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RAPID DETECTION OF ENTEROTOXIC AND EMETIC *BACILLUS CEREUS* STRAINS USING MULTIPLEX POLYMERASE CHAIN REACTION (MPCR) IN EGYPT

By

Gamal, M.A. [1], Mohamed, H.M.H. [2], Elhariri, M. [3] and Mohamed, Kh.F.[4]

[1] Ph.D. Researcher in Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt.

[2] Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt.

[3] Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt.

[4] Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt.

ABSTRACT

Very different toxins are responsible for the two types of gastrointestinal diseases caused by *Bacillus cereus*: the diarrhoeal syndrome is linked to nonhemolytic enterotoxin NHE, hemolytic enterotoxin HBL, and cytotoxin K, whereas emesis is caused by the action of cereulide. The used primers in multiplex PCR allowed the amplification of enterotoxin genes from strains, which were previously only detected by Southern blot analysis. The forward and reverse primers were each located in two different genes of the corresponding operons, targeting two toxin genes in a single reaction. The primers for detection of emetic toxin producers were directed against a part of the cereulide synthetase essential for cereulide production. The relative prevalence of toxin genes in relation to the total number of *B. cereus* isolates was 28.57% (8/28) for *hbl*, 100% (28/28) for *nhe*, 82% (23/28) for *cyt K* and 53.57% (15/28) for *ces*.

Key words:

(B.cereus- NHE - HBL- cytotoxin K- cereulide- Multiplex PCR).

INTRODUCTION

Bacillus cereus group comprises the following seven closely related species: *B. cereus, B. thuringiensis, B. anthracis, B. weihenstephanensis, B. mycoides, B. pseudomycoides* and *B. cytotoxicus,* which can be divided into seven phylogenetic groups **(Guinebretiere *et al.*, 2010)**. Due to its wide distribution in the environment, *B. cereus* is an ever-present problem in

a broad range of foods including but not limited to cereal products, rice, vegetables, pasta, meat, milk, liquid egg, herbs, texturing agents and spices **(Martínez *et al.,* 2010)**. *B. cereus* is a Gram positive rod-shaped aerobic, endospore-forming bacterium. *B.cereus* causes two types of food-borne intoxications. One type is characterized by nausea and vomiting and abdominal cramps and has an incubation period of 1 to 6 hours. This is the "short-incubation" or emetic form of the disease. The second type is manifested primarily by abdominal cramps and diarrhea with an incubation period of 8 to 16 hours. This type is referred to as the "long-incubation" or diarrheal form of the disease **(Granum, 2009).** Emesis is connected to a heat-stable depsipeptide toxin called cereulide which is also acid resistant and induces swelling of mitochondria in Hep-2 cells, respiratory distress and occasional death **(Ladeuze *et al.,* 2011).** The diarrheal food poisoning is caused by heat-labile enterotoxins such as: nonhemolytic enterotoxin (NHE), Enterotoxin FM (EntFM), Hemolysin BL (HBL) and cytotoxin K (CytK), are the main toxins and therefore often used for the detection of enterotoxic strains **(Kim *et al.,* 2012).** The five different enterotoxins causing the diarrheal type of food poisoning are HBL, NHE, enterotoxin-T, cytotoxin-K, and enterotoxin FM. Among these, the HBL complex consists of three types of proteins encoded by the *hblC, hblD* and *hblA*. NHE is also composed of three components encoded by the *nheA, nheB* and *nheC*. Enterotoxin-T, cytotoxin-K, and enterotoxin FM consist of a single protein encoded by the *bceT*,*cytK*,and *entFM* respectively**(Sergeev*et al.,* 2005)**.Cereulideis synthesized enzymatically via genomic non-ribosomal peptide synthetase (NRPS) sequences **(Dommel *et al.,* 2010)**. Thus, the current study was designed to shed light on the following points:

1- Molecular Characterization of toxin genes of *B. cereus* isolated from meat, meat products and poultry products in EGYPT.

2- Investigating the prevalence of toxin genes occurred in retrieved *B. cereus* isolates.

