of this assay in detection of *B. cereus* HBL in Thailand may be result in false-negative, due to the DNA sequence variation among *B. cereus* species between local isolates and standard strain. Therefore, the *hbl* genes from *B. cereus* isolated in Thailand should be investigated and compared with *B. cereus* ATCC 14579.

Objectives of this study were to detect HBL encoding gene profiles and HBL production by *B. cereus* isolates. The results from this study are useful for further study to develop a rapid test system for detection of HBL-producing *B. cereus* isolates.

MATERIALS AND METHODS

Bacillus cereus

All of *B. cereus* isolates used in this study were used for detection the presence of *hbl* genes by multiplex polymerase chain reaction and the production of HBL enterotoxin by HBL blood plates and BCET-RPLA test kit. Eight *B. cereus* isolates were isolated by Boonchaisuk[16]. Ninety *B. cereus* isolates were kindly provided by Division of Food Analysis, National Institute of Health (Department of Medical Science (DMSC), Ministry of Public Health, Thailand).

B. cereus ATCC 14579 which was kindly provided by Dr. Daniel Zeigler (Bacillus Genetic Stock Center, The Ohio State University, Columbus, USA) was used as a positive control. *B. cereus* NC 1291 clinical isolate , which was reported that it gave cereulide positive results with Hep-2 cell vacuolation test and mitochondria respiratory assay, was kindly provided by Prof. Norio Agata (Nagoya City Public Health Research Institute, Japan). *B. cereus* INRA C15 food borne isolate, which was reported to give positive results in PCR reaction with *hbl, nhe* and *cytK* genes, was kindly provided by Marie-Helene Guinebretie`re (Institute National de la Recherche Agronomique, France).

Culture condition

For DNA preparation, *B. cereus* isolates were grown in Luria-Bertani (LB) broth at 37°C for overnight with continuous shaking. Then 1% of the overnight culture was inoculated in LB broth and further incubated at 37°C for 3 h with shaking to collect cells in mid-exponential phase.

Genomic DNA preparation

Genomic DNA of *B. cereus* used as template for polymerase chain reaction (PCR) was isolated by phenol-chloroform extraction method[17].

Detection of Hemolysin BL genes by multiplex PCR

The *hbl* enterotoxin gene profiles of one hundred *B. cereus* isolates were detected by multiplex PCR. The primers HD1 F - HD1 R, HB1 F - HB1 R, HC2 F - HC2 R and HA3 F - HA3 R gave a product size of 807, 986, 884 bp and 622 bp, were applied to detect *hblD, hblB, hblC* and *hblA* genes in multiplex PCR, respectively. The sequences of primers were shown in the Table 1. PCR primers HD1 F - HD1 R and HA3 F - HA3 R were selected to amplify the fragment of *hblD* and *hblA* genes of *B. cereus*[14]. The other primers, HB1 F- HB1 R and HC2 F-HC2 R, were designed based on the known sequence of *B. cereus* A6 and the *hbl* operon sequence GenBank accession number AJ237785 of *B. cereus* ATCC 14579 reported by Økstad *et al*.[18]

Total DNA was extracted by boiling method as described by Hansen and Hendriksen[19]. A 25 µl of PCR mixture consisted of 200 µM of each dNTP, 3 mM $MgCl_2$, 200 nM of each primer, excepted for 400 nM of HC2 F-HC2 R primers, 1 unit of DyNazyme™ II DNA polymerase (Finnzymes, Finland) and 1X Mg^{2+}-free DyNAzyme™ II buffer containing 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl and 0.1% Triton® X-100. The volume of PCR mixture was adjusted with nuclease free water.

Throughout the investigation, PCR analysis of non-hemolytic enterotoxin A (*nheA*) gene gave a 500 bp product with primers NheA F (5′- TACGCTAAGGAGG GGCA-3′) and NheA R (5′- GTTTTTATT-GCTTCATCGGCT -3′)[19] was used as a control of DNA quality. Genomic DNAs of *B. cereus* ATCC 14579, prepared by boiling method and phenol-chloroform method, were also used as control of experimental procedure and reagent quality. The PCR reaction was carried out with a pre-heat at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 5 min. Amplified products were analyzed by 1.5% agarose gel electrophoresis.

