A study of Biofilm formation & Metallo-β-Lactamases in Pseudomonas aeruginosa in a tertiary care rural hospital

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***Abstract*-** Background and Aims: Pseudomonas aeruginosa is an opportunistic pathogen. The appearance of the Metallo-β-Lactamases genes and their spread among bacterial pathogens is a matter of concern with regard to the future of antimicrobial therapy.Antimicrobial resistance is an innate feature of bacterial biofilms. Biofilm formation is higher in MDR strains**.** The present study was undertaken with the aim to find Prevalence of Metallo-β-Lactamases among isolates forming Biofilm, Antibiotic resistance pattern of the isolates and their correlation with biofilm producer. The study was carried out in the Tertiary Care Hospital from the period of February 2013 to August 2013. Total of 60 Multidrug resistant (MDR) Pseudomonas aeruginosa isolated from 638 clinical samples were identified by standard microbiological techniques & the isolates were further tested for Antibiotic susceptibility testing.
Results:Of 60 Multidrug resistant (MDR)Pseudomonas aeruginosa, the Metallo-β-Lactamases was seen in 30%& Meropenem resistance was seen in 16.66 %.Highest prevalence of Metallo-β-Lactamases was seen in Pus 41.66% followed by Urine (33.33%) blood (23.07%), sputum (20%), Miscellaneous (30%). Biofilm formation was seen in in 65%. Higher antibiotic resistance was seen in strong biofilm producers as compared to the negative biofilm producers.In our study P. aeruginosa showed (56.67%) resistance to ceftazidime,Cefoperazone(61.67%), netilin (78.33%), ticarcillin (61.67%). P.aeruginosa showed higher sensitivity to Amikacin (83.33%),Meropenem(81.67%),Cefepime (66.67%),tobramycin(80%).

Conclusion: In our study Amikacin & Meropenem demonstrated maximum sensitivity against pseudomonas species. Therefore, use of these antibiotics should be restricted to severe nosocomial infections, in order to avoid rapid emergence of resistant strains.

***Index Terms*-** Antibiotic resistance, Biofilm formation, MDR, Metallo-β-Lactamases, Pseudomonas aeruginosa
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I. INTRODUCTION

Pseudomonas aeruginosa is an epitome of opportunistic nosocomial pathogen; it is aerobic Gram-negative bacillus, highly versatile microorganism able to tolerate low oxygen conditions. It can survive with low levels of nutrients and grow in temperatures ranging from 4-42°C. [1] These characteristics allow it to attach itself and survive on medical equipment and on other hospital surfaces, which favors the beginning of infections in immunocompromised patients.[1, 2]
P. aeruginosa can cause pneumonias, urinary tract infections and bacteremia's as well as causing high morbidity and mortality in patients with cystic fibrosis due to chronic infections that eventually cause pulmonary damage and respiratory insufficiency. Infections due to P. aeruginosa are difficult to eradicate because of their elevated intrinsic resistance as well as their capacity to acquire resistance to different antibiotics.[3]
Biofilms have an enormous impact on healthcare, and are estimated to be associated with 65% of nosocomial infections [4]
A biofilm is a structured consortium of bacteria embedded in a self-produced polymer matrix consisting of polysaccharide (EPS), protein and DNA. Bacterial biofilms cause chronic infections because they show increased tolerance to antibiotics and disinfectant chemicals as well as resisting phagocytosis and other components of the body's defense system. Characteristically, gradients of nutrients and oxygen exist from the top to the bottom of biofilms and these gradients are associated with decreased bacterial metabolic activity and increased doubling times of the bacterial cells; it is these more or less dormant cells that are responsible for some of the tolerance to antibiotics. Biofilm growth is associated with an increased level of mutations as well as with quorum-sensing-regulated mechanisms. Conventional resistance mechanisms such as chromosomal lactamase, up regulated efflux pumps and mutations in antibiotic target molecules in bacteria also contribute to the survival of biofilms. Biofilms can be prevented by early aggressive antibiotic prophylaxis or therapy and they can be treated by chronic suppressive therapy**.** [5]
The components of the EPS involved in the formation of P. aeruginosa biofilm are encoded mainly by different genes located in three independent operons: algU, psl, and pel. [6-8].Type IV pili (T4P) produced by P. aeruginosa shows twitching motility. These have been associated with biofilm formation, an essential event in host colonization. [8-10] these filamentous structures located at one pole of the bacteria are involved in various mechanisms such as adherence to human cells, formation of microcolonies, bacterial aggregation, phage receptor, evasion of the immune response and cellular signaling. . [11, 12]
Antimicrobial resistance is an innate feature of bacterial biofilms. [(13)] Many studies have shown that biofilm formation is higher in MDR strains [14]

