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Research Article

Incidence of community acquired ESBL-producing bacteria among asymptomatic University students in Anambra State, Nigeria

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ABSTRACT

This study was conducted to investigate the incidence of community acquired extended-spectrum β-lactamase (ESBL)-producing bacteria among asymptomatic students of Nnamdi Azikiwe University, Awka, Anambra State, South-East Nigeria. A total of 102 non-duplicate strains of *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were isolated from fecal samples (n=273) collected from the participating students. The isolates were subjected to antimicrobial susceptibility tests to determine their antimicrobial resistance profile. Their multiple antibiotic resistance (MAR) indices were also evaluated. Screening of the isolates for possible ESBL production was carried out by disk diffusion test using cefotaxime and ceftazidime disks. ESBL-production by the resistant strains was confirmed using the double-disk synergy test. Most of the isolates were found to be multi-drug resistant, as all *K. pneumoniae* and *P. aeruginosa* strains (100%), and 98.4% of the *E. coli* strains, had MAR indices ≥0.2. A total of 22 ESBL-producing bacterial species were confirmed, and the frequency of *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates among the ESBL-producing bacteria were n=20 (90.9%), n=2 (9.1%), and n=0 (0.0%) respectively. The total number of ESBL-producing bacterial strains isolated accounted for 8.1 % of the entire sample population. Although this prevalence rate may not indicate an alarming situation, it is important that the proliferation of ESBL-producing bacteria in the community be contained, since a high incidence of ESBL-producing organisms will create significant therapeutic problems in the near future. There is therefore need to develop strategies to reduce their spread in the community especially through monitoring, surveillance and proper detection protocol.

Keywords: Extended Spectrum β-Lactamase (ESBL); Antibiotic resistance; Gram-negative bacteria; Asymptomatic; Nigeria.

1. INTRODUCTION

β-lactamases are the most common mechanism of resistance to beta-lactam antibiotics including the third-generation cephalosporins to which extended spectrum β-lactamase plays a huge role among *Enterobacteriaceae* [1, 2]. Though there are different diverse β-lactamases, the extended spectrum β-lactamases (ESBLs) have been known to be of very high clinical importance. Infections caused by ESBL-producing organisms are difficult to manage for several reasons. First, empiric therapy consisting of β-lactam antimicrobials is often ineffective; and second, these organisms tend to also be resistant to other classes of antimicrobials including fluoroquinolones and aminoglycosides [1].

ESBLs capable of degrading the cephalosporins and monobactams are among the most important resistance determinants emerging in *Enterobacteriaceae* worldwide. ESBLs are β-lactamases capable of conferring bacterial resistance to the penicillins, first-, second-, and third-generation cephalosporins, and aztreonam by hydrolysis, excluding the cephamycins and carbapenems. These enzymes hydrolyze extended-spectrum cephalosporins such as ceftazidime or cefotaxime, as well as monobactams (aztreonam), and are inhibited by β-lactamase inhibitors [1, 3-5].

ESBLs have been reported worldwide in many different genera of *Enterobacteriaceae* and *Pseudomonas aeruginosa*. However, ESBL production has been previously reported to be most common in *Klebsiella* spp. and *Escherichia coli* [6-8]. It has become very important to study the prevalence of ESBL-producing organisms because of their increasing antimicrobial resistance and the decreasing number of new drugs available against such organisms.

Though the first detection of ESBLs in Nigeria is still not known and a national study for the actual prevalence of ESBL-producing bacteria in Nigeria is lacking, some reports have shown the increasing prevalence of ESBL-producing bacteria in some parts of the country [9-18].

The implications of the prevalence of ESBL-producing organisms in Nigerian communities cannot be overlooked. The wide and irrational use of antibiotics, especially the broad spectrum β-lactams, in our local communities allow for the emergence and spread of resistant strains of bacteria that render available drugs ineffective for treatment. Keeping in view the economic and clinical importance of ESBL-producing bacteria, our study was conducted to investigate the incidence of community acquired ESBL-producing bacteria among healthy and asymptomatic university students in Anambra State, South-East Nigeria.

