

Detection of enterotoxin targeted entFM and hblA genes by inoculating *Bacillus cereus* (Strain BC1) into ready-to-eat food (RTF) and drink samples using polymerase chain reaction (PCR)

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Article history

Received: 12 February 2013

Received in revised form:

19 March 2013

Accepted: 20 March 2013

Abstract

Detection of enterotoxin by targeting entFM and hblA genes in *Bacillus cereus* BC1 strain inoculated into ready to eat food (RTF) and drink samples using polymerase chain reaction (PCR) was conducted. The *B. cereus* BC1 strain was confirmed as a Bacillus diarrhoeal enterotoxin (BDE) when tested by a commercially available Enzyme-linked immunosorbent assay-BDE immunoassay (ELISA-BDE immunoassay, TECRA). In the specificity study, both enterotoxin genes were detected on chromosomal DNA of *B. cereus* BC1 strain by showing a specific band of 1269 bp (entFM) and 874 bp (hblA), respectively. However, none of the target genes were detected for the other 15 genomic DNA bacteria (*B. cereus* (ATCC 11779), *B. subtilis* (ATCC 6633), *Campylobacter jejuni* (ATCC 29428), *C. coli* (Jabatan Kimia Malaysia, JKM), *Clostridium perfringens* (ATCC 13124), *Enterobacter sakazaki* (ATCC 51329), *Escherichia coli* (ATCC 43888), *E. coli* (ATCC 11735), *Legionella pneumophila* (ATCC 33152), *Listeria monocytogenes* (ATCC 35967), *Salmonella typhi* (IMR), *S. enteritidis* (ATCC 13076), *S. typhimurium* (ATCC 14028), *Shigella flexneri* (ATCC 12022) and *Vibrio cholerae* bengal (Institute Medical Research (IMR), Malaysia) examined. The detection limit of both genes was 0.1 ng of genomic DNA. Thus, in the presence study it is evidence that the PCR analysis targeting enterotoxin of entFM and hblA genes are suitable and useful in detecting enterotoxic *B. cereus* in RTFs and drinks contaminated sample.

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Keywords

Bacillus cereus

BDE

PCR

entFM

hblA

Introduction

Bacillus cereus food poisoning is a major concern worldwide. This bacterium is an aerobic spore-forming bacterium and commonly found in soil. They are also can be isolated from raw meat, processed foods and vegetables and entered into the food chain either through contaminated food or water. Food poisoning from the past outbreaks include boiled and fried rice, vegetables, cooked meats, soups, and raw vegetable sprouts (FDA 2012). Contamination of these bacteria may take place when foods are prepared and inadequate refrigeration before serving.

Two types of sickness have been recognized to the consumption of foods contaminated with *B. cereus*. The first is characterized by abdominal pain and non-bloody diarrhea, occurs within 4-16 hours after ingestion. The second is an acute attack of nausea and emetic (vomiting), occurs within 1-5 h after eating of contaminated food (Granum *et al.*, 1996; Kotiranta *et al.*, 2000; Ehling-Schulz *et al.*,

2004). The former is known by Bacillus Diarrhoeal Enterotoxin (BDE) due to its ability to produce many proteolytic enzymes and toxic to animals and humans (Mäntynen and Lindström, 1998).

Bacillus cereus produces one emetic toxin (ETE) which can cause both diarrhea and vomiting; and three different enterotoxins of HBL complex that is hemolytic enterotoxins (hblA, hblC, and hblD gene), Nhe complex (nheA, nheB, and nheC genes) and EntK. All three enterotoxins are cytotoxic and cell membrane active toxins that will make holes or channels in membranes (Charve *et al.*, 2012). Mäntynen and Lindström (1998) used hemolysin hblA and bceT gene for rapid test of enterotoxic *B. cereus*. While, Chaves *et al.* (2012) used hblA, hblC, hblD, nheA, nheB, nheC, cytK, ces, and entFM genes for detecting *B. cereus* in coffee as food poisoning vehicle. The entFM appears to be common to and *B. cereus* and *Bacillus thuringiensis* strains. It also shown that the entFM gene was detected in most outbreak-associated strains due to it was suspected to

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cause at high doses fluid accumulation in rabbit and mouse ligated intestinal loop tests (Agata *et al.*, 1995; Tran *et al.*, 2010). 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* bacteria contamination in food to estimate the lowest genomic DNA present can be detected in food. The *B. cereus* BC1 strain was used as genomic DNA source for detection limit determination and the bacteria cells were inoculated into 8 types of RTF and 2 type of drinks. The PCR analysis was conducted by targeting entFM and hblA genes in detecting enterotoxic *B. cereus* in RTFs and drinks sample.

