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Toxigenic Profiling and Antibiotic Resistance of *Bacllus cereus* Strains Isolated from Food Consumed By School Children In Ilorin Metropolis

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ABSTRACT

Background: Food poisoning is caused by toxin produced by pathogenic organisms like bacteria, viruses etc. These toxins are common causes of food borne illness. This study was carried out to determine the antibiotic susceptibility and molecular identity of enterotoxigenic *Bacillus cereus* strains isolated from food consumed by school children in Ilorin metropolis

Methods: Bacterial isolates that were identified as Bacillus cereus (OQ235070), *Bacillus cereus* (OQ235071), *Bacillus cereus* (OQ235072), *Bacillus cereus* (OQ235073), *Bacillus thuringiensis* (OQ235074), *Bacillus cereus* (OQ235075) and *Bacillus cereus* (OQ235076) were obtained from culture collection from Microbiology laboratory, Kwara State University, Nigeria were used for this research. Antibiotic susceptibility test was carried out on the isolates using standard technique. Detection of resistance gene, plasmid profiling and curing, and detection of enterotoxigenic genes were done using standard microbiological methods.

Results: All the isolates were resistant to minimum of two antibiotics before and after plasmid curing. A total of 98 % of the isolates were susceptible to ofloxaxin, centamincin, pefloxacin, and augmentin while 90 % of the isolates were resistance to amoxylin, streptomycin, and chloramphenicol. Plasmid profiling shows that the bacteria harbored mega plasmid with equal size of 5000 bp. Curing removed all plasmid, presence of enterotoxin virulence gene nhe A, B, C and hbl C, D were observed but hbl A was not found.

Conclusion: The Bacillus species studied were found to be multi-drug resistant and carried enterotoxigenic genes which were chromosomally borne.

Keywords: *Bacillus cereus*, Enterotoxigenic, Plasmid profiling, Plasmid curing, Food poisoning, School children

1.0 INTRODUCTION

Food has been considered to be an imperative requirement for the existence of human being or any other living organism; to obtain good health, to accomplish ones jobs and duties effectively manner, to recover from illnesses, to implement adequate growth and development of the children and to survive, food is a basic necessity that is required to get fulfilled [1]. Foodborne disease is a type of illness caused by eating contaminated food. The organisms that cause foodborne disease are bacteria, fungi, viruses and others. The toxins of these organisms are the common causes of food poisoning. Infectious organisms or their toxins can contaminate food during processing or production [2].

Among the microorganisms that cause food poisoning, some are particularly important regarding the frequency and seriousness of the disease. The most commonly encountered bacteria include those producing toxins that cause food poisoning, resulting in symptoms ranging from gastrointestinal disorders to paralysis and death, some of these organisms are *Clostridium perfingens*, *Clostridium botulinum, Staphylococcus aureus*, and *Bacillus cereus*. *Bacillus cereus* is a soil dweller that strives well in the adversity of habitats or a part of the intestinal floral of different animals. It can withstand heat and harsh environments by forming endospores resistant to heat, dehydration, and other physical stress [3].

Bacillus is a spore-forming, Gram-positive, motile, aerobic rod that grows well anaerobically [4]. It is a group of ubiquitous facultative anaerobes commonly found in the soil. The spores contaminate various foods and food products such as meat, eggs, and dairy products. Foodborne illness associated with the toxin produced by B. cereus can result in self-limiting diarrhea or vomiting [5].

Bacillus cereus produces toxins that cause two types of food poisoning, the emetic toxin and diarrhea toxin that cause vomiting and diarrhea, respectively. Foodborne illness associated with the toxin produced by Bacillus cereus can result in self-limiting diarrhea or vomiting. Toxins are small molecules, peptides, or proteins capable of causing disease on contact with the body tissues interacting with biological macromolecules such as enzymes. Bacillus cereus produces three types of enterotoxins which are hemolysin (hbl), non-haemolytic enterotoxin (nhe) and cytk [6].

Street-vended foods have nutritional and economic importance in many developing countries, such as Nigeria, as they provide readily available, cheap, and diverse sources of daily meals [7].In developing countries many ready-to-eat foods are sold on all the streets. According to the [8], 2.5 billion people worldwide eat street food daily. In Nigeria and many developing countries, primary sources of ready-to-eat foods (Street Foods-SF) are prepared and sold at public places such as schools, markets places, and streets. The SFs offer food at relatively cheaper rates and easily accessible places [9]. Food-borne disease is among the leading causes of morbidity and mortality [10]. According to a report by the WHO, in 2010, 600 million people fell ill globally, and 420,000 people died from FBDs [11]. Information on the investigation of antibiotic susceptibility patterns and detection of enterotoxic genes of this foodborne bacterium in food consumed by school children in this part of the country is scanty. Hence, this work aimed to investigate the antibiotic susceptibility pattern and molecular characterization of enterotoxigenic *B. cereus* strains isolated from food consumed by school children in the Ilorin metropolis.

