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Phenotypic Detection of Extended-spectrum Beta-lactamase-Producing *Escherichia coli* and *Klebsiella pneumoniae* Isolated from Hospital and Environmental Sources in Enugu Metropolis, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Background: Extended -Spectrum Beta- Lactamases (ESBLs) are enzymes that confer resistance to a wide range of β-lactam antibiotics, including penicillins, third-generation cephalosporins, and aztreonam, but not to cephamycins or carbapenems, and are blocked by beta-lactamase inhibitors.
Aim: To evaluate the antimicrobial susceptibility profiles of *Escherichia coli* and *Klebsiella pneumoniae* and to determine the prevalence of ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates from the hospital and environmental samples.
Methodology: The study was conducted from October 2020 to June 2021 in the Microbiological Laboratory of the University of Nigeria Teaching Hospital Ituku-Ozalla, Enugu. A total of 150 non-duplicate bacteria isolates were recovered from urine, wound swab, high vaginal swab, stool, sputum, and environmental sources. Isolates were identified and characterized using standard microbiological protocols. Antimicrobial susceptibility was performed using the Kirby-Bauer disc diffusion procedure. Phenotypic detection of ESBL production was determined using Double Disc Synergy Tvest.

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Results: *E. coli* isolates from hospital samples were highly resistant to cefuroxime (100 %), cefixime (100 %) augmentin (100%), ciprofloxacin (91%), and cefotaxime(86.6%). However, nitrofurantoin and imipenem were highly potent 80.6 % and 76.1% respectively. Among the 67 strains of *E. coli* from hospital samples, 32(47.8%) were found to be ESBL producers. Of the 60 *Klebsiella pneumoniae* hospital isolates tested, 27(45%) were found to be ESBL-producers. Of the 18 strains of *E. coli* from environmental isolates, 12(66.7%) were found to be ESBL producers. Out of only five *Klebsiella pneumoniae* from environmental samples tested, 4(80%) were found to be ESBL producers. A total prevalence of 75(50%) ESBL producers from the 150 isolates were found.
Conclusion: The findings of this study showed an alarming rate of 50% ESBL-producing *E. coli* and *Klebsiella pneumoniae* in Enugu Metropolis, Nigeria with a high antimicrobial resistance in both ESBL and non-ESBL-producing isolates.

Keywords: Antibiotic resistance; ESBL; Klebsiella pneumonia; Escherichia coli; phenotypic method.

1. INTRODUCTION

Broad-spectrum antibiotics with a β-lactam ring in their basic molecular structure are known as beta-lactams. They are one of the most often recommended antimicrobials for bacterial infections globally [1,2]. They are the most extensively used antibacterial agents due to their cost-effectiveness, convenience of use, and tolerability. The efficiency of these antibiotics has been reduced as a result of indiscriminate use, which has resulted in the development of resistant mechanisms in certain species of bacteria [3]. The production of β-lactamases is the most common strategy used by bacteria to resist the effects of antimicrobial drugs [4]. Repeated exposure of bacterial strains to a wide range of β-lactam antibiotics has resulted in the emergence and mutation of the gene coding β-lactamases in these bacteria, which has increased their activity against newly found β-lactam antibiotics [1,2].

Extended Spectrum Beta Lactamases (ESBLs) are enzymes that give resistance to a wide range of β-lactam antibiotics, including penicillins, third-generation cephalosporins, and aztreonam, but not to cephamycins or carbapenems, and are blocked by beta-lactamase inhibitors. These enzymes are inhibited by beta-lactam inhibitors including clavulanic acid, sulbactam, and tazobactam [2]. ESBLs are the main mechanism of acquired resistance in Gram-negative organisms with *Klebsiella pneumoniae* and *Escherichia coli* being the predominant ESBL-producing isolates [5].

