CTX-M-15 is Established in Most Multidrug-Resistant Uropathogenic Enterobacteriaceae and Pseudomonaceae from Hospitals in Nigeria

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β-Lactam antibiotics are widely used to treat urinary tract infections in Nigeria. This study aimed to determine the presence and characteristics of extended spectrum β-lactamases in commonly isolated uropathogenic Gram-negative bacteria (GNB) in Nigeria.

Fifty non-duplicate GNB isolates consisting of *Escherichia coli*, 19; *Klebsiella pneumoniae*, 21; and *Pseudomonas aeruginosa*, 10 were obtained from three tertiary hospitals in Nigeria. The antibiotic susceptibility testing of all isolates to a panel of antibiotics including minimum inhibitory concentrations (MICs) and extended spectrum β-lactamases was determined. Polymerase chain reactions and sequencing were used to detect β-lactam genes.

Polymerase chain reactions and sequencing identified varying extended spectrum β-lactamases (ESBLs) encoding genes for 24 isolates (48.0%). Cefotaximase-Munich (CTX-M) 15 was the dominant gene with 20/24 of the isolates positive at 83.3%; multiple genes (2 to 6 ESBL genes) were found in 20 of the isolates. The isolates encoded other genes such as CTX-M-14, 33.3%; sulfhydryl variable (SHV) variants, 58.3%; oxacillinase (OXA) variants, 70.8%; OXA-10, 29.2%; and Vietnamese extended β-lactamase (VEB) 1, 25.0%. There was no difference between the MIC_{50} and MIC_{90} of all the isolates.

The high-level multidrug resistance of uropathogens to third generation cephalosporins including other antibiotics used in this study is strongly associated with carriage of ESBLs, predominantly CTX-M-15, as well as CTX-X-M-14, OXA-10, and VEB-1.

Keywords: extended spectrum β-lactamases, uropathogens, antibiotics, resistance, Nigeria

Introduction

Extended spectrum β-lactamases (ESBLs) are enzymes produced by a variety of Gram-negative bacteria which confer an increased resistance to commonly used antibiotics, such as the penicillins and the cephalosporins [1]. These ESBLs are mostly plasmid mediated and efficiently hydrolyze oxyimino cephalosporins (ceftazidime, cefotaxime, and ceftriaxone) and monobactams. They are inhibited by β-lactamase inhibitors such as clavulanate, sulbactam, and tazobactam [2]. Plasmids coding for ESBLs may also carry additional β-lactamase genes as well as genes conferring resistance to other antimicrobial classes [3, 4]. This can limit the chemotherapeutic options for ESBL-producing pathogens and facilitates inter-species and intra-species dissemination of ESBLs [5]. As such, they are a worrying global public health issue as infections caused by such enzyme-producing organisms are associated with a higher morbidity and mortality with greater fiscal burden coupled with increasing prevalence rates worldwide and an ever diminishing supply in the antibiotic armamentarium, and these enzymes represent a clear and present danger to public health [1].

ESBLs have been found mainly in *Enterobacteriaceae*, particularly *Klebsiella* species and *Escherichia coli*, but have also been reported in other genera, such as *Citrobacter*, *Enterobacter*, *Morganella*, *Proteus*, *Providencia*, *Salmonella*, and *Serratia* and in *Pseudomonaceae*. In Nigeria urinary tract infection (UTI) is one of the most common diseases that make people seek medical attention with the majority of infections caused by *Enterobacteriaceae* and *Pseudomonaceae* [6]. Reports of multidrug-resistant isolates have increased during the last decade, probably as a result of the extensive use of broad-spectrum antibiotics. Cefotaximase-Munich (CTX-M)-type ESBLs have emerged among *E. coli* and *Klebsiella pneumoniae* and are now commonly isolated from UTIs, and often, these enzymes are carried by strains with a multidrug resistance phenotype. Multidrug resistance expressed by CTX-M-producing isolates from the community is often associated with the presence of multiple ESBLs genes, as well as aminoglycoside and quinolone resistance genes, thereby limiting the choice of effective antimicrobial drugs. Studies on the causative agents of UTIs in Nigeria and their susceptibility to antimicrobials are lacking; particularly, there is paucity of information on the characterization of ESBLs genes in *Enterobacteriaceae* and *Pseudomonaceae* from patients with UTIs. Therefore, the study aimed to determine the presence and characteristics of extended spectrum β-lactamases in commonly isolated uropathogenic GNB in Nigeria.

