Prevalence and Characterization of Carbapenemase Encoding Genes in Multidrug-Resistant Gram-Negative Bacteria from medical institutes of Lahore

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Abstract- The rapid spread of metallo-β-lactamase (MBL) producing clinical pathogens is a matter of great concern. The presence of MBL in hospitals poses more threat for public health as MBL positive isolates show resistance to most of the antibiotics. The current study was carried out to determine the prevalence of extended-spectrum β-lactamases (ESBLs) and metallo-β-lactamases (MBLs), particularly *bla*$_{VIM}$ in clinical multi-drug resistant isolates from two tertiary care hospitals in Pakistan. A total of 57 clinical isolates were included in the study where the isolates were collected from two public tertiary care hospitals in Lahore. The isolates were screened for ESBLs and MBLs production by phenotypic method and PCR was performed to detect the presence of *bla*$_{VIM}$ genes. Out of 57 clinical isolates, the resistance against meropenem was noted to be highest in MBL producing strains (96%) as well as among all the bacterial strains (82%). Of these 57 isolates, 32 (56%) displayed MBLs production as accessed by combined disc method. In MBLs producing organisms, PCR amplification confirmed 6 (18.7%) isolates carried the *bla*$_{VIM}$-1 gene, whereas *bla*$_{VIM}$-2 gene was not identified in any strain. The prevalence of ESBL producing organisms was recorded to be 87.5%. It is concluded that there is an increasing trend of MBL producing gram negative bacterial strains: *P. aeruginosa* > *K. pneumonia* > *E. coli* > *A. baumannii* >*P. vulgaris*, respectively. This study demonstrated the emergence of carbapenemase-producing Gram-negative pathogens implicated in healthcare-related infections.

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Index Terms- Metallo-β-lactamase, Carbapenemase, Multidrug-Resistant, *Pseudomonas spp.*

I. INTRODUCTION

The continuous worldwide emergence and increasing prevalence of bacterial resistance to multiple antimicrobial agents is a major threat to public health [1-5]. In bacterial antimicrobial resistance mechanisms, the production of carbapenemase is one of the most important mechanisms by which bacteria acquire carbapenem resistance.

Several types of Metallo-β-lactamases conferring resistance to carbapenem have been identified in Gram-negative bacteria, which are geographically widespread. These include (1) KPC and GES variants of Ambler class A, (2) IMP-, VIM-, SPM-, GIM-, NDM-, and FIM-type metallo-β-lactamases (MBLs) of Ambler class B, and (3) OXA variant enzymes of Ambler class D [6-8]. The incidence rates of MBL production among members of Gram-negative organisms especially, *Acinetobacter baumannii (A. baumannii), Pseudomonas aeruginosa* (*P. aeruginosa*) and *Enterobacteria* has significantly increased during last few years [9]. There are several reports on the emergence of VIM-producing *Klebsiella pneumonia* (VPKP) isolates across the world. VIM types (e.g. VIM11,VIM2) MBLs in Southern Europe and the Far East, Australia, North and South America, India and Iran, and IMP-type MBLs in Southeast Asia are predominant raising serious concern with regard to future of antibacterial chemotherapy [10] .

In Pakistan, the emergence of MBL-mediated resistance is of serious concern. Carbapenems are considered to be effective therapeutic agents against the highly resistant pathogens such as *Acinetobacter and Pseudomonas spp*. The existence of MBL positive isolates in hospital setting poses a therapeutic complication that would genuinely restrict the therapeutic options, as well as critical problems for infection control management. The appropriate detection and reporting of MBL-producing bacteria will be helpful to detect the spread of these multidrug-resistant isolates. Furthermore, screening of patients at time of admission or during prolonged hospital stay can help minimize these infections. The present study was planned to determine the resistance pattern of clinical isolates of *Enterobacteriaceae* and to detect the presence of metallo beta lactamases and carbapenemase gene *bla*$_{VIM}$ by phenotypic combined-disc test and polymerase chain reaction (PCR).

II. MATERIAL METHODS

2.1. Bacterial Strains

During the study, a total of 57 strains were collected from the two institutes of Lahore namely Institute of Nuclear Medicine and Oncology Lahore (INMOL) and Post Graduate Medical Institute Lahore (PGMI). Samples were taken from different specimens such as urine, pus, blood, sputum, cerebrospinal fluid (CSF), human vaginal swab (HVS), tracheal swab and endotracheal tube tip (ETT-tip). All bacterial strains were characterized as gram negative by the gram staining method and biochemical tests.