Table 1. PCR primers for detection of *hbl* genes of *B. cereus* isolates

Target gene	Primer codes	Sequence (5'-3')	Predicted size (bp)
hblA	HA3 F[a]	ACG AAC AAT GGA GAT ACG GC	622
	HA3 R[a]	TTG GTA GAC CCA AAA TAG CAC C	
hblB	HB1 F	TCC AGA TGT GAG TTT TGA AGG A	986
	HB1 R	GCG CAT TTG TTC CAG TCT TT	
hblC	HC2 F	TCG AAG TGA GAG GTA AGT AAA ATC C	884
	HC2 R	GCA GCC GGT TCA ATA ATT TC	
hblD	HD1 F[a]	ATA TTC ACC TTA ATC AAG AGC TGT CAC G	807
	HD1 R[a]	CCA GTA AAT CTG TAT AAT TTG CGC CC	

a: PCR primers based on Veld *et al.*[14]

*Base positions refered to the hbl operon sequence of *B. cereus* ATCC 14579 reported by Økstad *et al.*[18]

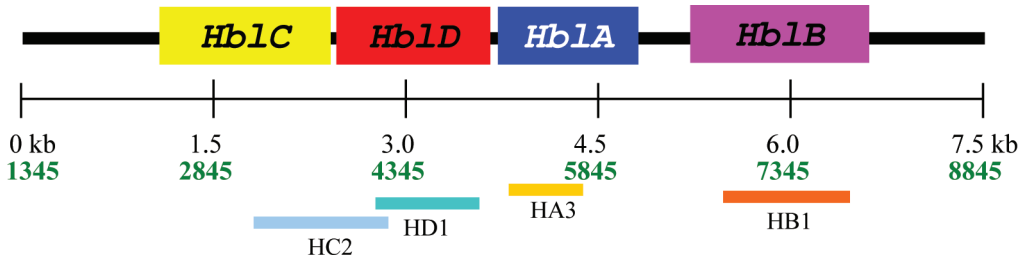

Figure 1. *Hbl* operon of *B. cereus* ATCC 14579 and positions of amplified fragments.

Detection of Hemolysin BL by HBL blood agar plates

The production of hemolysin BL enterotoxin of *B. cereus* isolates was demonstrated by discontinuous double hemolysis pattern on blood agar plates[13]. For preparation of HBL blood agar plates, defibrinated sheep blood (National Laboratory Animal Center, Mahidol University, Thailand) was centrifuged at 5,000 rpm 4°C for 10 min to isolate red blood cells (RBCs). The RBCs were washed in an equal volume of 0.15 M NaCl by centrifugation until the supernatant was colorless. Nutrient agar supplemented with 0.15 M NaCl was sterilized and allowed to cool to 50°C. Then, the washed defibrinated sheep blood and calf serum (Gibco BRL®, USA) were added to the cooled molten agar at the final concentrations of 5% and 2%, respectively, and poured into plates in 15-ml volumes.

B. cereus isolates were inoculated in 5 ml of Brain Heart Infusion broth supplemented with 0.1% glucose (BHIG) and incubated at 37°C for 18-20 h with 150 rpm shaking. The overnight culture was suspended by vortexing and inoculated on HBL blood agar plates by spot inoculation. After that, the plates were incubated at 24°C and were frequently observed between 12 and 72 h. *B. cereus* ATCC 14579 and *B. cereus* INRA C15 were used as positive control. *B. cereus* NC 1291 was used as negative control.

Detection of L_2 by reverse passive latex agglutination (RPLA)

The commercial kit, *Bacillus cereus* Enterotoxin Reverse Passive Latex Agglutination (BCET-RPLA) test kit (Oxford, England) was used to detect L_2 part of hemolysin BL transcribed by *hblC* gene, according to the manufacturer's instructions.