MATERIAL AND METHODS

Bacterial isolates:

A total number of 28 *Bacillus cereus* isolates DNA (isolated from meat, meat products and chicken products) was extracted and characterized by multiplex PCR by using oligonucleotide primers for detection of toxin genes (*hbl , nhe , cyt K* and *ces*) which are responsible for the production of (hemolytic enterotoxin HBL, nonhemolytic enterotoxin NHE, cytotoxin K and emetic toxin cereulide respectively).

Material used for multiplex Polymerase Chain Reaction (PCR):

Material used for extraction of DNA:

QIAamp DNA Mini Kit Catalogue no.51304

The QIAamp DNA Mini Kit provides silica-membrane-based nucleic acid purification from different types of samples. The spin-column procedure does not require mechanical homogenization, so total hands-on preparation time is only 20 minutes.

PCR Master Mix used for cPCR

Emerald Amp GT PCR mastermix (Takara) Code No. RR310A Contains:

A) Emerald Amp GT PCR mastermix (2x premix).

B) PCR grade water.

Oligonucleotide primers used in cPCR

They have specific sequence and amplify a specific product as shown in (Table 1).

Table (1): Oligonucleotide primers sequences: Source ***(Ehling-Schulz et al., 2006b)*** Metabion ***(Germany).***

Gene	Primer Sequence (5'-3')	Amplified product	Reference
hbl	**HD2 F (GTA AAT TAI GAT GAI CAA TTTC)**	**1091 bp**	***Ehling-Schulz et al., 2006b***
	HA4 R (AGA ATA GGC ATT CAT AGA TT)		
nhe	**NA2 F (AAG CIG CTC TTC GIA TTC)**	**766 bp**	
	NB1 R (ITI GTT GAA ATA AGC TGT GG)		
cytK	**CK F2 (ACA GAT ATC GGI CAA AAT GC)**	**421 bp**	
	CK R5 (CAA GTI ACT TGA CCI GTT GC)		
ces	**CesF1 (GGTGACACATTATCATATAAGGTG)**	**1271 bp**	
	CesR2 (GTAAGCGAACCTGTCTGTAACAACA)		

DNA* Molecular size *marker

Gel Pilot 100 bp plus ladder (cat. no. 239045) supplied from QIAGEN (USA).

Number of bands: 11 Size range: 100-1500 bp.

Material used for agarose gel electrophoresis:

Agarose 1 % (Sambrook *et al.*, 1989).

A multi-purpose, high gel strength agarose suitable for a wide range of molecular biology

techniques. As it has high gel strength and exclusion limits, multi ABgarose could effectively separate large DNA fragments with reduced running times.

Ethedium bromide solution 10 mg / ml (Sambrook *et al.*, 1989).

Tris borate EDTA (TBE) electrophoresis buffer (1x) (*Who, 2002*).

Extraction of *Bacillus cereus* DNA (according to QIAamp DNA mini kit instructions):

1- 20 µl QIAGEN protease were pipetted into the bottom of a 1.5 ml microcentrifuge tube. 200 µl of the sample was added.

2- 200 µl buffer AL were added to the sample, mixed by Pulse vortexing for 15 seconds. The mixture was incubated at 56˚C for 10 min.

3- The 1.5 ml microcentrifuge tubes were centrifugated to remove drops from the inside of the lid.

4- 200 µl ethanol (96%) were added to the sample, and mixed again by pulse vortexing for 15 seconds. After mixing, the 1.5 ml microcentrifuge tube was briefly centrifugated to remove drops from the inside of the lid.

5- The mixture from step 6 was carefully applied to the QIAamp mini spin column (in a 2ml collecting tube) without wetting the rim.The cap was closed, and centrifugated at 8000 rpm for 1 min. The QIAamp mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.

6- The QIAamp mini spin column was carefully opened and 500 ml buffer AW1 was added without wetting the rim. The cap was closed, and centrifugated at 8000 rpm for 1 min. The QIAamp mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.

7- The QIAamp mini spin column was carefully opened and 500 ml buffer AW2 were added without wetting the rim. The cap was closed, and centrifugated at full speed for 3 min.

8- The QIAamp mini spin column was placed in a new 2ml collection tube and the old collection tube was discarded with the filtrate. Centrifugation at full speed for 1 min was done.

