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Prevalence of Metallo-β-Lactamase *IMP* and *VIM* Producing Gram Negative Bacteria in Different Hospitals of Lahore, Pakistan

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ABSTRACT

Gram negative rod (GNR) infections cause a substantial amount of morbidity and mortality among hospitalized patients across the globe. Currently, β-lactam ring containing antibiotics predominantly carbapenems, are considered as last treatment option against multi-drug resistance GNR infections. However, the emergence of carbapenemases particularly metallo β-lactamases (MBLs) in bacteria have severely mitigated the efficiency of carbapenems. MBLs producing gram-negative bacteria have been reported from various hospital settings, worldwide. However, data is lacking in Pakistan regarding their frequency particularly among GNRs. Therefore, the present study aimed at determining the frequency of carbapenemase and β-lactamase producing GNRs recovered from different tertiary care hospitals of Lahore during January-December 2015. Additionally, existence of bla_{IMP} and bla_{VIM} carbapenems resistance determinant genes in carbapenems resistant isolates was also evaluated. The carbapenemase and β-lactamases production were evaluated by Modified Hodge test (MHT) and combined disks diffusion (CDD) method, respectively. The MBL producing clinical isolates were further subjected to PCR for the existence of bla_{IMP} and bla_{VIM} genes. The carbapenem resistant *A. baumannii* (n=32), *P.aeruginosa* (n=26), *K. pneumoniae* (n=19), *E. coli* (n=16), *C. ferundi* (n=04), *P. vulgaris* (n=02) and *E. cloacae* (n=01), were isolated from clinical samples of hospitalized patients. Out of these 100 carbapenem resistant isolates, 93 and 89 isolates were positive for cabapenemase and β-lactamase production, respectively. Notably, 3 (3.3%) of MBL producing strains harbor bla_{IMP} gene while 29 (32.5%) of MBL producing clinical strains were positive for bla_{VIM} gene. In a nutshell, several species of MBL-positive gram-negative rods are distributed broadly in different hospitals of Lahore region of Pakistan. The findings of the present study should be considered for planning strategies to treat and prevent the further spread of MBL-producing gram-negative rods infections.

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Authors' Contribution
JA performed the experiments and wrote the manuscript. SJ and SS designed the study. AW contributed in data analysing. IJ and FR helped in samples collection and NS reviewed the manuscript.

Key words
Gram negative rods, Carbapenemase, Metallo-β-lactamase, bla_{IMP}, bla_{VIM}.

INTRODUCTION

Gram-negative rods bacteria are considered as a major cause of community and hospital acquired infections (Sader *et al*., 2014). The most common disease causing gram-negative rods are *Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Bentley *et al*., 2013). Collectively, these bacteria are responsible for severe healthcare issues including pneumonia, urinary tract infections and bloodstream infections (Cardoso *et al*., 2014). The β-lactam ring containing antibiotics (β-lactams) which includepenicillins, cephalosporins, monobactams and carbapenems, are administered as last choice of treatment against gram-negative rods infections (Zeng *et al*., 2013). Theses β-lactams antibiotics bind and inactivate the penicillin-binding proteins (PBPs), which are mainly responsible for the development of the peptidoglycan layer of bacterial cell wall (Cho *et al*., 2014). Among β-lactams, carbapenems have been proven as most effective broad spectrum antibiotics due to the presence of carbapenem together with the β-lactam ring, which confers additional stability to the drug (Meletis, 2016). Very recently, gram-negative rods have been reported to harbor resistance against β-lactams including carbapenems which has become a serious health problem across the global (Ruppé *et al*., 2015). Understanding the β-lactams resistance mechanisms is of extreme importance

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in order to develop new anti-microbial agents or alternative tools for fighting against gram-negative rods challenge.

