Int.J.Curr.Microbiol.App.Sci (2016) *5*(10): 749-758

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 10 (2016) pp. 749-758**
Journal homepage: http://www.ijcmas.com

Original Research Article http://dx.doi.org/10.20546/ijcmas.2016.510.081

Phenotypic Characterization of ESBL, AmpC and MBL Producers among the Clinical Isolates of Multidrug Resistant *Pseudomonas aeruginosa*

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A B S T R A C T

Pseudomonas aeruginosa is a clinically troublesome gram negative pathogen that causes a wide range of opportunistic infections and nosocomial outbreaks. Metallo beta lactamase have recently emerged one of the most worrisome resistance mechanisms to hydrolyse all beta lactam agents including carbapenems. Drug resistance in turn leads to prolonged hospital stay and increased expenditure, which causes increased cross infections and poorer clinical outcomes. The present study investigated the prevalence of resistance mechanisms among Multi Drug Resistant *Pseudomonas aeruginosa* (MDRPA) clinical isolates fromclinical laboratories. One hundred and twenty MDR *P.aeruginosa* isolates were obtained from 400clinical samples from clinical laboratories. Antimicrobial susceptibility testing was performed by disk diffusion method and all these isolates were found to be MDR. All the isolates were subjected to different phenotypic assays to detect the production of enzymes such as ESBL, AmpC and MBL. Further, quantitative evaluation of biofilm production was carried out by microtiter plate assay, since many studies have shown positive correlation between MDR and biofilm formation. Of the 120 MDR *P. aeruginosa*, 45.83% were resistant to imipenem and 54.16% to meropenem. All the isolates were sensitive to polymyxin B. MBL production (58.33%) was found to be the predominant resistance mechanism followed by ESBL production (45.83%). None of them showed AmpC production. 93.33% of the strains produced abundant biofilms. *P. aeruginosa* was shown to be predominant nosocomial pathogen showing resistance to most of the available antibiotics including carbapenems. MBL is shown to be predominant mechanism for development of resistance in the present study.

Keywords

MDR,
Pseudomonas aeruginosa,
ESBL,
AmpC,
MBL.

Article Info

Accepted:
20 September 2016
Available Online:
10 October 2016

Introduction

Pseudomonas aeruginosa is a gram negative, non sporing, motile, aerobic bacilli cause of serious wound and surgical infections (Doggett, 1979). Infection caused by *Pseudomonas aeruginosa* is frequent amongst Hospital Acquired Infections (HAI). Further, acquired drug resistance is common in nosocomial isolates of Pseudomonas spp (Hemalatha *et al*., 2005). The mechanism of resistance to beta lactam antibiotics includes, the production of beta lactamase, reduced outer membrane

permeability, the altered affinity of target penicillin binding proteins, plasmid mediated resistance involving modifying enzymes (Cristina Lagatolla *et al*., 2004).

Metallo beta lactamases are class B beta lactamases. These require zinc or another heavy metal for their catalytic activity and their activities are inhibited by metal chelating agent such as EDTA and thiol based compounds (Johann *et al*., 2007). Acquired resistance is also reported by the production of plasmid mediated AmpC beta (β)- lactamase, Extended Spectrum Beta (β)–Lactamase (ESBL) and metallo beta (β)–lactamase (MBL) enzymes (Supriya Upadhyay *et al*., 2010). Carbapenems are often used as antibiotics of last resort for treating infections due to multi-drug resistant Gram-negative bacilli as they are stable against ESBL and AmpC β-lactamase (Tanzinah Nasrin *et al*., 2010). However, Acquired MBL in Pseudomonas spp have recently emerged as one of the most worrisome resistance mechanism because of their capacity to hydrolyze all beta (β)–lactam antibiotics including penicillins, cephalosporins and carbapenems, with the exception of aztreonam (Mehul *et al*., 2011). Based on molecular studies, cabapenem-hydrolyzing enzymes are classified into four groups A, B, C and D. The MBLs belong to group B. The genes responsible for MBL production may chromosomally or plasmid mediated and hence poses a threat for spread of resistance by gene transfer among the Gram-negative bacteria (Bush, 1998). MBL producing Gram negative bacilli, specially Pseudomonas spp, have been increasingly reported in Asia, Europe, Latin American and the United States.

