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CO-PRODUCTION OF EXTENDED-SPECTRUM BETA-LACTAMASES AND METALLO BETA-LACTAMASES AMONG MULTI-DRUG RESISTANT GRAM-NEGATIVE BACTERIA ISOLATES COLLECTED FROM TERTIARY HOSPITALS IN OYO STATE, NIGERIA

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ABSTRACT

Extended-spectrum beta-lactamases (ESBLs) and metallo beta-lactamases (MBLs) are compromising the chemotherapeutic use of cephalosporins and carbapenems respectively. This study investigated the burden of ESBLs and MBLs co-production among multi-drug resistant (MDR) Gram-negative bacteria collected from two tertiary hospitals in Oyo State. A total of 240 non-duplicated clinical isolates of *Escherichia coli*, *Klebsiella* spp. and *Pseudomonas* spp. were collected from the Microbiology units of two tertiary hospitals in Oyo State and their identities authenticated using standard identification techniques. Antimicrobial susceptibility testing was carried out by disc-diffusion method and isolates exhibiting resistance to ≥3 classes of antibiotics selected as MDR strains. ESBL and MBL production was detected by double-disc synergy test (DDST) and combined-disc-diffusion test (CDDT) respectively. Selected beta-lactamase genes were detected by PCR, amplicons sent out for sequencing and phylogenetic tree of the sequences constructed using Mega X software. MDR was exhibited by 43.8% of the isolates. ESBLs and MBLs were produced by 32.4% and 7.6% of the MDR isolates respectively. Co-production of ESBL and MBL was observed in 6.7% of the MDR isolates. *Bla*$_{CTX\text{-}M\text{-}15}$ (67.7%), *bla*$_{TEM\text{-}1}$ (55.9%), *bla*$_{SHV\text{-}1}$ (47.1%), co-existing *bla*$_{TEM}$ + *bla*$_{SHV}$, *bla*$_{TEM}$ + *bla*$_{CTX\text{-}M}$, *bla*$_{CTX\text{-}M}$ + *bla*$_{SHV}$ (each in 5.9%) and *bla*$_{CTX\text{-}M}$ +*bla*$_{TEM}$ + *bla*$_{SHV}$ (26.5%) were detected among the ESBL-producers. MBL genes were not detected among the MBL-producers. Only *bla*$_{TEM\text{-}1}$ sequences showed two different claudes on the phylogenetic tree. The occurrence of MDR isolates co-harbouring different classes of beta-lactamse genes observed in this study is of public health concern and hence, requires stricter control of antibiotic use.

Keywords: Extended-spectrum β-lactamases (ESBLs), metallo-β-lactamases (MBLs), multidrug resistance (MDR), gram-negative bacteria.

INTRODUCTION

Antibiotics resistance is a major health problem worldwide with serious impact on cost of care, morbidity, mortality and the length of hospital stay of patients (Carlet *et al.*, 2012, Prestinaci *et al*., 2015; Founou *et al*., 2017; Shrestha *et al*., 2018). The development of antibiotic resistance has been attributed to various factors including selective pressure imposed by use and overuse of antibiotics in human medicine, agriculture and veterinary medicine among others (Hao *et al*., 2014; Chang *et al*., 2015; Blaser, 2016). The overuse and misuse of antibiotics in both clinical and community settings has led to the emergence of multi-drug resistance among microorganisms which are occasionally implicated in many infectious outbreaks worldwide (Alabi *et al*., 2017; Chaw *et al*., 2018; Chokshi *et al*., 2019). The lack of frequent monitoring of the use of antibiotics and lack of routine assessments of the potencies and efficacies of the various brands of antimicrobial agents available in the pharmaceutical markets, particularly in developing countries, have led to increase in antibiotic resistance (Lucet *et al*., 1999; Ayukekbong *et al*., 2017; Chokshi *et al*., 2019). In most developing countries like Nigeria, drugs could be brought into the country illegally through the back door without evaluation by relevant regulatory bodies. Some unpatriotic citizens out of the quest to get rich overnight engaged in adulteration and counterfeiting of drugs, particularly antibiotics, and thus exerting a huge impact on the development of antibiotic resistance (Glass, 2014; Kelesidis and Falagas, 2015).

The beta-lactam antibiotics are group of antibiotics widely used in the treatment of infections in both community and clinical settings, and they are divided into narrow, broad and extended-spectrum beta-lactams. While it has been well established that the narrow and some of the broad-spectrum classes of these group of antibiotics are now mostly ineffective clinically due to the emergence of the conventional beta-lactamases (i.e. the class of enzymes that inactivates the narrow spectrum antibiotics), the extended-spectrum beta-lactam antibiotics, such as the third generation cephalosporins (cefotaxime, ceftazidime, ceftriaxone) and the monobactams (aztreonam), developed to overcome the resistance conferred by the conventional beta-lactamases are gradually becoming less effective against most bacterial pathogens due to the advent of superior beta-lactamases known as extended-spectrum beta-lactamases (ESBLs) that hydrolyses the beta-lactam ring structure of the antibiotics (Alabi *et al*., 2016). The carbapenems, which were beta-lactam antibiotics of last resort commonly used in the treatment of the extended-spectrum beta-lactamase (ESBL) producing bacteria pathogens are also gradually becoming ineffective against some of these

pathogens due to the development of a more superior beta-lactamases generally named carbapenemases part of which are the metallo beta-lactamases (MBLs) (Gaude and Hattiholli, 2013; Worthington and Melander, 2013; Levashov, 2014, Balsalobre *et al*., 2019, Mirza *et al*., 2019).