Resistance to multiple drugs is usually the result of combination of different mechanism in a single isolate. There is variety of mechanisms involved in the resistance of P. aeruginosa, among them 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, plasmid enzymatic modification. [15]

Carbapenemics (imipenem and meropenem) are broad-spectrum antibiotics used for the treatment of nosocomial infections caused by P. aeruginosa. Specific resistance to carbapenemics is attributed to the lack of porin permeability (OprD), an increase in the expression of the active expulsion pumps (MexAB-OprD) and to production of metalloenzymes. [15]

It has been demonstrated that MBLs require divalent cations, usually zinc, as metal co-factor for their enzymatic activity and no therapeutic option is known to be available to control MBLs .Three groups of MBL have been identified: class A (serines dependent and partially inhibited by clavulanic acid are inducible and nontransferable), class B (zinc dependent, inhibited by EDTA, inducible or associated with conjugative plasmids) and class C (oxacillinase). [15]

In India, the prevalence of MBLs ranges from 7.5% to 71% [16]

The present study was undertaken with the aim to find

- prevalence of Metallo β lactamases (MBLs)
- No. of isolates forming Biofilm
- Antibiotic resistancepattern of the isolates
- Correlation of biofilm producer & isolates producing Metallo-β-Lactamases (MBLs)
- Correlation of biofilm producer & antibiotic resistant pattern of the isolates.

II. MATERIAL AND METHODS

The study was carried out in the department of Microbiology, tertiary care hospital from the period of February 2013 to August 2013. A total of 60 Multidrug resistant (MDR) Pseudomonas aeruginosa isolated from clinical samples like pus/wound, blood, sputum, catheter tips and urine were identified by standard microbiological techniques. [17]

The Isolates were further tested for Antibiotic susceptibility testing by Kirby-Bauer disc diffusion method on Mueller Hinton agar as per CLSI Approved Standard M100-S17)**.** [18]Antibiotic disc were obtained from Hi-media Laboratories Pvt. Ltd, Mumbai, India. Multi-drug resistance among Pseudomonas aeruginosa is defined as non-susceptible to ≥ 1 agent in ≥ 3 antimicrobial categories**.** Table d [19]

III. PHENOTYPIC DETECTION OF MBL

Phenotypic detection of MBLs among the clinical isolates of P. aeruginosa was carried out using imipenem (10 µg) and imipenem (10 µg) +EDTA (750 µg) discs as described earlier [20.] The MBL producing isolates showed a greater than 7mm variation between the inhibition zone around imipenem discs alone and the inhibition zone around imipenem+ EDTA discs.

IV. THE BIOFILM FORMATION BY TUBE METHOD (TML)

A qualitative assessment of bifilm formation will be determined as described by Christensen et al. [21] TSBglu (10mL) was inoculated with loopful of isolates from overnight culture plates and incubated for 24 hours at 37°C. The tubes were decanted and washed with PBS (pH 7.3) and dried. Dried tubes were stained with crystal violet (0.1%). Excess stain was removed and tubes were washed with deionized water. Tubes were than dried in inverted position and observed for biofilm formation. Assays were performed in triplicate at three different times.

The data obtained was recorded and analysed by using appropriate statistical methods.

V. RESULTS

Of 60 Multidrug resistant (MDR) Pseudomonas aeruginosa isolated from 638 clinical samples, the Metallo-β-Lactamases was seen in 18 (30%) & Biofilm formation in 39(65%).

