# Detection of Extended Spectrum Beta-Lactamases in Gram Negative Bacilli from Clinical Specimens in a Teaching Hospital in South Eastern Nigeria.

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**SUMMARY**

Antimicrobial drug resistance seen among many gram-negative bacteria, especially those expressing the extended-spectrum β-lactamase (ESBL) enzymes that hydrolyze the expanded-spectrum cephalosporins has been on the increase. This has compromised treatment options and thus a threat to the containment of bacterial infections.

To determine the existence of the extended-spectrum β-lactamase enzymes in Nnewi, 250 clinical isolates of members of the family Enterobacteriaceae and *Pseudomonas species* from Nnamdi Azikiwe University Teaching Hospital, Nnewi were identified by conventional methods. These include *Klebsiella species* (96), *E. coli* (90),*Pseudomonas species* (37), *Enterobacter* species (13), *Proteus species* (6), *Citrobacter species* (5) and *Salmonella* species (3).

Antimicrobial drug susceptibility testing was carried out on all the isolates by the disc diffusion method. Extended Spectrum Beta- lactamases were detected by the double disc synergy test.

High level of antimicrobial resistance was noted in test organisms against some of the antimicrobial drugs: Ampicillin + Cloxacillin (93.2%), Tetracycline (90.8%), Streptomycin (82.4%), and Nalidixic acid (62%), and low level of resistance was observed against Ofloxacin (26.4%), Cefotaxime (28.8%) and Nitrofurantoin (28.8%).

One hundred and forty four isolates (57.6%) were suspected ESBL-producers judged by their resistance to any of the third generation cephalosporins used but 40 (16%) actually produced the extended spectrum beta- lactamase enzymes.

This shows the existence of Extended Spectrum Beta- Lactamase producing gram negative organisms in Nnewi. Considering the treatment difficulties, as well as the high cost of treatment associated with these organisms, concerted efforts are needed to contain their spread.

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**Keywords: Extended spectrum Beta- lactamases, Gram negative bacill**

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**INTRODUCTION**

The gram negative bacilli especially *Pseudomonas species* and members of the family enterobacteriaceae are common causes of infections of many parts of the body. They account for more than 50% of all isolates in nosocomial infections[1]. Resistance to a variety of antimicrobial drugs commonly used for the treatment of infections with these pathogens have been reported[2,3] . This phenomenon has compounded treatment of these infections and thus has become a challenge to global public health[4,5]. Many of the resistant cases have been attributed to the production of Extended Spectrum Beta- Lactamases (ESBLs). These are a group of enzymes that enable the bacteria possessing them to hydrolyze and thus confer resistance to expanded spectrum oxyimino- cephalosporins, penicillins and aztreonam among enterobacteriaceae and other gram- negative bacteria. It is indeed the largest source of resistance presently[6]. The ESBL enzymes are most commonly produced by *Klebsiella species* and *Escherichia coli*, but may also occur in other gram-negative bacteria including *Salmonella, Proteus, Pseudomonas, Citrobacter, Morganella, Serratia,*and *Shigella species* [7,8].

Many clinical laboratories are not fully aware of the importance of ESBL and a serious challenge facing clinical laboratories is that clinically relevant ESBL- mediated resistance is not always detectable in routine susceptibility tests[9-11]. The inability of the clinical laboratory to accurately detect and report ESBLs has resulted in avoidable therapeutic failures in patients[12-14], and outbreaks of multidrug resistant gram- negative pathogens that require expensive control efforts[15].

Despite the wide spread reports of the existence of ESBL and its clinical significance in Europe, America and Asia[16-18], there is limited report of its existence in sub- Saharan Africa, particularly in Nigeria[19]. The few reports of its presence in Nigeria were in Lagos and Ibadan, South-western Nigeria[2, 20 ,21], with little or no reports at all in other parts of Nigeria particularly the South-Eastern part of the country.

