Extended Spectrum Beta-lactamase Genes in Clinically Important Bacteria Isolated from Wastewater of Two Selected Tertiary Hospitals in Enugu, Nigeria

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Abstract: Hospital activities have resulted in increased discharge of untreated effluent into the environment, posing substantial risks to public health and the environment due to the presence of diverse harmful components, including extended spectrum β–lactamase (ESBL) producing bacteria harboring resistance genes, which are adding to the global crisis of antimicrobial resistance (AMR). This study aimed to assess the prevalence of ESBL genes in bacterial isolates from wastewater of two selected tertiary hospitals in Enugu State. A total of 20 samples were aseptically collected, transported and processed for bacteriological identification and susceptibility testing following standard procedures. Phenotypic and genotypic detection of extended spectrum beta lactamases (ESBL) were conducted following standard procedures. Screening for ESBL production was done by double disk synergy test and data obtained were analyzed using SPSS version 23. A total of 65 bacterial isolates, 41(63.1%) Gram-negative and 24(36.9%) Gram-positive, were detected from the samples. Out of these isolates, ESBL production was observed in five 5(81%) isolates of *Escherichia coli*, thirteen 13(81%) isolates of *Klebsiella* spp and one 1(20%) isolate of *Pseudomonas aeruginosa.* The ESBL encoding genes- *bla SHV, blaCTX-M, bla OXA, bla TEM* were found in varying levels among the *E. coli* and *Kleb.* spp isolates, while the *Pseudomonas aeruginosa* isolates were found to be harboring *bla-CTXM, bla OXA, bla TEM* genes, but *bla SHV* genes were absent. All the ESBL producers were multi-drug resistant, therefore proper treatment of hospital wastewater before discharge into the environment is highly recommended.
Key word: Antibiotics, bacteria, Enugu, extended spectrum beta lactamase (ESBL) genes**,** hospital wastewater

INTRODUCTION

The rise and dissemination of extended spectrum β -lactamases (ESBL) bacteria, once perceived as relatively harmless, have evolved into a significant resistance challenge afflicting healthcare system worldwide. This has given rise to a situation which threatens the efficacy of existing antibiotics used to combat bacterial infections, posing a serious threat to public health (Ovia *et al.,* 2023). Ja'afaru *et al*. (2023) revealed that the production of extended -spectrum β -lactamases is one of the ways bacteria have developed resistance to antimicrobial agents.

Extended spectrum beta lactamases (ESBLs) are a group of enzymes that possess the ability to deactivate the beta-lactam rings of penicillins, first, second and third generation cephalosporins, and aztreonam (Ejikeugwu *et al.,* 2016), but are inhibited by clavulanic acid, a beta – lactamase inhibitor (Bonnet, 2004; Rawat and Nair, 2010). Fernandes *et al.* (2014) identified several molecular variants of ESBLs designated as TEM-1, TEM-2, SHV, OXA, CTX-M and PER amongst others with the most prevalent types being TEM and SHV enzymes. The ESBLs are chromosomally or plasmid mediated and can easily be transferred from one bacterium to another via horizontal gene transfer (Ugah and Udeani, 2020). Therefore, organisms which produce ESBLs usually manifest resistance to multiple antibiotic classes (Giwa *et al.,* 2018), thereby posing very serious therapeutic challenges from limited treatment options, with severe, and in some cases, fatal clinical outcomes (Rawat and Nair, 2010).

Extended spectrum beta lactamases (ESBLs) are produced by Gram-negative bacteria and most strains producing them belong to the family Enterobacteriaceae, and often contain resistance determinants for other classes of antibiotics like the aminoglycosides, sulfonamides, fluoroquinolones tetracyclines, chloramphenicol, trimethoprim and sulphonamides which are readily transmissible from one strain of organism to another and between different species of Gram-negative bacteria (Munday *et al.,* 2004; Jacoby and Munoz-Price, 2005; Peirano and Pitout, 2010). Although these

transfers between bacteria can occur within the community, it is most often observed in healthcare facilities, and is a major challenge in nosocomial infections (Bello *et al.*, 2021). Oli *et al.* (2016) noted some factors that create suitable conditions for their spread within the hospital setting such as; poor hygienic practices in hospitals especially those in developing countries, indiscriminate antibiotics use, empirical antibiotic prescription and therapy not supported by the laboratory, absence of antimicrobial resistance surveillance programs and inadequate infection control practices.

