International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.9, No.11 pp 151-156, 2016

Determination of Haemolytic and Nonhaemolytic Genes Profiles of *Bacillus cereus* Strains Isolated from fried Rice samples by Polymerase Chain Reaction (PCR) technique

Nisreen Jawad

School of Chemical Sciences and Food Technology, Faculty of Science and TechnologyUniversiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia

Abstract : There are two types of sicknesses which are caused by *B. cereus* in human beings. diarrhoeal and emetic types. In these study the prevalence of *B. cereus* was conducted on cooked rice samples and the *B. cereus* isolates were examined there enterotoxigenic gene. Those fried rice samples were purchased from several restaurants in the area of Bangi, Kajang and Universiti Kebangsaan Malaysia, Selangor. A total of 70 samples have been analyzed for *B. cereus* contamination has been formed between 1.2×10^4 to 1.6×10^6 cfu/g cooked of 110 colonies of tentative *B. cereus* have been examined onto mannitol egg yolk polymyxin agar and Chromogenic *Bacillus cereus* Agar, and 35 colonies have been detected as *B. cereus* using biochemical test and partial sequence of 16S r DNA sequences analysis. The *B. cereus* isolates that are BC1 to BC35 have been characterized for hemolytic enterotoxin (HBL complex encoding gene *hblB*),and (non-haemolytic enterotoxin encoding gene *nheA*), 4 isolates have been found positive towards gene *hblB*, and 12 isolates were found positive towards *nheA* gene. Thus, in the presence study it is evidence that the PCR analysis targeting enterotoxin of *hblB* and *nheA* genes are suitable and useful in detecting enterotoxic *B. cereus* in fried rice contaminated samples.
Keywords :, *haemolytic, Bacillius cereus, isolated, fried rice, PCR technique.*

Introduction

The genus *Bacillus* is a heterogeneous collection of Gram positive, spore- bearing rods. Sporangial morphology is generally utilized for division of the genus into three particularly swells the sporangium, and those with spherical spores[1]. *B. cereus* often exists in different foods such as milk, dairy products, spices, cereals, meat cakes; desert, etc.[2] Milk and rice possibly are the two most frequently contaminated foodstuffs. It can also generally be identified in pasteurized milk, which causes spoilage due to the production of lipases and proteases. The psychotropic strains of *B. cereus* have led to a growing problem for the dairy industry[3].The genus *Bacillus* is extraordinarily large, with highly unpredictable (G+C) content, and a further subdivision into six subgroups has been suggested by[4]. The first explicated enterotoxin of *B. cereus* is now known as hemolysis BL (HBL). Previous names are diarrhoeagenic factor, fluid accumulation factor and vascular permeability factor[5]. NHE are two different Haemolysin BL (HBL) and Non-Haemolytic Enterotoxin enterotoxin complexes, each comprising three exoproteins. The proteins of NHE and HBL manifest definite degree of homology, with amino acid identities ranging from 18 to 44%[6]. Both HBL and NHE belong to general category of A-B toxins which includes two parts (A and B) each demonstrating different roles in toxin action. The B component attaches to molecules on the surface of target cells.The first sign of another enter toxigenic complex of *B.*

cereus was recognized in 1996. Examination of over 300 strains from different sources comprising strains from a number of outbreaks showed that HBL could not have been the causative agent in some of the outbreak cases. The cytotoxic consequences had to be assigned to a hitherto unknown enterotoxin[7]. The new three component enterotoxin was found after a food borne occurrence in Norway[8]. It appeared to cause symptoms similar to those resulted in by HBL, but it lacked the haemolytic activity. Even though they include several structural similarities, the cytotoxic potential of the two three component enterotoxins, HBL and NHE, were different[8]. Elucidation of the sequences of the genes provided sequences for primers for each of the three genes[7]. Later comparison of gene sequences of different strains let to improved primers for the identification of the separate genes[9].

Furthermore, average incubation period of 12 h is rat rather long for intoxication sensu stricto[3]. However, under certain circumstances, the pH in the stomach mayraise just enough to let the toxin pass unhurt to the small intestines[10]. These special conditions can take place with aged consumers and with an ingestion of large amount of foods, which besides can shelter the toxin molecule from the proteolytic gastric enzymes[10]. However, etiology of food poisoning caused by HBL and NHE, on the whole, follows the scenario of toxin mediated infection referring to the consumption of food harboring adequate counts of HBL/NHE producing *B. cereus*. Nhe forms transmembrane pores similar to Hbl and quickly disrupts the plasma membrane of intestinal epithelial cells. Detection of enterotoxic *B. cereus* using PCR in RTF contaminated food is essential because it is rapid and accurate when compared to the conventional method which required a lengthy time and less efficient. Rapid detection of *B. cereus* in foods is vital to facilitate the application of quality control measures to eliminate *B. cereus* from foods and enhance diagnosis of food poisoning outbreaks.

