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Direct detection of *Bacillus cereus* enterotoxin genes in food by multiplex Polymerase Chain Reaction

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Abstract

This study evaluated a direct multiplex PCR to detect food contamination with enterotoxigenic *Bacillus cereus (B. cereus)* in comparison with culture and multiplex gene detection using colonies. Detection of *B. cereus* enterotoxin genes was done on artificially contaminated and ready-to- eat market foods including cooked rice, pasteurized milk and cheese. Of the 108 food samples analysed, 51(47.2 % were found to be contaminated with enterotoxigenic *B. cereus* by culture and enterotoxin detection by multiplex PCR, but only 14(12.9%) of them were found to be contaminated with enterotoxigenic *B. cereus* by direct multiplex PCR. *B. cereus* enterotoxin genes were detected only in artificially contaminated and ready-to-eat market foods with bacterial counts of equal or more than 4000 ($4x10^3$)cfu/ml for both pasteurized milk and cheese and equal or more than 40,000 ($4x10^4$) cfu/g for cooked rice. Since high contamination of food with *B. cereus* (10^6 cfu/g) has been associated with food poisoning, this technique can be used to identify foods suspected to cause food poisoning without culture and identification of *B. cereus*. Detection of any of the enterotoxin genes will indicate contamination of foods with enterotoxigenic *B. cereus* group.

Keywords: *Bacillus cereus*, Enterotoxin genes, Multiplex PCR, Direct gene detection, food.

INTRODUCTION

Bacillus cereus food poisoning is a major concern worldwide. The poisoning is caused by toxigenic strains of *B. cereus*. These organisms are ubiquitous in the environment from where they contaminate food (Kramer and Gilbert, 1989; Granum et al. 1993; Granum and Lund, 1997). The organism causes emetic and diarrheal food poisoning syndromes. The diarrheal syndrome is due to hemolysin BL (Beecher *et al.*, 1995), a non-hemolytic enterotoxin (Lund and Granum, 1997) and a cytotoxin K (Lund *et al.*, 2000). The hemolysin BL consists of binding protein B (35 kda) encoded by *hblA* gene (Heinrichs *et al.*, 1993), lysing proteins L_1 (36 kda) and L_2 (45 kda) encoded by *hblC* and *hblD* genes respectively (Ryan *et al.*, 1997). Maximal expression of all HBL activities requires all three-protein components. The non-hemolytic enterotoxin has three proteins moieties B, L_1 and L_2, all of which are needed for maximun cytotoxicity. The three proteins are encoded by the three genes nheA, nheB and nheC respectively (Lindback *et al.*, 2004; Lund and Granum, 1997). The emetic syndrome is caused by a heat-stable depsipeptide cereulide that is formed during the late exponential to stationery growth phase and may be associated with sporulation (Mikami, 1994; Agata *et al.*, 1995). Cytotoxin K (CytK) of *B. cereus* is an important factor in severe food poisoning outbreaks, due to its heamolytic and cytotoxic activities (Lund *et al.* 2000, Hardy *et al.*, 2001). A variant of CytK has been designated as CytK-2, and the original CtyK as CytK-1 (Annette *et al.* 2004).

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Diagnosis of *B. cereus* food poisoning has traditionally relied on culture to identify *B. cereus* strains, detection of enterotoxins and cytotoxicity. Detection of *B. cereus* contaminating food involves use of several selective plating methods described by various authors (Holbrook and Anderson, 1980; Meira, *et al.*, 1995; Szabo *et al.*, 1984). The selection is based, on the ability of *B. cereus* to grow in the presence of polymyxin B and its lecithinase reaction (Holbrook and Anderson, 1980). Identification of enterotoxigenic *B. cereus* has mainly relied on biological and immunological methods that detect the ability of the strains to produce enterotoxin. The emetic toxin has low antigenicity which prevents its detection by immunological means. Detection of emetic - producing *B. cereus* rely on cell culture cytotoxicity assay. Culture and isolation of *B. cereus* with biochemical and confirmatory testing followed by enterotoxin detection, is time consuming requiring up to 3 days to perform.

