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Current Antibiotic Resistance Trends of Uropathogens from Outpatients in a Nigerian Urban Health Care Facility

Ifeanyi A. Onwuezobe*
Department of Medical Microbiology and Parasitology, University of Uyo, Akwa Ibom State, Nigeria

Ubong E. Etang
Department of Medical Microbiology and Parasitology, University of Uyo, Akwa Ibom State, Nigeria

Abstract

Background: The widespread use of antibiotics has resulted in emergence of community-acquired antibiotic resistance among uropathogens in outpatient's population. This constitutes an impediment in the management of urinary tract infection (UTI) in both community and hospital settings. **Objective:** The aim of this study was to determine the current antibiotic resistance trends, extended spectrum beta-lactamase (ESBL) production and plasmid profile of uropathogens from outpatients. **Methods:** A total of 370 mid-stream urine samples were collected and cultured by standard methods. Isolated uropathogens were identified using appropriate biochemical methods. The modified Kirby Bauer disk method was used for antibiotic susceptibility test. The ESBL-producing uropathogens were identified and their plasmid DNA extraction and curing were carried out by standard methods. **Results:** About 35.7% and 32.7% of uropathogens were multi-drug resistant and ESBL-producing respectively. There was higher prevalence of ESBL-production among isolates from female patients (62.5%) when compared to that from male patients (37.5%). The isolated uropathogens were most resistant to Cefotaxime, and most sensitive to Imipenem. Resistance to antibiotics by ESBL-producing uropathogens was found to be plasmid-mediated. **Conclusion:** Community acquired Uropathogens from outpatients were multidrug resistant due to ESBL production localized on plasmids, a probable cause of treatment failures experienced in Uyo.

Keywords: Outpatients; Uropathogens; Plasmid; Extended spectrum beta-lactamase; Urinary tract infection.

1. Introduction

The widespread use of beta-lactam antibiotics have led to the emergence of antibiotic resistant strains worldwide [1]. This has resulted in community-acquired plasmid-mediated resistance among uropathogens with grave clinical consequences. These include increase length of hospitalization, heath care cost and high treatment failure rates in patients with urinary tract infection (UTI) [2]. Usually, infection of the urinary tract results from the invasion and subsequent colonization of one or more parts of the urinary system such as the kidneys, the ureters, the urinary bladder and the urethra by pathogenic organisms either through the ascending or hematogenous routes [3].

The predominant pathogens implicated in the causation of UTI are mainly Gram negative aerobic bacilli such as *Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella spp, Enterobacter aerogenes, Citrobacter spp* and *Seratia spp*. Other uropathogens that are rarely implicated include *Staphylococcus aureus, Enterococcus spp, Staphyococcus saprophyticus* and *Salmonella spp* which may results in UTI following the colonization of the vagina and perianal skin [4, 5]. Many of these uropathogens have been reported to harbor plasmid-encoded ESBLs of different molecular sizes thus conferring resistance to those antibiotics that are commonly used to treat infections caused by them [6, 7].

Resistant uropathogens are increasingly difficult to treat, requiring alternative drugs or higher doses, both of which may be much toxic or exhorbitant [8]. Antibiotics are the main treatment for all UTIs. These are mostly beta-lactam drugs such as Penicillins, Cephalosporins, Carbapenems, Oxapenems, Monobactam and Cephamycins. In other cases, Aminogysides, Fluoroquinolones and urinary antiseptics (Nitrofurantoin) are also the drug of choice [9]. Despite the use of these medications, the rate of re-occurrence of infection still remains high due to increased level of antibiotic resistance worldwide [10]. The reason for this has been attributed to microbial mutation, selective pressure of antimicrobial use, societal and technological vicissitudes that enhance the development and transmission of drug resistant organisms [11].

Resistance to beta-lactam antibiotics is mostly enhanced by acquisition of beta-lactamase genes that are carried on mobile circular genetic elements such as plasmids and transposons [12]. However, research over the years has shown that plasmid-mediated resistance is by far the most common mechanism of transfer of resistant genes between pathogenic phenotypes [13]. The beta-lactamases are enzymes produced by many species of Gram positive and Gram negative bacteria that can compromise the efficacy of many beta-lactam antibiotics [14]. Some have extended spectrum of activity and are produced mostly by Gram negative bacilli such as *E. coli, K. pneumoniae, P, aeruginosa* and *P. mirabilis*, called extended spectrum beta-lactamases. These confer upon them additional ability to hydrolyze virtually all beta-lactam antibiotics including 3rd generation Cephalosporins with oxy-imino side chain and Monobactams [15]. Plasmids responsible for ESBL production often carry genes that code for resistance to other

