
Isolation, Characterisation, Antibiotic Susceptibility and Molecular Profile of Enterotoxigenic *Bacillus Cereus* from Fried Soya Bean Cake (Awara)

Farida Ishaq, Yahaya Oholi, Zakari Ladan

Department of Microbiology, Kaduna State University, Kaduna, Nigeria

Email address:

faridakauru@gmail.com (F. Ishaq)

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Abstract: A total of one hundred and fifty (150) “awara” samples were collected from different vendors in various parts of Kaduna metropolis and assessed for the presence of *Bacillus spp.* Three species of *Bacillus* (*Bacillus cereus*, *Bacillus subtilis*, and *Bacillus licheniformis*) were isolated on Mannitol Egg-yolk polymyxin Agar plates, characterized and identified from the “awara” samples by conventional method (Biochemical test). The occurrence of *Bacillus cereus*, *Bacillus subtilis* and *Bacillus Licheniformis* were 51 (34%), 22 (14.7%) and 13 (8.7%). All the samples tested contained high number of *Bacillus spp* where *Bacillus cereus* had the highest number. Statistical analysis using P-Values indicated statistically significant difference (P=0.001) in Kawo and Tudun wada while there was no statistically significant difference in Unguwan dosa (P=0.341). Isolates were found to possess the hblD enterotoxin produced by *Bacillus cereus*. The enterotoxin producing *Bacillus cereus* isolates were found to be susceptible to Pefloxacin (10µg), Ciprofloxacin (10µg), Streptomycin (10µg), Septrin (30µg) and Erythromycin (10µg). Resistance was observed against Ampiclox (30µg) while Gentamycin, (10µg), Zinnacef (20µg), Amoxicillin (30µg) and Rocephin (25µg) were found to be less susceptible respectively. All the “awara” samples were highly contaminated with *B. cereus* probably because its spores were very difficult to be eliminated from food materials by heat treatment. However, any outbreak can be effectively treated using the antibiotics that showed susceptibility.

Keywords: *Bacillus cereus*, Enterotoxin, Antibacterial Susceptibility, Multiple Antibiotic Resistance (MAR) Index

1. Introduction

Bacillus cereus is a Gram-positive, spore-forming microorganism capable of causing foodborne disease. At present three enterotoxins, able to cause the diarrheal syndrome, have been described: hemolysin BL (HBL), nonhemolytic enterotoxin (NHE) and cytotoxin K. HBL and NHE are othree-component proteins, whereas cytotoxin K is a single protein toxin. Symptoms caused by the latter toxin are more severe and may even involve necrosis. In general, the onset of symptoms is within 6 to 24 h after consumption of the incriminated food. *B. cereus* food poisoning is underestimated probably because of the short duration of the illness (~24 h) [30].

In 1887, *Bacillus cereus* isolated from air in a cowshed by Frankland and Frankland [11]. Since 1950, many outbreaks

from a variety of foods including meat and vegetable soups, cooked meat and poultry, fish, milk and ice cream were described in Europe. In 1969, the first well-characterized *Bacillus cereus* outbreak in the USA was documented. Since 1971, a number of *B. cereus* poisonings of a different type, called the vomiting type, were reported. This type of poisoning was characterized by an acute attack of nausea and vomiting 1–5h after consumption of the incriminated meal. Sometimes, the incubation time was as short as 15–30 min or as long as 6–12h. almost all the vomiting type outbreaks were associated with consumption of cooked rice. This type of poisoning resembled staphylococcal food poisoning [13].

The genus *Bacillus* is very diverse; it is presently divided into six subgroups based on spore morphology. *B. cereus* falls in the *Bacillus subtilis* group, and it is closely related to *B. anthracis*, *B. mycoides* and *B. thuringiensis*. Some argue,

because of the close relatedness, that the three later species should be classified as sub-species of *B. cereus*. *B. cereus* and *B. anthracis* are both recognized as pathogens, but the former is implicated with foodborne disease. *B. anthracis* can infect perorally, but is inefficient [38].

B. cereus is ubiquitous in nature. It is believed that the primary habitat of *B. cereus* is soil. It is found rather frequently in foods such as meats, poultry, milk, cereals, starches, herbs, and spices. Foods implicated with the diarrheal type of *B. cereus* poisoning include milk, vegetables, meat, and fish. Foods associated with the emetic type of poisoning include rice products, potato, pasta, and cheese products. Other foods such as sauces, pastries, soups, puddings, and salads were identified as vehicles in food poisoning outbreaks [13].