Thus, in this study we studied the detection limit of *B. cereus* bacterial contamination in food to estimate the lowest genomic DNA present can be detected in food. The *B. cereus* (BC1- BC35) strain was used as genomic DNA source for detection limit determination into fried rice samples. The PCR analysis was conducted by targeting *hlbB* and *nheA* genes in detecting enterotoxic *B. cereus* in fried rice samples.

Materials and Methods

Samples collection and preparing

For the purpose of sample collection and bacteria identification, a total of (n=70) cooked rice samples were bought randomly from different restaurants in Selangor, Malaysia from August 2013 until June 2014. All samples were immediately transported to the laboratory and were analyzed within 24 hours.

Isolation and morphological characterization

Samples were analyzed using the standard procedure for detection of *B. cereus*[11] with modifications described by[11]. A total of 25 g of each sample was placed in a stomacher bag added with 225 ml of Tryptic Soy Broth (TSB; Bacto™,) and homogenized in a stomacher (Interscience, France) for 60 s followed by incubation at 30°C for 12 h. The determination of *B. cereus* count has been done according to ISO 7932l2004 by the surface plating method with mannitol egg yolk polymyxin (MYP) agar (Oxide CM0929).The dilutions of the stomached fluid were prepared with Moreover, Tryptic Soy Broth (TSB; Bacto™).then 0.1 ml portions of each dilutions of the fluid were transferred into three tubes and incubated at 30°C for 18 to 24 h. A loopful of culture from each tube was streaked onto Mannitol Egg Yolk Polymyxin Agar Base (MYP; Difco) added with sterile Polymyxin B Selective Supplement (Difco) and sterile Egg-Yolk Tellurite Emulsion 20% (V/V) (Merck) which is a specific media for the isolation of the *B. cereus* and therefore identification is confirmed by microscopic. The biochemical tests were conducted as described by[12].

Isolation of genomic DNA

The bacterial DNA was extracted from 1 ml of the overnight culture grown of 30°C on orbital shaker (200 rpm) and purified using a DNA Extraction Kit (Promega). The concentration and purity of the extracted DNA were determined by absorbance at 260 nm and 280 nm using Maestro Nano Spectrophotometer (Maestro Gen, USA). The extracted DNA was stored at -20^{o}C until used.

PCR Amplification

1. Detection of Haemolysin BL (*hblB*) Genes by PCR Amplification

The detection of haemolysin BL *(hblB*) genes were conducted as described by[13] using primers as shown in (Table 1)which produced 237 bp and 250 bp amplicons, respectively. The PCR reaction was performed in a 25 µl volume containing of 12.5µl of DreamTaq™ PCR MasterMix (MyTACG Bioscience Enterprise,MY), 1µl forward and reverse 1µl of oligonucleotide primers (either *hblA* or *hblB*) 7.5 µl of sterile nuclease free water (NFW) and the 3 µl of (50-100 ng) DNA template. The amplicon was purified using a PCR purification kit (Promega, MY). Amplification was performed in an Eppendorf Mastercycler (Eppendorf, Germany) with a temperature program consisting of the initial denaturation at 95°C for 1 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing for 1 min at 55°C and polymerization at 72°C for 1 min. Final elongation was at 72°C for 10 min. Negative controls (NFW) were included in each PCR amplification, in order to verify the PCR efficiency and to detect contamination.

2. Detection Non-haemolysin (*nheA*) Gene by PCR Amplification

The detection of non-haemolysin BL (*nheA*) gene were conducted as described by[9] using primer as shown in Table 3.2 which produced 755 bp amplicons. The PCR reaction was performed in a 25 µl volume containing of 12.5 µl of DreamTaq™ PCR MasterMix (MyTACG Bioscience Enterprise,MY), 1µl forward and reverse 1µl of oligonucleotide primer (*nheA*) 7.5 µl of sterile nuclease free water (NFW) and the 3 µl of (50-100 ng) DNA template. The amplicon was purified using a PCR purification kit (Promega, MY). Amplification was performed in an Eppendorf Mastercycler (Eppendorf, Germany) with a temperature program consisting of the initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing for 1 min at 49°C and polymerization at 72°C for 1 min. Final elongation was at 72°C for 10 min Negative controls (NFW) were included in each PCR amplification, in order to verify the PCR efficiency and to detect contamination.

