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Determination of enterotoxin genes in *Bacillus cereus* from raw milk using multiplex polymerase chain reaction technique

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ABSTRACT

A Gram-positive bacterium, which can usually be found in cultivating plants and soil, causing it commonly universal is regarded as *Bacillus cereus*. It is said that most strains present in *B. cereus* is specifically called the primary cause of food poisoning illnesses which is characterised by either emetic or diarrheal syndrome, the most essential purpose of the current research was, therefore, to isolate and identify the enterotoxin genes in *B. cereus* through using a multiplex polymerase chain reaction (mPCR)technique. To achieve this aim, thus *B.cereus* was isolated from raw milk then cultured on mannitol egg yolk polymyxin agar and chromogenic *B. cereus* agar. The samples were then examined using biochemical investigation then established using partial sequencing of 16SrDNA. Among 40 samples, 20 of them were found to contain *hblC* and *hblD*, a hemolytic enterotoxin (HBL) complex encoding genes. The total mean of contamination samples in the *B. cereus* was proved to be higher (1.4×10^5 – 3.2×10^5 CFU/mL) than the acceptable detection level of *B. cereus* in dairy products ~10^3 CFU/mL. The isolates (10%) were shown to express *hblC* gene whereas the isolates (20%) were shown to express *the hblD* gene. The findings suggested that the detected enterotoxin genes of *B. cereus* which were found in raw milk may create a potential danger to the health of the public.

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INTRODUCTION

The gram-positive bacteria, *Bacillus cereus,* causes, has been found to cause critical food poisoning. *Bacillus cereus* has been identified in a forms spores and rod shape with the spores that can occur in the circumstances as hot and dry and continue being inactive for a long time (Drobniewski, 1993). *Bacillus cereus* is found to live in soil, though; it is identified in foodstuffs like dairy products, cornflakes (cereals), and something derived from cereals, as well as rice, dried foodstuffs, eggs, vegetables, spices and meats (Svensson *et al.,*2007). According to the FDA of the United States, food poisoning which is resulted from *B. cereus* is found in two various clinical disorders like diarrheal and emetic disorder. In the emetic category, between 0.5 and 6 h of ingestion lead to vomiting whereas in diarrhoeal type, between 8 and 16 h of consumption lead to diarrhoea and abdominal pain (Ehling-Schulz *et al.,* 2005). The diarrhoea caused by poisoning is resulted by heat-labile enterotoxins

which are generated while the vegetative development of *B. cereus* is made in the small intestine (Beecher *et al.,* 1995). Three various enterotoxins of *B. cereus* a rereported in which are engaged in food poisoning incidence. They contain two protein complexes as hemolysin BL (HBL) and non-hemolytic enterotoxin (NHE) as well as the single protein cytotoxic CytK (Agata *et al.,*1995). The occurrence of *B. cereus* in raw milk has been said to be potentially as a result of the fact that food handlers are mainly administered by migrant staffs or it is because of lack of enough education and knowledge with respect to food hygiene. The present study hence aimed at examining the pervasiveness of *B. cereus* which existed in raw milk through analysing the genetic variation among raw milk isolates of *B. cereus*. The variety of genes which is among *B. cereus* displays the locality and the association of *B. cereus* isolates which circulate in diverse settings. The findings of this study will be of extreme significance for examination and control.

MATERIALS AND METHODS

Collection and Preparation of Sample

To collect the data and to identify the bacteria, in this study, 40 raw milk samples were bought and collected at 9 pm from the dairy farms located in Selangor, in Malaysia.

Isolation and Morphological Description

In this study, the analysis of raw milk samples was performed on the basis of (Rhodehamel and Harmon's, 2001) research through conducting a standard process for identification of *B. cereus*. In this case, 225 mL from Tryptic Soy Broth (TSB, Oxoid) together with 25 mL of every given sample (raw milk) was situated in a stomacher bag, and they were homogenised for 60 s in a stomacher (Inter Science, France). The preparations of Serial dilutions were performed from 10^{-1} to10^{-6} by transmitting 1 mL from the homogenised sample (1:10 dilution) to 9 mL of TSB broth. Then, the serial dilutions were made up to 10^{-6}. Afterwards, one hundred µl (0.1 mL) was then transmitted into mannitol egg yolk polymyxin (MYP, Oxoid) agar which was then mixed with sterilized Polymyxin B (Difco), 20% (v/v) from Egg Yolk Tellurite Emulsion (Merck) as well as Chromogenic plate agar (CBC, France). A number of tests were carried out on these colonies which include gram staining, spore formation, as well as chemical investigations for example motility, VP, amylase (starch hydrolysis), nitrate reduction, catalase and beta- hemolysis on sheep blood agar, which was conducted and repeated three times. The biochemical investigations were carried out on the basis of the procedure described by (Roberts & Greenwood, 2003).

Isolation of Genomic DNA

For the purpose of this study, the bacterial DNA extraction was performed for overnight at 30°C through using culture grown 1 mL on an orbital shaker (200 rpm), to measure both of the absorbances at 260 nm and 280 nm. The extraction of DNA was then saved for further use.at -20°C.