For detection of *B. cereus* isolates, all of them were inoculated in 5 ml of Brain Heart Infusion broth supplemented with 0.1% glucose (BHIG) and incubated at 37°C for 18-20 h with 150 rpm shaking. The overnight cultures were centrifuged at 5,000 rpm 4°C for 5 min. Then, cell-free supernatant fluids were used as samples. Throughout the experiment, lyophilized *B. cereus* enterotoxin control (TD953) reconstituted by adding 500 µl of diluent (TD954) and shaking gently was used as positive control.

RESULTS

Detection of Hemolysin BL genes by multiplex PCR

One hundred of *B. cereus* isolates were detected for the presence of each *hbl* gene by multiplex PCR. The results showed that all of *B. cereus* isolates gave *nheA* gene positive. Among the one hundred of *B. cereus* isolates, 48 isolates showed positive results with *hblA*, *hblC* and *hblD* genes (48%), while 47 isolates showed positive results with all *hbl* genes (47%) including *hblB* gene as shown in Table 2. *B. cereus* isolate E82/44 gave positive results with *hblA*, *hblC* and *hblD* genes, but gave negative result with *hblB* gene. Some of the multiplex PCR results of *B. cereus* isolates were shown in Figure 2.

Detection of HBL by HBL sheep blood agar plates

All of *B. cereus* isolates showed beta-hemolysis that are characteristic of

B. cereus. The results showed that 42 of 100 *B. cereus* isolates gave positive discontinuous hemolysis (42%) as reported in the Table 2. In the 47 of multiplex PCR positive isolates with all *hbl* genes, the discontinuous hemolysis was observed in 41 isolates (87.2%). Exception *B. cereus* isolate E82/44, which gave multiplex PCR positive with *hblA*, *hblC* and *hblD* genes, also showed positive discontinuous hemolysis. The results between multiplex PCR and HBL sheep blood agar were correlated with the most *B. cereus* isolates. There were 6 isolates, however, which gave multiplex PCR positive with all *hbl* genes were not showed discontinuous hemolysis clearly (12.8%). Therefore, the results were reported as doubtful.

Detection of HBL by reverse passive latex agglutination (RPLA)

The cell-free culture supernatants of *B. cereus* isolates were subjected to detect for HBL enterotoxin production (L_2 part only) by *B. cereus* Enterotoxin-Reverse Passive Latex Agglutination (BCET-RPLA) test kit. The results as reported in the Table 2 showed that 48 of 100 *B. cereus* isolates gave positive agglutination (48%). The 47 of *B. cereus* isolates, which gave multiplex PCR positive with all *hbl* genes, showed correlation with RPLA test. Exception *B. cereus* isolate E82/44, which gave multiplex PCR positive with *hblA*, *hblC* and *hblD* genes, also showed positive agglutination in RPLA test.

