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Research article

Phenotypic Detection of Metallo-β-Lactamase (MBL) Enzyme in Enugu, Southeast Nigeria

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ABSTRACT

The current study was undertaken with the objective of investigating the occurrence of metallo-β-lactamase – producing bacteria in a tertiary hospital in Enugu, southeast Nigeria. Metallo-β-lactamases are beta-lactamase enzymes with unique ability to hydrolyze and confer resistance to the carbapenems which are the preferred drugs used for the treatment of infections caused by multidrug resistant Gram negative bacteria. Pathogenic bacteria producing metallo-β-lactamases (MBLs) are responsible for several infections in tertiary care hospitals around the world and carbapenem resistance due to these enzymes have been reported over the past few years. Ninetynine (99) non-duplicate clinical isolates of *Escherichia coli* (n=40), *Klebsiella pneumoniae* (n=39) and *Pseudomonas aeruginosa* (n=20) from specimens of patients were identified and tested for susceptibility to various antibiotics by disk diffusion method. Metallo-β-lactamase (MBL) production was detected phenotypically by disk potentiation test. Overall, metallo-β-lactamase was detected in 12.5% *E. coli*, 15.4% *K.pneumoniae* and 10% *P. aeruginosa* isolates, and our results have clearly shown for the first time the occurrence of MBL enzymes in this environment. Resistance of pathogens to the carbapenems mediated by metallo-β-lactamases puts under threat our ability to effectively treat infections caused by multidrug resistant bacteria. Prompt detection of drug resistant bacteria is essential in the proper treatment of affected patients. In addition, rational use of available antibiotics, review of antibiotic usage policies and proper infection control measures should be adopted by tertiary hospitalsin this environment in order to keep multidrug resistant bacterial infections under control.

Keywords: Resistance, MBLs, Carbapenems, Gram – negative bacteria

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INTRODUCTION

Metallo-β-lactamases (MBLs) are β-lactamase enzymes that hydrolyze and confer resistance on carbapenems, but are yet inhibited by chelating agents like ethylene-diamine tetra-acetic acid (EDTA) (Aibinu*et al*., 2007).They are a type of carbapenemases that require zinc ion (Zn^{2+}) as a cofactor for enzyme activity (Aibinu *et al*., 2007; Shahcheraghi *et al*., 2010).MBLs have become a serious public health problem with catastrophic consequences for the treatment of bacterial related infections(Bush, 1998). Their emergence and uncontrolled spread has put the use of the carbapenems under threat. The carbapenems including imipenem, meropenem and ertapenem are broad-spectrum antibiotics with high stability against most β-lactamase enzymes. They are the drug of choice for the treatment of infections caused by β-lactam resistant bacteria including those that produce extended spectrum enzymes(Bashir *et al*., 2011).The uncommon reduced susceptibility of bacterial pathogens to MBLs as reported in some quarters (Aibinu *et al*., 2007; Bashir *et al*., 2011; Chakraborty *et al*., 2010) is a call for concern. The growing resistance of organisms to the carbapenems is a risk to available antibiotics used for treating nosocomial infections(e.g. bacteremia, septicemia, and pneumonia in children). Their unprecedented presence in a clinical setting should be a source of worry to healthcare givers because they limit treatment options. Their prevalence have been widely reported from different parts of the world including India, North America, South America, Brazil, Australia and southwest Nigeria(Bashir *et al*., 2011; Dzierzanowska-Fangrat *et al*., 2005; Fiett *et al*., 2006). The increased utility of the carbapenems in clinical medicine may have necessitated their emergence and spread (Aibinu *et al*., 2007; Franco *et al*., 2010; Franklin *et al*., 2006).Early detection of MBL-producing bacteria is critical due to the worldwide increase in the occurrence, types and spread of MBLsin both the community and hospital settings, and a carbapenem-intermediate or resistant result arising from antibiotic susceptibility studies should raise the notion of a possible MBL production that warrants confirmation either phenotypically or genotypically (Feizabadi *et al*., 2006; Iroha *et al*., 2008; Kim *et al*., 2007; Overturf, 2010). There is a dearth of information on MBL-producing bacteria in our country. Therefore, we carried out this study to detect the possible production of MBL enzyme from clinical isolates obtained from a tertiary hospital in Enugu, southeast Nigeria over a one year period.

