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RESEARCH ARTICLE

STUDY OF METALLO BETA LACTAMASE PRODUCTION AND BIOFILM FORMATION OF PSEUDOMONAS AERUGINOSA IN CLINICAL ISOLATES

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ABSTRACT

Background: Pseudomonas aeruginosa is one of the most common pathogens causing nosocomial infections. Metallo-β lactamases (MBL) have recently emerged as one of the most troublesome resistance mechanisms owing to their capacity to hydrolyze all β-lactams including carbapenems..
Objectives: To know the antibiotic susceptibility pattern in Pseudomonas aeruginosa isolates. Also, to detect MBL production and biofilm forming ability of Pseudomonas aeruginosa from various clinical isolates from our hospital. **Materials and methods:** The present study was conducted in the department of Microbiology, Yenepoya medical college, Mangalore, Karnataka for a period of three months (December 2017 to February 2018). Pseudomonas aeruginosa was identified from the clinical samples by standard microbiological techniques. The isolates were further subjected for antibiotic susceptibility testing by Kirby-Bauer disc diffusion method on Mueller Hinton agar. Phenotypic detection of Metallobetalactamse (MBL) was carried out by Combined Disk Diffusion Technique (IPM CDST) using imipenem (10µg) and imipenem (10µg) +EDTA (750µg) discs. Biofilm formation was detected using Tissue Culture Plate method. **Results:** In our study, total of 155 isolates of P.aeruginosa were collected. Most of the isolates were collected from pus samples(52.2%), followed by sputum & ET tip (20%). Maximum resistance was observed for ceftazidime (38.7%) and ciprofloxacin(34.8%) whereas polymyxin B(7.7%) and piperacillin-tazobactum(20.6%) showed lowest resistance. 19(12.2%) isolates exhibited ≥ 7 mm zone size enhancement indicating a positive MBL test.MBL production was seen highest among isolates from pus (42.1%),followed by urine(31.5%). MBL isolates were most sensitive to Polymyxin B (57.8%), followed by piperacillin – tazobactum 8(42.1%). 114(73.5%) isolates were strongly biofilm positive, 20(12.9%) were moderate and 21 (13.5%) were weak isolates. Out of 19 MBL producers, 14(73.6%) were strong biofilm forming isolates and 5 (26.3%) were moderate biofilm forming isolates. **Conclusion:** Though the prevalence of MBL in our study is lower when compared to other recent studies, an association between biofilm production and MBL production was observed. Routine detection of MBLs will ensure optimal patient care and timely introduction of appropriate infection control practices. The detection of biofilm production among P. aeruginosa may help in introducing newer techniques based on interference with biofilm formation.

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INTRODUCTION

Pseudomonas aeruginosa is one of the most common pathogens causing nosocomial infections. It is an aerobic, motile, non fermentative gram-negative bacillus which can survive on medical equipments and other hospital surfaces, favouring emergence of hospital acquired infections. It is responsible for 10% of hospital acquired infections (Hancock, 1996). *P. aeruginosa* has been implicated in diverse clinical manifestations such as pneumonia, urinary-tract infection, bacteremia, and skin and soft-tissue infections in severe burns cases and in infections among immunocompromised individuals. Infections due to *P.aeruginosa* are difficult to eradicate because of high intrinsic resistance as well as the capacity to acquire resistance to various antibiotics (Breidenstein, 2011). There are variety of

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mechanisms involved in the resistance of *P.aeruginosa* such as over expression of efflux pump, acquisition of Extended-Spectrum β-Lactamases (ESBLs) and Metallo-β-Lactamases (MBLs), target site or outer membrane modification, porin mutations, and enzymatic modifications (Chaudhary, 2013). Metallo-β-lactamases (MBLs) are carbapenemases which require zinc at the active site and hydrolyze virtually all β-lactam antibiotics. Metallo-β-lactamase (MBL) producing *P.aeruginosa* has emerged as threat in the recent years. Early detection is essential for providing appropriate antimicrobial therapy and also to prevent their dissemination[4]. Currently, no standardized guidelines are available for MBL detection and though PCR is the gold standard for MBL detection, its accessibility is restricted to reference laboratories (Sarkar, 2006).