*Corresponding Author

classes of drugs, e.g. Aminoglycosides. Thus, antibiotic options in the treatment of UTI caused by ESBL-producing organisms are extremely limited [16]. The genes that code for ESBL production are derived from the classical TEM-1, TEM-2 and SHV-1 genes by mutations that alter the amino acid configurations around these beta-lactamases' active site. Currently, there is an increasing number of ESBLs not of TEM or SHV lineage such as the CTX-M and OXA-beta-lactamases [17]. The most widespread CTX-M enzymes are the CTX-M-14, CTX-M-13, and CTX-M-2 mostly active on Ceftazidime. The CTX-M-15 is mostly found in E. coli and is widely prevalent in the community [17]. The OXA-beta-lactamases can hydrolyze Oxacillin and Cloxacillin and related anti-staphylococcal penicillins. They are mostly found in *P. aeruginosa* [18].

The rapid and accurate detection of ESBLs production in uropathogens is imperative to guide proper antibiotic selection for empiric treatment and appropriate control measures of infection. Although there are many techniques for ESBL detection and confirmation, the Clinical Laboratory Standard Institute [19] has described the disk susceptibility test methods for initial screening and phenotypic confirmation of ESBL production by DDST. This method depend on detecting synergy between Clavulanic acid and Cephalosporin(s) which serve as substrates for the ESBL genes [20].

In Nigeria, there have been reports on antibiotic resistance among ESBL-producing uropathogens. However, few studies have been done in Uyo on ESBL production by uropathogens from clinical samples of in-patients [21]. Therefore, there is the need to have data on the co-acquisition of multi-resistant plasmids and ESBL resistance in uropathogens isolated from urine samples of outpatients in Uyo. This study therefore is aimed at determining the antibiotic resistance patterns, extended spectrum beta-lactamase (ESBL) production and plasmid profile of uropathogens from outpatients in an urban health care facility in Uyo, Nigeria.

2. Materials and Methods

This is a descriptive cross-sectional study carried out at the University of Uyo Teaching Hospital in Akwa Ibom State, Southern Nigeria for a period of 8 months.

A total of 370 consented patients attending the General Outpatient Department (GOPD) of the hospital were recruited in this study.

Ethical Consideration**:** Ethical approval was obtained from the Ethical Review Board of the hospital before the commencement of this study.

Sample Collection**:** Midstream urine samples were collected aseptically from outpatients in a sterile universal container. The samples were labelled and transported to the Microbiology Laboratory for analysis within 2 hours of collection.

Culture and Isolation of Uropathogens: A sterile calibrated wire loop was used to inoculate BA and CLED agar plates. The plates were incubated at 37^{o}C for 24 hours and then examined for growth.

Biochemical Identification of Isolated Bacterial Uropathogens**:** Pure bacterial cultures were Gram stained to differentiate Gram positive uropathogens from Gram negative ones. Isolated Gram negative uropathogens were identified biochemically using Microbact 24E (Oxoid, United Kingdom) while Gram positive uropathogens were identified biochemically using Coagulase, Catalase, Salt tolerant and Bile Esculin tests. Isolates were quality controlled using *E. coli* (ATCC 29522) strain and *S. aureus* (ATCC 29523) strain for Gram negative and Gram positive organisms respectively.

Antibiotic Susceptibility Test**:** Isolates prepared in suspension equivalent to 0.5 McFarland turbidity standard were used, and inoculated on Mueller Hinton Agar (MHA) and standard antibiotic disks including Ceftazidime (30µg), Cefotaxime (30µg), Aztreonam (30µg), Ofloxacin (5µg), Imipenem (10µg) and Gentamicin (10µg) (Oxoid, UK), were placed appropriately in accordance with the Modified Kirby Bauer Disk Diffusion method. Zones of inhibition were interpreted in accordance with the Clinical Laboratory Standard Institute [19].

Detection of Extended Spectrum Beta-Lactamase**:** Isolates that showed inhibition zone size of ≤22mm with Ceftazidime (30µg), ≤27mm with Cefotaxime (30µg) and ≤27mm with Aztreonam (30µg) were further tested for ESBLs production.