Microbial resistance as a result of ESBL production was first reported in Europe and subsequently in the United States in a single isolate of *Klebsiella ozaenae* (Bradford, 2001) while MBL was initially reported in a clinical isolate of *Serratia marcescens* conferring resistance to imipenem (Jiang *et al*., 2018). Since then, they have emerged in other countries of the world including Nigeria among Gram-negative bacteria particularly the Enterobacteriaceae (Mendonca *et al*., 2007; Olowe *et al*., 2012) and *Pseudomonas* spp. (Oberoi *et al*., 2013; Kaur and Singh, 2018). Although, the genes coding for these enzymes are mostly borne on resistance plasmids and thus are transferable to other genera or species (Bradford, 2001; Kaur and Singh, 2018; Teklu *et al*., 2019), some have been reported to be on the chromosome (Mahrouki *et al*., 2011; Song *et al*., 2011).

The ESBL enzymes are known to be ineffective against the cephamycin (cefoxitin, cefotetan) and carbapenem (imipenem, meropenem, ertapenem) classes of the beta-lactam antibiotics and hence, the use of carbapenems as the antibiotic of last resort against the ESBL producing bacteria (Teklu *et al*., 2019). The emergence of the carbapenemases or metallo beta-lactamases (MBLs) known to hydrolyse the carbapenems and all other beta-lactams, thereby conferring resistance against the antibiotics, is of serious clinical and therapeutic problem (Pitout and Laupland, 2008; Bora *et al*., 2014; Nepal *et al*., 2017; Kaur and Singh, 2018).

The scenario of having a microorganism producing different variants of ESBL or MBL and even co-producing both will definitely be of significant public health concern because of their effects on the therapeutic options and treatment outcomes. This study, therefore, screened MDR Gram-negative bacteria genera from two selected tertiary hospitals in Oyo state, for the production of ESBLs and MBLs and further determined the occurrence of ESBL and MBL genes among these bacteria isolates.

MATERIALS AND METHODS

Ethical Approval

There was no direct contact or an invasive procedure involved in this study and thus ethical approval is not required. Clinical isolates that are already isolated and characterized from patients that visited the out-patient sections of the teaching hospitals were collected from the microbiology laboratories of the two institutions involved in the study.

Study design and setting

This was a laboratory-based cross-sectional study involving Microbiology laboratory units of the two tertiary hospitals in Oyo State: University College Hospital (UCH), Ibadan and Ladoke Akintola University of Technology Teaching Hospital (LTH), Ogbomoso, Oyo State, Nigeria. The Gram-negative clinical isolates used in this study were collected using convenient sampling technique from the Microbiology laboratory units of the two tertiary hospitals and the research bench work conducted at the Molecular and Microbiology laboratory of the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Ibadan, Nigeria.

Collection of isolates

A total of 240 non-duplicated Gram-negative bacteria were collected between September and November, 2018 by convenience sampling on Nutrient agar (Oxoid Ltd, UK) slants at the Microbiology laboratories of University College Hospital (UCH), Ibadan and Ladoke Akintola University of Technology Teaching Hospital (LTH), Ogbomoso, Oyo state, Nigeria and transported to the main laboratory of the Department of Pharmaceutical Microbiology, University of Ibadan within 12 hours of collection for incubation at 37ºC for 24 hours. From the individual laboratory records, the entire isolates included in this study were from patients who visited the out-patient clinic of the hospitals and the isolates were recovered previously from different specimens such as urine, wound, ear swabs, sputum, catheter tips, pus, semen, aspirate, blood, high vagina swabs, stool, eye swabs and pleural fluid.

Confirmation of the identities of isolates

After 24 hours of incubation of the isolates collected on slants, they were sub-cultured on different selective media to observe their cultural characteristics and purity. Presumptive *E coli* collected were sub-cultured on MacConkey and Eosin Methylene Blue Agar (Oxoid Ltd, UK) to observe for the pink and green metallic sheen colonies respectively, characteristic of *E coli*. Presumptive *Pseudomonas* spp. were sub-cultured on cetrimide agar which permits the growth of the bacteria only. Presumptive *Klebsiella* spp. were sub-cultured on MacConkey agar to observe for the pink, mucoid colonies, characteristic of the bacteria. All the streaked plates were incubated in an inverted position for 24 hours at 37ºC.The emergent pure colonies were further subjected to standard biochemical test to confirm their identities. Confirmed isolates were streaked on fresh agar slants, incubated at 37ºC for 24 hours and kept at -4ºC for further studies.

Antibiotics susceptibility test

The confirmed isolates were subjected to antimicrobial susceptibility testing against eleven antibiotics using the disc-diffusion method on Mueller-Hinton agar. The selected antibiotics are amoxicillin-clavulanic acid (20/10µg), cefotaxime (30µg), ceftazidime (30µg), cefoxitin (30µg), cefepime (30µg), aztreonam (30µg), gentamicin (10µg), ciprofloxacin (5µg), imipenem (10µg), meropenem (10µg) and ertapenem (10µg). Briefly, an overnight culture of each isolate was suspended in sterile normal saline and the turbidity adjusted to 0.5 McFarland standard by dilution. With the aid of sterile cotton swab, each bacterial suspension was inoculated on the dried-surface of Mueller-Hinton agar plate to form a single cell layer lawn. With the aid of a sterile forcep, each of the antibiotic disc was transferred on the inoculated agar plates at equidistance, allowed to stand on the bench for pre-incubation diffusion for 1 hour and then incubated in an inverted position for 24 hours at 37ºC. The Clinical Laboratory Standard Institute (CLSI, 2017) breakpoints for interpreting antibiotic susceptibility test results as resistant, intermediate or sensitive was used and isolates that exhibited resistance to three or more classes of antibiotics were selected as multidrug resistant (MDR) isolates (Magiorakos *et al*., 2011).

Phenotypic detection of ESBL production

All isolates that exhibited resistance to all the third generation cephalosporins based on the CLSI breakpoint guideline were screened for ESBL production by double-disc synergy test (DDST). Briefly, bacterial suspension of overnight culture diluted to 0.5McFarland standard was inoculated on to the dried-surface of Mueller-Hinton agar plate by surface spreading as described previously above. With the aid of sterile forcep, discs (Oxoid Ltd, UK) of cefotaxime (30µg), ceftazidime (30µg) and cefepime (30µg) were placed at 20mm distance centre-to-centre around amoxicillin-clavulanic acid (20/10µg) disc, allow for pre-incubation diffusion for 1hour and then incubated in an inverted position for 24 hours at 37ºC. Isolates that exhibited enhanced susceptibility to one or more of the cephalosporin antibiotics (i.e. enhanced zone of inhibition) were selected as potential producers of ESBL.

