International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304
Vol.8, No.7, pp 193-198, 2015

Determination of haemolytic and Ematic Genes Profiles of *Bacillus cereus* Strains Isolated from cooked Rice samples by Polymerase Chain Reaction (PCR) technique

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Abstract: The aim of this study has been an investigation of the presence of *Bacillus cereus* and detection of enterotoxigenic genes in cooked rice samples through utilizing a PCR technique. In this study the providence of *B.cereus* was carried out to cooked rice samples and the *B.cereus* isolates were investigated for enterotoxigenic gene. The cooked rice samples were purchased from several restaurants in the area of (Bangi, Kajang and UKM) Selangor, Malaysia. A total of 70 samples have been analyzed. *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 tested 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 distinguished for hemolytic enterotoxin (HBL complex encoding gene (hblD), and ematic (ces) gene toxin. 12 isolates have been reported to be positive towards hblD, None of the *B. cereus* isolates have been found positive towards ematic(ces) gene. Therefore, the presence of *B. cereus* and their enterotoxigenic genes in cooked rice samples can be regarded as a potential risk for public health.

Keywords: *haemolytic ,ematic, B. cereus, isolated, cooked rice, PCR technique.*

Introduction

Bacillus cereus is a Gram-positive, spore-bearing rod that is widely distributed in the environment, including soil, plant materials and many types of foods, particulary those of plant origin; however, it is also frequently isolated from meat, eggs and dairy products[1]. An ubiquitous, spore-forming bacterium, *B. cereus* has been recognized to cause food spoilage producing two distinct types of toxins that differ in the major symptoms induced in humans. *B. cereus* is regarded as the etiologic agent of two types of food-borne disease which result in an infection causing vomiting and diarrhoea as the main clinical symptoms. Both *B. cereus*-derived diseases are caused by toxins: the diarrhoeal type happens due to protein toxins which are formed in the intestinal tract by growing organisms (enterotoxins) and the emetic toxin type results from a peptide that is preformed in the food (emetic toxin or cereulide)[2,3] Within 12 h of food consumption, the enterotoxin disease type has been characterized by diarrhoea. The emetic disease type has usually been characterized by vomiting and nausea within two hours after consumption of the suspected food. For both types of the disease, clinical signs last approximately 24 h[1]. The *B. cereus* group contains six different species: *B. cereus*, *B. mycoides*, *B. thuringiensis*, *B. anthracis, B.weihenstephanensis* and *B. pseudomycoides*[4,5]. Because of its ability to form spores, *B. cereus* is cap able of surviving a wide range of stress conditions such as those occure in certain foods. *B. cereus* can be isolated from a wide variety of foods, such as rice and pasta, milk and dairy products, infant

foods, meat products, spices, fresh vegetables, seafood, and ready to eat foods. Moreover, *B. cereus* manifests one of the main pathogens in mass catering, since its elimination is not guaranteed by pasteurization and sanitation procedures[6]. Food poisoning by *B. cereus* can either be resulted in an infection or an intoxication, which leads to a diarrheal or an emetic type of illness, respectively. Foods which are generally related to diarrheal food poisoning include meat products, soups, vegetables, sauces and dairy products. On the other hand, foods which are related to the emetic type of syndrome are mostly rice and pasta[7]. The diarrheal type of illness is regarded to be caused by the production of enterotoxins by *B. cereus* in the human small intestine after consuming contaminated food[7]. The emetic type of illness has been caused by producing the emetic toxin cereulide by *B. cereus* in foods before consumption and it causes nausea and vomiting [6] . As these types of toxins can be produced by all the other members of the *B. cereus* group, specifically by *B. thuringiensis*, *B. mycoides* and *B. weihenstephanensis*, food poisoning can even be caused by these different species[8]. in general, symptoms which are caused by *B. cereus* food poisoning are considered as mild, and therefore *B. cereus* food poisoning is probably under reported. Detection of toxin genes of *B. cereus* has been suggested by several authors in terms of polymerase chainreaction PCR primers of toxin subunits because the toxins were cloned and sequenced[9]. This study therefore was an attempt to detect the hemolytic enterotoxin gene (hblD) and ematic gene (ces) identified and molecularly confirmed isolates of *B. cereus* through *PCR* technique.

Materials and Methods

Sample collection

In the present study, a total of (n=70) cooked rice samples have been bought randomly from different restaurants in Selangor, Malaysia from August 2013 until June 2014. All samples have been immediately transported to the laboratory and have been analyzed within 24 hours.