Rapid detection of *B. cereus* in food is important to facilitate the application of quality control measures to eliminate *B. cereus* from food and enhance diagnosis of food poisoning outbreaks (Swaminathan and Feng, 1994; Rambabu and Kaiser, 2005). The advent of gene probe and PCR techniques has allowed the development of molecular techniques by which particular bacterial strains can rapidly be identified without the need for isolating pure cultures. Among the molecular methods that have been used in food microbiology, polymerase chain reaction has proved to be more rapid and cost effective (Bej *et al*. 1994; Rambabu and Kaiser 2005; Ombui *et al*. 2005). The PCR method has been used to detect various food pathogens (Cohen 1993; Tsen *et al*. 1994; Hill 1996; Klotz *et al*. 2003; Ombui *et al*. 2005).

The occurrence of DNA sequences for *hblA* , *hblC* and *hblD* genes encoding for hemolysin BL proteins, *nheA, nheB* and *nheC* genes for non-hemolytic enterotoxin proteins, CytK gene for cytotoxin K and a PCR fragment specific for emetic toxin strains of *B. cereus* allows for rapid detection of enterotoxic *B. cereus* using PCR amplification assay. To detect all the *B. cereus* enterotoxin genes, DNA can be extracted from bacterial strains and individual genes amplified and detected in separate reaction tubes. However, this increases reagent mixing errors and chances of contamination as well makes the whole exercise lengthy and costly. Recent efforts are towards establishing more rapid multiplex PCR techniques that can ensure production of results in a single reaction mix. Yang *et al.* (2005) described a multiplex PCR method for simultaneous detection of all the *B. cereus* enterotoxin genes and emetic-specific sequences using bacterial colonies. This as well requires lengthy bacterial culture and isolation. This study was aimed at developing a more rapid multiplex PCR assay method to detect simulteneously all the enterotoxin genes directly in foods.

MATERIALS AND METHODS

Bacterial strains

The following bacterial strains were used in this study; reference strains of *Bacillus cereus* NCTC 11145 that had *hblA, hblC, hblD, nheA, nheB* and *nheC* genes, *Bacillus cereus* strain BC 68 that had, *nheA, nheB, nheC* and emetic toxin genes. Other reference strains including *Escherichia coli* ATCC 25922, *Salmonella typhimirrium* 7222569-1, and Oxford *Staphylococcus aureus* were used to determine the specificity of the multiplex PCR assay.

DNA primers

DNA primers used to screen for the presence of *hblA, hblD, hblC* and *nheA, nheB, nheC* and emetic toxin genes in *B. cereus* shown in table 1 *[Supplementary data]* were described by Yang *et al*., (2005) and Ehling-Schulz *et al*. (2004). These primers were synthesized by Invitrogen® Co. USA.

Multiplex PCR Assay of *B. cereus* enterotoxin genes using colonies

A multiplex PCR was carried out using single colonies of *B. cereus* standard strains NCTC 11145, and isolate BC 68 by combining the seven primer pairs in one reaction tube to determine the suitability of a multiplex PCR assay to detect the respective *B. cereus* enterotoxin genes. One pure colony was suspended in 50µl distilled water, boiled for 10 minutes at 98^0C in a water bath and the suspension centrifuged for 3 minute at 15000 rpm. Five micro-liters of this DNA extract was amplified in a 25µl reaction mix containing the seven primer pairs, 12µl of supermix (Invitrogen, USA) containing 0.5U of Taq polymerase and

hexanucleotide. Amplification was done using 30 cycles of denaturation at 95^0C for 30s annealing at 60^0C for 30s and extension at 72^0C for 45s followed by a final extension at 72^0C for 7 minutes using a PCR minicycler (PTC-150 MJ Research, Inc. USA). PCR products were analyzed using 1.5% agarose gel electrophoresis in 0.5 X Tris boric ethylenediaminetetraacetic acid disodium salt (TBE) containing 0.5 µg/ml ethidium bromide buffer. Twenty micro-liter portions of PCR products was mixed with 4.0µl 6X gel loading dye and loaded onto gel submerged in 1 X TBE. A 100 bp molecular weight marker (Invitrogen, USA), was used to determine the approximate molecular weight of PCR products. A constant voltage of 10 V/cm was applied and the amplified fragments allowed to migrate until appropriate band separation was achieved. A UV transilluminator (Vilber Laurmat, France) was used to visualize bands relative to the molecular weight marker and results were documented by photography using a digital Polaroid camera (PDZ 2300Z, Polaroid, China).

