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Carbapenem Resistance among Order Enterobacterales from Tertiary Hospital in Bauchi State Nigeria

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Keywords:
AMR: Antimiçrobial resistance,
CRE Carbapenem resistant Enterobacterales,
PCR; polymerase chain reaction;
ESBL: Extended spectrum beta lactàmase,
CPE: Carbapenamase producing Enterobacterales.

ABSTRACT
The burden of antimicrobial resistance (AMR) is rapidly growing across antibiotic classes, with increased detection of isolates resistant to carbapenems. Data on the prevalence of carbapenem resistance in developing countries is limited; therefore, in this study, the study determined the prevalence of carbapenem resistance genes among order Enterobacterales isolated from clinical specimens in Bauchi State. A total of 315 isolates were analyzed for carbapenem resistance genes. For each isolate, two different PCR assays were performed, allowing for the detection of the major carbapenem resistance genes, using the primers blaCTX, blaCMY, blaTEM and blaNDM. CTX-types 19(22.3%) were the most predominant gene followed by TEM-typeswith 9(10.5%) incident. The genes were predominantly detected in *E. coli* 22 (25.8%), followed by *K. pneumonia* 10(11.7%). The report of this study have demonstrated for the first time the prevalence and co-existence of Carbapenamase producing carbapenem resistance (NDM) and non carbapenamase producing carbapenem resistance (CTX,TEM and CM-Y) from *K. pneumonia* in Bauchi State. This finding revealed the resistance rate of ampicillin, Amoxiclav, ceftazidime as 79.4%, 46.9% and 32.0% respectively. Imipenem and Meropenem were found to be considerably active to most of the isolates but not to CRE. The highest CRE was observed from CP-CRE 28.2% followed by ESBL producing CRE 16.5% and AmpC producing CRE 22.3%. Coexistence of betalactamases occurs from 32.9 % isolates from which 24.7% were ESBL, Ampicillin and Carbenicillin (AmpC) betalactamases producers while 8.2% isolates were ESBL and carbapenamases producers with *K. pneumoniae* carbapenemase producing species.

INTRODUCTION

One of the main reasons for the emergence of antibiotic resistanceworldwide is over-the-counter availability of antibiotics. With the incidences rising alarmingly, antimicrobial resistance poses severe challenges to the general public and the medical fraternity. Antimicrobial resistance accounts for a significant proportion of the global morbidity and mortality rates associated with bacterial infections. The most important contributor to multi-drug resistance (MDR) is Gram-negative bacteria. Recently, Multidrug resistance focus has been placed on Carbapenem-resistant Gram-negative bacteria. The World Health Organization (WHO) lists carbapenem resistance among order Enterobacterales as priority antimicrobial pathogens that pose significant threats to human health (Muhammad *et al*., 2020).

Enterobacterales are known to be the most common and well known order in the phylum proteobacteria. The members of its four families have been implicated as pathogens in humans capable of causing both community and nosocomial infections (Cunha *et al.*, 2016; Demiraslan *et al*., 2017).Carbapenem antibiotics are effective drugs for the treatment of multidrug-resistant gram-negative bacilli, particularly those producing extended-spectrum β -lactamase enzymes, as well as a broad range of Gram-positive bacteria. Just like other beta-lactam antibiotics, they act by binding to the penicillin-binding proteins, inhibiting the synthesis of the bacterial cell wall (Bharadwaj *et al*., 2018). Cabapenem resistance Enterobacterales are defined as enterobacterales that are resistant or intermediate to one or more carbapenem antibiotics by various mechanisms, and some of their species, such as Proteus, *Providencia, and Morganella species*, demonstrate an intrinsically elevated minimum inhibitory concentration (MIC) to imipenem (Kitchel *et*

al., 2009; Mohammed *et al*., 2015). A vast majority of carbapenem resistance genes in Enterobacterales are transmitted in tandem with other resistance genes, resulting in multidrug-resistant organisms. The resistance genes were primarily carried on plasmids, which allowed resistance genes to be exchanged between Enterobacterales and other Gram-negative bacteria, propagating resistant strains (Borer *et al*., 2017).