PCR Amplification

PCR amplification is managed in a 25 µL reaction volume which contains a combination of PCR Master Mix, nuclease-free water using as a negative control, as well as forward and reverses oligonucleotide primer (1st Base, Malaysia) together with the DNA extraction as it is clarified by the producer's guidelines as designated in Table 1. The mixture is generated in the tubes, 0.2 mL sterilized PCR. In addition, the control which has been negative was performed by the substitution of the DNA extraction with that of NFW and the positive control was carried out using *B. cereus* ATCC 11778 DNA. Gradient Thermocycler (Eppendorf, Germany) was used to perform a PCR reaction. These PCR samples then were put under amplification test based on the agenda which contain the initial activation of heat initial denaturation for 3 minutes at 94°C which was followed by 25 cycles of denaturation for 1 minute at 94 °C, annealing for 1 minute at 50°C as well as polymerization for 1 min at 72°C. Following that, the last elongation was performed for 10 minutes at 72°C.NFWwastaken into account in each PCR amplification, so as to validate the PCR effectiveness and to identify contamination (Devereux & Wilkinson, 2004).

Detection of Haemolysin BL (*hblC* and *hblD)* Genes by (PCR) Amplification

The identification of haemolysin BL (*hblC* and *hblD*) genes were carried out through what described by Çadirci *et al.,* (2013) using the primers as indicated in Table 2 which produced 411 bp and 205 bp amplicons respectively. The reaction of PCR was carried out in a 25 µl volume which contains 12.5 µl of DreamTaq™ PCR MasterMix (MyTACG Bioscience Enterprise, MY), 1µl forward and reverse 1µl of oligonucleotide primers (either *hblC* or *hblD*) 7.5 µl of sterile NFWas well as the 3 µl of (50-100 ng) DNA template. The purification of the amplicon was performed by using a PCR purification kit (Promega, MY). The amplification was also carried out in an Eppendorf Mastercycler (Eppendorf, Germany) through using a temperature plan which includes the primary denaturation for 5 minute at 95°C and then was continued by 30 denaturation cycles at 94°C for 1 minute, annealing for 1 min at 50°C and polymerization for 1 minute at 72°C. Further, the last elongation was then carried out at 72°C for 10 minutes. NFW was taken into account

Table 1: Characteristics of PCR primers used for the study

Target gene	Primer codes	Primer sequences (5'-3')	Product size (bp)	References
16S rDNA	*16S rDNA F*	AGAAGTTTGATCCTGGCTCAG	711 bp	(Devereux & Wilkinson et al. 2004)
	16S rDNA R	AAGGAGGTGATCCAGCCGCA		
hblC	*hblC R*	CGAAAATTAGGTGCGCAATC	411 bp	(Moravek et al. 2004)
	hblC F	TAATATGCCTTGCGCAGTTG		
hblD	*hblD R*	AGGTCAACAGGCAACGATTC	205 bp	(Moravek et al. 2004)
	hblD F	CGAGAGTCCACCAACAACAG		

Table 2: Isolation strains in this study and GenBank accession similarity for 16S rDNA sequences

Number of strains	Description	Identity (%)	Accession No.
BCM1	*Bacillus cereus*	99%	CP017060.1
BCM2	*Bacillus cereus*	98%	MG027637.1
BCM3	*Bacillus cereus*	99%	EF036537.1
BCM4	*Bacillus cereus*	99%	CP022044.2
BCM5	*Bacillus cereus*	99%	MF988719.1

in every PCR amplification. It was done to validate the PCR effectiveness and to identify contamination.

Gel Electrophoresis

In this stage following PCR amplification, electrophoresis method on 1.5 % (w/v) agarose gel in 1X TAE buffer. Next, At 100 V for 40 minutes was applied to separate the extracted DNA from the bacteria. After that, Maestrosafe™ Nucleic Acid was applied to pre-stain the gel and also GeneRuler™ 1 kb DNA ladder and 100 bp (Fermentas, MY) were used as DNA size marker. At last, UV trans-illuminator Gel Documentation System (Syngene, UK) was performed to examine and capture all gels.

RESULTS OF THE STUDY

Isolation of *Bacillus cereus*

The range of *B. cereus* isolates was reported as $1.4\times10^5 - 3.2\times10^5$ CFU/mL in raw milk samples. All the isolates of *B. cereus* (n =20) showed that the same of biochemical characteristics of 100% of isolates could make acidic fermentation from lactose, fructose and glucose. Nonetheless, no one of them was able to utilise mannitol, mannose, arabinose and xylose (Roberts& Greenwood, 2003). The isolates also demonstrated positive findings on citrate, the reduction of nitrate into nitrite, motile, starch hydrolysis, and catalase. The findings which were negative on oxidase and Indol test. The light microscope was used to diagnose the cells characterized by a rod shape.