The production of β-lactamases is considered as most important mechanism associated with β-lactams resistance in gram-negative rods (Sharma *et al*., 2005). β-Lactamases are a diverse set of enzymes that catalyze the hydrolysis of β-lactam ring, thereby deactivating β-lactam antibiotics (Zeng *et al*., 2013). Until now, over 900 types of β-lactamases have been identified which on the basis molecular structures are categorized into four different classes: A, B, C and D (Babic *et al*., 2006; Bush, 2010). The class A, C and D comprised of serine type enzymes having serine at their active site while class B enzymes require divalent cations (usually zinc ions) for their enzymatic activity, hence, called as metallo β-lactamases (MBLs) (Faghri *et al*., 2014). Based on the position within the bacterial genome, MBLs are categorized into naturally and acquired MBLs. The naturally occurring MBLs are usually encoded by the bacterial chromosome whereas acquired MBLs are positioned on the bacterial transferable genetic elements such as integrons, transposons and plasmids (Walsh *et al*., 2005). Approximately, eight different types of acquired MBLs have been described that include IMPs, VIMs, SPM-1, GIM-1, AIM-1, SIM-1, NDM-1, and DIM-1 (Poirel *et al*., 2010). Among them, bla_{IMP} and bla_{VIM} are most potent and prevalent MBLs in gram-negative rods (Walsh, 2011).The dissemination of these antibiotic resistance determinants genes by horizontal transfer, is documented as a prime source of acquired resistance in gram-negative rods to B-lactams including carbapenems (imipenem,meropenem, panipenem, biapenem, ertapenem and doripenem) (Livermore *et al*., 2011). In addition, genes that code these enzymes mutate frequently due to the heavy pressure of antibiotic use, which subsequently leads to production of newer enzymes having broader activity (Majiduddin *et al*., 2002). Therefore, it is vital to understand the presence and molecular characteristics of MBLs in gram-negative rods circulating in various clinical settings.

Many studies across the globe have reported the detection of MBLs particularly bla_{IMP} and bla_{VIM} in clinically important gram-negative rods such as *Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosae* and *Acinetobacter baumannii* (Shibata *et al*., 2003; Kiffer *et al*., 2006; Diab *et al*., 2013; Aghamiri *et al*., 2014; Xu *et al*., 2015). However, very limited data is available about the existence of these disastrous bacterial resistance genes in gram-negative rods causing severe infections in hospitalized individuals of Pakistan. Therefore, the present study aimed at determining the frequency of bla_{IMP} and bla_{VIM} genes in clinical isolates of MBL producing gram-negative rods. The present study might be helpful in designing novel strategies to prevent the further spread of MBL-producing bacteria ultimately controlling the carbapenems resistant gram-negative rods infection.

MATERIALS AND METHODS

Collection and identification of bacterial isolates

Clinical samples comprising of pus, urine, blood, sputum, broncho-alveolar lavage (BAL) and central venous catheter (CVC) tips from various patients hospitalized in different tertiary care hospitals of Lahore during the period of January to December 2015, were cultured for the isolation of gram-negative bacterial strains.

The bacterial isolates were identified by conventional morphology and biochemical based tests. Bacterial strains were sub-cultured on blood and MacConkey agar and plates were incubated at 37°C overnight aerobically. The colony morphology and culture characteristics were studied to identify bacterial species. The isolates were further confirmed employing Analytical Profile Index 20 (API 20 system) (BioMerieux, France).

Antimicrobial susceptibility determination

Antimicrobial resistance of the study isolates against carbapenem antibiotics (imipenem and meropenem) was performed by Kirby-Bauer disc diffusion method according to Clinical Laboratory Standards Institute (CLSI) 2015 guidelines. For this, bacterial strains were grown on Mueller-Hinton agar (Oxoid UK) and zone of inhibition was measured around the imipenem (10µg) and meropenem (10µg) antibiotic disk. The isolates were classified into susceptible, intermediate or resistant according to the NCCLS guidelines. *E. coli* (ATCC 25922), *Pseudomonas aeruginosae (*ATCC 27853) and *K. pneumonia* (ATCC 700603) were used as susceptible, intermediate and resistant control strains.

Phenotypic detection of carbapenemases

Modified Hodge Test (MHT) was performed to screen the carbapenem resistant isolates for the production of carbapenemase according to guidelines of Clinical Laboratory Standard Institute (CLSI) 2015. For this, carbapenem susceptible strain *E. coli* ATCC 25922 was grown on the Mueller Hinton agar (MHA) and 10 ug disk of imipenem and/or meropenem was placed in the middle of the plate. The test (clinical isolates) and control strains were streaked from the disc towards edges of the plate. After 18 h incubation at 37°C, a clover leaf type indentation at the intersection of test organism showed a positive result.

Table I.- Distribution of bacterial isolates in clinical specimens.