2.0 METHODOLOGY

2.1 Culture Collection

Bacillus species isolated from food consumed by school children within the Ilorin metropolis and identified as Bacillus cereus (OQ235070), *Bacillus cereus* (OQ235071), *Bacillus cereus* (OQ235072), *Bacillus cereus* (OQ235073), *Bacillus thuringiensis* (OQ235074), *Bacillus cereus* (OQ235075) and *Bacillus cereus* (OQ235076) were collected from the Microbiology laboratory of Kwara State University, Malete, Nigeria, and used for this research.

2.2 Antibiotic Sensitivity Test

Method of [12] was used for antibacterial susceptibility testing (AST). Susceptibility testing was performed by Kirby-Bauer disk diffusion technique according to criteria set by [13]. The inoculum was prepared by picking colonies of test organisms with a sterile wire loop and suspended in sterile normal saline. The density of suspension inoculated was determined by comparison with the opacity standard on McFarland 0.5 % solution. A 24-hours-old culture was diluted with a sterile physiologic saline solution (Ps; 0, 85 % w/v sodium chloride) concerning the 0.5 Macfarland standards to achieve an inoculum of approximately 1.5x108cfu/ml. This was uniformly seeded over a Mueller Hinton agar (Oxoid) plate, exposed to a concentration gradient of antibiotic diffusing from antibiotic-impregnated paper discs into the agar me-

dium, and then incubated at 37 ^{0}C for 18 hours. Diameters of the zones of inhibition around the discs were measured to the nearest millimeter and classified as sensitive, intermediate, and resistant according to the standardized Table [13]. The antibiotic drugs doses used were ampicillin (10µg), ciprofloxacin (5µg), norfloxacin (10ug), cephalothin (30g), gentamicin (10pg), tetracycline (30µg), cotrimoxazole (25µg), chloramphenicol (30µg), doxycycline (30µg), naldixic acid (15µg), ceftriaxone (30□g), Penicillin G (10IU), erythromycin (15g), and vancomycin (30□g) (Oxoid). This test was repeated after the plasmid curing of the Bacillus species studied.

2.3 Plasmid Profiling and Agar Gel Electrophoresis

Plasmid extraction was carried out using the method described by [14]. The integrity of the extracted plasmid was checked on a 1 % Agarose gel run to confirm amplification. The buffer (1XTAE buffer) was prepared and subsequently used to prepare 1 % agarose gel. The suspension was boiled in a microwave for 5 minutes. The molten agarose was allowed to cool to 60 °C and stained with 3µl of 0.5 g/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray, and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 minutes to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Two microliters (2 µl) of 10X blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 10µl of each PCR product and loaded into the wells. The 100-3000bp DNA ladder was loaded into the well. The gel was electrophoresed at 120V for 45 minutes visualized by ultraviolet trans-illumination, and photographed. The sizes of the PCR products were estimated by comparison with the mobility of the molecular weight ladder that was run alongside experimental samples in the gel.

2.4 Plasmid Curing

Plasmid curing was done to identify the location of resistance genes on a plasmid or chromosomal encoded in the B. cereus. The methods of [15] were used. A stock solution of 10 % SDS was prepared by the addition of 10 g SDS to 100 ml of nutrient broth. The pH was adjusted to 7.8 - 8.0. The mixture was steamed for 1 hour and kept aside as stock. The overnight cultures of selected isolates in Lysogeny Broth (LB) were diluted 100 fold, and 0.5 ml volume of each isolate was transferred aseptically into a fresh 30 ml volume of nutrient broth. The cultures were incubated with shaking for 3 hours, and SDS stock solution was added to give the required final concentration of 1 % (w/v). The cultures were incubated at 37 ^{0}C with mild agitation for 72 hours. The cured bacteria cells were tested for antibiotic susceptibility.