MATERIALS AND METHODS

Study Design and Population. This was a cross-sectional laboratory based study involving 315 isolates belonging to the Enterobacterales order, collected from December 2021 to December 2022 from clinical samples in a tertiary Hospital, Bauchi state, Northern, Nigeria. The isolates were from Stools (42), Urine (219), HVS (8), ECS (3) catheter tips (6), Sputum (21) and wound swabs (12)

Sub culturing and Disk Diffusion Susceptibility Testing

Isolates were subculture on MacConkey agar and then susceptibility testing was done according to standard guideline (CLSI, 2020). The antibiotics used (Oxoid, Ltd. Basingstoke, UK) were as follows: Ceftazidime (30µg), Cefotaxime (30µg), Gentamicin (10µg), Ciprofloxacin (5µg), Ampicillin (10µg), Agumentin (30µg), Imipenem (10µg) and Meropenem (10µg) and susceptibility results were interpreted based on the CLSI 2020 guideline.

Plasmid Curing Assay

Plasmid curing was carried out in order to determine the location (plasmid borne or chromosomal) of the drug resistance markers as described by Ojo *et al*., 2014. The curing analysis of the isolates was performed using 0.1mgmL-1 of acridine orange. Isolate were grown for 24h at 37^0C in Mueller-Hinton broth containing 0.1mgmL-1 acridine orange. The broth was agitated to homogenize the content and a loopful of the broth medium was inoculated on Mueller Hinton Agar (MHA) plates and antibiotics sensitivity testing was carried out. The antibiotic disc used for sensitivity testing after curing with acridine were Ceftazidime (30µg), Cefotaxime (30µg), Gentamicin (10µg), Ciprofloxacin (5µg), Ampicillin (10µg), Amoxy-Clav(30µg), Imipenem (10µg) and Meropenem (10µg) (Le -Page *et al*., 2016; CLSI, 2020; Hassan *et al*., 2020).

Polymerase chain reaction for amplification Carbapenem resistance Genes

DNA extraction: Bacterial genome extraction by boiling method, One colony from fresh bacteria was solved in 300 ml distilled water and homogenized with vortex and kept on thermo block in 100° C for 10 min, and then centrifuged in 12,000 rpm for 10 min and the supernatant containing genome was used for PCR Amplification

Amplifications of carbapenam resistance genes: Multiplex Polymarase chain reaction (MPCR) was employed for concomitant detections of blaKPC, bla VIM, blaOXO-48, and blaNDM, *bla*TEM, *bla*SHV and *blaCMY-2* genes using specific pair of primers (Tseng *et al*., 2023).

Table 1: Primer sets for amplification of carbapenem resistance determine genes (Zhou *et al*., 2022; Tseng *et al*., 2023).

	Detection of carbapenem resistant genes		
Genes Targeted	Primers F:Forward R: Reverse	Primer sequence (5′→3′)	PCR product Size (bp)
*bla*SHV	SHV-F	CGCCTGTGTATTATCTCCCT	300
	SHV-R	CGAGTAGTCCACCAGATCCT	
blaOXA-48-like	OXA-F	GCGTGGTTAAGGATGAACAC	438
	OXA-R	CATCAAGTTCAACCCAACCG	

blaKPC	KPC-F	CGTCTAGTTCTGCTGTCTTG	798
	KPC-R	CTTGTCATCCTTGTTAGGCG	
BlaIMP	IMP-F	GGAATAGAGTGGCTTAAYTCTC	232
	IMP-R	GGTTTAAYAAAACAACCACC	
blaNDM-1	NDM1-F	TGCCGAGCGACTTGGCCTTG	379
	NDM1-R	ACCGATGACCAGACCGCCCA	
blaTEM	TEM-F	TCAACATTTCCGTGTCG	800
	TEM-R	CTGACAGTTACCAATGCTTA	
blaCMY-2	CMY-2-F CMY-2-R	CCAGAACTGACAGGCAAACA CCTGCCGTATAGGTGGCTAA	