Phenotypic detection of MBL production

Bacterial isolates that exhibited resistance to all or any of the three carbapenem antibiotics used in this study were screened for the production of MBL using the combined-disc-diffusion test (CDDT) method as described by Lee *et al.*(2003) and Pitout *et al.* (2005). The CDDT was performed using discs of imipenem (10µg), meropenem (10µg) and ertapenem (10µg) alone and a second set of the discs to which 750 µg EDTA has been added to make carbapenem+EDTA combined discs. An increase in the zone diameter of ≥7 mm around any or all of the carbapenem+EDTA discs when compared to that of the carbapenem discs alone was considered positive for MBL production.

Template DNA extraction

The DNA of the ESBL- and MBL-producers was extracted by boiling method as described by Mendonca *et al* (2007). Briefly, pure colonies of the ESBL- and MBL-producer were dispersed each into 500µL of sterile distilled deionized water in an eppendorf tube. The inoculum was vortexed to give homogenous bacterial suspension. The suspension was then centrifuge at 10 RCF for 3mins and the supernatant was carefully discarded leaving the bacterial suspension. A 100 µL of fresh sterile deionized water was added into the bacterial cell pellet and re-suspended by vortexing. A small hole was made on the cap of the eppendorf tube and then boiled at 100^{O}C for 15mins. The eppendorf tube was then centrifuged for 5mins at 10 RCF and 50 µL of the supernatant was picked with a sterile micropipette into a new sterile eppendorf tube. The DNA was stored at -20^{O}C for PCR analysis.

PCR detection of ESBL and MBL genes

For amplification of all the beta-lactamase genes, a reaction volume of 20 µL containing 10 µL of pre-mixed master mix (Inqaba Biotech, SA), 8 or 7µL of sterile distilled deionized water (for simplex and multiplex reactions respectively), 0.5µL of each of the forward and reverse primers and 1µL of the template DNA was prepared. For *bla*$_{\text{CTX-M}}$ gene (Forward: TTT GCG ATG TGC AGT ACC AGT AA and Reverse: CGA TAT CGT TGG TGG TGC CAT A), conditions for the simplex PCR protocol was 5 minutes at 94^{0}C followed by 31 cycles of 1_minute at 94^{0}C, 1_minute at 56^{0}C, 1 minute at 72^{0}C and concluded by 10 minutes at 72^{0}C. For *bla*$_{\text{TEM}}$ (Forward: GAGTATTCAACATTTTCGT and Reverse: ACCAATGCTTAATCAGTGA) and *bla*$_{\text{SHV}}$ (Forward: TCGCCTGTGTATTATCTCCC and Reverse: CGCAGATAAATCACCACAATG), conditions for the multiplex PCR protocol include 5 min at 94°C, followed by 30 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 1.5 min. For *bla*$_{\text{NDM-1}}$ gene (Forward: GGTTTGGCGATCTGGTTTTC, Reverse: CGGAATGGCTCATCACGATC), the PCR conditions was 10 min at 94°C; 36 cycles of amplification consisting of 30 sec at 94°C, 40 sec at 52°C, and 50 sec at 72°C; and 5 min at 72°C for the final extension. For *bla*$_{\text{IMP}}$ (Forward: TTTCATATGGCAGAGTCTTTGCCAGATT, Reverse: ATCCTAGAAATTTAGTTGCTTGGTT) and *bla*$_{\text{VIM}}$ (Forward: ATGTTCAAACTTTTGAGTAAG, Reverse: CTACTCAACGACTGAGCG), multiplex PCR conditions include 95°C for 5 min, followed by 30 cycles at 95°C for 1 min, annealing at 45°C for 1 min, an extension at 68°C for 1 min, ending with incubation for 5 min at 68°C. For *bla*$_{\text{KPC}}$ gene (Forward: ATGTCACTGTATCGCCGTC, Reverse: CTCAGTGCTCTACAGAAAACC), the PCR conditions include 5 min at 95°C, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 30sec, extension at 72°C for 1 min 30 sec and final extension at 72°C for 10 min. The PCR products were visualized on 1.5% Agarose gel stained with EZ vision and visualized with UVP® GelMax® UV Gel Imager. The expected amplicon sizes of *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{NDM-1}}$, *bla*$_{\text{VIM}}$, *bla*$_{\text{IMP}}$ and *bla*$_{\text{KPC}}$ are 857, 768, 543, 621, 741, 801 and 872 bp respectively. The amplicons were sent out to Inqaba Biotechnology Laboratory, South Africa, for sequencing. Three sequences each, of *bla*$_{\textit{TEM-1}}$ (Accession Nos.: MT636821.1; MT636815.1 & NG050145.1), *bla*$_{\textit{SHV-1}}$ (Accession Nos.: MK482385.1; MK099071.1 & FJ668802.1) and *bla*$_{\textit{CTX-M-15}}$ (Accession Nos.: KY640567; KY640574.1 & NG048935.1) genes were selectively downloaded from genbank on NCBI website and combined with the sequences of the amplicons from this study to construct a phylogenetic tree by the neighbor-joining method to compare the sequences using the Molecular Evolutionary Genetics Analysis (MEGA) X software version 10.0.4.

RESULTS

Out of the 240 isolates collected, 24.2% (58/240) were from Ladoke Akintola University of Technology teaching hospital (LTH) while 75.8% (182/240) were from University College Hospital (UCH). The isolates were recovered from urine (32.9%), wounds (20.8%), ear swabs (11.3%), sputum (8.3%), catheter tips (4.6%), pus (4.2%), semen (3.8%), aspirate (3.3%), blood (2.9%), vagina swabs (2.5%), stool (2.5%), eye swabs (1.7%) and pleural fluid (1.3%) with higher percentage of each of the bacterial specie collected from UCH as presented in table 1.

