



# **Preliminary Studies on the Detection of *Bacillus cereus* and Its Toxins: Comparing Conventional and Immunological Assays with a Direct Polymerase Chain Reaction Method**

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## **Author's contribution**

*The sole author designed, analysed and interpreted and prepared the manuscript.*

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## **ABSTRACT**

**Aims:** Experiments were conducted to evaluate the specificity and rapidity of the application of conventional, immunoassay and direct Polymerase Chain Reaction (dPCR) techniques in the detection of *Bacillus cereus* and its toxins in contaminated rice.

**Place and Duration of Study:** Department of Life Sciences, Glasgow Caledonian University, UK, between May 2015 and December 2015.

**Methodology:** Conventional testing for the presence of *B. cereus* and associated toxins was achieved using Polymixin Egg-yolk Mannitol Agar (PEMBA) culture plates while immunoassays were conducted using a commercially available Reverse Passive Latex Agglutination (TD0950 BCET-RPLA, Oxoid, UK) kit. Direct PCR was used to detect the HBL-E gene in the food samples and the PCR amplicons were visualised after separation by gel electrophoresis.

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**Results:** Total Mean *B. cereus* count was recorded as  $5.3 \times 10^7$  cfu / g of the rice sample from the PEMBA plate culture. The PEMBA method was the least in terms of rapidity of assay completion. Further assays such as the RPLA and dPCR assays were tested for potential in overcoming this limitation. Results from the immunological study showed that sample agglutination with sensitised latex beads was only positive up to the  $10^{-2}$  dilution which is an indication of the presence of the Haemolysin BL enterotoxin (HBL-E). The PCR assay had the lowest limit of detection ( $5.3 \times 10^6$  cfu / g) thereby suggesting that the method has very low sensitivity for the bacteria. However, the PEMBA method was more sensitive but had highest detection limit (100 cfu / g) than the RPLA assay ( $5.3 \times 10^5$  cfu / g).

**Conclusion:** Although the dPCR had the advantage of producing results within a short time, it was less sensitive to *B. cereus* than RPLA and PEMBA assays. A development of improved highly sensitive assay for the toxin has the potential to enhance food safety.

**Keywords:** Toxins; PCR; detection; *Bacillus cereus*; culture; immunoassay; contamination.

## 1. INTRODUCTION

Many *Bacillus species* can be isolated from soil, growing plants and foods, including rice, fresh vegetables, meat products, pasteurised milk and dairy products, spices, dried foods and from the blood, urine and skin of humans [1]. However, food poisoning arising from *B. cereus* is more frequently associated with the consumption of rice and rice-based products [2,3]. Research has revealed the levels of cells of *B. cereus* to be greater than  $10^3$  cfu/g in both cooked and uncooked rice and in some cereal products [4]. Toxins produced by *B. cereus* have also been identified to cause illness due to their ability to induce necrosis of human tissues and / or gastrointestinal infections [5]. Two distinct forms of food poisoning (emetic and diarrhoeal-type syndromes) have been associated with *Bacillus cereus* [4]. The emetic syndrome is characterised by nausea and vomiting within 1 – 5 h of ingestion of contaminated food while the diarrhoeal-type syndrome results in diarrhoea and abdominal pain 8 – 16 h after the ingestion [6].

Conventionally, the PEMBA culture technique is applied in *B. cereus* detection in food samples. PEMBA is a selective medium for *B. cereus* isolation and enumeration. However, the technique takes much longer time for achievement of results (usually between 24 – 48 h) [6]. Polymixin, an antibiotic present in the PEMBA promote the inhibition of the growth of other pathogenic bacteria species although it allows the growth of other *Bacillus species* which may act as contaminants thereby decreasing sensitivity. It is worthy of note that the ability of *B. cereus* to give an opaque halo of egg-yolk phospholipids precipitate around peacock blue colonies enhances its identification in contaminated foods [7].

In recent times, most commonly applied techniques for diagnosis involve the identification of HBL-E, either through specific protein detection by an immunoassay or at the genomic level by PCR [8,9]. Currently, the use of the dPCR was recognised as a more reliable means of diagnosing HBL-E [10,11]. However, lower sensitivity levels were also observed due to high degree of molecular heterogeneity in genes of components of HBL-E [12]. Although detection systems based on the dPCR are both specific and accurate, they are only able to identify potential HBL-E producing bacteria that may not always correlate to the expression of toxin components by the organism [13]. The dPCR assay involves the application of PCR in the detection of genes of DNA taken from a colony of the isolated strain on PEMBA agar plate culture or from bacteria directly taken from the food sample, if highly sensitive PCR machine is used. A high count of *B. cereus* ( $1 \times 10^3$  bacteria per mL or gram of food) need to be present (although this is usually common in *B. cereus* poisoning).

