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Pattern of Esbls in Uro-Pathogens Obtained from a Nigerian Tertiary Hospital

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: Multidrug resistance remains a challenge in the treatment of Urinary Tract Infections (UTI) in Nigeria, a key factor being the occurrence of ESBL producers. Earlier reports have emphasized the occurrence of major ESBLs, little is known about the minor subtypes' occurrence in this regard.

Objective: This study sought to evaluate the occurrence of major and minor ESβL producers among a cohort of uropathogens collected from a Nigerian Teaching Hospital using molecular techniques.

Material and Methods: Cultures from 1000 UTI positive urine specimens were collected from the hospital laboratory between May 2015 and December 2017. All samples were subjected to standard isolation culturing techniques and identified. They were further tested for susceptibility to 8 antibiotics. Of these, gram-negative isolates with presumptive ESBL production were evaluated for confirmatory ESBL production using Chromogenic ESBL agar (Oxoid) and Agar Dilution tests. One hundred and twenty-five of them were evaluated for the presence of six ESBL genes (TEM, SHV, CTX-M 15, PER, GES, VEB) using Multiplex PCR/Agarose Gel Electrophoresis.

Results: The results revealed that 40 out of 97 (41%) detected ESBL genes were of the ESBL minor category (VEB, PER, and GES). These ESBL producers were also observed to be resistant to at least five of the 8 antibiotics tested.

Conclusion: More attention should be paid to the emergence minor ESBL producers among uropathogens in this environment as they represent a potential underlying influence on the observed treatment failure in the treatment of UTI.

Keywords: Urinary tract infections; Extended-Spectrum β-lactamase; Multiple drug resistance

INTRODUCTION

"Urinary Tract Infection" (UTI) is a term used to describe the microbial activity and its effects on the urinary system. UTIs represent one of the most commonly encountered infections in clinical visits globally (Shaifali et al., 2012). In the last century, UTI treatment outcomes were successful with the use of antibiotics such as the Penicillins (Fleming, 1980), Trimethoprim/Sulphamethoxazole, and Nitrofurantoin among others (Dodd et al., 1944; Huovinen, 2001). However, the emergence of antibiotic resistance in the last few decades has made the treatment of UTI increasingly difficult. It is further complicated by the evolution of definite patterns observed to express simultaneous resistance to at least antibiotics, hence the term multiple drug resistance.

Notable among the enzymes responsible for different multidrug resistance patterns reported is the Extended-Spectrum β-lactamase (ESβLs), a group of enzymes encountered in gram-negative bacteria that confer resistance to the Expanded Spectrum Cephalosporins (e.g., ceftriaxone, ceftazidime, and cefotaxime) (Giske et al., 2009). These enzymes are genetically controlled, the genes of which are borne on plasmids known to carry genes that mediate resistance to other classes of antibiotics. The clinical expression of this multidrug resistance pattern leads to a narrowing of the effective antibiotic options available. This leads to an increasing difficulty in the treatment of UTI.

An examination of scientific reports on ESβLs shows that earlier subtypes have been reported in many regions around the world. The first of them was TEM-3, which had evolved from point mutations of TEM-1,

named after the patient Temoniera from whom it was isolated (Datta and Kontomichalou, 1965) in Greece. SHV-2 was reported next, being a mutant of SHV-1, a sulphyhydyl variant of TEM (Liakopoulos et al., 2016). Matsumoto in 1986 isolated FEC-1 from a fecal sample in Japan, which was later rechristened as CTX-M in Munich, Germany (after the observed preferential cefotaximase activity) (Bonnet, 2004). Seven years later, Hall reported the occurrence of OXA ESβLs in Turkey, named after their preferential hydrolysis of Oxacillin (Hall et al., 1993).