Infections caused by ESBL-producing pathogens are especially challenging because they typically co-resist other antimicrobial classes, limiting the antibiotic options available for treatment [6]. Current surgery, instrumentation, a long hospital stay, nosocomial transmission of ESBL-producing organisms by hospital employees, and drug exposure, especially extended-spectrum beta-lactam antibiotics, are all known risk factors for ESBL-producing bacterium infection [7]. The plasmid-encoded extended-spectrum beta-lactamases (ESBLs) are easily transmitted from one bacteria to another by horizontal gene transfer [6]. As a result, most ESBL isolates are resistant to antimicrobials other than beta-lactams, such as aminoglycosides, fluoroquinolones, tetracyclines, and nitrofurans (e.g. nitrofurantoin) and trimethoprim/ sulphamethoxazole. It has proven extremely challenging to manage these multidrug-resistant infections [8]. These resistant strains place a huge burden on society, such as greater mortality rates, longer hospital stays, and higher healthcare costs [6].

As a result of the rising prevalence of ESBL-producing bacteria, laboratory diagnostic approaches that can accurately and quickly detect the presence of these enzymes in clinical isolates are in high demand. The Clinical and Laboratory Standard Institute (CLSI) as well as European Committee on Antimicrobial Susceptibility Testing (EUCAST) proposed a two-step phenotypic strategy for finding ESBL producers, with confirmatory tests afterward. The initial screening can be done using a broth microdilution or a disc diffusion approach, whereas the confirmatory test depends on the addition of beta-lactamase inhibitors to increase the inhibition zone [2, 9, 10]. The number of ESBL-producing organisms is fast increasing, and they are quickly becoming a major challenge in the field of infectious disease prevention and control. Most clinical microbiological laboratories in Enugu state like in many developing countries do not perform ESBL tests [11]. As a result, it is critical to routinely detect ESBL-producing organisms in the laboratory, as failure to do so may result in therapeutic failure as well as increased morbidity and death in patients infected with ESBL-producing bacteria. This work was therefore designed to assess the prevalence of ESBL-producing *E. coli* and *Klebsiella pneumoniae* isolated from hospital and environmental sources in Enugu state and also assess their antimicrobial susceptibility profiles.

2. MATERIALS AND METHODS

2.1 Study Design

The study was conducted from October 2020 to June 2021 in the Microbiological Laboratory of the University of Nigeria Teaching Hospital Ituku-Ozalla. A total of 600 non-duplicate bacterial isolates were collected from samples processed in the microbiology laboratories of 3 referral hospitals and private laboratories in Enugu metropolis including University of Nigeria Teaching Hospital, Ituku-Ozalla, National Orthopedic Hospital Enugu, Enugu State Teaching Hospital, Emmanuel Research Laboratory, and Mac-Chuks Diagnostic Laboratory. A total of 150 isolates were recovered from urine, wound swab, high vaginal swab, stool, sputum, and environmental sources (water, soya milk, and Zobo). Isolates were identified and characterized using standard microbiological protocols. Antimicrobial susceptibility was performed using the Kirby-Bauer Disc Diffusion method, those showing reduced susceptibility to two or three of the third generation cephalosporins were further confirmed phenotypically for ESBL-production using the Double Disk Synergy method.

2.2 Inclusion Criteria

Non-duplicate pure cultures of *E.coli* and *Klebsiella pneumoniae* were used in this work.

2.3 Exclusion Criteria

All isolates that were not confirmed as *E.coli* and *Klebsiella pneumoniae* and all duplicate cultures were excluded.

2.4 Cultivation of the Bacteria Isolates

The isolates were randomly collected from different sources. They were preserved on the nutrient agar slants and taken to the Microbiology Laboratory of University of Nigeria Teaching Hospital.

2.5 Identification of Bacteria Isolates

The isolates were re-activated and cultured primarily on MacConkey agar medium and incubated at 37^0C for 24 hrs. They were identified based on their gram reactions and other biochemical tests according to the method of Cheesbrough [11]. *Escherichia coli* are gram-negative rods, indole, and methyl red positive, citrate negative, urea negative, motile, and gas and acid producers from lactose, glucose, and mannitol.

Klebsiella pneumoniae are gram-negative bacilli, indole negative, methyl red negative, VP positive, citrate positive, oxidase negative, and catalase-positive [11 Patel]. Conventional biochemical tests and API 20E confirmatory system were used to confirm the isolates.

2.6 Phenotypic Detection of ESBL

ESBL testing involves two important steps. The first is a screening test with an indicator cephalosporin for the detection of specific zone diameters to identify isolates that are likely to be harboring ESBL. The second is the confirmatory test for synergy between an oxyimino cephalosporin and clavulanate, distinguishing isolates with ESBL from those that are resistant for other reasons [2, 14].

