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Phenotypic Detection of Metallo-B-lactamase Enzyme in Enugu, Southeast Nigeria

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Abstract: Pathogenic microorganisms producing metallo-B-lactamases are an emerging public health problem as they have unique ability to hydrolyze and confer resistance to the carbapenems which are the most potent drugs used for the treatment of infections caused by multidrug resistant bacteria including those that produce extended spectrum B-lactamases (ESBLs). The irrational use of the carbapenems is believed to have facilitated the emergence and spread of metallo-B-lactamases in both the community and hospital environment. Ninety nine (99) non-duplicate clinical isolates of *Escherichia coli* (n=40), *Klebsiella pneumoniae* (n=39) and *Pseudomonas aeruginosa* (n=20) were identified and tested for susceptibility to various antibiotics by disk diffusion method. Metallo-B-lactamase production was detected phenotypically by Disk Potentiation test and Re-Modified Hodges test.The resistance of test bacteria to different antibiotics was evaluated by multiple antibiotic resistance index (MARI). Metallo-B-lactamase was detected in 12.5 % *E. coli*, 15.4 % *K.pneumoniae* and 10 % *P.aeruginosa* isolates by the Disk Potentiation test while Re-Modified Hodges test detected MBL in 12.5 % *E. coli*, 7.7 % *K. pneumoniae* and 15 % *P.aeruginosa*. Themultiple antibiotic resistance index results showed that *E. coli*, *K. pneumoniae* and *P. aeruginosa* had multiple antibiotic resistance indexes of 0.2-0.6, 0.2-1.7 and 0.1-0.4 respectively. These findings clearly showed that pathogenic bacteria in this region are multiply resistant and produce metallo-B-lactamase enzymes in substantial proportions. There is need for proper and regular monitoring of antimicrobial resistancein order to avert their degrading effects on already available drugs. Further molecular studies are required to characterize the types of metallo-B-lactamases present in this environment.

Key words: MBLS · Carbapenems · Gram-Negative Bacilli · Nigeria

INTRODUCTION

Metallo-B-lactamases (MBLs) are B-lactamase enzymes that confer and hydrolyze carbapenems and a host of other B-lactam antibiotics, but are yet inhibited by chelating agents such as ethylene-diamine tetra-acetic acid (EDTA) [1].They are a type of carbapenemases that require zinc ion (Zn^{2+}) as a cofactor for enzyme activity [1,2].MBLs which hydrolyze and render inefficacious a wide variety of B-lactam antibiotics especially the carbapenems have become a serious public health problem with catastrophic consequences for the treatment of bacterial related infections, [3]and their uncontrolled emergence and spread amongst clinically important bacteria (e.g. *Escherichiacoli*, *Pseudomonasaeruginosa* and *Klebsiellapneumoniae*) has put the use of the carbapenems under threat. The carbapenems (e.g. imipenem and meropenem) are broad-spectrum antibiotics with high stability against most B-lactamase enzymes (e.g. extended spectrum B-lactamases) and they are the drug of choice for the treatment of infections caused by multidrug resistant bacteria [4]. MBL degrading activity on B-lactams is dependent on the interaction of the B-lactam drug with Zinc ions in the active site of MBL enzymes and this is the reason there is, why metal chelators such as EDTA is used in inhibitor-based MBL confirmation tests because these chelating agents block/inhibit MBL activity [2, 3]. The uncommon

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reduced susceptibility of bacterial pathogens to the carbapenems due to MBL productionas reported in some quarters [3-5] is a call for concern owing to their multidrug resistant patterns which limits therapeutic options. This is a threat to available antibiotics used for treating nosocomial infections (e.g. bacteraemia, septicemia and wound infections) because the presence of an MBL limits treatment options for patients [2]. Their prevalence have been widely reported from different parts of the world including India, North America, South America, Brazil, Australia and Southwest Nigeria [4-10]. Early detection of MBL-producing bacteria is critical due to the worldwide increase in the occurrence, types and spread of MBLs (including other drug-resistant pathogens) in both the community and hospital settings [11]. A carbapenem-intermediate or resistant result arising from antibiotic susceptibility studies should raise the notion of a possible MBL production that warrants confirmation [11-17] either phenotypically or genotypically in order to guide therapy effectively. There is a dearth of information on MBL-producing bacteria in Southeast Nigeria where this work was conducted, though MBL-producing pathogens have been reported in Lagos State, Southwest Nigeria [7]. Therefore, this presumptive study was carried out to detect the possible production of MBLs from clinical isolates obtained from the University of Nigeria Teaching Hospital (UNTH), Ituku-Ozalla in EnuguState, Southeast Nigeria over a one year period.

MATERIALS AND METHODS

Microorganisms: In the present day study, 99 non-duplicate clinical isolates of Gram-negative bacilli (20 *P. aeruginosa*, 39 *K. pneumoniae* and 40 *E. coli*) were recovered from clinical specimens of patients who attended the University of Nigeria Teaching Hospital (UNTH), Ituku-Ozalla in Enugu state, Southeast Nigeria from January to December 2011. Identification of the clinical isolates was performed on the basis of Gram staining, motility, colony morphology on growth media and specific biochemical tests as per standard microbiological characterization procedures [16].