The discovery of one ESβL type in a region often led to searches by researchers in other regions for related patterns, most cases in which they were found to exist. The resulting global representation of these subtypes formed the basis for classifying them as major ESβLs. Many more ESβLs subtypes have been reported, but their spread across geographical regions was much more limited. Hence, they were designated as minor ESβLs (Naas et al., 2008). GES type ESBL was first reported in a *K. pneumoniae* strain isolated from a one-month-old child in a hospital in French Guiana, South America, in 1998, hence the name GES. VEB type ESBLs were first reported in Vietnam from a four-month-old child in 1999. PER ESBLs were first reported in France in 1991and were named after the organism from which it was isolated, *Pseudomonas aeruginosa*. Other minor ESBLs reported are BES (of Brazilian origin- 1996), SFO (named after *Serratia fonticola*- 1988), TLA (from the Tlahuicas Indian tribe- 1991. BEL-1 (named after Belgium in 2005).

Although several studies in Nigeria have reported the occurrence of major ESBL types in UTI, the extent of its occurrence has not provided sufficient basis for the soaring rates of multiple drug resistance in uropathogens, hence the need to search for the possibility of more ESBL subtypes. The study aimed at determining the occurrence of selected Major and Minor ESβL types present in a cohort of uropathogens obtained from a Nigerian Tertiary Hospital.

METHODOLOGY

Collection, Isolation and Identification of isolates

Having obtained written ethical approval from the Institutional Ethics and Research Committee (IRB/IEC/0004553), 1000 positive urine cultures from patients were collected from the hospital laboratory between May 2015 and december 2017 . the samples were collected on freshly prepared Nutrient Agar plates and incubated at 37ºC for up to twenty four hours. The cultures were transferred to the Pharmaceutical Microbiology Lab, Department of Pharmaceutics, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife. Here, the organisms in each culture were isolated and identified using CLED agar, Hicrome® UTI agar, and other supporting standard biochemical tests.

Antibiotic susceptibility tests

Susceptibility to eight antibiotics, namely Nitrofurantoin (100µg); Ciprofloxacin (5µg); Streptomycin (10µg); Erythromycin (15µg); Trimethoprim/Sulphamethoxazole (1.25µg/23.75µg); Cefotaxime (30µg); Meropenem (10µg); Cefpodoxime (10µg) was tested using disc-diffusion method. Briefly, about four to five colonies of each isolate taken from an overnight nutrient agar plate culture were introduced into 10ml sterile water using a sterile platinum loop. The suspension was mixed thoroughly using a spin mixer. The suspension was after that adjusted to a turbidity of 0.5 McFarland standard (A625nm =0.09). A sterile cotton tipped applicator (Sterilin Ltd, Middlesex, UK) was inserted into the suspension and was used to swab the entire surface of over-dried Mueller Hinton agar uniformly . The inoculated plates were incubated at 37ºC for 20 minutes for the acclimatization and growth of the inoculums (BSAC). Antibiotic discs (Oxoid) were then lightly but firmly pressed onto the surface of the plates equidistant to each other using a pair of sterile forceps, and the plates were refrigerated at 4ºC for 30min to ensure adequate diffusion of antibiotics. The control strain used in this study was *E. coli* ATCC 25922. All plates were incubated at 37ºC for 18h. The inhibition zone diameters obtained were measured in millimeters and interpreted according to CLSI recommendations table 2A (2018).

Detection of ESβL production

All gram-negative uropathogens were further screened for phenotypic ESβL expression using two separate methods. All gram-negative isolates resistant to Cefotaxime and Cefpodoxime in the Antimicrobial Susceptibility tests were selected for screening. Two methods were used in the presumptive and confirmatory screening of Extended Spectrum β-lactamase.