This study was therefore carried out to detect the presence of the extended-spectrum beta- lactamases among gram- negative bacilli from clinical specimens in Nnewi, South-Eastern Nigeria, using the double disc synergy procedure.

**MATERIALS AND METHODS**

The study area is Nnewi, Anambra State, Nigeria. The town is the location of the Nnamdi Azikiwe University Teaching Hospital, a tertiary health care facility that serves as a referral centre for Anambra and neighboring states.

**Sample and sampling technique**

Two hundred and fifty gram-negative bacterial isolates from the Microbiology laboratory of the hospital were used for the study from October, 2006 to March, 2007. The isolates were obtained from the following clinical specimens: urine, urethral swabs, vaginal swabs, semen, sputum, stool, ear swab, wound swab and Pus.

The organisms were identified to the genus level by standard microbiological methods.

They include: *Klebsiella spp.* (96,) *Escherichia coli* (90), *Pseudomonas spp.*(37), *Enterobacter spp.* (13), *Citrobacter spp.* (5), *Proteus spp.*6, and *Salmonella spp (*3)

Control strains were *Escherichia coli* (ATCC 6571) and *Pseudomonas aeruginosa* (ATCC 27853)

Suspension of the test organism was prepared in sterile peptone water to the turbidity of 0.5 McFarland standard No 1. The suspension was then inoculated evenly over the surface of Mueller-Hinton agar plate and antimicrobial disks were placed on the plates. The plates were incubated at $35^0$C over night. The zones of inhibition were read and interpreted as: Sensitive (s), Resistant (R) or Intermediate (I) according to standard interpretative chart.

**Antimicrobial susceptibility testing**

Antimicrobial susceptibility test was performed on each of the isolates by disc diffusion method as recommended by the National Committee for Clinical Laboratory Standards, now Clinical Laboratory Standards Institute, CLSI[22] .

The antimicrobial susceptibility disks used include: Ceftazidime (CAZ) 30µg, Cefotaxime (CTX) 30µg, Ceftriaxone (CRO) 30µg, Amoxicillin plus Clavulanic acid (AMC) 30µg,(all from Oxoid Laboratories, UK), then, Ampicillin plus cloxacillin (APX) 30µg, Streptomycin (STR) 25µg, Gentamycin (GEN) 10µg, Ofloxacin (OFL) 5µg, Ciprofloxacin (CPX) 5µg, Nitrofurantoin (NIT) 20µg, nalidixic acid (NAL) 30µg, Colistin (COL) 5µg, Tetracycline (TET) 25µg (from Abtek Biologicals Ltd USA)

**Detection of Extended-spectrum Beta-Lactamase**

The presence of Extended- Spectrum Beta- Lactamase (ESBL) was detected by the Double Disk Synergy Test (DDST)[17,23,24]. A suspension of the test organism was inoculated on Mueller- Hinton agar.

A disk containing 30µg Amoxicillin plus Clavulanic acid was placed centrally on the plate. Disks containing Ceftazidime, Cefotaxime and Ceftriaxone were placed round the Amoxicillin + Clavulanic acid disk at a distance of 20mm (centre to centre) from the latter. The plates were incubated over night at $35^0$C. The patterns of zones of inhibition were noted. Isolates that exhibited a distinct shape/size with potentiation towards Amoxicillin + Clavulanate disk were considered ESBL producers.

**RESULTS**

A total of 250 clinical isolates of gram- negative bacteria (Pseudomonas species and members of the family enterobacteriaceae) from urogenital, stool, respiratory tract and throat swab specimens, ear swab, pus, wound or aspirate were tested. Two hundred and three isolates were from urogenital tract, 18 from respiratory tract, 5 from stool, 19 from wound/pus/ aspirate and 7 from ear swab as in Table 1. Varying degrees of resistance was noticed with the various groups of antimicrobial drugs used. Among the cephalosporins, 113 of all the isolates were either resistant (34.4%) or of intermediate susceptibility (10.8%) to ceftazidime, where as ofloxacin had the least resistant isolates compared to the 13 antimicrobial drugs used.