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 disk diffusion method in line with the ClinicalLaboratory 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. The antibiotic susceptibility results were interpreted as per the CLSI guidelines [17].

Screening for MBL production: The screening for metallo-B-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, 18]. Isolates showing reduced susceptibility to any of the carbapenems (IPM and MEM) were suspected to produce MBL enzyme and warrants a phenotypic confirmation test [4, 15].

Disk Potentiation Test: MBL production was confirmed phenotypically on IPM and MEM resistant isolates by a previously described method with little modifications [8]. 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). Sterilized 0.5M EDTA (10 µl) were each added aseptically to one IPM disk and one MEM disk using a micropipette. The plate(s) were incubated aerobically at 37°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. If the zone of inhibition of imipenem + EDTA disks or meropenem + EDTA disks compared to imipenem or meropenem disks without EDTA respectively is greater than 7 mm, then the test bacterium can be phenotypically inferred to be MBL positive [11, 19].

Re-Modified Hodges Test (MHT): The MHT was performed on Mueller-Hinton (MH) agar (Oxoid, UK) plates on all bacterial isolates that showed reduced susceptibility to any of the carbapenems (imipenem or meropenem) according to a previously described method [4]. A strain of *E. coli* ATCC 25922 (adjusted to a 0.5 McFarland turbidity standard)was inoculated on the surface of a MH agar plate and the plate was allowed to dry for 10 mins. A 10 µg imipenem disk (Oxoid, UK) was placed aseptically at the center of the MH agar plate and the test organism was streaked heavily from the

edge of the plate towards the edge of the central disk (10 µg imipenem). Positive MHT result was confirmed phenotypically if the growth of the test bacterium was observed within the inhibition zone of the 10 µg imipenem disks, thus giving a cloverleaf effect or distorted zone of inhibition [4, 10]

Multiple antibiotic resistance index (MARI): MARI was determined by a previously described method [25]. This was evaluated using the formular:MARI = a/b, where "a" is the number of antibiotics to which the bacteria was resistant to and "b" is the total number of antibiotics to which the resistant bacteria has been evaluated byantibiotic susceptibility testing.

RESULTS

During the study period (January to December 2011), 99 non-duplicate clinical isolates of Gram-negative bacteria (40 *E. coli*, 39 *K. pneumoniae* and 20 *P. aeruginosa*) were recovered from clinical specimens of patients who attended the University of Nigeria Teaching Hospital (UNTH), Ituku-Ozalla in Enugu state, Southeast Nigeria. Table 1 shows the site distribution of all the isolates employed for this study while Table 2 shows theirantimicrobial susceptibility patterns. 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. 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-B-lactamase (MBL) production in the potential MBL-producers was detected phenotypically. 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 while the Re-Modified Hodges test detected MBL in 5 *E. coli*, 3 *K. pneumoniae* and 3 *P. aeruginosa* bacterial isolates. Overall, a high degree of resistance to the tested antibiotics was noted amongst the bacteria isolates, especially to the third-generation cephalosporins where the resistance rates for CTX were (*E. coli* 45 %, *K. pneumoniae* 61.5 %, *P. aeruginosa* 50 %) and for 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

Table 1: Source distribution of the clinical bacterial isolates

Specimens	*K. pneumoniae* n (%)	*E. coli* n (%)	*P. aeruginosa* n (%)	Total
Urine	13 (23.2)	34 (60.7)	9 (16.1)	56
Ear swab	1 (14.2)	0 (0)	6 (85.7)	7
Pleural aspirate	1 (100)	0 (0)	0 (0)	1
Sputum	18 (94.7)	0 (0)	1(5.3)	19
High vaginal swab	0 (0)	1 (100)	0 (0)	1
Wound swab	6 (42.9)	5 (35.7)	3 (21.4)	14
Conjunctival swab	0 (0)	0 (0)	1 (100)	1
Total	39 (39.4)	40 (40.4)	20 (20.2)	99

Table 2:*In vitro* antimicrobial susceptibility pattern of the bacterial isolates

	E. coli(n=40)		*K. pneumoniae*(n=39)		*P. aeruginosa* (n=20)	
	S	R	S	R	S	R
Drugs	n(%)	n(%)	n(%)	n(%)	n(%)	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)

Table 3: 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 testno (%)	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)

Table 4: Multiple antibiotic resistance index (MARI) of resistant *E. coli*, *K. pneumoniae* and *P. aeruginosa* clinical isolates

Isolate no.	MARI
E28	0.6
K58	0.2
K30	0.6
K82	0.3
E60	0.6
E09	0.2
P66	0.4
K49	0.2
K51	0.7
P45	0.2
K52	0.7
K09	0.7
K10	0.7
P11	0.1

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). The results of the multiple antibiotic resistance index (MARI) of resistant isolates in our study are shown in Table 4. The antibiotic resistant *Escherichia coli* isolates have MARI of 0.2 to 0.6 while the antibiotic resistant *Klebsiella pneumoniae* isolates have MARI of 0.2 to 0.7. For antibiotic resistant *Pseudomonas aeruginosa* isolates, its MARI was 0.1 to 0.4.