Gene detection in artificially contaminated food by multiplex PCR

After establishing the suitability of the multiplex assay to simultaneous detect the seven *B. cereus* enterotoxin genes using colonies, the assay was repeated using DNA templates prepared directly from artificially contaminated food samples. Initially, a sensitivity test of the multiplex PCR was done to determine the level of detection of bacterial contamination of food with *B. cereus.* Three types of foods used in this study were, pasteurized milk, cheese and cooked rice. The foods were artificially contaminated by spiking them separately with *B. cereus* strains NCTC 11145 and BC 68 at varying bacterial concentrations and then prepared for gene detection by multiplex PCR amplification as described below under each food type. Non-spiked foods were included as controls. PCR conditions were optimized for direct genes detection in food.

Boiled Rice

Ten grams of rice that was negative for *B. cereus* were homogenized using a stomacher 400 (Seward Medical, London, UK) for 60 seconds in 90ml of 0.1% tyrptone soya broth (TSB) (Oxoid, UK), supplemented with 100U/ml polymyxin B. *Bacillus cereus* strains NCTC 11145 and BC 68 were grown on *B. cereus* selective agar (BCSA) overnight at 35°C. A spiking bacterial suspension was prepared by suspending one colony of the overnight culture in 3mls of the rice homogenate as diluent. The bacterial suspension was serially diluted in the same homogenate to final concentrations ranging from 10^{-1} to 10^{-8}. One millilitre of homogenate for every dilution was then centrifuged at 15,000 rpm for 10 min and deposits containing cells re-suspended in 1.0 ml of sterile distilled water. The bacterial deposits were washed 4 times in distilled water to remove any *Taq* DNA polymerase inhibitors present. After the last wash, the deposit was resuspended in 200µl DNAse/RNAse free sterile distrilled water, boiled for 10 minutes at 98^0C in a water bath and centrifuged at 15,000 rpm for 5 minutes to release the bacterial DNA. The supernatant containing bacterial DNA was transferred into a new tube and stored at -20^0C, from which 2µl aliquots were used as template in the multiplex PCR assay. Suitable controls including non-spiked rice homogenate, PBS buffer, PCR reagents without template, were employed to check for any false-positive reactions. The number of *B. cereus* in each dilution was determined by inoculation of 0.1ml of each dilution onto the surface of *B. cereus* selective agar plate (Oxoid, Unipath, U.K), and incubated at 35^0C for 24 hours before enumeration of the *B. cereus* population.

Cheese

Ten grams of soft cheese free of *B. cereus* was homogenized using a stomacher 400 (Seward Medical, London, UK) in 90 ml of tryptone soya broth (TSB) supplemented with 100U per ml polymyxin B and grown on BCSA overnight at 35°C. A spiking bacterial suspension was prepared by suspending one colony of the overnight culture of *B. cereus* strain NCTC 11145 and BC 68 in 3mls of the cheese homogenate as the diluent. The bacterial suspension was serially diluted in the same cheese homogenate to final concentrations ranging from 10^{-1} to 10^{-8}. Five hundred microlitres of each dilution was transferred to a new tube and, 1000 µl of lysis buffer (0.5% *N*-laurylsarcosine, 50 mM Tris-Cl, 25 mM EDTA, pH 8.0) added to the mixture. After vortexing for 1 min, the mixture was centrifuged at 15,000 rpm for 5 min. The pellet was re-suspended in 200 µl of lysis buffer containing 4 µl of proteinase K (2 mg/ml). After incubation for 1 h

at 37^{0}C, 300 µl of NaI solution (6 M NaI in 50 mM Tris-Cl, 25 mM EDTA, pH 8.0) and 500 µl of isopropanol was added to the suspension and then centrifuged at 15,000 rpm for 5 min. The pellet was washed with 35% isopropanol, dried for a short time, and then suspended in 20 - 200 µl of sterile distilled water for use as template in multiplex PCR assay. Suitable controls including media, PCR reagents without template, and *B. cereus* purified DNA were employed to check for any false-positive and false-negative reactions. The number of *B. cereus* in each dilution was determined by inoculation of 0.1ml of each dilution onto the surface of *B. cereus* selective agar plate (Oxoid, Unipath, U.K), and incubated at 35^{0}C for 24 hours before enumeration of the *B. cereus* population.