MATERIALS AND METHODS

Microorganisms: In the present study, 99 non-duplicate isolates of Gram-negative bacilli (20 *P. aeruginosa*, 39 *K. pneumoniae* and 40 *E. coli*) were recovered from clinical specimens of patients that attended a tertiary hospital in Enugu, southeast Nigeria from January to December 2011. Identification of the isolates was performed as per standard microbiological characterization techniques (Vandepitte *et al*., 2003).

Antimicrobial susceptibility testing: Susceptibility profile of all the test isolates (maintained at 0.5 McFarland turbidity standards) were determined on Mueller Hinton (MH) agar (Oxoid, UK) plate(s) by the Kirby-Bauer disk diffusion method in line with the Clinical and Laboratory Standards Institute (CLSI) guidelinesusing antibiotic disks containing: sulphamethoxazole-trimethoprim (SXT:25µg), ciprofloxacin (CIP:5µg), gentamicin (CN:10µg), ofloxacin (OFX:5µg), imipenem (IPM:10µg), meropenem (MEM:10µg), amoxicillin-clavulanic acid (AMC:20/10µg), ceftazidime (CAZ:30µg) and cefotaxime (CTX:30µg) (Oxoid, UK). *K. pneumoniae* ATCC 700603, *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 (Oxoid, UK) were used as quality control organisms for antibiotic resistance (CLSI, 2005).

Screening of MBL: The screening for metallo-β-lactamase (MBL) production in all test isolates was undertaken on MH agar (Oxoid, UK) plate(s) using IPM (10µg) and MEM (10µg) antibiotic disks (Oxoid, UK) as described previously [8] and with little modifications. Isolates showing reduced susceptibility to any of the carbapenems (IPM and MEM) were suspected to produce MBL enzyme, and warrants a phenotypic confirmation test using a chelating agent(Bashir *et al*., 2011; Overturf, 2010).

Disk potentiation test: MBL production was confirmed phenotypically on IPM and MEM resistant isolates by a previously described method [8] with little modifications. Briefly, two IPM (10µg) and two MEM (10µg) antibiotic disks (Oxoid, UK) were each placed at a distance of 25mm apart on MH agar plate(s) already inoculated with the test isolate (maintained at 0.5 McFarland turbidity standards). 10µl of sterilized 0.5M EDTA were each added aseptically to one IPM disk and one MEM disk using a micropipette. The plate(s) were incubated aerobically at 37^{o}C for 24hrs, and the zones of inhibition around the IPM and MEM disks impregnated with EDTA and those without EDTA were recorded and compared. An increase of ≥7mm in zone diameter of either the IPM+EDTA disk or IPM alone or MEM+EDTA disk and MEM alone was taken to be positive (phenotypically) for the presence of

MBL in the test isolate(s). EDTA was tested alone on the isolate(s) before incorporation into the antibiotic disk(s) in order to ensure that it did not inhibit the test organism, and cause a false positive result (Feizabadi *et al*., 2006; Bashir *et al*., 2011).

RESULTS

During the study period (January to December 2011), 99 non-duplicate isolates of Gram-negative bacteria (40 *E. coli*, 39 *K. pneumoniae* and 20 *P. aeruginosa*) were recovered from clinical specimens of patients that were admitted or attended a tertiary hospital in Enugu, south-east Nigeria.

Table 1: Source distribution of the clinical isolates

Specimens	*K. pneumoniae* n (%)	*E. coli* n (%)	*P. aeruginosa* n (%)	Total
Urine	13 (13.1)	34 (34.3)	9 (9.1)	56
Ear swab	1 (1.0)	0 (0)	6 (6.1)	7
Pleural aspirate	1 (1.0)	0 (0)	0 (0)	1
Sputum	18 (18.2)	0 (0)	1(1.0)	19
High vaginal swab	0 (0)	1 (1.0)	0 (0)	1
Wound swab	6 (6.1)	5 (5.1)	3 (3.0)	14
Conjunctival swab	0 (0)	0 (0)	1 (1.0)	1
Total	39 (39.4)	40 (40.4)	20 (20.2)	99

Table 1 shows the site distribution of all the isolates employed for the present day study. The antimicrobial susceptibility patterns of all the isolates are shown in Table 2. All bacterial isolates were highly resistant to sulphamethoxazole-trimethoprim (SXT), showing resistance rates of 100%, 84.6% and 97.5% for *P. aeruginosa*, *K. pneumoniae* and *E. coli* isolates respectively. This was closely followed by ciprofloxacin (CIP) and ofloxacin (OFX), both fluoroquinolones (Table 2).