The appearance of MBL genes and their spread among bacterial pathogens is a matter of concern with regard to the future antimicrobial chemotherapy (Walsh *et al*., 2005). Further, due to increase occurrence and types of these multiple βlactamase enzymes, early detection is crucial, the benefits of which includes implementation of proper / optimal antibiotic therapy particularly in critically ill and hospitalized patients, infection control policy and to control the spread of resistance (Richet *et al*., 2001). Thus, our study was undertaken to detect the MBL (metallo-β-lactamase) positive isolates of *Pseudomonas aeruginosa* from different clinical samplesfrom clinical laboratories.

Materials and Methods

The present study was carried out on *P.aeruginosa* obtained from various clinical samples from clinical laboratories during the period March 2015 to February 2016. One hundred and twenty MDR *P. aeruginosa* isolates were obtained from 400 clinical samples. The samples from which the strains were isolated include blood, Pus, Urine, Broncho alveolar lavage and endotracheal aspirates and tissues. Repeat isolates were excluded from the study. All the samples were processed for isolation and antibiotic sensitivity. Isolation of organism was done by streaking the samples on MacConkey's agar and Blood agar plates. Further identification was done by gram staining (gram negative bacilli), catalase (positive), oxidase(positive), and pigment production (positive), hanging drop preparation(motile) was done. ATCC *P. aeruginosa* 27853 strain was used as quality control reference strain for all experiments with satisfactory results.

Antibiotic sensitivity testing method was performed by Kirby Bauer method. Antibiotics included in the study are piperacillin (75µg), Piperacillin/Tazobactam (100/10µg), Ceftazidime (30µg), Cefaperazone (75µg), Ceftriaxone (30µg), Imipenem (10µg), Meropenem(10 µg),

Gentamicin(10µg), Amikacin(30µg), Norfloxacin(10µg), Ciprofloxacin(5µg), Cefaperazone/Sulbactam (75/25µg), Tobramycin (10µg), Netilmicin (30µg), Polymyxin B (300U), gatifloxacin (5ug).

All the MDR *P. aeruginosa* were subjected to different phenotypic assays to detect the production of enzymes such as ESBL, AmpC and MBL, which are implicated for causing multiple drug resistance. Phenotypic confirmatory test for ESBL production was performed by placing ceftazidime (30µg) and ceftazidime + clavulanic acid disc. Detection of Metallo-ß-lactamases production test was carried out by combined disc diffusion test by placing 2 imipenem discs one with 0.5M EDTA and the other plain are placed on the surface of the agar plate approximately 30mm apart. AmpC detection was done using AmpC discs method.

Microtitre plate assay for Biofilm production since many studies have shown positive correlation between biofilm and multiple drug resistance, quantitative evaluation of biofilm production by *P. aeruginosa* isolates was carried out by microtiter plate assay. Biofilm negative E. coli isolate from our collection and ATCC *P. aeruginosa* 27853 were used as negative and positive controls respectively. Based on the OD values, the extent of biofilm formed by the clinical isolates were classified as follows,

- OD ≤ ODc - Non adherent
- ODc<OD ≤ 2 × ODc- Weakly adherent
- 2 × ODc< OD4 × ODc- Moderately adherent
- 4 ×ODc< OD - Strongly adherent (16).