Materials and Methods

Bacterial Isolates and Study Sites. A total of 50 non-duplicate unbiased GNB isolates consisting of *E. coli*, 19; *K. pneumoniae*, 21; and *Pseudomonas aeruginosa*, 10 were obtained from three tertiary hospitals in Nigeria. The distribution of the isolates from the hospitals is as follows: hospital I, 24; hospital II, 16; and hospital III, 10; these hospitals are located at Ibadan, Ogbomoso, and Osogbo, respectively, in South West Nigeria. Each

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isolate was obtained from a patient with significant bacteriuria diagnosed of varying specific and general UTIs ranging from pyelonephritis, cystitis, prostatitis, and so on. All isolates were speciated using Analytical Profile Index (API) 20E strips (BioMerieux, France) and conventional biochemical tests. The study was carried out at the Molecular Biology Laboratory of Department of Biomedical Science, Ladoke Akintola University of Technology, Ogbomoso, Osogbo campus.

Antibiotic Susceptibility Testing. The antibiotic susceptibility patterns of all the isolates to a panel of antibiotics were determined by the disk diffusion method (disks from Oxoid, UK) using Mueller-Hinton agar according to Clinical Laboratory Standards Institute (CLSI) guidelines [7]. Resistant isolates were further selected for susceptibility testing to β-lactam drugs such as ceftriaxone, ceftazidime or Amoxycillin-clavulanic acid using the agar doubling dilution method [8]. All runs included the control organisms such as E. coli (NCTC 10418) for Enterobacteriaceae and P. aeruginosa (NCTC 10662) for Pseudomonas species.

Phenotypic Detection of β-lactamases. Broth cultures of each test strain as well as ampicillin-resistant E. coli strain NCTC 10418 (carrying pUC18) were incubated overnight in 5 ml of Luria–Bertani broth. Then, 200 μl of the resulting overnight culture was transferred into the wells of a microtiter tray, and sterile broth was included as a negative control. Then, 10 μl of nitrocefin solution (Fisher Scientific, Loughborough, UK) prepared according to the manufacturer's instructions was added to each well. β-Lactamase production was inferred when the broth turned red within 30 min of addition of nitrocefin as directed by the manufacturer.

Detection of Extended-spectrum β-lactamases by the Double-disk Diffusion Test (DDDT). Suspensions of each test strain were prepared in sterile water to give an inoculum equivalent to a 0.5 McFarland standard before being used to inoculate the surface of Mueller-Hinton agar plates [8]. ESBL-positive K. pneumoniae ATCC 700603 and ESBL negative E. coli ATCC 25922 control strains were used in these experiments. A 30-μg ceftazidime disk (the best indicator for Temoneira (TEM)- and SHV-derived ESBLs) was placed on the left of each plate and a 30-μg cefotaxime disk (the best indicator for CTX-M types) was placed on the right; an AMC (20/10 μg) (Oxoid Ltd., Basingstoke, UK) was placed in the center between the other disks. The disks were placed 25–30 mm apart, center-to-center. Following overnight incubation in air at 37 °C, ESBL production was inferred when the zone of inhibition around the ceftazidime and cefotaxime disks was expanded by ≥5 mm by the presence of clavulanic acid.

Amplification of ESBL Genes. Polymerase chain reaction (PCR) was used to detect genes encoding resistance to β-lactams (blaOXA, blaSHV, blaCTX-M, blaVEB, blaPER, blaOXA-10, blaOXA-48, blaKPC, blaNDM, blaGES, blaVIM) as previously described (Table 1) [9, 10]. Amplimers resulting from these PCR reactions were sequenced at Institute of Microbiology and Infection,

University of Birmingham, Birmingham, UK to confirm the identity and specific variant of each gene identified and sequences were aligned to known reference sequences using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/help/index.html).