Sixty-six (26.6%) of the isolates were resistant to Ofloxacin and 31 (12.4%) were intermediate (Table 2). Among the aminoglycosides, 132 (52.8%) of the isolates were resistant to Gentamycin and 36 (14.4%) were intermediate it. Furthermore, 123 isolates (49.2%) were resistant to Amoxicillin + Clavulanic acid, while 31 isolates (12.4%) were intermediate to the drug. Most of the isolates were resistant to Ampicillin+Cloxacillin (233; 93.2%) and Tetracycline (227; 90.8%) (Table 3).

Of all the isolates 224 (89.6%) were resistant to e" 5 of the 13 antimicrobial drugs used. The antibiogram also shows that 65 (26%) of all the isolates were resistant to all three cephalosporins used, 38 (15.2%) were resistant to a single cephalosporin, and 41 (16.4%) were resistant to any two of the three cephalosporins. One hundred and six (42.4%) of the isolates were susceptible to all the three cephalosporins (table 4 and picture 1).

One hundred and forty four (57.6%) isolates were resistant to one, two or the three cephalosporins used. Forty of these (16%) of all isolates were ESBL-producing (Table 4). None of the 6 isolates of *Proteus species* produced ESBL.

Among the 40 ESBL-producing isolates, 32 (80%) were detected by Ceftazidime, 26 (65%) by Cefotaxime and 24 (60%) by Ceftriaxone, 13 (32.5%) of the ESBL-producing isolates showed synergy with only one of the three cephalosporins, while 27 (67.5%) of them showed synergy with either two or all three cephalosporins. Picture 3. Eighteen (7.2%) of all the isolates

**Table 1: Isolates and their sources**

| Isolates | Urogenital tract | Respiratory tract +Throat | Stool | Wound/pus/ Aspirate | Ear swab | Total |
|---|---|---|---|---|---|---|
| *Klebsiella* | 80 | 13 | - | 3 | - | 96 |
| *E. coli* | 85 | 1 | 2 | 2 | - | 90 |
| *Pseudo* | 20 | 3 | - | 8 | 6 | 37 |
| *Enterobacter* | 10 | - | - | 2 | 1 | 13 |
| *Proteus* | 5 | - | - | 1 | - | 6 |
| | *Citrobacter* | 3 | 1 | - | 1 | - 5 |
| *Salmonella* | - | - | 3 | | - | 3 |
| Total | 203 | 18 | 5 | 19 | 7 | 250 |