2.5 Detection of Enterotoxin Genes in Isolates by PCR

DNA was isolated from a day-old culture of the Bacillus species by boiling in Luria-Bertani medium, as described by [16]. All strains were examined for the presence of the hbl *CDA* and the nhe ABC genes with primers designed by [17], as listed in Table 1. The toxin extraction was done using BCET-RPLA (reversed passive latex agglutination) kit (Oxoid. Hampshire, UK) prepared according to the manufacturer's instructions. Toxin assay was done using PCR, as described by [6].

Table 1. Polymerase Chain Reaction (PCR) Primers Used for Selection of Enterotoxigenic Genes

Primers	Target genes	Primer Sequence (5'-3')	Product size (bp)
hblA1	*hblA*	GTGCAGATGTT-	320
hblA2		GATGCCGAT ATGCCACTGCGTG-	
L1A	*hblD*	GACATAT AATCAAGAGCTGTCAC-	430
L1B		GAAT CACCAATTGAC-	
L2A	*hblC*	CATGCTAAT AATGGTCATCGGAACTC-	750
L2A		TAT CTCGCTGTTCTGCTGTTA	
nheA-S	*nheA*	AT TACGCTAAGGAGGGGCA	500
nheA-A		GTTTTTATTGCTTCATCG	
nheB-S	*nheB*	GCT CTATCAGCACTTATGG-	770
nheB-A		CAG ACTCCTAGCGGTGTTCC	
nheC-S	*nheC*	CGGTAGTGATTGCTGGG	582
nheC-A		CAGCATTCGTACTT-	
cytk-F	*cytK*	GCCAA GTAACTTTCATT-	505
	cytK-R	GATGATC GAATACATAAATAATT- GGT	

Adopted from Park *et al.,* [REF]

3.0 RESULTS

3.1 Antibiotic Susceptibility Pattern of Isolates

The antibiogram pattern of the isolates is presented in Tables 2a and 2b. All the isolates were resistant to one or more drugs of different structural classes. All the isolates were found to be susceptible to Augmentin (71.4%), Ofloxacin (100%), Cotrimoxazole (85.7%), Tarvid (85.7%), Reflacine (100 %), and Ciprofloxacin (100 %) with a varying magnitude of the zone of inhibition (mm), but the isolates were resistant to Amoxyllin (71.4 %), Streptomycin (57.1%), Chloramphenicol (57.4%), Nalixidic acid (100 %) Erythromycin, (100%), Gentamycin (100%), and Ampicillin (100%).

Table 2b. Antibiotic Susceptibility Pattern of *Bacillus cereus* before and after Curing

TEST ORGNS	AMX	OFL	STR	CHL	CEF	GEN	PEF	COT	CPX	ERY
B. cereus 1_before	8	20	0	10	0	0	12	15	24	0
B. cereus 1_after	8	20	0	10	0	0	12	15	24	0
B. cereus 2_before	0	15	0	0	0	0	10	18	12	6
B. cereus 2_after	0	15	0	0	0	0	10	18	12	0
B. cereus 3_before	17	29	7	10	8	0	26	27	16	0
B. cereus 3_after	17	29	0	10	8	0	26	27	16	0
B. cereus 4_before	0	16	10	0	0	0	13	13	16	0
B. cereus 4_after	0	16	10	0	0	0	13	14	10	0
B. cereus 5_before	0	13	0	0	10	0	10	0	14	0
B. cereus 5_after	0	14	0	0	10	0	10	0	15	0
B. cereus 6_before	0	28	0	15	11	0	6	19	26	0
B. cereus 6_after	0	28	0	15	0	0	6	19	25	0
B. cereus 7_before	13	26	0	10	0	0	15	21	14	0
B. cereus 7_after	13	37	0	10	0	0	15	20	14	0

Interpretation based on CLSI 92011) 0-7 resistance, 8-12 mild susceptibility, 18-above highly susceptible; B 1-7 are the Bacillus species studied