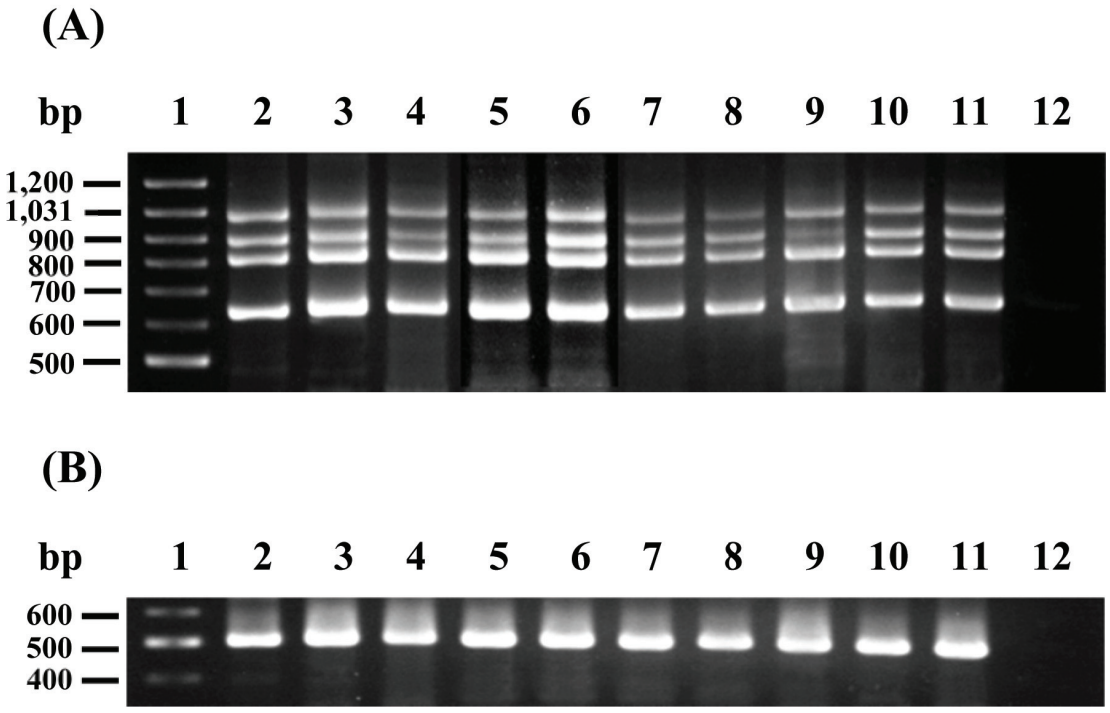

Figure 2. 1.5% agarose gel electrophoresis of multiplex PCR of *hbl* genes and PCR reaction of *nheA* gene. (A) Multiplex PCR of *B. cereus* isolates by using HA3 F-R, HB1 F-R, HC2 F-R and HD1 F-R primers for detection of *hblA*, *hblB*, *hblC* and *hblD* genes resulting in amplified fragment sizes of 622, 986, 884 and 807 bp, respectively. (B) PCR reaction of *nheA* gene of *B. cereus* isolates by using NheA F-R primers resulting in amplified fragment size of 500 bp.

Lane 1 : GeneRuler™ 100 bp DNA ladder plus
Lane 2 : *B. cereus* ATCC 14579 phenol-chloroform prepared genomic DNA
Lane 3 : *B. cereus* ATCC 14579 boiling prepared genomic DNA
Lane 4 : *B. cereus* A6 boiling prepared genomic DNA
Lane 5 : *B. cereus* E83/44 boiling prepared genomic DNA
Lane 6 : *B. cereus* H82/44 boiling prepared genomic DNA
Lane 7 : *B. cereus* K142/45 boiling prepared genomic DNA
Lane 8 : *B. cereus* DMSC 12126 boiling prepared genomic DNA
Lane 9 : *B. cereus* DMSC 15995 boiling prepared genomic DNA
Lane 10 : *B. cereus* DMSC 17004 boiling prepared genomic DNA
Lane 11 : *B. cereus* INRA C15 boiling prepared genomic DNA
Lane 12 : Negative control (nuclease free water)

Table 2. Detection of *hbl* genes and HBL enterotoxin production of *B. cereus* isolates

B. cereus isolate codes	PCR[a]	Multiplex PCR[a]				RPLA test[b]	HBL blood agar plates[c]
	nheA	*hblA*	*hblB*	*hblC*	*hblD*		
ATCC 14579	+	+	+	+	+	>128	+
E82/44	+	+	–	+	+	4	+
A4	+	+	+	+	+	16	+
K142/45, DMSC 12060, DMSC 17004	+	+	+	+	+	128	+
INRA C15, A3, A5, A6, A7, E83/44, E113/45, G194/44, G231/44, G265/45, H3/44, H82/44, H84/44, H183/44, K140/45, K143/45, P8/44, P44/44, N120/44, N122/44, DMSC 11929, DMSC 11949, DMSC 11950, DMSC 11952, DMSC 11953, DMSC 12126, DMSC 12127, DMSC 12833, DMSC 12836, DMSC 12128, DMSC 12632, DMSC 14924, DMSC 15942, DMSC 15995, DMSC 16007, DMSC 16018, DMSC 16028	+	+	+	+	+	>128	+
G267/45	+	+	+	+	+	128	+/–
A8, P43/44, DMSC 11948 DMSC 12059, DMSC 16064	+	+	+	+	+	>128	+/–
NC 1291, A1, A2, E80/44, E81/44, E209/44 E211/44, E4/45, E115/45, E131/45 G35/44, G36/44, G157/44, G189/44 G193/44, G266/45, H192/44, H193/44, H196/44, K166/44, K155/45, K169/45, K171/45, N106/44, N118/44, P4/44, P5/44, DMSC 11927, DMSC 11928, DMSC 11951, DMSC 11954, DMSC 12004, DMSC 12061, DMSC 12062, DMSC 12063, DMSC 12834, DMSC 12835, DMSC 14617, DMSC 14684, DMSC 14707, DMSC 14708, DMSC 14709, DMSC 15300, DMSC 15401, DMSC 15402, DMSC 15940, DMSC 15993, DMSC 16143, DMSC 16314, DMSC 16686, DMSC 16700, DMSC 16840,	+	–	–	–	–	0	–
Total (%)d	100	48	47	48	48	48	42