Materials and Methods

Bacteria

Bacillus cereus BC1 strain and *Campylobacter coli* were supplied by Chemistry Department (JKM), Malaysia. *B. cereus* (ATCC 11778), *B. subtilis* (ATCC 6633), *Campylobacter jejuni* (ATCC 29428), *Clostridium perfringens* (ATCC 13124), *Enterobacter sakazaki* (ATCC 51329), *Escherichia coli* (ATCC 43888), *Legionella pneumophilla* (ATCC 33152), *Listeria monocytogenes* (ATCC 35967), *S. enteritidis* (ATCC 13076), *S. typhimurium* (ATCC 14028) and *Shigella flexneri* (ATCC 12022) were purchased from American Type Culture Collection (ATCC). While, *Salmonella typhi* and *Vibrio cholerae* bengal were supplied by Institute Medical Research (IMR), Malaysia.

Bacillus Diarrhoeal Enterotoxin (BDE) confirmation using TECRA kit

Bacillus cereus (BC-1) was confirmed using TECRA kit. This bacteria were inoculated onto Mannitol-Egg yolk-polymixin medium and incubated at 37°C for 24 hours. *B. cereus* colonies were shown pink in color and surrounded by precipitate zone which indicated that lecithinase producer. Five colonies were then further tested using ELISA kit (TECRA) to confirm the enterotoxic *B. cereus* colonies were belong into *Bacillus* Diarrhoeal Enterotoxin (BDE) group. A single colony was selected and named as *B. cereus* BC1 strain. This strain was used in artificial contamination in RTFs and drinks sample.

DNA extraction

The genomic DNA of bacteria was extracted

using Epicentre (Epicentre, USA) extraction method as described by the manufacturers with modification and known as JKM M 3050 standard method for bacteria DNA extraction.

Detection of hblA gene

Detection of hblA gene and PCR condition was conducted as described by Granum *et al.* (1996). A pair of primer was used as sequences described hblA for forward, 5'- GCT AAT GTA GTT TCA CCT GTA GCA AC -3' and hblA for reverse 5'- AAT CAT GCC ACT GCG TGG ACA TAT AA -3'. The assay was performed in a 50 µl volume containing 0.2 µl of 1.5 unit Taq polymerase (Promega), 10 mM Tris-HCL, 50 mM KCL, 1.5 mM MgCl₂ (Promega), 25 µl of 10X PCR reaction buffer, 5 µl of 10 ng of DNA, 0.2 µl of 1.5 µM dNTPs, 5 µl of 10 pM each of the forward and reverse primers (Helix Biotech) and 10 µl of deionized water. Amplification was performed in Thermal-cycler (BioRad) with a temperature program consisting of the initial denaturation at 94°C for 30 second followed by 5 cycles of denaturation at 70°C for 1 min and 72°C at 1.3 min. The PCR amplication was then change to 35 cycles and DNA denatured at 94°C for 30 second, annealing for 1 min at 65°C, and polymerization at 72°C for 1.3 minute. Final elongation was at 72°C for 7 minute. The amplification products were analyzed by electrophoresis in a 1.5% (w/v) agarose 1X TBE (0.1 M Tris, 0.1 M boric acid, 0.2 mM EDTA) at 120 V for 60 minutes. Gels were stained with ethidium bromide. The amplified fragments were visualized with UV transilluminator (Alpha Imager TM2200). The 100 bp and 1 Kb DNA ladder (Promega) was used as a DNA size marker.

Detection of entFM gene

Detection of entFM gene and PCR condition was conducted as described by Martinez-Blanch *at al.* (2009). A pair of primer was used as sequences described entFM for forward, 5'- ATG AAA AAA GTA ATT TGC AGG -3' and entFM for reverse 5'- CGT GCA TCT GTT TCA TGA AA-3'. The assay was performed in a 50 µl volume containing 0.2 µl of 1.5 unit Taq polymerase (Promega), 10 mM Tris-HCL, 50 mM KCL, 1.5 mM MgCl₂ (Promega), 25 µl of 10X PCR reaction buffer, 5 µl of 10 ng of DNA, 0.2 µl of 1.5 µM dNTPs, 5 µl of 25 pM each of the forward and reverse primers (Helix Biotech) and 10 µl of deionized water. Amplification was performed in Thermal-cycler (BioRad) with a temperature program consisting of the initial denaturation at 94 °C for 1 min followed by 30 cycles of denaturation at 70°C for 20 second, annealing at 52°C for 20

second and polymerization at 72°C for 7 minute. The amplification products were analyzed by electrophoresis in a 1.5% (w/v) agarose 1X TBE (0.1 M Tris, 0.1 M boric acid, 0.2 mM EDTA) at 120 V for 60 minutes. Gels were stained with ethidium bromide. The amplified fragments were visualized with UV transilluminator (Alpha Imager TM2200). The 100 bp and 1 Kb DNA ladder (Promega) was used as a DNA size marker.