2.7 Antimicrobial Susceptibility Testing

A suspension of the tested isolates was made using a loop-full of the colony in a freshly prepared normal saline to achieve cell turbidity equivalent to 0.5 McFarland standards. The inoculums were spread on Mueller Hinton agar plates. The antimicrobials were aseptically placed on the surface of Mueller Hinton agar using sterile forceps. The plates were incubated at 37^oC for 24 hours. Antibiotics discs used were cefuroxime (30μg), cefixime (5μg), ceftaxidime (30μg), augumentin (10μ), gentamicin (10μg), ofloxacin (5μg), ciprofloxacin (5μg), nitrofurantion(300μg), cefotaxime (30μg), cefoxitin (30μg) and imipenem (10μg). The inhibitory zone diameters were measured across the disc and the results were evaluated using Clinical and Laboratory Standard Institute guidelines [14].

The isolates that were resistant to any of the third-generation cephalosporins were then confirmed for ESBL production using the double-disc synergy test method.

2.8 Double Disc Synergy Test (DDST)

A suspension of suspected ESBL-producing *E. coli* and *Klebsiella pneumoniae* isolates was adjusted to the 0.5 McFarland turbidity standards and aseptically inoculated on Mueller-Hinton agar (Oxoid, UK) plates using sterile swab sticks. With the help of a template, a combination disc of amoxicillin-clavulanic acid, AMC (20/10µg) was placed at the center of the plate, and cefotaxime (30µg), ceftriaxone (30µg), and ceftazidime (30µg) were placed on either side of the central disc (AMC-20/10µg) at a distance of 15mm apart. At 37°C, the plates were incubated for 18 to 24 hours. After incubation, an increase in the zone of inhibition for either of the cephalosporins (CEF and CTX) towards the centrally placed AMC (20/10µg), phenotypically confirms ESBL production in the tested isolate (2, 14).

2.9 Statistical Analysis

SPSS for Windows version 22 was used for all statistical analyses (SPSS, Chicago, IL, USA). Categorical variables were described using descriptive statistics (frequencies and percentages). At a 95 % confidence interval, one-way analysis of variance (ANOVA) and Student t-test were employed to compare mean differences between and among groups. P-value ≤ 0.05 is considered statistically significant.

3. RESULTS

Of the 600 isolates from clinical and environmental sources, 150 isolates were confirmed; 65(43.3%) were *Klebsiella pneumoniae* and 85(56.7%) were *E. coli*. Figs. 1 and 2 show the frequency distribution of *Escherichia coli* and *Klebsiella pneumoniae* isolated from different samples. Urine samples yielded the highest number of *Escherichia coli* and *Klebsiella pneumoniae* with a total of 34 (40%) isolates and 35 (53.5%) isolates respectively, while stool sample yielded the lowest number of *E. coli* isolates, environmental isolates yielded the lowest number of *Klebsiella pneumoniae*.