Double Disc Synergy Test (DDST)**:** The phenotypic confirmation of ESBL production was done using DDST. Mueller Hinton Agar plate was inoculated with the prepared suspension of presumptive bacteria isolate with turbidity equivalent to 0.5 McFarland standard. Amoxicillin/clavulanate (20µg/10µg) disk was placed at the center of MHA plate. Ceftazidime (30µg) and Cefotaxime (30µg) disks were placed 20mm away from the edge of the Amoxicillin/clavulanate disk. The plate was incubated at 37^{o}C aerobically for 24 hours. The observation of Cephalosporin/clavulanate synergy was interpreted as positive for ESBL production.

Plasmid DNA Extraction and Gel Electrophoresis**:** Plasmid DNA from ESBL-producing uropathogens was extracted using Plasmid Miniprep Kit (D4036, D4037, D4019 and D4020) method. Isolates were subcultured on Luria Bethany (LB) broth and incubated at 37^{o}C for 24hrs. About 600µl of the isolate was transferred into a 1.5ml microcentrifuge tube and lysed with 100µl of 7x lysis buffer, neutralized with 350µl cold neutralization buffer and vortexed for 5 seconds. 200µl of Endo-wash buffer was added to the supernatant in the Zymo-Spin IIN column and about 400µl of Zyppy-wash buffer was also added to the column and centrifuged at 11,000rpm for 30 seconds respectively. Subsequently, 30µl of Zyppy elution buffer was added and centrifuged at 11,000rpm for 15 seconds to elute the plasmid DNA. The eluted DNA was further subjected to nanodropping. The extracted plasmid DNA were separated by gel electrophoresis in 1% (w/v) agarose gel submerged in TBE 0.5x (Tris/Borate/EDTA) buffer and the bands were visualized under the view of a UV trans-illuminator stained with ethidium bromide and then photographed with the aid of a polaroid camera.

Plasmid Curing**:** Plasmid curing of ESBL-producing uropathogens was done according to the published method by Virtual Amrita Laboratories Universalizing Education [22]. About 2-3 colonies of the isolates was inoculated into a 5ml LB broth containing 50µl of ethidium bromide curing agent in a sterile bijou bottle and incubated at 37°C for 24hrs. After incubation, this was subcultured onto MHA plate by pour plate method. The plate was allowed for 3-5minutes to dry with the lid in place. Appropriate antibiotic disks were placed and the plate incubated for at 37°C for 24hrs. After incubation, susceptibility test result was read and interpreted following standard interpretive criteria.

Data Analysis**:** Data obtained were analysed using SPSS version 20.0. Pearson Chi-square test was used to established significant relationship between variables at 95% confidence level. P-value < 0.05 was accepted as statistically significant.

3. Results

The antibiotic susceptibility pattern of uropathogens, showed Imipenen (71.2%) as the most sensitive antibiotic and Cefotaxime (83.7%) as the most resistant (table 1). *Klebsiella ascorbata*, *Klebsiella ornithinolytica* and *Enterobacter aerogenes* were most eliminated by the included antibiotics (about100% sensitivity). Of the 14 different strains of bacterial pathogens isolated, 5(35.7%) were multi-drug resistant while, about 16 (32.7%) of the 49 isolated Gram-negative bacteria, produced ESBL. The ESBL production was more prevalent with *Klebsiella spp* (46.2%) followed by *E. coli* (42.1%) (table 2). The frequency of these Uropathogens producing ESBL to the gender of the patients, revealed that female outpatients 10(62.5%) had more when compared to male outpatients 6(37.5%), (Figure 1). The result of the agarose electrophoresis of the plasmids extracted from the ESBL-producing Gram-negative isolates showed that all the 16 ESBL-producing uropathogens, possessed single sized plasmid of same molecular weight of 1100bp (figure 2).

Table-1. Antibiotic Susceptibility Pattern of Isolated Uropathogens to Selected Antibiotics