2. Materials and Methods

Glass ware: All glass wares used are thoroughly cleaned with ethanol and rinsed with distilled water before use.

2.1. Sampling Site and Collection

Samples of awara were purchased from three (3) locations namely: Kawo, Tudun wada and Unguwan dosa from the production points and hawkers at each part of the sampling area within Kaduna metropolis in a clean polythene bag (50 from each location), and were immediately transported to Food and Industrial Microbiology research laboratory of the Department of Microbiology, Kaduna State University.

2.2. Preparation of Mannitol Egg-Yolk Polymyxin Agar (MYP)

Exactly 21.5 g of the mannitol egg-yolk polymyxin agar base was suspended in 450ml of distilled water; it was mixed well and dissolved by heating with frequent agitation. The medium was then boiled for one minute until complete dissolution. Sterilization in autoclave was done at 121°C for 15 minutes. The medium was then allowed cool to 45-50°C, after which one vial of polymyxin-B supplement and egg-yolk suspension were added, mixed well and then dispensed into sterile petri dishes. The prepared medium was stored at 8-15°C for use during cultivation [15].

2.3. Enumeration and Identification of *Bacillus Cereus*

Stock samples of awara were prepared by weighing and blending 25g of each “awara” in 225 ml of sterile 0.1% buffered peptone water. The homogenate were subjected to ten-fold serial dilutions by transferring 1ml of the homogenate into 9 ml of sterile 0.1% buffered peptone water. From this serial dilution, 1ml of 10^{-5} , 10^{-6} and 10^{-7} dilutions were plated on freshly prepared mannitol Egg-Yolk polymyxin agar (MYP), a highly selective medium for *Bacillus cereus* developed by Holbrook and Anderson, 1980 [15]. The inoculated agar plates were incubated at 37°C for 24hrs. The isolates were examined for growth and all samples showed growth within 24hrs of incubation. Colonies

that appeared pink, surrounded by whitish zones of clearance were presumed to be *B. cereus*. Colonies that appeared yellow were presumed to be *B. subtilis*, those that appeared creamy were presumed to be *B. licheniformis*. Colonies of *Bacillus cereus* grown on MYP are pink and lecithinase positive, but other bacteria are not inhibited and can interfere with isolation of *Bacillus cereus* [34]. The presumptive isolates were identified by morphological characteristics. The morphological test includes appearance of cell, shape and pigmentation. Further identification of the organisms using biochemical characteristics are Gram's reaction, position of spores, motility, oxidase, indole production, nitrate reduction, citrate, catalase, coagulase, methyl red voges proskaur and lecithinase [27]. The presumptive *Bacillus cereus* were confirmed using the PCR technique.

2.4. Genomic DNA Extraction of the Bacterial Isolate

High salt based method [38] was followed for genomic DNA extraction. Reagents used for genomic DNA extraction were TE buffer (Tris-EDTA), 10% (w/v) sodium dodesyl sulfate (SDS), 20 mg/ml proteinase K, 5M NaCl, CTAB/NaCl solution, 24:1 chloroform/ isoamyl alcohol, 25:24:1 phenol/chloroform/isoamyl alcohol, isopropanol and 70% ethanol [23].

The tubes containing the cell pellet (approximately 500µg each test tube) were used for DNA extraction. The cell pellet was resuspended in 567µl TE buffer by repeated pipetting. 30µl of 10% SDS was added in all the tubes. However to observe the effect of different types of lysis agents on the overall extraction procedure of Gram positive bacteria, lysis steps were carried out with 0, 3 and 6µl of proteinase- K respectively. For the entire step, DNA extraction was carried out in triplicate. 100µl 5M NaCl was added and mixed thoroughly and 80µl CTAB/NaCl.

Solutions were added to each tube and then the mass was mixed and incubated for 10 min at 65°C. Equal volume chloroform/isoamyl alcohol was added, mixed and microcentrifuged (MIKRO 2, Hettich, Germany) at 13000 rpm for 4 min. The supernatants were then transferred to a fresh tube, and 0.6µl of isopropanol was added and mixed gently and kept for 1 day at -20°C for DNA precipitation. DNA pellets were then obtained by centrifuging at 13000 rpm for 4 minute, and then dried and resuspended in 100µl TE buffer [23].