2. MATERIALS AND METHODS

2.1. Study area and population

The study population comprised of students of Nnamdi Azikiwe University studying at the university campuses located at Agulu, Mbaukwu and Awka, Anambra State, Nigeria. The participants were adult male and female students between the ages of 18-26 years. Inclusion criteria were: (1) the participant is ≥18 years; (2) healthy or appear healthy at physical examination; (3) have no history of previous ESBL infection; (4) have not been hospitalized in the last 6 months. Consent forms and questionnaires indicating demographics and medical history of the individual were filled by the participating students.

2.2. Collection of samples

A total of 273 stool samples were randomly collected over a twelve (12) month period (March 2012-February 2013) from consenting university students spread across the three campuses of the university.

2.3. Isolation and identification of microorganisms

For bacterial isolation and identification, various cultural, staining, and biochemical testing procedures were carried out as previously described [19, 20]. A loopful of each stool sample was inoculated into respective test tubes containing 5 ml of freshly prepared nutrient broth (Oxoid, UK) and incubated at 35°C for 24 h. Bacterial growth was indicated by the turbidity of the broth culture. Using a wire loop, suspensions from the turbid solution was plated aseptically onto MacConkey and cetrimide selective agars (Oxoid,

UK) plates and incubated at 35°C for 24 h. Suspected colonies of *Escherichia coli* and *Klebsiella pneumoniae* isolates were subcultured onto freshly prepared MacConkey agar plates, while suspected *Pseudomonas aeruginosa* isolates were subcultured onto cetrimide selective agar plates to obtain pure cultures. For confirmation, Gram staining and con-ventional biochemical testing techniques including indole test for suspected *E. coli*, citrate and malonate utilization tests for suspected *K. pneumoniae*, and oxidase test for suspected *P. aeruginosa* were carried out.

2.4. Antimicrobial Susceptibility Test (AST)

AST of the isolates was carried out using the modified Kirby-Bauer disk diffusion method described by Cheesbrough [19] and the Clinical and Laboratory Standard Institute (CLSI) [21]. Mueller-Hinton (MH) agar (Oxoid, UK) was prepared according to the manufacturer's instructions, and transferred into 90 mm diameter sterile Petri dishes to a depth of 4 mm. The surface was lightly and uniformly inoculated using a sterile cotton wool swab in three directions rotating the plate approximately 60^0C, to ensure even distribution. Prior to inoculation, the swab stick was dipped into bacterial suspension having visually equivalent turbidity to 0.5 McFarland standards. Excess liquid from the sterile cotton-wool swab dipped in the bacterial suspension was removed by turning the swab stick against the side of the tube. The plates were covered and allowed to dry on the bench before applying the discs. Antibiotic discs were placed on the agar plate within 15 minutes of inoculation of isolates. Inoculated plates were incubated at 37 ^{o}C for 24 hours. On the next day, plates were read by taking measurement of zone of inhibition using a meter rule; *E. coli* ATCC 25922 was used as a negative control. Antibiotic disks used include cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), imipenem (10 µg), meropenem (30 µg), tetracycline (30 µg), erythromycin (15 µg), cefpodoxime (10 µg), amoxycillin-clavulanic acid (20/10 µg), sulphamethoxazole-trimethoprim (25 µg), ciprofloxacin (5 µg), and gentamicin (10 µg) (Oxoid, UK).