Buelow *et al*. (2017) recognized hospital wastewater as a reservoir for ESBL genes, which can be disseminated into the environment, posing a risk to human health. Several cases of ESBL producing bacteria have been reported across the world in both clinical and nonclinical samples in Nigeria, Japan, South Korea, and Egypt (Ejikeugwu *et al*., 2013; Zorgani *et al.,* 2017). The ESBL-producing bacteria are responsible for the cause of community onset of urinary tract infections (UTIs) (Zorgani *et al.,* 2017). To date there is paucity of information regarding the actual disease burden and frequency of ESBL producing bacteria in Nigeria, hence this study was carried out to evaluate the presence and prevalence of extended spectrum β -lactamases genes in some clinically significant bacteria isolated from wastewater of two Tertiary Hospitals in Enugu, Nigeria.

MATERIALS AND METHODS

Sample site and collection: The wastewater samples were collected from four (4) different wastewater outlets of University of Nigeria Teaching Hospital (UNTH) and National Orthopedic Hospital Enugu (NOHE) Nigeria, with four sampling points designated: NOHE Female Medical Ward (NFMW), NOHE Amenity Ward (N-AW), UNTH Post Natal Ward (U-PNW) and UNTH Male Medical Ward (U-MMW). Samples were collected into sterile, clean dry universal containers that were tightly capped immediately after sampling and then transported to the laboratory for microbial analysis within 1–2 h from the collection time.

Isolation and characterization of test bacteria: The test samples were inoculated on chocolate agar (CHA), mannitol salt agar (MSA), cetrimide agar (CTA), Eosin - methylene blue agar (EMB), blood agar (BA), and MacConkey agar plates (MA) according to the method described by Cheesbrough (2000). These plates were incubated at 37^0C for 24 hours, and bacterial colonies on the plates were observed for shape and colour after the first 24 hours, then isolated, Gram-stained, and studied microscopically. Isolates that fermented lactose and had a greenish metallic sheen were suspected to be *Escherichia coli* and were subsequently confirmed by testing for indole production, methyl red, voges proskae, and citrate consumption (IMVIC) with other Enterobacteriaecae, using the method described by Fawole and Oso (2004). Catalase production and coagulase positive tests were performed on colonies with yellow zones on Mannitol salt agar culture. Catalase, coagulase, nitrate, oxidase, indole production, methylred test, Voges-Prauskaur, citrate utilization test, use of Kligler Iron Agar **(**KIA) (for double sugar fermentation and H_2S formation), pigment synthesis, and motility tests were performed on all other isolates (Cheesbrough, 2000).

Extended spectrum beta lactamase (ESBL) screening and confirmatory test: The ESBL production was phenotypically confirmed in all the bacteria clinical isolates by the double disk synergy test (DDST) method (Ejikeugwu *et al.,* 2013; Ejikeugwu *et al*., 2016). Double disk synergy test was performed as a standard disk diffusion assay on Mueller-Hinton (MH) agar plates (Oxoid, UK) as suggested by Clinical Laboratory Standard Institute (CLSI, 2011). Antibiotic disks of amoxicillin-clavulanic acid (20/10 µg) was placed at the center of the MH agar plate, and antibiotic disks containing two third generation cephalosporins (3GC) cefotaxime (30 µg) and ceftriaxone (30 µg) was each placed at a distance of 15 mm from

the central disc (amoxicillin/clavulanic acid). The plates were incubated at 37^0C for 18-24 hours. Extended spectrum beta lactamase production was inferred phenotypically when the zones of inhibition of the cephalosporins (Cefotaxime 30 µg or ceftazidime 30 µg) was expanded by the amoxicillin/clavulanic acid disk (20/10 µg). However, a ≥ 5 mm increase in the diameter zone of inhibition for either of the cephalosporins tested in combination with amoxicillin/clavulanic acid versus its zone when tested alone confirmed ESBL production phenotypically (Bradford, 2001; Ejikeugwu *et al.*, 2016).

Molecular characterization of ESBL genes: The ESBL-producing isolates were subcultured in 5 ml of nutrient broth and incubated overnight at 37^0C for molecular characterization of ESBL genes. The whole chromosomal DNA was extracted by boiling following the procedure described by Queipo-Ortuno *et al*. (2008). The PCR amplification with specific primers targeting blaCTX-M, blaTEM, blaOXA and blaSHV genes were done using a thermocycler. Polymerase chain reaction (PCR) products were sequenced, and sequence analysis was performed using bioinformatics tools. All gene sequences were compared with data of the GenBank (NCBI) database to identify the exact b-lactamase genotype and presented in Table 1.