**Table 2: Overall susceptibility pattern to the cephalosporins and quinolones**

| Isolate/Drugs | *Kleb.* | *E. coli* | *Pseudo.* | *Entero.* | *Prot.* | *Citr* | *Salmo* | Total |
|---|---|---|---|---|---|---|---|---|
| CAZ | | | | | | | | |
| S | 54(56.3%) | 51(56.7%) | 22(59.5%) | 5(38.5%) | 3 | 0 | 2 | 137(54.8%) |
| I | 12(12.5%) | 8(8,9%) | 5(13.5%) | 1(7.7%) | 1 | 0 | 0 | 27(10.8%) |
| R | 30(31.3%) | 31(34.4%) | 10(27%) | 7(53.8%) | 2 | 5 | 1 | 86(34.4%) |
| CTX | | | | | | | | |
| S | 60(62.5%) | 64(71.1%) | 19(51.4%) | 9(69.2%) | 3 | 1 | 2 | 158(63.2%) |
| I | 12(12.5%) | 2(2.2%) | 4(10.8%) | 1(7.7%) | 1 | 0 | 0 | 20(8%) |
| R | 24(25%) | 24(27.8%) | 14(37.8%) | 3(23.1%) | 2 | 4 | 1 | 72(28.8%) |
| CRO | | | | | | | | |
| S | 54(56.3%) | 60(66.7%) | 14(37.8%) | 6(46.2%) | 4 | 0 | 2 | 140(56.0%) |
| I | 19(19.8%) | 5(5.6%) | 6(16.2%) | 3(23.1%) | 1 | 0 | 0 | 34(13.6%) |
| R | 23(24%) | 25(27.8%) | 17(45.9%) | 4(30.7%) | 1 | 5 | 1 | 76(30.4%) |
| OFL | | | | | | | | |
| S | 63(65.6%) | 50(55.6%) | 23(62.2%) | 8(61.5%) | 5 | 2 | 2 | 153(61.2%) |
| I | 9(9.4%) | 12(13.3%) | 7(18.9%) | 2(15.4%) | 1 | 0 | 0 | 31(12.4%) |
| R | 24(25%) | 28(31.1%) | 7(18.9%) | 3(23.1%) | 0 | 3 | 1 | 66(26.4%) |
| CPX | | | | | | | | |
| S | 53(55.2%) | 47(52.2%) | 23(62.2%) | 8(61.5%) | 5 | 1 | 1 | 138(55.2%) |
| I | 4(4.2%) | 10(11.1%) | 2(5.4%) | 2(15.4%) | 0 | 0 | 0 | 18(7.2%) |
| R | 39(40.6%) | 33(36.7%) | 12(32.4%) | 3(23.1%) | 1 | 4 | 2 | 94(37.6%) |
| NAL | | | | | | | | |
| S | 32(33.3%) | 24(26.7%) | 13(35.1%) | 5(38.5%) | 1 | 0 | 0 | 75(30%) |
| I | 6(6.3%) | 9(10%) | 2(5.4%) | 1(7.7%) | 1 | 1 | 0 | 20(8%) |
| R | 58(60.4%) | 57(63.3%) | 22(59.5%) | 7(53.8%) | 4 | 4 | 3 | 155(62%) |

Kleb. = *Klebsiella spp*, Pseudo. = *Pseudomonas spp*, Entero. = *Enterobacter spp*, Prot. = *Proteus spp*, Citro. = *Citrobacter spp*, Salmo. = *Salmonella spp*. R= RESISTANT, I= INTERMEDIATE AND S= SUSCEPTIBLE, CAZ = Ceftazidime, CTX =Cefotaxime, CRO = Ceftriaxone, OFL = Ofloxacin, CPX = Ciprofloxacin, NAL = Nalidixic acid.