2.2. Antibiotic Susceptibility Test

Antibiotic susceptibility of the bacterial strains was determined by the Kirby-Bauer disc diffusion method [11]. The antibiotic disc used were ciprofloxacin (5µg), meropenem (10µg), imipenem (10µg), cefotaxime (30µg), ceftazidime (30µg), cefepime (30µg) and ceftriaxone (30µg).

2.3. Detection of ESBL Producing Bacteria

In all bacterial isolates, ESBL production was determined by double disc synergy test (DDST) as illustrated previously [12]. Synergy was determined between a third-generation cephalosporin antibiotic disc alone and in combination with clavulanic acid. A disc of ceftazidime (30 µg) alone and a ceftazidime+clavulanic acid disc (30µg) were used in this test. The experimental plates were incubated overnight at 37 °C. Difference in the diameter of zones of inhibition with and without clavulanic acid was measured.

2.4. Detection of Metallo-β-Lactamases (MBL)

The phenotypic detection of the carbapenemase production was performed by the modified Hodge test as described by CLSI [13]. Briefly, the imipenem / imipenem + EDTA disc method was performed for detection of metallo-beta-lactamase. The isolates with an increase in inhibition zone diameter produced by imipenem + EDTA disc compared with imipenem were considered positive for metallo-beta-lactamase production.

2.5. DNA Extraction and PCR

DNA was isolated from MBL producing gram negative bacterial strains by using the modified protocol of alkaline lysis method [14]. The Amplification of *bla*$_{VIM-1}$ and *bla*$_{VIM-2}$ genes was carried out by performing PCR assays. The primer sequences used for *bla*$_{VIM1}$ and *bla*$_{VIM-2}$ gene amplification was described previously [15].

III. RESULTS

Collected bacterial samples were identified as *P. aeruginosa* (n= 40), *Klebsiella pneumonia* (*K. pneumonia*) (n=10), *A. baumannii* (n= 7), *Proteus vulgaris* (*P. vulgaris*), (n=1) and *Escherichia coli* (*E. coli*) (n=6). The most frequently isolated bacterial strain was *P. aeruginosa* (Table 1).

3.1. Antibiotic Resistance Pattern of Isolated Strains

Antibiotic resistance pattern for carbapenems, 3rd and 4th generation cephalosporins and floroquinolones was determined. Comparison of resistance pattern in different bacterial strains isolated from pus, Ett-tip, urine, sputum, CSF, blood, tracheal swab and HVS against different antibiotics was shown in (Figure 1). Among all isolated bacterial strains, highest resistance was observed against meropenem whereas lowest for imipenem. Most of the strains (n=48) were multidrug resistant conferring resistance to two or more drugs. In this study, *P. aeruginosa* showed highest multidrug resistance followed by *K. pneumonia, E. coli, A. baumannii and P. vulgaris* respectively (Figure 2).

3.2. Detection of ESBL and MBL

About 39 % of bacterial strains (n=22) were found to be ESBL producers while 61% strains were non-ESBL producers (Figure 3a). Bacterial strains isolated from pus samples were found to be highest ESBL producers. Distribution between ESBL producers and non-producers (P≥0.005) was found significant. However, distribution of ESBL producers isolated from male and female patients was found non-significant (p≥0. 1). Similarly among 57 bacterial strains, 32 (56%) were MBL producers (Figure 3b). Distribution of metallo beta lactamase producing and non-producing strain isolated from different clinical specimens was significant (p≥0.005). Among 32 MBL positive gram negative bacterial strains, 27 (84%) showed resistant to ceftazidime, while the highest resistance was shown against meropenem in 28 (87%) isolates. The percentage distribution of MBL producing and non-producing gram negative bacterial strains was shown in Table 2. *A. baumannii* displayed the highest MBL production (77%) followed by *P. aeruginosa* (64%) in this study. The sensitivity pattern of MBL producing and non-producing bacterial strains with the sensitivity profile of all gram negative bacterial isolates was compared and shown in Table 2. The resistance against meropenem was noted to be highest in MBL producing strains (96%) as well as among all the bacterial strains (82%).

3.3. PCR Amplification of *Bla*$_{VIM}$ Genes in Bacterial Strains

Out of 32 MBL producer bacterial strains, 6 (18.7%) isolates carried the *bla*$_{VIM}$-1 gene, whereas *bla*$_{VIM}$-2 gene was not identified in any strain (Figure 4). The product size of the *bla*$_{VIM}$-1 gene was 920bp. Distribution of bacterial Isolates showing VIM detection by PCR included *P. aeruginosa* (n=4), 1 *K. pneumoniae* (n=1) and *A. baumannii* (n=1) strains and shown in Table 3.