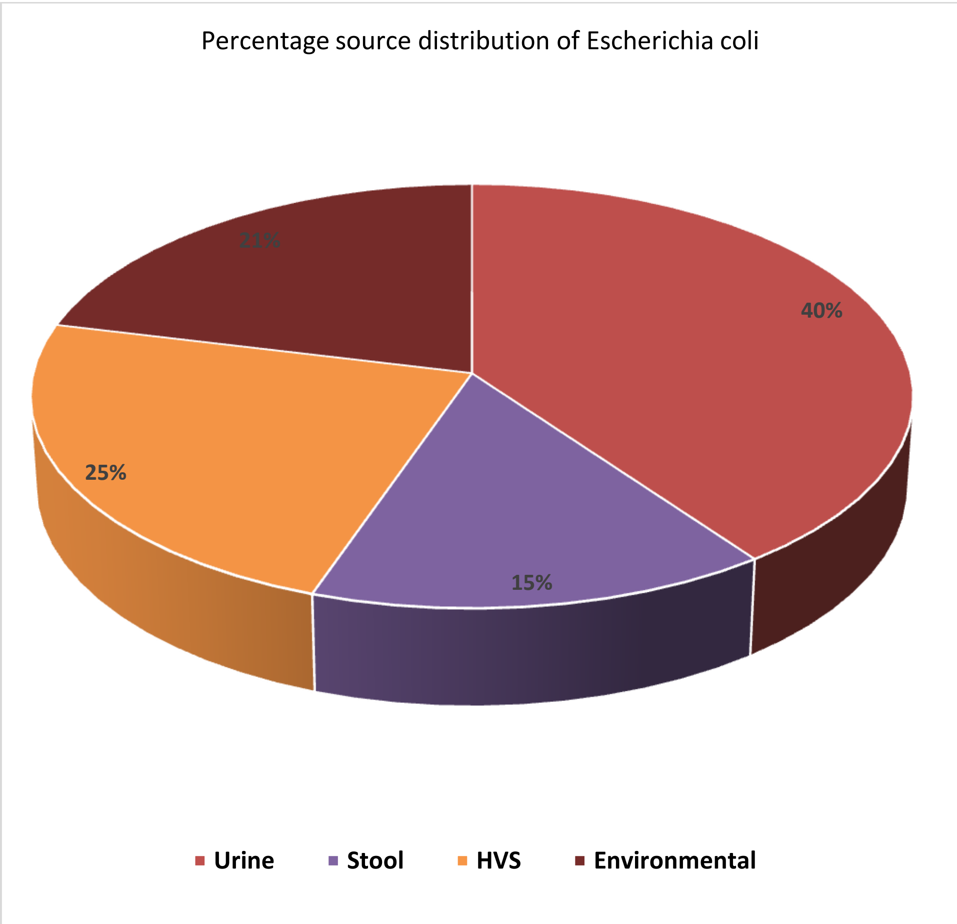

Fig. 1. Percentage distribution of *Escherichia coli* examined

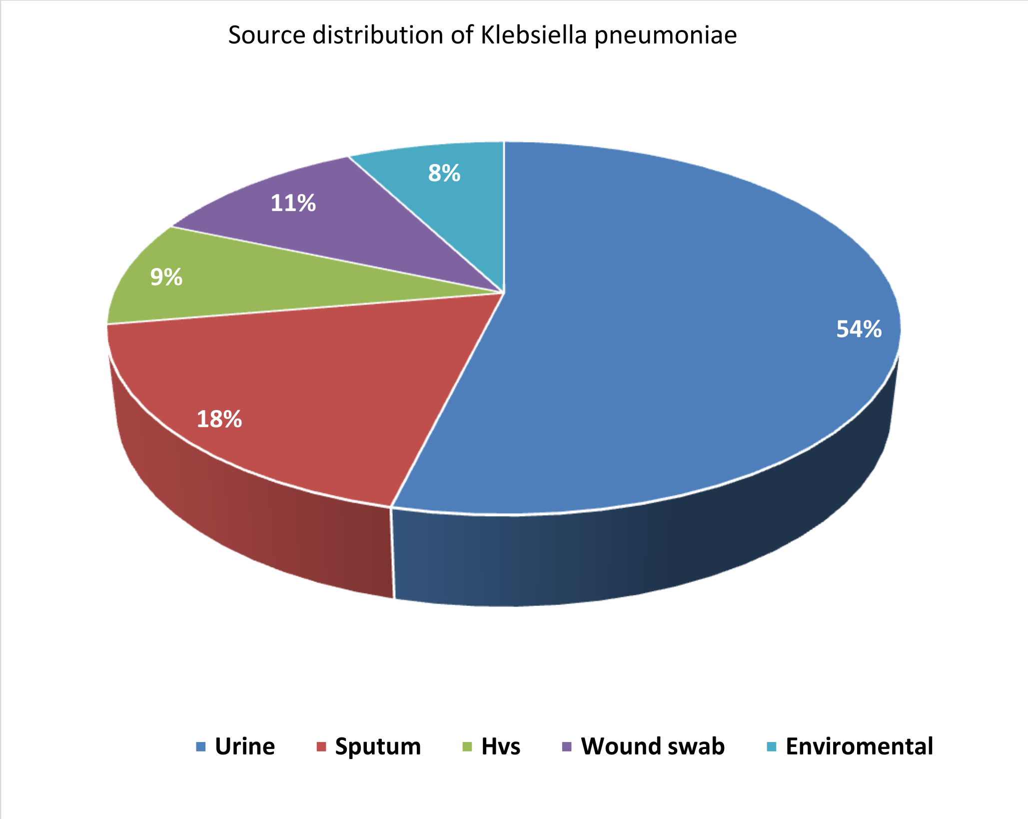

Fig. 2. Percentage Source distribution of *Klebsiella pneumoniae*

Table 1. Percentage susceptibility and resistance of *Escherichia coli* isolates from the hospital and environmental samples