RESULTS

Table 2 shows the different isolates. Among the bacterial genera isolated namely *Klebsiella* spp and *E. coli* were the most prevalent Gram-negative bacteria (GNB) accounting for 16 (24.6 %), and 6 (9.2%) isolates respectively. Phenotypically, ESBL production was observed in five isolates of *E. coli*, thirteen strains of *Klebsiella* spp and one isolate of *P. aeruginosa* (Table 3). However, no presence of ESBL enzymes in *Enterobacter* spp, *Salmonella* spp, *Proteus mirabilis and Serratia marcescens* was detected. The ESBL positive isolates were found to be resistant to antibiotics in the class of aminoglycosides, cephalosporins, macrolides, penicillins and fluoroquinolones (Tables 4).

Table 1: Extended spectrum beta lactamase gene sequence of forward and reverse primers for multiplex PCR technique

Gene target(s)	Primer sequence (5' to 3', as synthesized)	Expected amplicon size (bp)
blaTEMF	5'AAACGCTGGTGAAAGTA3'	500
blaTEMR	5'AGCGATCTGTCTAT3'	
blaOXA-F blaOXA-R	5'ACACAATACATATCAACTTCGC3'	650
	5'AGTGTGTTTAGAATGGTGATC3'	
blaCTX-M F blaCTX-M R	5'CGCTTTGCGATGTGCAG3'	750
	5'ACCGCGATATCGTTGGT3'	
blaSHV F blasSHV R	5'ATGCGTTATATTCGCCTGTG3'	850
	5'TGCTTTGTTATTCGGGCCAA3'	

Note: All primers were synthesized by Inqaba Biotec™, Pretoria, South Africa

Table 2: Different bacterial isolates from the Hospital wastewaters

Bacterial isolates	Number isolated (%)	Sources
Klebsiella spp	16 (24.6)	N-AW, U-PNW, N-FMW and U-MMW
Escherichia coli	6 (9.2)	N-AW, U-PNW, N-FMW and U-MMW
Enterobacter spp	6 (9.2)	N-AW, U-PNW, N-FMW
Pseudomonas aeruginosa	5 (7.7)	N-AW, U-PNW
Salmonella spp	3(4.6)	N-AW, U-PNW
Proteus spp	2(3.1)	N-FMW
Serratia marcescens	3(4.6)	N-FMW and U-MMW
Staphylococcus aureus	8 (12.3)	N-AW, U-PNW, N-FMW and U-MMW
Coagulase-ve staphylococci	6(9.2)	N-AW, U-PNW, N-FMW and U-MMW
Streptococcus pneumoniae	4 (6.2)	U-MMW, N-FMW
Enterococcus faecalis	6 (9.2)	N-AW, U-PNW, N-FMW and U-MMW

Key: N-AW, NOHE Amenity Ward; U-PNW, UNTH Post Natal Ward; N-FMW, NOHE Female Medical Ward; U-MMW, UNTH Male Medical Ward

Table 3: Phenotypic detection of ESBL in test isolates

Organism	Number of isolates tested	Number of ESBL positive isolates (n%)	Number of ESBL negative isolates (n%)
E. coli	6	5(83)	1(17)
Klebsiella spp	16	13(81)	3(19)
P. aeruginosa	5	1(20)	4(80)
Enterobacter spp	6	0(0)	6(100)
Salmonella spp	3	0(0)	3(100)
Proteus mirabilis	2	0(0)	2(100)
Serratia marcescens	3	0(0)	3(100)

Table 4: Prevalence of ESBL genes in the test isolates

Bacteria	Number of ESBL positive isolates	Number of Isolates (%) habouring				Antibiotics resistant to
		bla SHV	*bla-CTX-M*	*bla OXA*	*bla TEM*	
E. coli	5	4(80)	5(100)	5(100)	5(100)	Cephalosporins
Klebsiella spp	13	12(92)	13(100)	13(100)	10(77)	Fluoroquinolones, Penicillins
P. aeruginosa	1	0(0)	1(100)	1(100)	1(100)	Macrolides, aminoglycosides