IV- DISCUSSION

In gram negative bacterial species the increasing resistance against carbapenems and extended spectrum betalactams is a major issue primarily in restricted number of bacteria and now increasing rapidly. Multidrug resistance (MDR) has been increasingly observed in many gram negative bacteria as a result of the extensive use of several antibiotics [16]. The present study confirmed that clinically isolated strains of *P. aeruginosa* (89%), *A. baumannii, E. coli* and *K. pneumoniae* (75%) were multidrug resistant with highest resistance against meropenem, cefotaxime, ceftriaxone, ceftazidime, cefepime, ciprofloxacin and imipenem respectively. Among cephalosporins, highest resistance was found against cefotaxime (78%), followed by ceftriaxone and ceftazidime (77%) respectively. In case of fluoroquinolones, ciprofloxacin depicted higher (71%) resistance. Lowest rate of resistance was observed against imipenem.

In our study, the overall prevalence of extended spectrum beta lactamases in isolated bacterial strains was 39%. Similar prevalence rate (40%) was reported from Pakistan in 2005 [17] whereas the prevalence of ESBL was found to be 71% in another study [18]. Among *P. aeruginosa* isolates, 21% were ESBL producers. Low prevalence of ESBL producing *P. aeruginosa* were reported (18, 19). Likewise significant prevalence of MBLs (50%) in *P. aeruginosa* and *Acinetobacter spp.* was reported from India [20] and other studies [21-23]. In the present study, 64% imipenem resistant *P. aeruginosa* and 66% *A. baumannii* were metallo beta lactamase producers.

P. aeruginosa has been one of the most important and chief nosocomial pathogens in the health-care settings. Among the bacterial strains tested in this study, *P. aeruginosa* was frequently isolated strain and found to be highly resistant to the third and fourth generation cephalosporins. Higher rates of resistance to cefotaxime, imipenem, ceftazidime, ceftriaxone and ciprofloxacin was reported in a study from Iran [24]. Among *P. aeruginosa*, maximum rate of resistance was observed against meropenem 92%. In present study, the prevalence of multi drug resistance (MDR) in *P. aeruginosa* isolates was 92% which is higher than reported (24) and also comparable to other studies from Iran, Malaysia and Pakistan [19, 25, 26]. Moreover, increasing trend in bacterial resistance has also been observed in this study as compared to previous reports from Pakistan. In the present study, the resistance to imipenem was also highest in *P. aeruginosa* (78%) followed by *A. baumannii* (66%).

In the present study, *bla*$_{VIM-1}$ gene was detected in 6 (18.7%) isolates while none of *bla*$_{VIM-2}$ gene was detected in bacterial strains that is comparable to another study conducted from Pakistan who reported 20% prevalence [28]. However, another study conducted in two hospitals of Pakistan showed that none of the isolate carry *bla*$_{VIM}$ gene [29]. Similarly, higher *bla*$_{VIM}$ gene prevalence rate of 97.2% from France [15] and 51.4% of *bla*$_{VIM}$ */bla*$_{IMP}$ genes in metallolactamase producing bacteria from India has been reported [16]. The above mentioned results confirmed different level of resistance in Pakistan in contrast to other parts of the world.

V- CONCLUSION

From the present study, it is concluded that there is an increasing trend of MBL producing gram negative bacterial strains. This study demonstrated the emergence of carbapenemase-producing Gram-negative pathogens implicated in healthcare-related infections. In addition to this, the antimicrobial drugs are now losing their efficacy due to the spread of antimicrobial resistance. Moreover resistance to antibiotic treatment and transfer to other gram negative bacteria will constrain our remedial options. Accurate identification of carbapenem-resistant bacterial pathogens is essential for patient treatment, as well as the development of appropriate contamination control measures to limit the rapid spread of pathogens.

