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Research Article

Faecal Carriage of AmpC Producing *E. coli* and *Klebsiella* Spp

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Abstract

AmpC-producing organisms can act as a hidden reservoir for ESBLs; the high-level expression of the AmpC β-lactamases may mask the recognition of the ESBLs. In recent years, the world has seen a surge in *Enterobacteriaceae* resistant to broad-spectrum beta-lactam antibiotics due to the production of Extended-Spectrum Beta-Lactamases (ESBLs) or Plasmid-mediated AmpC (pAmpC) enzymes. The aim of this study was to determine faecal carriage of AmpC producing *enterobactercae* among students of Ambrose Alli University, Ekpoma, Edo State. Fecal samples were collected from 102 students of AAU who accepted to participate in the study, who had not been recently hospitalized and had no recent exposure to antibiotics (≥ 3 months): all are from healthy individuals (students of AAU). Of the 102 faecal samples analysed, 46 were lactose fermenters which were assessed for AmpC. In this current study, the prevalence of fecal carriage of AmpC genes among HCWs was 28.26%. To our knowledge, this is the first study investigating the prevalence of AmpC-producing *E. coli* isolated from stool samples healthy subjects in this axis. This study demonstrated a prevalence of colonization by multi-drug resistant, non-clonally-related AmpC positive *E. coli* and *Klebsiella spp*. Isolates among male and female university students in AAU Ekpoma. Colonization of AmpC in the population of study was not related to prior antimicrobial consumption or hospitalization.

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Introduction

The widespread and inappropriate use of antibiotics has resulted in a significant increase in antibiotic resistant bacteria worldwide [1]. A major contributor to this increasing resistance is the production of inactivating enzymes, in particular Extended Spectrum Beta Lactamases (ESBLs). There is no agreement on the exact definition of ESBLs. However, ESBLs are commonly defined as beta-lactamases that confer resistance to the penicillins; first, second, and third-generation cephalosporins and monobactams by hydrolysis of these antimicrobials [2]. In addition, these enzymes are inhibited by beta-lactamase inhibitors such as clavulanic acid [3]. Multidrug resistance is increasingly seen in many *Gram-negative bacteria* as a result of widespread use of various antibiotics [1,2,4,5]. Carbapenems are currently the treatment of choice for serious infections, with fluoroquinolones being and an alternative choice, if the organism is shown to be susceptible. Infections with ESBL producing bacteria are not only extremely challenging to treat, but are also associated with increased mortality [2,6].

In recent years, the world has seen a surge in *Enterobacteriaceae* resistant to broad-spectrum beta-lactam antibiotics attributed to the production of Extended-Spectrum Beta-Lactamases (ESBLs) and/or plasmid-mediated AmpC (pAmpC) enzymes [7,8]. ESBLs are, by the classical definition, inhibited by clavulanic acid [9] whereas pAmpC enzymes are not [10]. The plasmid location of ESBL and pAmpC genes facilitates their spread via horizontal gene transfer [11].

Escherichia coli are not only constituents of the commensal gut flora but also common opportunistic pathogens often implicated in urinary tract [3,12]. They frequently harbor ESBL- and pAmpC-encoding genes. Broad-spectrum beta-lactamase production is associated with increased morbidity and mortality in both high and low/middle-income countries [13,14]. To the best of our knowledge, there is no published data from Nigeria on the prevalence of community acquired faecal carriage of AmpC producing organisms in adults or children.

Data on the epidemiology of AmpC-producing *Enterobacteriaceae* in Africa are still limited. The majority of publications report on the prevalence of ESBL-producing *Enterobacteriaceae* in clinical samples, and there are only few studies on colonization [15,16]. Furthermore, there is a predominance of reports from Northern and Western Africa [17,18] and only individual studies from Eastern and Southern

Africa [19,20] (excluding South-Africa). The aim of this study is to determine the prevalence of faecal colonization with ESBL- producing *enterobactercea* (*E. coli* and *Klebsiella* spp) among healthy university students in Ambrose Alli University, Ekpoma, Edo State, Nigeria and to analyze the resistance phenotype, AmpC resistant gene content and clonal relatedness of isolates.

Materials and Methods

The study population of this study comprises of students of Ambrose Alli University (AAU), Ekpoma, Edo State. A total of one hundred and two (102) students of AAU, Ekpoma, Edo State were recruited for this study which consist fifty-one (51) male and fifty-one (51) female students within the age group of 18 - 30 years.

Sample Selection/Collection: Faecal samples were collected from 102 students of AAU who accepted to participate in the study, who had not been recently hospitalized and had no recent exposure to antibiotics (≥ 3 months): all are from healthy individuals (students of AAU). Of the 102 faecal samples analysed, 46 were lactose fermenters which were assessed for AmpC.