Results and Discussion

A total of 120 clinical isolates of MDR *Pseudomonas aeruginosa* identified from Clinical Microbiology laboratories for a period of one year were included in this study. Among these 0ne hundred and twenty isolates from MDRPA infection, male were found to be more predominant 71 (59.16%) than females 49 (40.83%). 40%(48) between 41 to 60 years of age group, 25%(30) between 21 to 40 years of age group, same number of cases 30 (25%) among the age group above 61 years and 12(10%) in less than 20 years of age. MDR *Pseudomonas aeruginosa* infection were mainly from RTA injuries 49(40.8%), Sepsis 9(7.5%), cellulitis 3(2.5%), COPD 6(5.83%), CVA & Pneumonia 14(11.6%), throat infection 4(3.3%), non-healing wound 4(3.3%), infectious bedsore 13(10.8%), DM foot 2(1.66%), fever 1(0.83%), UTI 1(0.83%), RT foot ulcer 1(0.83%), hip dislocation 7 (5.83%), 5(4.1%)infectious sutures and severe LR 4(3.3%).

Different types of specimens were, endotracheal (ET) aspirates 48(40%), wound swab 42(35%), followed by blood 20(16.66%), Broncho alveolar lavage (BAL)4(3.3%), urine 3(2.5%) and tissue 3(2.5%) respectively. Table (1)

All one hundred and twenty cases of MDRPA isolates were found to be 100% sensitive to Polymyxin B. They showed highest resistance towards Ciprofloxacin 93.33%(112) and tobramycin 84.16%(101), ninety eight isolates (81.66%) resistance to Ceftriaxone, eighty percent (96) resistance to ceftazidime, 79.16% (95) resistance togatifloxacin, 76.66% (92) resistance towards Cefoperazone, 72.5% (87) to Cefoperazone/Sulbactam and 70.83% (85) towards gentamycin. Seventy four isolates (61.66%) showed resistance to Piperacillin,

55% (66) were resistant to Netilmicin and 65(54.16%) resistant to meropenem 52.5% (63) resistance were noticed for Amikacin, 59 (49.1%) isolates shows resistant towards Piperacillin/Tazobactam. These strains showed resistance of 45.83% (55) towards Imipenem and none of them were resistant to Polymyxin B. Among these one hundred and twenty MDRPA strains table (2).

MIC for meropenem ranged from 0.5µg/ml to >64µg/ml. Sixty five of one hundred and twenty (54.16%) isolates of MDRPA were found to be resistant to meropenem. The isolates were categorized resistant if the MIC value was more than 8µg/ml. fifty four (45%) isolates showed sensitive MIC value (≤4µg/ml) and 1 (0.8%b) isolates showed intermediate MIC value (8µg/ml). All the isolates showed lower MIC of 0.5µg/ml to 1µg/ml for polymyxin B (Break point MIC for *Pseudomonas aeruginosa* ≤2µg/ml to ≥8µg/ml) and none of these isolates showed resistant or intermediate MIC values.

Seventy isolates (58.33%) were found to produce MBL and only fifty five (46%) isolates showed ESBL production, none of the isolates showed AmpC production. Twenty (16.66%) isolates were found to be negative for phenotypic production of all β-lactamases and 25(20.83%) shows both ESBL and MBL production (table-3). Almost all isolates (93.33%) from our collection were biofilm producers, wherein 75% of the isolates were strongly adherent, 8% moderately adherent and 11% weakly adherent. The percentage of non-adherent cells or biofilm negative isolates was found to be a meager of 7%.

Pseudomonas aeruginosa is the leading cause of nosocomial infections, including pneumonia, urinary tract infections, and bacteremia. *P.aeruginosa* exhibits intrinsic resistance to several antimicrobial agents. However, acquired resistance to anti-pseudomonal β-lactams such as ticarcillin, piperacillin, ceftazidime, cefepime, aztreonam and carbapenems considered as deterrent weapon that can be a major challenge in managing MDRPA infections, especially while it is associated with co-resistance with other classes of drugs namely aminoglycosides andfluoroquinolones. Several mechanisms can contribute to the acquired β-lactam resistance in *P.aeruginosa*, that includes production of β-lactamases, the upregulation of efflux systems, and decreased outer membrane permeability. With respect to β-lactamase production, acquired extended-spectrum β-lactamases (ESBL) and Metallo-β-lactamases are the predominant emerging resistance mechanisms in *P.aeruginosa*. The present study aimed at elucidating major resistance mechanisms in MDRPA infections. We were encountered with more isolates producing MBL when compared to other mechanisms in MDRPA infections.