Table 1: Specimen wise distribution – Metallo β- Lactamases & Biofilm formation

Specimen	No of isolates	Metallo-β-Lactamases positive	Biofilm formation
Urine	15	5 (33.33%)	6S 4W 5N
Blood	13	3 (23.07%)	4S 3W 6N
Pus	12	5(41.66%)	6S 1W 5N
Sputum	10	2(20%)	5S 2W 3N
Miscellaneous	10	3(30%)	6S 2W 2N

TOTAL	60	18(30%)	27S 12W 21N

S: Biofilm production Strong, W: Biofilm production Weak, N: Biofilm production Negative

Table 2: No of isolates forming biofilm & Metallo-β-Lactamases

Biofilm formation	No of isolates	Metallo-β-Lactamases positive isolates
Strong	27	14 (51.85%)
Weak	12	4 (33.33%)
Negative	21	0
Total Isolates	60	18

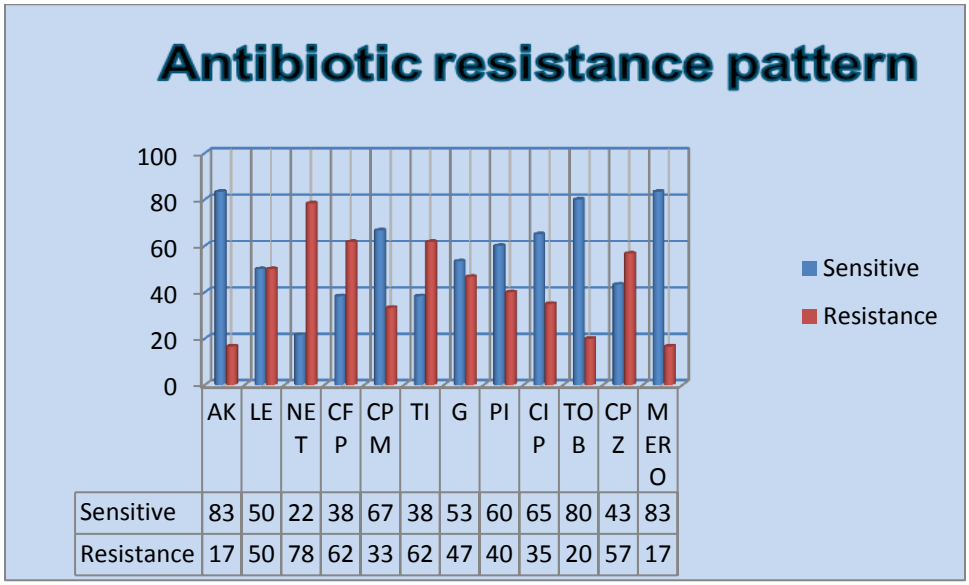
Table showing higher percentage of Metallo-β-Lactamases in strong Biofilm forming isolates

Table 3: Biofilm formation & antibiotic resistant pattern of the isolates

Antibiotic tested	Biofilm formation					
	Strong %		Weak %		Negative %	
	S	R	S	R	S	R
Amikacin	81.48	18.52	75	25	90.47	9.52
Levofloxacin	25.93	74.07	66.66	33.33	71.43	28.51
Netilin	00	100	16.66	83.33	52.38	47.62
Cefoperazone	18.52	81.48	50	50	57.15	42.85
Cefepime	74.07	25.93	75	25	52.38	47.62
Ticarcillin	37.04	62.96	33.33	66.66	42.85	57.15
Gentamycin	51.85	48.15	58.33	41.66	52.38	47.62
Piperacillin	51.85	48.14	75	25	61.90	38.09
Ciprofloxacin	37.04	62.96	83.33	16.66	90.47	9.52
Tobramycin	70.37	33.33	83.33	16.66	90.47	9.52
Ceftazidime	37.04	62.96	41.66	58.33	52.38	47.62
Meropenem	85.18	14.81	83.33	16.66	80.95	19.05

Table showing higher antibiotic resistance in Strong Biofilm producer as compared to the strain not producing biofilm