In the current study, samples analysed were of the diarrhoeal-type syndrome which is caused by the production of about five different enterotoxins of which HBL-E is the most common. Detection of this toxin in the suspected food source is necessary in order to facilitate the identification of outbreaks of food poisoning caused by *B. cereus*. Several (*in vivo* and *in vitro*) tests, including the mouse lethality test, the rabbit ileal loop test, the vascular permeability reaction, and cell culture assays are required to detect *HBL* producing strains. Most of these methods however, show limitations, particularly with regard to specificity and sensitivity, and are hardly applicable for the detection of *HBL* toxin producing *B. cereus* [14].

The objective of the current study therefore was to compare, in terms of rapidity and specificity, the application of conventional, immunoassay and direct PCR techniques in the detection of *Bacillus cereus* and its HBL enterotoxin.

## 2. MATERIALS AND METHODOLOGY

### 2.1 Sample Collection

The sample evaluated in this study was leftovers of cooked rice consumed by a patient manifesting diarrhoeal symptoms of food poisoning in an undisclosed hospital within the City of Glasgow, Scotland, UK.

### 2.2 Sample Preparation

To the 5 g of original rice suspension sample to be analysed, 45 mL of peptone water was added. This was followed by blending in a stomacher bag using a Stomacher Lab Blender 400 AS2878 (Seward Laboratory, London, UK) for 60 sec to allow for the formation of a homogeneous suspension. The bag was thereafter left to stand in a beaker for 10 min so as to allow the debris present in its content to settle. Four bottles containing 9 mL each of peptone water and four PEMBA agar plates were labelled for  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$  and  $10^{-5}$  dilutions of the rice suspension. Using a 1 mL pipette, 1 mL of the supernatant from the stomacher bag was removed and transferred to the  $10^{-2}$  labelled bottle. With the aid of a fresh pipette tip, 1 mL was removed from the  $10^{-2}$  bottle after mixing and transferred to the  $10^{-3}$  labelled bottle. This serial dilution procedure was conducted up to the  $10^{-5}$  labelled bottle.

### 2.3 Site of Experiment

The study was conducted under aseptic conditions in the Food Microbiology Research Laboratory, Department of Health and Life Sciences, Glasgow Caledonian University, UK.

### 2.4 Experimental Materials

#### 2.4.1 Conventional *B. cereus* cultures

*B. cereus* selective agar -PEMBA (Oxoid, UK), peptone Water, Stomacher bag, beakers, pipette and pipette tips, sterile plates, sterile spreaders, Bunsen burner and incubator were used for the selective culture.

#### 2.4.2 Direct PCR of sample for HBL enterotoxin

PCR Master mix containing dNTPs, taq polymerase enzyme (1 unit), magnesium ions and PCR primers (25 pMol of each) specific to the HBL enterotoxin, all in a PCR buffer were used. DNAase- and RNAase- free distilled water, hyperladder IV, electrophoresis buffer chamber, agarose gel casting stand (Fischer Scientific, Leicestershire, UK), molten agarose containing green midori, water bath, sticky tape, LMS-20 Transilluminator (BioDoc Imaging System, Cambridge, UK), TAE Buffer, Flow Gen Emission Power Pack, heating block (Techne Dri-block DB-2A), bench microfuge (MSE 2990 Micro Centaur, Ralston Scientific / Analytical Services, Larnarkshire), disposal pot, sterile, UV irradiated Eppendorf tubes, Taq enzyme was supplied in ready mix buffer and PCR machine were used for this assay.

#### 2.4.3 HBL-E detection using immunoassay

ATD0950BCET-RPLA Toxin Detection kit [7] was used for this assay. The kit had a round bottomed micro-titre plate, sterile peptone water, sensitised latex beads (pre-coated with an antibody capable of recognising HBL-E), unsensitised latex beads (not pre-coated with an antibody capable of recognising the HBL-E) and a sticky plastic cover. The kit was stored in a refrigerator at 4°C before use.