2.5. Screening of bacterial isolates for possible ESBL production

To screen all the *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates for the production of ESBL enzymes, single antibiotic disks comprising cefotaxime (30 µg) and ceftazidime (30 µg) were placed aseptically at a distance of 30 mm apart on MH agar plates that was previously swabbed with standardized inoculum of the test bacterium. The plates were allowed for about 30 mins for pre-diffusion of the antibiotics; and these were incubated for 18-24 hrs at 37^{o}C. After the incubation, the zones of inhibition were measured and recorded to the nearest millimeter using a meter rule. ESBL production was inferred or suspected if any of the test bacteria showed reduced susceptibility or is resistant to any one of the third generation cephalosporins (cefotaxime and ceftazidime) as per the breakpoints of CLSI [21].

2.6. Multiple Antibiotic Resistance (MAR) Index

Multiple antibiotic resistance (MAR) index (number of antibiotics to which test isolate displayed resistance divided by total number of antibiotics to which the test organism has been evaluated for sensitivity/resistance) for each test isolate was calculated according to the method described by Riaz et al. [22].

2.7. Confirmation of ESBL production by Double Disk Synergy Test (DDST)

ESBL production was confirmed in the *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates by the double disk synergy test (DDST) method as previously described [21]. DDST was performed as a standard disk diffusion assay on MH agar plates. Standardized bacteria suspension was aseptically swabbed on the MH agar plates. Amoxycillin-clavulanic acid disc (20/10 µg) was placed at the centre of the plate, and cefotaxime (30 µg) and ceftazidime (30 µg) discs were each placed at a distance of 15 mm (centre to centre) from the amoxycillin-clavulanic acid disc. The plates were incubated at 37^{o}C for 18-24 hrs. ESBL production was confirmed phenotypically when a difference of ≥5 mm increase in the inhibition zone diameter for

the zones of inhibition of the cephalosporins (cefotaxime and ceftazidime) tested alone and in combination with amoxicillin-clavulanic acid was observed.

3. RESULTS

3.1. Isolation and identification of microorganisms

A total of 273 fecal samples from the university students were analyzed. Of the 273 samples analyzed, 102 non-duplicate bacterial isolates comprising *E. coli* (n=63), *K. pneumoniae* (n=20), and *P. aeruginosa* (n=19) were isolated.

3.2. Antimicrobial resistance profile of the community-derived isolates

The *E. coli* isolates showed high resistance of 76.2, 82.5, and 79.3% to cefpodoxime, tetracycline, and erythromycin respectively. The *E. coli* isolates showed least resistance to imipenem (6.3%) followed by ciprofloxacin (14.3%) and cefotaxime (14.5%). All *P. aeruginosa* isolates were completely resistant (100%) to cefpodoxime, meropenem, amoxicillin-clavulanic acid and erythromycin, but were highly susceptible to imipenem, gentamicin and ciprofloxacin with a percent resistance of 5%. *K. pneumoniae* isolates like *P. aeruginosa* showed complete resistance (100%) to cefpodoxime, and were highly resistant to meropenem (90%), sulfamethoxazole-trimethoprim (75%), tetracycline (85%) and erythromycin (90%). Generally, imipenem showed the highest antibacterial activity against the tested isolates, followed by ciprofloxacin and gentamicin (Table 1). It can be observed in Table 2 the isolates were found to be multi-drug resistant as all *K. pneumoniae* and *P. aeruginosa* strains (100%), as well as 98.4% of the *E. coli* strains had MAR indices of 0.2 and above.

Table 1. Antibiotic resistance profile of isolates.

Antibiotics	Resistance (%)		
	E. coli	***K. pneumoniae***	***P. aeruginosa***
Ceftazidime	20.6	30	11
Cefotaxime	14.5	25	32
Ceftriaxone	34.9	25	16
Cefpodoxime	76.2	100	100
Imipenem	6.3	0	5
Meropenem	58.7	90	100
Amoxycillin-clavulanic acid	28.6	40	100
Gentamicin	20.6	15	5
Ciprofloxacin	14.3	15	5
Sulfamethoxazole-trimethoprim	54	75	84
Tetracycline	82.5	85	79
Erythromycin	79.3	90	100

Table 2. Multiple Antibiotic Resistance (MAR) Index of isolates.