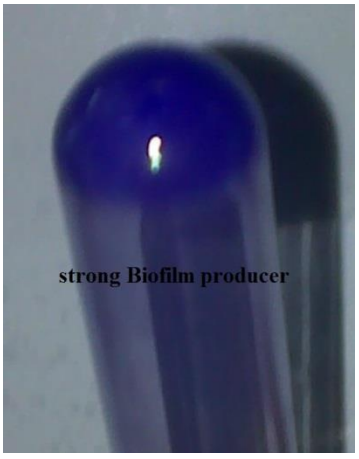
Chart 1: Antibiotic resistance pattern



	AK	LE	NET	CFP	CPM	TI	G	PI	CIP	TOB	CPZ	MERO
Sensitive	83	50	22	38	67	38	53	60	65	80	43	83
Resistance	17	50	78	62	33	62	47	40	35	20	57	17

Figure:

1A: strong Biofilm producer



1B: Weak Biofilm Producer

1C: Non Biofilm Producer

VI. DISCUSSION

Pseudomonas aeruginosa is a common nosocomial pathogen, notorious for its multidrug resistance (MDR) and life threatening infections in critically ill patients.Lately, carbapenems are being used as the last resort antimicrobial to treat serious infections due to MDR P. aeruginosa. [22] In a few Indian studies, the rate of carbapenem resistance in P. aeruginosa has been reported to vary from 12-37 % [23]

In our study, Meropenem resistance was seen in 16.66 %(10/60), Metallo-β-Lactamases was seen in 18 (30%).Highest prevalence of Metallo-β-Lactamases was seen in Pus 41.66%) followed by Urine (33.33%) blood (23.07%), sputum (20%), Miscellaneous (30%).

Shashikala in their study reported a prevalence rate of resistance to imipenem/ meropenem of 10.9% among P. aeruginosa isolates.[24] whereas Sachinkumar in their study reported resistance to Carbapenems in P. aeruginosa of 53.96% [25]

In a study carried out by Varaiya, et al on Incidence of metallo beta lactamase producing Pseudomonas aeruginosa in ICU patients they found 25% of Pseudomonas aeruginosa were found to be carbapenem resistant and 20.8% were found to be MBL producers. Overall 36 % patients responded to gatifloxacin, 42 % responded to piperacillin/tazobactam while 14 % responded to combination of gatifloxacin and piperacillin/tazobactum. [26] Viren A Javiya in their study reported notable resistance of (19.64%) to P. aeruginosa against carbapenems. [27]

In relation to carbapenems, the samples were more resistant to imipenem than to meropenem. Variations in the resistance rates between these antibiotics have been previously describe by Alicia Valéria Zaranza whereas in a study in Brazil, described higher resistance to meropenem. This susceptibility difference among carbapenems is explained by several resistance mechanisms, such as loss of proteins of external membrane OprD, that causes resistance to imipenem and not to meropenem; superexpression of efflux systems; and carbapenemase production. [28]

In our study P. aeruginosa showed (56.67%) resistance to ceftazidime, Cefoperazone (61.67%), netilin (78.33%), ticarcillin (61.67%). Similar is the finding of Bijayini Behera et al who reported 70% resistant to ceftazidime, 75% to piperacillin, 59% to piperacilin/tazobactam, 89% to ticarcillin/clavulinic acid, 82% to cefoperazone, 74% to amikacin, 81% to cefepime, 71% to levofloxacin, 79% to ciprofloxacin and 69% to aztreonam. [22]

Carlos J et al in their study reported P. aeruginosa showed (75%) sensitivity to amikacin; (61%) gentamycin; (77%) tobramycin and (100%) resistance to Ceftriaxone, Cefoxitin, Ampicillin, Cefazolin, Trimeth-Sulfameth. They also reported resistance to Ceftazidime(67%); Ciprofloxacin(75%); Levofloxacin (80%) this is similar to our study. [14]

In our study P. aeruginosa showed higher sensitivity to Amikacin (83.33%), Meropenem(81.67%) , Cefepime (66.67%), tobramycin (80%). similar is the finding of Viren A Javiya et al, Neils et al who demonstrated maximum sensitivity to amikacin against pseudomonas species. [27, 5]

In our study Biofilm formation was found in 39(65%). Strong biofilm producer was shown by 27/60(45%); weak biofilm producer in 12/60(20%). (Fig 1A, 1B) Alicia Valéria Zaranza in their study showed biofilm production by the Congo Red Agar method in 52.0% & biofilm formation on polystyrene microplates, from 86.0% strains. Among them 22.1% were strongly adhered, 47.7% were moderate and 30.2% were weakly adhered. [28] Carlos J et al reported biofilm formation in P. aeruginosa in 83% of clinical strains & that biofilm formation was prevalent among isolates with a MDR phenotype. [14] In our study we found higher antibiotic resistance in strong biofilm producers as compared to the negative biofilm producers.