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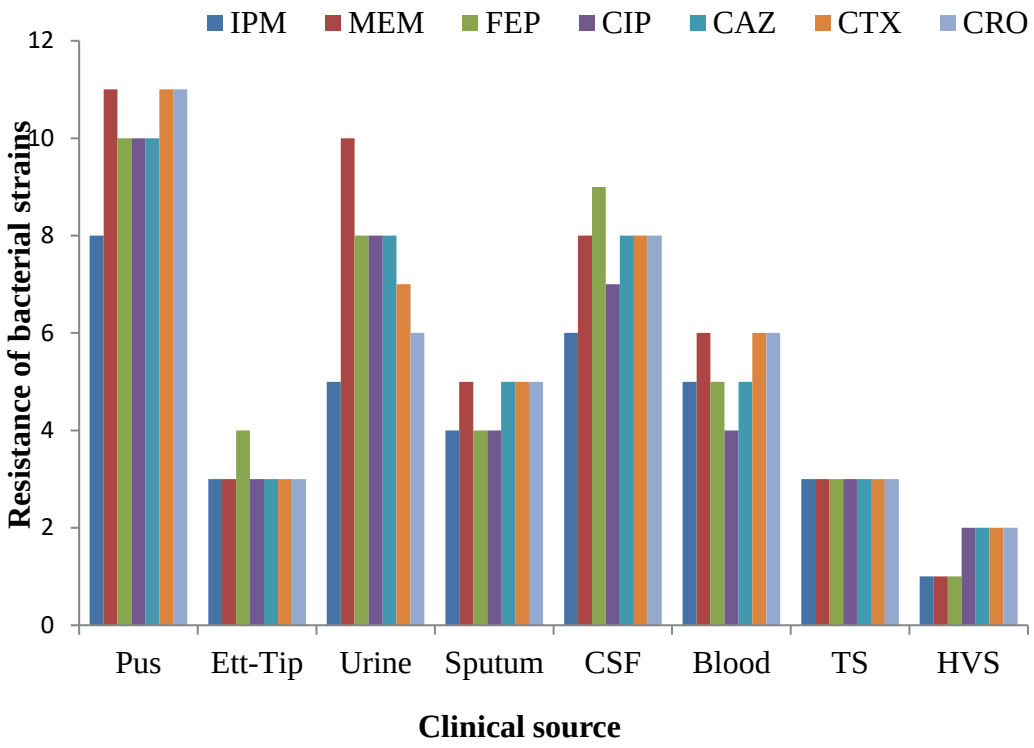
Table 1: Distribution of MBL producing and non-producing bacterial strains isolated from different clinical specimens

Clinical Source	MBL +ive	MBL –ive	p value
Pus	8	6	0.005
Blood	4	1	
Sputum	4	3	
Tracheal swab	3	0	
CSF	6	3	
HVS	0	3	
Ett-tip	3	1	
Urine	4	8	
Total	32	25	

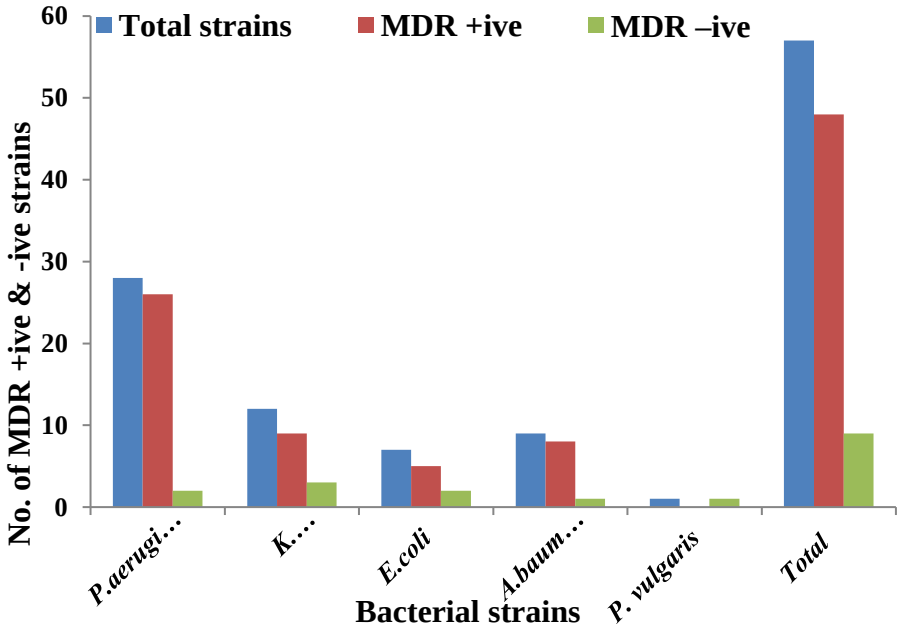
Table2: Distribution of MBL producing and non-producing Gram negative bacterial strains and prevalence of MBL genes (bla_{VIM}) amplified by Polymerase Chain Reaction.

Bacterial strains	Total	MBL +ive Strains	MBL –ive Strains	MBL genes (bla_{VIM-1}) detected
P. aeruginosa	28	18 (64 %)	10 (35%)	4 (22%)
K. pneumonia	12	6 (50%)	6 (50%)	0(0%)
A. baumannii	9	7 (77%)	2 (23%)	1(16%)
E. coli	7	1 (14%)	6 (85%)	1(14%)
P. vulgaris	1	0	1 (100%)	0(0%)



Imipenem (IPM); Meropenem (MEM); Cefepime (FEP); Ceftazidime (CAZ); Cefotaxime (CTX); Ceftriaxone (CRO)

Figure 1: Resistance pattern of bacterial strains isolated from different clinical sources against antibiotics. Results were interpreted according to CLSI guidelines (CLSI, 2009).