For amplification, 5 μL of DNA template was added to Accupower gold multiplex PCR premix tubes containing 200 μM of dNTP mixtures (Roche, Switzerland), 0.4 μM of each primer, 2.5 U Taq polymerase (Invitrogen, Germany), and appropriate buffer (0.2 μM MgCl2, 2.5 μM KCL, 0.5 μ 10% Tween 20, 1 μL of Gelatin, and 3.8 μL of purified water). The amplification was done using PTC 100 thermocycler machine (Hain Life science GmbH, Nehren, Germany). For *bla*VIM, *bla*KPC, *bla*NDM, and *bla*OXA-48, the programmed was denatured at 95^0C for 45 seconds, annealing at 94^0C for 1 40 second, and elongation at 72^0C for a 40 second. For *bla*IMP the same programmed was used except that the annealing temperature was adjusted to 45^0C for 60 seconds. The cycles were repeated 40 times and all primer sets had a final extension of 72^0C for 10 minutes. Quality control was performed with each run using the test organisms, negative and positive control strains *K. pneumoniae* ATCC BAA-1706 and *K. pneumoniae* ATCC BAA-1705 respectively (Zhou *et al*., 2022**)**.

Agarose Gel Analysis of genomic DNA by electrophoresis

Five micro liters of PCR products were analyzed by electrophoresis in 1.5% agarose stained with ethidium bromide (Sigma Aldrich) to detect the specific amplified product under UV transilluminator and photographed DNA band of each amplicon was compared with appropriate base pair (bp) plus DNA mass maker.

Ethical Issues: The protocol for this study was submitted to the Health Research Ethics Committee (HREC) of Bauchi State, Nigeria, for review and approval before the commencement of data collection.

Ethical approval: with reference number (MOH/GEN/S/1409/1)/NREC/03/11/19B/2020/36 was obtained.

RESULTS AND DISCUSSION

Three hundred and fifteen (315) Enterobacterales were isolated. The most predominant species isolated were *Escherichia coli* and *K. pneumoniae*, with a prevalence of 161 (51.1%) and 75 (23.8%), respectively. Other species identified were *Shigellaspp* 9 (2.9%), *Klebsiella ozone* 6 (1.9%), *E. aerogenes* 11 (4.1%), C. *freundii* 5 (1.5%), *Citrobacter sedlakii* 5 (1.5%), *Proteus mirabilis* (2.9%), *Serratia marcescens* 5 (1.5%), *Morganella morganii* 4 (1.2%), *Hafniaalvei* (4; 0.9%), *E. cloacae* 3 (0.9%), *Raoultella terrigena* 5 (1.5%), *Salmonella spp* 12 (3.8%), and *Serratia odorifera* 11 (0.3%) as shown Table 2. Resistance profile of the isolates identified shows that 179 were multidrug resistant, 64 (20.6 %) isolates have reduced susceptibility to meropenem while only 91 (28.8%) isolates have reduced susceptibility to Imipenem after screening using carbapenem antibiotic.

The Prevalence of Carbapenem resistance Genes were 33 (38%) out of 85carbapenem resistant phenotypeand were positive for one or more of the carbapenem resistance genes. The genes identified were CTX, TEM, CMY and NDM, CTX-types were the most predominant carbapenem resistant genes detected in 19(22%), followed by TEM -types 9(10.5%), NDM-

Types 4 (4.7%) andCM-Y –types1 (1.2%), these genes were either detected along or with more than one gene in one bacterial isolates. gene (Table 4).

The plasmid curing assay revealed that most of Carbapenem resistant Enterobacterales isolated in this study were plasmid mediated since 71. 8% of the isolates showed zones of inhibition (cured) when tested against the selected antibiotics after curing while 28.2% remained resistance (no zone of inhibition) after curing (plasmid not cured) indicating chromosomal borne resistance.