Excluding cefotaxime, cefoxitin and ertapenem against *Pseudomonas* spp., the results of the antibiotic susceptibility test showed that 73.8% of the isolates were resistant to amoxicillin-clavulanic acid, 58.8% to gentamicin, 49.6% to cefotaxime, 48.8% to aztreonam, 47.9% to ciprofloxacin and 45.4% to ceftazidime. Resistances to imipenem, meropenem and ertapenem were exhibited by 14.2%, 8.3% and 7.9% of the isolates (Table 2) In this study, 43.8% of the isolates exhibited MDR phenotype cumulatively. including 28.8%, 53.8% and 48.8% of the *Pseudomonas* spp., *Klebsiella* spp. and

E. coli isolates respectively (Figure 1). ESBL production was detected in 32.4% of the MDR isolates cumulatively, including 33.3%, 44.2% and 8.7% of the *E coli*, *Klebsiella* spp. and *Pseudomonas* spp. resistant to the cephalosporins respectively. Production of MBL was detected in 7.6% of the MDR isolates cumulatively, including 7.7%, 4.7% and 13% of the *E coli*, *Klebsiella* spp. and *Pseudomonas* spp. resistant to the carbapenems (Figure 2). Co-production of ESBL and MBL was observed phenotypically in 6.7% of the MDR isolates (Figure 3). The PCR screening for the presence of ESBL and MBL genes detected 67.7% of the isolates harbouring $bla_{\text{CTX-M}}$, 55.9% harbouring bla_{TEM} and 47.1% habouring bla_{SHV} among the ESBL producers. Relatively among each of the genera, 50% of the ESBL producing *Pseudomonas* spp. harboured bla_{TEM} and $bla_{\text{CTX-M}}$, 53.8% of ESBL producing *E coli* harboured $bla_{\text{CTX-M}}$ while *Pseudomonas* spp. and *E coli* did not harbour bla_{SHV} (Figure 4). Co-existence of bla_{TEM} +bla_{SHV}, bla_{TEM}+bla_{CTX-M} and bla_{CTX-M}+bla_{SHV} was detected in 5.9% of the ESBL producers in each case while 26.5% co-harboured bla_{TEM}+bla_{SHV}+bla_{CTX-M} genes. Coexistence of bla_{TEM}+bla_{SHV}, bla_{CTX-M}+bla_{TEM} and bla_{CTX-M}+bla_{SHV} was detected each in 10.5% of the ESBL producing *Klebsiella* spp. while bla_{TEM}+bla_{SHV}+bla_{CTX-M} coexisted in 31.6% and 23.1% of ESBL producing *Klebsiella* spp. and *E coli* respectively (Figure 4). The ESBL genes and MBL enzymes detected in this study are harboured by isolates mostly from urine (37.1%), wound (31.4%), catheter tip (8.6%), stool (5.7%), sputum (5.7%), aspirate (5.7%), HVS (2.9%) and blood (2.9%) (Table 3). Analysis of the amplicons' sequences on National Center for Biotechnology Information (NCBI) BLAST website identified bla_{TEM} as $bla_{\text{TEM-1}}$, bla_{SHV} as $bla_{\text{SHV-1}}$ and $bla_{\text{CTX-M}}$ as $bla_{\text{CTX-M-15}}$.The PCR amplification of the selected MBL genes (bla_{IMP}, bla_{VIM}, $bla_{\text{NDM-1}}$ and bla_{KPC}) did not give amplicons. The phylogenetic tree reflected close relationship in the sequences of each type of the ESBL genes sequenced and those downloaded from genbank on the NCBI website. However, for the bla_{TEM-1} genes, out of the 19 sequences, 5 (26.3%) showed closed similarities with the three downloaded bla_{TEM-1} sequences while 14 (73.7%) showed closed similarities but with some level of differences from the downloaded and the five sequences as shown in figure 5.

Table 1: Distribution of isolates among the two tertiary hospitals and specimen types

Variables		N/% (N = 240)	Distribution (n/%) by hospitals and specimens		
			Pseudomonas spp.	*Klebsiella* spp.	*Escherichia coli*
Hospital	LTH	58 (24.2)	12 (20.6)	23 (39.7)	23 (39.7)
	UCH	182 (75.8)	68 (37.4)	57 (31.3)	57 (31.3)
	Total (N/%)	240 (100.0)	80 (33.3)	80 (33.3)	80 (33.3)
Specimen	Urine	79 (32.9)	19 (24.1)	16 (20.3)	44 (55.6)
	Wound swab	50 (20.8)	19 (38)	21 (42)	10 (20)
	Ear swab	27 (11.3)	23 (85.2)	3 (11.1)	1 (3.7)
	Sputum	20 (8.3)	0 (0)	20 (100)	0 (0)
	Catheter tip	11 (4.6)	8 (72.7)	1 (9.1)	2 (18.2)
	Pus	10 (4.2)	0 (0)	4 (40)	6 (60)
	Semen	9 (3.8)	2 (22.2)	3 (33.3)	4 (44.4)
	Aspirate	8 (3.3)	1 (12.5)	5 (62.5)	2 (25)
	Blood	7 (2.9)	3 (42.8)	2 (28.6)	2 (28.6)
	HVS	6 (2.5)	2 (33.3)	2 (33.3)	2 (33.3)
	Stool	6 (2.5)	0 (0)	0 (0)	6 (100)
	Eye swab	4 (1.7)	2 (50)	2 (50)	0 (0)
	Pleural fluid	3 (1.3)	1 (33.3)	1 (33.3)	1 (33.3)

Legend: LTH – Ladoke Akintola University of Technology Teaching Hospital; UCH – University College Hospital

Table 2: The percentage resistance pattern of the clinical isolates against selected antibiotics