Detection limit of oligonucleotide primers

The detection limit of entFM and hblA oligonucleotide primers were examined using *B. cereus* BC1 strain genomic DNA. The PCR assay condition was similar as described in PCR amplification using different oligonucleotide primers with different concentration of genomic DNA ranging from 0.1-30 ng.

Detection of enterotoxin entFM and hblA gene using *Bacillus cereus* BC1 strain inoculated in ready-to-eat foods (RTF) and drink samples

A total of 8 types RTF such as rice, chicken curry, donut, fried noodle, karpap, fried kwee tiow, chicken with coconut milk and fish with chili paste were inoculated with *B. cereus* BC1 strain at a concentration of 1×10^4 CFU/ml of 100 g food samples. Two types of drink namely; orange juice and plain water were also inoculated with *B. cereus* cells at a similar concentration of 50 ml solution. All samples were incubated at 37°C for 2 hours. Each food sample was weighted at 25 g in a stomacher bag and added 225 ml of TSB Broth. The sample was homogenized using Stomacher Lab blender for 1 minute and incubated at 37°C for 24 hours. One ml of incubated samples were placed into microcentrifuge tube and centrifuged at a low speed of 200 rpm for 3 min to remove the insoluble food particles. The supernatant was transferred into a new microcentrifuge tube and centrifuged at a maximum speed of 10,000 rpm for 1 min to obtain pellet cell. DNA extraction was done using JKM M 3050 standard method for bacteria DNA extraction. The genomic DNA concentration was then diluted up to 0.1 ng. The PCR analysis for targeting the enterotoxin of entFM and hblA genes were then conducted as method mentioned above.

Results and Discussion

The presence of *B. cereus* in foods source was traditionally relied previously on selective medium based on the ability of *B. cereus* grown onto culture contained polymixin B and its lecithinase reaction. Isolation, biochemical test, confirmation and enterotoxin detection is time consuming requiring

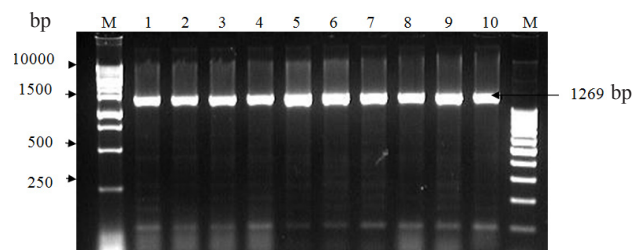


Figure 1. Detection limit of entFM gene from genomic DNA of *Bacillus cereus* (Strain BC1). Lane M: 1 kb ladder; Lane 1: 30 ng; Lane 2: 20 ng; Lane 3: 10 ng; Lane 4: 5 ng; Lane 5: 2 ng; Lane 6: 1 ng; Lane 7: 0.5 ng; Lane 8: 0.1 ng; Lane 9: 0.1 ng; and Lane 10: 0.1 ng.

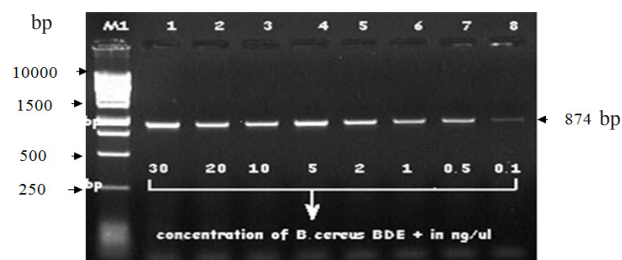


Figure 2. Detection limit of hblA gene from genomic DNA of *Bacillus cereus* (Strain BC1). Lane M1: 1 kb ladder; Lane 1: 30 ng; Lane 2: 20 ng; Lane 3: 10 ng; Lane 4: 5 ng; Lane 5: 2 ng; Lane 6: 1 ng; Lane 7: 0.5 ng; and Lane 8: 0.1 ng.

