Molecular detection of bla_{VIM} Metallo-β-lactamase producing clinically isolated *Pseudomonas aeruginosa* from tertiary care hospital, Faisalabad

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Abstract: Metallo-β-lactamases (MBLs) producing *Pseudomonas aeruginosa* are major threat for public health. They produce resistance against various antibiotics and remain low or no therapeutic options. A total of 200 clinical isolates of *P. aeruginosa* were collected from tertiary care hospital, Faisalabad. Isolates were sub-cultured on basic and selective media and confirmed by API 20NE. Phenotypic detection of carbapenamase, MBLs, antibiogram and MIC were determined as per CLSI guidelines. Molecular detection of bla_{VIM} was performed using specific primers by PCR. Among 200 *P. aeruginosa*, majority (n=82) were isolated from pus samples followed by 28 from tracheal aspirates and 27 from sputum. Out of 110 (55%) MDR *P. aeruginosa*, 12 (11%) were positive for MHT and MBLs and bla_{VIM} was identified in MBL positive isolates. Antibiogram revealed that all the isolates were resistant to β-lactam drugs including carbapenems followed by 95% to levofloxacin, 67% to doxycycline and more effective drugs were tigecycline and colistin. MIC value for imipenem drug was 16μg/mL and 8μg/mL against 6 and 5 isolates respectively while MIC value for meropenem against 6 and 3 isolates were 8μg/mL and 16μg/mL respectively. Our study concluded the high prevalence of bla_{VIM} producing *P. aeruginosa* in our clinical settings.

Keywords: *P. aeruginosa*, MDR, MBL, bla_{VIM}.

INTRODUCTION

Pseudomonas aeruginosa are becoming emerging threat to public health sector around the globe particularly in developing countries like Pakistan. *P. aeruginosa* are the notorious nosocomial pathogens that are associated with morbidity and mortality despite the consequences of suitable antimicrobial therapy (Hirsch and Tam, 2010). According to the Centers for Disease Control and Prevention (CDC), *P. aeruginosa* are ranked as 4th commonest nosocomial pathogens and difficult to treat due to the acquisition of antimicrobial resistant genes against different antibiotics (Khan *et al*., 2015). Metallo-β-lactamases (MBLs) are the potent β-lactam enzymes that are not only hydrolyze the β-lactam drugs including carbapenems but also other classes of antibiotics such as aminoglycosides and quinolones. Carbapenem drugs are considering the last resort to treat the infections caused by such pathogens (Qamar *et al*., 2014). Among 612 MBLs, 60 bla_{VIM} variants have been identified so far (Naas *et al*., 2017). *P. aeruginosa* acquires resistant genes such as bla_{VIM} due to the acquisition of mobile genetic elements such as plasmids, integrons and transposons (El-Domany *et al*., 2017). These enzymes are encoded as gene cassettes and reside with other resistance genes within integron structures that are associated with transposons and can insert on the bacterial chromosome or within plasmids which become a sources of transfer to other bacteria and lead to emergence of multidrug resistant (MDR) pathogens (Malkocoglu *et al*., 2017).

The bla_{VIM} types MBLs in the carbapenem resistant *P. aeruginosa* isolates have been found from different subcontinents such as Africa (Chouchani *et al*., 2011, Sefraoui *et al*., 2014, Zafer *et al*., 2014, Mathlouthi *et al*., 2015), Europe, Middle East and Asia (Corvec *et al*., 2006, Al Bayssari *et al*., 2014, Rojo-Bezares *et al*., 2014). A study from Islamabad, Pakistan reported that one *E. coli* isolated from pediatric patient co-harbored NDM-1 and VIM-2 (Qamar *et al*., 2015). However, another study from Pakistan revealed the presence of 14 bla_{VIM} from clinically isolated *P. aeruginosa* (Nahid *et al*., 2013). There are many studies available on the prevalence of bla_{VIM} among *Enterobacteriaceae* family, however, few or no data available on *P. aeruginosa* from Pakistan so far. Therefore, we designed this study to determine the spread of bla_{VIM} type in clinical isolates of *P. aeruginosa* from tertiary care hospital in Faisalabad metropolis.