**Table 3: Cumulative susceptibility pattern to other antimicrobials**

| Isolate/Drugs | *Kleb.* | *E. coli* | *Pseudo.* | *Entero.* | *Prot.* | *Citro.* | *Salmo.* | Total |
|---|---|---|---|---|---|---|---|---|
| APX | | | | | | | | |
| S | 9(9.4%) | 2(2.2%) | 1(2.7%) | 0(0) | 1 | 0 | 0 | 13(5.2%) |
| I | 2(2.1%) | 0(0) | 2(5.4%) | 0(0) | 0 | 0 | 0 | 4(1.6%) |
| R | 85(88.5%) | 88(97.8%) | 34(91.9%) | 13(100%) | 5 | 5 | 3 | 233(93.2%) |
| AMC | | | | | | | | |
| S | 44(45.8%) | 35(38.9%) | 8(21.6%) | 4(30.8%) | 1 | 2 | 2 | 96(38.4%) |
| I | 13(13.5%) | 14(15.6%) | 2(5.4%) | 1(7.7%) | 1 | 0 | 0 | 31(12.4%) |
| R | 39(40.6%) | 41(45.6%) | 27(73%) | 8(61.5%) | 4 | 3 | 1 | 123(49.2%) |
| NIT | | | | | | | | |
| S | 57(59.4%) | 56(62.2%) | 16(43.2%) | 10(76.9%) | 2 | 1 | 2 | 144(57.6%) |
| I | 11(11.6%) | 12(13.3%) | 6(16.2%) | 2(15.4%) | 1 | 1 | 1 | 34(13.6%) |
| R | 28(29.2%) | 22(24.4%) | 15(40.5%) | 1(7.7%) | 3 | 3 | 0 | 72(28.8%) |
| GEN | | | | | | | | |
| S | 41(42.7%) | 20((22.2%) | 16(43.2%) | 4(30.8%) | 0 | 0 | 1 | 82(32.8%) |
| I | 17(17.7%) | 11(12.2%) | 3(8.1%) | 2(15.5%) | 3 | 0 | 0 | 36(14.4%) |
| R | 38(39.6%) | 59(65.6%) | 18(48.6%) | 7(53.8%) | 3 | 5 | 2 | 132(52.8%) |
| COL | | | | | | | | |
| S | 44(45.8%) | 37(41.1%) | 16(43.2%) | 6(46.2%) | 4 | 3 | 1 | 111(44.4%) |
| I | – | – | – | – | – | – | – | – |
| R | 52(54.2%) | 53(58.0%) | 21(56.8%) | 7(53.8%) | 2 | 2 | 2 | 139(55.6%) |
| TET | | | | | | | | |
| S | 11(11.6%) | 2(2.2%) | 3(8.1%) | 2(15.4%) | 0 | 0 | 1 | 19(7.6%) |
| I | 1(1.0%) | 3(3.3%) | 0(0) | 0(0) | 0 | 0 | 0 | 4(1.6%) |
| R | 84(87.5%) | 85(94.4%) | 34(91.9%) | 11(84.6%) | 6 | 5 | 2 | 227(90.8%) |
| STR | | | | | | | | |
| S | 18(18.8%) | 6(6.7%) | 3(8.1%) | 5(38.5%) | 0 | 0 | 0 | 32(12.8%) |
| I | 4(4.2%) | 5(5.6%) | 3(8.1%) | 0(0) | 0 | 0 | 0 | 12(4.8%) |
| R | 74(77.1%) | 79(87.8%) | 31(83.8%) | 8(61.5%) | 6 | 5 | 3 | 206(82.4%) |

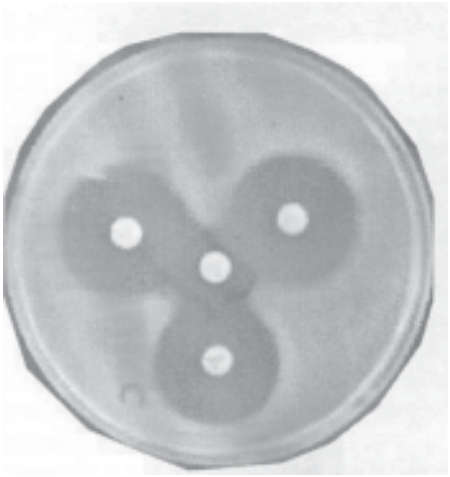
Kleb. = *Klebsiella spp*, Pseudo. = *Pseudomonas spp*, Entero. = *Enterobacter spp*, Prot. = *Proteus spp*, Citro. = *Citrobacter spp*, Salmo. = *Salmonella spp*. R= RESISTANT, I= INTERMEDIATE AND S= SUSCEPTIBLE, AMC = Amoxicillin + Clavulanic acid, APX = Ampicillin + Cloxacillin, STR = Streptomycin, GEN = Gentamycin, NIT = Nitrofurantoin, COL = Colistin, TET = Tetracycline.