DISCUSSION

Antimicrobial resistance to the carbapenems (e.g. imipenem and meropenem) mediated by metallo-B-lactamase (MBL) enzymes has tremendous clinical implications since the carbapenems are usually the last treatment options for bacterial infections caused by multidrug resistant organisms (e.g. producers of extended spectrum B-lactamases) [20]. In Nigeria, especially in the SouthEastern part where this study was conducted, information regarding MBL-producing bacteria is scarce, non-existent or has not been reported. The emergence and spread of multidrug resistant bacteria with acquired resistance to various B-lactams is a therapeutic problem with growing worldwide concern [6, 10]. In this study, the antibiogram of all the isolates was investigated. These results of *in vitro* antimicrobial susceptibility profiles of the clinical isolates included in this study 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 [21-23]. Thesusceptibility of the isolates in this study to amoxicillin-clavulanic acid, AMC (Table 2)isalso 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 [24]. However, these rates are 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% [25]. 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). MBL production was confirmed phenotypically by Disk Potentiation test and Re-Modified Hodges test. The observed prevalence of MBLs in *E. coli* isolates as obtained in this study (Table 3) is lower than similar studies conducted in India where the prevalence of MBLs in *E. coli* isolates tested was 28.5% [1] and in Australia where some*E. coli* isolates tested positive for phenotypic MBL production [11]. The prevalence of MBLs in *K. pneumoniae* isolates from this study (Table 3) is in contrast to the results obtained in India and Europe, where *K. pneumoniae* isolates showed 36.6% production of MBL [1, 11]. According to previous studies, [10, 12, 20]. MBL production is found to be more prevalent and propagated first in *P. aeruginosa* isolates before appearing in Enterobacteriaceae including *E. coli* and *K. pneumoniae* isolates. The prevalence of MBL in *P. aeruginosa* isolates in thisstudy (Table 3) corresponds to similar studies conducted in Mumbai and Kashmir, where the prevalence rates of MBL production in *P. aeruginosa* isolates were 20.8% and 11.66% respectively [8, 19]. MBL producing organisms are reported across

South America, Southern Europe and Southeast Asia and have also been recently reported in Southwest Nigeria [7, 9, 10]. However, the prevalence of MBL production in our *P. aeruginosa* isolates (Table 3) is lower than results obtained in Brazil (22.77 %,), India (51.28 %) and Iran (53.2 %) [1, 4, 14]. In the South Eastern part of Nigeria where this work was conducted, there is a dearth of data regarding the prevalence of MBL production, but a report from Southwest Nigeria showed that 4 out of 97 *P. aeruginosa* isolates tested for metallo-B-lactamase enzymes produced MBLs phenotypically [7].In this study, could be reported the first phenotypic MBL detection amongst Gram-negative bacilli from clinical specimens of patients from University of Nigeria Teaching Hospital (UNTH), Ituku-Ozallain Enugu, Southeast Nigeria (Table 3). Considering the prevalence of MBL production in our study, it seems likely that it resulted from the irrational and uncontrolled usage of wide spectrum antibiotics. This scenario might in no doubt allowed bacteria to develop resistance through selective pressure mounted on them over time as a result of unreasonable use of these drugs and possible poor infection control practices in both the community and hospital environments [11]. Secondly, the acquisition of antibiotics (including broad spectrum drugs such as carbapenems) over-the-counter in this region coupled with the uncontrolled usage of these agents in livestock and animal husbandry purposes may have given power to the development of this all important enzymes (MBLs) in our environment. Our multiple antibiotic resistance index (MARI) showed that the *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates in this study were all resistant to more than five antibiotics.However, our MARI results are concordant with other claiming that Gram-negative bacteria including *E. coli*, *P. aeruginosa* and *K. pneumoniae* that produce B-lactamase enzymes (MBL inclusive) are resistant to multiple classes of antibiotics used in clinical medicine [6, 10-12].

CONCLUSION

Conclusively, these findings clearly showed that bacterial pathogens expressing MBL phenotypes actually exist in Enugu, Southeast Nigeria and might be responsible for some hospital-acquired and community-acquired infections. The clinical microbiology laboratory is paramount to tackling multidrug resistance through timely detection and reporting and as such should be proactive in promptly and accurately detecting pathogens producing MBLs in addition to other antibiotic degrading enzymes produced by pathogenic bacteriaas a way of impeding their emergence and spread within a given hospital community.It will also be of importance to incorporate MBL detection in the laboratory routine of hospitals in this region since no hospital in the SouthEastern part Nigeria where this work was conducted currently looks out for MBL producing bacteria in their laboratory work.

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