9- The QIAamp mini spin column was placed in a clean 1.5 ml microcentrifuge tube and the collection tube containing the filtrate was discarded. The QIAamp mini spin column was carefully opened and 100 µl buffer AE were added. The QIAamp mini spin column was incubated at room temperature (15-25˚C) for 1 min, and then centrifugated at 8000 rpm for 1 min.

Preparation of multiplex PCR Master Mix according to Emerald Amp GT PCR mastermix (Takara) Code No. RR310A kit:

Component	Volume/reaction
Emerald Amp GT PCR mastermix (2x premix)	25 µl
PCR grade water	9 µl
Forward primer (20 pmol)	1 µl each
Reverse primer (20 pmol)	1 µl each
Template DNA	8 µl
Total	50 µl

Temperature and time conditions of the two primers during mPCR are shown in (Table 2).

Table (2): Cycling conditions of the different primers during cPCR

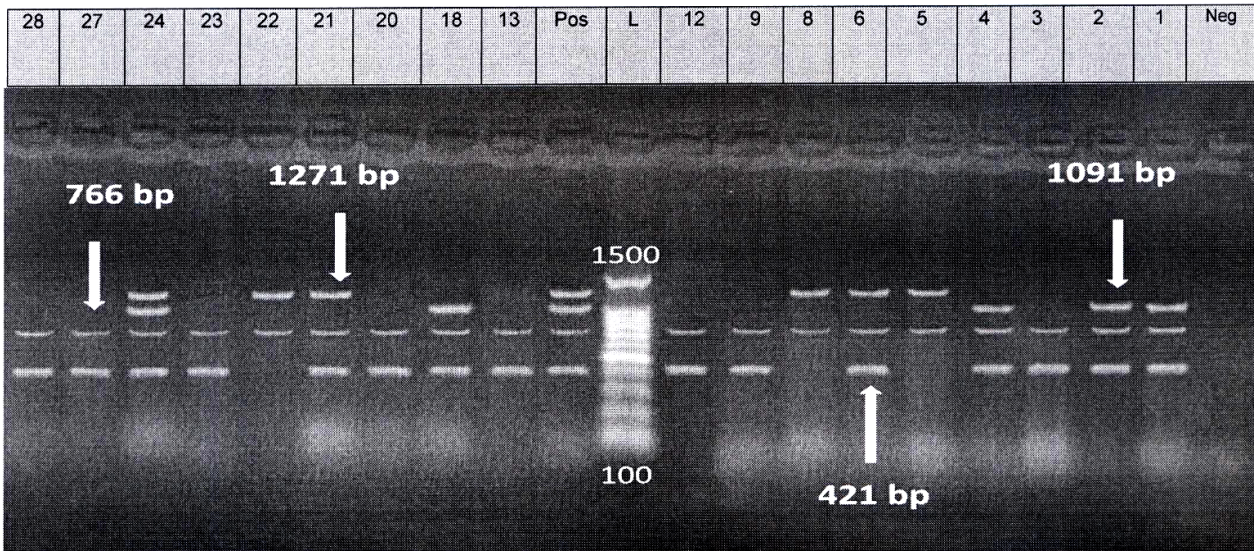
Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
95˚C 5 min.	94˚C 30 sec.	49˚C 1 min.	72˚C 1 min.	35	72˚C 10 min.

According to **(Sambrook *et al.,* 1989**):Electrophoresis grade agarose (1 g) was prepared in 100 ml TBE buffer in a sterile flask, it was heated in microwave to dissolve all granules with agitation, and allowed to cool at 70˚C, then 0.5µg/ml ethedium bromide was added and mixed thoroughly. The warm agarose was poured directly in gel casting apparatus with desired comb in apposition and left at room temperature for polymerization. The comb was then removed, and the electrophoresis tank was filled with TBE buffer. 40 µl of each PCR product samples, negative control and positive control were loaded to the gel. The power supply was 1-5 volts/cm of the tank length. The run was stopped after about 30 min and the gel was transferred to UV cabinet. The gel was photographed by a gel documentation system and the data was analyzed through computer software.