2.5. PCR Assay of Genomic DNA Using HBLD (Primer) and Gel Electrophoresis

The DNA extracts and controls were amplified with primers, 200mM of each dNTP (Promega), 10 mM KCl PCR buffer, 2 mM MgCl₂ and 1.0U *Taq* polymerase (Bioline). Amplification conditions for the PCR were as follows: 5 min at 94°C to denature the DNA, followed by 40 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and strand extension at 72°C for 2 min on a programmable thermal controller. The amplified PCR products were separated by electrophoresis on a 1.5%

agarose gel with 0.5% ethidium bromide in 1xTAE buffer and DNA ladder were visualized under ultraviolet (UV) light photographed by Gel Doc 2000 [4].

2.6. Antibiotic Susceptibility

Bacillus cereus isolates were tested for their sensitivity to antibiotics by means of disc diffusion method [29]. The organisms were investigated using antibiotic disc containing: Pefloxacin (10µg), Gentamycin (10µg) Ampiclox (30µg) Zinnacef (20µg), Amoxicilin (30µg), Rocephin (25µg), Ciprofloxacin (10µg), Streptomycin (30µg), Septrin (30µg), and Erythromycin (10µg). The antibiotic discs were spaciouly placed on Mueller-Hinton agar plates previously seeded with overnight broth cultures of *Bacillus cereus* isolates. The plates were incubated at 37°C for 18-24hours. The plates were incubated at 37°C for 18-24hours. The different zones of inhibition were measured to the nearest millimetre and interpreted as sensitive (greater or equal to 18mm), moderate sensitive (13 to 17mm) and resistant (less than 13mm) based on the interpretation by Clinical Laboratory standard Institute (CLSI) [5, 6, 33].

Table 1. Cultural and Morphological Appearance of *Bacillus Spp* Isolated from Awara from Kawo, Tudun wada and Unguwan Dosa.

Number of samples	Colonial morphology	Cellular morphology	Gram's reaction	Probable organism
51	pinkish colonies	Rods	Positive	<i>Bacillus cereus</i>
22	yellow colonies	Rods	Positive	<i>Bacillus subtilis</i>
13	creamy colonies	Rods	Positive	<i>Bacillus licheniformis</i>

The percentage (%) ocurrence of *Bacillus spp* isolates from the three sample areas showed that *Bacillus cereus* had the highest percentage 40%, followed by *Bacillus subtilis* 16% and *Bacillus licheniformis* (6%) from Kawo samples. This was closely followed by Tudun wada samples in which

2.7. Multiple Antibiotic Resistant (MAR) Index

The multiple antibiotic resistant (MAR) index was determined for the isolates by dividing the number of antibiotics to which the isolate is resistant by the total number of antibiotics tested [29].

$$\text{MAR index} = \frac{\text{Number of antibiotics isolate is resistant to}}{\text{Total number of antibiotics tested}}$$

3. Results

A total of one hundred and fifty (150) "awara" samples were purchased from various selling outlets in kaduna metropolis and were analysed, three species of *Bacillus* which include *Bacillus cereus*, *Bacillus subtilis*, and *Bacillus licheniformis* showed morphological appearance. All the *Bacillus cereus* isolated in the cause of this research revealed an irregular to round edge, flat, rough and dry bright pink colonies with zones of egg yolk precipitate on the selective agar (MYP) compared to other *Bacillus spp* identified. They are rod shaped and gram positive. Table 1.

Bacillus cereus had (38%), followed by *Bacillus subtilis* (12%), and *Bacillus licheniformis* (8%), and lastly Unguwan dosa samples in which *Bacillus cereus* had (24%), *Bacillus subtilis* (16%), and *Bacillus licheniformis* (12%) respectively.

Table 2. Percentage (%) ocurrence of *Bacillus spp* Isolated from Awara from Kawo, Tudun wada and Unguwan dosa.