RAPD PCR Typing. The epidemiological relationships between multiple strains of E. coli, K. pneumoniae, and P. aeruginosa carrying ESBLs were analyzed according to species by randomly amplified polymorphic DNA (RAPD). The primers sequence and PCR running conditions used were according to Vogel et al. [11] modified to use 1 μl of 100 μmol of primers at a final concentration of 0.02 μmol [6]. The experiment was repeated twice for each species to ensure reproducibility. DNA fingerprints were compared by visual inspection to assign similar banding patterns to the same RAPD type.

Statistics Analysis. Data were analyzed using the statistical package within Microsoft Excel. χ^2 was used to determine the association between distribution of ESBL genes and hospitals. In this case, p value less than 0.05 was considered to be significant.

Results

Antimicrobial Susceptibility Testing. A total of 50 clinical isolates of GNB were obtained from three tertiary hospitals in South West Nigeria. These isolates were obtained from patients suffering from UTIs with significant bacteriuria. API and other biochemical tests characterized these GNB to be E. coli, K. pneumoniae, and P. aeruginosa. The results of antimicrobial susceptibility testing showed that colistin was more susceptible at 78% followed by imipenem, 72%. The cephalosporin class of antimicrobial agent such as ceftriaxone recorded 32% with ceftazidime having 30%. Surprisingly, tigecycline had the least susceptibility of 0% followed by ciprofloxacin 2% (Table 2). The minimum inhibitory concentrations (MICs) of ceftriaxone, ceftazidime, and amoxycillin-clavulanic acid showed that there

Table 2. Summary of antimicrobial disk susceptibility testing of 50 bacterial isolates

Antibiotics (μg/ml)	Sensitive	Intermediate	Resistance
Imipenem (10)	36 (72)	4 (8)	10 (20)
Meropenem (10)	21 (42)	12 (24)	17 (34)
Amoxycillin-clavulanic acid (30)	9 (18)	5 (10)	37 (74)
Ceftazidime (30)	15 (30)	5 (10)	30 (60)
Cefotaxime (30)	10 (20)	3 (6)	37 (74)
Cefpodoxime (10)	11 (22)	2 (4)	37 (74)
Ceftriaxone (30)	16 (32)	2 (4)	32 (64)
Nalidixic acid (30)	8 (16)	2 (4)	40 (80)
Ciprofloxacin (5)	1 (2)	7 (14)	42 (84)
Chloramphenicol	19 (38)	1 (2)	30 (60)
Colistin	38 (78)	0 (0)	11 (22)
Sulfonamide	6 (12)	1 (2)	43 (86)
Tigecycline	0 (0)	3 (6)	47 (94)

Numbers in parentheses are percentages

Table 1. Primer used for the amplification of β-lactamase genes

Forward primer	Sequence (5′-3′)	Reverse primer	Sequence (3′-5′)	Annealing temp. (°C)	Product size (bp)
CTX-M-1	GACGATGTCACTGGCTGAGC	CTX-M-1	AGCCGCCGACGCTAATACA	60	499
CTX-M-9	GCTGGAGAAAAGCAGCGGAG	CTX-M-9	GTAAGCTGACGCAACGTCTG	60	293
SHV	AGGATTGACTGCCTTTTTG	SHV	ATTTGCTGATTTCGCTCG	56	393
OXA	ATATCTCTACTGTTGCATCTCC	OXA	AAACCCTTCAAACCATCC	50	216
OXA-10	GTCTTTCGAGTACGGCATTA	OXA-10	ATTTTCTTAGCGGCAACTTAC	52	600
OXA-48	TTCGGCCACGGGAGCAAATCAG	OXA-48	GATGTGGGCATATCCATATTCATCGCA	56	240
PER-1	ATGAATGTCATTATAAAAGC	PER-1	AATTTGGGCTTAGGGCAGAA	51	590
VEB	CGACTTCCATTTCCCGATGC	VEB	GGACTCTGCACCAAATACGC	55	604
KPC	ATGTCACTGTATCGCCGTCT	KPC	TAGACGGCCAACACAATAGG	56	785
NDM	TTGATGCTGAGCGGGTG	NDM	CTGTCCTTGATCAGGCAGC	56	578
VIM	AGTGGTGAGTATCCGACAG	VIM	ATGAAAGTGCGTGGAGAC	56	261
GES	CGGTTTCTAGCATCGGGACACAT	GES	CCGCCATAGAGGACTTTAGCACAG	58	263

