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Genotypic detection of metallo-Beta-Lactamases among multidrug resistant *Klebsiella pneumoniae* isolated from urine samples of UTI patients

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Abstract

Metallo-beta-lactamases producing bacteria are of public health concern, owning to the fact that bacteria that harbour genes for their production are notably resistant to several antibiotics. This study was carried out to genotypically investigate the prevalence of MBL-production in multidrug resistant *K. pneumoniae* isolated from urine samples of UTI patients attending a tertiary teaching hospital in Awka, Anambra State, Nigeria. Two hundred (200) mid-stream urine samples were collected from UTI patients, and were analyzed using standard microbiological procedures. Antibiotic susceptibility testing (AST) of the isolates was carried out using Kirby-Bauer disc diffusion method and combination disc test was employed to phenotypically screen the isolates for MBL production. The genotypic screening was performed by Polymerase chain reaction (PCR), using specific primers. Fifty-one (51) *K. pneumoniae* isolates were recovered from the urine samples. AST revealed that the isolates exhibited high resistance to Amoxicllin-clavulanic acid (100%), Ampiclox (98.04%), Imipenem (98.04%), Cefotaxime (96.08%), Cefuroxime (96.08%), Nitrofurantoin (70.59%), and were relatively susceptible to Ofloxacin (54.91%), Gentamycin (50.98%) and Levofloxacin (47.06%). A 79.02% of the *K. pneumoniae* isolates showed resistance to more than two classes of antibiotics, thus termed multidrug resistance strains. Of the 38 (41.76%) isolates of *K. pneumoniae* that were resistant to imipenem, 19 (20.88%) were confirmed as MBL-producers phenotypically, while 19 (20.88%) were non-MBL producers. Genotypically, *bla*VIM (21.98%) was the most detected MBL gene, followed by *bla*OXA-48 (18.68%) and *bla*IMP (15.38%). This study revealed the prevalence of multidrug resistant and MBLs producing *K. pneumoniae* in urine samples of the selected UTI patients.

Keywords: Multidrug resistance; Metallo-beta-lactamases; *Klebsiella pneumoniae*; Genotypic detection; Urinary tract infections

1. Introduction

Metallo-beta-lactamases (MBLs) are carbapenem-hydrolyzing enzymes that have the exceptional ability to hydrolyze the carbapenems antibiotics including imipenem, meropenem, ertapenem, e.t.c [1]. Carbapenems are often the last line of drugs for the treatment and management of multidrug resistance infections, but unfortunately, carbapenem resistance is progressively spreading among Gram-negative clinical isolates including *Klebsiella pneumoniae* [2]. MBLs also confer variable range of resistance to beta-lactam antibiotics, with the exception of monobactams, thus limiting the treatment options for treating life-threatening bacterial infections such as urinary tract infections (UTIs) [3]. MBLs producing bacteria have been isolated globally, and more than 80 types of MBLs have been identified worldwide, with over 75% occurring as plasmid-encoded enzymes [4]. Among them, *bla*VIM, *bla*IMP, *bla*NDM, and *bla*OXA types of enzymes are the major variants reported globally [2, 5].

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Urinary tract infections (UTIs) are one of the most frequent human bacterial infections worldwide, occurring both at the community and hospital settings, with a high rate of casualty and financial cost [6]. Among the Enterobacteriaceae, *Klebsiella pneumoniae* has been reported as one the major MBL-producers, and the increasing prevalence in urinary tract infections is of global health concern [7]. It is estimated that about 150 million cases of UTIs occur globally every year, with 50% of women and 12% of men experiencing at least one symptomatic UTI during their lives and about 25% of affected women have recurrent UTI [8]. The emergence of Metallo-Beta-Lactamases producing organisms are promoted by factors such as extensive and indiscriminate use of antibiotics both in the hospital and community, suboptimal infection prevention and control practices, use of urinary catheters, presence of immunosuppressive condition, along with the horizontal transmission of resistance gene-carrying plasmids among bacterial strains [7]. However, it is crucial to provide up-to-date resistance patterns of these organisms and their molecular mechanisms of resistance, which will help to guide proper diagnosis and treatment by healthcare practitioners. Therefore, this study was carried out to genotypically investigate the prevalence of MBL-production in multidrug resistant *K. pneumoniae* isolated from urine samples of UTI patients attending a tertiary teaching hospital in Awka, Anambra State, Nigeria.