Antibiotics Discs Used														
Isolated uropathogens	**CAZ (%)**		**CTX (%)**		**IPM (%)**		**ATM (%)**		**CN (%)**		**OFX (%)**		**MEAN (%)**	
	S	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**
Gram Negative														
E. coli	9	7		10	14	3	9	10	9	10	8	10	9	8
n=19(30.6%)	47.4	36.8	21.1	52.6	73.7	15.8	47.4	52.6	47.4	52.6	42.1	52.6	47.4	42.1
K. pneumoniae	3	4	0	3	5	1	4	3	4	3	4	3	3	3
n=7(11.3%)	42.9	57.1	0.0	42.9	71.4	14.4	57.1	42.9	57.1	42.9	57.1	42.9	42.9	42.9
K. oxytoca	1	2	0	3	3	0	1	2	1	2	1	2	1	2
n=3(4.8%)	33.3	66.7	0.0	100	100	0.0	33.3	66.7	33.3	66.7	33.3	66.7	33.3	66.7
K.ornithinolytica	0.0	2	0	2	1	1	0	2	0	2	0	2	0	2
n=2(3.2%)	0	100	0.0	100	50.0	50.0	0.0	100	0.0	100	0.0	100	0.0	100
K. ascorbata	1	0	1	0	1	0	1	0	1	0	1	0.0	1	0
n=1(1.6%)	100	0.0	100	0.0	100	0.0	100	0.0	100	0.0	100	0	100	0.0
P. aeruginosa	2	1	2	1	2	0	2	1	2	1	0	3	2	1
n=3(4.8%)	66.7	33.3	66.7	33.3	66.7	0.0	66.7	33.3	66.7	33.3	0.0	100	66.7	33.3
P. mirabilis	1	3	0	4	2	3	1	4	3	2	2	3	2	3
n=5(8.1%)	20	60.0	0.0	80.0	40.0	60.0	20.0	80.0	60.0	40.0	40.0	60.0	40.0	60.0
E. aerogenes	0	1	0	1	0	0	0	1	0	1	0	1	0	1
n=1(1.6%)	0	100	0.0	100	0.0	0	0.0	100	0	100	0.0	100	0	100
E. agglomerans	2	3	1	3	3	1	1	4	1	4	1	4	2	3
n=5(8.1%)	40.0	60.0	20	60.0	60.0	20.0	20.0	80.0	20.0	80.0	20.0	80.0	20.0	60.0
H. alvei	0	1	0	1	0	0	0	1	0	1	0	1	0	0
n=1(1.6%)	0.0	100	0.0	100	0	0.0	0.0	100	0	100	0.0	100	0	0
M. morgannii	1	1	0	1	2	0	1	1	1	1	1	1	1	1
n=2(3.2%)	50.0	50.0	0.0	50.0	100	0.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Gram Positive														
S. aureus	0	6	0	6	0	3	0	4	0	6	0	6	0	5
n=6(9.7%)	0.0	100	0.0	100	0.0	50.0	0.0	66.7	0.0	100	0.0	100	0.0	83.3
S. saprophyticus	0	3	0	3	2	1	2	1	1	1	1	1	1	2
n=3(4.8%)	0.0	100	0.0	100	66.7	33.3	66.7	33.3	33.3	33.3	33.3	33.3	33.3	66.7
Enterococcus spp	0	3	0	3	2	2	0	2	0	4	0	4	0	3
n=4(6.5%)	0	75.0	0.0	75.0	50.0	50.0	0.0	50.0	0.0	100	0.0	100	0.0	75
Total	20.0	37	8	41	37	15	22	36	23	38	19	41		
n=62(100.0%)	**35.1**	**64.9**	**16.3**	**83.7**	**71.2**	**28.8**	**37.9**	**62.1**	**37.7**	**62.3**	**31.7**	**68.3**		

Key: CAZ=Ceftazidime, CTX=Cefotaxime, IPM=Imipenem, ATM=Aztreonam, CN=Gentamicin, OFX=Ofloxacin, S=Sentitive, R=Resistant.

Table-2. Distribution of Extended Spectrum Beta-lactamases (ESBLs) in Gram negative Urinary Isolates by Double Disk Synergy Test (DDST)

Organisms	**No. of isolates**	**No. of ESBL-producers (%)**
Klebsiella spp	13	6(46.2)
Escherichia coli	19	8(42.1)
Enterobacter spp	6	0(0.0)
Proteus mirabilis	5	1(20.0)
Morganella morgannii	2	0(0.0)
Hafnia alvei	1	0(0.0)
Pseudomonas aeruginosa	3	1(33.3)
Total	49	16(32.7

Figure-1. Comparative distribution of ESBL-producing isolates according to gender