MATERIALS AND METHODS

Collection of bacterial isolates

Prior to start the study, ethical approval for this study was taken from the Ethical Review Committee, Government College University, Faisalabad. A total of 200 clinical *P. aeruginosa* isolates were collected from different source of specimens such as pus swabs, urine and blood from

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hospitalized patients from tertiary care hospital, Faisalabad during January to June, 2017.

Identification of the isolates

Bacterial isolates were sub-cultured on Blood agar, MacConkey agar and Cetrimide agar (Oxoid, UK) and plates were incubated at 37°C overnight aerobically. Isolates were identified on the basis of their colony morphology and culture characteristics. Further confirmation of the isolates was carried out using API 20NE (BioMerieux, France).

Antibiotic susceptibility testing

Antibiotic susceptibility testing of the clinical isolates was done by Kirby-Bauer disc diffusion assay using Mueller Hinton agar (MHA) (Oxoid, UK) as per CLSI 2016 guidelines. The implanted antibiotic discs (Oxoid, UK) were cefixime, cefoperazone, ceftrizone, cefepime, amoxicillin-clavulanic acid, aztreonam, levofloxacin, ciprofloxacin, amikacin, piperacillin-tazobactam, doxycycline, imipenem, meropenem, tigecycline and polymyxins-B. Interpretation of the zone of inhibition was carried out according to CLSI guidelines (Patel *et al*., 2016).

Minimum inhibitory concentration (MIC)

MIC (μg/mL) of imipenem and meropenem drugs were performed against selected isolates using E-tests (Thermo Fisher Scientific, UK). The results were interpreted as per CLSI 2016 breakpoints (Patel *et al*., 2016).

Modified hodge's test (MHT)

This test is used for the detection of carbapenemase producing bacteria according to CLSI guidelines. Briefly, fresh cultures of clinical isolates were spread on MHA (Oxoid, UK) plates. A meropenem (10μg) disc was placed in the center of the plate. Organism was streaked in a straight line from the edge of the disk to the edge of the plate and plates were incubated overnight at 35°C. A cloverleaf like indentation showed that the isolate was carbapenamase producer (Patel *et al*., 2016).

Double disc synergy test

This test is used for the detection of MBL producing *P. aeruginosa*. Briefly, test organism was inoculated onto MHA plates. Two imipenem (10μg) and meropenem (10μg) discs were placed and 10 μl of 0.5M EDTA was added on each imipenem and meropenem discs and plates were incubated at 35°C. Isolates were considered positive for MBL if the EDTA discs showed >5mm zone of inhibition compared to non EDTA discs (Qamar *et al*., 2018).

Polymerase chain reaction

Bacterial DNA was isolated using commercially available GeneJET Genomic DNA Purification Kit (Thermo Scientific, UK). The bla_{VIM} type of MBL was amplified using specific set of primer (F-5'-GTTTGGTCGCATA TCGCAAC-3'and R-5'-AAT GCGCAGCACAGGA TAG-3'). PCR amplification was carried out with following the conditions; initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 10 seconds, annealing at 52°C for 30 seconds and extension at 72°C for 10 second, final extension at 72°C for 10 mints and total cycles were 36. Amplicons were separated on 1.5% agarose gel containing ethidium bromide under UV in Gel Documentation (Bio-Rad, UK).

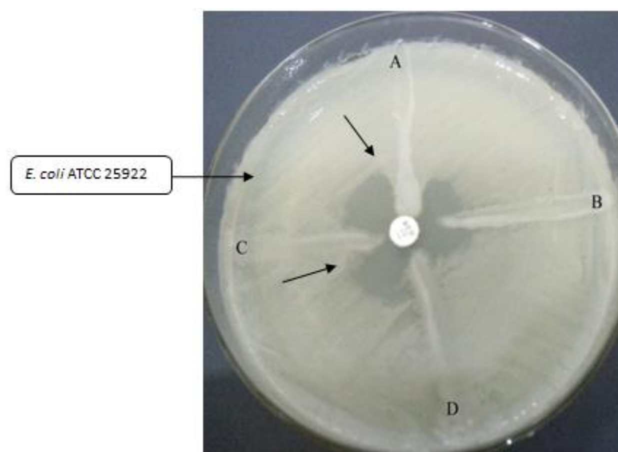

Fig. 1: shows the MHT for the detection of carbapenemase producing *P. aeruginosa*. Black arrows show cloverleaf like indentation. A: Positive control, B: Negative control, C and D: tested organisms