The phenotypic techniques makes use of enzyme's zinc dependence by using chelating agents such as EDTA(ethylene diamine tetra acetic acid) or 2-Mercaptopropionic acid, to inhibit its activity (Jangyan, 2008). Another important factor contributing to the pathogenesis of P.aeruginosa in causing fatal infections is its potential to form biofilms on biotic and abiotic surfaces (Karatuna, 2010). A biofilm is a structured consortium of bacteria embedded in a self-produced polymer matrix consisting of exopolysaccharide (EPS), protein and DNA. Biofilms can be formed by bacteria and fungi. Bacteria commonly involved in the synthesis of biofilms include *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus viridans*, *Escherichia coli*, *Enterococcus faecalis Klebsiella pneumoniae*, *Proteus mirabilis* and *Pseudomonas aeruginosa* (Donlan, 2001). The bacterial populations in biofilms are more resistant to antibiotics and host-mediated clearance strategies, giving rise to chronic infections that are difficult to treat. Biofilms are estimated to be associated with 65% of nosocomial infections (Niels Hoiby, 2010). Hence we have conducted this study to know the antibiotic susceptibility pattern in *P.aeruginosa* isolates. Also, to detect MBL production and biofilm forming ability of *Pseudomonas aeruginosa* from various clinical isolates from our hospital.

MATERIALS AND METHODS

- This was a prospective cross sectional study conducted in a tertiary care hospital of Karnataka, India from December 2017 to February 2018. A total of 155 isolates of *P.aeruginosa* were collected from various clinical samples like pus, swab, blood, sputum and urine.
- *Pseudomonas aeruginosa* was identified from the clinical samples by standard microbiological techniques like Gram staining, motility test, oxidase test, culture on blood agar and MacConkey agar, biochemical reactions like citrate, triple sugar iron test and others (Koneman, 2006).

Antimicrobial susceptibility testing

The isolates were further subjected for antibiotic susceptibility testing by Kirby-Bauer disc diffusion method on Mueller Hinton agar as per Clinical and Laboratory Standards Institute guidelines 2017 (M100-S27)[11]. Using *P. aeruginosa ATCC* 27853 as quality control, all the isolated *P. aeruginosa* strains were tested for their sensitivity against a panel of antimicrobials as follows: Amikacin (30µg), Ciprofloxacin (5µg), Ceftazidime (30µg), Cefepime (30µg), Piperacillin (100µg), Piperacillin-Tazobactum (100/10µg), Imipenem (10µg), Meropenem (10µg), Polymyxin B (50units), Tobramycin (10µg) and Netilimicin (30µg) (Microxpress, Tulip Diagnostics, Goa).

MBL detection

Phenotypic detection of MBLs among the clinical isolates of *P. aeruginosa* was carried out in our study by Combined Disk Diffusion Technique (IPM CDST) using Imipenem (10 µg) and Imipenem (10 µg) +EDTA (750 µg) discs as described by Yong *et al*. (2002). A 0.5 M EDTA solution was prepared by dissolving 18.61 g of EDTA in 100 ml of distilled water and adjusting its pH 8.0 by using NaOH. The mixture was sterilized by autoclaving. The test organism was inoculated on Mueller-Hinton agar as recommended by the CLSI guidelines (2017). Two Imipenem (10 µg) discs were placed on the surface of an agar plate at distance of 25 mm and 10 µl of prepared EDTA solution was added to one of the Imipenem discs to obtain the desired concentration of 750 µg. The zone of inhibition of Imipenem and Imipenem with EDTA discs were compared after 16 to 18 hrs of aerobic incubation at 37°C. In the combined disc test, if the increase in inhibition zone of Imipenem with EDTA disc was ≥7 µm than the Imipenem disc alone, it was considered as MBL positive.

Detection of biofilm formation

Biofilm formation was detected using tissue culture plate (TCP) method as described by Christensen's et al. (Christensen, 1985). Isolates from fresh agar plates were inoculated in brain heart infusion (BHI) broth with 2% sucrose and incubated for 18–24 hrs at 37°C. The broth with visible turbidity was diluted to 1 in 100 with freshly prepared BHI broth. Individual wells of flat bottom polystyrene plates were filled with 0.2 ml of the diluted cultures, and broth without cultures served as a control to check sterility and nonspecific binding of the medium. These plates were incubated for 24 hrs at 37°C. After incubation, the content of the well was gently tapped to remove the cultures; later the microtiter plate was washed 4 times with 0.2 ml of phosphate buffer saline (PBS pH 7.2) to remove free-floating "planktonic" bacteria. Biofilms formed by adherent "sessile" organisms in plate were fixed using sodium acetate (2%) for half an hour and stained with crystal violet (0.1% w/v) for half an hour. Excess stain was rinsed off by thorough washing with deionized water and plates were kept for air drying. Adherent bacterial cells usually formed a biofilm on all side wells and were uniformly stained with crystal violet . Optical densities (OD) of stained adherent bacteria were determined with an Enzyme Linked Immunosorbent Assay auto reader at wavelength of 570 nm (OD 570 nm) and were graded as per Christensen et al. (Christensen, 1985) as shown in Table 1. These OD values were considered as an index of bacteria adhering to the surface and forming biofilms.