A. Presumptive determination of ESβL producers by Minimum Inhibitory Concentration to Cefotaxime

The procedure involved an initial screening using one of the five expanded spectrum cephalosporins (Cefpodoxime, Cefotaxime, Ceftazidime, Cefazolin, and Aztreonam) as recommended in Table 3A (CLSI-M100-S24, 2018. Duplicate plates of Mueller Hinton agar containing 0, 1, 2, 4, 8, 16, 32, and 64µg/ml concentrations of Cefotaxime 1, 2, 4, 8, and 16µg/ml concentrations of Cefpodoxime were inoculated with

dilutions of an overnight nutrient broth culture of isolates adjusted to a 0.5 McFarland turbidity standard using a 48- multipoint inoculator. Results were taken after 20 h incubation at 37ºC with *E. coli* ATCC 25922 as negative control strain and MH agar positive control (without cephalosporin) and negative growth control plates with cephalosporin but no organisms). The lowest concentration of Cefotaxime inhibiting growth in both replicates was taken as the MIC, and the isolates with MIC ≥2µg/ml for Cefotaxime and ≥ 8µg/ml for Cefpodoxime were interpreted as presumptive ESβL producers.

B. Confirmatory determination of ESβL production

i. **Chromogenic agar**- The present study used 10 x 90mm size Brilliance® ESβL agar Ready-Poured Plates (Oxoid- PO5302A) for confirming the status of the presumptive ESβL producers. A loopful of overnight broth cultures of specimens were streaked onto the plates and were incubated in inverted position for 24 h at 37ºC. All plates with no growth were re-incubated for an additional 24 h. Plates with growth were interpreted as ESβL positive, and the identities of the samples were re-confirmed based on the color of the growing colonies: *E. coli*- blue or pink; *Klebsiella*, *Enterobacter*, *Serratia* and *Citrobacter* (KESC)- green; *Proteus spp*- brown halo; *P. aeruginosa*- colorless. The negative control strain in this study was *E. coli* ATCC 25922, while *K. pneumoniae* ATCC 700603 was used as a positive control.

ii. **Agar dilution** - Cefotaxime- MH plate concentrations of 0.25mg/L to 64mg/L in doubling dilutions (9 concentrations) were prepared. To an identical set was added 4mg of clavulanic acid in each of the plates. Overnight broth cultures of isolates adjusted to a 0.5 McFarland standard were transferred onto the plates using a multi-inoculator. The plates were left on the bench for 15minutes to absorb the culture, after which the plates were reverted and incubated at 37ºC for 18 h. The plates were examined, each plate compared with its clavulanic acid counterpart, and MIC reduction values ≥3 two-fold concentration was taken as an indicator for ESBL production (CLSI, 2018).

1. Detection of genetic determinants of ESβL production

Six ESβL gene subtypes were screened for in this study- three major (TEM, SHV, CTX-M) and three minor (VEB, GES, PER) ESβLs (see Table 1). Corresponding primers were adopted from previous literature and confirmed using the BLAST search tool (NCBI). One hundred and twenty-five of the confirmed ESβL producers were randomly picked and subjected to a multiplex protocol of Polymerase Chain Reaction/ Pulsed-field Gel Electrophoresis. Briefly, a loopful of bacteria was suspended in 700µL of sterile distilled water, and the resulting suspension was boiled for 10 minutes. It was centrifuged at 7000xg for 4 minutes at 4ºC. It was cooled on ice for 10 minutes and centrifuged again for 3 minutes at 8000xg. It was returned to the ice pack until ready for use. A 2µL volume of supernatant from the mixture was retrieved and placed in a PCR tube containing a mix of ingredients- 0.5µL of each primer (x12), 25µL of master mix (Inqaba Biotec, South Africa) and 17µL of Molecular Grade water. The PCR protocol employed in the study is as shown below:

Hot start/ denaturation 94ºC for 1 minute, then, 94ºC for 30 seconds for 35 cycles: Annealing temperature 59ºC for 30 seconds for 35 cycle. Extension temperature 72ºC for 1 minute for 35 cycles, then for 6 minutes. Samples were held at 4ºC afterward. PCR products (amplicons) were run on a 2% agarose gel under the following conditions: 90 volts, 400 amps for 35 minutes. Gels were examined using G-box® gel analyzer.