Antibiotics	Resistance pattern (n/%)			
	Pseudomonas spp. (n = 80)	*Klebsiella* spp. (n = 80)	*Escherichia coli* (n = 80)	Total Resistance (N=240)
Amoxicillin-clavulanic acid (AMC) – 20/10µg	79 (98.8)	34 (42.5)	36 (45)	177 (73.8)
Cefotaxime (CTX)- 30µg	ND	63 (78.8)	55 (68.8)	119 (49.6)
Ceftazidime (CAZ) - 30µg	10 (12.5)	56 (70)	41 (51.3)	109 (45.4)
Cefepime (CFP) - 30µg	13 (16.3)	42 (52.2)	35 (43.8)	90 (37.5)
Cefoxitin (FOX) - 30µg	ND	34(42.5)	22 (27.5)	56 (23.3)
Azetreonam (AT) - 30µg	12 (15)	58 (72.5)	44 (55)	117 (48.8)
Gentamicin (GN) - 10µg	46 (57.5)	63 (78.8)	43 (53.8)	141 (58.8)
Ciprofloxacin (CIP) - 5µg	26 (32.5)	51 (63.8)	47 (58.8)	115 (47.9)
Imipenem (IMP) - 10µg	8 (10)	13 (16.3)	15 (18.8)	34 (14.2)
Meropenem (MEM) - 10µg	5 (6.3)	6 (7.5)	9 (11.3)	20 (8.3)
Ertapenem (ETP) - 10µg	ND	7 (8.8)	12 (15)	19 (7.9)

Legend: ND: Not Done

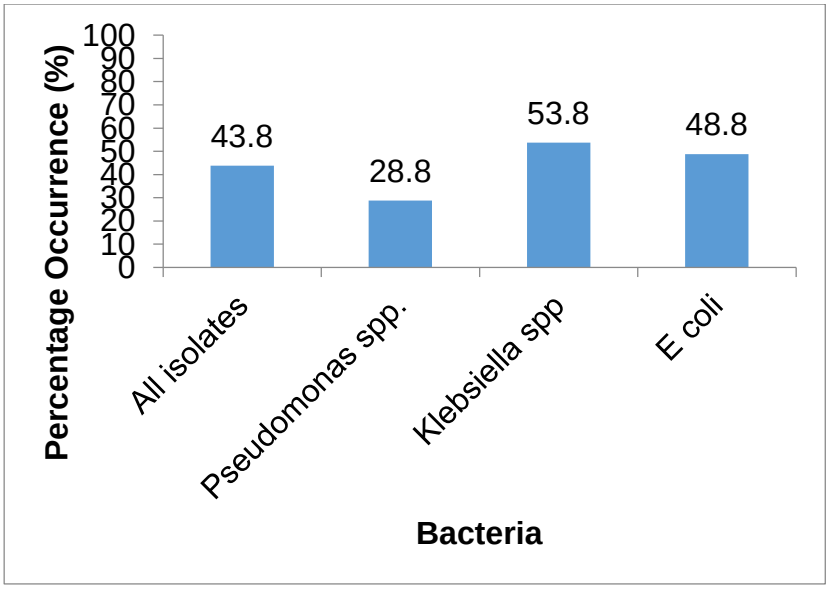

Figure 1: Percentage distribution of MDR phenotype among the isolates

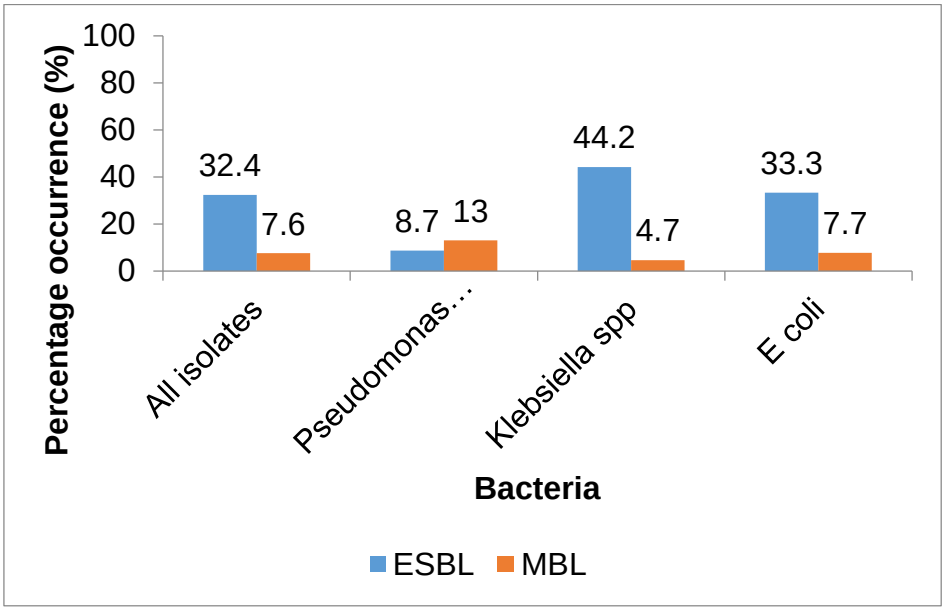

Figure 2: Percentage distribution of ESBL and MBL producers among the MDR isolates

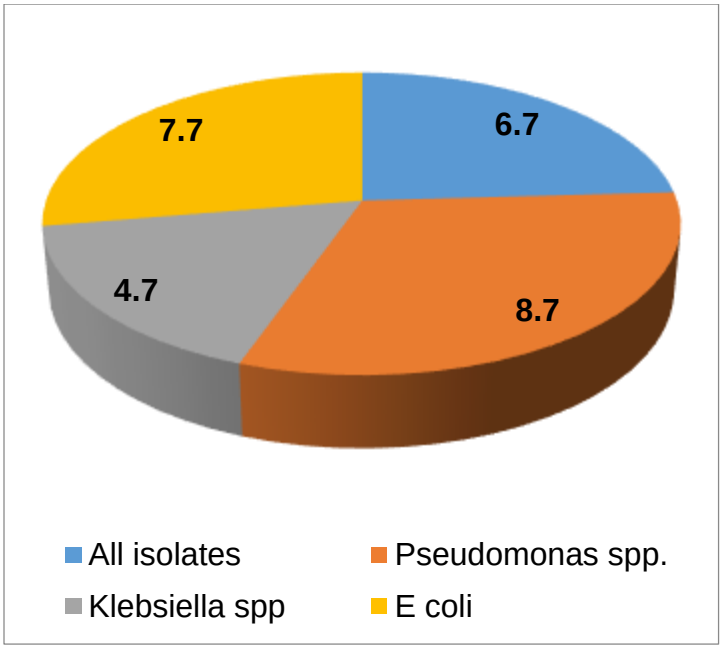

Figure 3: Percentage distribution of co-producers of ESBL and MBL among the MDR isolates