Gel Electrophoresis

The extracted DNA from the bacteria were separated by electrophoresis technique on 1.5 % (w/v) agarose gel in 1X TAE buffer (40 mMTris-acetate, 1 mM EDTA, pH 8.0) (1st Base, Malaysia) at 100 V for 40 min. following PCR amplification. The gel was pre-stained with Maestrosafe™ Nucleic Acid (V-Bio Science, Malaysia) while GeneRuler™ 1 kb DNA ladder and 100 bp (Fermentas, MY) were used as DNA size marker. Finally, all gels were viewed and captured by UV trans-illuminator Gel Documentation System (Syngene, UK).

Table 1: Oligonucleotide primers used for detection of *Bacillus cereus* toxin genes

Target gene	Primer codes	primer sequences (5'-3')	Product size (bp)	References
hblB	*hblB F* *hblB R*	AATATGTCCCAGTACACCCG AAGCAATGGAATACAATGGG	250	9
nheA	*nheA F* *nheA R*	ACGAATGTAATTTGAGTCGC GTTAGGATCACAATCACCGC	755	9

Results and Discussion

Enterotoxin Detection

Determination of enterotoxin genes was conducted for haemolytic enterotoxin (HBL) complex encoding gene (*hblB*), and non haemolytic (*nheA*).All genomic DNA of *B. cereus* isolates (BC1 to BC35) were exacted and examined for haemolytic enterotoxin (HBL) complex encoding gene (*hblB*). Of 35 *B. cereus* isolates 4 isolates positive towards *hblB* and 12 isolates positive towards *nheA*. The existence of enterotoxin genes *hblB B. cereus* isolates from fried rice samples were 4(11%), and *nheA* 12 (34%), respectively. The *B. cereus* which revealed to be positive towards *hblB* gene (11%) would generate amplicons of 250 bp in size (Figure 1), The result has been in contrast with[14]. which were slightly high when compared to *B. cereus* from positive towards *hblB* 7/31 (22) gene in *B. cereus* isolated from baby food with rice and milk-based. They reported 4/ 25 (32 %),

were positive towards *hblB* gene from baby food with added wheat and milk based, and 1/18(16%) from baby food with wheat, honey and milk based, and 1/10 (10%) from baby food with wheat, banana, and milk based[15]. reported a total of Fifty-two strains of *B. cereus* isolated from a variety sources of food (milk, dairy product, spices and rice salad) analyzed, were reported to be positive towards 2/52 (3%) *hblB* gene .

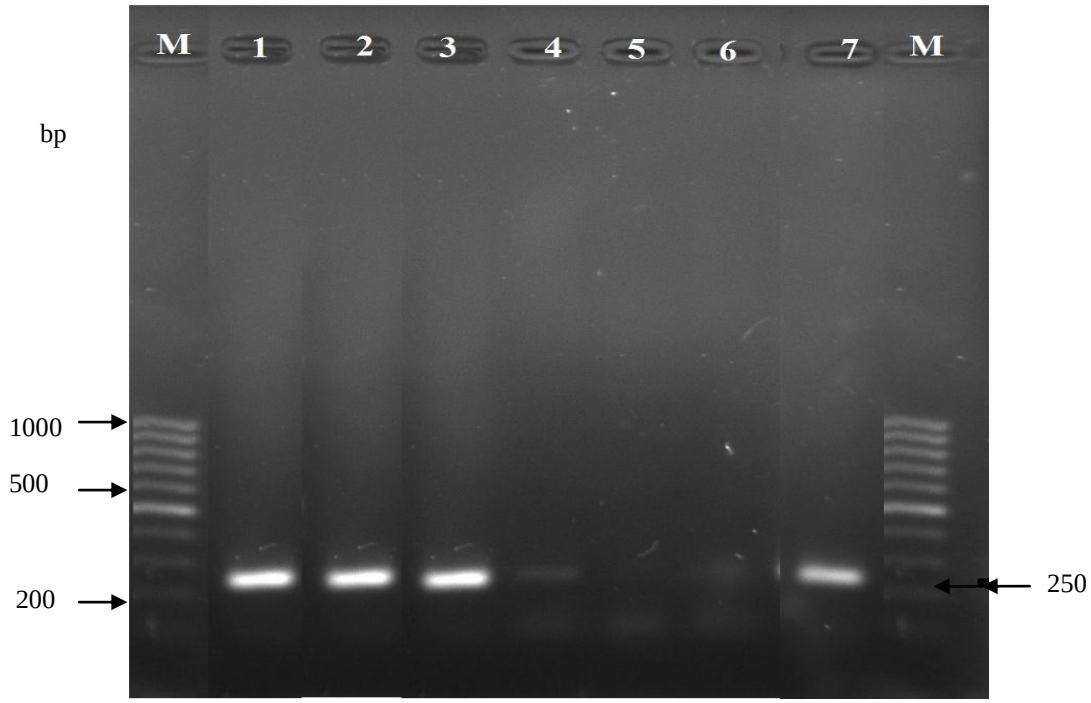

Figure1:Amplicons of *hblB* gene detected in *Bacillus cereus* strains by polymerase chain reaction (PCR) technique. M: 100 bp ladder; Lane 1: positive control; Lane 2– 7: *B.cereus* isolates (BC3, BC7, BC22, BC27)

