MOLECULAR DETECTION OF OXA-48-LIKE CARBAPENEMASE-PRODUCING ESCHERICHIA COLI FROM CLINICAL LABORATORIES IN LAGOS, NIGERIA

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ABSTRACT

The global rising report of extended spectrum beta lactamase (esbl) as well as carbapenem resistance (cr) is alarming. This increase is of serious health concern as the choice of treatment is limited. We therefore investigated the incidence of esbl and cr among urinary isolates obtained from clinical laboratories in lagos state. Fifty-eight urine samples were collected and tested. Their antibiotic susceptibilities and esbl production were determined using disk diffusion method. According to the european committee of antimicrobial susceptibility testing (eucast), out of the 58 isolates tested, the most inhibitory was meropenem and the kleast effective ciprofloxacin. The results panned out as meropenem (91.4%), ertapenem (74.1%) ceftazidime (44.8%), ciprofloxacin (15.5%), gentamicin (20.7%), cefotaxime (31%) and amoxicillin/clavunate acid (31%). Esbl production was found in 4 (6.9%) of the 58 isolates. Also, 6.9% were found to be carbapenem non-susceptible with only 5.1% expressing oxa-48 type carbapenemase while 1.7% expressed both oxa-48 type carbapenemase and esbl. Among the carbapenems, meropenem had the highest activity followed by ertapenem (74.1%). The detection of oxa- 48 type carbapenem in e. Coli poses threats to the community hence adequate screening methods for early detection is imperative to aid prevent and control their dissemination

KEY WORDS: Urinary *Escherichia Coli***,** Carbapenems, Extended Spectrum Beta Lactamase.

INTRODUCTION

Escherichia coli is a harmless Gram negative rod shaped bacteria commonly found in the lower intestine as a normal flora of the gut but has become a major clinical concern. Some strains associated with diarrhoea, meningitis, sepsis and urinary tract infections are able to grow as commensals in the human gut (Alteri and Mobley, 2012; Toval *et al*., 2014). Previously, infections caused by *E.coli* were effectively managed with antibiotics, however, the emergence of rapid development of antibiotic resistance in hospitals, and in the community has become a major challenge in hospitals as well as in the community (Hoffmann *et al*., 2011). Treatment options have been restrictive because of the growing number of strains carrying extended-spectrum β-lactamases (ESBL) and plasmid-mediated AmpC β-lactamases (Pitout *et al*., 2005). Among the carbapenems, imipenem, meropenem, and ertapenem are sometimes the most potent to combat severe infection caused by

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ESBL-positive *E. coli*. A group of β-lactamases, Carbapenemases produced by some bacterial pathogens considerably hydrolyze imipenem, meropenem, and ertapenem and a wide range of other β-lactam antibiotics thereby conferring resistance to carbapenems (Hong *et al*., 2005; Queenan and Bush, 2007). Thus, the frequent use of carbapenems has led to an increasing number of carbapenem resistant organisms worldwide. The most common carbapenemases include veronica integron metallo-beta-lactamases types (VIM), imipenemase (IMP) types, *Klebsiella pneumoniae* carbapenemase (KPC), oxacillinase-48 (OXA-48), and New Delhi metallo-beta-lactamase-1 (NDM-1), encoded by carbapenem resistance determining genes *bla*VIM, *bla*IMP, *bla*KPC, *bla*OXA-48, and *bla*NDM, respectively (Nordmann *et al*., 2011). Originally, the carbapenemases were described in a few organisms and limited to specific geographical locations, but they have become a global concern since the 2000s (Zavacski et al., 2013). Several studies have documented the prevalence of carbapenem resistance Enterobacteriaceae especially within the clinical settings (Chakraborty *et al.*, 2010). Although carbapenem resistance has been rarely reported in *E. coli* (Hong *et al*., 2005), its occurrence is attributed to an outer-membrane porin deficiency and the expression of a plasmid-mediated class C β-lactamase (Stapleton *et al*., 1999; Davies *et al*., 2011).

This study was therefore aimed at detecting the occurrence of carbapenemases among *E. coli* strains isolated from urine samples from males and females from clinical laboratories in Lagos, Nigeria.

MATERIALS AND METHODS

STUDY POPULATION

Fifty eight non-duplicate urine samples from both males (n=34) and females (n=24) attending private laboratories at Oshodi and Victoria Island, Lagos State were collected over a period of two months (July–August 2015) and analysed at the Department of Medical Microbiology and Parasitology, College of Medicine, University of Lagos, Lagos. Identification of the isolates was done using colonial morphology, Gram staining procedure and VITEK® 2 compact ((BoMerieux Vitek, Missouri).