Table 2: In vitro antimicrobial susceptibility pattern of the isolates

Drugs	*E. coli* (n=40)		*K. pneumonia* (n=39)		*P. aeruginosa* (n=20)	
	S n(%)	R n(%)	S n(%)	R n(%)	S n(%)	R n(%)
CTX	22(55)	18(45)	15(38.4)	24(61.5)	10(50)	10(50)
CAZ	28(70)	12(30)	24(61.5)	15(38.5)	14(70)	6(30)
SXT	1(2.5)	39(97.5)	6(15.4)	33(84.6)	0(0)	20(100)
CN	27(67.5)	13(32.5)	22(56.5)	17(43.6)	14(70)	6(30)
OFX	14(35)	26(65)	18(46.1)	21(53.8)	7(35)	13(65)
CIP	15(37.5)	25(62.5)	16(41.1)	23(59)	10(50)	10(50)
IPM	38(95)	2(5)	34(87.2)	5(12.8)	20(100)	0(0)
MEM	38(95)	2(5)	36(92.3)	3(7.7)	20(100)	0(0)
AMC	18(45)	22(55)	30(76.9)	9(23.1)	8(40)	12(60)

Out of the 99 Gram-negative bacilli (GNB) screened for MBL production using imipenem (10µg) and meropenem (10µg) disks, only 27 bacterial isolates (4 *P. aeruginosa*, 12 *K. pneumoniae* and 11 *E. coli*) were suspected as potential MBL producers (Table 3). Metallo – β – lactamase (MBL) production in the potential MBL-producers was detected by disk potentiation test. As shown in Table 3, MBL was phenotypically detected in 5 *E. coli*, 6 *K. pneumoniae* and 2 *P. aeruginosa* isolates using the inhibitor (EDTA) based assay method.

Table III. MBL prevalence among *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates as determined by screening and disk potentiation tests

Clinical bacterial isolates	No of tested isolates	Potential MBL producer's no (%)	Confirmed MBL producers by disk potentiation test no (%)	Re-Modified Hodges test no (%)
E. coli	40	11(27.5)	5(12.5)	5 (12.5)
K. pneumoniae	39	12(30.8)	6(15.4)	3 (7.7)
P. aeruginosa	20	4(20)	2(10)	3 (15)
Total	99	27(27.3)	13(13.1)	11 (11.1)

DISCUSSION

Bacterial resistance to various antimicrobial agents and even the carbapenems has tremendous clinical implications and, they limit treatment options for multidrug resistant infections (Shahcheraghi *et al*., 2010; Bush, 1998; Bashir *et al*., 2011). In Nigeria, especially in the southeastern part where this study was conducted, information regarding MBL-producing bacteria is scarce – as there are no published presumptive studieson the subject. The emergence and spread of multidrug resistant bacteria with acquired resistance to various β-lactams is a therapeutic problem with growing worldwide concern(Franklin *et al*., 2006). In our study, the antibiogram of all the selected isolates was investigated. Overall, a high degree of resistance to multiple classes of antibiotics used was noted amongst the test bacteria, especially to the third-generation cephalosporins where the resistance rates were CTX (*E. coli* 45%, *K. pneumoniae* 61.5%, *P. aeruginosa* 50%) and CAZ (*E. coli* 30%, *K. pneumoniae* 38.5%, *P. aeruginosa* 30%) (Table 2). To SXT and CN, the resistance rates of the *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates were 97.5%, 84.6%, 100% and 32.5%, 43.6%, 30% respectively (Table 2). The resistance rates of the *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates to the fluoroquinolones used in our study, OFX and CIP were 65%, 53.8%, 65% and 62.5%, 59%, 50% respectively. For the carbapenems used, IPM and MEM, the resistance rates of the *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates were 5%, 12.8%, 0% and 5%, 7.7%, 0% respectively (Table 2). All these results were comparable to similar studies done in Tehran in 2006, in Nigeria in 2008 and in Poland between 2001-2002, where high rates of resistance amongst *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates for varying antibiotics including CTX, CAZ, CN, SXT, CIP, OFX, IPM and MEM was reported (Aibinu *et al*., 2007; Bashir *et al*., 2011; Dzierzanowska-Fangrat *et al*., 2005; Iroha *et al*., 2008). *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates showed reduced susceptibility to AMC at a rate of 55%, 23.1% and 60% respectively (Table 2). This rates however, is in line with studies conducted in Pakistan, where resistance rates of *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates to AMC were 11.5%, 20.6% and 71.4% respectively (Chakraborty *et al*., 2010), and in contrast to studies conducted in Lahore where the resistance rates to AMC where *E. coli* 80.2%, *K. pneumoniae* 78.9% and *P. aeruginosa* 90.2%(Javeed *et al*., 2011). Among all the antibiotics tested, the carbapenems (IPM and MEM) were the most active, and this was followed by CN, CAZ, CTX and AMC (Table 2).

