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PHENOTYPIC DETECTION OF MULTI-DRUG RESISTANT EXTENDED SPECTRUM *Beta-Lactamase*-PRODUCING GRAM-NEGATIVE CLINICAL BACTERIA IN HEALTH CARE FACILITIES IN AKWA IBOM STATE, NIGERIA

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ABSTRACT

The prevalence of multi-drug resistant (MDR) extended spectrum β-lactamase (ESBL) Gram-negative bacteria have become a global concern. This constitutes a challenge in the therapeutic management of patients in both hospital and community settings. In order to achieve an effective treatment option, a more robust laboratory technique that combines both routine antimicrobial susceptibility testing and phenotypic confirmatory test for the identification of prevalent species producing ESBL as well as their antibiotic susceptibility pattern is essential. The aim of this study was to investigate the prevalence of multi-drug resistant ESBL-producing Gram-negative bacteria and assess their antibiotic susceptibility in Akwa Ibom State, Nigeria. A total of 420 clinical samples (urine, n = 310; wound, n = 110) were aseptically collected from consented patients attending University of Uyo Teaching Hospital (UUTH) and General Hospital Iquita (GHI) for laboratory investigation. Isolated Gram-negative bacteria were identified using Microbact 24E (Oxoid, UK), antibiotic susceptibility testing (AST) was done using the modified Kirby-Bauer disc diffusion method while ESBL-producing bacteria were determined by Double Disk Synergy Test (DDST). Gram-negative bacterial growth was detected in 114 (27.1%) cases. The overall prevalence of Gram-negative bacteria was 29% while the most common bacterial strains causing UTI and pyogenic infection were *P. aeruginosa* (17.2%), followed by *Klebsiella* spp.(15.6%), *Enterobacter spp.* (15.6%) and *E. coli* (12.3%). Antibiotics such as Imipenem, Amoxicillin – clavulanic acid, Gentamicin and Ofloxacin showed 100% sensitivity to the most commonly isolated pathogens in patients with these infections. Overall, 119 (50.4%) were ESBL-producers. The highest ESBL production was observed for *K. ozanae*(66.7%), *P. gergoviae* (66.7%), *Enterobacter clocae* (62.5%), *P. agglomerans* (60%), *E. coli* (42.9%), *P. mirabilis* (50%) and *P. aeruginosa* (33.3%). ESBL-producing Gram-negative bacteria showed a high level of multi-drug resistance (68.9%) compared to non-ESBLs. The high prevalence of MDR in Gram-negative bacteria in community and hospital settings is the probable cause of treatment failures experienced among UTI and pyogenic patients in our study area.

INTRODUCTION

The increasing prevalence of antibiotic-resistant Gram-negative bacteria causing clinical infections constitutes a significant public health concern worldwide. For decades, antibiotics have been used successfully to treat infections caused by bacteria. However, in the past few years, consistent abuse of antibiotics has resulted in the emergence of multi-drug resistance around the world (Saipriya *et al.,* 2018). In recent times, it has been reported that approximately 700,000 people globally die as a result of antimicrobial resistance (AMR) yearly, and it has been forecasted that this number may reach 10 million by 2050 (O'Neill, 2016).

At present, antibiotics have been the most successful form of chemotherapy developed in the 20th century, for the treatment of pathogenic bacterial infections (Banin *et al.,* 2017). However, the most widely used class of antibiotics are the β-lactam drugs and account for almost 65% of antibiotic usage worldwide. These drugs have been classified into six main groups based on the chemical structure of the β-lactam ring which includes Penicillins, Cephalosporins, Carbapenems, Monobactams, Cephamycins, and β-lactamase inhibitors. Their mechanism of action is to block cell wall synthesis by preventing accurate working of the Penicillin binding protein (PBP), which has a principal role in the synthesis of the bacterial cell wall, thus resulting to cellular death. Incidentally, there has been an upsurge in resistance to this class of antibiotics (Malloyand Campos, 2011).