Organism	n	Pus	Urine	Blood	Tissue	CVC tip	Sputum	BAL	HVS
A. baumannii	32	18%	3%	2%	2%	3%	1%	1%	2%
P. aeruginosa	26	22%	2%	1%	-	-	1%	-	-
K. pneumoniae	19	6%	9%	4%	-	-	-	-	-
E. coli	16	6%	8%	1%	1%	-	-	-	-
C. freundii	4	3%	1%	-	-	-	-	-	-
P. vulgaris	2	2%	-	-	-	-	-	-	-
E. cloacae	1	-	1%	-	-	-	-	-	-
Total	100	57%	24%	8%	3%	3%	2%	1%	2%

Phenotypic detection of MBLs

MBLs production by carbapenem resistant isolates was first evaluated phenotypically by using combined disks diffusion method (CDD) as described previously (Nahid *et al*., 2013). Briefly, study isolates were cultured on MHA and two 10 ug disks of each imipenem and meropenem were placed opposite to each other near the periphery of the plate. One disk of each imipenem and meropenem was treated with 10 ul of 0.5 M EDTA, while other two disks, each imipenem and meropenem, were remained untreated. After 18-24 h incubation at 37°C, the zone of inhibition was compared in EDTA treated and untreated imipenem and meropenem disks. An increase of ≥7 mm in the zone of inhibition manifested the positive results.

Molecular identification of MBL genes

The genomic DNAs were extracted from all phenotypically MBLs producing strains by using TIANamp Genomic DNA extraction (TIANGEN Biotech Beijing, Co., Ltd.). For bla_{IMP} and bla_{VIM} detection, the extracted DNAs were subjected to PCR using primer sequences already described in the literature (Qamar *et al*., 2017). Briefly, forward (5`-CTACCGCAGCAGAGTCTTTG-3`) and reverse (5`-AACCAGTTTTGCCTTACCAT-3`) primers were used for bla_{IMP} detection. Whereas, bla_{VIM} was detected by using forward (5`AGTGGTGAGTATCCGACAG3`) and reverse (5`ATGAAAGTGCGTGGAGAC3`) primers. The PCR conditions were used as follows; initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at (52°C and 55°Cfor bla_{IMP} and bla_{VIM} genes, respectively) for 40 sec, primary extension at 72°C for 1 min and final extension at 72°C for 10 min. The amplified PCR products were separated on 1.5% agarose gel by gel electrophoresis and visualized under UV light for the evaluation of molecular size of amplified products.

Statistical analysis

Results were analyzed with the SPSS (version 20.0, SPSS Inc). The detection of bla_{IMP} and bla_{VIM} genes in isolates was presented as percentages in the Tables.

RESULTS

In the present study, altogether 100 Gram-negative rods: *A. baumannii* (n=32), *P aeruginosae* (n=26), *K. pneumoniae* (n=19), *E. coli* (n=16), *C. ferundi* (n=04), *P. vulgaris* (n=02) and *E. cloacae* (n=01), were isolated from clinical samples. It is worth mentioning here that majority of the clinical isolates (57%) were recovered from pus followed by 24% from urine, 8% from blood, 3% from central venous catheter (CVC) tips, 2% from sputum and 1% from broncho-alveolar lavage (BAL). The distribution of bacterial isolates in clinical specimens is enlisted in Table I. All of these isolates manifested resistance against carbapenem (imipenem and meropenem) antibiotics.

The Modified Hodge Test for the phenotypic detection of carbapenemase production revealed that out of 100 carbapenem resistant isolates, 93 (93%) isolates were positive for carbapenemase production. The representative carbapenemase producing and non-producing isolates are shown in Figure 1A. On the other hand, 89 (89%) carbapenem resistant isolates were found phenotypically positive for MBL production since they manifested increased zone of inhibition in the presence chelating agent (EDTA), indouble disc diffusion method. A representative MBL producing Gram negative *A. baumannii* isolate is shown in Figure 1B.

The MBL producing isolates were further subjected to PCR for the detection of bla_{IMP} and bla_{VIM} genes the presence of which is considered as foremost mechanism of carbapenem resistance among Gram-negative rods. The bla_{IMP} positive samples showed amplification products of 261 bps. Similarly, the amplification of 587 bps PCR product from carbapenem resistant isolates indicated that those isolates harbor bla_{VIM} gene. In nutshell, PCR data showed that only 3 (3.3%) of MBL producing strains harbor bla_{IMP} gene. Among those bla_{IMP} positive isolates,

two strains were of *P. aeruginosae* and one of *E. coli*. However, none of *A. baumannii* or and *K. Pneumoniae* strain was found harboring bla_{IMP} gene. Unlike bla_{IMP}, elevated bla_{VIM} gene positivity 29 (32.5%) was observed among MBL producing clinical isolates. Among those 29 bla_{VIM} positive isolates, 12 (37.5%) strains were of *A. baumannii,* 11 (42.3%) of *P. aeruginosa,* 4 (21.1%) of *K. pneumonia* and 2 (12.5%) of *E. coli.* Table II briefly describes the distribution of bla_{IMP} and bla_{VIM} genes in MBL producing Gram-negative rods under this study.