**Table 4: Resistance pattern to the three cephalosporins only**

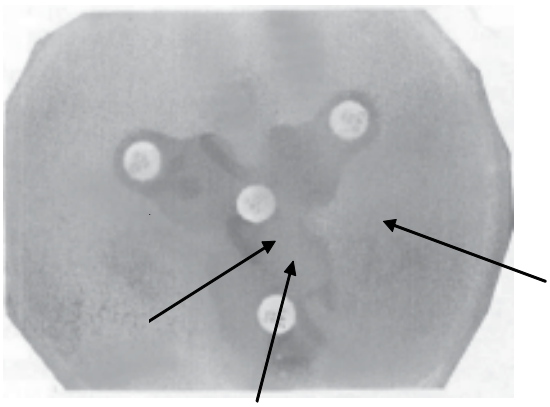
| ISOLATES | CAZ ONLY | CTX ONLY | CRO ONLY | CAZ, CTX | CAZ, CRO | CTX, CRO | CAZ, CTX, CRO | NONE | ESBL producers |
|---|---|---|---|---|---|---|---|---|---|
| *Klebsiella*(96) | 5 | 3 | 6 | 4 | 7 | 3 | 26 | 42 | 11(11.4%) |
| *E.coli*(90) | 11 | 2 | 3 | 3 | 6 | 2 | 19 | 44 | 18(20%) |
| *Pseudomonas* (37) | 1 | 2 | 2 | 0 | 5 | 7 | 9 | 11 | 5(13.5%) |
| *Enterobacter* (13) | 2 | 0 | 1 | 0 | 2 | 0 | 4 | 4 | 2(15%) |
| *Proteus*(6) | 0 | 0 | 0 | 1 | 0 | 0 | 2 | 3 | 0 |
| *Citrobacter*(5) | 0 | 0 | 0 | 0 | 1 | 0 | 4 | 0 | 3 |
| *Salmonella*(3) | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 1 |
| Total | 19 | 7 | 12 | 8 | 21 | 12 | 65 | 106 | 40(16%) |

CAZ = Ceftazidime, CTX =Cefotaxime, CRO = Ceftriaxone ESBL= Extended Spectrum Beta-lactamase.

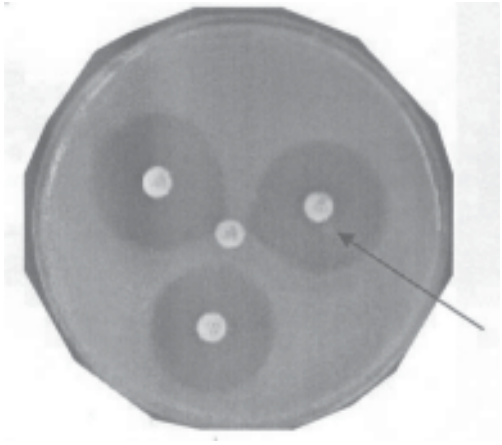
(11 of which were ESBL-producing) were resistant to all 13 antimicrobial drugs used. Another 19 (7.6%) isolates were resistant to all but one antimicrobial drug. Seven (7) of these 19 isolates were also ESBL-producing. 39 (97.5%) of the 40 ESBL-producing isolates were resistant to e" 7 antimicrobial drugs. 13 (32.5%) of the 40 ESBL-producing isolates were susceptible to Colistin and 16 (40%) were susceptible to at least one of the quinolones.



**Picture 1: Susceptible to all 3 cephalosporins**



**Picture 2: ESBL-producing Synergy with all 3 cephalosporins Arrows indicate synergy**



**Picture 4: Discrete colony within the large inhibition zone of CTX**

## DISCUSSION

From this study, *Klebsiella species* and *Escherichia coli* were the most frequently isolated gram-negative organism from clinical specimens especially from the urogenital tract. It was also observed that *Pseudomonas species* caused significant urinary tract infections as well as infections of wound and external ear

None of the isolates tested was susceptible to all the antimicrobial drugs used in the study. Two hundred and forty four isolates showed significant multi-drug resistance from 5 to all 13 antimicrobial drugs used. Three isolates were resistant to only one of the 13 antimicrobial drugs; another 2 were resistant to only 2 antimicrobial drugs. Five isolates were resistant to only 3 antimicrobial drugs and 26 isolates were resistant to only 4 antimicrobial drugs. Of particular note is an *E. coli* strain isolated from the stool of a year old child with diarrhoea that was resistant to all but one antimicrobial drug (Ceftriaxone). The quinolones (Ofloxacin and Ciprofloxacin) have up to 26.4% and 37.6% resistance respectively among the isolates and this is a cause for concern because many clinicians fall back on the quinolones for the treatment of gram-negative pathogens in the face of multi-drug resistance[25]. Increased resistance to ciprofloxacin had been reported earlier in Nigeria by Aibinu *et al*,[20], who discovered that 18% of all ESBL-producing *Enterobacter species* were resistant to Ciprofloxacin. In another report, Aibinu *et al* [21] discovered a contrasting situation with