There are different mechanisms through which β-lactam resistance can occur. These includes generation of efflux pumps, changes in the production of outer membrane porins, alterations of PBPs, and the production of β-lactamases for inactivating antibiotics (Paterson, 2000). Of these mechanisms, the production of β-lactamases is the most prevalent source of resistance to β-lactam antibiotics which are produced extra cellularly by Gram-positive bacteria and in the periplasmic space by Gram-negative bacteria. These enzymes are capable of inactivating the β-lactam antibiotics by binding covalently to their carbonyl section and hydrolyzing the β-lactam ring thus enabling β-lactam resistance (Eiamphungporn *et al.,* 2018). To date, many β-lactamases have been reported to be produced by diverse microorganisms, such as Penicillinases, Extended-spectrum β-lactamases (ESBLs), Cephalosporinases (AmpC), Metallo-β-lactamases (MBLs), and Carbapenemases (KPCs). Among these, ESBL-producing Gram-negative bacteria are very important and have attracted the attention of researchers owing to their lethal hydrolyzing effect and delayed treatment outcome (Hansen *et al.,* 2016).

ESBLs are β-lactamases enzymes with the capability to hydrolyze β-lactam antibiotics containing Penicillins, Aztreonam, as well as the first to fourth-generation Cephalosporins, with the exception of Cephamycins, Moxalactam, and Carbapenems (Rajivgandhi *et al.,* 2018). Furthermore, they are inhibited by β-lactamase inhibitors, such as Clavulanic acid, Tazobactam, and Sulbactam (Rajivgandhi *et al.,* 2018). The most worrisome part is that, ESBL-producing organisms are also able to induce resistance to some classes of antibiotics other than β-lactams including Aminoglycosides, fluoroquinolones, Chloramphenicol and Trimethoprime sulfamethoxazoles (Taghizadeh *et al.,* 2018).

ESBLs are mostly produced by Gram-negative bacilli, especially those belonging to Enterobacteriaceae family, but not limited to oxidase-positive Gram-negative bacteria (Olusolabomi *et al.,* 2011). ESBL-producing Enterobacteriaceae cause a variety of hospital and community-acquired infections such as bloodstream infections, wound infections, respiratory tract infections, and urinary tract infections (Teklu *et al.,* 2019). Urinary tract infections (UTIs) and pyogenic (wound) infections are very common infectious diseases that occur in a high proportion of the population and are a serious concern in the healthcare system (Moustafa *et al.,* 2018). Presently, the outbreak of infections caused by ESBL-producing Gram-negative pathogens is becoming increasingly frequent (Baziboroun *et al.,* 2018). The ESBLs are plasmid-encoded enzyme. The location of these ESBL genes contributes to their spread via the horizontal gene transfer to similar and different species of bacteria (Stadler *et al.,* 2018). The prevalence of ESBL-producing isolates depends on some factors including species, geographic region, hospital, group of patients and type of infection, and extensive overuse of antibiotics (Shakya *et al.,* 2017; McNulty *et al.,* 2018).

As a result of the emergence of expanding antibiotic resistance among Gram-negative bacteria and the high distribution of ESBL producing isolates in clinical samples, the recognition of the prevalent species that produce this enzyme as well as their antibiotic susceptibility pattern using appropriate diagnostic techniques is necessary for each community and hospital to select the most effective therapeutic options. Although there are many techniques for ESBL detection and confirmation, the Clinical Laboratory Standard Institute (CLSI, 2020) described the disk

susceptibility test methods for initial screening and phenotypic confirmation of ESBL production by Double Disk Synergy Test (DDST). This method depends on detecting synergy between Clavulanic acid (Augmentin) and either of the two Cephalosporins (Ceftazidime, Cefpodoxime and Cefotaxime) which invariably serve as substrates for the ESBL. The DDST phenotypic method is cost effective, easy to perform and requires 2 days to complete (Cormican *et al.,* 1996). Thus, the aim of this study was to determine the prevalence of multi-drug resistant ESBL-producing Gram-negative bacteria and their antibiotic susceptibility pattern isolated from urine and wound samples of patients attending UUTH and GHI in Akwa Ibom State, Nigeria.

MATERIALS AND METHODS

Study Design and Population: This was a cross sectional hospital-based study of patients (in- and out-patients) attending the Surgical, Paediatric, General Outpatient Department (GOPD) and Orthopaedic clinics and wards of the UUTH at Uyo and GHI. Patients of all age groups and gender seen at the clinics and wards of the hospitals were recruited following informed consent.