Milk

Ten millilitres UHT milk was mixed with 9.0 ml TSB, supplemented with 100U polymyxin B. *Bacillus cereus* strains NCTC 11145 and BC 68 were grown on BCSA overnight at 35°C. A spiking bacteria suspension was prepared by suspending one colony of the overnight culture in 3mls of same milk as diluent. The bacterial suspension was serially diluted in the same milk to final concentrations ranging from 10^{-1} to 10^{-8}. Five hundred microlitres of the mixture was transferred to a new tube and, 1000 µl of lysis buffer (0.5% *N*-laurylsarcosine, 50 mM Tris-Cl, 25 mM EDTA, pH 8.0) added to the mixture. After vortexing for 1 min, the mixture was centrifuged at 15,000 rpm for 5 min. The pellet was re-suspended in 200 µl of lysis buffer containing 4 µl of proteinase K (2 mg/ml). After incubation for 1 h at 37^{0}C, 300 µl of NaI solution (6 M NaI in 50 mM Tris-Cl, 25 mM EDTA, pH 8.0) and 500 µl of isopropanol was added to the suspension and then centrifuged at 15,000 rpm for 5 min. The pellet was washed with 35% isopropanol, dried for a short time, and then suspended in 20 - 200 µl of sterile distilled water for use as template in multiplex PCR assay. Suitable controls including media, PCR reagents without template, and *B. cereus* purified DNA were employed to check for any false-positive or false-negative reactions. The number of *B. cereus* in each dilution was determined by inoculation of 0.1ml of each dilution onto the surface BCSA plate (Oxoid, Unipath, U.K), and incubated at 35^{0}C for 24 hours before enumeration of the *B. cereus* population.

Direct gene detection in ready -to- eat foods by multiplex PCR assay

Rice samples

Ten grams of rice were homogenized using a stomacher 400 (Seward Medical, London, UK) for 60 seconds in 90ml of 0.1% tyrptone soya broth (TSB) (Oxoid, UK) supplemented with polymyxin B giving a final concentration of 100U per ml. The homogenate was incubated for 4 hr at 35^{o}C. One millilitre of the 4 hr pre-incubated homogenate was centrifuged at 15,000 rpm for 5 minutes and the deposit washed 4 times in sterile distilled water to remove PCR-inhibitors in the sample. After the last wash the deposit was resuspended in 200µl DNAse, RNAse free sterile distrilled water, boiled for 10 minutes at 98^{0}C in a water bath and centrifuged at 15,000 rpm for 5 minutes to release the bacteria DNA. The supernatant containing bacterial DNA was transferred into a new tube and stored at -20^{0}C, from which 2µl aliquots were used as template in the multiplex PCR assays.

Enumeration and isolation of *B. cereus* from boiled rice samples

Enumeration of *B. cereus* in rice samples was done by inoculating 0.1ml of pre-incubated rice homogenate onto the surface of BCSA plate and incubated at 35^{0}C for 24 - 48 hrs. Plates were examined for typical *B. cereus* colonies after 24 and 48 hrs. Colonies on BCSA that appeared crenated and about 5 mm in diameter with a turquoise to peacock blue colour surrounded by a good egg yolk precipitate of the same colour were counted. One typical *B. cereus* colony was purified and confirmed as *B. cereus* using the rapid confirmatory staining procedure developed by Holbrook and Anderson (1980), and tested for carriage of enterotoxin genes using colony multiplex PCR assay as a confirmatory system.

Direct gene detection in milk samples

Ten millilitres of pasteurized milk was mixed with 9.0 ml TSB, supplemented with 100U polymyxin B and incubated for 6 -15 h at 35^{o}C. Five hundred microlitres of the 6h and 15h pre-incubated milk transferred to a new tube and, 1000 µl of lysis buffer (0.5% *N*-laurylsarcosine, 50 mM Tris-Cl, 25 mM EDTA, pH 8.0) added to the mixture. After vortexing for 1 min, the mixture was centrifuged at 15,000 rpm for 5 min. The pellet was re-suspended in 200 µl of lysis

buffer containing 4 µl of proteinase K (2 mg/ml). After incubation for 1 h at 37^{0}C, 300 µl of NaI solution (6 M NaI in 50 mM Tris-Cl, 25 mM EDTA, pH 8.0) and 500 µl of isopropanol was added to the suspension and then centrifuged at 15,000 rpm for 5 min. The pellet was washed with 35% isopropanol, dried for a short time, and then suspended in 20 µl of sterile distilled water from which 2µl aliquots were used as template in the multiplex PCR assays.