### 2.5 Methodology

#### 2.5.1 Selective *B. cereus* PEMBA plate culture

From each dilution (starting with  $10^{-5}$  and then working down to  $10^{-1}$ ), 100  $\mu$ L was pipetted out (using a micropipette and separate micropipette tip for each dilution) and transferred to the previously labelled PEMBA plates. Sterile spreaders were used to spread the 100  $\mu$ L uniformly in the plates which were then left to dry before being inverted and incubated for 48 h at 30°C. After this period of incubation, the count of *B. cereus* per gram of sample (cfu/g) was determined by selecting a plate that produced between 30 and 300 typical *B. cereus* colonies having its characteristic appearance and then adjusting colony count to the appropriate dilution factor.

#### 2.5.2 Direct PCR for HBLE

The PCR method used was a slightly modified version of that adopted by [15,16] using primer M13 (50-GAGGGTGGCGGCTCT-30) as

according to [17]. To sterile UV irradiated Eppendorf tubes, 1 mL of the original stomached rice sample devoid of debris and 1 mL each of the sample dilutions prepared in Experiment 1 above was transferred and tubes were centrifuged at the maximum speed for 5 min on a bench microfuge (MSE Micro Centaur, Ralston Scientific / Analytical Services, Larnarkshire, UK) so as to pellet the bacteria. The supernatant was thereafter discarded by carefully pipetting it off and then re-suspending the bacteria by mixing with 200  $\mu$ L of DNAase- and RNAase- free distilled water using a micropipette and pipette tips with filters so as to prevent contamination with extraneous DNA. Again, the tubes were centrifuged at maximum speed for 5 min in order to pellet the washed bacteria. The water was pipetted off and the bacteria was re-suspended by mixing thoroughly in 50  $\mu$ L of DNAase- and RNAase- free distilled water. Samples were transferred to a heating block to allow them boil for 15 min so as to lyse the bacteria and release DNA. In the suspension from each tube, 5  $\mu$ L of DNA was taken and added to the PCR tubes. Additional PCR tubes containing 1  $\mu$ L each of the positive control DNA and the negative control DNA were also taken. To each of these PCR tubes, 25  $\mu$ L of the PCR master mix was added quickly. The PCR reaction tubes were then immediately placed in the PCR machine held at 4°C. The PCR reaction was ran for 35 cycles including: step 1 in which they were allowed to heat at 95°C for 5 min; followed by 35 cycles of step 2 involving heating at 95°C for 1 min; then step 3 by heating at 58°C for 40 secs; step 4 by heating at 72°C for 60 sec and then the final heating step at 72°C for 5 min. After the running of samples in the PCR machine, the PCR reaction tubes were then held at 4°C until when removed and stored at -20°C before analysing for a specific band of 840 bp DNA amplification by agarose gel electrophoresis.

### 2.5.3 Procedure for Agarose gel electrophoresis

A 50 mL of molten agarose containing midori green (2  $\mu$ L of green dye) was poured into the agarose gel casting stand with the gel comb in place and after it has been taped at either end with a sticky tape. The gel was allowed to set for about 20 min after the removal of any bubble present and the tape was removed and gel transferred on the cast to the electrophoresis buffer chamber and then covered with TAE buffer to about 0.5 cm on top. 20  $\mu$ L of each PCR sample was added to separate wells (labelled)

and 5  $\mu$ L of hyperladder IV was added to another well (also labelled). The gel apparatus was then appropriately connected to the power pack and the gel run at 100 V for about 50 min, stopping the power when the blue dye front in the samples reaches nearly the end of the gel. The buffer was then drained off and gel lifted from the cast and taken to the gel doc reader for visualisation under UV light and a photograph of the image captured.

### 2.5.4 HBL-E testing through immunoassay technique

The immunoassay procedure was also used to test sample dilutions for HBL-E using the procedure by [7]. In this assay enterotoxin control served as the positive control while sterile peptone water was used as the negative control. To wells 1 and 2 of row F of a round bottomed micro-titre plate, 25- $\mu$ L aliquot of the  $10^{-5}$  dilution of the rice suspension sample was pipetted out and added. To wells 1 and 2 of row E of the micro-titre plate, 25- $\mu$ L aliquot of the  $10^{-4}$  dilution of the rice suspension sample was pipetted out and added. This same procedure was followed until all the sample dilutions with the original stomached suspension ( $10^{-1}$ ) were added to their corresponding wells up 1 and 2 of row B. To wells 1 and 2 of row G, 25  $\mu$ L of the enterotoxin control was added. To wells 1 and 2 of row H, 25  $\mu$ L of sterile peptone water was added. Thereafter, 25  $\mu$ L of sensitised latex beads was carefully added to all wells in rows B to H of column 1. 25  $\mu$ L of the unsensitised (control) latex beads was also carefully added to rows B to H of column 2. The micro-titre plate was then covered with a sticky plastic cover and then incubated for 24 h undisturbed at room temperature. After this incubation period, the sticky plastic lid was carefully removed (so as not to disturb any pellets of beads) from the assay plate. The plate was thereafter examined in order to determine whether there was a pellicle of beads (positive result) or a pellet of beads (negative result) in each well. Results were then tabulated and presented as contained in the subsection following.