Study Area: The study was carried out at two health facilities, UUTH and GHI in Akwa Ibom State. Akwa Ibom State is an oil-rich state located in the Southern part of Nigeria, with diverse geography and climate ranging from arid to tropical. The state has a vast oil deposits and play host to the International oil giant, Exxon Mobil. It has diverse cultural background and language. The main ethnic groups are Ibibio, Annang, Oron, Eket and Obolo. Their major occupation is fishing and agriculture. The state has a land area of 6,900 sq Km located between Cross River, Abia and Rivers on the sandy coastal plain of the Gulf of Guinea. It is bordered on the south by the Atlantic Ocean which stretches from Ikot Abasi to Oron. Akwa Ibom State lies between latitude 40º32' and 50º53' North; and longitudes 70º25' and 80º25' East with a population of 5,450,758 (2016 population census).

Ethical Approval and Informed Consent: Approval for the study was obtained from the Ethical Review Board of the University of Uyo Teaching Hospital before commencement of study. Informed consent was obtained from patients before collection of samples.

Data Collection: Demographic and clinical data of subjects were collected from consented patient case notes/folders and laboratory request forms.

Sample Size Determination: The size of the sample of patients studied was determined using the EPI info 7 STATCAL for population survey, based on prevalence rate of 47.1% (Azekhueme *et al.,* 2015), precision of 5% and a confidence level of 95%. The calculated minimum sample size was 382. Hence, the required sample size with 10% attrition rate used for the study was 420.

Sample Collection, Culture and Identification of Bacterial Isolates: The samples collected for analysis were urine and superficial wound swabs. Standard operating procedures were maintained during sample collection and in all subsequent laboratory analysis. Samples were cultured on Eosin Methylene Blue (EMB) agar and incubated for 18-24 hrs at 37^{o}C for isolation of Gram-negative bacteria. Purification of isolated bacteria was done on Nutrient agar following standard procedure.

Identification of isolated organisms were done using Microbact24E (Oxoid, UK) according to manufacture guidelines. Isolates were stored in microvials containing brain heart infusion (BHI) broth containing 25% glycerol at 4^{o}C until ready for further analysis and -70^{o}C for longer storage.

Antimicrobial Susceptibility Testing (AST): Antimicrobial susceptibility test was performed according to CLSI guidelines (CLSI, 2020) using the modified Kirby-Bauer disc diffusion method to evaluate the sensitivity of the isolated Gram-negative bacilli to Ciprofloxacin (5µg), Ampicillin (10µg), Cefpodoxime (10µg), Gentamicin (10µg), Aztreonam (30µg),

Tazobactam/piperacillin (30µg), Ceftazidime (30µg),Ceftriaxone (30µg), Tetracycline (10µg), Imipenem (10µg) Co-trimoxazole (25µg), Cefepime(30µg) and Ofloxacin (10µg) (Oxoid, UK).

Phenotypic Detection of ESBL-producing Strains Using the Double Disk Synergy Test (DDST): The isolates were tested by disc diffusion method for their susceptibility to the third generation Cephalosporins: Cefpodoxime (10µg), Ceftazidime (30µg), Aztreonam (30µg), and Ceftriaxone (30µg)). Isolates with zone diameters indicating suspicion for ESBL production, that is (Cefpodoxime: ≤17mm, Ceftazidime: ≤22mm, Aztreonam: ≤27mm, Ceftriaxone: ≤25mm) were subjected to the Double Disk Synergy Test as earlier described in previous study by Azekhueme *et al.,(*2015). Briefly, a suspension of each of the test organism from an overnight culture adjusted to match the 0.5 MacFarland standard was inoculated on the surface of each of the molten Mueller Hinton agar plates using a sterile swab. A combination disc of Amoxycillin (20µg) plus Clavulanic acid (10µg) was placed at the center of each inoculated Mueller Hinton agar plate. Ceftriaxone (30µg) and Ceftazidime (30µg) single discs were then placed 15mm (center to center) from the Amoxycillin/Clavulanic disc and incubated at 37^{o}C overnight. Interpretation of test as Resistant (R) and Susceptible (S) was done according to CLSI (2020) guidelines and interpretive criteria.

RESULTS AND DISCUSSION

Table 1 shows the culture result of clinical samples obtained from subjects attending UUTH and GHI. Of the 420 samples collected, 81 urine samples and 33 wound samples were culture positive on Eosin Methylene Blue (EMB) agar giving the percentage culture positivity of 26.1% and 30%, respectively. This gives an overall percentage of 27.1% from a total of 114 positive samples.