Antibiotics	Hospital Sample (n=67)		Enviromental Samples (n=18)	
	Susceptible	Resistant	Susceptible	Resistant
Cefixime	0 (0%)	67 (100%)	0 (0%)	18 (100%)
Ceftazidime	11 (16.4%)	56 (83.4%)	0 (0%)	18 (100%)
Cefuroxime	0 (0%)	67 (100%)	0 (0%)	18 (100%)
Cefoxitin	47 (70.1%)	20 (29.9%)	12 (66.7%)	6 (33.3%)
Ofloxaxin	11 (16.4%)	56 (83.3%)	12 (66.7%)	6 (33.3%)
Augmentin	0 (0%)	67 (100%)	0 (0%)	18 (100%)
Ciprofloxacin	6 (9.0%)	61 (91%)	4 (22.2%)	14 (77.8%)
Cefotaxime	9 (13.4%)	58 (86.6%)	0 (0%)	18 (100%)
Gentamicin	20 (29.9%)	47 (70.1%)	12 (66.7%)	6 (33.3%)
Nitrofuratoin	54 (80.6%)	13 (19.4%)	14 (77.8%)	4 (22.2%)
Imipenem	51 (76.1%)	16 (23.9%)	12 (66.7%)	6 (33.3%)
	P<0.0007*		*P*<0.0001*	

Table 1 shows the percentage susceptibility and resistance of *Escherichia coli* isolates from the hospital and environmental samples. *Escherichia coli* isolates from hospital samples were all (100%) resistant to Cefixime, cefuroxime, and Augmentin. Reduced frequency of susceptibility of these isolates was also recorded against Ceftazidime (16.4%), Ciprofloxacin (9.0%), Cefotaxime (13.4%), and gentamycin (29.9%). There was high susceptibility of the organisms to cefoxitin, imipenem, and nitrofurantoin, 70.1%, 76.1%, and 80.6% respectively. Statistically, the chi-square test revealed a significantly higher proportion of resistant isolates than sensitive isolates of hospital *Escherichia coli* to the tested antibiotics ($p<0.05$). All *Escherichia coli* isolates from environmental samples were (100%) resistant to Cefixime, Ceftazidime, cefuroxime, Augmentin, and Cefotaxime. Reduced susceptibility of these isolates was also recorded against ciprofloxacin (22.2%). Statistically, the chi-square test revealed a significantly higher proportion of resistant isolates than sensitive isolates on environmental *Escherichia coli* to the tested antibiotics (p<0.05, X^2=93.23).

Table 2 shows the percentage susceptibility and resistance of *Klebsiella pneumoniae* isolates from the hospital and environmental samples. *Klebsiella pneumoniae* isolates from hospital samples were completely (100%) resistant to Augmentin. Reduced susceptibility of these isolates was also recorded against Cefixime (16.7%), Ceftazidime (13.3%), Cefuroxime (10%), Cefotaxime (10%), Ofloxacin (23.3%), Ciprofloxacin (25%), and Nitrofurantoin (23.3%). The isolates showed moderate susceptibility to Gentamicin (36.7%) and Cefoxitine (35%) and with high susceptibility to Imipenem (75%). Statistically, the chi-square test revealed a significantly higher proportion of resistant isolates than sensitive isolates of hospital *Klebsiella pneumoniae* isolates to the tested antibiotics (p<0.05, X^2=128.3). For environmental samples, *Klebsiella pneumoniae* isolates from environmental samples were completely (100%) resistant to Cefixime, Ceftazidime, cefuroxime, Augmentin, and Nitrofurantoin. Moderate susceptibility of these isolates was also recorded against Cefoxitin (40%) and Cefotaxime (40%). Ofloxacin and Gentamicin were highly potent (100%). Imipenem had 60% susceptibility. Statistically, the chi-square test revealed a significantly higher proportion of resistant isolates than sensitive isolates of environmental *Klebsiella pneumoniae* to the tested antibiotics (p<0.05, X^2=34.26)