SUSCEPTIBILITY TESTING

Susceptibility testing was done for t8he following 7 antibiotics: meropenem (10µg), ertapenem (10µg), gentamicin (30µg), amoxicillin/ clavulanic acid (20/10µg), ceftazidime (30µg), ciprofloxacin (5µg), cefotaxime (30µg). Susceptibility of all

isolates was determined by the modified Kirby Bauer disk diffusion method on Mueller-Hinton agar (Oxoid, U.K) at 35 ± 2^0C following the zone size criteria as recommended by the European Committee of Antimicrobial Susceptibility Testing (EUCAST, 2013) guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance. *E. coli* ATCC 25922 was used during testing for quality assurance.

PHENOTYPIC DETECTION OF EXTENDED SPECTRUM β-LACTAMASES

The double-disc synergy test (DDST) was performed on isolates showing resistance (zone diameter ≥12mm) to temocillin and no synergy observed as increase in zone diameter with 10μg meropenem disk/tablet and inhibitors of carbapenemase and AmpC β-Lactamases. Double disk synergy test was performed by placing ceftazidime (30 µg) and cefotaxime (30 µg) at a distance of 20mm (center to center) from a disk with amoxicillin-clavulanic acid (30 µg). Ceftazidime was used as an indicator for TEM and SHV derived ESBLs, while cefotaxime was used as indicator for CTX-M types. A positive result was indicated when the inhibition zones around any of the cephalosporin disks are augmented in the direction of the disk containing amoxicillin-clavulanic acid (Wang *et al*., 2011).

PHENOTYPIC SCREENING FOR THE CARBAPENEMASE PRODUCTION

The susceptibility of the carbapenem resistant isolates was also tested in which isolates with reduced susceptibility to meropenem and imipenem (diameter of zone of inhibition <13mm) by disk diffusion method were screened for the production of carbapenemase. This was carried out phenotypically using the ROSCO diagnostica Neo-Sensitabs, an inhibitor combination disc test composed of KPC-type carbapenemase and OXA-48 carbapenemase (98015) constituting the following antibiotics; meropenem 10 µg (Disc A), meropenem 10 µg + boronic acid (KPC and AmpC inhibitor), (Disc B), meropenem 10 µg + cloxacillin (AmpC inhibitor), (Disc C), meropenem 10 µg + dipicolinic acid (Metallo-β-Lactamase inhibitor), (Disc D), temocillin 30 ug (only in the OXA-48 Confirm kit) (Disc E). The interpretation of the test was as follows: The zone of inhibition of disc A was compared to the zones of inhibition of each of the carbapenem-plus-inhibitor disc (B, C, and D). If tablet B showed a zone difference of ≥5 mm from disc A, the organism was recorded as demonstrating MBL activity. If disc C showed a zone difference ≥4 mm from tablet A, the organism was recorded as demonstrating KPC activity. If disc D showed a zone difference of ≥5 mm from tablet A, then resistance

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to meropenem was due to AmpC production. However if disc D showed a zone difference of ≥3 mm from tablet A, then meropenem resistance was due to ESBL production. No zone of inhibition around the Temocillin (30 µg) disc was a presumptive indication of OXA-48 production (Day *et al*., 2013).

DETECTION OF CARBAPENEMASE ENCODING GENES

Total DNA was extracted from all strains by boiling method as described previously (Kilic *et al*., 2004). A multiplex Polymerase chain reaction was carried out to identify OXA-48, OMP-35 and KPC using the primer pair listed in Table 1. Briefly, the PCR reaction was carried out using the Solis Biodyne 5X HOT FIREPol Blend Master mix. PCR in 20 µl of a reaction mixture, and the reaction concentration was brought down from 5X concentration to 1X concentration containing 1X Blend Master mix buffer Buffer (Solis Biodyne), 2.0 mM $MgCl_2$, 200µM of each deoxynucleoside triphosphates (dNTP) (Solis Biodyne), 20 pMol of each primer (JENA, Germany), 2 units of Hot FIREPol *DNA* polymerase (Solis Biodyne), Proofreading Enzyme, 5µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture.