The isolates of *B. cereus* have been numbered20 on 16SrDNAsequences were analyzed as illustrated in Figure1. The amplicons were transferred to MyTACG Bioscience Enterprise laboratory, and 16SrDNAsequenced data were evaluated comparing it with the National Center for Biotechnology Information (NCBI). GenBank databases apply. Table 2 shows that the 16SrDNAsequences of *B. cereus* isolates were examined through a comparison of these sequences from Gen Bank database.

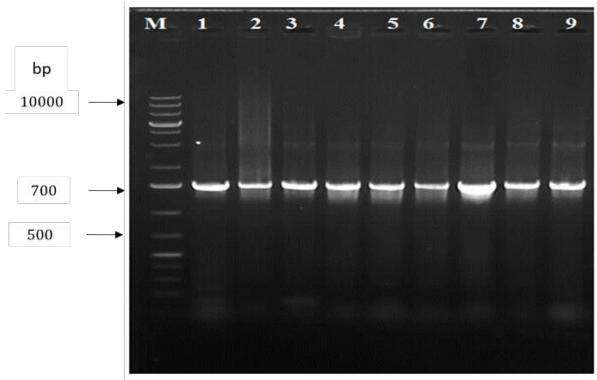

Figure 1: Amplicons of Bacillus cereus isolates using 16s rDNA universal primers on 1.5 % (w/v) agarose gel. Lane M: 1 kb DNA ladder, Lane1-8: B. cereusisolates and Lane 9: Positive control

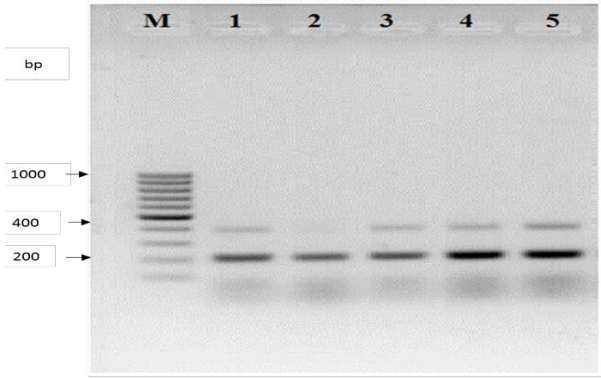

Figure 2: Amplicons of Bacillus cereus isolates using hblC and hblD genes 1.5 % (w/v) agarose gel. Lane M: 100 bp DNA ladder, Lane 2-5: B. cereus isolates

The toxigenic profiles of *B. cereus* which were isolated from raw milk were evaluated by multiplex

PCR assay for the existence of toxigenic genes associated with enterotoxin diseases. Multiplex PCR assay according to (Choo *et al.,* 2007; Çadirci *et al.,*2013) demonstrated that the *B. cereus* was positive towards *hblC* gene (10%) and that produced amplicons of 411 bp in size which is shown in (Fig 1). *Bacillus cereus* which was demonstrated as positive against *hblD* gene (20%) would produce amplicons of 205 bp in size as illustrated in (Fig 2).

DISCUSSION

Whereas the findings of this study were incompatible with that study described by (Sandra *et al.,* 2012), the occurrence of *B. cereus* in the samples was reported to be lower with ranged from< 3 to 1100 MPN/g. The findings were in agreement with (Blanco's *et al.,* 2009) study. They detected eight samples test with various kinds of foodstuffs (onion, sweet potato, fried rice, boiled rice, celery and coriander) which were reported to be contaminated with *B. cereus* at different concentration levels ranging from 140 to 3.9×10^6 CFU/g. Correspondingly, (Sandra *et al.,* 2012) (King *et al.,* 2007) reported that the dehydrated potato samples between 10 and 40% were infected with *B. cereus.* (Kim *et al.,* 2004) found that the *B. cereus* was infected with 17 samples of raw sprout products that represent of 70.6% then 118 seafood samples that represent of 13.6% as well as 140 dried red pepper samples that represent of 84.3%.

Moreover, as the findings indicated, the results were incompatible with (Çadirci *et al.,* 2013) that have to some higher amount while compared to *B. cereus* positive against *hblD* gene dessert samples 6(30 %), (Andre *et al.,* 2013) reported that the total of food 260 samples (milk and dairy products) were examined, 34 (54%) were shown to be positive against *hblD* gene. Rather *et al.,*2011) showed that the analysed food samples that include both meat products and raw meats were 150 whereas the positive demonstrating against *hblD* were 66%.

CONCLUSION

The spoilage potential of *B. cereus* was on the basis of some main issues such as bacterial concentration which exist in dairy products (raw milk). In this study, the findings designated that 50% of raw milk samples which were found from optional milk farms in Selangor were contaminated with *B. cereus* strains. Some of the *Bacillus cereus* spp. Belonging to one of the most ubiquitous foodborne pathogens. Bacterial growth leads to various toxins production; therefore, food containing consumption $>10^3$ bacteria per gram may cause emetic and diarrhoeal syndromes. A quick and sensitive bacterial investigation technique is found to be important for food integrity.

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