Enumeration and Isolation of *B. cereus* from Pasteurized Milk

Enumeration of *B. cereus* in milk samples was done by inoculating 0.1ml of pre-incubated milk mixture onto the surface of BCSA plate and incubated at 35^{0}C for 24 - 48 hrs. Plates were examined for typical *B. cereus* colonies after 24 and 48 hrs. Typical *B. cereus* colonies on BCSA were then counted. They were then tested for carriage of enterotoxin genes using colony multiplex PCR assay as described above as a confirmatory system.

Direct gene detection in cheese samples

Ten grams of cheese was homogenized in 90ml of 0.1% tyrptone soya broth (TSB) (Oxoid, UK), supplemented with 100 U/ml polymyxin B for 60 seconds, using a stomacher 400 (Seward Medical, London, UK). The cheese homogenate was incubated for 6 -15 h at 35^{o}C. Five hundred microlitres of the 6h and 15h pre-incubated cheese homogenate was transferred to a new tube and, 1000 µl of lysis buffer (0.5% *N*-laurylsarcosine, 50 mM Tris-Cl, 25 mM EDTA, pH 8.0) added to the mixture. After vortexing for 1 min, the mixture was centrifuged at 15,000 rpm for 5 min. The pellet was re-suspended in 200 µl of lysis buffer containing 4 µl of proteinase K (2 mg/ml). After incubation for 1 h at 37^{0}C, 300 µl of NaI solution (6 M NaI in 50 mM Tris-Cl, 25 mM EDTA, pH 8.0) and 500 µl of isopropanol was added to the suspension and then centrifuged at 15,000 rpm for 5 min. The pellet was washed with 35% isopropanol, dried for a short time, and then suspended in 20 µl of sterile distilled water from which 2µl aliquots were used as template in the multiplex PCR assays.

Enumeration and Isolation of *B. cereus* from Cheese

Enumeration of *B. cereus* in cheese samples was done by inoculating 0.1ml of pre-incubated cheese homogenate onto the surface of BCSA plate and incubated at 35^{0}C for 24 - 48 hrs. Plates were examined for typical *B. cereus* colonies after 24 and 48 hrs. Typical *B. cereus* colonies on BCSA were then counted and tested for carriage of enterotoxin genes using colony multiplex PCR assay as described above, as a confirmatory system.

RESULTS

Specificity of the multiplex PCR

Escherichia coli ATCC 25922, *Salmonella typhimirrium* 7222569-1, and Oxford *Staphylococcus aureus* colonies had no PCR products in the multiplex PCR assay (Fig. 1). The colony multiplex PCR results gave distinctly amplified PCR products, with bands clearly visible in gel electrophoresis. Results of multiplex PCR were confirmed with singleplex PCR (data not shown).

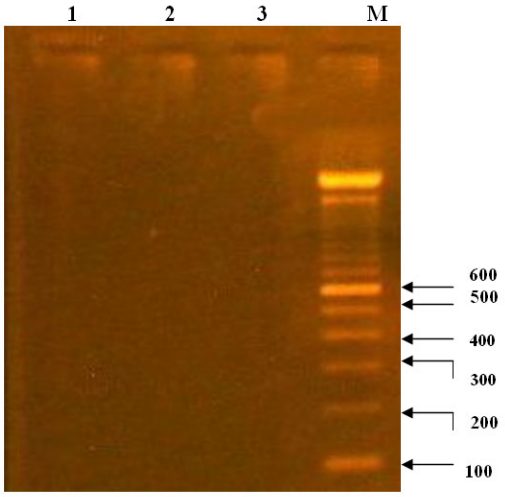

Figure 1: Agarose gel electrophoresis for the negative control bacterial strains. Lane 1: No genes detected from *Escherichia coli* ATCC 25922. Lane 2: No genes detected from *Salmonella typhimurium* 7222569-1. Lane 3: No genes detected from Oxford *Staphylococcus aureus*. Lane 4: 100 bp Molecular weight maker

Gene detection in artificially contaminated food by multiplex PCR

B. cereus genes were detected starting from bacterial counts of 4000 cfu and above for both pasteurized milk and cheese, while for rice it was 40,000 cfu and above. No genes were detected at the lower concentrations for the respective foods. Fig. 2a and Fig. 2b shows gel

electrophoresis of PCR products of enterotoxin genes in artificially contaminated rice and UHT milk respectively.