RESULTS

Results of multiplex Polymerase chain reaction (multiplex PCR):

The DNA of 28 *Bacillus cereus* isolates was extracted and characterized by multiplex PCR by using oligonucleotide primers for detection of toxin genes *(hbl,nhe, cyt K* and *ces)* which are responsible for the production of (hemolytic enterotoxin HBL, nonhemolytic enterotoxin NHE, cytotoxin K and emetic toxin cereulide respectively). The isolated isolates ultraviolet transluminated. The photo (1) of 18 *Bacillus cereus* isolates showed that, the lane (L) is the DNA ladder; the lanes (1, 2, 3, 4, 5, 6, 8, 9, 12, 13, 18, 20, 21, 22, 23, 24, 27 and 28) are the DNA of *Bacillus cereus* isolates. Lane (Neg) is the negative control. Lane (Pos): positive control. The photo (2) of 10 *Bacillus cereus* isolates showed that, the lane (L) is the DNA ladder; the lanes (7, 10, 11, 14, 15, 16, 17, 19, 25 and 26) are the DNA of *Bacillus cereus* isolates. Lane (Neg) is the negative control. Lane (Pos): positive control. All isolates were positive for at least two toxin genes. The relative prevalence of toxin *genes* in relation to the total number of *Bacillus cereus* isolates was 28.57% (8/28) for *hbl*, 100% (28/28) for *nhe*, 82% (23/28) for *cyt K* and 53.57% (15/28) for *ces* as shown in (Table 3).



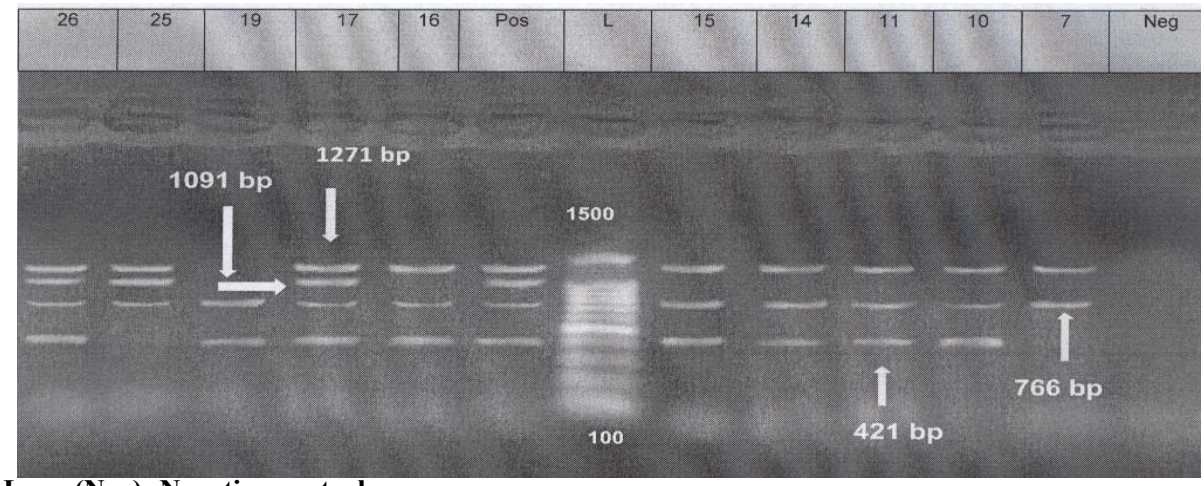
Lane (Neg): Negative control

Lane (Pos): positive control

Lane (L): DNA ladder

Lanes (1, 2, 3, 4, 5, 6, 8, 9, 12, 13, 18, 20, 21, 22, 23, 24, 27 and 28): *Bacillus cereus* isolates

Photo (1): Agarose gel electrophoresis showing amplification of (1091 bp, 766 bp, 421 bp and 1271 bp) for toxin genes *hbl, nhe, cyt K* and *ces* respectively.



Lane (Neg): Negative control

Lane (Pos): positive control

Lane (L): DNA ladder

Lanes (7, 10, 11, 14, 15, 16, 17, 19, 25 and 26): *Bacillus cereus* isolates

Photo (2): Agarose gel electrophoresis showing amplification of (1091 bp, 766 bp, 421 bp and 1271 bp) for toxin genes *hbl, nhe, cyt K* and *ces* respectively.

Table (3): The relative prevalence of toxin genes in relation to the total number of *B. cereus* isolates.

