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Brief Communication

Bacillus cereus containing *nheA, hblC* and *cytk* enterotoxin genes is associated with acute childhood gastroenteritis in Nigeria

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ABSTRACT

Bacillus cereus is rarely implicated when diarrheal cases in children are diagnosed in developing countries due to the lack of molecular methods to identify its enterotoxigenic genes. We report that out of 62 enterobacteria isolated from 70 stool samples collected from children hospitalized at the Mile 4 Hospital, Ebonyi State, Nigeria, 24 isolates were identified as *B. cereus* based on 16SrRNA gene sequence. The enterotoxins genes *nheA* and *cytK2* were detected in 23 out of the 24 isolates, while *hblC* was detected in 19 isolates. *B. cereus* may be responsible for greater number of yearly incidences of acute childhood gastroenteritis in Nigeria.

1. Introduction

Bacillus cereus produces different virulence factors generally associated with foodborne illness, causing two types of gastrointestinal diseases: the emetic and diarrheal syndrome [1,2]. Emesis is caused by a cyclic peptide toxin known as cereulide synthesized by non-ribosomal peptide synthetase encoded by *cesA* (*cer*) and *cesB* [3]. Three different enterotoxins are associated with the diarrheal syndrome: the hemolytic enterotoxin BL (HBL) encoded by *hblA*, *hblC* and *hblD*, the non-hemolytic enterotoxin (NHE) encoded by *nheA*, *nheB* and *nheC* and the cytotoxin K (CytK), also called hemolysin IV, encoded by *cytK1* or *cytK2* [4,5]. In Nigeria as well as in other developing countries, reports of children diarrhea cases associated to *B. cereus* are scarce [6–8]. This scenario might change once molecular testing to determine the bacterial species causing the symptoms and disease becomes a routine [9]. Here, we investigated the bacterial species implicated in cases of acute childhood gastroenteritis in Nigeria and the type of enterotoxins involved.

2. Methodology

Seventy stool samples were collected from children hospitalized (0–5years) at the paediatric ward of Mile 4 Hospital, Abakaliki, Ebonyi State, Nigeria, from January to March 2020. Inclusion criteria were children aged 5 years and below, children with diarrhea alone or with diarrhea and vomiting and children with no antibiotic treatment before admission. Children with only vomiting and previous antibiotic treatment before admission were not included. Stool samples were collected at the children's hospital admission and processed according to the standard guidelines provided for laboratory diagnosis of enteric pathogens, including antibiotic susceptibility evaluation with disc diffusion testing [10]. Purified bacterial DNA from single colonies was used as template for polymerase chain reaction (PCR) amplification of 16S rRNA gene V3–V4 region, emetic gene (*cesA)* [11] and enterotoxin genes (*entFM, nheA, cytK2, hblC, and bceT)* as previously described [12–15]. PCR amplicons were analysed by agarose gel electrophoresis and subjected to Sanger dideoxy sequencing. Amplicon sequences were BLASTn searched against the Genbank/NCBI [16]. Concatenated multiple sequence alignment using CLUSTAL 2.1 algorithm was performed with

Abbreviations: HBL, hemolytic enterotoxin BL; NHE, non-hemolytic enterotoxin; TSB, trypticase soy broth; PCR, polymerase chain reaction; TBE, Tris Borate-EDTA.

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enterotoxin sequences from 5 randomly selected samples, where all four enterotoxin genes (*cytK, nheA, hblC* and *entFM*) were present, and with *B. cereus* and closely related *B. thuringiensis* genes as references. The maximum likelihood tree was obtained using IQ-Tree v.1.6.12 [17] (Fig. S5). Clustal Omega v1.2.4 was also used to compute multiple sequence alignments of each set of genes using conserved domains and local sequence similarity information.

3. Results

Clinical symptoms like watery/frequent stools, increased temperature above 37 °C, dehydration and ill appearance were observed in all the sampled children. Out of the 70 stool samples collected from hospitalized children, 62 bacteria colonies were isolated. Sequencing of the 16S rRNA V3–V4 amplicon identified 24 isolates (38.7%) as *Bacillus* sp (Table 1). Using disc diffusion test to evaluate antibiotic susceptibility (data not shown), we verified that the 24 isolates (100%) were susceptible to both levofloxacin and imipenem. While 23 (96%) were susceptible to ciprofloxacin and amoxicillin-clavulanate, 1 (4%) was intermediately resistant. Thirteen isolates (54%) were resistant to cefepime, cefotaxime and ceftazidime and while 11 (46%) were susceptible. Twelve (50%) isolates were resistant to ampicillin while the other half were susceptible. Fifty percent of the isolates were resistant to ceftriazone while 11 (46%) and 1 (4%) were susceptible and intermediately resistant, respectively. The non-hemolytic enterotoxin gene *nheA* was detected in 23 (95.8%) out of the 24 *Bacillus* sp isolates while the haemolytic enterotoxin gene *hblC* was detected in 19 isolates (79.1%). The single component toxin *entFM* and the cytotoxin *cytK* were detected in 24 (100%) and 23 (95.8%) *Bacillus* sp isolates, respectively. The gene *bceT* and the *B. cereus* emetic toxin gene *cesA* were not detected in the 24 isolates (Table 2). The sequence alignments of each set of genes revealed high similarity to the *B. cereus* enterotoxin reference genes (Figs. S1–S4) with no variations within the conserved regions. These results provide further evidence that the *Bacillus* isolates are indeed *B. cereus* carrying enterotoxin genes, but not the emetic toxin gene.