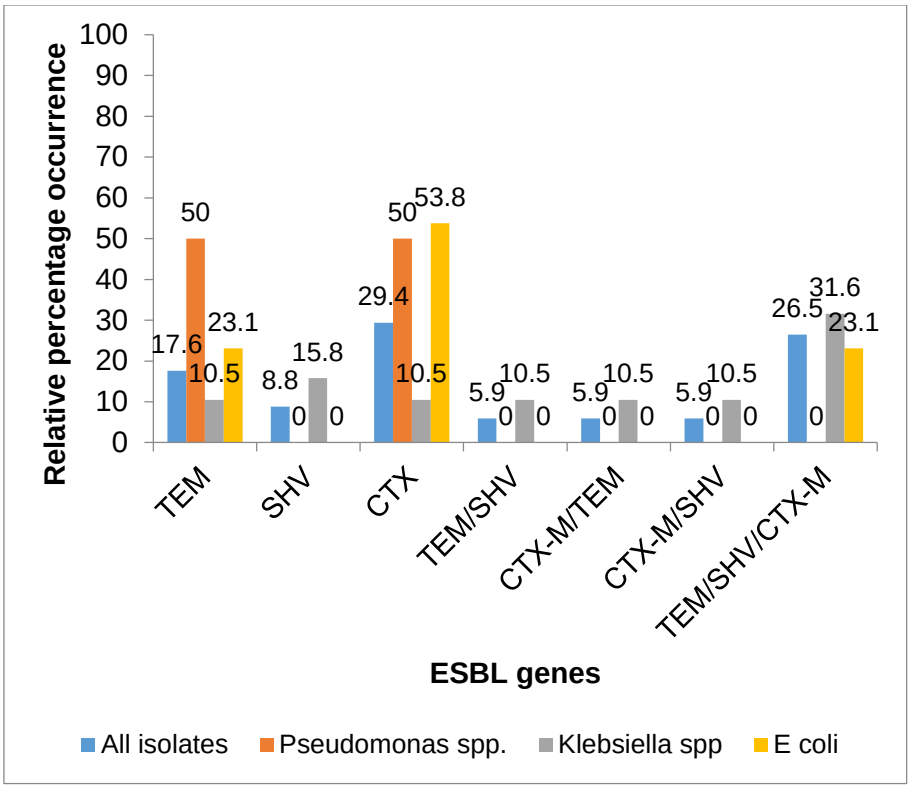

Figure 4: Relative percentage distribution of ESBL genes among the producing isolates

Table 3: Distribution of MDR, ESBL, MBL phenotypes and the beta-lactamase genes among the clinical isolates and the specimens in the two tertiary hospitals

Isolate ID	Hosp.	Sample	Phenotypes			Genotypes
			MDR	ESBL	MBL	Genes
EC008	LTH	Urine	+	+	-	TEM
EC009	LTH	Wound	+	+	-	CTX-M
EC012	LTH	Urine	+	+	-	TEM,SHV,CTX-M
EC200	LTH	Urine	+	+	-	CTX-M
EC030	LTH	Wound	+	+	-	CTX-M
EC033	UCH	Urine	+	+	+	TEM,SHV, CTX-M
EC046	UCH	Urine	+	+	+	TEM,SHV,CTX-M
EC057	UCH	HVS	+	+	-	CTX-M
EC064	UCH	Wound	+	+	+	CTX-M
EC080	UCH	Urine	+	+	-	TEM
EC084	UCH	Stool	+	+	-	CTX-M
EC467	UCH	Wound	+	+	-	CTX-M
EC963	UCH	Urine	+	+	-	TEM
KL005	LTH	Sputum	+	+	-	CTX-M
KL120	LTH	Aspirate	+	+	-	SHV
KL012	LTH	Stool	+	+	-	CTX-M
KL140	LTH	Urine	+	+	+	TEM,SHV,CTX-M
KL200	LTH	Wound	+	+	-	TEM
KL022	LTH	Wound	+	+	-	SHV
KL250	LTH	Aspirate	+	+	-	TEM
KL025	LTH	Blood	+	+	-	TEM,SHV,CTX-M
KL077	UCH	Urine	+	+	-	TEM,SHV,CTX-M
KL081	UCH	Sputum	+	+	+	SHV,CTX-M
KL433	UCH	Wound	+	+	-	SHV
KL435	UCH	Urine	+	+	-	TEM,SHV,CTX-M
KL698	UCH	Urine	+	+	-	TEM,SHV,CTX-M
KL786	UCH	Catheter tip	+	+	-	TEM,SHV,CTX-M
KL860	UCH	Wound	+	+	-	TEM,SHV
KL887	UCH	Catheter tip	+	+	-	TEM, CTX-M
KL889	UCH	Wound	+	+	-	TEM,CTX-M
KL914	UCH	Wound	+	+	-	SHV,CTX-M
KL926	UCH	Urine	+	+	-	TEM,SHV
PS088	LTH	Wound	+	+	+	TEM
PS918	UCH	Urine	+	+	+	CTX-M
PS436	UCH	Catheter tip	+	-	+	-