The prevalence of MDR *P. aeruginosa* was found to be 33.33% in our investigation, which is lesser in accordance with a recent study from India that showed the predominance rate of 44%. However, comparatively lesser prevalence of MDRPA (26.7%) responsible for burn wound infections in Iran (Antonopoulo *et al*., 2007). Likewise, one more investigation from India reported 22% MDRPA and 4% Pandrug resistant *P. aeruginosa*, wherein, we recorded a higher level of MDRPA incidence. Factors such as age and sex among MDRPA infection were found to have significant association with MDRPA, wherein the incidence was more among the age groups between 41 and 60 (40%) and males being predominant (59.16%) which is the likely case in earlier reports. Possibly, it may be due to high incidence of road traffic accidents among males, leading to hospitalization thereby high incidence of *P.*

aeruginosa infection through catheterization. Earlier investigations have reported the major source of MDRPA to be sputum, tracheostomy specimen, pus, respiratory tract, surgical sites and endotracheal aspirate (Aggarwal *et al*., 2008; Shanthi *et al*., 2009). In the present study, the major source of MDRPA was found to be endotracheal aspirate (40%),followed bywound swabs (35%), implying that wound infections and respiratory tract infections are most significant infections caused by MDRPA in most of the hospitals. The major risk factors were prolonged hospitalization followed by patients on Foleys catheter. Foot infections and surgical site infections were found to be common source of MDRPA among the diabetic patients. MDRPA isolates showed markedly high-level resistance towards ciprofloxacin (93.33%), tobramycin (84.16%), followed by ceftriaxone (81.66%), ceftazidime (80%), gatifloxacin (79.16%), cefoperazone (76.66%), cefoperazone/ sulbactam (72.5%), gentamicin, (70.83%) piperacillin (61.66%). Sixty three isolates (52.5%) showed resistance to amikacin and 49.1% resistance was noticed for piperacillin/ tazobactam combination which is likely in the very recently published reports (Priyanka *et al*., 2016). Among carbapenems, imipenem and meropenem resistance was observed to be fifty five isolates (45.83%) and sixty five isolates (54.16%) respectively which is likely case in recently published reports (Samira *et al*., 2014). None of the isolates were resistant to polymyxin B. MIC for meropenem ranged from 0.5µg/ml to >64µg/ml. sixty five of 120 (54.16%) isolates of MDRPA were found to be resistant to meropenem. The isolates were categorized resistant if the MIC value was more than 8µg/ml. fifty four (45%) isolates showed sensitive MIC value (≤4µg/ml) and 1 (0.8%) isolates showed intermediate MIC value (8µg/ml). All the isolates showed lower MIC of 0.5µg/ml to 1µg/ml for polymyxin B (Break point MIC for *Pseudomonas aeruginosa* ≤2µg/ml to ≥8µg/ml) and none of these isolates showed resistant or intermediate MIC values.

Among various mechanisms of resistance, MBL and ESBL enzymes were found to be more effective and the incidence of MBL production in *P.aeruginosa* has been reported to be 10-30% from different clinical setups in India (Navaneeth *et al*., 2002). Previous studies have shown a incidence of MBL (47%), AmpC (50%) and ESBL (13.3%) among the MDRPA isolates tested (Morten *et al*., 2001). In another study shows MBL (36%) among MDRPA were isolated. In our study, we have observed a little high prevalence of MBL (58.33%), ESBL (45.83%) producers and 20.83% of MDRPA isolates were found to produce both MBL and ESBL, which appears to be significant.