Table 1: Culture result of clinical samples obtained from 420 subjects on EMB Agar

Sample type	No. of samples	Positive (%)	Negative (%)
Urine	310	81(26.1)	229(73.9)
wound	110	33(30.0)	77(70.0)
Total	**420**	**114(27.1)**	**306(72.9)**

EMB = Eosin Methylene Blue

Table 2: Frequency distribution of Gram-negative bacterial isolates in the study area by type of clinical sample

S/N	**Isolate**	**Urine sample (%)**	**Wound sample (%)**	**Total (%)**
1.	*Escherichia spp.*	10(11.8)	5(13.5)	15(12.3)
2.	*Pentoea agglomerans*	4(4.7)	1(2.7)	5(4.1)
3.	*Enterobacter spp.*	13(15.3)	6(16.2)	19(15.6)
4.	*Klebsiella spp.*	14(16.5)	5(13.5)	19(15.6)
5.	*Proteus spp.*	6(7.1)	6(16.2)	12(9.8)
6.	*Acinetobacter spp.*	3(3.5)	1(2.7)	4(3.3)
7.	*Citrobacter spp.*	6(7.1)	1(2.7)	7(5.7)
8.	*Pseudomonas aeruginosa*	9(10.6)	12(32.4)	21(17.2)
9.	*Kluyvera ascorbata*	2(2.4)	-	2(1.6)
10.	*Raoultella ornithinolytica*	2(2.4)	-	2(1.6)
11.	*Pluralibacter gergoviae*	3(3.5)	-	3(2.5)
12.	*Salmonella spp.*	5(5.9)	-	5(4.1)
13.	*Hafnia alvei*	2(2.4)	-	2(1.6)
14.	*Stenotrophomonas maltophilia*	1(1.2)	-	1(0.8)
15.	*Serratia spp.*	4(4.7)	-	4(3.3)
16.	*Lellottia amnigena*	1(1.2)	-	1(0.8)
	Total	**85(69.7)**	**37(30.3)**	**122(100)**