Table 2: Clinical isolates by samples studied

Entero bacteriacea	**Urine**	**Stool**	**Sputum**	**Wound Sw**	**ECS**	**HVS**	**C. Tips**	**U. Swab**	
E. coli	124 (14.8)	22(6.5)	6 (4.2)	0 (0.0)	1 (2.3)	5(14.7)	1 (2.9)	2(25.0)	161(10.7
K. pneumo	54 (6.3)	0 (0.0)	5 (3.5)	9(17.3)	2 (4.6)	2(5.8)	3 (8.8)	1(0.3)	75(5.0)
*Shigella*spp	4 (0.4)	3 (0.8)	1 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.9)	0(0.0)	9(0.6)
K. ozaenae	4 (0.4)	0 (0.0)	1 (0.7)	1 (1.9)	0 (0.0)	0 (0.0)	0 (0.0)	0(0.0)	6 (0.4)
E. aerogen	5(0.5)	4 (1.1)	4 (2.8)	0(0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0(0.0)	13(0.9)
C. freundii	4 (0.4)	0 (0.0)	1 (0.7)	0(0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0(0.0)	5 (0.3)
C. sedlakii	2(0.2)	1 (0.2)	1 (0.7)	0(0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0(0.0)	5 (0.3)
P. mirabilis	8 (0.9)	0 (0.0)	1 (0.7)	1 (1.9)	0 (0.0)	0 (0.0)	1 (2.9)	0(0.0)	9 (0.6)
S. marcesc	5 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0(0.0)	5 (0.3)
M. morgan	2 (0.2)	0 (0.0)	1 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0(0.0)	3(0.2)
H. alvei	2 (0.2)	0 (0.0)	1 (0.7)	0 (0.0)	0 (0.0)	1(2.9)	0 (0.0)	0(0.0)	4(0.2)
S. odorifer	0 (0.0)	0 (0.0)	0 (0.0)	1.(1.9)	0 (0.0)	0 (0.0)	0 (0.0)	0(0.0)	1 (0.6)
E. cloacae	0 (0.0)	2 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0(0.0)	2 (0.1)
R. terrigen	3 (0.4)	0 (0.0)	2 (0.4)	2 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0(0.0)	5 (0.3)
S. spp	2 (0.2)	10 (2.9)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0(0.0)	12 (0.8)
Total	219(25.7)	42 (12.4)	21 (14.8)	12 (23)	3 (6.9)	8 (23.5)	6(17.6)	2(25.0)	315(21.0

ECS: Endocervical swab; HVS: high vaginal swab; C. Tips: Catheter tips; Urethral swab

Table 3. Antibiotics Susceptibility Profile of Enterobacterales from the study area

S/N	**Antibiotics**	**Code**	**Concentration**	**Sensitive**	**Intermediate**	**Resistance**
1	Ampicillin	AMP	10 µg	61 (19.4)	4 (1.3)	250 (79.4)
2	Amoxy-clav	AMC	20 µg	217 (68.9)	1 (0.3)	97 (30.8)
3	Cefoxitin	FOX	10µg	254 (82.2)	3 (0.9)	58 (18.1)
4	Cefotaxime	CTX	30 µg	232 (73.7)	1 (0.3)	82 (26.0)
5	Ceftazidime	CAZ	30 µg	223 (70.8)	4 (1.3)	88 (27.9)
6	Gentamicin	GEN	10 µg	227 (72.1)	1(0.3)	87 (27.6)

7	Ciprofloxacin	CIP	5 µg	191 (60.6)	1(0.3)	123 (39.4)
8	Imipenem	IPM	10 µg	250 (79.4)	1(0.3)	64(20.6)
9	Meropenem	MEM	10 µg	221 (70.2)	3(0.9)	91 (28.8)

AMP, AMC, CAZ, CIP, FOX, IMI, and MEM stand for ampicillin, amoxicillin/clavulanic acid, ceftazidime, gentamicin, ciprofloxacin, Imipenem, and meropenem, respectively.

Table 4. Prevalence of AmpC, ESBL and Carbapenamase producing isolates from some different Hospitals studied

Isolates	**No. of isolate**	**bla CTX-M**	**Bla TEM**	**bla NDM**	**Bla SHV**	**Bla CM-Y**	**bla VIM**	**bla KPC**	**Total %**	**bla genes**
E .coli	44	14	7	1	-	0	-	-	22	CTX-M, TE, NDM
K. Pneumoniae	29	4	2	3	-	1	-	-	10	CTX-M, TEM,CMY, NDM
Shigellaspp	2	-	-	-	-		-	-	-	-
K. Ozaenae	2	-	-	-	-	-	-	-	-	-
E. aerogenes	3	1	-	-	-	-	-	-	1	CTX-M, TEM
P. Mirabilis	1	-	_	-	-	-	-	-	-	-
S. Marcescens	1	-	_	-	-	-	-	-	-	-
Salmonella spp	3	-	-	-	-	-	-	-	-	-
Total	85	19	9	4	0	1	0	0	3	-

Table 5. Antibiotics Susceptibility Testing of Meropenem (10µg) on the isolates before and after curing with acredine orange.