Sample	ID	Results			
		hbl	***ces***	***cytK***	***nhe***
Lunchon 1	**1**	+	-	+	+
Raw meat 1	**2**	+	-	+	+
Panne 1	**3**	-	-	+	+
Nuggets 1	**4**	+	-	+	+
Minced 1	**5**	-	+	-	+
Panne 2	**6**	-	+	+	+
Panne 5	**7**	-	+	-	+
Chicken burger 1	**8**	-	+	-	+
Minced 2	**9**	-	-	+	+
Panne 6	**10**	-	+	+	+
Minced 3	**11**	-	+	+	+
Chicken burger 2	**12**	-	-	+	+
Panne 3	**13**	-	-	+	+
Nuggets 4	**14**	-	+	+	+
Chicken burger 5	**15**	-	+	+	+
Nuggets 5	**16**	-	+	+	+
Minced 4	**17**	+	+	+	+
Chicken burger 3	**18**	+	-	+	+
Lunchon 3	**19**	-	-	+	+
Nuggets 2	**20**	-	-	+	+
Chicken burger 4	**21**	-	+	+	+
Lunchon 2	**22**	-	+	-	+
Nuggets 3	**23**	-	-	+	+
Panne 4	**24**	+	+	+	+
Panne 7	**25**	+	+	-	+
Lunchon 4	**26**	+	+	+	+
Raw meat 2	**27**	-	-	+	+
Raw meat 3	**28**	-	-	+	+
Total	**28**	**8**	**15**	**23**	**28**
Percentage		**28.57%**	**53.57%**	**82%**	**100%**

DISCUSSION

Microbial infections are a leading cause of morbidity and mortality in humans worldwide. Every year, microbial infections cause more than 100 million illnesses worldwide **(Alwan, 2010).**Microbial illnesses are orchestrated by an array of virulence factors that contribute to the pathophysiology and survival of the pathogen in the host **(Finlay and Falkow, 1997)**. These factors include cell surface proteins, receptors, and adhesions that facilitate host colonization; capsular polysaccharides that confer protection from host immune system, and microbial toxins that cause host tissue damage **(Wu *et al.,* 2008).**Microorganisms control in meat products is the major concern in the preparation of high quality foods **(Jo *et al.,* 2004)**. *Bacillus cereus* strains were observed to produce an emetic and diarrhoeal enterotoxins beside other virulence factors including phospholipase protease and hemolysin, one of which cereolysin is a thiol activated hemolysin. These virulence factors may be contributed to enteric and non-enteric diseases **(Drobniewski, 1993)**. *B. cereus* emetic toxin has been associated with life threatening acute conditions such as fulminant liver failure and rhabdomylosis **(Mahler *et al.,* 1997)**. This toxin is unique among enterotoxins since it is resistant to proteolytic degradation, pH extremes and elevated temperatures surviving 121^{o}C for 90 minutes **(Granum *and* Lund, 1997)**. **(Lund *et al.,* 2000)** reported for the first time that the cytotoxic *gene cytK* of *B. cereus* (a clinical isolate) was the only cause of severe food poisoning outbreak that killed three people. They also reported that *Cyt K* toxin had necrotic and hemolytic action and was completely different from other *B. cereus* enterotoxins. **(Svensson *et al.,* 2007)** observed that, the primers used for *nheA* and *hblC* were not able to detect some strains with gene polymorphisms. Therefore, the PCR results cannot be interpreted separately. They can be used to find strains that are TECRA or BCET-RPLA negative but do have the gene, i.e., are potentially toxigenic. New primers for the *nhe* and *hbl* genes have been developed and validated after this investigation as a result of the observations of gene polymorphisms **(Ehling-Schulz *et al.,* 2006a).** Multiplex PCR for rapid diagnosis of *B. cereus* toxin *genes* by using forward primer with sequence (5’ GTA AAT TAI GAT GAI CAA TTTC3’) and reverse primer with sequence (5’ AGA ATA GGC ATT CAT AGA TT 3’) for *hbl*, forward primer with sequence (5’ AAG CIG CTC TTC GIA TTC 3’) and reverse primer with sequence (5’ ITI GTT GAA ATA AGC TGT GG 3’) for *nhe*, forward primer with sequence (5’ ACA GAT ATC GGI CAA AAT GC 3’) and reverse primer with sequence (5’ CAA GTI ACT TGA CCI GTT GC 3’) for *cyt K*, forward primer with sequence