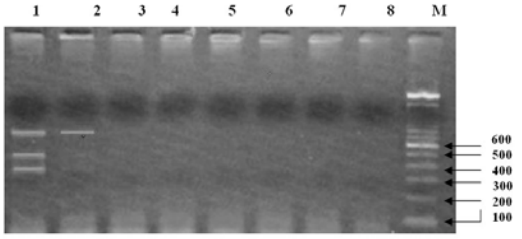

Figure 2a: Agarose gel electrophoresis of PCR products of enterotoxin genes detected in rice spiked with varying concentrations of *B. cereus* strain BC68. Lane 1: Three genes, emetic strain-specific sequence (635 bp), nheA (475 bp), and nheB (328 bp) detected from 400, 000 CFU Lane 2: Only emetic strain-specific sequence (635 bp)detected from 40, 000 CFU, Lanes 3 to 8: No genes detected from 4000 CFU, 400 CFU, 40 CFU, pre-enriching broth, non-spiked rice homogenate, and buffer used to prepare rice homogenate respectively. Lane M: 100-bp Molecular weight marker

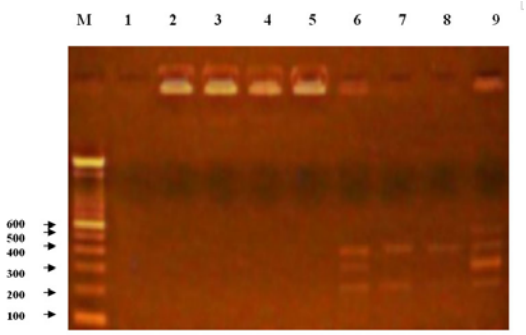

Figure 2b: Agarose gel electrophoresis of PCR products of enterotoxin genes detected in UHT milk spiked with varying concentrations of *B. cereus* strain NCTC 11145. Lanes 1 to 5: No genes detected from 400 CFU, 40 CFU, non-spiked milk, PBS buffer and pre-enrichment broth respectively, Lane 6: Three genes, hblC (386 bp), nheB (328 bp) and hblA (237bp) detected from 400, 000 CFU. Lane 7: Two genes, hblC (386 bp), and hblA (237bp) detected from 40,000 CFU. Lane 8: One gene, hblC (386 bp) detected from 4,000 CFU: Lane 9: Four genes, nheA (475 bp), hblC (386 bp), nheB (328 bp) and hblA (237bp) detected from *B. cereus* strain NCTC 11145 using colonies; Lane M: 100-bp molecular weight marker. The sensitivity level was established to be at 4,000 CFU.

Gene detection and enumeration of *B. cereus* in ready -to- eat foods

Of the 108 food samples analysed, 51(47.2 %) were found to be contaminated with enterotoxigenic *B. cereus* by culture and enterotoxin detection by the confirmatory colony multiplex PCR, but only 14(12.9%) of them were found to be contaminated with enterotoxigenic *B. cereus* by direct multiplex PCR. Out of the 36 cheese samples analysed, 8 (22.2%) were found to be contaminated with enterotoxigenic *B. cereus* by culture and the confirmatory colony multiplex PCR technique, while no sample was found to be contaminated by direct multiplex PCR. Of the 36 samples of pasteurized milk analysed, 12 (33.3%) were found to be contaminated with enterotoxigenic *B. cereus* by culture and confirmatory colony multiplex PCR while only 4(11.1%) samples were found to be contaminated by direct multiplex PCR. Eighty six percent of cooked rice were found to be contaminated with enterotoxigenic *B. cereus* by culture and detection by confirmatory colony multiplex PCR but only 27.8% of them were found to be contaminated by direct multiplex PCR (Table 2).