Out of 27.3% of Gram-negative bacilli (GNB) that were potential MBL producers in our study, 11 were *E. coli*, 12 were *K. pneumoniae* and 4 were *P. aeruginosa* isolates (Table 3). MBL production was confirmed phenotypically by disk potentiation test in 12.5% *E. coli* (n=5), 15.4% *K. pneumoniae* (n=6) and 10% *P. aeruginosa*(n=2) isolates (Table 3). The observed prevalence of MBLs in *E. coli* isolates in our study (12.5%) is lower than similar studies conducted in India where the prevalence of MBLs in *E. coli* isolates tested was 28.5% and in Australia where all 6 *E. coli* isolates tested positive for MBL production phenotypically (Bashir *et al*., 2011; Franklin *et al*., 2006). The prevalence of MBLs in *K. pneumoniae* isolates from our study (15.4%) is in contrast to the results obtained elsewhere, where *K. pneumoniae* isolates showed 36.6% production of MBL (Aibinu *et al*., 2007; Feizabadi *et al*., 2006). MBL production according to reports is found to be more prevalent and propagated first in *P. aeruginosa* isolates before appearing in *Enterobacteriaceae* including *E. coli* and *K. pneumoniae* isolates (Franklin *et al*., 2006; Feizabadi *et al*., 2006). The prevalence of MBL in *P. aeruginosa* isolates in our study (10%) corresponds to similar studies conducted in Kashmir, where a prevalence of MBL production in *P. aeruginosa* isolates were 20.8% and 11.66% respectively (Bashir *et al*., 2011). MBL producing organisms are distributed across South America, southern Europe and Southeast Asia, and it has also been recently reported in southwestern Nigeria (Aibinu *et al*., 2007). However, the prevalence of MBL production in our *P. aeruginosa* isolates (10%) is lower than results (22.77%, 51.28% and 53.2%) obtained in Brazil, India and Iran respectively (Franco *et al*., 2010; Feizabadi *et al*., 2006; Chakraborty *et al*., 2010). In Nigeria, there is a dearth of data regarding the prevalence of MBL production, but a report from south-western Nigeria showed that 4 out of 97 *P. aeruginosa* isolates tested for MBL production

produced MBLs phenotypically (Aibinu *et al*., 2007). Nevertheless, a national prevalence data on MBL producing bacteria is still lacking in Nigeria.
In the current study, we reported the first phenotypic MBL detection amongst Gram-negative bacilli (*E. coli* 12.5%, *K. pneumoniae* 15.4%, *and P. aeruginosa* 10%) from clinical specimens of patients that attended a tertiary hospital in southeast Nigeria. Considering the prevalence of MBL production amongst the tested bacteria in our study, it seems very likely that it must have resulted from uncontrolled usage and administration of extended spectrum drugs. This scenario might in no doubt allowed bacteria to develop resistance through selective pressure mounted on them over time as a result of irrational use of these drugs and possible poor infection control practices.

CONCLUSION

Conclusively, our findings therefore, clearly showed that bacterial pathogens recovered from clinical specimens in this tertiary hospital produce metallo-β-lactamase (MBL) enzymes. Such bacterial isolates are resistant to penicillins, cephalosporins, and some non-beta-lactam antibiotics used for treating Gram-negative infections in our hospitals. Further molecular studies are critical to characterize our MBL phenotypes and to compare same with genes already isolated from other parts of the globe using molecular epidemiology techniques. Rational use of available drugs, strict antibiotic policy, antibiotic resistance surveillance and proper infection control measures are advocated in order to keep multidrug resistant bacteria at bay.

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