DISCUSSION

Hospitals play an important role in the preservation and promotion of a citizen's health. However, Azuma *et al.* (2020) reported that hospital operations cause the formation of diverse mixes of inorganic, organic, and microbiological components, which are typically discharged into the environment as wastewater effluents without previous treatment. This study evaluated the presence of ESBL-producing bacteria in hospital wastewaters, and the prevalence of ESBL encoding genes in sample isolates. Understanding the distribution of ESBL-producing bacteria is crucial for guiding empirical antibiotic therapy and implementing infection control measures in healthcare settings. In the present study, a total of 65 bacterial strains were isolated from the four different wards. Among the bacterial genera isolated were *Klebsiella* spp and *E. coli*. The two isolates appeared to be the most prevalent Gram-negative bacteria (GNB) accounting for 16(24.6%), and 6(9.2%) of the isolates respectively. This is consistent with the findings of Mirkalantari *et al*. (2020), that reported *E. coli* to be the most dominant when urine samples were investigated. However, studies have reported conflicting results regarding the predominant bacteria isolated from various clinical samples, with Ogefere *et al.* (2015); Andrew *et al.* (2017) identifying *K. pneumoniae* as the most common species in their works, Jain *et al*. (2003) reported *Enterobacter* as the most prevalent genus, and Pandey *et al*. (2020), recorded *Bacillus* spp, *Staphylococcus* spp, and *Streptococcus* spp as the most common bacteria in hospital wastewater. These variations in results might be due to differences in sampling sites, prescription patterns of healthcare practitioners in the areas, isolation procedures, amongst other factors. Among the tested species in this study, *E. coli* and *Klebsiella* spp exhibited the highest rates of ESBL positivity, with 83% and 81% of isolates being ESBL-positive, respectively. This is consistent with the study of Zhang *et al*. (2009) who recorded a significant incidence of ESBL-producing *E. coli* bacteria in hospital wastewaters. The high prevalence of ESBL production in these organisms highlight the urgent need for vigilant surveillance and antimicrobial control efforts to mitigate the spread of resistant strains and optimize therapeutic strategies. Contrastingly, *Pseudomonas aeruginosa* an opportunistic pathogen associated with hospital-acquired infections, particularly in immunocompromised individuals and those with cystic fibrosis

exhibited a lower rate of ESBL positivity, with only 20% of isolates being positive. On the other hand, the ESBL-producing *P. aeruginosa* strains are less common compared to other Gram-negative bacteria. Livermore (2009) revealed that their emergence poses a significant threat to immunocompromised patients and those with indwelling medical devices. *Enterobacter* species and the other Enterobacteriaceae tested (*Salmonella* species, *Proteus mirabilis,* and *Serratia marcescens*) displayed a striking absence of Extended spectrum beta lactamase production in this study. This may be interpreted as a favourable finding from an infection control perspective. Woodford *et al*. (2014) documented that the absence of ESBL production in these species does not negate their clinical significance or their potential to acquire resistance mechanisms through horizontal gene transfer or mutational events (Ugah and Udeani, 2020). The data presented in this study revealed that there was prevalence of ESBL genes among various bacterial isolates, along with their associated resistance profiles. Notably, all ESBLpositive *E. coli* isolates harboured genes encoding for bla-SHV, bla-CTX-M, bla-OXA, and bla-TEM, conferring resistance to cephalosporins. This corroborates the findings of Olutayo and Abimbola (2016), thus indicates a diverse repertoire of ESBL enzymes within this species. This observation also aligns with the report of Obasi *et al.* (2017), and other previous studies documenting the widespread dissemination of bla-CTX-M, bla-OXA, and bla-TEM genes among clinical *E. coli* isolates, contributing to the global burden of multidrug-resistant infections (Cantón and Coque, 2006; Adeyankinnu *et al.,* 2014; Raji *et al.,* 2015; Bello *et al.,* 2021). Similarly, *Klebsiella* species exhibited a high prevalence of ESBL genes with majority of the ESBL-positive strains harbouring bla-SHV, bla-CTX-M, bla-TEM and bla-OXA genes, conferring resistance to fluoroquinolones, and penicillin. This conforms with the reports of Clarivet *et al*. (2016), and Azuma *et al*. (2022) who also recorded the bla-SHV enzymes predominated in *Klebsiella* spp. These findings underscore the importance of vigilant surveillance and infection control practices, particularly in healthcare settings where *Klebsiella* spp. are prominent contributors to nosocomial infections (Bush and Fisher, 2011). The association of ESBL genes with resistance to macrolides and aminoglycosides underscores the complex interplay between different classes of antibiotics and the potential for cross-resistance to develop (Livermore, 2002). All of these findings point to the existence of a large number of MDR bacteria with ESBL genes in hospital settings. However, unlike other ESBLs, the bla-CTX-M family is made up of a diverse and complicated set of enzymes which are now significantly more common in Enterobacteriaceae than other forms of ESBLs in Europe and other areas of the world (Azuma *et al*., 2022).

CONCLUSION

This study found a substantial population of both Gram negative and Gram positive multiantibiotic resistant bacteria in wastewater effluents from the two tertiary hospitals used. Most ESBLs, were found in higher proportions in *Klebsiella* spp, and *E. coli*. Some or all of the GNB recovered from the four hospital wards have ESBL producing genes. A major public health worry is that multi-drug resistant isolates from hospital wastewaters serve as a reservoir of resistance genes that could be transferred to other vulnerable bacteria. In view of these findings, proper treatment of hospital wastewater is recommended before discharge into the environment.

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