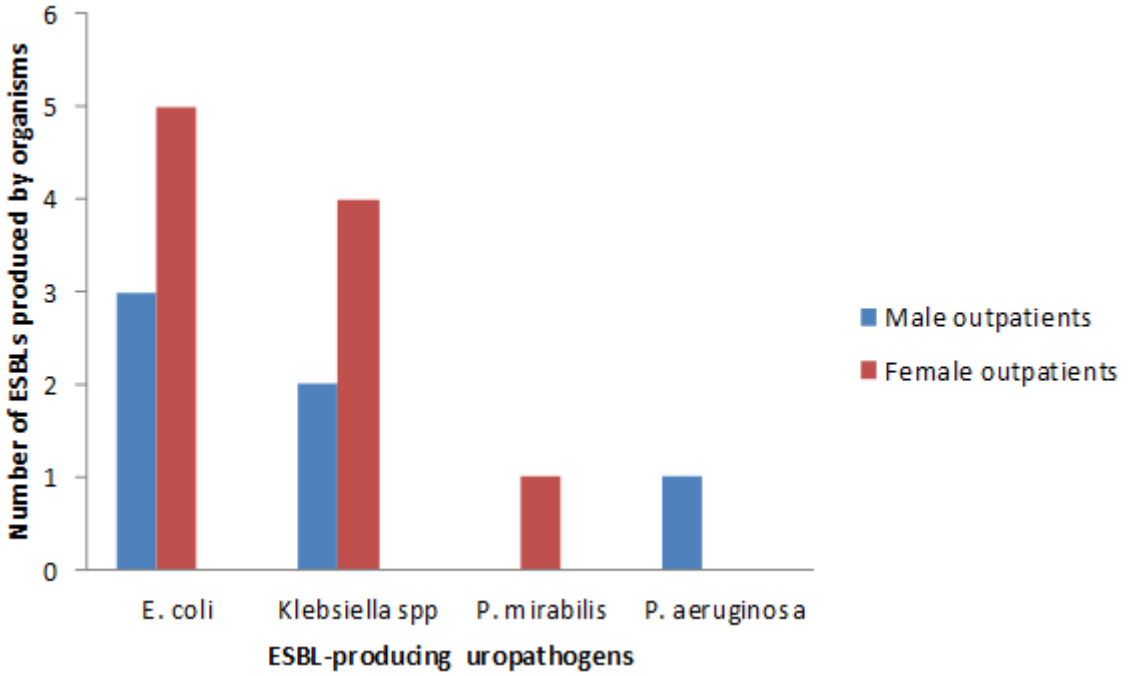
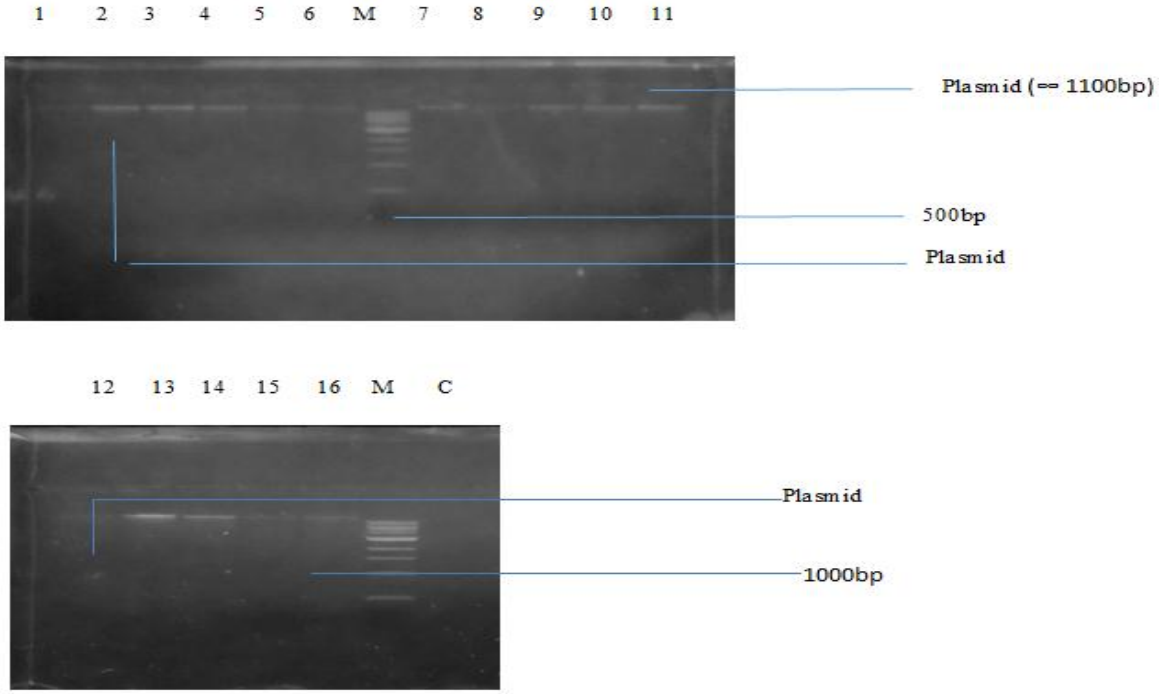