CRE	**No of CRE before curing**	**No. of cured Plasmid by Acridine Orange**	**No.of uncured Pasmid**
E .coli	44	36	8
K. Pneumoniae	29	18	11
K. Oxytoca	2	2	0
K. Ozaenae	2	1	1
E. aerogenes	3	2	1

P. Mirabilis	1	1	0
S. Marcescens	1	0	1
Salmonella spp	3	1	2
Total	85	61(71. 8)	24(28.2)

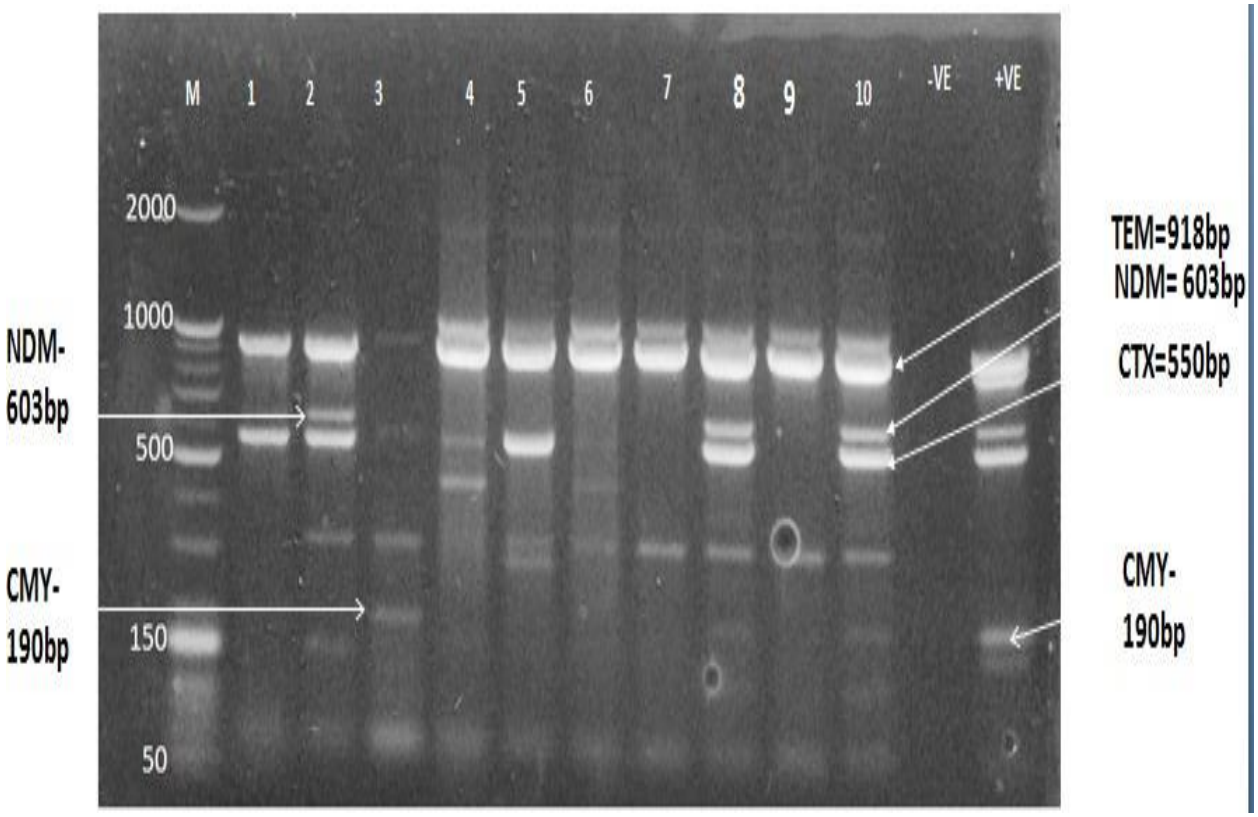

Figure 1 Amplified products of TEM, CTX-M, FOX and NDM genes.

1.5% Agarose gel analysis of PCR amplified fragment of (A) bla CTX-M (550bp) using CTX-M-F and CTX-M-R primers, (B) blaTEM (918bp) using TEM-F and TEM-R primers (C) FOX (190) using blaFOX-F and blaFOX-R (D) bla NDM (603p) using the NDM-F and NDM-R primers. The lanes 1-10 represent the PCR products of the CRE genes from *Enterobacterales* isolate. Lane M is the molecular weight ladder (DNA markers) spanning from 50-2000bp; NC and PC are PCR products of negative and positive controls respectively.