Sampling Area	Number of samples Positive for <i>Bacillus cereus</i>	Number of sample positive for <i>Bacillus subtilis</i>	Number of sample positive for <i>Bacillus licheniformis</i>
Kawo	20 (40%)	8 (16%)	3 (6%)
Tudun Wada	19 (38%)	6 (12%)	4 (8%)
Ungwan Dosa	12 (24%)	8 (16%)	6 (12%)
Total	51 (34%)	22 (14.7%)	13 (8.7%)
Chi-Square Value	4.471	0.727	2.154
P-Values	0.107	0.695	0.341

Gel Electrophoresis of PCR Product of *Bacillus cereus*

The gel electrophoresis showed the band (Plate 1) obtained from PCR and the amplicon size is 829 base pairs, and primer used was hb1D. Plate 1

Antibacterial Susceptibility Testing of the Enterotoxigenic *Bacillus cereus*

Table 3 showed the antibacterial susceptibility testing of the enterotoxin producing *Bacillus cereus*. The *Bacillus cereus* isolates were found to be susceptible to Pefloxacin, Ciprofloxacin, Streptomycin, Septrin and Erythromycin. Resistance was observed against Ampiclox while Gentamycin, Zinnacef, Amoxicilin and Rocephin were found to be less susceptible.

Table 3. Antibacterial Susceptibility testing of the Enterotoxigenic *Bacillus cereus* using 10 Commercially Available Antibiotics.

S/N	Antibiotics	Concentration	Categories
1	Pefloxacin	10µg	+++
2	Gentamycin	10µg	++
3	Ampiclox	30µg	-
4	Zinnacef	20µg	++
5	Amoxicilin	30µg	++
6	Rocephin	25µg	++
7	Ciprofloxacin	10µg	+++
8	Streptomycin	30µg	+++

S/N	Antibiotics	Concentration	Categories
9	Septrin	30µg	+++
10	Erythmycin	10µg	+++

Key: +++ = Sensitive, ++ = Moderate sensitive and - = Resistance

Multiple Antibiotic Resistance (MAR) Index

$$\text{MAR index} = \frac{\text{Number of antibiotics isolate is resistant to}}{\text{Total number of antibiotics tested}}$$

$$\text{MAR} = \frac{1}{10} \text{ Therefore, MAR} = 0.1$$

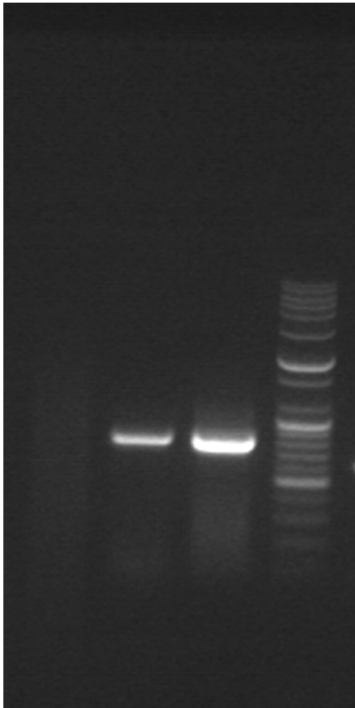


Figure 1. Gel electrophoresis of HBLD gene amplified by PCR, indicating the target DNA fragment was successfully amplified in the 2 samples. (Molecular weight marker, M 100 bp DNA ladder, lane 1 and 2, *Bacillus cereus* DNA).

4. Discussion

Bacillus cereus is becoming an important food poisoning organism because of its widely distribution in nature. *Bacillus cereus* could be transmitted to other foods when contaminated food products are used as constituents in the processing of these foods. The isolation of *Bacillus cereus* in all awara samples implies extreme contamination and potential health risk of these street food samples.

The cultural characteristics of the *Bacillus spp* isolated in this work (table 1) showed that *Bacillus cereus* appeared pinkish; *Bacillus subtilis* yellowish, while *Bacillus licheniformis* creamy. This correspond with the work of Okonlawon [24], who isolated *Bacillus spp* from Nigerian Traditional Condiments and Odeyele [25], who also isolated *Bacillus spp* from street food.

The microscopic results obtained from all species of *Bacillus* showed that they were gram positive and rod in shape. This agrees with the findings of Okonlawon [24], who

detected *Bacillus spp* such as *Bacillus cereus*, *Bacillus subtilis*, *Bacillus pumilus* and *Bacillus licheniformis* from four Nigerian condiments (Iru, Dawadawa, Ogiri and Okpehe) as all these species were found to be gram positive rods and motile.

The biochemical characteristics of the *Bacillus spp* isolated in this work were confirmed [27]. *Bacillus spp* isolated in this work include, *Bacillus cereus*, *Bacillus subtilis* and *Bacillus licheniformis* and *Bacillus cereus* were found to be catalase positive, citrate positive, indole negative, lecithinase production positive, nitrate reduction positive, motility positive, methyl red negative and voges proskauer positive. These results are in agreement with the results of the study by Kotiranta [16], Ouoba [28] and Catia [3].

