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ABSTRACT

Objectives: **To investigate the potential use of DNA techniques in epidemiological diagnosis of *Bacillus cereus* food poisoning.**
Subjects: **Fifty six *B. cereus* isolates from milk were studied.**
Design: **The 56 *B. cereus* isolates were characterised into enterotoxin positive (27 isolates) and enterotoxin negative (29 isolates) using reverse passive latex agglutination technique.**
Setting: **Plasmid and genomic DNA were isolated from all the *B. cereus* isolates. The plasmid DNA was analysed by gel electrophoresis, while genomic DNA was used for restriction endonuclease and toxin gene analyses.**
Main outcome measures: **Plasmid profile analysis, restriction endonuclease analysis of genomic DNA, and test for *bceT* and *hblA* genes by polymerase chain reaction and gene probing.**
Results: **Seventy two per cent of the isolates contained one to five plasmids of molecular sizes between 0.1 to 60 mDa. Restriction analysis of genomic DNA gave different restriction patterns among enterotoxin positive and enterotoxin negative isolates. A polymerase chain reaction assay detected *bceT* gene in 41.1% of the isolates, 16% of which tested positive for enterotoxin with *B. cereus* enterotoxin reverse passive latex agglutination (BCET- RPLA) kit, while *hblA* gene was detected in all the enterotoxin positive isolates. *BceT* and *hblA* gene probes detected the respective genes in all the isolates that also tested positive for toxin genes by polymerase chain reaction.**
Conclusion: **DNA techniques provide an alternative approach to the diagnosis of enterotoxigenic *B. cereus.***

INTRODUCTION

Bacillus cereus food poisoning manifests as emetic and diarrhoeal syndromes. The two syndromes are caused by the emetic and diarrhogenic strains of *B. cereus* respectively. The emetic strains produce an emetic toxin (cerulide) that is responsible for the emetic syndrome(1), while the diarrhoeal strains produce two types of enterotoxins both of which are three protein complexes namely, haemolysin(2), and a non-haemolytic enterotoxin(3). Some strains produce both of the enterotoxins, while other strains produce either of them(3,4). A third single component enterotoxin(5) produced by other strains of *B. cereus* has not been shown to cause food poisoning(6). The genes coding for the three enterotoxins have been cloned and sequenced(5-9).

Methods that have been used in the diagnosis of *B. cereus* diarrhoeal food poisoning rely on production of detectable amounts of enterotoxin. They cannot be used for studying strains that do not produce readily detectable enterotoxins or those strains that have silent genes(10). DNA techniques have been shown to have a potential in the diagnosis of food poisoning. They have been used to detect toxin genes in *Staphylococcus aureus*(10,11) and *Clostridium perfringens*(12) using specific nucleotide sequences as probes. These techniques are free of problems associated with immunological methods such as co-agglutination in serotying, or the need to select precise environmental growth conditions for optimal toxin elaboration(10-12). This study investigated the potential of DNA techniques in detecting enterotoxins from *B. cereus* strains.

MATERIALS AND METHODS

Fifty six *B. cereus* isolates from milk were used. Diarrhoeal enterotoxin producing *B. cereus* strain NCTC 11145, the emetic strain NCTC 11143 and *Bacillus cereus* toxin gene *(bceT)* positive strain DSM 4384 were used as reference strains. The study isolates were characterised as enterotoxin positive (27 isolates) and enterotoxin negative (30 isolates) using *Bacillus cereus* enterotoxin reverse passive latex agglutination (BCET-RPLA) test kit (Oxoid, Unipath, UK) according to the manufacturer's instructions.

Plasmid profile analysis: Plasmid DNA was isolated from *B. cereus* as described by Birnboim and Doly(13) and analysed by gel electrophoresis on 1.0% agarose as described by Meyers *et al*(14).

Isolation and restriction endonuclease analysis of genomic DNA: Genomic DNA was isolated from *B. cereus* strains according to Ausubel *et al*(15), and digested with the following restriction enzymes; Hind III, *EcoRI, Sau3A, MboI* and *DraI*. The products of digestion were analysed by gel electrophoresis on a 1% agarose.