Frequency of MAR Index			MAR Index
E. coli ***n* (%)**	***K. pneumoniae*** ***n* (%)**	***P. aeruginosa*** ***n* (%)**	
0 (0.00%)	0 (0.00%)	0 (0.00%)	0
1 (1.59%)	0 (0.00%)	0 (0.00%)	0.1
13 (20.63%)	1 (5.00%)	0 (0.00%)	0.2
17 (26.98%)	1 (5.00%)	1 (5.27%)	0.3
12 (19.05%)	7 (35.00%)	3 (15.79%)	0.4
15 (23.81%)	7 (35.00%)	10 (52.63%)	0.5
5 (7.94%)	4 (20.00%)	4 (21.05%)	0.6
0 (0.00%)	0 (0.00%)	0 (0.00%)	0.7
0 (0.00%)	0 (0.00%)	0 (0.00%)	0.8
0 (0.00%)	0 (0.00%)	0 (0.00%)	0.9
0 (0.00%)	0 (0.00%)	1 (5.26%)	1.0

3.3. Confirmation of ESBL-producing isolates

DDST was used for the phenotypic confirmation of ESBL production by the isolates. An increase of ≥5 mm was observed in the IZDs produced by the cephalosporins (with amoxicillin-clavulanic acid) against the ESBL producing organisms compared to the individual IZDs of any of the cephalosporins (without amoxicillin-clavulanic acid) (Figure 1). This was consistent with the CLSI's specifications for the double disc synergy test for the confirmation of ESBL production [21].

A total number of 102 Gram negative isolates (encompassing *E. coli*, *K. pneumoniae*, and *P. aeruginosa*) were isolated from the sample population (n=273). From these isolates, 22 ESBL-producing bacteria strains were confirmed, which is 8.1% of the total sample population. Of the total number of Gram negative isolates (n=102), 21.6% (n=22) were confirmed ESBL-producers. A majority of the ESBL-producers were *E. coli* (n=20, 90.9%), followed by *K. pneumoniae* (n=2, 9.1%). ESBL-production was not detected among the *P. aeruginosa* isolates (Figure 2).

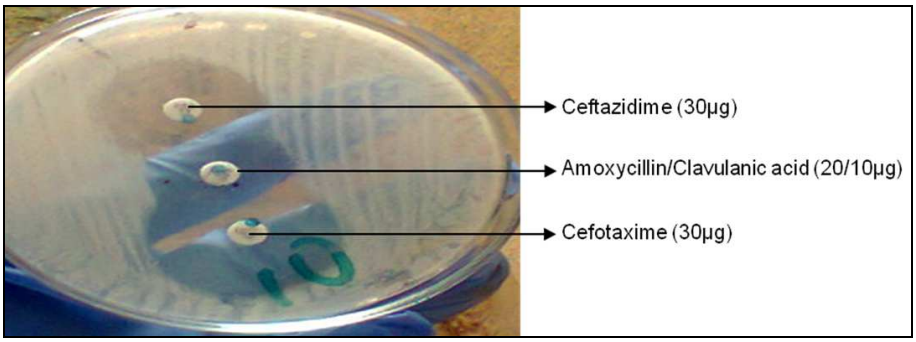

Figure 1. A picture of ESBL-producing isolate after the double-disc synergy testing (DDST); showing the keyhole effect notable for ESBL production in the phenotypic test.