Thermal cycling was conducted in an MJ Research PTC- 100 thermal cycler (GMI Inc, MN, USA) under the following conditions. Initial denaturation was done at 95°C for 15 minutes followed by 35 amplification cycles of 30 seconds at 95°C; 1 minute at 58°C and 1 minute 30 Seconds at 72°C. This was followed by a final extension step of 10 minutes at 72°C. The amplification product was separated on a 1.5% agarose gel and electrophoresis was carried out at 80 V for 1 hour 30 minutes. After electrophoresis, DNA bands were visualized by ethidium bromide staining. 100 bp DNA ladder was used as DNA molecular weight standard.

STATISTICAL ANALYSIS

Statistical analysis was carried out using the software programs Epi info and WHONET 5.6. Differences in sensitivity and specificity of the tests were analyzed with the chi square test. Variables with P value ≤ 0.05 were recorded as being significant while variables with P value >0.05 were not significant. Results were presented as odds ratios (ORs) and 95% confidence intervals (95% CI).

RESULTS

A total of 58 urinary isolates of *E. coli* collected from private clinical laboratories in Lagos state were investigated for the presence of extended spectrum beta lactamase (ESBL) and carbapenemase producing *E. coli* strains. All the 58 isolates

were subjected to antimicrobial testing and interpreted as resistant or susceptible using the European Committee on Antimicrobial Suceptibility Testing (EUCAST, 2013) guidelines. The isolates showed varied susceptibility patterns to the various antibiotics tested with the highest suceptibility observed in meropenem (91.4%) and the lowest in Ciprofloxacin (15.5%). Among the carbapenems, the isolates were more susceptible to meropenem (91.4%) than ertapenem (74.1%). The other suceptibility activities showed ceftazidime (44.8%), ciprofloxacin (15.5%), gentamicin (20.7%) and amoxicillin/clavunate acid (31%) (Table 2) (Fig, 1). A total of 8(13.8%) out of the 58 isolates produced ESBL of which 5(62.5%) of them were carbapemen (OXA-48) resistant. Carbapenemases were detected in the 5 isolates with all displaying OXA-48 *bla* gene but none of the isolates produced KPC nor OMP-35. Overall, the prevalence of the OXA-48 was higher in males (58.6%) than the females (41.4%) however, there was no significant association between the the subjects (P= 0.5714).

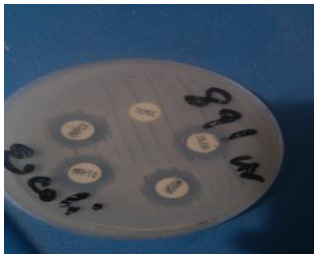

Fig. 1. Urine isolate of E.coli expressing OXA-48 type carbapenemase. Note the no zone of inhibition around the Temocillin (30 µg) disc, a presumptive indication of OXA-48 production.

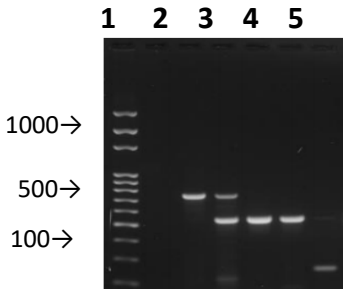

Fig. 2. Agarose gel electrophoresis showing representative strains of Carbapenemase genes in the lanes. Lane 1 is the the 100bp ladder.

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Table 1. List of Primers used for the Identification of Carbepenemase Genes

Gene	Primer	Product size (bp)	(Poirel *et al*., 2003)
OXA-48-F	5'- AACGGGCGAACCAAGCATTTT-3'	585	(Poirel *et al*., 2003)
OXA-48-R	5'-TGAGCACTTCTTTTGTGATGGCT-3'		(Kaczmarek *et al*., 2006)
OMP-35-F	5'-CAG ACA CCA AAC TCT CAT CAA GGG-3'	587	(Kaczmarek *et al*., 2006)
OMP-35-R	5'-AGA ATT GGT AAA CGA TAC CCA CG-3'		(Hindiyeh *et al*., 2008)
KPC-F	5'-GAT ACC ACA TTC CGT CTG G-3'	246	(Hindiyeh *et al*., 2008)
KPC-R	5'-GCA GGT TCC GGT TTT GTC TC-3'		

Table 2. Resistance patterns of ESBL *E. coli* strains isolated from urine samples of males.