The *B. cereus* which revealed to be positive towards *nheA* gene (34%) would generate amplicons of 755 bp in size (Figure 2), The result has been in contrast with[16] which were slightly high when compared to *B. cereus* positive towards 13/31 in *B. cereus* isolated from baby food with rice and milk based positive towards (41%), They reported 9/25 (36%), were positive towards *nheA* gene was from baby food added with wheat and milk, 9/18 (50%),were baby food with wheat, honey, and 6/10 (60%) were milk based and baby food with wheat, banana, and milk based. Reported[17] a total 30 samples coffee analyzed, (70.6%), were reported to be positive towards *nheA* gene. Rather et al. (2011) reported a total of 150 food samples (raw meats and meat products) analyzed, were (96.61%) reported to be positive towards *nheA* gene. Reported[13] a total of 63 strains of *B. cereus* isolated from daily dessert samples (36 from pasteurized milk, 15 from powdered milk and 12 form UHT milk) analyzed,19 (95%). were reported to be *nheA* gene. reported a total of Fifty-two strains of *B. cereus* isolated from variety sources of food (milk, dairy product, spices and rice salad) analyzed, 6 / 52(78%), were reported to be positive towards *nheA* gene .

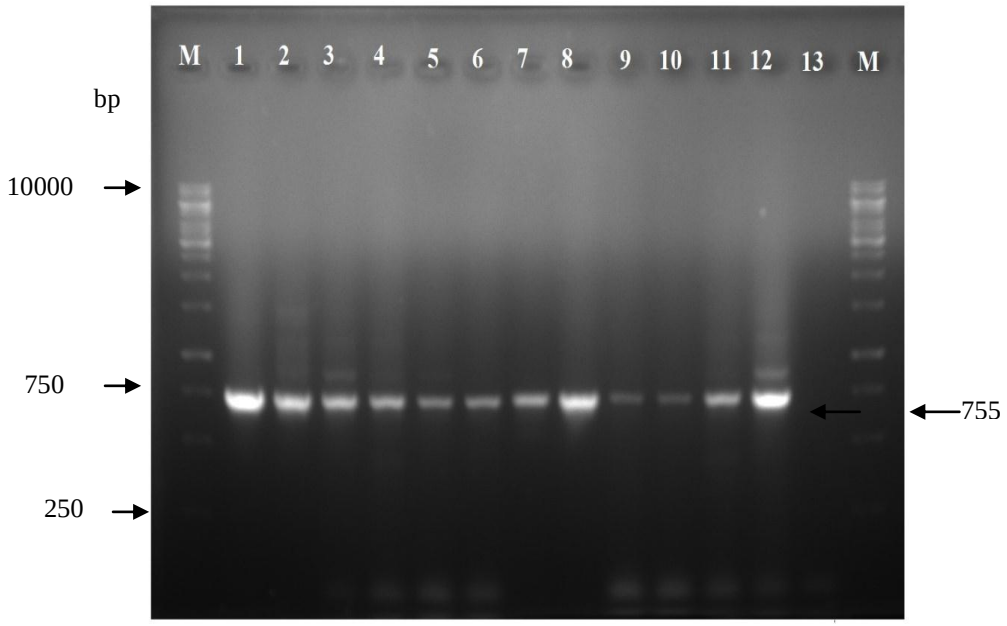

Figure 2: Amplicons of *nheA* gene of *B. cereus* strains by by polymerase chain reaction (PCR) technique. M: 1 kb ladder; Lane. 1: positive control; 2 –12: *B. cereus* isolates (BC3, BC4, BC11, BC12, BC14, BC21, BC23, BC26, BC28, BC29, BC33, BC34)

Conclusion

It is an evidence that direct detection of both genes (*hblB* and *nheA*) using PCR analysis is preliminarily useful, since this technique is rapid and simple to identify foods suspected to cause food poisoning of enterotoxigenic *B. cereus.* Therefore, the presence of *B. cereus* and their enterotoxigenic genes in fried rice samples can be considered a potential risk for public health.