Isolation and morphological characterization

Samples have been analyzed, using the standard procedure for the detection of *B. cereus* [10]with modifications which have been explained in details by[11.] Generally a total of 25 g of each sample has been put in a stomacher bag and it has been added with 225 ml of Tryptic Soy Broth (TSB; Bacto™,), and it has been homogenized in a stomacher (Interscience, France) for 60 s followed by incubation at 30°C for 12 h. *B. cereus* count has been determined on the basis of ISO 7932l2004 by the surface plating method with mannitol egg yolk polymyxin (MYP) agar (Oxide CM0929). In addition, the dilutions of the stomached fluid have been prepared. Tryptic Soy Broth (TSB; Bacto™). 0.1 ml portions of each dilutions of the fluid have then been transmitted into three tubes and incubated at 30°C for 18 to 24 h. Then a loopful of culture from each tube has been streaked onto Mannitol Egg Yolk Polymyxin Agar Base (MYP; Difco) and added with sterile Polymyxin B Selective Supplement (Difco) and sterile Egg-Yolk Tellurite Emulsion 20% (V/V) (Merck) which is a particular media for the isolation of the Rough and bright pink colonies with a zone of egg yolk precipitation, and use chromogenic medium (chromagar™ *B. cereus* base)add supplement(France) have then been transmitted to nutrient agar slants, *B. cereus*. Identification is consequently confirmed by microscopic tests. The biochemical tests have been conducted as described by[12].

Isolation of genomic DNA

The bacterial DNA has been extracted from 1 ml of the overnight culture grown of 30^0C on orbital shaker (200 rpm) and then it has been purified through using a DNA Extraction Kit (Promega). The concentration and purity of the extracted DNA have been established by absorbance at 260 nm and 280 nm through using Maestro Nano Spectrophotometer (Maestro Gen, USA). Then, the extracted DNA has been stored at -20°C until it can be used.

2.4 PCR Amplification

PCR amplification has been operated in a 25 µl reaction volume containing a mixture of DreamTaq™ PCR Master Mix (Fermentas), forward and reverse oligonucleotide primer (1st Base, Malaysia), nuclease free water (NFW) and the extracted DNA as it is explicated by the manufacturer's instruction. All this mixture has been produced in a 0.2 ml sterile PCR tubes.Moreover, negative control has been provided through substituting the extracted DNA with nuclease free water (NFW) while positive control which used *Bacillus cereus* ATCC

11778 DNA. PCR reaction has also been carried out through utilizing Eppendorf Gradient Thermocycler (Eppendorf, Germany). The PCR samples have been subjected to amplification on the basis of the program which comprises the initial heat activation at 94°C for 3 minutes, and with 25 cycles have been designed as follows: 94°C for 1 minutes, 50°C for 1 minutes, 72°C for 2 minutes ,and a final extension 72°C for 10 minutes(hblD-PCR 1), initial denaturation at 95°C for 5 min, followed by 30 cycles including denaturation at 94°C for 1 min, annealing at 55°C for 1 min (ces-PCR 2). A final extension has also been carried out at 72°C for 10 min. Then, the amplified PCR product (amplicon) has then stored at -20^{o}C for electrophoresis purpose. The extracted DNA from bacteria have then been taken apart through utilizing 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 minutes. The gel has been pre-stained with Maestrosafe^{TM} Nucleic Acid (V-Bio Science, Malaysia) while GeneRuler^{TM} 1 kb and 100 bp DNA ladder (Fermentas) has been utilized as DNA size marker. Finally, all gels have been observed and captured by UV trans-illuminator Gel Documentation System (Syngene, UK).

Table 1: Characteristics of PCR primers used for detection of *B. cereus* toxin gens. used in this study.

Traget gene	Primer codes	primer sequences (5'-3')	Product size (bp)	References
HblD	*hblD F* *hblD R*	AGGTCAACAGGCAACGATTC CGAGAGTCCACCAACAACAG	205 bp	[13]
Ces	c*es F1* c*es R2*	GGTGACACATTATCATATAAGGTG GTAAGCGAACCTGTCTGTAACAACA	665bp	[14]