a PCR and multiplex PCR reported as: +, PCR product of the expected size was observed; –, no PCR product was observed.
b Results of RPLA test were reported as the last dilution of sample that gave a positive agglutination from 0 to 128 according to the manufacturer's instructions. Samples, which were reported at zero dilution, were considered as negative.
c HBL blood agar plate results reported as: +, discontinuous hemolysis pattern was observed; –, continuous hemolysis pattern was observed; +/–, continuous hemolysis pattern was observed but discontinuous hemolysis pattern was not clearly visible (doubtful).
d The total of positive results in all tests reported as the percentage and calculated by excluding the result from B. cereus strain ATCC 14579.

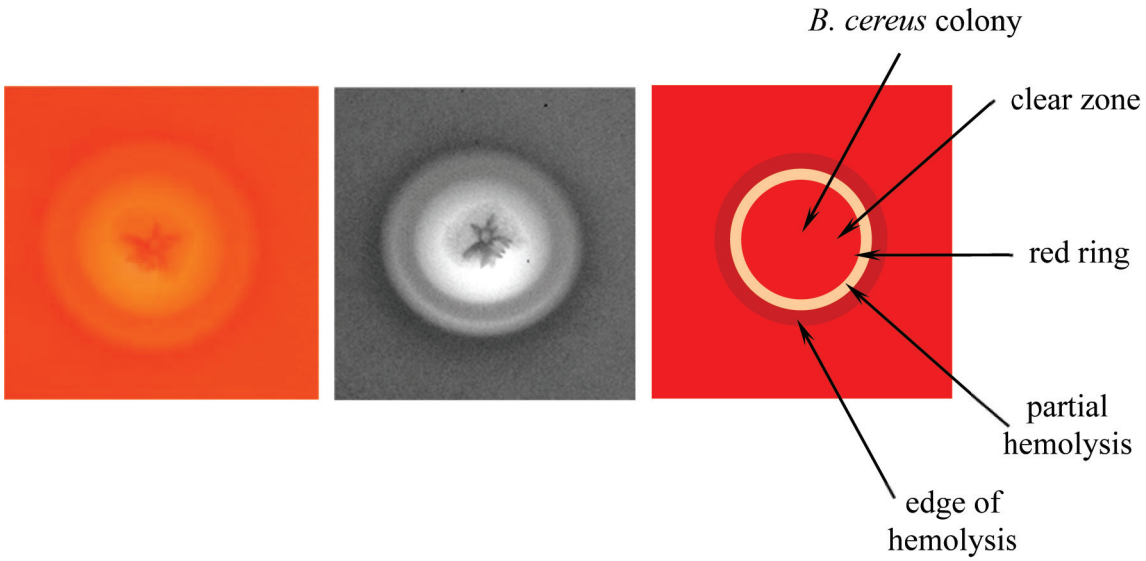

Figure 3. The discontinuous hemolysis of *B. cereus* in HBL blood agar plate.