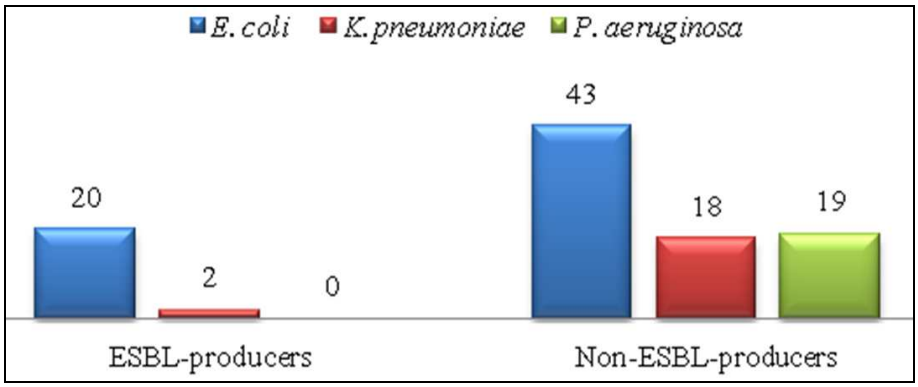

Figure 2. Total ESBL-producers and non-ESBL-producers among the community isolates.

3.4. Geographic and gender distribution of the samples, total isolates and ESBL-producing isolates

A total number of 273 samples were collected from the three locations Agulu, Awka, and Mbaukwu. The highest percentage of the samples came from Agulu (63.4%), followed by Mbaukwu (24.5%) and Awka (12.1%) (Table 3). A total of 102 non-duplicate bacterial isolates comprising of *K. pneumoniae*, *E. coli* and *P. aeruginosa* strains were isolated from the samples; and 52.0%, 17.6%, and 30.4% of the total number of isolates were from Agulu, Awka and Mbaukwu respectively. The distribution of the ESBL-producing bacteria from the three locations follows the same pattern as the distribution of the total number of samples and isolates from the locations.

Table 3. Geographical distribution of samples, total isolates and ESBL-producing isolates.

Source of samples	Number of samples [n (%)]	Number of isolates [n (%)]				Number of ESBL-producing isolates [n (%)]			
		EC	KP	PA	Total	EC	KP	PA	Total
Agulu	173 (63.4%)	43 (42.15%)	9 (8.82%)	1 (1.0%)	53 (52.0%)	16 (72.7%)	0 (0.0%)	0 (0.0%)	16 (72.7%)
Awka	33 (12.1%)	7 (6.86%)	3 (2.94%)	8 (7.8%)	18 (17.6%)	0 (0.0%)	2 (100.0%)	0 (0.0%)	2 (9.1%)
Mbaukwu	67 (24.5%)	13 (12.74%)	8 (7.84%)	10 (9.8%)	31 (30.4%)	4 (18.2%)	0 (0.0%)	0 (0.0%)	4 (18.2%)
Total	273 (100.0%)	63 (100.0%)	20 (100.0%)	19 (100.0%)	102 (100.0%)	20 (100.0%)	2 (100.0%)	0 (0.0%)	22 (100.0%)

KP: *K. pneumoniae*, EC: *E. coli*, PA: *P. aeruginosa.*

Table 4. Gender distribution of samples, total isolates and ESBL-producing isolates.

Gender	Gender distribution of samples collected [n (%)]				Gender distribution of isolated bacteria [n (%)]				Gender distribution of ESBL-producing isolates [n (%)]			
	Agulu	Awka	Mbaukwu	Total	EC	KP	PA	Total	EC	KP	PA	Total
Males	41 (23.7%)	20 (60.6%)	17 (25.4%)	78 (28.6%)	22 (34.9%)	8 (40.0%)	7 (36.8%)	37 (36.3%)	2 (10.0%)	1 (50.0%)	0 (0.0%)	3 (13.6%)
Females	122 (64.7%)	10 (30.3%)	41 (61.2%)	173 (63.4%)	35 (55.6%)	10 (50.0%)	11 (57.8%)	56 (54.9%)	16 (80.00%)	1 (50.0%)	0 (0.0%)	17 (77.3%)
Unknown	10 (5.8%)	3 (9.1%)	9 (13.4%)	22 (8.1%)	6 (9.5%)	2 (10.0%)	1 (5.3%)	9 (8.8%)	2 (10.0%)	0 (0.0%)	0 (0.0%)	2 (9.1%)
Total	173 (100.0%)	33 (100.0%)	67 (100.0%)	273 (100.0%)	63 (100.0%)	20 (100.0%)	19 (100.0%)	102 (100.0%)	20 (100.0%)	2 (100.0%)	0 (0.0%)	22 (100.0%)