up to 3 days to perform results. In this study, we confirmed that the *B. cereus* strain BC1 were as a Bacterial Diarrhoeal Enterotoxin (BDE) using ELISA-BDE immunoassay (TECRA). The result was consistent with polymerase chain reaction (PCR) analysis where the *B. cereus* strain BC1 showed positive toward *B. cereus* enterotoxin by targeting two genes of entFM gene and hblA. The amplicon produced from PCR analysis indicated, specific band of 1269 bp (entFM) and 874 bp (hblA), respectively on agarose gel (Figure 1 and 2). None of the DNA genomic bacteria (*B. cereus* (ATCC 11779), *B. subtilis* (ATCC 6633), *Campylobacter jejuni* (ATCC 29428), *C. coli* (Jabatan Kimia Malaysia, JKM), *Clostridium perfringens* (ATCC 13124), *Enterobacter sakazaki* (ATCC 51329), *Escherichia coli* (ATCC 43888), *E. coli* (ATCC 11735), *Legionella pneumophila* (ATCC 33152), *Listeria monocytogenes* (ATCC 35967), *Salmonella typhi* (IMR), *S. enteritidis* (ATCC 13076), *S. typhimurium* (ATCC 14028), *Shigella flexneri* (ATCC 12022) and *Vibrio cholerae* bengal (Institute Medical Research (IMR), Malaysia) tested were positive toward both genes, indicated both gene were specific for enterotoxigenic *B. cereus*. The result was in general agreement with Martinez-Blanch *et al.* (2009) and Granum *et al.* (1996) whose reported the Bacterial Diarrhoeal Enterotoxin (BDE) would produced a specific band of 1269 bp (entFM) and 874 bp (hblA), respectively. Similar findings were also reported by Agata *et al.* (1995) and Mantynen

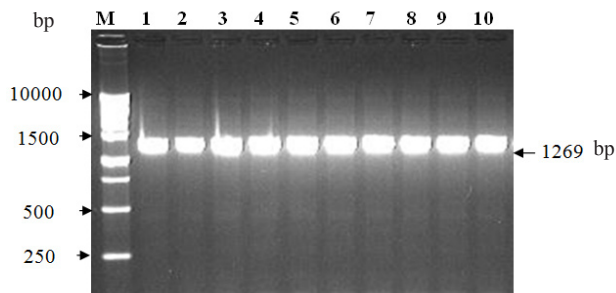


Figure 3. Detection of entFM gene in various type of ready to foods (RTFs) and water samples. Lane M: 1 kb ladder ; Lane 1: Rice; Lane 2: Orange juice, Lane 3: Chicken curry; Lane 4: Donut; Lane 5: Fried noodle; Lane 6: *Karipap*; Lane 7: Fried Kwee tiow; Lane 8: Water; Lane 9: chicken with coconut milk and Lane 10: Fish with chilli paste.

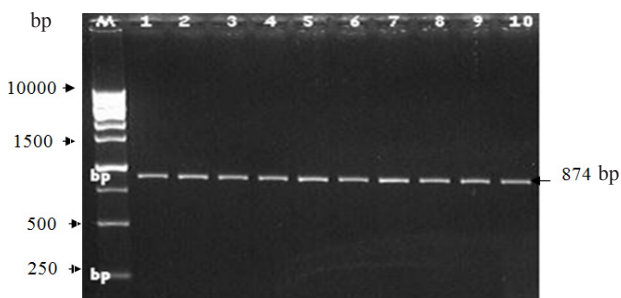


Figure 4. Detection of hblA gene in various type of ready to eat foods (RTFs) and water samples. Lane M: 1 kb ladder ; Lane 1: Rice; Lane 2: Orange juice, Lane 3: Chicken curry; Lane 4: Donut; Lane 5: Fried noodle; Lane 6: *Karipap*; Lane 7: Fried Kwee tiow; Lane 8: Water; Lane 9: chicken with coconut milk and Lane 10: Fish with chilli paste.

and Lindstrom (1998) which were able to detect two enterotoxin genes (entFM and hblA) from contaminated enterotoxigenic *B. cereus* in food.

Detection limit of both genes were conducted using various concentrations DNA of *B. cereus* strain BC1 through PCR analysis. The result showed the lowest concentrations of genomic DNA detected using PCR assay for both genes were up to 0.1 ng/ul (Figure 1 and 2). Study has been extended to artificially contaminated in variety of Ready-to-eat (RTE) food samples and drinks with *B. cereus* strain BC1. The genomic DNA extracted from RTFs and drinks were diluted to up 0.1 ng/ul, prior PCR analysis. As shown in Figure 3 and 4, *B. cereus* enterotoxin genes (entFM and hblA) were consistently detected in repeated experiments, using the above genomic DNA concentration. Similar observation has been reported by Yang *et al.* (2005) and Omboi *et al.* (2008) whose reported the direct detection of toxigenic *B. cereus* targeted enterotoxin genes in artificially contaminated foods using multiplex PCR. Thus, in the presence study combination of both gene primers or more, are suggested in PCR analysis (multiplex PCR) in detecting both genes (entFM and hblA) and

other possible enterotoxin genes in *B. cereus*. It is an evidence that direct detection of both genes (entFM and hblA) 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*.

Acknowledgement

We are gratefully thanks to Department of Chemistry Malaysia, Ministry of Sciences, Technology and Innovation Malaysia (MOSTI) for their assistance in this research.

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