2. Materials and methods

2.1. Sample collection

A total of 200 mid-stream urine samples were collected from UTI patients attending Chukwuemeka Odumegwu Ojukwu University Teaching Hospital (COOUTH), Amaku, Awka, Anambra State. Verbal informed consents were obtained from all patients and a research questionnaire filled out by each patient before specimen collection. The ethics of the study was confirmed and approved by the Health Research and Ethics Committee of Chukwuemeka Odumegwu Ojukwu University Teaching Hospital (COOUTH), Amaku, Awka, with Reference No: COOUTH/HREC/ETH.C/VOL.1/FN:04/317. Mid-stream urine samples were collected using sterile wide-mouth universal containers, labeled properly and transported to the Laboratory of Pharmaceutical Microbiology and Biotechnology Department, Nnamdi Azikiwe University, Agulu Campus, within 2-3 hours of samples collection for microbial analysis.

2.2. Isolation and identification of bacterial isolates

A loopful of each urine sample were aseptically inoculated into Nutrient broth tubes, and incubated at 37 °C for 24 hours. After 24 hours of incubation, presence of bacterial growth was indicated by cloudiness/turbidity in the tubes. Then, each broth culture was sub-cultured in MacConkey media. After incubation, the plates were observed for visible growth and examined for their colonial appearance such as colour, size, elevation and consistency. Discrete mucoidal smooth pink colonies suspected to be *K. pneumoniae* were sub-cultured by streaking on a fresh MacConkey agar medium and incubated at appropriate conditions. Identification and characterization of the isolates were carried out using Gram-staining techniques and selected biochemical tests including; citrate utilization test, indole test, oxidase test, catalase test, as described by Cheesebrough [9].

2.3. Antibiotics susceptibility testing

The antibiotic susceptibility testing of the test isolates was performed using Kirby-Bauer disc diffusion method on Muller-Hinton agar (MHA) as reported by Khan *et al*. [10]. A total of 12 antibiotics from six different classes were used against the isolates, and they include; Amoxicillin-clavulanate (30 µg), Cefotaxime (25 µg), Imipenem (10 µg), Ofloxacin (5 µg), Gentamycin (10 µg), Nalidixic acid (30 µg), Nitrofurantoin (300 µg), Cefuroxime (30 µg), Ceftriaxone (30 µg), Ampiclox (10 µg), Cefixime (5 µg), and Levofloxacin (5 µg). After incubation at 37 °C for 24 hrs, the inhibition zone diameters (IZDs) were recorded and interpreted according to Clinical Laboratory Standard Institute (CLSI) performance standard for antimicrobial disk susceptibility test [11]. Isolates that showed resistance to two or more classes of antibiotics were termed multi-resistance strain [12].

2.4. Phenotypic screening of isolates for Metallo-Beta-lactamases (MBLs) production

Isolates that were resistant to carbapenem antibiotics (imipenem (10 µg) and meropenem (10 µg), with inhibition zones of diameter ≤ 23 mm for both antibiotics on presumptive screening test were further screened for the confirmation of metallo beta-lactamases production using Combination Disk Test (CDT) as recommended by CLSI [11].

Briefly, plates of MHA were inoculated with standardized suspension of the isolates using sterile swab sticks, and allowed to dry. Using a sterile needle, two discs of imipenem (10 µg) were placed 30 mm apart on the MHA plates. Then using a sterile micropipette, 5 µL of 5 M EDTA neutralized solution was introduced to one of the imipenem disc, labelled properly and allowed to stand for 20 minutes. The plates were incubated at 37 °C for 24 hours. After incubation, isolates

that showed an increase of ≥ 7 mm in the zone of inhibition around the imipenem + EDTA disc as compared to the imipenem disc alone were considered as MBLs- producers [13].

2.5. Genotypic screening of the isolates for MBLs producing genes

Bacterial isolates that showed high resistance to carbapenem antibiotics, which also tested positive in the phenotypic screening test for MBLs production, were screened genotypically for MBL genes.

2.5.1. DNA extractions

DNA extractions were carried out using ZR Fungal/Bacterial DNA miniprep™ (Zymo Research, Catalog no: D6005) by following the manufacturer's instructions.

2.5.2. PCR Amplification of genomic DNA for MBL producing genes

The DNA isolated from the test isolates were subjected to Polymerase chain reaction (PCR) using specific primers for MBLs genes (VIM, IMP, OXA_{48}) as shown in Table 1. The PCR reaction were performed in a final volume of 25 µL of the amplification mixture containing; Nuclease free water (8.5 µL), Taq 2X master mix (New England Biolab: M0270) (12.5 µL), 10 µM of each forward primer (1 µL), 10 µM of each reverse primer (1 µL) and DNA sample (2 µL). The PCR products were separated on 2% agarose gel electrophoresis stained with 10µL EZ Vision DNA stain, and visualized under ultraviolet (UV) light.