Table.1

Table.1 Kind of samples and their numbers

SAMPLES	TOTAL NO.
ET aspirate	48 (40%)
BAL	4 (3.3%)
Blood	20 (16.66%)
Wound swab	42 (35%)
Tissue	3 (2.5%)
Urine	3 (2.5%)

Table.2

Table.2 Antibiotic resistance among isolates of MDR *P.aeruginosa*

ANTIBIOTICS	RESISTANT	SENSITIVE	INTERMEDIATE
piperacillin (75ug)	74 (61.66%)	46 (38.33%)	0 (0%)
piperacillin-tazobactam (100/10ug)	59 (49.1%)	54 (45%)	7 (5.83%)
ceftazidime(30ug)	96 (80%)	15 (12.5%)	9 (7.5%)
cefoperazone(75ug)	92 (76.66%)	9 (7.5%)	19 (15.83%)
ceftriaxone(30ug)	98 (81.66%)	9 (7.5%)	13 (10.83%)
imipenem(10ug)	55 (45.83%)	65 (54.16%)	0 (0%)
meropenem(10ug)	65 (54.16%)	54 (45%)	1 (0.8%)
gentamicin(10ug)	85 (70.83%)	35 (29.16%)	0 (0%)
amikacin(30ug)	63 (52.5%)	44 (36.66%)	13 (10.83%)
ciprofloxacin(5ug)	112 (93.33%)	4 (3.3%)	4 (3.3%)
cefoperazone-sulbactam(75/25ug)	87 (72.5%)	11 (9.16%)	22 (18.33%)
tobramycin(10ug)	101 (84.16%)	19 (15.83%)	0 (0%)
netilmicin(30ug)	66 (55%)	54 (45%)	0 (0%)
gatifloxacin(5ug)	95 (79.16%)	18 (15%)	7 (5.83%)
PolymyxinB(300U)	00 (0%)	120 (100%)	0 (0%)

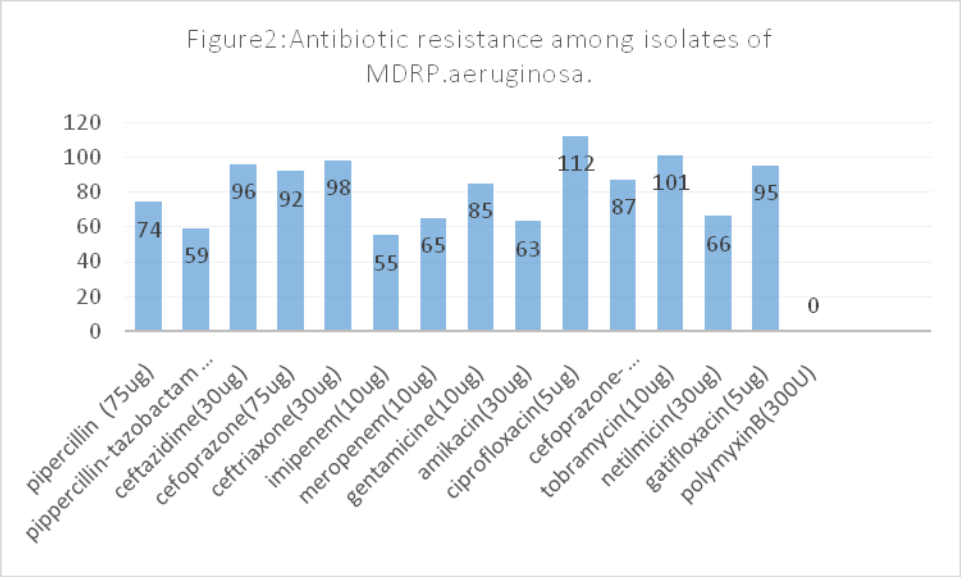
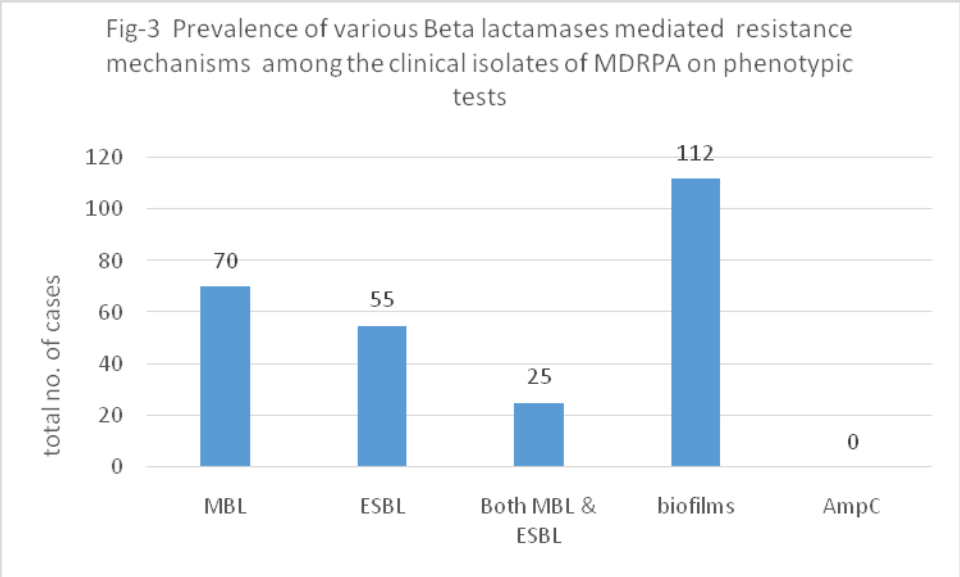