Fig. 3 shows that out of the 67 isolates of *Escherichia coli* from hospital samples tested for phenotypic detection of ESBL, only 32 (47.8%) were confirmed to produce ESBL while 35 (52.2%) were confirmed to be ESBL non-producers. Out of the 18 isolates of *Escherichia coli* from environmental samples tested for phenotypic detection of ESBL, only 12 (66.7%) were confirmed to produce ESBL while 6 (33.3%) were confirmed to be ESBL non-producers. Statistically, there was no significant difference in the proportion of ESBL producers and non-producers in hospital and environmental isolates of *Escherichia coli* (p>0.05, X^2=1.195).

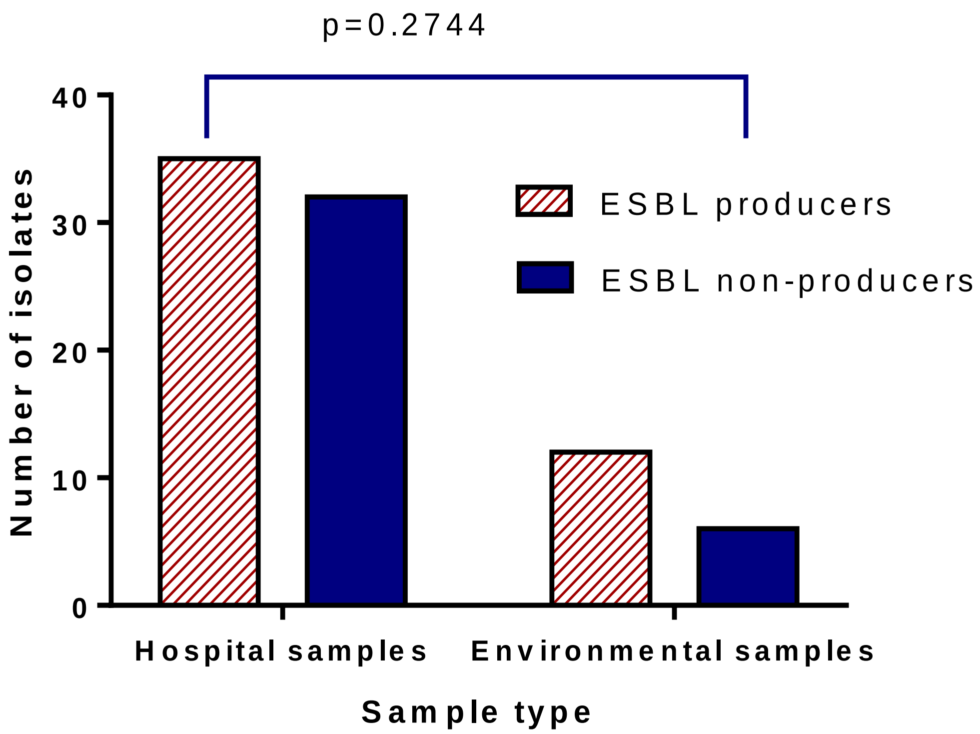

Fig. 3. Distribution of ESBL producers and non-ESBL producers among *Escherichia coli* isolates

Table 2. Percentage susceptibility and resistance of *Klebsiella pneumoniae* isolates from the hospital and environmental samples