Results and Discussion

A total of 70 cooked rice (n=70) samples have been got from three various locations of Kajang (25), Bangi (25), and UKM (20), respectively. A total of 43.3% samples of cooked rice have known to be positive for *B. cereus* from Kajang (48.6%), Bangi (28.6%) and UKM's cafeteria (22.9%) samples have been found to be positive. *B. cereus* contamination has been the average number of *B. cereus,* between 1.2×10^4 to 1.6×10^6 cfu/g in these cooked rice samples cfu/g. All these *B. cereus* isolates (n =35) manifest similar biochemical characteristics[15] : Those isolates also have been reported as having positive results on citrate but not hydrolyze urea and coagulase , decrease nitrate into nitrite, , motile, 10% isolation into starch hydrolysis, catalase, Indol test. On the other hand, the cells morphology which were viewed under microscope have been demonstrated as rod shape (Table 2).The finding was consistent with polymerase chain reaction (PCR) analysis in which the *B. cereus* strain (BC2,BC7,BC10,BC13,BC15,BC20,BC24,BC27,BC28,BC29,BC30) isolates manifested being positive toward *B. cereus* enterotoxin by targeting two genes of hblD gene and ces. The amplicon generated from PCR analysis showed particular band of 205 bp (hblD) and 665 bp (ces), respectively on agarose gel (Figure 1).The results indicate that *hblD* 34.3% (12/35) (fig. 1) of *B. cereus* isolates comprise enterotoxic HBL complex which encode genes. The ces gene has not been realized in any sample (Table3).One of the most recent ones has been developed by[16] in India manifesting raw and pasteurized milk and the detection of HBL genes. In that investigation the percentages of detection for *hblA*, *hblC* and *hblD* have been around 70% for the tested samples. Another study conducted in Thailand by[17] utilizing milk demonstrated detection percentages of *hblA, hblC* and *hblD* genes around 60%. In Brazil[18], investigated different types of food, comprising products, for the presence of *hblA, hblC* and *hblD* genes the genetic detection has been lower than 40% for *hblA* and *hblC* and *hblD* has been found in 70.6% of the isolates which were examined. In all these investigations, including this present one, the gene with the higher detection rate has been *hblD*, indicating that, in milk and dairy products, *hblD* is the most extensively distributed HBL gene of *B. cereus.* In Malaysia, some investigations on biosafety of *B.cereus* have also been conducted (e.g. ready to eat cereals, chocolate, honey, milk by[19].

Therefore, It has been an indication that direct detection of both genes (hblD and ces) utilizing PCR analysis is preliminarily helpful, since this technique has been rapid and simple to recognize foods suspected to cause food poisoning of enterotoxigenic *B. cereus.*

Table 3. ***Prevalence of enterotoxin genes of Bacillus cereus isolates.***

Samples Positive/Total (%)	**No.of Isolates**	**hblD**	**Ces**
Kagan	BC1- BC17	6 (17%)	**0**
Bangi	BC18-BC28	4 (11.4)	**0**
UKM	BC29-BC35	2 (5.7%)	**0**
Total = 35	BC1 -BC35	12 (34.3%)	**0**

Table 2: Morphology and biochemical test of *Bacillus cereus* in cooked rice samples.

Citrate utilization	coagulase	Urea hydrolysis Urea	Anaerobic growth	Gram stian	Shape	Starch hydrolysis	Catalase	Indol	Motility	Nitrate reduction	No.of isolat
+	-	-	+	ve+	Rod	+	+	-	+	+	BC 1
+	-	-	+	ve+	Rod	+	+	-	+	+	BC 2
+			+	ve+	Rod	+	+	-	+	+	BC 3
+	-	-	+	ve+	Rod	+	+	-	+	+	BC 4
+	-	-	+	ve+	Rod	+	+	-	+	+	BC 5
+			+	ve+	Rod	+	+	-	+	+	BC 6
+	-	-	+	ve+	Rod	+	+	-	+	+	BC 7
+	-	-	+	ve+	Rod	+	+	-	+	+	BC 8
+			+	ve+	Rod	+	+	-	+	+	BC 9
+	-	-	+	ve+	Rod	+	+	-	+	+	BC 10
+	-	-	+	ve+	Rod	-	+	-	+	+	BC 11
+			+	ve+	Rod	-	+	-	+	+	BC 12
+	-	-	+	ve+	Rod	-	+	-	+	+	BC 13
+	-	-	+	ve+	Rod	-	+	-	+	+	BC 14
+			+	ve+	Rod	-	+	-	+	+	BC 15
+	-	-	+	ve+	Rod	-	+	-	+	+	BC 16
+	-	-	+	ve+	Rod	-	+	-	+	+	BC 17
+			+	ve+	Rod	-	+	-	+	+	BC 18
+	-	-	+	ve+	Rod	-	+	-	+	+	BC 19
+	-	-	+	ve+	Rod	-	+	-	+	+	BC 20
+			+	ve+	Rod	-	+	-	+	+	BC 21
+	-	-	+	ve+	Rod	-	+	-	+	+	BC 22
+	-	-	+	ve+	Rod	-	+	-	+	+	BC 23
+			+	ve+	Rod	-	+	-	+	+	BC 24
+	-	-	+	ve+	Rod	-	+	-	+	+	BC 25
+	-	-	+	ve+	Rod	-	+	-	+	+	BC 26
+			+	ve+	Rod	-	+	-	+	+	BC 27
+	-	-	+	ve+	Rod	-	+	-	+	+	BC 28
+	-	-	+	ve+	Rod	-	+	-	+	+	BC 29
+			+	ve+	Rod	-	+	-	+	+	BC 30
+	-	-	+	ve+	Rod	-	+	-	+	+	BC 31
+	-	-	+	ve+	Rod	-	+	-	+	+	BC 32
+			+	ve+	Rod	-	+	-	+	+	BC 33
+	-	-	+	ve+	Rod	-	+	-	+	+	BC 34
+	-	-	+	ve+	Rod	-	+	-	+	+	BC 35