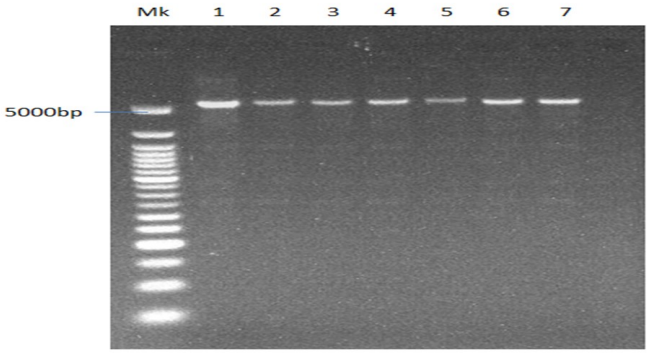


Figure 1. Presence of High Molecular Weight Plasmid in *Bacillus species*

Note: 1 to 7 represents the *Bacillus species*

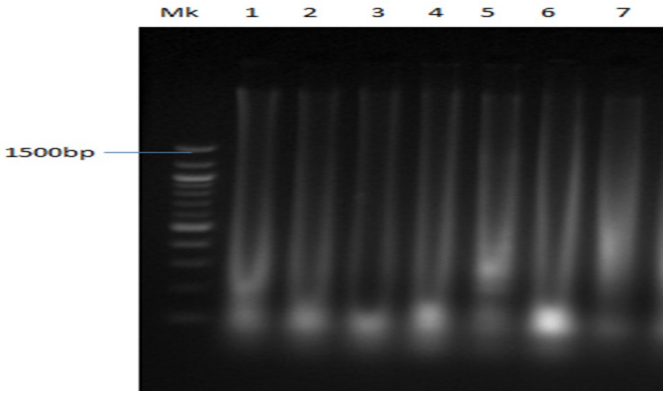


Figure 2. Degraded Plasmid in Bacillus species after been Cured with Ethidium bromide ; *Note*: 1 to 7 represents the Bacillus species

Table 2a. Antibiotic Susceptibility Pattern of *Bacillus cereus* before and after Curing

TEST ORGNS	OFX	REF	CPX	AU	CN	S	QEP	NA	SXT	PN
B. cereus 1_before	0	10	16	0	0	12	0	0	0	0
B. cereus 1_after	0	10	16	0	0	12	0	0	0	0
B. cereus 2_before	22	23	0	15	0	0	0	0	0	6
B. cereus 2_after	22	23	0	0	0	0	0	0	0	10
B. cereus 3_before	21	18	22	14	14	0	20	0	17	10
B. cereus 3_after	21	18	22	14	14	0	20	0	17	0
B. cereus 4_before	19	17	10	0	0	0	0	0	0	0
B. cereus 4_after	19	17	10	0	0	0	0	0	0	0
B. thutingensis 5_before	15	18	24	12	7	0	0	0	13	0
B. thutingensis 5_after	15	18	24	12	7	0	0	0	10	0
B. cereus 6_before	14	18	21	16	0	20	15	0	12	0
B. cereus 6_after	14	18	21	16	0	20	15	0	12	0
B. cereus 7_before	18	14	26	19	0	13	0	0	11	10
B. cereus 7_after	18	14	26	19	0	13	0	0	11	10

Interpretation based on CLSI 92011) 0-7 resistance, 8-12 mild susceptibility, 18-above highly susceptible
B 1-7 are the *Bacillus* species studied

3.2 Plasmid Profiling

The DNA from B. cereus strains indicated that all the isolated strains harbored mega plasmid equal to 5000 bp, as presented in Figure 1.

3.3 Plasmid Curing

Plasmids were successfully removed from the DNA of the isolated Bacillus cereus after being cured with ethidium bromide (Figure 2).

3.4 Molecular Identification of Enterotoxin

Multiplex PCR show the presence of non haemolysis enterotoxin (nhe) virulence

genes in the isolates, presence of 480, 740, and 560 bp indicates the presence of nhe A, B, and C, respectively (Figure 3a and b). The presence of haemolytic BL (hbl) enterotoxin virulence genes was also detected in the isolates; the presence of 750 and 430 bp indicates the presence of hblC and hblD, respectively. The gene for hblA was not found. The result is presented in Figures 3a and b.

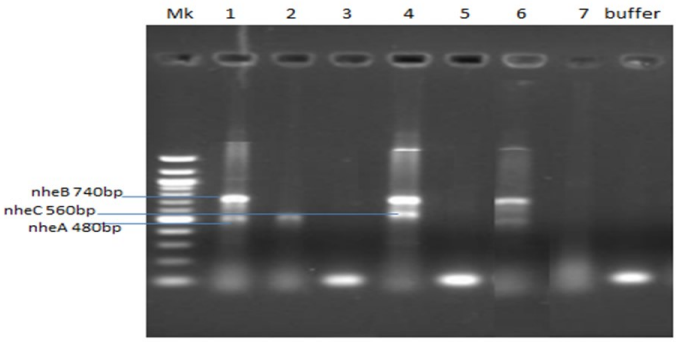


Figure 3a. Molecular Identification of the Enterotoxin (nhe type)