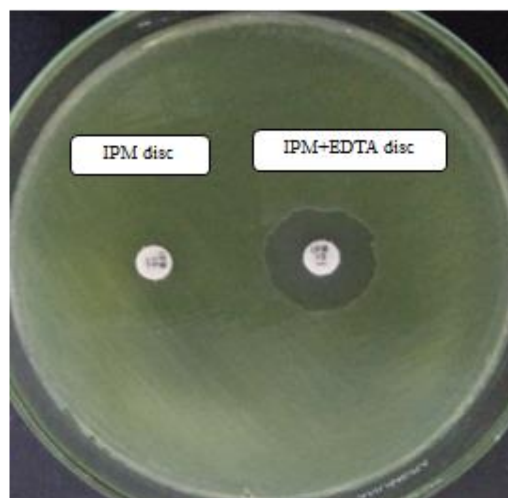

Fig. 2: shows double disc synergy test for the detection of MBL producing *P. aeruginosa*. IPM+EDTA disc showed large zone of inhibition as compared to non-EDTA IPM disc.

RESULTS

Out of 200 *P. aeruginosa;* 82 (41%) were isolated from pus samples, 28 (14%) from tracheal aspirates, 27 (13.5%) from sputum, 26 (13%) from ear swabs, 25

(12.5%) from urine and 12 (6%) from blood samples. Male to female ratio was 1.5:1. Out of 200 clinical isolates, 110 (55%) were MDR *P. aeruginosa* and among these 12 (11%) were positive for MHT and MBLs (Fig. 1 & 2). The bla_{VIM} was identified in all the MBLs producing *P. aeruginosa* (fig. 3). The bla_{VIM} positive *P. aeruginosa* were mainly identified from pus samples (n=10) followed by 1 from urine and 1 from blood sample.

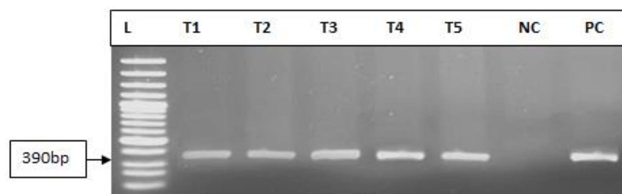

Fig. 3: shows the amplicon size of bla_{VIM} (390bp). NC: negative control, PC: positive control, L: ladder (100bp), T1 to T5; tested organisms

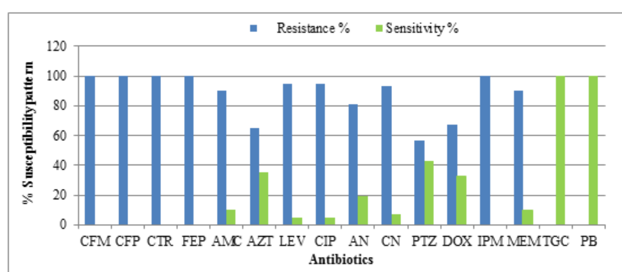

Fig. 4: Antimicrobial susceptibility of blaVIM positive isolates of *P. aeruginosa*

CFM: Cefixime; CFP: Cefoperazone; CTR: Ceftrizone; FEP: Cefepime; AMC: Amoxicillin-clavulanate; ATM: Aztreonam; LEV: Levofloxacin; CIP: Ciprofloxacin; AN: Amikacin; CN: Gentamicin; PTZ: Piperacillin-tazobactam; DOX: Doxycycline; IPM: Imipenem; MEM: Meropenem; TGC: Tigecycline; PB: Polymyxins B.

Antimicrobial susceptibility testing of bla_{VIM} producing *P. aeruginosa* revealed that all the isolates were resistant to all the β-lactam drugs including cefoperazone, cefixime, cefepime, ceftriaxone, imipenem and meropenem while 95% to levofloxacin and ciprofloxacin, 67% to doxycycline and 65% to aztreonam. However, least resistance was observed against tigecycline and colistin (Fig. 4).