4. Discussion

The distribution of the toxin genes we have observed agrees with other studies that investigated enterotoxin gene profile in *B. cereus* isolates from food or environmental samples [1]. The prevalent rate of HBL, NHE and CytK toxins has been shown to be respectively, 40–92%, 95–98% and 50–80% [18,19] which is similar to our observations for *nheA* (95.8%) and *hblC* (79.1%) although somewhat different for *cytK* (95.8%). The two variations of the *cytK* gene, which encodes CytK, are *cytK1* (which encodes the most poisonous CytK variant) and *cytK2* (which encodes the less toxic and more common CytK variant) [15]. One of the limitations of this study is the screening of only the *cytk2* without including the more toxic *cytk1*. The lack of detection of *cer* emetic toxin gene(*cesA*) in our study may be attributed to the exclusion criteria that preclude children with vomiting only. Moreover, although *B. cereus* is widespread, strains carrying emetic toxin are not commonly found in the environment [20]. A single source outbreak caused by emetic group belonging to the *B. paranthracis* species has been reported [21].

Food is the commonest source through which *B. cereus* enters the gut since it is a ubiquitous soil bacterium which can easily be carried through dust particles, contaminating food samples [22]. From 860 ready-to-eat foods samples in China, 35% were positive for *B. cereus*, out of which *entFM* was detected in all isolates while 7% harboured emetic *ces*(*cer*) gene [23]. Moreover, from 360 meat and milk products in Egypt, the predominant *B. cereus* enterotoxins genes were *nheA*(100%), followed by *cytK*(61.11%) and *hblC*(5.56%) [24], similar to the pattern of our results, except that while we observed *entFM* in all the isolates, 33.33% was observed in their study. In Nigeria, *B. cereus* has been isolated from diarrheal stools and food samples [6,7,25], but molecular detection of enterotoxin genes was not reported in any of the studies.

For the first time, a molecular detection approach has been employed and showed that *B. cereus* with enterotoxin genes *nheA*, *hblC* and *cytK* may be responsible for greater number of yearly incidences of acute childhood gastroenteritis that goes unreported in Nigeria. These yearly incidences of acute childhood gastroenteritis that occur in Nigeria coincide with the “dry season” (December to March) characterized by Harmattan haze. Dust particles which may act as source of transmission of enterotoxigenic *B. cereus* are carried from the soil into food particles. This transmission can be prevented through proper cooking, covering of food and proper hygienic methods. Since more than half of the isolates were resistant to up to three antibiotics, universally accepted treatment options [26] should be followed rather than empirical treatment [27] which may enhance evolution of resistant strains.

Table 1
Taxonomic identification of 62 stool isolates based on 16S rRNA V3–V4 sequence.

Taxonomic identification	Number of isolates	GenBank accession of 16S rRNA V3–V4 sequences
Bacillus sp	24	OP650106, OP650107, OQ472527, OP650104,OP650100-OP650102, OP650098, OQ472528,OP650095, OP113777-OP113790, OP650092
Alcaligenes sp	23	OP650109,OP650103,OP650099, OP650097,OP650094, OP650091, OP164754,OP131838-OP131851
Lysinibacillussp	2	OP219795, OP216525
Enterococcus faecalis	2	OP650093, OP132418
Providencia sp	2	OP132417, OP132416
Bacterium	1	OP650108
Proteus sp	2	OP132419,OP650096
Psychrobacter	1	OP216524
Brevundimonassp	1	OP219796
Burkholderiasp	1	OP650092
Lost sample	3	N/A

Table 2
PCR detection of enterotoxin genesin*Bacillus sp* isolates.

Toxin Gene	Number of *Bacillus* sp isolates	GenBank accession of enterotoxin gene sequences from 5 randomly selected samples
nheA	23	LC732104, LC732106, LC732108, LC732112, LC732116
hblC	19	LC732105, LC731860 LC732109, LC732113, LC732117
entFM	24	OP618834, LC732107, LC732110, LC732114, LC732118
cytK2	23	LC731453, OP618833, LC731454, LC732111, LC732115
bceT	0	N/A
cer (cesA) (emetic toxin)	0	N/A

CRediT authorship contribution statement

Ebuka E. David: Conceptualization, Investigation, Methodology, Formal analysis, Writing-original draft, Writing-reviewing & editing, Funding acquisition. Ikechuku O. Igwenyi: Conceptualization, Supervision. Ifeanyichukwu R. Iroha: Conceptualization, Supervision. Layla F. Martins: Methodology, Formal analysis. Guillermo Uceda-Campos: Data curation, Formal analysis. Aline M. da Silva: Supervision, Writing-original draft, Writing-reviewing &editing, Funding acquisition.

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Ethical statement

Ethical approval was obtained from the hospital Ethics Review Committee clearance number RE/M4H/48/19. Parental consents for each child were obtained and all personal information was kept confidential.

Data availability

Amplicon sequences obtained in this work were deposited in NCBI GenBank with accession numbers listed in Tables 1 and 2

Declaration of competing interest

Authors Declare No Competing Interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijmmb.2024.100666.

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