Note: 1 to 7 represents the *Bacillus species*

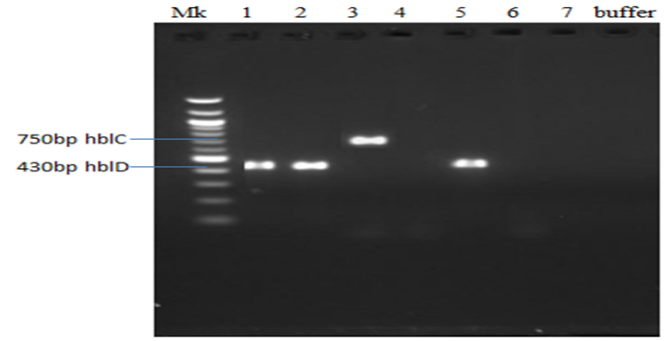


Figure 3b. Molecular Identification of the Enterotoxin (hbl type)

Note: 1 to 7 represents the *Bacillus species*

Table 3. Enterotoxin Production of Strains *Bacillus cereus* Isolated

Isolate	nheA	nheB	nheC	hblA	hblC	hblD
B. cereus 1	+	+	-	-	-	+
B. cereus 2	-	-	-	-	-	+
B. cereus 3	-	-	-	-	+	-
B. cereus 4	+	+	+	-	-	-
B. thuringensis 5	-	-	-	-	-	+
B. cereus 6	+	+	-	-	-	-
B. cereus 7	-	-	-	-	-	-

Detection of enterotoxin in the *Bacillus cereus* strains by PCR.

The enterotoxin genes that were detected: hblD-haemolysinblD gene, hblC-haemolysinblC gene, hblA-haemolysinblA gene, nheA-non-haemolyticeaA gene, nheB - non-haemolyticeaB gene, nheC- non-haemolyticeaC gene.

4.0 DISCUSSION

The public health significance of *Bacillus cereus* is of high concern, given the organism being implicated in several food poisoning outbreaks worldwide [18]. Food sold to school children falls under the category of street-vended food of ready- to-eat status. However, these foods' safety and microbiological quality should be the priority, since they do not receive any further heat treatment before consumption. The effect of the food poisoning caused by these bacterial isolates essentially on children cannot be over-emphasized [10]. All the Bacillus strains examined were found to be multidrug resistant being, resistant to more than two antibiotics. The overall implication of this finding is the inability of these antibiotics to cure infections caused by the organisms after consumption of food invariably.

In this study, all the isolates manifested resistance to more than three antibiotics, six (85.7 %) of the seven isolates were resistant to erythromycin, (100%), gentamycin (100%), nalixidic acid (100%), and ampicillin (100%) amoxicillin (71.4), chloramphenicol (57.4%), and streptomycin (57.1%). Previous studies have reported a similar trend on antibiotic susceptibility patterns of enteropathogenic food-borne B. cereus [19]. Some other authors reported that B. cereus strains were generally resistant to penicillin, ampicillin, cephalosporins, and trimethoprim and susceptible to clindamycin, erythromycin, chloramphenicol, vancomycin, imipenem, aminoglycosides and ciprofloxacin [20]. The report of [21] also supported the observed pattern of resistance in this study. However, [20] mentioned that antibiotic susceptibility of *Bacillus cereus* strains to streptomycin, chloramphenicol, erythromycin, and ciprofloxacin was high but was less susceptible to ampicillin, ampiclox, cotrimoxazole, cloxacillin. Variations in the percentages of susceptibility recorded may be due to the differences in the sources of isolates and culture parameters, genomic composition, and the overall widespread use of antibiotics in the environment [20,21]. The development of drug resistance has been traced to the indiscriminate use of these drugs in medical and veterinary practice to treat infections coupled with abuse and misuse of the drugs in society; these practices have led to DNA mutation and acquiring resistance genes among bacterial isolates [22]. Furthermore, antibiotic-resistance gene transfer is very common through horizontal gene transfer among bacteria. The knowledge of the pattern of antibiotic resistance of Bacillus cereus from foods is useful in epidemiological stud-

ies, but its effectiveness decreases due to the negligence in utilization [21]. However, it is noteworthy that the enterotoxic *B. cereus* strains examined were highly susceptible to ofloxacin (100%), cotrimoxazole (85.7%), ciprofloxacin (100%), and reflacine (100%). This observation agrees with the submission by [23]. Antibiotic resistance by strains of *B. cereus* is not unusual and may be caused by the production of beta-lactamase enzyme by *B. cereus* strains [24].