were no differences between the MIC_{50} and MIC_{90} of all the isolates. MIC_{50} and MIC_{90} of *K. pneumoniae*, *E. coli*, and *P. aeruginosa* were >128 µg/ml having 100% resistance in some cases (Table 3). These results indicate that the uropathogenic isolates were highly resistant strains.

Detection of Extended Spectrum β-lactamases. Twenty-one (42%) of the isolates were ESBL producers (Table 4). Polymerase chain reactions and sequencing identified varying ESBLs encoding genes for 24 isolates (48.0%). CTX-M-15 was the dominant gene with 20/24 of the isolates positive at 83.3%; multiple genes (2 to 6 ESBL genes) were found in 20 of the isolates. The isolates encoded other genes such as CTX-M-14, 33.3%; SHV variants, 58.3% (all SHV variants were encoded as a multiple gene); oxacillinase (OXA) variants, 70.8%; OXA-10, 29.2%; and Vietnamese extended β-lactamase (VEB) 1, 25.0%. Of these, 11/19 (57.9%) *E. coli*, 11/21 (52.4%) *K. pneumoniae*, and 2/10 (20%) *P. aeruginosa* carried these respective ESBL genes. However, none of the isolates was positive for any of the carbapenemase genes tested in this study. The ESBL genes were found in the three hospitals and the isolates in varying proportions including the clinical diagnosis. However, no association was found between hospitals and distribution of ESBL genes ($X^2 = 1.32$; $p = 0.85$; $p > 0.05$).

Typing of Isolates. The degree of clonality among the resistant isolates revealed high diversity among all the isolates tested with no identical RAPD or banding patterns observed except lanes 3 and 5 (Figure 1). This suggests that the resistance is underpinned by spread of resistance genes and not expansion of a dominant clone(s).

Discussion

CTX-M-15 was the dominant ESBL gene, followed by OXA, SHV, CTX-M-14, OXA-10, and VEB-1 in frequent order. This is consistent with the present antimicrobial resistance situation among *Enterobacteriaceae* where the CTX-M family has replaced TEM and SHV types and became the dominant ESBL in most parts of the world including Nigeria, where they are prevalent both in the hospital and community settings [6, 12, 13]. In Morocco, CTX-M was detected in 6 out of 7 ESBL-producing *E. coli* with a predominance of CTX-M-15 (6/6) [14], while in Cameroon all the ESBL-*E. coli* strains isolated from stool samples of women with UTIs contained group 1 CTX-M enzymes [15]. Similarly, CTX-M-15 dominated ESBLs in *Enterobacteriaceae* isolated from environmental samples in a hospital in Tunisia [16]. The high distribution of CTX-M-15 type ESBLs among these isolates

Table 3. Minimum inhibitory concentrations (MICs) of the 50 bacterial isolates

Isolate, n	Antimicrobial agents	MIC (0.06–128 µg/ml)		Sensitive	Intermediate	Resistant
		MIC_{50}	MIC_{90}			
Escherichia coli, 19	Ceftriaxone	>128	>128	0 (0.00)	1 (5.2)	18 (94.7)
	Ceftazidime	>128	>128	0 (0.00)	3 (15.7)	15 (78.9)
	Amoxycillin-clavulanic acid	>128	>128	0 (0.00)	0 (0.00)	19 (100)
Klebsiella pneumoniae, 21	Ceftriaxone	>128	>128	1 (4.8)	0 (0.00)	20 (95.0)
	Ceftazidime	>128	>128	0 (0.00)	0 (0.00)	21 (100)
	Amoxycillin-clavulanic acid	>128	>128	0 (0.00)	0 (0.00)	21 (100)
Pseudomonas aeruginosa, 10	Ceftriaxone	>128	>128	0 (0.00)	0 (0.00)	10 (100)
	Ceftazidime	>128	>128	0 (0.00)	0 (0.00)	10 (100)
	Amoxycillin-clavulanic acid	>128	>128	0 (0.00)	0 (0.00)	10 (100)