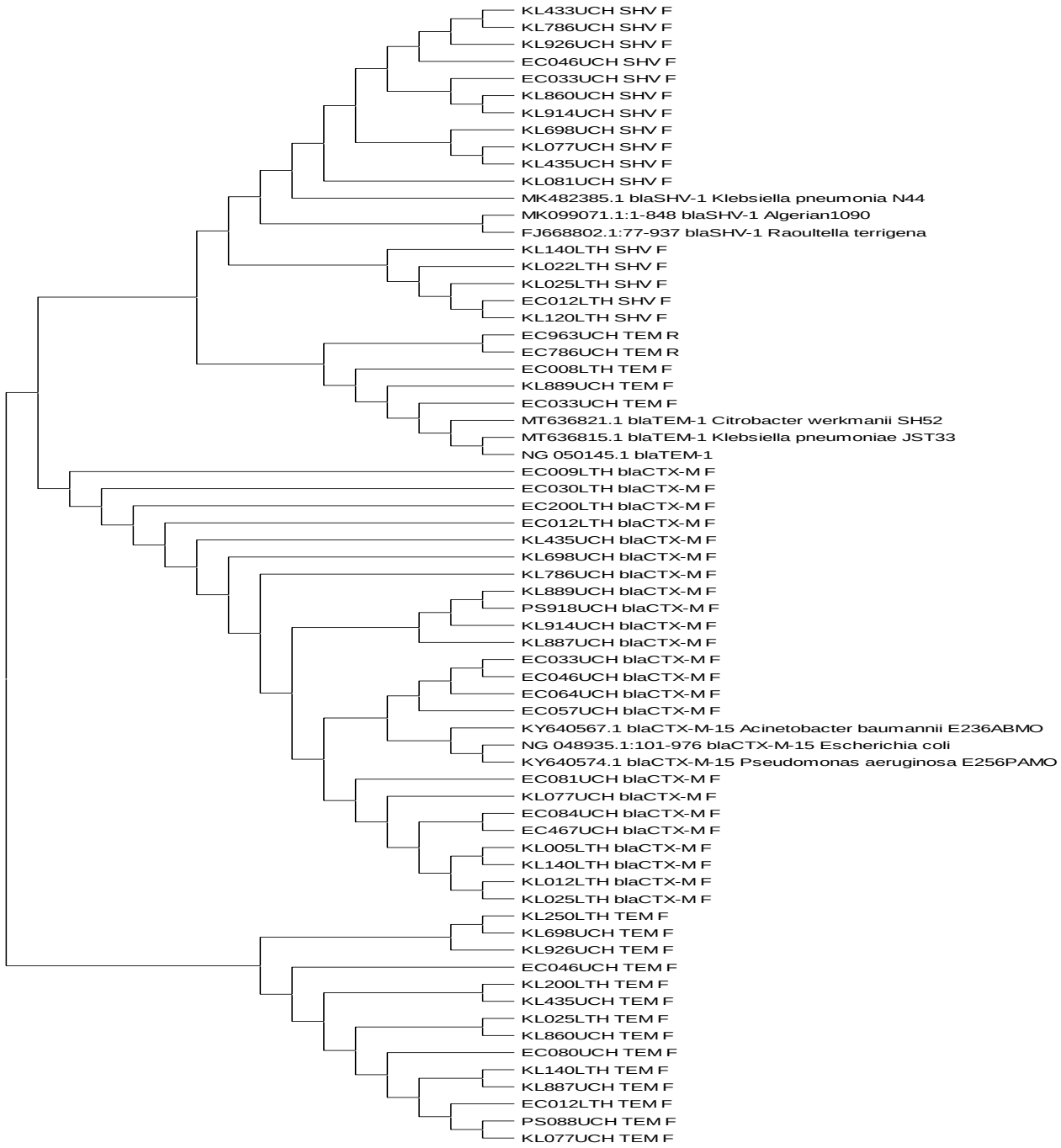

Figure 5: Phylogenetic tree showing evolutionary relationships of the ESBL sequences

DISCUSSION

Gram-negative bacteria exhibiting multiple resistance to several classes of antibiotic are on the increase and cut across almost all genera and species, most especially among the Enterobacteriaceae and Pseudomonadaceae (Leverstein-van Hall *et al*., 2003; Lynch III *et al*., 2013; Yadav *et al*., 2015; Cilloniz *et al*., 2019). The ability of the individual microorganism toacquire resistance determinants through mobile genetic elements and transfer the resistance traits among themselves coupled with the principle of selective pressure as a result of misuse or overuse of broad and extended-spectrum antibiotics have encouraged the spread of multiple antibiotic resistance among these microorganisms (Thanner *et al*., 2016; Manyi-Loh *et al*., 2018). The increase in the use of beta-lactam antibiotics, particularly the broad and extended-spectrum beta-lactams in both community and clinical infectious cases have partly contributed to the high prevalence of ESBL and MBL producing bacteria causing infections worldwide (Rawat and Nair, 2010; Teklu *et al*., 2019). In this study, three most commonly encountered bacteria genera in most infectious cases were selected for collection and the highest percentage were recovered from urine (32.9%), most especially *E coli* (55.6%). This is not surprising as Enterobacteriaceae in particular, are part of the leading causes of urinary tract infection (UTI), particularly among

female subjects due to the proximity of their anus to the urinary tract compared to the male subjects (Behzadi *et al*., 2010).