Table 3: Antibiotic Susceptibility Profiles of ESBL-producing Isolates

Organism	**Antibiotics discs (µg/disc)**												
	CN (%)	**TET (%)**	**ATM (%)**	**CIP (%)**	**SXT (%)**	**CTX (%)**	**CRO (%)**	**OFX (%)**	**FEP (%)**	**AMC (%)**	**CAZ (%)**	**CPD (%)**	**IPM (%)**
E. coli (n=14)	9(64.3)	1(7.1)	1(7.1)	0(0)	3(21.4)	0(0)	4(28.6)	14(100)	4(28.6)	12(85.7)	5(35.7)	4(28.6)	5(35.7)
E. fergusonii (n=1)	1(100)	0(0)	0(0)	1(100)	1(100)	0(0)	1(100)	0(0)	1(100)	1(100)	1(100)	0(0)	1(100)
P. agglomerans (n=5)	2(40)	1(20)	3(60)	0(0)	1(20)	0(0)	2(40)	5(100)	1(20)	3(60)	1(20)	2(40)	0(0)
P. aeruginosa (n=21)	19(90.5)	13(61.9)	0(0)	9(42.9)	0(0)	1(4.8)	1(4.8)	17(81.0)	15(71.4)	4(19.0)	6(28.6)	2(9.5)	21(100)
E. cloacae (n=16)	9(56.3)	3(18.8)	2(12.5)	0(0)	0(0)	1(6.3)	5(31.3)	6(37.5)	5(31.3)	7(43.8)	4(25.0)	5(31.3)	1(6.3)
E. hormaechei (3)	0(0)	2(66.7)	0(0)	0(0)	3(100)	0(0)	0(0)	3(100)	0(0)	2(66.7)	1(33.3)	0(0)	2(66.7)
K. ascorbata (2)	1(50.0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	2(100)	0(0)	0(0)	0(0)	0(0)
P. gergoviae (n=3)	2(66.7)	2(66.7)	0(0)	0(0)	0(0)	0(0)	1(33.3)	2(66.7)	1(33.3)	1(33.3)	1(33.3)	1(33.3)	1(33.3)
C. koseri (n=4)	3(75.0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	4(100)	1(25.0)	0(0)	1(25.0)	1(25.0)	1(25.0)
S. diarizonae (n=1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(100)	0(0)	1(100)	0(0)	0(0)	0(0)
S. houtenae (n=4)	1(25.0)	2(50.0)	0(0)	0(0)	1(25.0)	0(0)	0(0)	4(100)	1(25.0)	0(0)	1(25.0)	1(25.0)	0(0)
P. mirabilis (n=10)	9(90.0)	6(60.0)	2(20.0)	0(0)	4(40.0)	0(0)	1(10.0)	10(100)	0(0)	4(40.0)	2(20.0)	3(30.0)	0(0)
P. penneri (n=2)	1(50.0)	1(50.0)	0(0)	0(0)	1(50.0)	0(0)	0(0)	2(100)	0(0)	1(50.0)	1(50.0)	1(50.0)	0(0)
Hafnia alvei (n=2)	2(100)	0(0)	0(0)	0(0)	1(50)	0(0)	0(0)	2(100)	0(0)	0(0)	0(0)	1(50)	0(0)
S. liquefaciens (n=2)	1(50)	1(50)	0(0)	1(50)	1(50)	0(0)	1(50)	1(50)	0(0)	1(50)	0(0)	0(0)	1(50)
S. marcescens (n=2)	2(100)	2(100)	0(0)	0(0)	0(0)	0(0)	0(0)	2(100)	0(0)	0(0)	1(50)	0(0)	1(50)
A. baumannii (n=3)	3(100)	2(66.7)	0(0)	0(0)	1(33.3)	0(0)	2(66.7)	2(66.7)	0(0)	2(66.7)	1(33.3)	2(66.7)	3(100)
A. lwoffi (n=1)	1(100)	0(0)	0(0)	0(0)	0(0)	0(0)	1(100)	0(0)	1(100)	0(0)	0(0)	0(0)	1(100)
K. aerogenes (n=1)	1(100)	1(100)	0(0)	0(0)	1(100)	0(0)	0(0)	1(100)	0(0)	1(100)	0(0)	1(100)	0(0)
K. pneumoniae(n=8)	5(62.3)	6(75.0)	1(12.5)	0(0)	3(37.5)	0(0)	2(25.0)	4(50.0)	0(0)	8(100)	5(62.3)	4(50.0)	0(0)
K. oxytoca (n=7)	5(71.4)	2(28.6)	3(42.9)	0(0)	1(14.3)	0(0)	0(0)	6(85.7)	0(0)	1(14.3)	1(14.3)	1(14.3)	1(14.3)
K. ozanae (n=3)	0(0)	0(0)	0(0)	0(0)	2(66.7)	0(0)	0(0)	2(66.7)	0(0)	2(66.7)	0(0)	0(0)	0(0)

KEY: CN = Gentamicin; TET = Tetracycline; ATM = Aztreonam; CIP = Ciprofloxacin; SXT = Sulfonamide/Trimethoprim; CTX = Cefotaxime; CRO = Ceftriaxone; OFX = Ofloxacin; FEP = Cefepime; AMC = Amoxicillin/Clavulanic Acid; CAZ = Ceftazidime; CPD = Cefpodoxime; IPM = Imipenem

Table 3 shows the susceptibility profiles of all the Gram-negative bacterial isolates that produced extended spectrum beta-lactamase (ESBL). The results show that, for the most commonly encountered clinical isolates, OFX, AMC, CN and OFX were 100% effective against *E. coli*, *K. pneumoniae,A. baumannii* and *P. mirabilis*, respectively. Also, IMP was 100% effective against *P. aeruginosa* and *A. baumannii* isolates.