The occurrence of *Bacillus cereus* in this study showed that most of the samples examined were contaminated with *Bacillus cereus* having 51 (34%), occurrence followed by *Bacillus subtilis* having 22 (14.7%) and *Bacillus licheniformis* having 13 (8.7%) occurrence, which as a result, revealed a high incidence of *Bacillus cereus*. This agrees with the findings of Okonlawon [24], who isolated *Bacillus spp* from four different local condiments (Iru, Dawadawa, Ogiri and Okpehe). The isolates were identified as *Bacillus cereus*, *Bacillus subtilis*, *Bacillus pumilus* and *Bacillus licheniformis*, where *Bacillus cereus* had the highest occurrence of 13 (38.24%) followed by *Bacillus subtilis* 12 (35.30%), *Bacillus pumilus* 4 (11.76%) and *Bacillus licheniformis* 5 (4.70%). The results of this research are also in agreement with the results of Ugwu [36], who also detected *Bacillus cereus* from rice, meat and fish samples. *Bacillus cereus* occurred in 123 (35.14%) of rice samples, 111 (31.71%) of meat samples and 116 (33.14%) of fish samples. Ugwu [36] reported that, the presence of *Bacillus cereus* in food and food products could be as a result of the ubiquity of its spores to survive dried storage which means that most raw and ready to eat foods may contain *Bacillus cereus*. Odeyele [25] equally isolated *Bacillus spp* from street food which include *Bacillus cereus*, *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus firmus*. *Bacillus cereus* was found to occur in 84 (56%) samples, *Bacillus subtilis* occurred in 36 (24%), *Bacillus firmus* and *Bacillus licheniformis* equally occurred in 15 (10%) samples. *Bacillus cereus* spores occur widely in foods and all kinds of foods have been implicated in *Bacillus cereus* food poisoning [9, 14].

The occurrence of *Bacillus cereus* in higher proportion than other species in awara samples may be due to the fact that *Bacillus cereus* is more adaptable to a wider varying environment than other species. The high incidence of *Bacillus cereus* in this study could be explained by the ubiquitous distribution of this organism and its ability to form endospores [16, 21] coupled with poor storage

conditions, the unsanitary and largely unhygienic nature of the food preparations and services areas as foods are good indicators of the state of environment in which they are prepared or served [8, 26].

Molecular identification of *Bacillus cereus* enterotoxin genes by PCR revealed that, *Bacillus cereus* possessed the gene encoding the hblD enterotoxins and were detected among the *Bacillus cereus* isolates and a product size of 829 base pair was obtained. This corresponds with the work of Guinebretiere [12], who detected *Bacillus cereus* hblD enterotoxin gene from food and obtained a product size of 829 base pair.

This also agrees with the work of Basil [2], who also isolated *Bacillus cereus* from milk and milk products and were tested for the hblD enterotoxin genes in 11 isolates and found out that 90.9% possessed the hblD genes. These results are also in line with the work of Fayaz [10], who detected *Bacillus cereus* from meat and detected the hblD genes. About 28 isolates possessed one or more genes of hbl complex with an overall incidence of 63.63% of this complex. Montanhini [22], also isolated *Bacillus cereus* from dairy products and found out that *Bacillus cereus* strains incubated at 30°C produced the hbl enterotoxin. Same results were reported by Dufrenne [7], whose study found out that 100% of psychrotrophic *Bacillus cereus* strains produced the hbl enterotoxin. Therefore, this contamination is recognized to be a hazard to consumers. Lopez and Alippi [18], also worked on *Bacillus cereus* from food and reported that out of 132 *Bacillus cereus* isolates, all the four hbl genes (ABCandD) were detected in 55 strains (42%). According to Lindback and Granum [19], about 40% of *Bacillus cereus* strains harbor the hblACD genes responsible for the hbl codification. For this reason, these enterotoxins proteins are considered to be the most important produced by these microorganisms.

The effective use of quality control and safety measures during production/manufacture besides creating awareness among food handlers and consumers becomes necessary to ward off the menace of *Bacillus cereus* and other food poisoning organisms [10].