Test for bceT gene by PCR and hybridisation with 741 base pair probe: Two DNA primers were designed from the published gene sequence(5) and PCR performed using genomic DNA as template. The forward primer corresponded to position 1176-1199 (5'..TTA TCA ACA GCG CGT ATC GGT ..3') and the reverse primer corresponded to the compliment of positions 1894-1916 (5'...ATA CAC ATG CAA ATG ATG CTC CGG AC..3'). A 741 base pair PCR product from strain DSM 4384 was isolated from agarose, labelled with the non-radioactive digoxigenin-II-dUTP labelling kit using a random primed DNA labelling procedure(16), and used as probe to test for *bceT* gene in *B. cereus* strains.

Test for hblA gene by PCR and DNA probe: Two primers, 311 bases apart were designed from the published *hblA* sequences(7) and used for PCR amplification. The 17 base forward primer (5' ..TGC AGA TGT TGA TGC CG - 3') corresponded to the positions 672 to 688 in the *hblA* gene. The reverse 17 base primer (5'..CCC GCA AGA TCC CTG AT...3') corresponded to the complement bases of positions 1011 to 1027 in the *hblA* gene. Amplification was carried out in a DNA thermocycler and the expected 355 base pair products of the PCR reaction were analysed by agarose gel electrophoresis. The PCR product was isolated from the gel, purified and labelled with the non-radioactive digoxigenin-II-DUTP labelling kit using a random primed DNA labelling procedure(16), and used as probe to test for *hblA* gene in *B. cereus* isolates.

RESULTS

Plasmid and restriction profile analysis: A variety of plasmids were found in 71.7% of the isolates. These isolates contained between 1 and 5 plasmids of different molecular sizes ranging between 0.1 and 60 MDa. The diarrhoeal prototype strain NCTC 11145 and strain DSM 4384 had no plasmids, while the emetic strain NCTC 11143 had two plasmids of molecular sizes 3.9 and 3.7 megadaltons. The isolates were grouped into 20 plasmid profile groups according to the molecular sizes of plasmids they carried, with those with no plasmids in one profile group. Table 1 shows the plasmid profiles of the 59 *B cereus* strains. Plasmids were detected in 52.3% of enterotoxin positive and 31.3% of enterotoxin negative isolates. There was no apparent relationship between ability of *B. cereus* to produce enterotoxin and presence of plasmids as there was no specific plasmid profile associated with enterotoxin producing strains.

Restriction digest patterns of genomic DNA from both enterotoxin positive and enterotoxin negative isolates differed among themselves for all the restriction enzymes used. There was no common restriction endonuclease digest pattern associated with a specific enterotoxin. However, some isolates produced similar restriction patterns with one particular enzyme but differed when digested with a different restriction enzyme. This was attributed to the fact that enterotoxin producing strains are not necessarily genetically similar.

Table 1

Plasmid profiles of 59 Bacillus cereus isolates

Plasmid profile	No. of isolates	Molecular weights of plasmids (MDa)
1	16	None
2	1	0.1
3	1	3.4
4	6	3.7
5	2	3.9
6	3	35.8
7	11	60
8	1	1.5, 0.1
9	1	3.7, 2.5
10	1	4.8, 3.7
11	2	3.9, 3.7
12	1	60, 35.8
13	3	35.8, 3.7
14	3	60, 3.7
15	2	60, 4.8, 37
16	1	60, 4.8, 3.9
17	1	35.8, 4.8, 3.7
18	1	35.8, 4.8, 3.9
19	1	35.8, 4.8, 4.5, 3.7
20	1	2.6, 3.7, 4.6, 58, 60

The table includes reference strain NCTC 11143 that had two plasmids of molecular weight 3.9 and 3.7 MDa, and NCTC 11145 and DSM 4384 strains which had no plasmids

Detection of bceT gene and hybridisation with 741 bp DNA probe: The *bceT* gene was detected by PCR in 41.1% of *B. cereus* isolates including the reference strain DSM 4384. Both the strains NCTC 11145 and NCTC 11143 did not carry the *bceT* gene. A dot blot hybridisation with the 741 DNA probe detected *bceT* gene in all the isolates that were positive by PCR. There was no correlation between enterotoxin production and presence of *bceT* gene ($p=0.2561$). The *bceT* gene was found in both enterotoxin positive and enterotoxin negative strains. The gene was also absent in some enterotoxin positive strains.