Numbers in parentheses are percentages

Table 4. Carriage of β-lactamase genes and their minimum inhibitory concentrations

ID no.	Clinical diagnosis	Isolate	MIC (µg/ml)			ESBL phenotype (DDST)	ESBLs genes
			CAZ	CRO	AMC		
AR7	UTI in pregnancy	*E. coli*	>128	>128	>128	−	CTX-M-15, OXA
AR8	UTI	*E. coli*	>128	>128	>128	−	OXA
AR11	Pelvic inflammatory disease	*E. coli*	128	>128	>128	−	OXA
AR12	Prostate enlargement	*E. coli*	>128	>128	>128	−	CTX-M-15, CTX-M-14
AR20	UTI	*E. coli*	>128	>128	>128	+	CTX-M-15, CTX-M-14, SHV, OXA, OXA-10, VEB-1
AR28	Cystitis	*E. coli*	>128	>128	>128	+	CTX-M-15, SHV, VEB-1, OXA
AR31	Prostatitis	*E. coli*	16	128	>128	+	CTX-M-15, CTX-M-9, SHV, OXA and OXA-10
AR33	Pelvic inflammatory disease	*E. coli*	8	8	>128	−	OXA, SHV and OXA-10
AR51	UTI	*E. coli*	>128	>128	>128	+	CTX-M-15
AR65	UTI	*E. coli*	>128	>128	>128	+	CTX-M-15, CTX-M-14, OXA and OXA-10
AR73	Urosepsis	*E. coli*	64	>128	>128	+	CTX-M-15, SHV
AR2	Prostatitis	*K. pneumoniae*	32	>128	>128	−	CTX-M-15, SHV, OXA
AR16	Chronic kidney disease	*K. pneumoniae*	>128	>128	>128	−	CTX-M-15, SHV, OXA
AR17	Burn injury	*K. pneumoniae*	>128	>128	>128	−	SHV, OXA
AR18	UTI	*K. pneumoniae*	>128	>128	>128	+	CTX-M-15, CTX-M-14, SHV, OXA, OXA-10, VEB-1
AR23	UTI	*K. pneumoniae*	>128	>128	>128	−	CTX-M-15, SHV, OXA
AR32	Glomerulonephritis	*K. pneumoniae*	128	>128	>128	−	CTX-M-15
AR54	Prostate enlargement	*K. pneumoniae*	>128	>128	>128	+	CTX-M-15, CTX-M-14, SHV, OXA
AR55	Urosepsis	*K. pneumoniae*	>128	>128	>128	+	CTX-M-15, CTX-M-14, SHV, OXA, OXA-10, VEB-1
AR71	Prostatitis	*K. pneumoniae*	>128	>128	>128	−	CTX-M-15, CTX-M-14
AR78	Pelvic inflammatory disease	*K. pneumoniae*	128	>128	>128	+	CTX-M-15, CTX-M-14
AR79	UTI	*K. pneumoniae*	32	>128	>128	+	CTX-M-15, SHV-
AR26	Pyelonephritis	*P. aeruginosa*	>128	>128	>128	+	CTX-M-15, SHV, OXA and VEB-1
AR68	Pyelonephritis	*P. aeruginosa*	>128	>128	>128	+	CTX-M-15, OXA, OXA-10, VEB-1