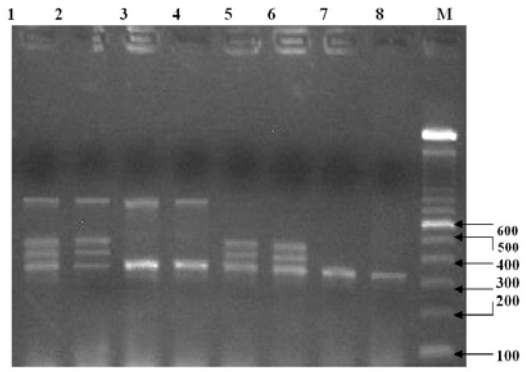

Figure 3: Agarose gel electrophoresis of PCR products of *B. cereus* enterotoxin genes detected in food by colony and direct multiplex PCR. Lane 1: Four genes, (635 bp), nheA (475 bp), hblD (436) and hblC (386 bp) detected in rice by colony multiplex PCR. Lane 2: Four genes, (635 bp), nheA (475 bp), hblD (436) and hblC (386 bp) detected in rice by direct multiplex PCR. At 40,000 cfu/ml. Lane 3: Two genes, (635 bp), and hblC (386 bp) detected in rice by colony multiplex PCR Lane 4: Two genes, (635 bp), and hblC (386 bp) detected in rice by direct multiplex PCR at 40, 000 cfu/ml Lane 5: Three genes, nheA (475 bp), hblD (436) and hblC (386 bp) detected in milk using colony multiplex PCR. Lane 6: Three genes, nheA (475 bp), hblD (436) and hblC (386 bp) detected in milk using direct multiplex PCR at 4,000 cfu/ml. Lane 7: One gene, hblC (386 bp) detected in milk by colony multiplex PCR.

The samples that were positive on culture followed by multiplex PCR but were negative on direct multiplex PCR had few organisms that were lower than the assays detection levels, 4000 cfu/ml for cheese and milk, and 40,000 cfu/g for rice. On incubation of food samples at 35^{o}C for 4hrs, 6hrs, and 15 hrs, counts were still below the detection limit upto 6 hrs and still low for many of the samples after 15 hrs. However, foods that had initial bacterial counts of more than 150cfu/ml attained counts that were considered to be within the multiplex PCR

Table 2: Detection of Enterotoxigenic *Bacillus cereus* in Market Ready-to-Eat Foods Using Both Colony and Direct Multiplex PCR.

No. and types of food	No. of samples with *B. cereus* by culture	No. of samples positive for gene detection by colony multiplex PCR	No. of samples positive for gene detection by direct multiplex PCR
Cheese N=36	10 (27.8%)	8 (22.2%)	0
Milk N=36	12 (33.3%)	12 (33.3%)	4 (11.1%)
Rice N=36	31 (86.1%)	31 (86.1%)	10 (27.8%)
TOTAL N=108	53 (49.1%)	51 (47.2%)	14 (12.9%)

detection limit after 15 hrs incubation. However, those that had initial counts below 150 cfu/ml had counts still below the detection limit after 15h incubation. Fig. 3 shows an agarose gel electrophoresis of PCR products of *B. cereus* enterotoxin genes detected directly in foods by multiplex PCR and confirmed by colony multiplex PCR.

DISCUSSION

In this study, evaluation was done to determine if a multiplex PCR assay can be applied directly to food to detect contamination with enterotoxigenic *B. cereus* without the need to go through the lenghty culture, colony purification and gene detection procedure. Results of this study indicate that it is possible to use direct multiplex PCR in detecting foods contaminated with enterotoxigenic *B. cereus*. PCR detection of genes directly in food had previously been tried but problems of food substances inhibiting PCR assays have hindered progress in this area (Shoichi *et al.* 1999). We succeeded in developing a multiplex PCR for detection of seven *B. cereus* enterotoxin genes directly in processed food by combining selective pre-enrichment and DNA extraction for both milk and cheese and selective pre-enrichment plus boiling for cooked rice. However, a certain threshold bacterial load is required to have a positive gene detection. We found the detection limit to be 4000 ($4x10^3$) cfu/ml for cheese and pasteurized milk and 40,000 ($4x10^4$) for cooked rice. It was not possible to detect the *B. cereus* genes in foods that had counts below these detection limits. Selective pre-enrichment of food samples greatly enhanced the sensitivity of the PCR assay by increasing the bacterial load in the sample. The failure to detect all the PCR products with direct multiplex polymerase chain reaction, at low bacteria counts, could be attributed to inhibition effects of the *Taq* polymerase by food components. It was observed that some of the genes would be detected at a higher bacterial load but become undetectable at lower bacterial load, while other genes were detectable at both higher and lower bacterial load. Genes that were detected at lower bacterial load were thought to occur in higher copy number than those genes that were not detectable at lower bacterial load, hence less amplification of some of the genes rendering them undetectable in gel electrophoresis (Lindback *et al.* 2004).

The direct multiplex PCR assay technique was used to test the level of contamination with *B. cereus* of 108 ready-to-eat foods. Of the 108 samples tested, only 12.9% of them had counts above the detection limits and were positive for *B. cereus* enterotoxins. However, 47.2% of the food samples including those detected by direct multiplex PCR assay were found to be contaminated with enterotoxigenic *B. cereus* by culture and colony multiplex PCR assay. On incubation of food samples at 35^{o}C for 4hrs, 6hrs, and 15 hrs, it was noted that only foods with initial bacterial counts of more than 150cfu/ml could attain counts that are considered to be within the multiplex PCR detection limit after 15hrs, while those that had initial counts below 150 cfu/ml had counts still below the detection limit after 15 hrs incubation.