Bacterial Isolates: Fresh stools samples were collected (between July 2021 and September 2021) in a sterile, clean, wide-mouthed plastic and leak proof container by a clean wooden applicator stick for microbiological analysis and contained in the coproculture pots were stored in the icebox and transported to the Department of Medical Microbiology, Ambrose Alli University, Ekpoma. The samples were cultured on CLED agar plates (Oxoid, Basingstoke, UK) and then incubated for 18-20 hours at 37°C. *E. coli* was isolated and identified according to conventional microbiological methods [21]. The sample containers were labeled properly with the date of collection and participant name/ID.

All samples were cultured on CLED agar with and without ceftriaxone (1 mg/L) for selection of third-generation cephalosporin-resistant isolates. 0.5 g of faecal sample was suspended in 5 mL of sterile 0.9% saline. Each suspension was seeded on CLED agar supplemented with rocephin at 1 mg/L to select the *Enterobacteriaceae* resistant to the Third-Generation Cephalosporins (3GC). After seeding, the plates were incubated for 48h at 37°C. One colony representing each distinct colonial morphotype was isolated from supplemented CLED agar and further analyzed by gram coloration. After 24 hours, the colonies suspended in 0.9% saline to yield a turbidity equivalent to that of the 0.5 McFarland standards appropriate were prepared for the biochemical identification and the antibiotic susceptibility testing. The Biochemical identification was carried out, which constitutes a standardized system of identification of *Enterobacteriaceae*.

Identification of Bacterial Isolates: Identification of the isolates were done by the following standard microbiological techniques which involved morphological appearance of the colonies, Gram's staining reactions, indole test, and other biochemical properties as required.

Phenotypic Screening for AmpC B-Lactamase-Producing Strains: *E. coli* and *Klebsiella* isolates were tested for β-lactamase production using the Kirby-Bauer disk diffusion method. The antibiotics used in the study included a second-generation cephalosporin (cefoxitin, 30 µg); third-generation cephalosporins (ceftazidime, 30 µg); a fourth-generation cephalosporin (cefepime, 30 µg); carbapenem (imipenem, 10 µg); cefpodoxime impregnated with boronic acid was placed in the center and on either side of the disk, cefpodoxime were used for detection of any synergy [22,23]. For quality control, *E. coli* ATCC 25922 was used [24].

Isolates with one or more of the following criteria were considered to be potential AmpC and were listed for confirmation of AmpC production by the combined disks method. The criteria included an inhibition zone to cefpodoxime ≤ 22 mm, boronic acid disc ≤ 26mm, extension of the zone of inhibition of any cephalosporins towards boronic acid by the disk diffusion method [23]. Isolates resistant to cefoxitin (inhibition zone < 18 mm), in addition to being resistant to one or more of the third-generation cephalosporins and being intermediate or resistant to amoxicillin-clavulanic acid were considered as putative AmpC β-lactamase producers [22,25].

Test for AmpC-β-Lactamase Production: AmpC-β-lactamase enzyme production was detected by the 3-aminophenylboronic acid inhibitor-based detection method which was performed by inoculating on MHA and placing a disk containing 30 µg of cefpodoxime (Hi-Media) plus 400 µg of boronic acid onto the agar. Inoculated plates were incubated overnight at 37°C. An organism that demonstrated a zone diameter around the disk containing cefpodoxime and boronic acid that was 5mm or greater than the zone diameter around the disk containing cefoxitin were considered an AmpC-β-lactamase producer [26-28].

Preparation of Disks Containing Boronic Acid: About 120 mg of phenylboronic acid (benzeneboronic acid; Sigma-Aldrich, Milwaukee, Wis.) was dissolved in 3 ml of dimethyl sulfoxide. Three milliliters of sterile distilled water were added to this solution. Twenty microliters of the stock solution were dispensed onto disks containing 30 µg of cefpodoxime. Disks were allowed to dry for 30 min and used immediately or stored in airtight vials with desiccant at 4°C and at − 70°C [26].

Antibiotic Susceptibility Testing: The antimicrobial susceptibility tests were performed using the Kirby-Bauer disk diffusion method on Nutrient agar (HiMedia, India) as per CLSI recommendations [29]. The antibiotics tested in this study ceftazidime (30 µg), cefotaxime (30 µg), cefoxitin (30 µg), cefepime (30 µg), imipenem (10 µg), meropenem (10 µg) respectively. All the antibiotics used were purchased from Rehoboth Pharmacy in Ekpoma, Edo State, Nigeria. Interpretation of antibiotic susceptibility results was made according to standard interpretative zone diameters suggested in CLSI guidelines [29]. In this study, if the isolates were resistant to at least three classes of first-line antimicrobial agents, they were regarded as MDR (MultiDrug Resistance) [30].