KP: *K. pneumoniae*, EC: *E. coli*, PA: *P. aeruginosa.*

As more samples were collected from Agulu (n=173, 63.4%), followed by Mbaukwu (n=67, 24.5%), and then Awka (n=33, 12.1%), the highest percentage of ESBL-producing isolates was from Agulu (n=16, 72.7%), followed by Mbaukwu (n=4, 18.2%), and then Awka (n=2, 9.1%). Of the 22 ESBL-producing isolates, *E. coli* was the most prevalent (n=20, 90.9%), followed by *K. pneumoniae* (n=2, 9.1%). No ESBL-producing *P. aeruginosa* strain was detected. A rate of 72.7% of the *E. coli* isolates that were found to be ESBL-producers were isolated from Agulu, 18.2% were from Mbaukwu, and none from Awka. ESBL-producing *K. pneumoniae* (n=2, 100%) was reported only from samples emanating from Awka (Table 3).

Of the total 273 fecal samples collected from the university students, 173 (63.4%) samples were received from female students, and 78 (28.6%) were from male students. Twenty two (8.1%) students did not indicate their genders (Table 4). A total of 36.3% of all the bacterial isolates comprising *K. pneumoniae*, *E. coli* and *P. aeruginosa* strains were from males, and 54.9% were from females. The distribution of the ESBL-producing bacteria amongst the female and male students also follows the same pattern as the distribution of the total number of samples and isolates amongst the male and female students.

As more samples were collected from the female participants (n=173, 63.4%) compared to their male counterparts (n=78, 28.6%), the highest percentage of ESBL-producing isolates was from the female students (77.3%), while 13.6% was recorded for the male students. Eighty percent of the *E. coli* isolates that were found to be ESBL-producers were isolated from the female students, and 10.0% were from the males. There was an equal distribution of ESBL-producing *K. pneumoniae* (50%) amongst the female and male students (Table 4).

4. DISCUSSION

The antibiotic resistance profile of the isolates reveals that the isolates were generally resistant to the different classes of antibiotics tested. The isolates however, exhibited very high resistance rates to cefpodoxime, meropenem, tetracycline, erythromycin, and sulfamethoxazole-trimethoprim. Lower levels of resistance to ciprofloxacin and gentamicin were recorded among the community isolates. The isolates showed least resistance only to imipenem, a drug known for its potent and broad spectrum antimicrobial activity. The complete susceptibility of the isolates to imipenem could be attributed to the low-rate use of the drug, since is extremely expensive and not readily available in the Nigerian market, and is used as a last option in serious infections when all other antimicrobial drugs have failed.

Majority of the isolates were found to be multi-drug resistant with MAR index ≥2 (Table 2). Riaz et al. [22] stated that a MAR index >0.2 indicates that the organism may have originated from an environment where antibiotics are over used. The observed resistance to these drugs is a probable indication of earlier exposure of the isolates to the antibiotics, which may have enhanced their multidrug-resistance development.

From the 102 Gram negative bacteria isolated from the sample population (n=273), a total number of 22 (8.1 %) ESBL-producing bacteria strains were confirmed. The ESBL-producing isolates observed in this study were *E. coli* and *K. pneumoniae.* This is in agreement with other studies that reported the expression of the ESBL enzyme by both species [1, 23, 24].

Tansarli et al. [7] indicated that Nigeria, together with several other African countries with low human development index (Rwanda, Kenya, Nigeria, Central African Republic, Benin, Senegal, Malawi and Tanzania), have prevalence rates of ESBL-producing organisms from both clinical or community sources varying from 3.8% to 22.8%. Their report showed that the proportion of ESBL-producing isolates among the *Enterobacteriaceae* may not be high in Africa, but is certainly not negligible [7].