S/N	ID	ESBL	C	Type of C	CAZ	CIP	ETP	GEN	MEM	AMC	CTX
1	1	+	-		24	26	28	18	25	6	6
2	6	-	-		30	27	27	16	25	22	30
3	9	-	-		26	6	29	6	28	6	30
4	12	-	-		15	6	29	6	26	6	6
5	13	-	-		15	30	28	17	27	6	30
6	587	+	-		26	20	26	19	28	9	26
7	588	-	-		22	6	25	9	28	14	22
8	640	-	-		16	6	22	8	24	26	22
9	672	-	-		26	24	28	20	28	20	26
10	718	-	-		14	7	24	6	28	14	6
11	729	-	-		6	6	25	6	26	12	6
12	740	-	-		30	6	33	7	31	12	7
13	786	-	-		18	25	26	20	26	20	24
14	788	-	-		24	6	28	7	28	16	28
15	792	-	-		14	10	25	6	26	20	14
16	808	-	-		16	6	30	20	36	7	7
17	809	-	-		6	16	9	9	15	20	10
18	817	-	-		12	6	25	8	25	12	6
19	849a	-	-		14	6	28	11	26	14	8
20	849b	-	-		14	12	24	8	26	14	12
21	880	-	-		16	6	25	9	25	12	12
22	891	+	+	OXA 48	6	6	19	7	22	16	6
23	902a	-	-		40	12	30	8	30	22	36
24	911	-	-		28	7	36	6	30	18	30
25	913	-	-		20	10	29	6	28	16	12
26	914	-	-		24	6	22	7	26	16	26
27	956a	-	-		28	6	30	7	32	12	30
28	957	-	-		12	6	25	12	26	6	6
29	958	-	-		30	6	31	7	34	16	32
30	959	-	-		18	6	39	7	32	20	12
31	973	+	+	OXA-48	6	6	15	8	18	6	6
32	975	+	+	OXA-48	6	6	15	9	20	6	6
33	976	+	+	OXA-48							
34	979	-	-		19	6	27	8	28	8	19

Table 3. Resistance patterns of ESBL *E. coli* strains isolated from urine samples of females.

S/N	ID	ESBL	C	Type of C	CAZ	CIP	ETP	GEN	MEM	AMC	CTX
1	59	+	+	OXA-48	12	18	17	15	20	20	14
2	319	-	-		23	22	27	15	27	14	22
3	324	+	-		22	6	26	6	28	9	22
4	518	-	-		19	6	29	17	24	20	15
5	558	-	-		11	10	20	6	19	9	6
6	585	-	-		12	6	24	6	24	10	6
7	717	-	-		14	6	24	8	28	14	6
8	737	-	-		1	1	3	1	28	20	8
9	773	-	-		28	14	29	10	28	20	12
10	791	-	-		14	7	24	10	28	6	7
11	860	-	-		30	7	26	7	30	7	7
12	864	-	-		28	20	30	20	30	20	32
13	885	-	-		28	26	32	20	30	14	30
14	898	-	-		26	6	30	6	27	20	23
15	902b	-	-		32	6	22	7	34	18	32
16	910	-	-		24	20	30	16	28	14	28
17	933	-	-		24	17	27	18	30	12	28
18	955	-	-		12	14	29	6	27	16	8
19	956b	-	-		20	6	30	7	40	20	14
20	961	-	-		14	6	29	11	30	14	8
21	962	-	-		22	6	27	6	27	14	24
22	977	-	-		12	6	23	10	24	22	22
23	982	-	-		28	26	32	20	32	20	32
24	986	-	-		28	26	30	20	28	22	28

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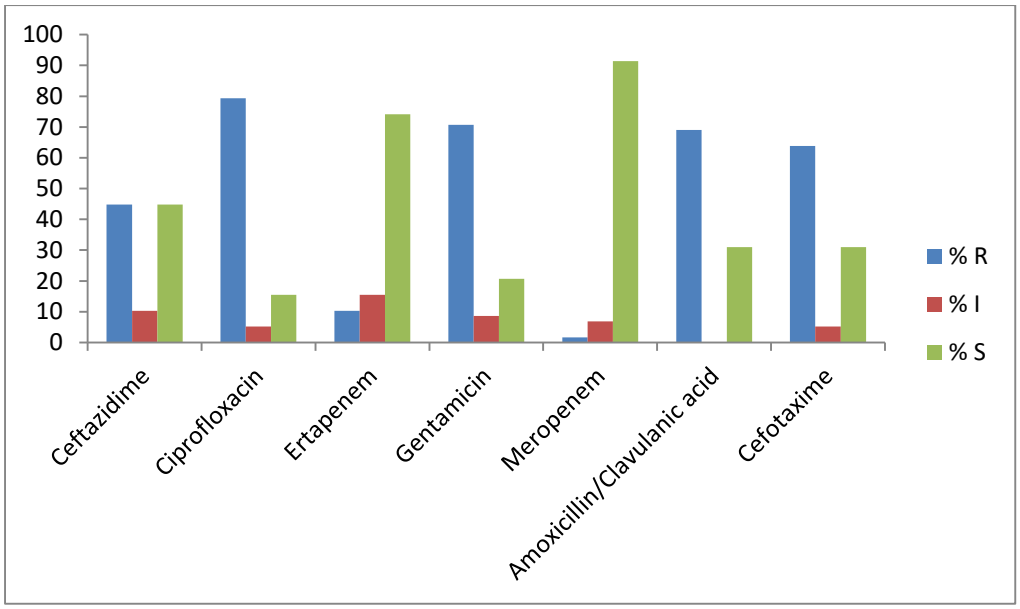