The high number of bacteria cells required to contaminate food for direct multiplex PCR detection could be as a result of the presence of

substances that inhibit the PCR by reducing the amplification efficiency. These substances probably interfere with the cell lysis stage, capture and degrade the isolated nucleic acids, and inactivate the thermostable DNA polymerase in use (Abu Al-Soud and Radstrom 1998). Various inhibitors have been described (Abu Al-Soud and Radstrom 1998), proteinases in milk and cheese degrades DNA polymerase, heme found in blood blocks the active sites of polymerase while phenol and detergents used in DNA isolation cause denaturation of DNA polymerase. Other inhibitors include bile salts and complex polysaccharides in feces, humic substances in soil and urea in urine (Abu Al-soud and Radstrom 1998).

In this study milk, cheese and rice samples spiked with known amounts of toxigenic *B. cereus* cells were subjected to two methods previously described (Shoichi *et al.* 1999, Sou-ichi 1995) with some modifications. The modification included, selective enrichment of the target bacteria by supplementing the pre-incubating broth culture with polymyxin B, which selectively allows multiplication of *B. cereus* in the presence of other food contaminants. The alcohol/NaI DNA precipitation method reported by Sou-ichi (1995) was found to be applicable for milk and cheese but not for rice. The method reduced proteinase, the PCR inhibitor and improved gene detection. Proteinase K, though a DNA polymerase inhibitor was used in DNA preparation from bacteria cells contaminating food samples. NaI effectively removes the proteinases in milk and cheese, and the incorporated proteinase K thus reducing the inhibition effect in the prepared DNA template (Abu Al-soud and Radstrom 1998). However, cooked rice formed small precipitates that could not be separated from the Alcohol/NaI precipitated DNA, thus making this procedure unsuitable for use in rice (Sou-ichi, 1995). For this reason the boiling method reported by Shoichi *et al*. (1999) was used for rice with some modification. The modification of the boiling method involved skipping the two filtration steps. It involved washing an aliquot of the sample severally followed by boiling. The technique gave similar results (10^4 CFU per gram at 4 hour pre-incubation) as those described by Shoichi *et al.* (1999). The modification reduced the time needed to carry out the test and the cost of materials required without compromising on the sensitivity of the assay. The method was however ten times less sensitive compared to the DNA precipitation with alcohol/NaI. This shows that the PCR inhibitors were not completely eliminated and hence the need for more work in this area.

The specificity of direct multiplex PCR was tested using *Escherichia coli*, *Salmonella typhimullium* and *Staphylococcus aureus*. All the three microorganisms did not yield any detectable PCR products on gel electrophoresis, thus indicating that the test is specific for *B. cereus* strains.

Results of this study indicate that it is possible to apply the multiplex PCR technique directly to suspected foods to diagnose cases of *B. cereus* food poisoning. This is because poisoning occurs after consumption of foods with more than 10^6 *B. cereus* per gram (Jeffery and Stanley, 2001). The food poisoning *B. cereus* counts are way above this established PCR detection limit. The sensitivity levels for the direct multiplex PCR for the three types of foods under study were far below the numbers of *B. cereus* organisns known to cause food poisoning. The detection levels for both cheese and milk were at 4000 ($4x10^3$) cfu per ml or gram and cooked rice at 40,000 ($4x10^4$) cfu per gram. This means that the technique can be used to identify foods that could have caused *B. cereus* food poisoning without the need to culture and isolate the organisms.

For purposes of quality control of processed foods, the multiplex PCR assay can also be applied directly to the food and any food sample that is found positive for gene detection shall be deemed to contain no less than 4000 cfu/g or per ml. Negative samples may be re-incubated at 35ºC for 15 hrs to confirm absence of *B. cereus* in the food sample. Pre-incubation allows multiplication of *B. cereus* present in food to detectable levels.

The study concluded that multiplex polymerase chain reaction can be used to test for contamination of foods with enterotoxigenic *B. cereus* group by simultaneous detection of all the enterotoxin genes directly in food. The technique was found to be rapid, specific and cost effective with results obtained in one day. It is recommended that the detection levels be improved to detect bacterial genes in foods with much lower bacterial load as well as target genes with few copy numbers.

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