Table 1. List of Primers used in the study

Primer	Sequence	Band size	Reference
bla SHV (F)	AGCCGCTTGAGCAAATTAAAC	786	(Strauß et al., 2015)
bla SHV (R)	GTTGCCAGTGCTCGATCAGC		
bla TEM (F)	CATTTCCGTGTCGCCCTTATTC	846	(Decré et al., 2010)
bla TEM (R)	CCAATGCTTAATCAGTGAGGC		
bla CTX-M -15(F)	GGCGATCCGCGTGATACCAC	262	(Jajic-Bencic et al., 2009)
bla CTX-M -15 (R)	GTGAGGATGAAGATATCTCC		
bla GES (F)	CTGGCAGGGATCGCTCACTC	604	
bla GES (R)	GGTTTCCGATCAGCCACCTCTCA		(Trung et al., 2015)
bla PER (F)	CAGTGTGGGGGCCTGACGAT	731	(Trung et al., 2015)
bla PER (R)	CTGAGCAACCTGCGCAATRATAGCTT		
bla VEB (F)	GATGGTGTTTGGTCGCATATCGCAAC	391	(Trung et al., 2015)
bla VEB (R)	CATCGCTGTTGGGGTTGCCCAATTTT		

RESULTS AND DISCUSSION

Seven hundred and twenty-two gram-negative bacteria were isolated from 1000 urine cultures collected in this study. Of these, 206 gram-negative bacteria were found to be ESβL producers (> a quarter of all the gram-negative uropathogens isolated in this study). All the isolates tested were resistant to at least five antibiotics, while 119 were resistant to all 8 antibiotics used in this study (Figure 1).