Table 1. Classification of bacterial adherence by TCP method

Mean OD values	Adherence	Biofilm formation
<0.120	None	None / weak
0.120-0.240	Moderately	Moderate
>0.240	Strong	High

RESULTS

In our study, total of 155 isolates of *P.aeruginosa* was collected during the study period of 3 months. Most of the

isolates were collected from pus samples 81(52.2%), followed by sputum & ET tip 32(20%), blood17 (11%), swab14 (9%) and urine11 (7%). The antibiotic resistance pattern of the *P.aeruginosa* isolates is depicted in Table 2. Maximum resistance was observed for ceftazidime (38.7%) and ciprofloxacin (34.8%) whereas polymyxin B (7.7%) and piperacillin-tazobactum (20.6%) showed lowest resistance.

Table 2. Antibiotic susceptibility patterns

Antibiotic	No of isolates sensitive (%)	No Of Isolates Resistant (%)
Amikacin	105(67.7%)	50 (32.2%)
Ciprofloxacin	101(65.1%)	54 (34.8%)
Ceftazidime	95(61.2%)	60 (38.7%)
Cefepime	105(67.7%)	50(32.2%)
Piperacillin	105(67.7%)	50 (32.2%)
Piperacillin -Tazobactum	123(79.3%)	32 (20.6%)
Imipenem	119(76.6%)	36 (23.2%)
Meropenem	118(76.1%)	37(23.8%)
Polymyxin B	143(92.2%)	12(7.7%)
Tobramycin	120(77.4%)	35 (22.5%)
Netilimicin	120(77.4%)	35 (22.5%)

All the 155 isolates were tested for MBL production by Combined Disk Diffusion Technique (IPM CDST). Of these 19(12.2%) isolates exhibited ≥ 7 mm zone size enhancement indicating a positive test.MBL production was seen highest among isolates from pus (42.1%), followed by urine (31.5%), sputum (15.7%), swab (5.2%) and blood (5.2%) as shown in Table 3. In the 19 MBL isolates, the antibiotic showing maximum sensitivity is Polymyxin B (57.8%), followed by piperacillin –tazobactum 8(42.1%). Almost all of the isolates were resistant to ciprofloxacin (94.7%), cephalosporins (89.4%) and carbapenems (84.2%). Biofilm production by tissue culture plate method performed on all the *P.aeruginosa* isolates, 114(73.5%) was strongly positive, moderate among 20(12.9%) and weak for 21 (13.5%) isolates. The strong biofilm producers were mostly isolated from pus samples. The specimen wise distribution of biofilm production is depicted in Table 4.

Table 3. Specimen wise distribution of MBLs

Specimen	MBL	
	No of isolates	Percentage
Pus (81)	8	42.1%
Swab (14)	1	5.2%
Sputum/et tip(32)	3	15.7%
Blood (17)	1	5.2%
Urine (11)	6	31.5%
Total (155)	19	12.2%

Table 4. Specimen wise distribution of biofilm production

SPECIMEN	BIOFILM FORMATION		
	HIGH	MODERATE	WEAK
PUS (81)	58 (71.6%)	11(13.5%)	12(14.8%)
SWAB(14)	13(92.8%)	1(7.1%)	0 (0%)
SPUTUM/ET TIP (32)	21(65.6%)	6(18.7%)	5(15.6%)
ET TIP(7)	4(57.1%)	2(28.5%)	1(14.2%)
BLOOD(17)	16(94.1%)	1(5.8%)	0(0%)
URINE(11)	6(54.5%)	1(9%)	4(36.3%)
TOTAL(155)	114(73.5%)	20(12.9%)	21(13.5%)

Out of 19 MBL producers, 14(73.6%) were strong biofilm forming isolates and 5 (26.3%) were moderate biofilm forming isolates. None of the weak biofilm producers were positive for MBL production (Table 5).