Table 1 Primer sequences and PCR conditions used for the amplification of the MBLs genes

MBL genes	Primer sequences (5' – 3')	Annealing Temp (°C)	No. of cycles	Amplicon size (bp)
VIM (F)	GATGGTGTTTGGTCGCATA	56	36	250
VIM (R)	CGAATGCGCAGCACCAG			
IMP (F)	GGAATAGAGTGGCTTAAYTCT	56	36	232
IMP (R)	CGGTTTAAYAAAACAACCACC			
OXA_{48} (F)	GCGTGGTTAAGGATGAACAC	56	36	550
OXA_{48} (R)	CATCAAGTTCAACCCAACCG			

Keynote: F = Forward primer, R = Reverse primer.

3. Results

3.1. Sample distribution based on the socio-demographical and clinical characteristics of the study participants

Table 2 showed the distribution of the samples based on the socio-demographical and clinical characteristics of the study participants. Out of 200 urine samples collected for this study, a greater number of 142 (71 %) samples were collected from women having UTI as their primary diagnosis, while 58 (29 %) samples were collected from men. The age group 19-30 years (42.5 %), had the highest prevalence of UTI, followed by age group 31-40 years (28.5 %), while the age group 0-18 years had the least occurrence (4.5 %). Forty-nine (24.5 %) and 151 (75.5 %) urine samples were collected from in-patients and out-patients respectively. Sixteen (27 %) out of the 58 men that have UTI, have had recurrent UTI, while 53 (37.32 %) out of the 142 women that have UTI, have had recurrent UTI.

Table 2 Samples distribution based on the socio-demographical and clinical characteristics of the study participants

S/N	Characteristics	Category	Frequency (%)
1	Age	0 - 18	9 (4.5 %)
		19 – 30	85 (42.5 %)
		31 – 40	57 (28.5 %)
		41 – 60	28 (14 %)
		≥ 61 & above	21 (10.5 %)
			Total = 200 (100 %)
2	Gender	Male	58 (29 %)
		Female	142 (71 %)
			Total = 200 (100 %)
3	Patient Setting	In-patients	49 (24.5 %)
		Out-patients	151 (75.5 %)
			Total = 200 (100 %)
4	Re-current UTI	Male	16 (27 %)
		Female	53 (37.32 %)
			Total = 69 (34.5 %)

3.2. Prevalence of the study isolates based on the socio-demographical data of the study participants

A total of 51 clinical isolates of *K. pneumoniae* were recovered from 200 urine samples of UTI patients attending COOUTH, Awka Anambra State. The age group 19-30 years had the highest prevalence of the test isolates 23 (45.10 %), followed by the age group 31-40 years 11 (21.57 %), while the least prevalence was seen in the age group 0-18 years 2 (3.92 %). The statistical comparison of the prevalence of the study isolates within the age-group of the study participants shows that, there is no significant difference as $p > 0.05$. Forty (78.43 %) out of the total 51 (100 %) isolates were recovered from the female patients, while 11 (21.57 %) were recovered from the male patients as shown in Table 3.

Table 3 Prevalence of the study isolates based on the socio-demographical data of the study participants

S/N	Characteristics	Category	*K. pneumoniae* (n = 51)
1	Age	0 - 18	2 (3.92 %)
		19 – 30	23 (45.10 %)
		31 – 40	11 (21.57 %)
		41 – 60	8 (15.69 %)
		≥ 61 & above	7 (13.73 %)
2	Sex	Male	11 (21.57 %)
		Female	40 (78.43 %)
3	Patient Setting	In-patients	9 (17.65 %)
		Out-patients	42 (82.35 %)

3.3. Antibiotic susceptibility testing of the isolates to multi-antibiotics

The antibiotic susceptibility testing of the isolates showed that the *K. pneumoniae* isolates were highly resistant to Amoxicillin-clavulanic acid (100 %), Ampiclox (98.04 %), Imipenem (98.04 %), Cefotaxime (96.08 %), Cefuroxime

(96.08 %), Cefixime (94.12 %), and Ceftriaxone (76.47 %). Whereas, 54.91 %, 50.98 %, 47.06 % and 33.33 % of the isolates were susceptible to Ofloxacin, Gentamycin, Levofloxacin, and Nalidixic acid respectively (Table 4). Also, 79.02% of the isolates showed multiple resistant to more than two classes of antibiotics, thus termed multidrug resistant strains.