Acknowledgments

The author thanks Associate. Prof. Sahilah Abd Mutalib for their fruitful discussions and support, this research was supported by the School of Chemical Sciences and Food Technology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM), Malaysia.

References

1. Harwood, C.R. & Cutting, S.M. *Molecular Biological Methods for Bacillus*. England, John Wiley and Sons, Inc., 1990,27–74.
2. Svensson, B., Monthan, A., Guinebretiere, H.M., Nguyen, C. & Christiansson, A. Toxin production potential and the detection of toxin genes among strains of *Bacillus cereus* group isolated along the dairy production chain. *International ,Dairy Journal.*, 2007.17: 1201-1208.
3. Granum, P.E. & Lund, T. *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiology Letters.*, 1997,157: 223-228.
4. Priest, F.G. Systematics and ecology of *Bacillus.*In *Bacillus subtilis* and other gram-positive bacteria, 3-15. In. Sonenshein, A.L., Hoch J.A. & Losick, R: *American Society for Microbiology*, Washington., 1993,D.C.USA.
5. Sutherland, A.D. & Limond, A.M. Influence of pH and sugars on the growth and production of diarrhoeagenic toxin by *Bacillus cereus*. *Journal of Dairy Research.*, 1993,60: 575-580.
6. Arnesen, L.P.S., Fagerlund, A. & Granum, P.E. From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiology Reviews.*, 2008,32: 579-606.
7. Granum, P. E., O' Sullivan, K. & Lund, T. The sequence of the non-haemolytic enterotoxin operon from *Bacillus cereus*. *FEMS Microbiology Letters*., 1999, 177(2):225- 229.
8. Lund, T. & Granum, P.E. Characterisation of a nonhaemolytic enterotoxin complex from *Bacillus*

cereus isolated after a foodborne outbreak. *FEMS Microbiol Lett.*, 1996,141: 151–156.
9. Guinebretière, M.H., Broussolle, V. & Nguyen-The, C. Enterotoxigenic profiles of food-poisoning and food-borne *Bacillus cereus* strains. *Journal of Clinical Microbiology*., 2002,40: 3053-3056.
10. Wijnands, L.M., Dufrennne, J.B. & van Leusden, F.M. The pathogenic mechanism of the diarrhoeal syndrome caused by *Bacillus cereus*. (Rep. No. RIVM 250912001). Bilthoven.The National Institute for Public Health and the Environment (RIVM)., 2002,The Netherlands.
11. Lee, H.Y., Chai, L.C., Tang, S.Y., Jinap, S., Ghazali, M.,M.H. Application of gaseous ozone to inactivate *Bacillus cereus* in processed rice. *Journal of Food Process Engineering.*,2011,34 (6); 2220- 2232.
12. Rhodehamel, E.J. & Harmon, M.S. *Bacillus cereus.* In *Bacteriological Analytical Manual*. 8th (ed.) Arlington., 2001,FDA; AOAC.
13. Çadirci. Ö., Gücükoğlu, Ali., Terzi, G., Kevenk, T.O. & Alişarli, M. Determination of enterotoxigenic gene profiles of *Bacillus cereus* strains isolated from dairy desserts by Multiplex PCR. *Journal of the Faculty of Veterinary Medicine* Kafkas Univ. *http://vetdergi.kafkas.edu.tr*., 2013, 19 (5): 869-874.
14. Rahimi, E., Abdos, Fahimeh., Momtaz, H., Baghbadorani, Z. & Jalali, M. *Bacillus cereus* in infant foods: prevalence study and distribution of enterotoxigenic virulence factors in Isfahan Province, Iran. *The Scientific World Journal*., 2013, 1-5. http://dx.doi.org/10. 1155/2013/292571
15. Souad, M., Nozha, C., Noël, G., Michel, G. & Mustapha, L. Enterotoxigenic Profiles of psychrotolerant and mesophilic strains of the *Bacillus cereus* group isolated from food in Morocco. *International Journal of Engineering Research and Applications*., 2013,3: 964-970.
16. De Matos, C., Da Cunha, O., de Souza, C., De Mello, S. & Brantes, P. Detection of enterotoxins produced by *B. cereus* through PCR Analysis of ground and roasted coffee samples in Rio de Janeiro, Brazil. *Cienciae Tecnologia de Alimentos.*, 2011,31(2): 443 - 449.
17. Rather, M.A., Aulakh, R.S.. Gill, J.P.S., Rao, T.S., Hassan. M.N. Direct Detection of *Bacillus cereus* and its Enterotoxigenic Genes in Meat and Meat Products by Polymerase Chain Reaction. *Journal of Advanced Veterinary Research*,. 2011,1:99-104.

*****