The 8.1% prevalence rate of ESBL-producing organisms in our study population may not indicate an alarming situation, but there is need for it to be curtailed since a high incidence of ESBL-producing organisms will create significant therapeutic problems in the near future.

Several studies in Nigeria have shown that abuse and indiscriminate use of antibiotics by people practicing self-medication are partly responsible for the high prevalence of multidrug resistance and

ESBL-producing bacteria in both community and hospital acquired infections [18, 25, 26]. In our study, majority of the study participants indicated that they have at one time or the other taken antibiotics without prescription, especially for ailments such as, typhoid, UTI, cough, and sore throat to mention a few. It can be inferred that the occurrence of multidrug-resistant and ESBL-producing bacteria among the university students may be attributed to the abuse and indiscriminate use of antibiotics by the students in this region. In Nigeria, antibiotics can be relatively inexpensive, sold usually over the counter even without prescription, and can be readily abused or used indiscriminately. Moreover, the indiscriminate proliferation of patent medicine outlets that makes these drugs easily accessible in the community, as observed in the study locations, may have also contributed to the proliferation of these resistant bacteria in the community. This should be of great health concern to university communities across Nigeria, as well as the entire Nigerian population.

ESBL detection and prevalence studies in Nigeria are majorly undertaken by researchers in the academia. However, this have been quite limited to the educational/research institutions without the translation of the findings into a template that can be used in our hospitals for the accurate detection of ESBL from clinical isolates, as well as for the development of policies that will guide the proliferation, use, and abuse of antibiotics in the non-hospital environment.

According to Shaikh et al. [27] and Coque *et al.* [28], the detection of ESBL production is of paramount importance both in hospital and community isolates. Therefore, infection-control practitioners and clinicians need the clinical laboratory to rapidly identify and characterize different types of resistant bacteria. Methods should be improved to efficiently detect and track those bacterial clones and plasmids that constitute the major vehicles for the spread of ESBL-mediated resistance. An improvement is needed in the methods for detecting multidrug-resistant ESBL producers that express a low level of resistance to β-lactams or might contain silenced antibiotic resistance genes not detectable by standard susceptibility testing protocols. The use of broad spectrum cephalosporins and fluoroquinolones in humans should be urgently limited to cases in which other therapeutic alternatives according to evidence-based guidelines are not possible. Limiting antimicrobial use may curtail the selection and persistence of predominant ESBL clones and the probable dissemination of conjugative plasmids among strains, thus decreasing not only the number of potential ESBL donors but also the accumulation of antibiotic resistance genes on common genetic elements. There is also need for national and supranational public health efforts to implement surveillance, epidemiologic, environmental health, and policy-making components on the use of antibiotics. These will certainly contain the spread of ESBL-producing organisms and prevent the emergence of new incidences of diseases caused by ESBL-producing organisms.

5. CONCLUSION

This study has revealed the presence of multidrug-resistant, ESBL-producing organisms among healthy university students of Nnamdi Azikiwe University community, Anambra State, Nigeria. The proliferation of ESBL-producing organisms in the community will create significant therapeutic problems in the near future if not curtailed. There is need to develop effective and innovative strategies to reduce their spread in the community.

ETHICAL APPROVAL

Ethical approval for this research was obtained from the ethical committee of Anambra State University Teaching Hospital, Amaku, Awka, Nigeria (Ref no. ANSUTH/AA/ECC/40).

CONSENT

All participants gave written informed consent to participate in this study.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between all authors. COE designed and supervised the study. CRC, PME and NTU managed the laboratory analyses. CRC and PME managed the data analysis and literature searches, and prepared the first draft of

the manuscript. ICA, CPE and DOA revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

TRANSPARENCY DECLARATION

The authors declare that there is no conflict of interest regarding the publication of this article.

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