Table 5. Number of isolates forming both biofilm and MBL

Biofilm formation	No of Isolates	Mbl Positive Isolates
High	114	14 (73.6%)
Moderate	20	5(26.3%)
Weak/none	21	0
Total	155	19

DISCUSSION

P.aeruginosa is a known nosocomial pathogen that causes severe morbidity and mortality in hospitalized patients. In our study most of the isolates were collected from pus (61.2%), followed by sputum /ET tip (20%), blood (11%) and urine (7%). This is similar to other studies like Andhale JD et al (Andhale, 2016) where 63.3% isolates were from pus and 20% from urine. In a study by Rajat et al. (Rajat, 2012) maximum isolates (71%) are isolated from pus/swab followed by 16% from urine and 12% from sputum, 3% from other samples, showing that *P.aeruginosa* is isolated from pus specimens more often than others. Increasing resistance to different anti-pseudomonal drugs particularly among hospital strains, has been reported world-wide and this is a serious therapeutic problem in the management of cases. The susceptibility patterns of *P.aeruginosa* to the eleven tested anti-microbial agents varied among the isolates investigated. In our study maximum resistance was observed for ceftazidime (38.7%) and ciprofloxacin (34.8%) whereas polymyxin B (7.7%) and piperacillin-tazobactum (20.6%) showed lowest resistance. In the study done by Dash M et al (Dash, 2014), *P.aeruginosa* was highly resistant to ceftazidime 77.7%, cefepime 64.8%, piperacillin 45%. In the study by Devi PVet al[17] in 2015 it was reported that *P.aeruginosa* isolates were least resistant to Polymyxin B (8%). Acquired metallo-β lactamases (MBL) have recently emerged as one of the most troublesome resistance mechanisms owing to their capacity to hydrolyze all β-lactams including carbapenems and also because their genes are carried on plasmids, allowing easy dissemination.

In recent years, MBL genes have spread from *P. aeruginosa* to members of *Enterobacteriaceae,* hence the occurrence of an MBL positive isolate in hospital environment poses not only a therapeutic problem, but is also a serious concern for infection control management. In India prevalence of MBLs range from 7 – 65% [18]with a recent study reporting 34% occurrence (Castanheira, 2009). In our study the prevalence of MBL producing P.aeruginosa strains was 12.2%, which is similar to studies conducted by Attal RO et al (11.4%) (Attal, 2010), Navneeth et al (12%) (Navneeth, 2002), and Rajput A et al. (11%) (Rajput, 2012), respectively from different parts of India. MBL production in our area is relatively less in comparison with other studies. There is wide variation in antibiotic susceptibility pattern of MBL-producing *Pseudomonas* in different studies. In the 19 MBL isolates, the antibiotic showing maximum sensitivity is Polymyxin B (57.8%),followed by piperacillin –tazobactum 8(42.1%). Devi PV et al. (Devi, 2015), had demonstrated maximum sensitivity of MBL isolates to polymyxin B which is similar to our study. Chand et al (Chand, 2016) reported that MBL-producing *Pseudomonas* was most susceptible to piperacillin-tazobactam combination (43.48%).Of these MBL producing isolates, 4 were pandrug resistant, for which a sensitive antibiotic could not be determined. In our study, biofilm formation was detected by tissue culture plate method, which is considered as the gold standard for biofilm detection. Among the 155

isolates, 114 (73.5%) isolates were strong biofilm producers, 20(12.9%) were moderate biofilm producers. This finding is in concordance with Kaur et al. (Kaur, 2013) where biofilm production was seen in 65% of the isolates. Saxena et al (Saxena, 2015). Reported that 53.5% of the pseudomonas isolates were strong biofilm producers.

The strong biofilm producers were mostly isolated from pus samples, which is similar to other studies (Kaur, 2013 and Saxena, 2015). Out of 19 MBL producers, 14(73.6%) were strong biofilm forming isolates and 5 (26.3%) were moderate biofilm forming isolates. None of the weak biofilm producers were positive for MBL production Thus, our study demonstrates a strong association between MBL production and biofilm formation, attributing biofilm to its etiological role. Kaur et al. (Kaur, 2013), reported that 51.8% of strong biofilm producers and 33.33% of weak biofilm producers were positive for MBL production. In another similar study by Saxena et al, (Saxena, 2015) 56.2% isolates carried MBL genes with biofilm formation indicating that there is a strong relationship between biofilm formation and the production of MBL genes.

Conclusion

To conclude, in our study it was observed that most of the isolates are sensitive to the anti pseudomonal antibiotics indicating that the occurrence of multidrug resistant isolates is comparatively lesser in our hospital. Thus the overuse of carbapenemases should be restricted to prevent the emergence of resistance in the future. Also it can be reemphasized that the antibiotic policies should be formulated hospital wise rather than blindly following the universal guidelines and continual monitoring is required to update the antibiotic policies. Though the prevalence of MBL in our study is lower when compared to other recent studies, an association between biofilm production and MBL production was observed. This shows that biofilm is still the most important virulence factor in *Pseudomonas aeruginosa*. Routine detection of MBLs will ensure optimal patient care and timely introduction of appropriate infection control procedure. The detection of biofilm production among *P.aeruginosa* may help in introducing newer techniques based on interference with biofilm formation.

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