Fig. 3. Antibiotic resistant patterns of *E. coli* from urine samples.

CAZ=ceftazidime, **CIP=**ciprofloxacin, **ETP=** ertapenem, **GEN=gentamicin, MEM=**meropenem**, AMC=**amoxicillin/clavulanic acid, **CTX**=cefotaxime

DISCUSSION

Multidrug resistant bacteria in both the community and hospital settings has become a major threat globally, gradually reducing the choice and efficiency of available antibiotics used for the treatment of infectious diseases. Extended spectrum beta lactamase (ESBL) production is a key factor which is responsible for the resistance of pathogenic bacteria to extended spectrum antibiotics (especially the 3rd generation cephalosporins).

This study showed a high rate of resistance of *E.coli* isolates to some selected antibiotics (ciprofloxacin, gentamicin, amoxicillin/clavulanic acid, cefotaxime). A high rate of resistance was recorded for ciprofloxacin (79.3%) which was followed by gentamicin (70.7%), amoxicillin/clavulanic acid (69%) and cefotaxime (63.8%). Ceftazidime and amoxicillin-clavulanic acid showed less activity than these.
Previous studies have also reported high prevalence resistance of *E. coli* strains to ciprofloxacin, cefotaxime and ceftazidime (Eze, 2012; Hsu *et al*., 2007; Ejikeugwu

et al., 2012). Nevertheless, the high sensitivity of the carbapenems (ertapenem and meropenem) to the isolates showed that despite the resistance exhibited by some strains, the drugs are still the most potent and effective antibiotics against the *E. coli* strains used in our environment. Previous studies (Spanu *et al*., 2002; Jyothsna *et al*., 2011) have also reported the good antibiotic activity of the carbapenems on Gram negative bacteria. The detection of ESBL producing *E. coli* is of great importance owing to the clinical significance of the organisms. Studies have shown that ESBLs are associated with co-resistance to other antibacterial classes thus organisms harbouring them become resistant to multiple antibiotics which usually leads to therapeutic failures (Nordmann, 2011). Carbapenems on the other hand, are considered drugs of choice for the treatment of extensive drug resistant bacteria. In this study, a prevalence of 8.6% was recorded just like similar reports of low prevalence between the range of 0.04% to 2.8% seen in Spain, China, Germany as well as in Morocco, (Miro *et al*., 2013; Hu *et al*., 2014; Ehrhard *et al*., 2014; El Wartiti *et al*., 2014). However, it contradicts the high prevalence ranging from 35% to 43.2% observed in Uganda, Tanzania and Greece (Okoche *et al*., 2015). The low prevalence observed in this study may be due to sample bias as well as the source of the sample from which was from the community. It has been reported that these organisms are more prevalent in hospital settings.

Previous studies in Nigeria and elsewhere had reported a high incidence of metallo beta-lactamases (MBL) (Ejikeugwu *et al*., 2012; Yusuf *et al*., 2012) but in this study, only OXA-48 *bla* gene was detected. However, it is similar to study by Wartiti (2012) and co-workers who isolated it in conjunction with other *bla* genes such as the KPC and the MBL. This study highlights the dissemination of carbapenemase producers, OXA-48-types, in two different neighbourhoods in Lagos. The results suggest caution as reports have shown that the coexistence of different ESBLs and carbapenemase resistance genes on mobile genetic elements (such as plasmids) in the same bacterial isolate can lead to the widespread transfer of these elements between the identical species, as well as different species. This will ultimately result in failed antimicrobial therapy. Moreso, the detection of carbapenemases in laboratory is challenging because phenotypic tests are time consuming and difficult to interpret and the molecular methods are usually not available in routine diagnostic laboratories. Additionally, the growing number of new carbapenemases makes molecular tests not ideal for the initial detection of carbapenemase production.

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