Table 4 Antibiotic susceptibility pattern of *Klebsiella pneumoniae* to multi-antibiotics

		***Klebsiella pneumoniae* (N = 51)**			
	Antibiotics	**Abbrev.**	**Susceptible (S)**	**Intermediate (I)**	**Resistant (R)**
	Combined B-lactam				
1	Amoxicillin-clavulanic acid (30 µg)	AUG	0(0%)	0(0%)	51(100%)
2	Ampiclox (10 µg)	ACX	1(1.96%)	0(0%)	50(98.04%)
	Cephems				
3	Cefotaxime (25 µg)	CTX	1(1.96%)	1(1.96%)	49(96.08%)
4	Cefuroxime (30 µg)	CXM	0(0%)	2(3.92%)	49(96.08%)
5	Ceftriaxone (30 µg)	CRO	7(13.73%)	5(9.80%)	39(76.47%)
6	Cefexime (5 µg)	ZEM	2(3.92%)	1(1.96%)	48(94.12%)
	Carbapenems				
7	Imipenem (10 µg)	IMP	0(0%)	1(1.96%)	50(98.04%)
	Aminoglycosides				
8	Gentamycin (10 µg)	GN	26(50.98%)	5(9.80%)	20(39.22%)
	Fluoroquinolones				
9	Nalidixic acid (30 µg)	NA	17 (33.33 %)	3(5.88%)	31(60.79%)
10	Levofloxacin (5 µg)	LBC	24(47.06%)	1(1.96%)	26(50.98%)
11	Ofloxacin (5 µg)	OFX	28(54.91%)	4(7.84%)	19(37.25%)
	Nitrofurans				
12	Nitrofurantoin (300 µg)	NF	10(19.61%)	5(9.80%)	36(70.59%)

3.4. Phenotypic screening of the isolates for MBLs production.

Out of the 51 isolates of *K. pneumoniae* phenotypically screened for MBLs production, 38 (74.51 %) were resistant to imipenem on the MBLs presumptive screening, while only 19 (37.25 %) of the isolates were confirmed to be MBLs producer by Combination Disc Method (CDT). Whereas, 37.25 % of the isolates that were resistant to imipenem were not MBLs-producers.

3.5. Genotypic screening result of the isolates for MBL producing genes

Ten (19.61%) out of the 19 (37.25%) *K. pneumoniae* isolates that were phenotypically positive for MBL- production were screened for MBL-genes (*bla*VIM, *bla*IMP and *bla*OXA_{48}) by PCR using specific primers. The result revealed the prevalence of *bla*VIM in 19.61% of the isolates. *bla*IMP and *bla*OXA_{48} were detected in 17.65% and 13.73% of the isolates respectively. Whereas, 6 (12.09 %) of the *K. pneumoniae* isolates co-harboured the *bla*VIM + *bla*IMP + *bla*OXA_{48} genes simultaneously (Table 5). The predominant MBL-gene detected in this study was *bla*VIM.

Table 5 Genotypic screening result of the isolates for MBL- genes

K. pneumoniae (n=10)	
Screened MBL-genes	**Frequency (%)**
VIM	10 (19.61 %)
IMP	9 (17.65 %)
OXA_{48}	7 (13.73 %)
VIM+IMP	3 (5.88 %)
VIM+OXA_{48}	1 (1.96 %)
VIM+IMP+OXA_{48}	6 (11.76 %)

4. Discussion

The increasing and rapid spread of MBL-producing *Enterobacteriaceae*, particularly *K. pneumoniae* constitute serious threats to public health globally. *K. pneumoniae* is one of the major uropathogens that causes difficult-to-treat UTIs, and they contribute to treatment failures and increase morbidity and mortality in UTI patients [14]. The findings of this study revealed that UTI was more prevalent in females than males, with the prevalence ratio of 2:5. This could be as a result of the anatomical structure of the female urinary tract. Women have shorter urethra than men, which shortens the distance a bacterial ought to travel to reach the bladder [12]. This finding is similar with the report of Shakya *et al*. [15] and Barua *et al*. [16], who reported that ratio of UTI in men to women, were 2:6 and 2:7 respectively.

Our study recorded high prevalence of UTI within the age group 19-30 years (42.5 %), followed by the age group 31-40 years (28.5 %). This could be attributed to the fact that these age brackets lie within the sexually active individuals, and therefore have high chances of exposure to these organisms. This finding is closely related to the findings of Moue *et al*. [17], who reported high prevalence of UTI in patients between the age group of 21-40 years. Our study observed high prevalence of the study isolates in the age group 19-30 years (23/51), followed by 31-40 years (11/51), while the age-group 0-18 years (2/51) had the least prevalence of the study isolates.