The results of antibiotic susceptibility of the isolates showed that the susceptibility was determined by disc agar diffusion method. The enterotoxigenic *B. cereus* were susceptible to Pefloxacin, Ciprofloxacin, Streptomycin, Septrin and Erythromycin. Resistance was observed against Ampiclox, while Gentamycin, Zinnacef, Amoxicillin and Rocephin were observed to be less susceptible.

The findings of this study correspond with those obtained by other researchers [35]. The previous work have shown that antimicrobial susceptibility of *Bacillus cereus* were highly susceptible to Streptomycin, Chloramphenicol, Erythromycin, Ciprofloxacin and less susceptible to Ampicillin and Ampiclox [35].

This also agrees with the findings of Agwa [1], who reported that *Bacillus cereus* were highly resistance to Ampiclox and sensitive to Erythromycin, Chloramphenicol, Streptomycin and Ciprofloxacin and less sensitive to

Gentamycin [1]. Odeyele [25], also reported that all enterotoxigenic *Bacillus cereus* isolates were susceptible to Gentamycin and almost all to Ciprofloxacin, while Ampicillin, chloramphenicol and erythromycin showed resistance.

Turnbull [32] worked on 67 *Bacillus cereus* strains implicated in non-gastrointestinal infections and food poisoning and from the environment, reported that resistance to Ampicillin is constant while susceptibility to Erythromycin, Chloramphenicol is usually observed and also, susceptibility to Ciprofloxacin was uniform and it has been shown to be highly effective in the treatment of *Bacillus cereus* wound infections [32]. Weber [37] conducted studies on 54 *Bacillus cereus* strains isolated from blood cultures and showed that all strains tested were susceptible to Chloramphenicol, Gentamycin and Ciprofloxacin and many strains were susceptible to Erythromycin. Luna [20] also tested 42 *Bacillus cereus* isolates and reported results similar to those reported by Turnbull [32] and Weber [37]

In variance to my findings in final analysis of each of the reported studies of Turnbull [32], Weber [37] and Luna [20], small populations of *Bacillus cereus* isolates showed resistance to Erythromycin.

Variations in the zone of inhibition may be due to the differences in the concentrations of antimicrobial agents used, drug resistance transfer and the overall wide spread use of the antibiotics in the environment. The development of drug resistance may be due to the use of these drugs in the medical and veterinary practice to treat infections and misuse of the drugs resistance strains [1].

The multiple antibiotic resistances (MAR) index in this study is 0.1. MAR index gives an indirect suggestion of the probable source of the organism and MAR index greater than 0.2 indicates that the organism must have originated from an environment where there are no strict rules concerning prescriptions and usage of antibiotics [29, 33].

The antimicrobial resistance patterns of *Bacillus cereus* from foods is useful in epidemiological studies [17] as it provides adequate awareness about the safety of consumable foods contaminated with high level of potential microbes for food borne disease outbreak, resulting to a distribution that cannot be controlled and thus bringing about changes in public health and deterioration thereby increasing susceptibility rate [31].

5. Conclusions

This research succeeded in isolating three species of *Bacillus* which include *Bacillus cereus*, *Bacillus subtilis*, and *Bacillus licheniformis* from “awara” samples in Kaduna metropolis using the mannitol egg yolk polymyxin agar. All of them were gram positive rods, motile and spore formers. This research found that 51 (34%) of 150 of the *Bacillus* strains isolated from “awara” samples in kaduna metropolis belonged to *B. cereus* while *B. subtilis*, *B. licheniformis* were found in 22 (14.7%), and 13 (8.7%) respectively. These food samples are highly contaminated with *B. cereus*, as its spores

will not be eliminated from food materials by heat treatment. This research has also attempted to establish that potentiality of isolates of *B. cereus*, generally referred to as opportunistic foodborne pathogen, did harbour toxigenic traits as evidence by molecular studies where the isolate of *Bacillus cereus* possess the enterotoxin. The enterotoxigenic *Bacillus cereus* isolates were found to be susceptible to Pefloxacin, Ciprofloxacin, Streptomycin, Septrin and Erythromycin. Resistance was observed against Ampiclox while Gentamycin Zinnacef, Amoxicillin and Rocephin were found to be less susceptible. Based on the result of this research, the effective antibiotics against enterotoxigenic *B. cereus* strains isolated from “awara” in Kaduna metropolis are Pefloxacin, Ciprofloxacin, Steptomycin, Septrin and Erythromycin.

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