Table.3. Prevalence of various Beta lactamases mediated resistance mechanisms among the clinical isolates of MDRPA on phenotypic tests

	NEGATIVE	POSITIVE
MBL	50 (41.66%)	70 (58.33%)
ESBL	65 (54.16%)	55(45.83%)
Both MBL & ESBL	20 (16.66%)	25 (20.83%)
Biofilms	8 (6.66%)	112 (93.33)
AmpC	120 (100%)	00



Around 16.66 % of isolates did not show any of the mechanisms studied which might follow altogether different resistance mechanisms like formation of biofilms and/or cell wall permeability defects and efflux pump mechanisms. High percentage of biofilm producers were observed in our study, which may be due to the increase number of MDRPA isolates encountered. Morten Hentzer *et al*., earlier reported the strong correlation between biofilm formation and multiple drug resistance in Gram negative pathogens. Thus, biofilm formation appears to be one of the mechanisms among these strains to develop multi drug resistance, which is evident from the earlier reports from India.

In conclusion, in summary, MDR *P. aeruginosa* is a notable cause of hospital acquired infections and known to cause a wide spectrum of life threatening diseases..MBL production is the major cause for resistance to carbapenem group of antibiotics which are considered to be effective drugs for treatment of infections caused by *P.aeruginosa*. These organisms are resistant to almost all commonly available antibiotics with limited treatment options. Forty sixpercent of isolates showed resistance to imipenem and 54% to meropenem, which is an "alarming sign", since carbepenems were the present drug of choice. Furthermore, 93% of isolates had the ability to form biofilm that might aid in the

persistence of MDRPA thereby imparts resistance. Routine detection of MBLs will ensure optimal patient care and timely introduction of appropriate infection control procedure.

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Statistical analysis

All the data were entered in Microsoft Excel sheet and the results were analyzed by SPSS software. (IBM, USA)

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How to cite this article:

Mohammed Ansar Qureshi and Rakesh Kumar Bhatnagar. 2016. Phenotypic Characterization of ESBL, AmpC and MBL Producers among the Clinical Isolates of Multidrug Resistant *Pseudomonas aeruginosa. Int.J.Curr.Microbiol.App.Sci.* 5(10): 749-758.
doi: http://dx.doi.org/10.20546/ijcmas.2016.510.081