Discussion

The most common species among all the families in this study were *Escherichia coli* followed by *Klebsiella pneumonia* (belonging to the family *Enterobacteriacea*) with the percentage prevalence of 161 (50.9%) and 75 (24.1%) respectively, this agreed with the study reported by Nuhu *et al.*, 2017 which shows that *E. coli* is the most common uropathogen. Accordingly, this finding indicates presence of significant species belonging to the families of *Yersiniaceae* and *Hafniaceae* in the study area thus, highlighted the occurrence of enteric bacterial infection among the study population Oli *et al.*, 2019; Olowo-okere*et al.*, 2019. The resistance profile according to this finding was 79.4%, 46.9% and 32% for Ampicillin, ciprofloxacin and Amoxyclav. The prevalence of 64(20.6%) Imipenem and 91(28.8%) meropenem resistance were confirmed as enzymatic resistance but the remaining (0.9%) isolates may be resistant from either active expulsion of carbapenem out of the periplasmic space after their entrance or by the diminished expression or loss of porin as described by Meletis, 2016, Gulumbe *et al.*, 2019. The plasmid curing assay revealed that most of CRE isolated in this study were plasmid mediated since 71. 8% of the isolates showed zones of inhibition (cured) when tested against the selected antibiotics while 28.2% showed no zone of inhibition (plasmid not cured) indicating chromosomal borne resistance gene. The above finding could generally explain the prevalence of carbapenem

resistant *Enterobacterales* emanating from the six different hospitals in Bauchi state, North Eastern Nigeria and also as a serious health threat because it may ease the spread of carbapenem resistance from one species to another as reported by Mohammed *et al.* 2020. This study demonstrated the prevalence of 33(38%) carbapenem-resistance genes among multidrug resistant Enterobacterales in Bauchi State. Most of the isolates harboring genes for the resistance originated from urine samples and Stool. Seven genes were identified as follows; 19(22.0%) blaCTX-M), and 9(10.5%) blaTEM, 4 (4.7%) bla NDM and 1(1.2%) blaCMY, but absence of the other three (3) genes (blaKPC, blaIMP and blaOXA-48).

Our finding is in agreement with the prevalence study reported from Sokoto, North western Nigeria in which the prevalence of 38.0% CRE were also reported although carbapenamase genes were the most prevalent (MBL-positive) (Olowo-okere *et al*. 2019). The study is in disagreement with the study of Abba *et al*., 2019) in Banue state, Nigeria in which they found that 11.8% of CRE from bla TEM variants.

This finding recommend routine testing for carbapenem resistance among the order Enterobacterales in tertiary Hospital and other health facilities in developing countries where there is high prevalence of Enterobacterial infections. In addition, other antibiotics such as colistin and tigecycline should be tested to provide alterative treatment to these isolates.

CONCLUSION

In conclusion, the study revealed the occurance rate of 21.0% for members of the four families (*Enterobacteriaceae, Hafniaceae, Morganellaceae* and *Yarsiniaceae*) of the order Enteroacterales in Bauchi State, Nageria, .with *E. coli* and *K. pneumoniae* as the most prevalent species isolated and the resistance profile of Ampicillin, Amoxiclav, Ceftazidime was found to be 79.4%, 46.9% and 32.0% respectively. Imipenem and Meropenem were found to be considerably active to most of the isolates but not to Carbapenem producers. The highest CRE was observed from CP-CRE 28.2% followed by ESBL producing CRE 16.5% and AmpC producing CRE 22.3%. Coexistence of betalactamases occurs from 32.9 % isolates from which 24.7% were ESBL, ampicillin and carbenicillin (AmpC) betalactamases producers while 8.2% isolates were ESBL and carbapenamases producers with *K. pneumoniae* carbapenemase producing species. This study identified the presence of four genes as follows; bla NDM, blaCMY, blaCTX-M, and blaTEM responsible for CRE but absence of the other tree (3) genes (blaKPC, blaIMP and blaOXA-48) out of seven genes study. However *bla*TEM, *bla*CTX-M are more prevalent than *bla*CMY and *bla*NDM which enhances the antimicrobial resistance of the isolated carbapenem resistant Enterterobacterales. The most predominant bacteria encoding these geses from this study was *E. coli* which has 22 (50.0%).

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