Figure-2. Agarose gel electrophoresis indicating the various plasmid bands of isolated bacteria from urine. Lane 1- 16 represents the isolates. Lane M represents the Quick-Load 1kb molecular ladder while lane C is the negative control



4. Discussion

The increasing spread of ESBL-producing uropathogens is posing a serious threat in the treatment of infections especially urinary tract infection which, treatment is mostly empiric in an outpatient setting in our locality. These pathogens primarily exert their antibiotic resistance against beta-lactam antibiotics by producing plasmid-mediated enzymes called extended spectrum beta-lactamases. The uropathogens isolated in this study exhibited varying levels of resistance to the antibiotics tested. The most isolated uropathogens was *E. coli* 19(30.6%) followed by *Klebsiella pneumoniae* 7(11.3%), *Staphylococcus aureus* 6(9.7%), *Proteus mirabilis* 5(8.1%), *Enterobacter agglomerans complex* 5(8.1%) and *Enterococcus spp* 4(6.5%). This result is close to that obtained in a similar study carried by Ouno, *et al.* [23] who reported *E. coli* as the most predominant isolate from urine. These pathogens are known to mostly consist of the outer genital and periurethral bacteria reflecting the gut flora [23]. In all cases, *E. coli* is mostly implicated in 70-80% of UTI, while other unusual ones may occur following antibiotic therapy, surgery or the presence of obstruction or renal stones [24].

In order to reduce the incidence of community-acquired antibiotic resistance in UTI outpatient's population, regular reporting of sensitivity patterns of UTI pathogens and other diseases is imperative. In this study, the most

resisted antibiotic by the isolated uropathogens is Cefotaxime (83.7%), a mostly parenteral antibiotic. This is even higher than the resistance against Ofloxacin (68.3%), one of the most abused antibiotics which can easily be obtained without prescription from pharmacy outlets in Nigeria. This may not be unconnected with the fact that there is a shift from oral drugs to injectable due to poor treatment result experienced by many in the treatment of UTI. The overall resistance of 62.1% by the uropathogens against Aztreonam is even more worrisome as the drug not only expensive but also not commonly available. Nevertheless, this susceptibility pattern has been observed and reported in a similar study by Oluremi, *et al.* [25].

Obviously, the most effective antibiotic was Imipenem which showed 71.2% sensitivity to the isolated uropathogens. The possible reason for this may be due to its limited use and abuse by patients. However, this result is consistent with other studies [26, 27] which recorded high sensitivity of Imipenem to isolated uropathogens including those that were multi-drug resistant (MDR). The resistance rate of 62.3% seen with Gentamicin (aminoglycoside) is not far-fetched, as this may be attributed to its widespread use in hospitals and health-care centres coupled with their availability, accessibility and cheapness [28].

The prevalence of multi-drug resistant uropathogens in this study was 35.7%. This was recorded in UTI caused by *Enterobacter aerogenes, Hafnia alvei, S. aureus, K. ornithinolytica, and Enterococcus spp*. especially as they were resistant to 3 or more antibiotics at a time [29]. Multi-drug resistant *E. coli, S. aureus* and *Enterococcus spp* have also been reported in some studies [30, 31].

The overall prevalence of extended spectrum beta-lactamase (ESBL) in this study was 32.7% which is higher than earlier report of 20.0% from our center [21] and from other studies [32, 33] This may not be unrelated to the acquisition of ESBL producing strains by outpatients and consequently the poor empirical treatment outcomes seen in them.

The plasmid profiles of all ESBL-producing isolates revealed single sized plasmids of high molecular weight (1100bp). The result is however different from that obtained by Motayo, *et al.* [33] who reported 11.8kbp to 35.5kbp range of plasmid sizes among the study isolates and may be due to the different mechanisms of resistance by these organisms to antibiotics. More so, the Plasmid curing of the resistant plasmids of *E. coli, P. mirabilis, Klebsiella spp* and *P. aeruginosa* isolates producing ESBL showed increased zone diameter (of at least 25mm) of the antimicrobial agents to the test organisms (Plate 2).

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Declaration of Conflicting Interests

There is no conflict of interests.

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