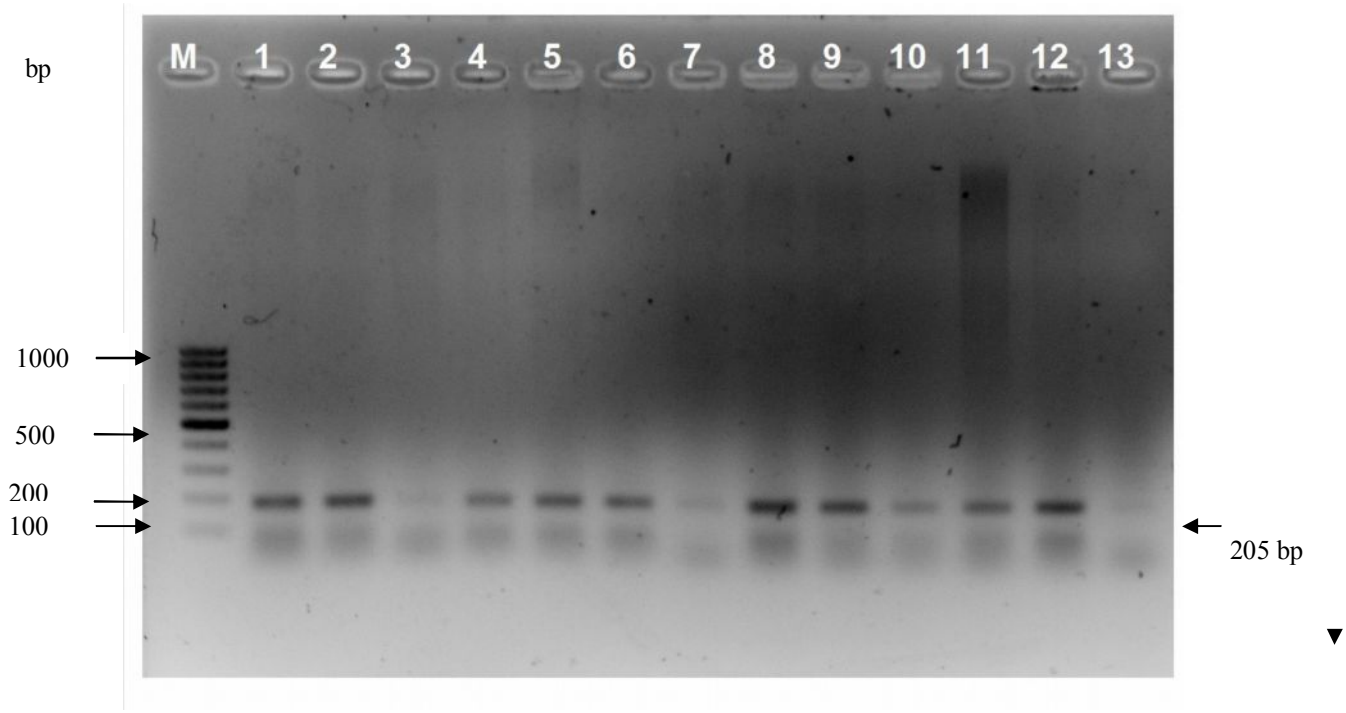

Figure 1 : Demonstration of *hblD* gene of *B. cereus* strains by PCR technique. M: Marker; 1: positive control;2 –13: *B. cereus*(BC2,BC7,BC10,BC13,BC15,BC20,BC24,BC27,BC28, BC29,BC30) isolates.

Conclusion

The data which has been demonstrated and explicated in the present study indicates a high expression of the diarrheal toxin HBL of *Bacillus cereus* isolated in cooked rice samples manifesting that the pathogenic strains of the microorganism have very well adapted for toxins production on these products. Raw and cooked rice have been discovered to be contaminated with *B. cereus*. The spores of this microorganism would not be devastated by heat treatment (such as boiling or frying). Foods kept at ambient temperature let the spores germinate and grow fast and consequently cause food poisoning. In general, food poisoning takes place as a result of poor hygiene and/or food handling practice. As almost all samples have been kept at room temperature for several hours before consumption, *B. cereus* has been detected in all samples. Therefore, it is essential to train food handlers concerning their responsibilities for food safety and educate them on personal hygiene policies and crucial practices for having safe food handlings.

Acknowledgement

The author thanks Associate. Prof. Sahilah Abd Mutalib and Prof. Dr. Aminah Abdullah for their fruitful discussions and support, The present research project has been supported by the School of Chemical Sciences and Food Technology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM), Malaysia.

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