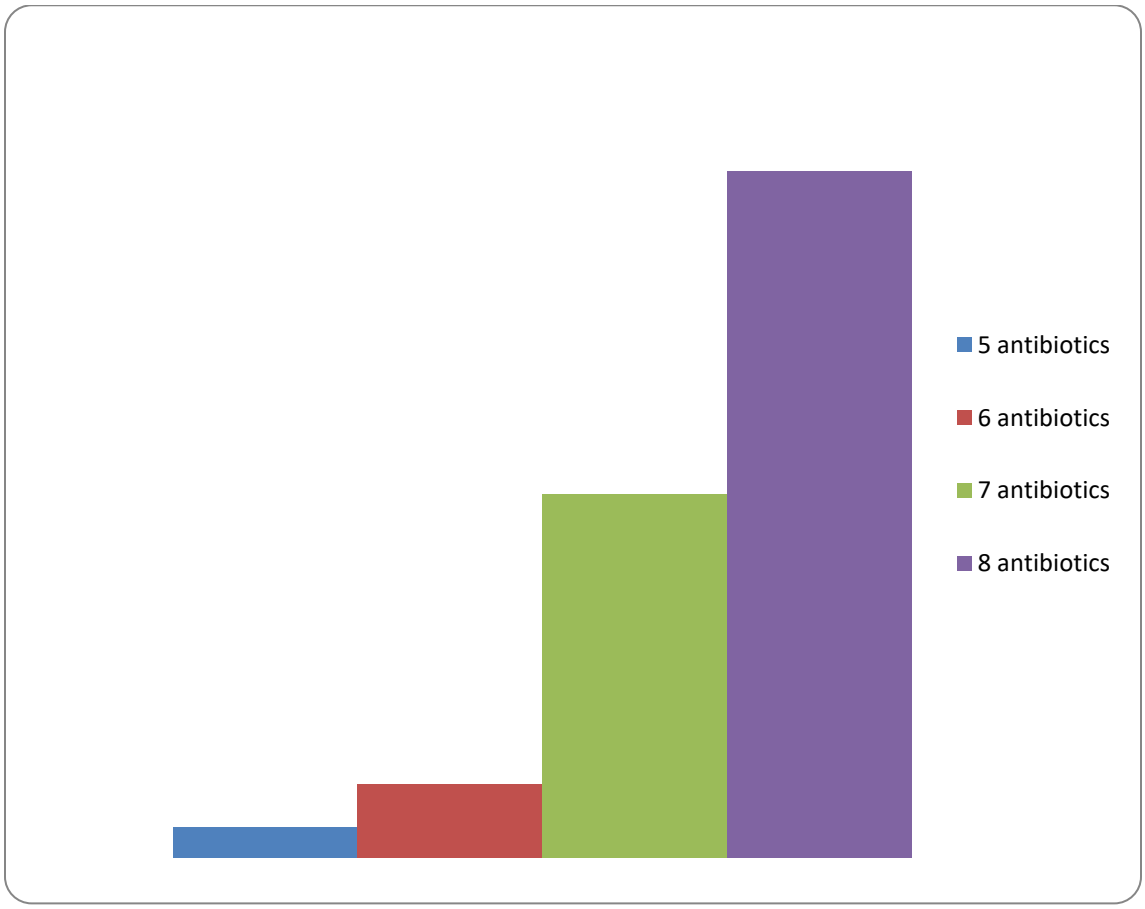

Figure 1. Pattern of multidrug resistance of ESBL producers to antibiotics used in the study

Also, ESBL production was detected in 6 species of gram-negative bacteria isolated in this study (Figure 2). *Klebsiella spp.* was the highest occurring ESBL producer (76), closely followed by *E. coli* (72). Other ESBL producers were detected in *Pseudomonas aeruginosa* (35), *Proteus mirabilis* (14), *Enterobacter aerogenes* (6), and *Citrobacter freundii* (2).

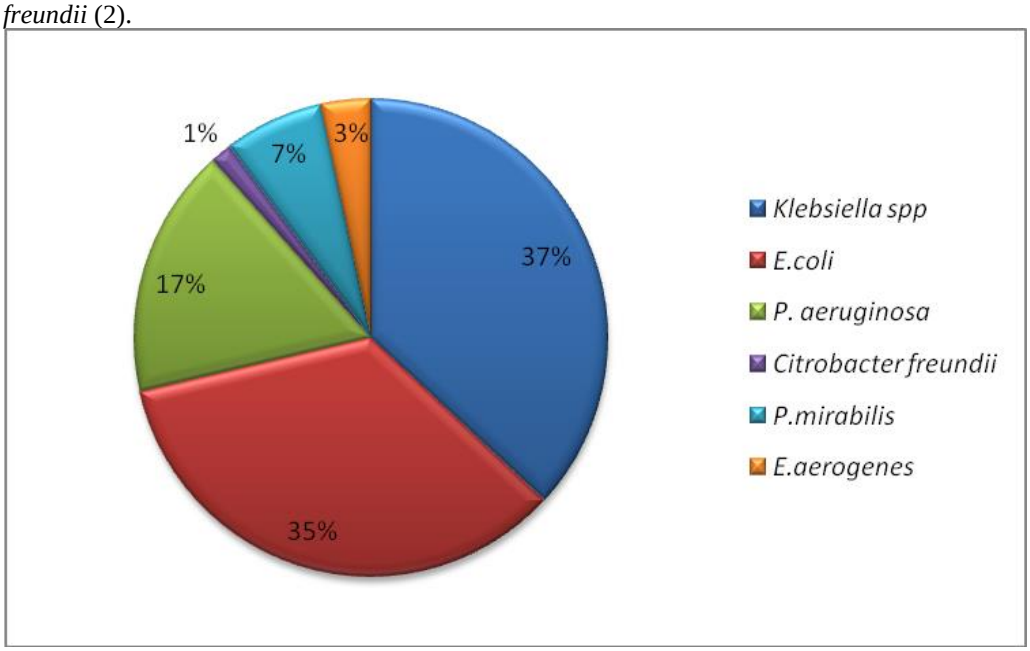

Figure 2 - Proportion of species involved in ESBL production in the study (n = 206)