*Klebsiella pneumoniae* in which all ESBL-producing *Klebsiella pneumoniae* was susceptible to Ciprofloxacin. Very recently, Doi *et al* [26] reported two different isolates of community acquired *E. coli* that were resistant to ciprofloxacin and other drugs in USA. Paterson *et al* [27] had reported that globally 18% of all ESBL- producers were resistant to ciprofloxacin. But today, 7 years after this report, this present study found 37.6% resistance to Ciprofloxacin among ESBL producers in Nnewi. This means that the resistance phenomenon is on the increase. This increasing resistance to several antimicrobial drugs is due to inappropriate usage of antimicrobial drugs (such as over use, misuse, suboptimal dosage and non compliance with treatment duration) which leads to selection pressure.

This study has identified the presence of extended-spectrum â-lactamase among clinical isolates in Nnewi. Forty (16%) of the 250 isolates tested were ESBL producers. *Escherichia coli* has the highest number of ESBL-producing isolates with 18 (29%) out of the 90 isolates tested followed by *Klebsiella* species follow with 11 (11.4%) out of the 96 isolates showing ESBL-production. This conforms to the findings of Kumar *et al* [27] that more *E. coli* than *Klebsiella* possess ESBL. For the rest of the isolates, *Enterobacter species* had 2 out of the 13 isolates (15.4%) producing ESBL. Aibinu *et al* [20] had earlier reported 8 out of 40 isolates (20%) of *Enterobacter species* that produced ESBL enzymes in Lagos Nigeria. It is worthy of note that no ESBL-producing isolate was discovered among the 6 isolates of *Proteus species* tested. Out of the 40 ESBL-producing isolates, 80% (32) were detected by Ceftazidime, 65% (26) by Cefotaxime and 60% (24) by Ceftriaxone. This means that Ceftazidime is the best ESBL screening agent among the three Cephalosporins used in the study.

A total of 144 isolates were resistant to at least one â-lactam antibiotics. By NCCLS definition, these are suspected ESBL producers. Only 40 (27.8%) of these were actually ESBL producers. The reason may be that other resistant enzymes other than ESBL such as'Inhibitor-Resistant â-lactamases' (IRT) could have been present in these ESBL-non-producing but â-lactam resistant isolates. Some strains have also been reported as producing both ESBL and the inhibitor-resistant â-lactamase AmpC which prevents the recognition of the ESBL phenotypically. It should be noted that except one of the 40 ESBL-producing isolates that was resistant to only 4 antimicrobial drugs, the rest showed resistance to 7 or more antimicrobial drugs. This conforms to the findings of Aibinu *et al* [4] who reported that all ESBL-producing *Klebsiella pneumoniae* are multi-drug resistant. Pope *et al*[29] also reported an ESBL- producing *Klebsiella pneumoniae* that co-produced KpC carbapenemase that was resistant to all antibiotics used. While 13 (32.5%) of the 40 ESBL-producing isolates showed synergy with only one cephalosporin, 27 (67.5%) showed synergy with either two or all three cephalosporins. This may suggest the presence of multiple enzymes in the isolates, as the cephalosporinase that hydrolyzes Ceftazidime may be different from that which hydrolyzes Cefotaxime and Ceftriaxone[28].

In conclusion, there is the existence of Extended Spectrum Beta Lactamase producing gram negative organisms in Anambra State, Nigeria. Health care practitioners are therefore advised to be careful on the use and abuse of antimicrobials to minimize the spread. The study detected the ESBL phenotypes and efforts are on the way to determine the ESBL genotypes.

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