Antibiotics	Hospital Sample (n=60)		Enviromental samples (n=5)	
	Susceptible	Resistant	Susceptible	Resistant
Cefixime	10 (16.7%)	50 (83.3%)	0 (0%)	5 (100%)
Ceftazidime	8 (13.3%)	52 (86.7%)	0 (0%)	5 (100%)
Cefuroxime	6 (10%)	54 (90%)	0 (0%)	5 (100%)
Cefoxitin	21 (35%)	39 (65%)	2 (40%)	3 (60%)
Ofloxaxin	14 (23.3%)	46 (76.7%)	5 (100%)	0 (0%)
Augmentin	0 (0%)	60 (100%)	0 (0%)	5 (100%)
Ciprofloxacin	15 (25%)	45 (75%)	3 (60%)	2 (40%)
Cefotaxime	6 (10%)	54 (90%)	2 (40%)	3 (60%)
Gentamicin	22 (36.7%)	38 (63.3%)	5 (100%)	0 (0%)
Nitrofuratoin	14 (23.3%)	46 (76.7%)	0 (0%)	5 (100%)
Imipenem	45 (75%)	15 (25%)	3 (60%)	2 (40%)
	$P<0.0007^*$ $X^2=128.3$		$P=0.0002^*$ $X^2=34.26$	

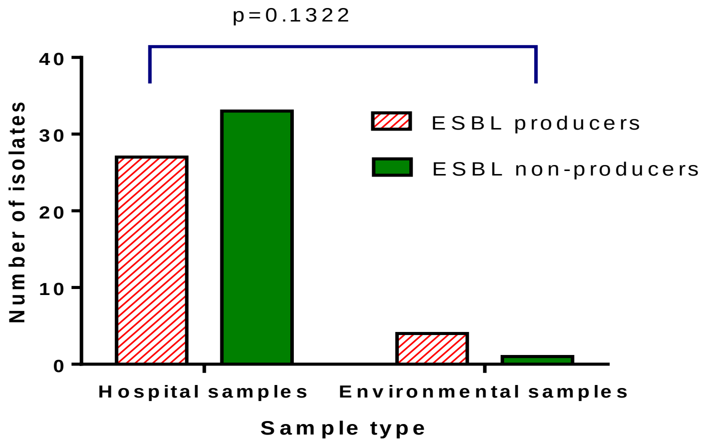

Fig. 4. Distribution of ESBL producers and non-ESBL producers among *Klebsiella pneumoniae* isolates

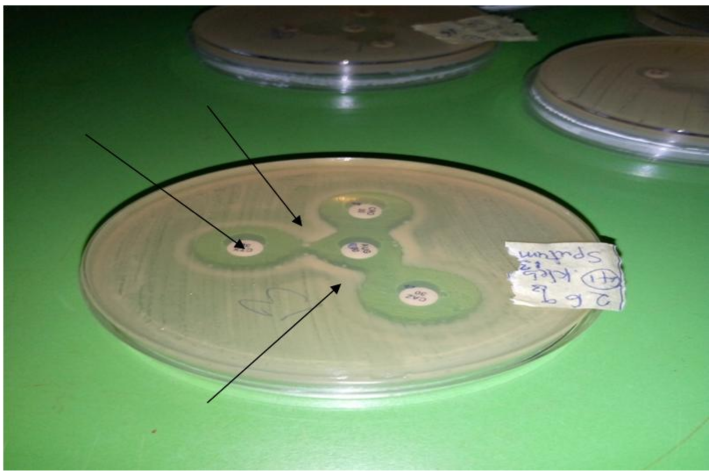

Fig. 5. Double Disc Synergy Test showing the Zone of Inhibition of Ceftazidime(CAZ), Cefotaxime (CTX), and Ceftriaxone (CTR) moving towards the Amoxicillin/ Clavulanic Acid (AMC) disc confirming ESBL Producer

Fig. 4 shows that of the 60 isolates of *Klebsiella pneumoniae* from hospital samples tested for phenotypic detection of ESBL, only 27 (45%) were confirmed to produce ESBL while 33 (55%) were confirmed to be ESBL non-producers. Out of the 5 isolates of *Klebsiella pneumoniae* from environmental samples tested for phenotypic detection of ESBL, 4 (80%) were confirmed to produce ESBL while 1 (20%) was confirmed to be ESBL non-producer. Statistically, there was no significant difference in the proportion of ESBL producers and non-producers in hospital and environmental isolates of *Klebsiella pneumoniae* ($p>0.05$, $X^2=2.266$).

Fig. 5 shows the result of the positive Double Disc Synergy tests of *Klebsiella pneumoniae.* Three third generation cephalosporins were used namely Ceftazidime(CAZ), Cefotaxime (CTX), and Ceftriaxone (CTR) and centrally located Amoxicillin/ Clavulanic Acid (AMC) disc.