One hundred and twenty-five ESBL producers were randomly selected for PCR and Agarose gel electrophoresis. Only 61 of these isolates yielded bands indicating genes. Of these, 40 yielded a single gene, 10 produced 2; another 8 yielded 3; 2 produced 4, and one yielded 5. In all, 97 genes, each corresponding to at least one primer size used, were recorded in this study. Thirty-three of these genes were found in *E. coli*; 24 in *K. pneumoniae*; 12 in *Pseudomonas aeruginosa*; 10 in *Proteus mirabilis*; 8 in *Citrobacter freundii*; 6 in *Enterobacter aerogenes*, and 4 in *K. oxytoca* (Figure 3). Figure 4 presents a more concise picture of all the ESBL types found in the species in the study.

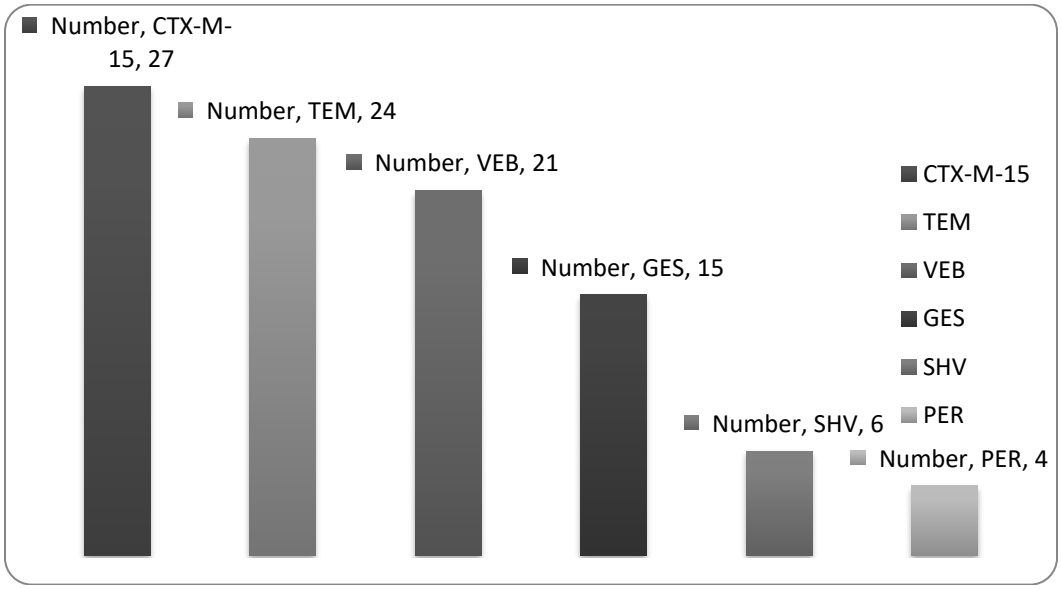

Figure 3- Chart showing frequency outcome of ESBL subtypes from PCR/PFGE

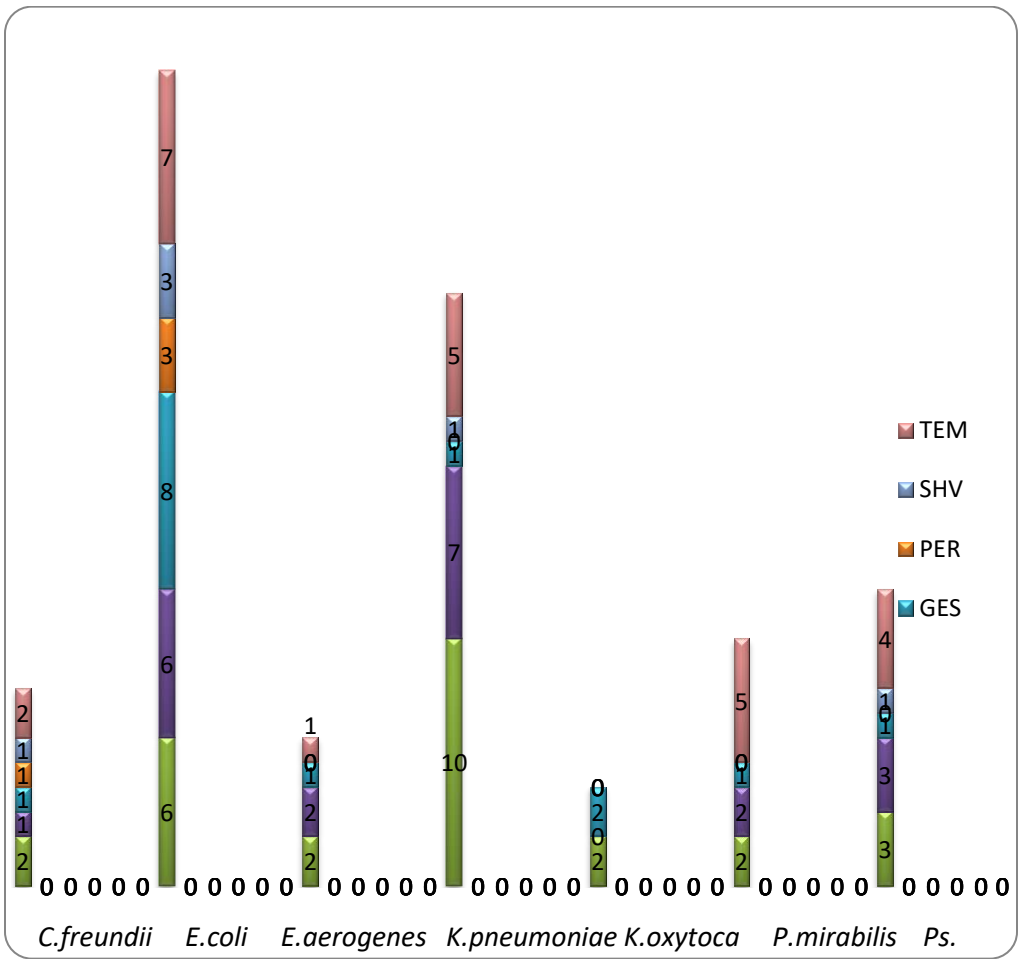

Figure 4 - Chart analysis of ESβL gene distribution among uroisolates at a glance

The availability of effective antibiotic options in the treatment of UTI in this environment is an issue of growing concern. As seen in this study, multidrug resistance among ESBL producing uropathogens is an established basis for UTI treatment failure. This finding only strengthens an already established description of ESBL producers being multidrug-resistant.

Furthermore, the observed distribution of the ESβL subtypes across gram-negative uropathogens suggests an already established dissemination across species in the environment - *E. coli*, and *Klebsiella pneumoniae* being in the lead. Earlier separate studies by Ali and Azekhueme and their research teams corroborate *E.coli* and *K. pneumoniae* as the two most prominent bearers of ESβL genes (Ali et al., 2017; Azekhueme et al., 2015). By implication, there exists the possibility of ESBL gene dissemination by other gram-negative bacteria in other health conditions. The implications of this could mean increased morbidity, and in some cases, mortality arising from infections caused by ESBL producing gram-negative bacteria.