MDR producing (n=48); MDR non-producing (n=9)

Figure 2: Multidrug resistance pattern in different bacterial strains. Results were interpreted according to CLSI guidelines (CLSI, 2009).

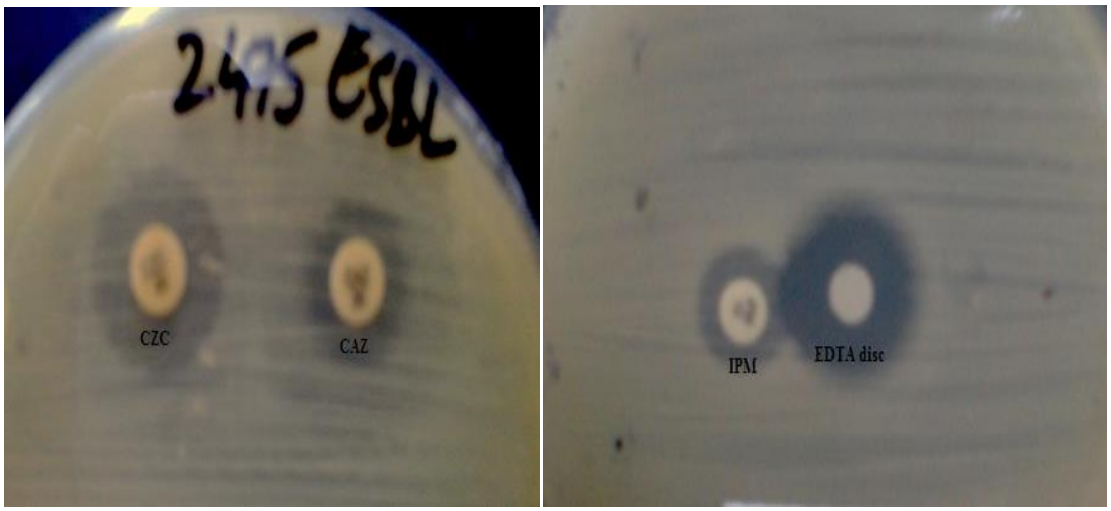


Figure 3. Phenotypic detection of carbapenemases. (A) Disc diffusion method for extended spectrum β-lactamases (ESBLs) detection. Ceftazidime (CAZ); Ceftazidime + Clavulanic acid (CZC) (B) Detection of metallo beta lactamases of gram negative bacterial strains by EDTA disc synergy test

Table 3: Comparison of sensitivity pattern of MBL producing and non-producing bacterial strains.

Antibiotics	Overall sensitivity of all bacterial strains			Sensitivity of MBL +ve bacterial strains			Sensitivity of MBL –ve bacterial strains		
	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
IPM	61	1.7	36	90	0	9	24	4	72
MEM	82	8.7	8.7	96	3	0	64	16	20
FEP	77	10	12	84	9	6	68	12	20
CIP	71	15	12	81	9	9	60	24	16
CAZ	77	10	12	93	0	6	56	24	20
CTX	78	14	7	93	3	3	60	28	12
CRO	77	19	3.5	87	12	0	64	28	8

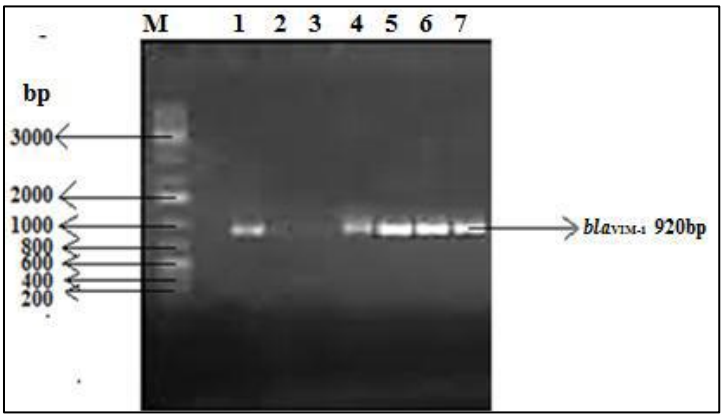


Figure 4: PCR of *bla*$_{VIM-1}$ gene. Product size: 920 bp; M- DNA ladder 200bp; Lane 1, 4, 5, 6, 7: Amplified product of *P. aeruginosa, K. pneumonia, A. baumannii bla*$_{VIM-1}$ gene