Table 4: Distribution of ESBL producers among the isolates by DDST in the study area

S/N	Isolates	No. screened	ESBL producers (%)
1.	*Escherichia coli*	14	6(42.9)
2.	*Escherichia fergusonii*	1	1(100)
3.	*Pentoea agglomerans*	5	3(60.0)
4.	*Enterobacter cloacae*	16	10(62.5)
5.	*Enterobacter hormaechei*	2	2(100)
6.	*Kluyvera ascorbata*	2	1(50.0)
7.	*Pluralibacter gergoviae*	3	2(66.7)
8.	*Citrobacter koseri*	4	4(100)
9.	*Citrobacter freundii*	3	0(0.0)
10.	*Stenotrophomonas maltophilia*	1	0(0.0)
11.	*Salmonella diarizonae*	1	1(100)
12.	*Salmonella houtenae*	4	4(100)
13.	*Proteus mirabilis*	10	5(50)
14.	*Proteus penneri*	2	2(100)
15.	*Hafnia alvei*	2	2(100)
16.	*Serratia liquefaciens*	2	1(50.0)
17.	*Serratia marcescens*	2	1(50)
18.	*Acinetobacter baumannii*	3	1(33.3)
19.	*Acinetobacter lwoffi*	1	1(100)
20.	*Lelliota amnigena*	1	0(0.0)
21.	*Klebsiella aerogenes*	1	1(100)
22.	*Klebsiella pneumonia*	8	2(25.0)
23.	*Klebsiella oxytoca*	7	1(14.3)
24.	*Klebsiella ozanae*	3	2(66.7)
25.	*Pseudomonas aeruginosa*	21	7(33.3)
	Total	**119**	**60(50.4)**

***Key: ESBL** = Extended Spectrum Beta-Lactamase; **DDST** = Double Disk Synergy Test*

Table 4 shows the distribution of ESBL-producing Gram-negative isolates in the study area. A total of 60 isolates were ESBL-producers with Enterobacter clocae (62.5%), E. coli (42.9%), P. aeruginosa (33.3%), P. agglomerans (60%), P. gergoviae (66.7%), P. mirabilis (50%) and K. ozanae (66.7%) being the most prominent ESBL producers. The overall prevalence of ESBL-producing Gram-negative isolates in the study area was 50.4%.

Table 5: Multi-drug Antibiotics Resistant Index (MARI) of ESBL-producing Isolates in the Study Area

S/N	Isolates	Antibiotics Resisted	MDR (%)	MARI
1.	*E. fergusonii*	CN, CPD	2(15)	0.15
2.	*K. aerogenes*	CIP, CTX, FEP, IMP	4(30.8)	0.31
3.	*S. diarizonae*	TET, CIP, SXT, CTX, CAZ, IMP	6(46.2)	0.46
4.	*A. lwoffi*	ATM, CIP, SXT, CTX, OFX, CPD	6(46.2)	0.46
5.	*P. agglomerans*	CN, TET, CIP, SXT, CTX, CRO, FEP, CPD	8(61.5)	0.62
6.	*K. ascorbata*	TET, ATM, CIP, SXT, CTX, CRO, CAZ, IMP	8(61.5)	0.62
7.	*P. penneri*	TET, CIP, SXT, CTX, CRO, AMC, CAZ, IMP	8(61.5)	0.62
8.	*K. pneumoniae*	CN, TET, ATM, CIP, SXT, CTX, CRO, FEP, IMP	9(69.2)	0.69
9.	*S. houtenae*	CN, ATM, CIP, SXT, CTX, CRO, FEP, AMC, IMP	9(69.2)	0.69
10.	*E. hormaechei*	CN, ATM, CIP, CTX, CRO, FEP, AMC, CAZ, CPD	9(69.2)	0.69
11.	*A. baumannii*	ATM, CIP, SXT, CTX, CRO, OFX, FEP, CAZ, CPD	9(69.2)	0.69
12.	*S. marcescens*	ATM, CIP, SXT, CTX, CRO, FEP, AMC, CAZ, CPD	9(69.2)	0.69
13.	*Hafnia alvei*	ATM, CIP, SXT, CTX, CRO, FEP, AMC, CAZ, IMP	9(69.2)	0.69
14.	*K. oxytoca*	CN, TET, CIP, SXT, CTX, CRO, FEP, AMC, CAZ, CPD	10(76.9)	0.80
15.	*P. mirabilis*	TET, ATM, CIP, SXT, CTX, CRO, FEP, CAZ, CPD, IMP	10(76.9)	0.80
16.	*E. coli*	CN, TET, ATM, CIP, SXT, CTX, CRO, FEP, CAZ, CPD, IMP	11(84.6)	0.85
17.	*P. aeruginosa*	CN, TET, ATM, SXT, CTX, CRO, OFX, FEP, AMC, CAZ, CPD	11(84.6)	0.85
18.	*S. liquefaciens*	CN, TET, ATM, CIP, SXT, CTX, CRO, FEP, AMC, CAZ, CPD, IMP	12(92.3)	0.92
19.	*K. ozanae*	CN, TET, ATM, CIP, SXT, CTX, CRO, FEP, AMC, CAZ, CPD, IMP	12(92.3)	0.92
20.	*C. koseri*	TET, ATM, CIP, SXT, CTX, CRO, OFX, FEP, AMC, CAZ, CPD, IMP	12(92.3)	0.92
21.	*P. gergoviae*	CN, TET, ATM, CIP, SXT, CTX, CRO, OFX, FEP, AMC, CAZ, CPD, IMP	13(100)	1
22.	*E. cloacae*	CN, TET, ATM, CIP, SXT, CTX, CRO, OFX, FEP, AMC, CAZ, CPD, IMP	13(100)	1