CTX-M-1, cefotaximase-Munich-1; OXA, oxacillinase; VEB, Vietnamese extended β-lactamase; MIC, minimum inhibitory concentration; EC, *Escherichia coli*; KP, *Klebsiella pneumoniae*; PA, *Pseudomonas aeruginosa*; DDST, double disc synergyst; +, positive; −, negative
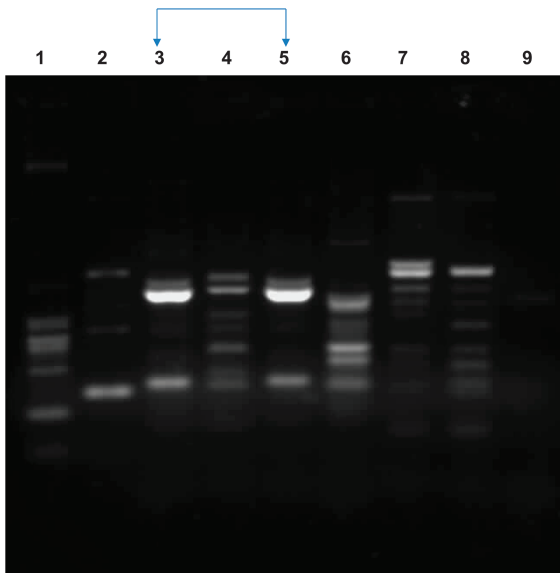
Figure 1. Representative RAPD typing of *E. coli* isolates showing different banding patterns. Lanes 1 to 9 showed the banding patterns of each isolates; no identical banding patterns were observed except for lanes 3 and 5

explains the high rate of resistance to cephalosporin such as cefotaxime, ceftriaxone, and ceftazidime in our study.

CTX-M-15 co-existed with either OXA-10 or VEB-1 in all the isolates of *E. coli, Klebsiella* species, and *P. aeruginosa*. VEB-1 was first described in an *E. coli* isolate from Vietnam in 1998 [17]. Ever since, it has been reported from various parts of the world in different species of *Enterobacteriaceae* [18], including Nigeria where Aibinu *et al.* reported the emergence of OXA-10, VEB-1, and Cephamycins (CMY) β-lactamases and mobile elements of clinical *Providencia* isolates from a catheter tip of a patient in Lagos [19]. To the best of our knowledge, this is the first report of VEB-1 and OXA-10 in clinical isolates of *E. coli*, *Klebsiella* species, and *P. aeruginosa* from Nigeria. OXA-10 is an important serine β-lactamase because it is inhibited only weakly by clavulanic acid. Interestingly, the risk of widespread ESBL-producing strains among uropathogens not only rendered oxyimino-cephalosporins ineffective but also pose a therapeutic challenge since they are frequently resistant to other kinds of antimicrobial drugs, including aminoglycosides, quinolones, and co-trimoxazole [20]. There is no association between ESBL genes in the isolates and the various hospitals suggesting that the resistance genes spread across without any peculiar determinant and the RAPD typing found no major clonal or epidemiological relationship which suggested the occurrence and dissemination of a plasmid bla_{ESBL}.

It is common knowledge that production of ESBLs is the most common mechanism of resistance to third-generation cephalosporins among *Enterobacteriaceae* including *K. pneumoniae* and *E. coli* and mediates multidrug resistance as shown in this study. There is high-level resistance to various other antibiotics including the unprecedented high-level resistance to tigecycline and the level of resistance to colistin and meropenem, a last resort carbapenem antibiotic against GNB. These findings have significant implications for the management of patients with urinary tract infections using third-generation cephalosporins or fluroquinolones, including carbapenems. Carbapenem resistance development in isolates producing CTX-M and other ESBLs due to selection of mutants lacking expression of outer membrane porins has been reported [21, 22]. Resistance of UTI pathogens to the panels of antibiotics may not be unconnected with their frequent prescription in the hospital, their easy availability in the community without prescription, and their relative affordability which make them subject to abuse.

Conclusions

The high-level multidrug resistance of uropathogens to third-generation cephalosporins including other antibiotics used in this study is strongly associated with carriage of ESBLs found in this study, predominantly CTX-M-15, as well as CTX-M-14, OXA-10, and VEB-1. A combination therapy is recommended for use guided by antimicrobial susceptibility testing, and we should furthermore exercise restraint in using or prescribing carbapenems or colistin to prevent selection of resistant isolates.

Authors' contribution

D.O.O. has the study concept, designed the work, interpreted the data, and prepared the article. O.A.T.A. codesigned, supervised, and prepared the article. M.A.W. codesigned and analyzed certain aspect of the data. A.S.O. supervised and also contributed in data analysis. O.M.O. analyzed the data and prepared the article. All authors have full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of interest

The authors declare no conflict of interest.

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