DISCUSSION

One hundred of *B. cereus* isolates were detected for the presence of each *hbl* gene by multiplex PCR. The PCR reaction of non-hemolytic enterotoxin A (*nheA*) gene of *B. cereus* isolates was also performed as a control of DNA quality. The results showed that all of *B. cereus* isolates gave *nheA* gene positive, which demonstrated that boiling prepared genomic DNA was sufficient to use for *hbl* detection by PCR. In previous study, Janyakhantikul[20] detected *hblA*, *hblD*, *bceT* and *nheA* genes of fifty *B. cereus* isolates. The primers used to detect *hblA*, *hblD* and *nheA* genes in that study were also used in this study. However, that study reported that *B. cereus* isolate G189/44 gave *nheA* gene negative. In this study, PCR condition of *nheA* gene was modified to similar with condition of multiplex PCR to facilitate operation in one time. Fortunately, this condition was also improved the sensitivity of NheA F and NheA R primers for amplification of *nheA* gene.

B. cereus isolate E82/44 gave positive results with *hblA*, *hblC* and *hblD* genes, but gave negative result with *hblB* gene. While Janyakhantikul[20] reported that, *B. cereus* isolate E82/44 gave positive result with *hblD* gene, but gave negative result with hblA gene. Furthermore, *B. cereus* isolate E83/44 was reported that it gave PCR negative both of *hblA* and *hblD* genes. In this study, it gave PCR positive with all of *hbl* genes. The results demonstrated that PCR condition used in this study provided more sensitivity of primers to detect *hblA* and *hblD* genes than the previous study.

From the result of multiplex PCR with *B. cereus* isolate E82/44 that only *hblB* gene was not detected, this isolate may either not contain *hblB* gene or has genetic variation in a region of primer(s) annealing site as described by Veld *et al*.[14]. This study was similar to the previous study by Guinebretie`re *et al*.[21] that *hblA*, *hblC* and *hblD* genes were observed but *hblB* gene could not detect by PCR. However, *hblB* gene was not included in detection of *hbl* genes in most of studies[19,22-23], since it has been reported that *hblA*, *hblC* and *hblD* genes are cotranscribed as operon, except *hblB* gene is still unclear its function[24].

Moreover, one hundred of *B. cereus* isolates were also detected for HBL enterotoxin production by discontinuous pattern on HBL sheep blood agar and BCET-RPLA test. The results correlated for 47 of 100 *B. cereus* isolates (47%) that were detected in multiplex PCR with all *hbl* genes and gave positive agglutination with RPLA test. However, 41 of

47 isolates (87.2%) produced discontinuous hemolysis on HBL sheep blood agar. The 52 of 100 isolates (52%) gave negative results in all of tests. Exception *B. cereus* isolate E82/44, which gave multiplex PCR positive with *hblA*, *hblC* and *hblD* genes and showed positive agglutination with RPLA test, also produced discontinuous hemolysis. This is possible according to the study indicated that only three hbl genes, *hblA*, *hblC* and *hblD* genes are required for the production of discontinuous hemolysis pattern on blood agar[10]. There were 6 of 47 isolates (12.8%), however, which gave multiplex PCR positive with all *hbl* genes and showed positive agglutination in RPLA test were not clearly produced discontinuous hemolysis. Therefore, the results were reported as doubtful. Beecher and Wong[13] described that the discontinuous hemolysis pattern appeared transiently for most isolates, because of the central continuous pattern expanded with increasing time and obscured the discontinuous pattern. In addition, the discontinuous pattern was masked by other hemolysin generated by *B. cereus*. It has been reported that the discontinuous hemolysis pattern on blood agar was only visible at a limited of time and this method was not deserved as a reliable tool for HBL detection[14]. Therefore, the discontinuous pattern not observed in these 6 isolates may be resulted from one or all of those reasons.

This study provided genetic and phenotypic informations of *B. cereus* isolates in Thailand including *hbl* gene profiles and HBL enterotoxin production. The potential multiplex PCR condition for detection all of *hbl* genes in *B. cereus* isolates by operation in one time was established.

Suggestion for further study should be emphasized on the sequence, analyse the hemolysin BL operon of *B. cereus* local isolates and compare with other sequences reported in the database, characterization, cloning, expression, purification and production of these recombinant proteins, for development a rapid test system using specific antibodies toward the hemolysins.

ACKNOWLEDGEMENTS

This study was supported by the Center for Medical Biotechnology, Ministry of Public Health, Thailand.

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