Out of 12 bla_{VIM} producing *P. aeruginosa,* MIC value for the imipenem antibiotic against 5 and 6 isolates were 8μg/mL and 16μg/mL respectively, however, 6 and 3 isolates were inhibited at 8μg/mL and 16μg/mL against meropenem drug respectively (fig. 5 and table 1).

DISCUSSION

Multidrug-resistant (MDR) *P. aeruginosa* are the leading pathogens frequently and cause nosocomial infections that lead to morbidity and mortality around the globe (Buhl *et al*., 2015). Over the last two decades, resistance of *Pseudomonas* spp. to antibiotics has been immensely increased therefore it's compelled to check regularly for best opinion about clinical outcome of various therapeutic options (Sarwar *et al*., 2013).

Fig. 5: shows the E-Test strip for the MIC (μg/mL) of *P. aeruginosa*

In present study, majority of the *P. aeruginosa* were identified from pus samples (n=82) and tracheal aspirates (n=28). These findings are in accordance with the previous studies (Ahmed, 2016, Mansoor *et al*., 2016). This could be recognized as various clinical conditions such as accident, surgery and burns lead to pus formation and acting as a main source of infection because it provides optimum condition for the growth of pathogens and dissemination of an infection (Procop *et al*., 2017).

Rising resistance in *P. aeruginosa* against β-lactams including carbapenems have reached alarming level. These pathogens produce resistance by different mechanisms mainly due to enzymes, efflux pumps and porin loss. Present study showed that *P. aeruginosa* were found highly resistance against all the β-lactam drugs including imipenem and meropenem while 95% to levofloxacin and ciprofloxacin and the most effective drug was colistin. A study from China also reported similar finding with 100% resistance to β-lactam drugs (Wang *et al*., 2010). Another study from Pakistan also documented 100% resistance to cephalosporins (Khan *et al*., 2014). However, two more studies stated 100% and 87% of *P. aeruginosa* resistance against carbapenems drugs (Rodriguez-Martinez *et al*., 2009, Hirsch and Tam, 2010). This high resistance could be due to irrational, broad spectrum uses of empirical antibiotics and self-medication in our locality.

Table 1: Minimum Inhibitory Concentration (μg/mL) of MDR *P. aeruginosa* (n=12)

Strains code	Imipenem (MIC: 0.002 - 32μg/mL)	Meropenem (MIC:0.002 - 32μg/mL)
P4	8	8
P5	16	8
P9	16	16
P12	8	≥32
P13	16	8
P14	16	≥32
P15	8	8
P16	16	≥32
P17	≥32	8
P26	8	16
P30	16	8
P44	8	16

The 11% bla_{VIM} producing *P. aeruginosa* was found in this study. In recent study from India, the prevalence of bla_{VIM} was reported to be 18.6% in carbapenem resistant Gram-negative bacteria. In Pakistan, there is few or no data available on the presence of bla_{VIM} producing *P. aeruginosa*, however, two studies one from Islamabad documented 25% presence of bla_{VIM} from *P. aeruginosa* and another study revealed that single isolate of *E. coli* was positive for $bla_{VIM.}$ This high emergence of bla_{VIM} gene in clinical isolates of *P. aeruginosa* mainly because of emergence of bla_{VIM} positive strains present major health problems as these strains show resistance against commonly used antibiotics, leaving the clinicians with limited options. We have identified inappropriate infection control practices. In our clinical settings major risk factors which are associated with spreading the resistant bacteria are contaminated hands of medical staff and poor sterilization of various hospital surfaces such as floor, door handles, sink and intravenous catheters. There is also ineffective infection control and substandard practices. Neonates with premature birth are more at risk due to lack of immune system and maternal antibodies (Hannan *et al*., 2013). Moreover, empirical therapy should be revised after every three months according to WHO criterion (Ofori-Asenso and Agyeman, 2016).

CONCLUSION

This study revealed the emergence of MBLs and bla_{VIM} producing *P. aeruginosa* in our clinical settings which can lead to treatment failure and prolong hospitalisation. Moreover, these pathogens can become a serious threat to public health and can cause morbidity and mortality. Therefore, surveillance studies should be conducted at national and local level in clinical settings to overcome this menace.

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