Detection of hblA gene by PCR and hblA gene probe: A PCR amplification assay detected the presence of *hblA* gene in enterotoxin producing reference strains DSM 4384 and NCTC 11145, and in all enterotoxin positive isolates. The gene was not detected in strain NCTC 11143 or in any enterotoxin negative isolates. A *hblA* probe detected *hblA* in all *B. cereus* strains including strains *B. cereus* DSM 4384 and NCTC 11145 but not in strain NCTC 11143.

DISCUSSION

Isolations of a large number of *B. cereus* strains from suspect food, vomits and stool of victims of a food poisoning outbreaks with similar plasmid profiles may indicate a common source of infection. In addition, identical plasmid

profiles would indicate a common source of infection and may be important in epidemiological investigation of *B. cereus* food poisoning outbreaks. For example, DeBuono *et al*(17), found all *B. cereus* strains isolated from stool of victims of a poisoning outbreak to be of the same biotype and serotype and all had identical plasmid profiles. However, in this study, although there was a variety of plasmids, no common plasmid profile was associated with a specific enterotoxin. This means that plasmid profile analysis may not be useful in detecting enterotoxigenic *B. cereus*.

Bacterial restriction endonuclease (BRENDA) DNA analysis could also not distinguish between enterotoxin positive and enterotoxin negative *B. cereus* isolates, as there was no common restriction pattern among *B. cereus* isolates to distinguish the two groups. However, since a single serotype may be involved in a given outbreak, BRENDA may be useful in the investigation of *B. cereus* food poisoning outbreaks. In such a case, *B. cereus* isolated from stool and vomits of outbreak victims and from food involved in an outbreak will have similar restriction patterns as they are of the same serotype. Lack of a common restriction pattern among isolates used in this study was due to the fact that the isolates were not from an outbreak (common source), but rather from milk drawn from a number of locations.

A polymerase chain reaction amplification assay and gene probing were found to be useful techniques in detecting enterotoxins from *B. cereus*. The occurrence of DNA sequences of the genes encoding the confirmed enterotoxins produced by *B. cereus* enables the development of DNA primers for amplification tests and production of DNA probes for gene probing. A PCR test with *hblA* in this study found *hblA* gene in enterotoxin positive, but not in enterotoxin negative strains. The presence of *bceT* gene in both enterotoxin positive and enterotoxin negative strains indicates that the *bceT* gene coded for a different enterotoxin. In this study, characterisation of the strains as enterotoxin positive and enterotoxin negative was by use of BCET -RPLA test kit that is based on one of the three proteins of hemolysin BL, which could not detect the protein coded by *bceT* gene.

In conclusion, the study established that *B. cereus* produce more than one enterotoxin and that the BCET-RPLA test kit cannot be used to detect all the enterotoxins from *B. cereus* alone because it is based on only one enterotoxin. DNA gene probing and PCR techniques provide alternative approaches to the diagnosis of *B. cereus* food poisoning by detecting the enterotoxin genes in *B. cereus* strains. The occurrence of DNA sequences encoding the haemolysin BL(7,8) and non-haemolytic enterotoxin(9) complexes will allow the design and preparation of DNA diagnostics for diagnosis of *B. cereus* diarrhoeal food poisoning. The techniques are, however, not without limitations. They have low sensitivity in identifying true positives and the presence of a single bacterial cell in food may falsely be identified by PCR as a case of food poisoning. These techniques are capable of detecting strains with silent enterotoxin genes and eliminate the need to select precise environmental growth conditions for optimal toxin production required for detection with biological and immunological techniques.

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