KEY: CN = Gentamcin; TET = Tetracycline; ATM = Aztreonam; CIP = Ciprofloxacin; SXT = Sulfonamide/Trimethoprim; CTX = Cefotaxime; CRO = Ceftriaxone; OFX = Ofloxacin; FEP = Cefepime; AMC = Amoxicillin/Clavulanic Acid; CAZ = Ceftazidime; CPD = Cefpodoxime; IPM = Imipenem

Table 5 indicates the multi-drug antibiotics resistant index (MARI) of ESBL-producing Gram-negative bacterial isolates in the study area. The result shows that all the 22 isolated bacterial species were MDR as they resisted at least 2 different classes of antibiotics. Two bacterial

species, *P. gergoviae* and *E. cloacae* showed 100% resistance to all the 13 antibiotics used and have MARI of 1. This was closely followed by *S. liquefaciens, K. ozanae* and *C. koseri*that resisted 12 out of 13 antibiotics used having MDR of 92.3% and MARI of 0.92.

Table 6: Prevalence of MDR Gram-negative Bacteria in Relation to ESBL Production

	No. of isolates	**MDR (%)**
ESBL	60	60(100)
Non-ESBL	59	22(37.3)
Total	**119**	**82(68.9)**

ESBL = Extended Spectrum Beta-Lactamase; MDR = Multi-drug Resistance

The prevalence of MDR Gram-negative bacteria in relation to ESBL production is shown in table 6. The result shows that, 100% of ESBL-producing isolates were multi-drug resistant while, 37.2% of Non-ESBL producers were multi-drug resistant. The overall prevalence of multi-drug resistant Gram-negative bacteria in the study area was 68.9%.

Presently, increasing production of extended spectrum beta-lactamase enzymes (ESBL) by Gram-negative bacteria have become a global public health concern because of inducing drug resistance to multiple classes of antibiotics (MDR) and making the treatment cumbersome. In order to unravel an appropriate treatment option, a more robust laboratory technique that combines both routine antimicrobial susceptibility testing and phenotypic confirmatory test for the identification of prevalent species which produce ESBL as well as their antibiotic susceptibility pattern is imperative. In this study, bacterial growth was detected in 114 (27.1%) of cases while a total of 122 Gram-negative bacterial isolates were recovered from clinical samples (urine and wound) from different groups of patients attending University of Uyo Teaching Hospital (UUTH) and General hospital Iquita, Oron (GHI). The prevalence of Gram-negative bacteria in the study area was 29%. More isolates were recovered from urine (69.7%) than wound samples (30.3%). The preponderance of uropathogens is in line with results from previous studies (Sageerabanoo *et al.,* 2015; Onwuezobe *et al.,* 2019; Ogefere *et al.,* 2019). A number of studies have reported urinary tract infection (UTI) to be the second most common infectious disease around the world caused by a wide range of microbial pathogens with Gram-negative bacteria constituting the most implicated uropathogens (Behzadi *et al.,* 2019). Therefore, the comparative high prevalence of uropathogens in this study may not be unconnected with the above stated reports.