Unlike earlier studies that addressed the occurrence of major ESβLs in the country, this study reports the highest incidence of minor ESβLs so far in Nigeria. Although there exists the proof of concept in this study that CTX-M 15, a major ESβL, represents the most commonly encountered ESβL in Nigerian hospitals (Ogbolu *et al*., 2018), the results above show that there is a >40% chance of encountering any of these minor ESβL subtypes (VEB, GES, PER) in this environment. The carriage of multiple ESβL subtypes by the isolates is also evident in this study as seen in 21 out of the 61 isolates that yielded. Co-carriage of multiple ESBL types is an established observation in previous studies (Alqasim et al., 2018). However, the importance of this observation lies in the increasing complexity in expression. Each ESβL type often expresses preferential substrate hydrolysis, each substrate being a member of the class of third-generation cephalosporins (Seyedjavadi et al., 2016). Multiple subtypes could mean resistance to all cephalosporins in question or multiple carriages of plasmids. Either way, it mediates higher expression of drug resistance.

Sixty-two ESBL producers did not yield any band. A plausible explanation for this is that the ESβL subtypes contained in them were not part of those that were screened. In other words, the ESBL types present in this cohort may exceed those that were screened for.

The emergence of minor ESBL types in this environment can be explained in two possible ways. Resistance genes in bacteria may be acquired either by a gene mutation or by a horizontal gene transfer. Although gene mutations are rare, they still occur in response to antibiotic pressure. The uncontrolled use of antibiotics largely drives the antibiotic pressure in this environment. In 2005, Okeke and other investigators (2005) reported that diverse pressure factors were responsible for driving antibiotic resistance in this environment. Among them are ineffective implementation of antibiotic protection policies, uncensored use of antibiotics, and lack of control in the prescription and sale of antibiotics. Research reports around the world have reported similar findings in countries where minor ESBLs were first reported (Ingle et al., 2017; Klein et al., 2018; Laxminarayan and Chaudhury, 2016). It is therefore possible that there is an ongoing emergence of an unrelated but identical ESBL type in the study environment.

On the other hand, resistance genes could spread by dissemination via extrachromosomal genetic elements. Unlike the earlier major ESBLs (TEM and SHV) which evolved by point mutations, these minor ESBLs are known to be effectively disseminated via extrachromosomal genetic elements hence facilitating their spread (Girlich et al., 2001; Naas et al., 2008a; Poirel et al., 2000). Earlier on, reports about the occurrence of minor ESBLs in the country have been fewer and more sporadic than major ESBLs. A probable reason for this is that the spread of minor subtypes was initially limited to the areas of the first mention. However, the advent of international travel has made the dissemination of genes across continents more complex. An interesting dimension to it is the growing medical tourism industry in favour of the Asian continent. The combination of quality healthcare and modest prices has led to a significant increase in visits to this region from all over the world (Beladi et al., 2015; Sen Gupta, 2015). According to a study by Langford and Schwartz (2018), there is 75% likelihood that 340 Africans out of every thousand will be colonized with ESβLs in Asia, the origin of VEB type ESβLs). Sampaio and Theiler (2015) have speculated that traffic from Low and Middle-income countries to South America (origin of GES) will increase, due to cheaper higher-education study options and low-cost vacation preferences (Maringe et al., 2013; Sampaio, 2015). These travel trends constitute the highest risk of the influx of these ESβL subsets.

The study encountered a few challenges. First, the number of ESBL types that were screened in the study was limited to what the available research materials could cover. The consequence of this was evident in the fact that some of the phenotypic ESBL producers did not yield any band, meaning that the ESBL type contained in them was not screened for. Besides, there may have been novel subtypes the discovery of which will require more sophisticated equipment. The limited supply of resources also informed the random selection of ESBL phenotypes for molecular investigation. Nevertheless, the prominence of *E. coli* and *K. pneumoniae* among ESBL producers remained evident in the study.

CONCLUSION

On a conclusive note, the reports about ESβL types in this environment may have been grossly underestimated, hence needing a more thorough assessment. The reality of a constantly evolving and expanding pattern of this multidrug resistance, for which healthcare professionals in this environment must be in constant awareness of in taking decisions in the treatment /management of UTI, must remain within the zone of ongoing research

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