(5' GGTGACACATTATCATATAAGGTG 3') and reverse primer with sequence (5'GTAAGCGAACCTGTCTGTAACAACA 3') for *ces*, which were designed according to **(Ehling-*Schulz et al.* 2006b)** who reported that, the designed primers allowed the amplification of enterotoxin genes from strains, which were previously only detected by Southern blot analysis. The forward and reverse primers were each located in two different genes of the corresponding operons, targeting two toxin genes in a single reaction. The forward primer, designed for the detection of the nhe complex, was located in *nheA* while the reverse primer was located in *nheB,* and primers for hbl were located in *hblD* and *hblA*, respectively. Oligonucleotide primers for *cytK* were directed at highly conserved regions of the toxin *gene* so as to detect both forms of *cytK* (*cytK-1* and *cytk-2*), recently described in a single reaction. The primers for detection of emetic toxin producers were directed against a part of the cereulide synthetase essential for cereulide production. The specificity of the assay was assessed using a panel of *B. cereus* strains with known toxin profiles and was successfully applied to characterize strains from food and clinical diagnostic labs as well as for the toxin gene profiling of *B. cereus* isolated from silo tank populations. The results showed that, the relative prevalence of toxin *genes* in relation to the total number of *B. cereus* isolates was 28.57% (8/28) for *hbl*, 100% (28/28) for *nhe*, 82% (23/28) for *cyt K* and 53.57% (15/28) for *ces*. **(Hwang and Park, 2015)** found that, the prevalence of *nhe gene* was 100 %, *hbl* was 84 % and *cytK* was 48 %. While the total prevalence of *B. cereus* was 50.5 %. Of the four *genes* encoding virulence factors investigated in this study, the *nhe gene* was present in 100% of strains belonging to *B. cereus* group. These results agreed with those reported by **(Stenfors Arnesen *et al.,* 2008)** who found *nhe genes* in all tested strains. However, recently **(De Santis *et al.,* 2008)** detected *nhe genes* in ca. 65% of *B. cereus* group strains isolated from sheep ricotta cheese and **(Martínez *et al.,*2009)** who found *nhe genes* in 76% of strains belonging to *B. cereus* .These results may be due to the variability in the *nhe* operons **(Stenfors Arnesen *et al.,* 2008)**. Although the majority of the isolates investigated have the potential to produce diarrhoeal toxins as they harbour the requisite genes, previous studies have determined the expression of toxin may not always occur as it is modulated by various factors including the composition of the growth substrate **(Ouhib *et al.,* 2006)**.72% of the isolates harboured the gene for cytotoxin K. None of the isolates carried ces gene. Emetic toxin production has been determined in previous studies to be rare **(Samapundo *et al.,* 2011)**. **(Altayar and Sutherland,**

2006) determined that cereulide was produced by only three of 177 *B. cereus* isolates recovered at 30 °C and only one of 148 isolates recovered at 7 °C.**(Guinebretière *et al.,* 2002)** found the cytK gene among 37% of 51 food-related strains and 73% of diarrhoeal strains. 52% of the mesophilic isolates were positive for *cytK* **(Svensson *et al.,* 2007). (Svensson *et al.,* 2007)** reported a very low prevalence of Ces (0.5%) is in the same *range* as in a previous investigation along the milk production chain **(Svensson *et al.,* 2006).** It has been reported that some isolates may cause poisoning with only 1 or 2 genes from among *hbl* or *nhe* component genes. Therefore, the presence of any one of the *hblC, hblD,* and *hblB* component genes coding for lytic component L2, L1, and binding component B of HBL toxin was considered positive for hbl. In addition, the presence of any one of the *nheA, nheB,* and *nheC* component genes coding for Nhe toxin was considered positive for nhe. Those were due to the reports that some isolates might cause poisoning with only 1 or 2 genes among *hbl* component genes or *nhe* component genes **(Ngamwongsatit *et al.,* 2008)**.

CONCLUSION

Nonhemolytic enterotoxin NHE was the predominant toxin gene among all detected toxin genes *(hbl, nhe, cyt K* and *ces)* by a percentage of 100%, while the lowest incidence was for *hbl* toxin gene by 28.57 %.

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