## 3. RESULTS AND DISCUSSION

### 3.1 Results

#### 3.1.1 Selective *B. cereus* plate count of sample

The  $10^{-4}$  PEMBA plate was selected after the incubation period since it was the only plate that

produced typical *B. cereus* colonies that were between the range of 30 and 300. The number of colonies in the selected plate was counted to be 53. The results showed that Total Mean *B. cereus* count was  $5.3 \times 10^7$  cfu / g contaminated cooked rice sample indicating the presence of the bacteria in high numbers. Previous researches [2,3,4,5] has shown that cooked rice is a major source of *B. cereus* and its HBL enterotoxins with values usually greater than 1000 colony forming units per gram sample [4]. Colony counts were however observed to be higher than values of  $3.5 \times 10^3$  cfu/g reported earlier for cooked rice samples under incubation conditions at 30°C for 24 h [6]. Limit of detection for *B. cereus* count using the PEMBA plate culture method was observed to be 100 cfu / g rice sample. This is an indication that the assay was highly sensitive for *B. cereus* cells but longer time was required for its completion. The PEMBA assay could not however detect the toxin thereby requiring the application of other assays.

### 3.1.2 Direct PCR for HBL-E detection

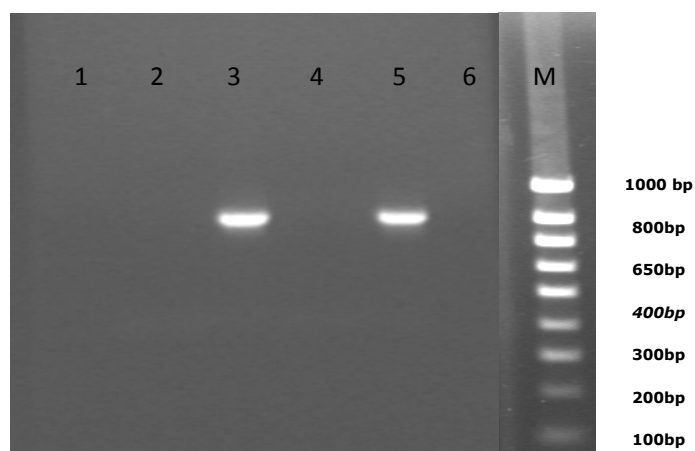
The identification of the *B. cereus* strain so as to show whether or not the strain is a producer of toxins was achieved through the direct PCR assay for HBL-E. The occurrence of an amplified

DNA band in the PCR sample is an indication that it has bound ethidium bromide which made it to be visible as a fluorescent band. The hyperladder IV on Lane M showed a ladder of DNA bands of known molecular size (bp) as represented in Fig. 1 below. The results on Fig. 1 showed that the original rice suspension ( $10^{-1}$ ) was visible as a specific fluorescent band DNA with amplification of amplicon size of 865 bp by agarose gel electrophoresis. This indicates that the rice suspension analysed contained *B. cereus* genes for HBL-E.

Total colony count for *B. cereus* from the PCR assay was observed to be equal to  $5.3 \times 10^6$  cfu/g. This was slightly less than that recorded from the PEMBA assay ( $5.3 \times 10^7$  cfu/g). The limit of detection was observed to be lowest in the dPCR assay for *B. cereus* cells and HBL-E as compared to the other two assay techniques tested.

### 3.1.3 RPLA immunoassay for HBL-E

A standard agglutination pattern from BCET-RPLA immunoassay [7] was used to enhance an understanding of pattern of agglutination from the current study by comparison with information on Fig. 2.