In this study, higher percentage of the isolates exhibited resistance to amoxicillin-clavulanic acid (73.8%) and gentamicin (58.8%) while lower resistance was observed against imipenem (14.2%), meropenem (8.3%) and ertapenem (7.9). This result was relatively similar to that described by Teklu *et al*. (2019) where 71.6% of the isolates exhibited resistance against amoxicillin-clavulanic acid, 43.4% against gentamicin and 5.2% against meropenem. However, Olowookere *et al* (2019), in a study carried out on Gram-negative bacteria isolates in a hospital in the Northwest of Nigeria reported lower level of resistance to amoxicillin-clavulanic acid (45.7%) and gentamicin (38.8%) but higher level of resistance to carbapenems (28.2%).Considering each of the genera, it was observed in this study that higher percentage of the *Klebsiella* spp. exhibited resistance against cefotaxime (78.8%), gentamicin (78.8%), ceftazidime (70%) and aztreonam (72.5%) while lower percentage exhibited resistance against meropenem (7.5%), Ertapenem (8.8%) and imipenem (16.3%) compared to the other genera. High percentage of the *E coli*isolates exhibited resistance mainly to cefotaxime (68.8%) with moderate percentage resistance against other antibiotics. However, higher percentage of *E coli* relatively exhibited resistance to meropenem (11.3%), ertapenem (15%) and imipenem (18.8%) compared to *Klebsiella* spp. in this study. Teklu *et al*. (2019) reported slightly similar antibiotic susceptibility profile against the *Klebsiella pneumoniae* isolates where 86.4% of the isolates were resistant to cefotaxime, 85.4% to ceftazidime, 70% to gentamicin and 10.7% to meropenem. However, contrary to this study, 23.3% of the *Klebsiella pneumoniae* isolates from the work of Teklu *et al.* (2019) were resistant to aztreonam as against 72.5% in this study. Also, 3.5% of the *E coli* isolates from the work of Teklu *et al.* (2019) were resistant to meropenem as against 11.3% resistance in this study. The *Pseudomonas* spp. isolates in this study exhibited highest percentage resistance against amoxicillin-clavulanic acid (98.8%) but lowest percentage resistance to the other antibiotics such as ceftazidime (12.5%), cefepime (16.3%), aztreonam (15%), ciprofloxacin (32.5%), imipenem (10%) and meropenem (6.3%) compared to the two other genera. However, Kaur and Singh (2018) gave a contrary report where the *Pseudomonas aeruginosa* isolated from different specimens from patients in intensive care unit (ICU) in Bathinda, India exhibited higher percentage resistance against ceftazidime (60.9%), cefepime (57.8%), aztreonam (55.2%), gentamicin (44.3%), ciprofloxacin (42.8%), meropenem (35.5%) and imipenem (28.6%) compared to the *Pseudomonas* spp. from this study. Higher percentage of the isolates in this study exhibited MDR (43.8%) phenotypes. When considering the individual genera, relatively, higher percentage of the *Klebsiella* spp. exhibited MDR phenotype (53.8%) compared to the two other genera. This scenario was also reported by Teklu *et al*. (2019) where among the 103 *Klebsiella pneumoniae* isolates collected 82.5% of them were MDR compared to the other genera among the426 isolates. Ugwu *et al*. (2020) in a study carried out on uropathogens in Southeastern Nigeria also reported *Klebsiella* spp. recording the highest MDR phenotype among the other clinical isolates. Olowookere *et al* (2019) reported lower (26%) MDR phenotype among the Gram-negative bacteria isolates from a hospital in the Northwest of Nigeria. Phenotypically, low level ESBL (32.4%) and MBL (7.6%) producers was recorded in this study with relatively, higher percentage of the *Klebsiella* spp. (44.2%) producing ESBL compared to the *E coli* (33.3%), and lesser percentage producing MBL (4.7%) compared to the *E coli* (7.7%). This was partly similar to the report of Ugwu *et al*. (2020) where phenotypically, 32.0% and 3.1% of the entire isolates produced ESBL and MBL respectively but with *E coli* producing higher percentage of both ESBL (27.6%) and MBL (5.2%) compared to the other isolates. However, Olowookere *et al* (2019) reported higher (38.0%) occurrence of MBL producers among the carbapenem resistance clinical Gram-negative bacteria from Northwest Nigeria with *Klebsiella* spp. (36.8%) being the highest producers compared to the other genera. Co-production of both ESBL and MBL was recorded in 6.7% of the isolates in this study which was highest among the *Pseudomonas* spp. (8.7%), followed by the *E coli* (7.7%) and then the *Klebsiella* spp. (4.7%). Co-existence of two beta-lactamase genes was recorded only in 5.9% of the *Klebsiella* spp. in this study with 31.6% of the *Klebsiella* spp. and 23.1% of the *E coli* isolates harbouring the three selected ESBL genes (bla_{TEM}+bla_{SHV}+$bla_{CTX\text{-}M}$). Similar scenario was also reported by Ugwu *et al*. (2020) among clinical isolates of *Klebsiella pneumoniae* and *E coli* from Southeastern Nigeria. Co-existence of bla_{TEM}+bla_{SHV}+$bla_{OXA\text{-}Like}$ genes was recorded among the *Klebsiella* spp. (21.4%) but only bla_{TEM}+bla_{SHV} was recorded among the *E coli* (5.2%) by Ugwu *et al*. (2020). In this study, isolates producing MBL was detected but PCR amplification of the target MBL genes did not produced amplicons. This suggests that the isolates are probably resisting the carbapenems via different mechanisms, or possibly, the types of MBL genes harboured by the isolates are different from the specific MBL genes targeted in this study. This however, requires further screening of the isolates for more of the MBL genes by PCR technique or to carry out whole genome sequencing and analysis of the MBL-producers so as to elucidate the exert mechanism(s) of resistance to the carbapenems. The evolutionary relationships expressed by the phylogenetic tree in this study revealed close relationship among all the $bla_{SHV\text{-}1}$ genes and among the $bla_{CTX\text{-}M\text{-}15}$ genes sequenced with their respective downloaded sequences. However, some level of evolutionary differences was observed among the $bla_{TEM\text{-}1}$ sequences. While 5 (26.3%) of the $bla_{TEM\text{-}1}$ sequences were similar to the three downloaded sequences, 14 (73.7%) had relatively different gene sequences compared with the downloaded and the five sequenced genes.

CONCLUSION

This study reveals the presence of MDR bacteria isolates from community acquired infections co-harbouring multiple types of ESBL genes and co-producing ESBLs and MBLs. This signified a serious public health threat, especially for the carbapenem resistance and thus required continuous surveillance and strict restrictions in the use of extended-spectrum beta-lactams and carbapenems for community based infections to prevent further increases and spread within the community.

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