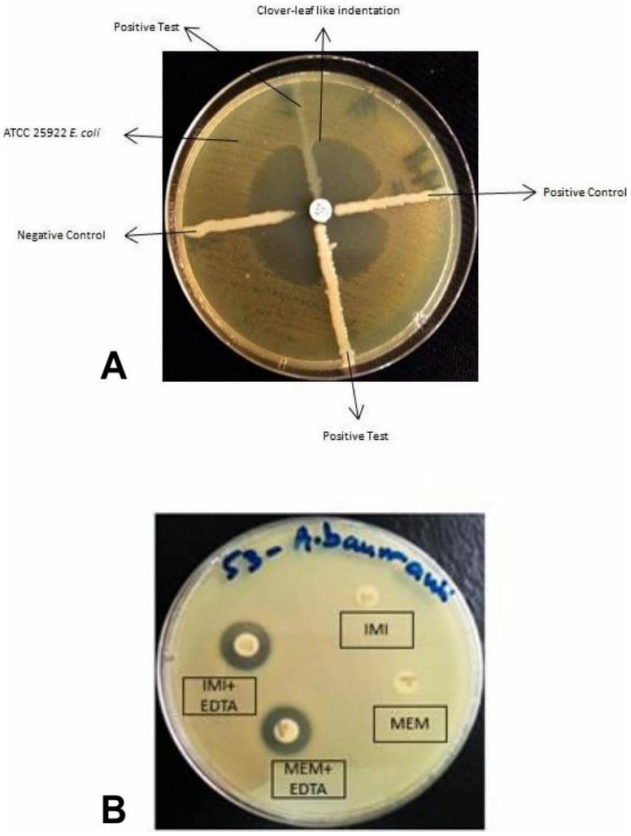

Fig. 1. Phenotypic detection of carbapenemase and metallo-β-lactamase production by Modified Hodge test (MHT) and Combined disks diffusion (CDD) method, respectively. A, The presence of clover leaf-type indentation at the intersection indicates the positive test organism while carbapenemase negative organism lack of the clover leaf-type indentation at the intersection; B, Positive results are shown by enlarged zone of inhibition in the presence of EDTA. IMI, Imipenem disc; MEM, meropenem disc; EDTA, ethylene-diamine tetra acetic acid.

DISCUSSION

Gram-negative bacteria, being responsible for high morbidity and mortality in hospitalized patients, are rapidly becoming resistant to maximum available antibiotics. Currently, β-lactam ring containing carbapenem antibiotics are used as last treatment option against multi-drug resistance bacterial infections (Rossolini, 2005). However, the emergence of carbapenemases particularly MBLs in resistant bacteria have severely mitigated the efficiency of carbapenems. Theses resistant determinant genes in bacteria can pass along genetic materials that allow other bacteria to become drug-resistant also (Sarhangi *et al.*, 2013). MBLs producing Gram-negative bacteria have been reported worldwide from various hospital settings. However, their frequency particularly among Gram-negative rods, has not been well described in Pakistan. Therefore, the present study was aimed at determining the frequency of carbapenemase and β-lactamases producing gram-negative bacteria recovered from different tertiary care hospitals of Lahore during January-December 2015. Additionally, existence of bla_{IMP} and bla_{VIM} genes in carbapenem resistant isolates was also evaluated.

Table II.- Distribution of bla_{VIM} and bla_{IMP} positive gram-negative rods.

Organism	n	bla_{VIM} (n=29)	bla_{IMP} (n=3)
A. baumannii	32	12 (37.5%)	-
P. aeruginosae	26	11 (42.3%)	1 (3.8%)
K. pneumonia	19	4 (21.1%)	-
E. coli	16	2 (12.5%)	2 (12.5%)

In the present study, *Acinetobacter baumannii* was observed as most frequent (32%) carbapenem resistant clinical isolate while previous studies conducted in Pakistan (Irfan *et al.*, 2008a), United states (Sader *et al.*, 2014) and Iran 32.08% (Mohammadi-Mehr and Feizabadi, 2011) described *E. coli* as most prevalent carbapenem resistant organism. This discrepancy may be due to the fact that we only studied imipenem and meropenem (carbapenem) resistance which is more pronounced in non-fermenting bacteria than members of *Enterobacteriaceae* (Irfan *et al.*, 2008b).