The antibiotic susceptibility testing showed that *K. pneumoniae* isolates were highly resistant to Amoxicillin-clavulanic acid (100%), Ampiclox (98.04%), Imipenem (98.04%), Cefotaxime (96.08 %), Cefuroxime (96.08%), Cefixime (94.12 %), Ceftriaxone (76.47 %) and Nitrofurantoin (70.59%), which might be as a result of MBLs production. And this may imply that using these classes of antibiotics to treat UTIs at our study area may result in treatment failure. This finding is in line with the reports of Mulu *et al*. [12], who reported high resistance among MBL-producing *K. pneumoniae* to Ampicillin (93.3%), Amoxicillin-clavulanic acid (58.7%), Cefotaxime (86.6 %), and Ceftazidime (86.6 %). Also, 54.91 %, 50.98 %, 47.06 % and 33.33% of the isolates were susceptible to Ofloxacin, Gentamycin, Levofloxacin, and Nalidixic acid respectively (Table 4). The antibacterial activities of the tested antibiotics against *K. pneumoniae* were in the order of Amoxicillin-clavulanic acid < Imipenem < Ampiclox < Cefotaxime < Cefuroxime < Cefixime < Ceftriaxone < Nitrofurantoin < Nalidixic acid < Levofloxacin < Gentamycin < Ofloxacin which also indicates that Ofloxacin can be prescribed for the empirical treatment of UTIs caused by *K. pneumoniae* in the study area. This finding is in line with the findings of Sikarwar and Batra [18] and Ugwu *et al*. [6], who recommended the use of fluoroquinolones particularly Ofloxacin for the treatment of UTIs, caused by *K. pneumonia*e, but contrary to the reports of Pandit *et al*. [19], Kebbeh *et al*. [20], and Pantha *et al*. [21], who reported 3rd generation cephalosporin and imipenem to be very effective in treatment of UTIs caused by Gram-negative uropathogens. However, 79.02% of the isolates showed multiple resistant to more than two classes of antibiotics, thus termed multidrug resistant strains.

In this study, imipenem resistance was detected in 38 (74.51%) of the isolates. Of the 38 isolates that were imipenem resistant, 19 (37.25%) were phenotypically confirmed to be MBL-producers using CDT method, while 19 (37.25%) were non-MBL producers. Similar finding was reported by Maduakor *et al*. [22], where imipenem resistance was observed in 27.7% isolates of *K. pneumoniae* isolated from urine samples in a tertiary hospital in Enugu State, Nigeria. Dissemination of MBL-producing bacteria could be attributed to the presence of multiple risk factors such as inappropriate use of broad-spectrum antibiotics, inappropriate prescription, long duration of hospital stay and transfer of MBL-resistance genes by transposable elements such plasmid in community and health care settings (Ugwu *et al*., 2020).

Molecular studies showed that the most prevalent MBL-gene detected in this study was *bla*VIM (19.61%), followed by *bla*IMP (17.65%) and *bla*OXA_{48} (13.65%). However, 5.88 % of the studied isolates co-haboured *bla*VIM + *bla*IMP, while 6 (12.09 %) of the isolates co-harboured *bla*VIM + *bla*IMP + *bla*OXA_{48} genes simultaneously (Table 5). Our findings is in line with the study of Odewale *et al*. [7], who reported *bla*VIM (43 %) as the most detected MBL-gene from clinical isolates *K. pneumoniae* in Southwest, Nigeria, but contrary to the study carried out by Ugwu *et al*. [6], in the same study area, they reported *bla*SPM as the most predominant MBL-gene. The high prevalence of MBL resistant genes detected in this study could be the reason for the high resistance to carbapenems recorded in the AST of the isolates, and could be due to the regular use and misuse of the antibiotics by the patients. The occurrence of multi-drug resistance *K. pneumoniae* isolates co-harbouring more than one MBL resistance genes observed in this study is of public health concern, and hence requires strict control of antibiotic use in the study area. This is of serious concern owing the fact that, it predisposes risk factors of transferring resistance to other species of bacteria [23].

5. Conclusion

Our research revealed the prevalence of multi-drug resistant (MDR), and Metallo-beta-lactamases (MBLs) producing *K. pneumoniae* in urine sample of the selected UTI patients. Fifty-one isolates of *K. pneumoniae* were successfully characterized from the urine samples, which showed high level of resistance to the tested antibiotics. Multidrug resistance was observed in 79.02% of the isolates, thus resulting in limited treatment options, while co-existence of more than one MBL gene was observed in 11.76% of the studied isolates. The most predominant MBL-gene was *bla*VIM. Hence, there is need to discontinue treatment based on empirical data, for effective treatment of UTI caused by MBLs producing bacteria.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that they have no conflict of interest relevant to this article.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

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