Methods of Analysis

The first stage after collection of the sample, it was inoculated into peptone water containing Rocephin antibiotics. Then Incubated for 18-24hrs, it was observed for turbidity, those with turbidity where then cultured/inoculated into a CLED agar plate containing Rocephin antibiotics of 4ug/ml. Then it was incubated for 18-24hrs at 37°C. The plate was observed for growth and only the lactose fermenters were used for analysis. (They appeared pink on the plate).

Plates demonstrating no growth in a primary examination were incubated for another 24 hrs. Growing organisms were identified by standard techniques. After selecting the lactose fermenters out biochemical tests were carried on them which include motility, indole test, urease test, and citrate test. From the fresh inoculated peptone water without antibiotics, a little was removed for storage so that it can be used later on for AmpC detection and antibiotic sensitivity.

Biochemical Characterisation and Identification: For the Gram-negative bacilli, overnight broth cultures was made for each by adding the colonies to sterilized peptone water and incubated for 24 hours at 37^{0}C and motility test was done to ascertain their motility. Indole test was done by adding some drops of Kovacs reagent (Paradimethylamino benzldehyde) to the overnight broth cultures. Development of red ring at the surface after ten seconds indicates positive reaction and such bacteria were regarded as *Escherichia coli*. For those colonies that were Lactose Fermenters and Oxidase negative, Urease test was performed on them. Having been positive to Urease and negative to Indole confirmed *Klebsiella pneumonia*.

Antimicrobial Susceptibility: Antimicrobial susceptibility was determined by the disk diffusion test, using Clinical and Laboratory Standards Institute (CLSI) guidelines [31]. Samples yielding bacteria that grew on CLED agar were initially identified as suspicious for AmpC. Isolates were screened for AmpC production by the double-disk synergy test in which a cefpodoxime (20 µg/10 µg) disk was placed in the centre with cefpodoxime with boronic acid (10 µg). Strains producing AmpC were defined as those showing synergism between BA and any one of CPD. The standard CLSI combined disk method involving CAZ and CTX with and without the inhibitor clavulanic acid (30 µg) (Mast Diagnostics, Merseyside, UK) was used to confirm the presence of ESBL [31]. AmpC production was indicated by an increase in zone size of more than 4mm with and without boronic acid. Quality control was done using *K. pneumoniae* ATCC 700603 (positive control) and *E. coli* ATCC 25922) (negative control).

Statistical Analysis

The result was presented using tables. Data was analyzed using the SPSS software. The percentage prevalence was calculated in each case. The Mean and Standard deviation of the results obtained was calculated. Chi Square and Percentage was used for the analysis using SPSS package version 18. Value with p<0.05 were considered statistically significant in this study. Study findings were explained in words and tables.

Results

Table 1 revealed the socio-demographic characteristics of the respondents. Age was categorized into two age groups; 16-20 and 21-25. Majority of the respondents were within ages 16-20 years; 32 (67.4%) and 21-25; 15 (32.6%). Regarding the isolates, 41 (89.1%) of the isolate was *E. coli*, while 5 (10.9%) was *Klebsiella*. Regarding the Ampc, 28 (60.9%) of the respondents tested negative, 15 (32.6%) were positive and nil; 3 (6.5%). The mean age of the respondents was 19.91±2.44.

Variables		Frequency (N=46)	Percentages (%)
Age (Years)	16-20	32	67.4
	21-25	15	32.6
	Total	46	100
Isolate	*E. coli*	41	89.1
	Klebsiella spp	5	10.9
	Total	46	100
Ampc	Positive	15	32.6
	Negative	28	60.9
	Nil	3	6.5
	TOTAL	46	100
Age (Mean ± SD)	19.91±2.44		

Table 1: Socio-Demographic Characteristics of the Study Population.

The results in Table 2 presented the isolate and AmpC levels of faecal carriage of AmpC producing *E. coli* and *Klebsiella Spp* among AAU students between male and female. The result showed that the isolate levels were significantly lower (p<0.05) in male subjects (1.00±0.00) as compared with female subjects (1.19±3.40). On the contrary, the levels of AmpC were not significantly lower (p>0.05) in male subjects (1.63±0.50) as compared to female (1.81±0.62).

Parameters		16-20 years (n=31)	21-25 years (n=15)	t-value	p value
Ampc	Positive	1.71±0.64	1.80±0.41	0.495	0.623
	Negative	1.10±0.30	1.13±0.35	0.366	0.716

Table 2: Comparison of isolate and AmpC levels of faecal carriage of AmpC producing *E. coli* and *Klebsiella Spp* among AAU students between male and female.