In this study, majority of the carbapenem resistant gram-negative rods (93%) were found carbapenemase positive. These results are comparable with the result of studies performed in India, Madagascar and Brazil where 94.4%, 89.8% and 88.2% carbapenemase positivity was described among gram-negative bacteria, respectively (Amudhan *et al.*, 2012; Andriamanantena *et al.*, 2010; Franco *et al.*, 2010). Likewise, 89% phenotypically MBL positivity was recorded in the present study which is in accordance to the studies conducted by Martins *et al.* (2014) (93.75%), Toval *et al.* (2015) (81.6%), Diab *et al.*

(2013) (82%) (Martins *et al*., 2014; Toval *et al*., 2015; Diab *et al*., 2013). However, in the current study, MBLs production rate among carbapenem resistant isolates was higher than the previous studies performed in Pakistan where 76% (Kaleem *et al*., 2010) and 81.87% (Nahid *et al*., 2013) MBLs production was reported among Gram-negative rods in 2009 and 2013, respectively. This time dependent gradual increase in MBLs detection rate is indicating continuous proliferation of MBL producing pathogens in hospitals of Pakistan.

In the present study, 3.3% MBL producing Gram-negative rods were found positive for bla_{IMP} gene. This finding is quite similar with the results of studies performed in Iran (3.48%) and India (2.08%) (Amudhan *et al*., 2012; Aghamiri *et al*., 2014). However, few studies also reported the higher prevalence of bla_{IMP} gene among gram negative rods. For instance, studies from Tanzania, Brazil and Japan reported bla_{IMP} gene positivity among gram-negative rods as 21.6%, 12.5 % and 13.33%, respectively (Mushi *et al*., 2014; Polotto *et al*., 2012; Zhao *et al*., 2009). The geographical variations could explain this disparity in results. Our study is also unique in a way that *Pseudomonas aeruginosa* harboring bla_{IMP} was first time described in Pakistan. Previously, only *E. coli* strain from Pakistan was reported to harbor bla_{IMP} (Nahid *et al*., 2013).

We observed that 32.5% MBLs producing Gram-negative rods carry bla_{VIM} gene which is also comparable with the studies conducted in Iran (33%) and Greece (37.6%) (Aghamiri *et al*., 2014; Psichogiou *et al*., 2008). However, previously in Pakistan, detection rate of bla_{VIM} gene among Gram-negative rods was observed as 25.1% (Nahid *et al*., 2013), which is lower than our study. Nonetheless, our study second the reports stating that bla_{VIM} is more prevalent than bla_{IMP}. Nahid *et al*.(2013) study is also c our finding in some other ways e.g. they found *Pseudomonas aeruginosa* as most frequent (42.42%) pathogen positive for bla_{VIM} while our study described *Acinetobacter baumannii as* most frequent (37.5%) organism harboring bla_{VIM}gene. This difference could be due to the fact that they did not include any *Acinetobacter baumannii*isolate in their study. Nevertheless, the present study is the first report on bla_{VIM} positive *Acinetobacter baumannii* in Pakistan.

The absence of bla_{IMP} and bla_{VIM} genes in rest of the carbapenem resistant isolates in this study beaconed that bla_{IMP} and bla_{VIM} genes are not sole source of carbapenem resistance in our locality. It can be speculated that other MBL genes such as SIM, GIM, SPM and NDM may contribute towards the resistance against carbapenems in gram-negative rods. The porin loss/mutations may also contribute towards carbapenem resistance as described earlier (Mushi *et al*., 2014).

CONCLUSION

It is concluded that several species of MBL-positive gram-negative rods are distributed broadly in different hospitals of Lahore region, Pakistan. These Gram-negative rods are carbapenem resistant and harbor bla_{IMP} and bla_{VIM} genes which are mainly responsible for carbapenem resistance. Notably, bla_{VIM} gene is more prevalent (32.5%) than bla_{IMP} gene (3.3%) in carbapenem resistant Gram-negative rods clinical isolates. The present study is the premier report for the detection of bla_{IMP} in *Pseudomonas aeruginosa* and bla_{VIM} gene in *Acinoetobacter baumannii* that may indicate the horizontal transfer of these genes within these organisms.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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