Bacterial antibiotics resistance patterns are sometimes associated with the presence of large plasmids and the ability of the plasmids conjugation process [25]. Large sizes of plasmid have been detected in bacterial isolates from different sources. Plasmids that can be transconjugated usually possess a high molecular weight thereby making these plasmids to harbor antibiotic resistance genes [26, 25]. This may increase their capacity to threaten human consumers since food-borne strains with resistance genes qualify them as potential human pathogens [25].

The gene(s) coding for antibiotics resistance may either be coded on plasmid and/or chromosomal DNA. All the strains examined were found to harbor plasmid of equal size of base pair. Plasmid curing is a proven strategy of removing resistance genes from bacteria when such were plasmid borne. Although curing of plasmid did not cure antibiotic resistance in this study, bacteria cured of plasmid still showed multiple antibiotic resistance patterns to the antibiotic tested. This observation indicates that resistance to most of the antibiotic is of chromosomal origin or on other mobile genetic elements that may help in the dissemination of resistant genes to other bacteria of human clinical significance [27] and not plasmid borne. This finding was also supported by the report of [20]. Some authors [28, 29] suggested that susceptible bacteria could acquire multi-drug resistance (MDR) plasmid during treatment with antibiotics that can induce and select for horizontal transfer and. It may be plausible to suggest that the possession of plasmid may have helped the strains to acquire their resistance through selective pressure from increased use and misuse of antibiotics [30, 31]

Two different enterotoxin protein complexes have been characterized in Bacillus cereus strains: emetic and diarrheal type (nhe and hbl) [21, 31]; production of emetic toxin is reported to be restricted to a specific group of B. cereus. In this study, nhe genes were found in most strains in a higher proportion than hbl genes. The results confirmed the previous assertion that in B. cereus two or more enterotoxins might be involved in causing food poisoning in humans [32, 33].

All seven strains studied except Bacillus cereus (OQ235076) had at least one enterotoxigenic gene or a component of hbl and nhe genes. The presence of at least one gene of the hbl or nhe complexes indicates that each entire operon might likely be present [27]. The percentage of hbl genes found in this study is higher than that reported by other authors for non-emetic *B. cereus* and food poisoning strains [30], although, nhe occurrence was similar to findings earlier reported elsewhere [33, 34, 21].

Expression of hbl genes has been shown to be regulated by several proteins, such as the PleR (Phospholipase C Regulator) [33] which controls most known virulence factors in *B. cereus* [34]. Studies have documented variations in the distribution of diarrhea toxigenic genes in *B. cereus* isolates from environmental and food samples [21, 24]. The non-detection of hbl A genes may be attributed to polymorphism among genes, a phenomenon where multiple forms of a single gene exist in an organism. The previous assertion by [24] reiterated that polymorphism among the genes is the likely explanation of the inability to detect all genes in some *B. cereus* isolates by PCR. However, high polymorphism has been reported in hbl genes in food associated with *B. cereus* emetic strains than diarrheal strains [32, 35]. A high occurrence of protein components and/or genes involved in diarrheal disease has previously been described for *B. cereus* from food [24]. Polymorphism among genes likely explains the inability to detect hblA genes by PCR. However, high polymorphism has been reported in hbl genes in food associated with *B. cereus* emetic strains than in diarrheal strains [32, 35]. A high occurrence of protein components and genes involved in diarrheal disease has previously been described for *B. cereus* from food [24]. Polymorphism among genes could be responsible for the inability to detect hblA genes by PCR in all of the toxin-producing *B. cereus* strains examined. Results obtained by [36] indicated a high degree of polymorphism in the hbl and nhe genes (within the primer annealing sites) of food-associated isolates. Production of all three components of the enterotoxin complexes hbl and nhe is confirmed to be necessary for biological activity [32].

From the results obtained in this work, it is concluded that the Bacillus cereus studied were multidrug-resistant, the resistance was chromosomally borne, and they harbor enterotoxigenic genes.

Recommendations

From the results of this work, it is recommended that food should be appropriately handled to avoid contamination with enterotoxigenic *B. cereus* strains, misuse of antibiotics in veterinary and other form of self-medication that can lead to drug abuse should be avoided in other to prevent development of drug resistance by this diseases causing organism.

Conflicts of Interest

The authors declare that there is no conflict of interests.

Authors' Contributions

TTA conceived and designed the study, contributed to data collection, data analysis tools, analysis of data and manuscript writing. **SA** contributed to study design and supervised the study. **MRA** co-supervised the study and contributed to manuscript writing. All authors approved the final copy of the manuscript.

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