The most frequently isolated Gram-negative bacterium in this study was *Pseudomonas aeruginosa* (17.2%). This was closely followed by *Klebsiella* spp. (15.6%), *Enterobacter* spp. (15.6%) and *E. coli* (12.3%). This finding differs from the results of earlier studies in Nigeria where *Escherichia coli* was ranked the highest in Uyo (Onwuezobe and Etang, 2018) and Benin (Ogefere *et al.,* 2019) while *Klebsiella* spp. was reported as the most frequently isolated organism in Kano (Yusuf *et al.,* 2013). These observed differences in the preponderance of isolated species may be due to the type and source of clinical sample used.

The antibiotic susceptibility testing of commonly prescribed antibiotics was accomplished for the most frequent pathogens found in this study. The significance of this was to reduce the incidence of acquired antibiotics' resistance in both in-and-out patients. In determining the antibiotic sensitivity patterns of isolated pathogens, *P. aeruginosa* and *A. baumannii*, the most commonly isolated pathogens in patients with pyogenic infections, showed 100% sensitivity to Imipenem, Amoxicillin/Clavulanic Acid (Augmentin) and Gentamicin, while *E. coli* and *P. mirabilis,* which are notable uropathogens were completely sensitive to Ofloxacin (100%). A high level of Ofloxacin (81.0%) against *P. aeruginosa*, Tetracycline (75.0%) against *K. pneumoniae* and Gentamicin (71.4%) against *K. oxytoca* was also obtained. The possible reason for the high sensitivity of these antibiotics as obtained in this study may be due to their limited use and abuse by patients. Most studies on the antibiotic susceptibility of urinary and pyogenic pathogens around the world have found similar results (ccc). For instance, in a similar study by Onwuezobe and Etang (2018) in Uyo, Nigeria, found a high sensitivity rate (100%) of Ofloxacin to *P. aeruginosa,* and Imipenem (73.7%) to *E. coli*, Illiyasu *et al.,* (2018) in Bauchi,

Nigeria reported 80.8% sensitivity of *E. coli* isolates to Imipenem while Gharavi *et al.,* (2021) in Iran reported a high sensitivity of Imipenem to *P. aeruginosa* isolates. In contrast, studies done by Sageerabanoo *et al*., (2015) reported high resistance of *Klebsiella* spp. and *E. coli* to extended-spectrum Cephalosporins such as Cefpodoxime, Cefotaxime and Ceftazidime including Aztreonam.

In this study, 60 isolates comprising of 25 different Gram-negative bacterial species were ESBL producers. The prevalence of ESBL-producing Gram-negative bacteria was 50.4%. This result is higher than earlier report from our center (20.0%) by Onwuezobe and Orok (2015), Kano (15.0%) by Yusuf *et al.,* (2013) and Benin by Ibadin *et al.,* (2018) where prevalence of 47.1% was obtained. However, the finding is comparatively lower than that obtained from a study using similar phenotypic method from Uganda where a prevalence of 89.0% was obtained. The most prominent ESBL-producers were *K. (ozanae) (*66.7%), *P. gergoviae* (66.7%), *Enterobacter clocae* (62.5%), *P. agglomerans* (60%), *E. coli* (42.9%), *P. mirabilis* (50%) and*P. aeruginosa* (33.3%). The prevalence of ESBL has been shown to vary based on geographical location and over time (Malloy and Campos, 2011; Shaikh *et al.,* 2015). This study therefore shows the existence of ESBL phenotypes among Gram-negative bacterial isolates causing clinical infections at the secondary and tertiary healthcare facilities. It is pertinent however to note that most isolates recovered are not routinely reported in routine screening for this enzyme despite the simplicity of the double disk synergy test (DDST). The implication of such results may pose a serious clinical concern in the management of patients harboring ESBL-producing pathogens.

Multidrug resistance of ESBL-producers emphasizes an overwhelming health and economic burden especially in undeveloped and developing countries as resistance narrows the therapeutic options leading to increased morbidity and mortality rates in community and hospital settings (Jit *et al.,* 2020). This result shows that all ESBL-producing Gram-negative bacterial isolates were MDR as they resisted at least 3 different antibiotics. This study reported 68.9% prevalence of multi-drug resistant Gram-negative bacteria. This result is comparatively higher than that obtained in other studies (Siwakoti *et al.,* 2018; Gharavi *et al.,* 2021; Abdelaziz *et al.,* 2021). The reason for this high prevalence of MDR in the Gram-negative bacterial isolates is not far-fetched as it is possibly not unconnected with the acquisition of ESBL by these pathogens.

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