**Fig. 1. Gel picture for the detection of HBL-E genes of *Bacillus cereus***

Key:

Lane 1,  $10^{-3}$  Master mix plus DNA suspension

Lane 2,  $10^{-2}$  Master mix plus DNA suspension

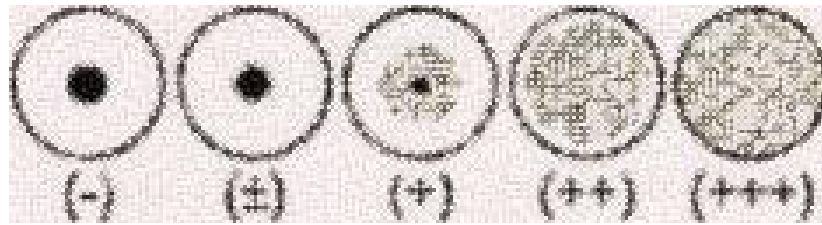
Lane 3,  $10^{-1}$  Master mix plus DNA suspension

Lane 4, No Sample

Lane 5, Positive Control DNA

Lane 6, Negative Control DNA

M - Hyperladder IV (Bioline)



**Fig. 2. Standard agglutination pattern from BCET-RPLA assay [7]**

From the assay, patterns classified as (+), (++) and (+++) were considered to be positive while others (-, ±) were considered to be negative. The positive results indicate the presence of a pellicle of beads while those showing negative results indicate the occurrence of a pellet of beads. The pellicle of beads is an indication of the presence of HBL-E.

Results from the analysis of sample testing for HBL-E by immunoassay are presented in Table 1 above. The results showed that sample agglutination with sensitised latex beads was only positive for the original stomached suspension ( $10^{-1}$  dilution) and the  $10^{-2}$  dilution. This gives an indication of the presence of the HBL-E in the  $10^{-1}$  and  $10^{-2}$  sample dilutions. The highest dilution of sample that therefore showed a positive result (pellicle) from this assay was the  $10^{-2}$  dilution. However, all sample dilutions, including both positive and negative controls, were negative in their agglutination with latex beads unsensitised as expected.

### 3.2 Discussion

#### 3.2.1 Conventional assay using PEMBA

Results obtained from the conventional method using PEMBA agar plate culture for *B. cereus* count indicates that the number of colony forming unit per gram of the rice sample is  $5.3 \times 10^7$ . This value is slightly higher than that reported by [15] in which the total aerobic count for *B. cereus*

from PEMBA plates incubated at between 10 and 18 °C for 6 days was observed to be about  $1.0 \times 10^6$  cfu/g and higher than values between  $1.2 \times 10^3$ –  $3.5 \times 10^3$  cfu/g reported by [18]. The difference observed could be attributed to the variation in the temperature and total period of incubation as opposed to the current experiment where PEMBA plates were incubated at 30°C for a one-week period before colonies were counted.

It has been reported in previous studies [19] that numbers equal to or greater than  $10^5$  cfu/g must be isolated in order to prove that a contaminated food consumed has caused food poisoning thereby leading to the production of diarrhoeal symptoms in the person suspected of food poisoning. The large number of *B. cereus* colonies ( $5.3 \times 10^7$ ) in the rice sample is therefore an indication of the occurrence of food poisoning from the bacteria. The most common food associated with the symptoms of food poisoning cases relating to *B. cereus* and its enterotoxins is rice. Spores produced by *B. cereus* are capable of surviving normal cooking temperatures and rapid growth and enterotoxin production may occur if food, for instance, rice is not cooled quickly and refrigerated [19].

A limitation of the conventional PEMBA agar plate culture method is that it is time consuming and has high limit of detection when compared to other methods. The minimum detectable *B. cereus* count using the undiluted sample was observed to be 100 cfu / g sample. This high

**Table 1. Agglutination for HBL enterotoxin by RPLA immunoassay**

Tested samples	Agglutination with sensitised latex beads	Agglutination with unsensitised latex beads
$10^{-1}$ dilution	Positive	Negative
$10^{-2}$ dilution	Positive	Negative
$10^{-3}$ dilution	Negative	Negative
$10^{-4}$ dilution	Negative	Negative
$10^{-5}$ dilution	Negative	Negative
Enterotoxin control	Positive	Negative
Sterile peptone water	Negative	Negative

detection limit and low specificity reduce its application in that it permits the growth and identification of other *Bacillus species*. Also, the ability of the *B. cereus* isolate to produce HBL toxin which is the most common toxin of the diarrhoeal-type syndrome in the current study or its ability to be directly detected in contaminated food samples is limited. However, the use of further tests such as the RPLA immunoassay and the Direct PCR assay was assessed as a means to overcome some or all of these limitations.