4. DISCUSSION

Enterobacteriaceae that produce ESBLs have become a major global issue. The widespread of ESBLs compromises the efficacy of broad-spectrum antibiotics, posing considerable treatment challenges and giving rise to poor clinical outcomes [15,16]. The high prevalence of ESBL isolates of *E. coli* and *Klebsiella pneumoniae* is not only seen in hospital isolates but also from environmental sources. Increased resistance to broad-spectrum cephalosporins in *E.coli* and *Klebsiella species* has been documented in numerous countries, largely due to the production of ESBLs [17,18]. A total of 150 isolate comprising85 *E.coli* and 65 *Klebsiellapneumoniae* were tested for ESBL production. The overall prevalence of ESBL-producers in our study was 75/150(50.0 %). The results of this study showed a comparatively high prevalence level of ESBL producers in our environment; self-medication, easy access to pharmacies, their usage without a doctor's prescription, and gaps in drug policy standards which are common in developing countries may be major contributors [17]. The high prevalence of ESBL of 50% calls for the need to implement a strong infection control plan.

Our 50% prevalence is higher than what was reported in an earlier study (35%) in the same hospital in 2019 [6]. The rise could be attributed to patients' uncontrolled antibiotic use, particularly beta-lactam drugs, as well as poor infection control techniques [6]. Nevertheless, our prevalence of 50% is lower than what was reported in other parts of the country: Sokoto North-West Nigeria. (100%) [19], Bauchi, North-East (82.3%) [8], and Anambra, South-East (61%) [20]. The explanation for the differences in the prevalence of ESBL-producing bacteria between studies could be due to local antibiotic prescribing patterns, widespread use of broad-spectrum antibiotics, especially third-generation cephalosporins, and the endemicity of drug-resistant infections in the area [21]. However, as documented elsewhere, the frequencies of ESBL in developed countries are quite low [22,23]. The difference might be due to infection control strategies in these countries. Moreover, our finding is higher than the prevalence reported in non-European countries such as Saudi Arabia (27%) [24], Nepal (40.3%) [21], India (41.07%) [25], Cambodia (44%) [26] and Turkey (41.4%) [27]; this variation in the prevalence may be due to the study population, methodology, and drug regulation policies.

In this research, the distribution of antibiotic resistance to β-lactams was comparable to that reported by Iroha et al [5], with nearly all the isolates being resistant to the beta-lactam antibiotics. In the clinical isolates of *E. coli*, nitrofurantoin, a bacteriostatic drug, showed a favorable susceptibility profile against both ESBL and non-ESBL isolates. However, because of its toxicity, it is mostly used to treat urinary tract infections and under specified conditions [28]. Most of the isolates showed decreased susceptibility to imipenem, 67.9% for *K. pneumoniae* and 71% for *E. coli*. Our results of antibiotic susceptibility pattern of imipenem (67.9% for *K. pneumoniae* and 71% for *E. coli*) are consistent with the previous studies of Motayo *et al*., who reported 62.5% for *E. coli* and 60% for *K. pneumoniae*[28]. Iliyasu*et al*, also reported higher susceptibility (80.8%) to imipenem [8]. In the treatment of multidrug-resistant *E. coli* and *Klebsiella pneumoniae* infections, imipenem remains the drug of choice. Although our research demonstrates a growing threat of up to 30.5 percent carbapenem resistance, routine antibiotic drug resistance surveillance must be prioritized [28]. The unrestricted use of drugs may increase antibiotic resistance. Antibiotic treatment options are significantly hampered due to resistance to routinely used antibiotics, leaving only a few reserve antimicrobials available.

5. CONCLUSION

The findings of this study showed an alarming rate of 50% ESBL-producing *E. coli* and

Klebsiella pneumoniae in Enugu Metropolis, Nigeria with a high antimicrobial resistance in both ESBL and non- ESBL- producing isolates.

To ensure quality health care and proper antibiotic administration (proper infection treatment and control) in Enugu state, ESBL phenotypic detection should be incorporated into antimicrobial susceptibility testing. To reduce the spread of ESBL-producing bacteria, it is imperative to develop suitable community and hospital antibiotic policies.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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