The results in Table 3 presented the isolate and AmpC levels of faecal carriage of AmpC producing *E. coli* and *Klebsiella Spp* among AAU students according to their age groups. The result showed that the levels of isolate were higher (p>0.05) within the age range of 21-25 years (1.13±0.35) as compared to 16-20 years (1.10±0.30). Also, levels of AmpC were higher (p>0.05) within age 21-25 years (1.80±0.41) as compared to 16-20 years (1.71±0.64) (Table 4).

Parameters		Male (n=19)	Female (n=27)	t value	p value
Ampc	Positive	1.63±0.50	1.81±0.62	1.066	0.292
	Negative	1.00±0.00	1.19±3.40	2.032	0.048

Table 3: Comparison of isolate and AmpC levels of faecal carriage of AmpC producing *E. coli* and *Klebsiella spp* among AAU students according to their Age Groups.

		Probable Microorganism	
		Escherichia coli	*Klebsiella spp*
Cultural characteristics	Shape	Rod	Rod
	Elevation	Convex	Raised
	Consistency	Mucoid	Mucoid
	Colour	Rose Pink in CLED	Light Pink in CLED
Biochemical analysis	Gram	-	-
	Catalase	-	-
	Coagulase	-	-
	Indole	+	-
	Motility	+	-
	Oxidase	-	-
	Citrate	-	+
	Urease	-	+
Sugar Fermentation	Glucose	A/G	A/G
	Maltose	A/G	A
	Sucrose	A	A
	Lactose	A/G	-

Table 4: Cultural Characteristics and Biochemical Analysis of Bacterial Isolates.

KEY: + = Positive NA = Nutrient agar A = Acid production G = Gas - = Negative CLED = Cysteine Lactose Electrolyte Deficient Agar

Discussion

AmpC-producing organisms can act as a hidden reservoir for ESBLs; the high-level expression of the AmpC β-lactamases may mask the recognition of the ESBLs [32]. The present study aimed to determine the prevalence of faecal carriage of AmpC producing *enterobactercae* among students of Ambrose Alli University, Ekpoma, Edo State, Nigeria by conventional microbiological methods. The evolution of antibiotic resistance is a continuous threat to human health worldwide [33]. *E. coli* may act as a reservoir of genes coding for antibiotic resistance and may also be responsible for a group of endogenous infections [1,2,5].

In this current study, the prevalence of faecal carriage of AmpC genes among HCWs was 28.26%. To our knowledge, this is the first study investigating the prevalence of AmpC-producing *E. coli* isolated from stool samples healthy subjects in this axis. These results are higher than those obtained from patients in other works; Naseer, Haldorsen, Simonsen & Sundsfjord, [34] reported a prevalence of 3.7% of AmpC among outpatients in Naseer et al. [34] reported a prevalence of 1.9% Czech Republic and Denmark (rates were1.1% and 2.4%, respectively) [35,36]. In 2008, carriage of AmpC-β-lactamase in the GastroIntestinal (GI) tract was detected in nearly 4% of healthy Danish army recruits. The same group was also demonstrated to carry ESBL-positive *Enterobacteriaceae* [37].

In the present study, AmpC-β-lactamase-producing strain was dominantly found in *E. coli* and *Klebsiella spp*. in healthy subjects. We conclude that despite the estimated prevalence of AmpC producing *Enterobacteriaceae* especially in healthy individuals is relatively high. They are certainly a reservoir for the resistance genes. These reservoirs should certainly be included in attribution studies for human infections. Furthermore, using selective media to isolate AmpC producing bacteria may reduce the chance to underestimate the prevalence of these bacteria.

Conclusion

This study demonstrated a prevalence of colonization by multidrug resistant, non-clonally-related AmpC positive *E. coli* and *Klebsiella spp*. Isolates among male and female university students in AAU Ekpoma. Colonization of AmpC in the population of study was not related to prior antimicrobial consumption or hospitalization. Similar studies should be done to further explore the epidemiology of multidrug resistant *Enterobacteriaceae* in the population as a whole.

Ethics Statement and Conflict of Interest Disclosures

Human Subjects: Consent was obtained from all participants in this study. Ambrose Ali University Health Research and Ethics Committee of Ambrose Ali University (A.A.U.), Ekpoma, Edo State, Nigeria issued approval AAU-HREC-02-32-2022.

Conflicts of Interest: In compliance with the ICMJE uniform disclosure form, all authors declare the following:

Payment/Services Info: All authors have declared that no financial support was received from any organization for the submitted work.

Financial Relationships: All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work.

Other Relationships: All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

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