### 3.2.2 HBL-E detection using RPLA immunoassay method

Haemolysin BL enterotoxin (HBL-E) is a unique membrane-lytic toxin from *B. cereus* and has a variety of toxic activities including haemolysis, cytotoxicity, vascular permeability, dermonecrosis, enterotoxicity, and ocular toxicity [10]. Previous studies by [6] have shown that HBL-E is present in most frequently consumed foods but mostly associated with cooked rice. The positive agglutination observed for the original stomached suspension ( $10^{-1}$  dilution) and the  $10^{-2}$  dilution with the sensitised latex beads indicates that HBL toxin is present in the sample. Results from the analysis of sample testing for enterotoxin HBL by immunoassay as presented in Table 1 above is an indication that the HBL enterotoxin present in the rice sample and responsible for the associated food poisoning symptom of diarrhoea manifested. The immunoassay technique is advantageous in the identification of the HBL enterotoxin from *B. cereus* contaminated food samples. The assay however has its limitation in its inability to identify genes of this enterotoxin from a *B. cereus* infected food sample as compared to the PCR method.

The negative agglutination noticed in all sample dilutions as well as the hyperladder IV, positive and negative controls with unsensitised latex beads is a reflection of the degree of sensitivity of the immunoassay procedure in the identification of the HBL-E gene from food samples. The unsensitised latex beads were not pre-coated with an antibody that is capable of recognising the HBL-E and thus did not demonstrate its presence in food samples contaminated with *B. cereus*. This therefore indicates that the RPLA immunoassay technique is a highly sensitive method in the identification of the HBL enterotoxin from contaminated samples. The BCET-RPLA assay was observed

to have lower limit of detection ( $5.3 \times 10^5$  cfu / g) than that of the PEMBA plate culture method (100 cfu / g). This was however slightly higher than that of the direct PCR assay ( $5.3 \times 10^6$  cfu / g) making the latter a more rapid but less sensitive technique for detection of *B. cereus* and its enterotoxins.

### 3.2.3 Direct PCR assay for *B. cereus* detection

The direct PCR assay was used to determine whether the strain of *B. cereus* present in the rice sample contains genes for expression of the HBL enterotoxin. PCR assay has been applied for rapid detection of enterotoxins genes in *Bacillus cereus* [20,21] as well as for direct detection of food contamination associated with enterotoxigenic *B. cereus* [22]. The amplicon size of 865 bp shown by the original rice suspension in the gel photo for the PCR assay is close to that reported by [18] indicating the presence of the HBL-E gene. Direct PCR analysis can be used as a routine detection method for the enterotoxin genes in a large variety of *B. cereus* strains [23]. It is a rapid method which only identifies the potential HBL producing *B. cereus* that may not always correlate to the expression of toxin components [13]. From the current study however, the method has a very low sensitivity for the HBL-E gene. Therefore, the use of combined methods such as immunoassays and PCR or other advanced methods may be necessary to detect the presence of toxin genes [23,24] both in a rapid and specific manner.

## 4. CONCLUSION

From the current study, it can be concluded that the contaminated food sample analysed tested positive for food *B. Cereus* poisoning using any of the assays evaluated. Colony counts from PEMBA ( $5.3 \times 10^7$  cfu / g) were considered high enough to cause food poisoning. *B. cereus* and its enterotoxins could be detected using conventional methods of plate cultures, immunoassays and PCR assays. However, a comparison of the conventional and immunoassay techniques with the direct PCR method applied in the detection of *Bacillus cereus* and its toxins suggests that the sensitivity of these methods is lowest for PCR, intermediate for immunoassays and highest for plate cultures. PEMBA agar plate culture technique is however highly limited in its inability to determine if the strain of *B. cereus* present in the contaminated food sample is a toxin producer or not. *B. cereus*

strains isolated from the cooked rice should be regarded as potential enterotoxin producers according to the results obtained from the dPCR and immunoassay. This corroborates findings by other researchers [20,21,22,23,24] using similar techniques thereby confirming these assays as being more specific for detecting the presence of HBL-E. The most rapid method of detection was the direct PCR method but this was however less efficient than other methods. It is therefore recommended that rice should be consumed as quickly as possible after cooking (usually within a day) and avoid storing at room or ambient temperatures in order to avoid proliferation of *B. cereus* cells and possible contamination by its HBL toxins.

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### COMPETING INTERESTS

The author has declared that no competing interests exist.

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