In our study 50% of the strains from Sputum were strong biofilm producers, 20% weak biofilm producers and among them 20% of the strains were Metallo –β- lactamases positive. Drenkard E, Ausubel FM in their study found that antibiotic-resistant phenotypic variants of P. aeruginosa with enhanced ability to form biofilms arise at high frequency both in vitro and in the lungs of CF patients. They also identified a regulatory protein (PvrR) that controls the conversion between antibiotic-resistant and antibiotic-susceptible forms. [29]

25% of the P. aeruginosa were isolated from urine of inpatients. Study by Lucchetti et al showed that P. aeruginosa was the main isolated agent causing infections in the urinary tract, and according to epidemiologic data, 35.0% to 45.0% of all acquired nosocomial infections are urinary and 80.0% are related to catheter use. [30] In our study P. aeruginosa were isolated from females (53.3%) and males (46.6%) respectively.

The high incidence of this bacterium in the ICU is probably due to the fact that P. aeruginosa is an opportunist pathogen that causes bacteremia in immunocompromised patients, burn victims, patients with urinary infections related to catheters use and nosocomial pneumonia, related to mechanical ventilation, especially in this unit. A remarkable feature in infections by P. aeruginosa acquired in the ICU is multiresistance. [27] In our study (23/60) 38.33% of the isolates were isolated from ICU.

Pseudomonas aeruginosa produces amature in vitro biofilm in 5–7 days. Development of an in vitro biofilm is initiated by planktonic (freely moving) bacteria that reversibly attach to a surface, At this stage, the bacteria are still susceptible to antibiotics The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of antibiotics to biofilm-growing bacteria may be up to 100–1000-fold higher compared with planktonic bacteria .Monotherapy with antibiotics such as lactams, which are only active against dividing P. aeruginosa cells, are therefore not very efficient at eradicating biofilm infections There is increased horizontal gene transmission in biofilms .These physiological conditions may explain why biofilm-growing bacteria easily become multidrug resistant by means of traditional resistance mechanisms against lactam antibiotics, aminoglycosides and fluoroquinolones, which are detected by routine susceptibility testing in the clinical microbiology laboratory where planktonic bacterial growth is investigated. [5]

Neil et al in their study reported Colistin is only antimicrobial active against the non-dividing central part of P. aeruginosa biofilms in vitro. Since the metabolically active surface layer of the biofilm is susceptible to ciprofloxacin in contrast to the dormant central part of the biofilm, combination therapy with ciprofloxacin and colistin was able to kill all cells in the biofilm in vitro. [5]

Antibiotic resistance is increasing at an alarming rate, leading to increased morbidity, mortality and treatment costs. A key factor in the development of antibiotic resistance is the inappropriate use of antibiotics. The medical fraternity needs to understand that antibiotics constitute a precious and finite resource. Unless conscious efforts are made to contain the menace of drug resistance, multi-drug resistant organisms, untreatable by every known antibiotic, may emerge, reversing the medical progress made by mankind and throwing us back to the pre-antibiotic era. [24]

VII. CONCLUSION

In our study Amikacin & Meropenem demonstrated maximum sensitivity against pseudomonas species. Therefore, use of these antibiotics should be restricted to severe nosocomial infections, in order to avoid rapid emergence of resistant strains.

Carbapenem resistance not only has enormous therapeutic implications, but is also important from the point of view of infection control. Such stains are known for rapid intra institutional spread and therefore, must be notified to infection control teams.

Higher antibiotic resistances were seen in strong biofilm producers are due so testing for biofilm formation.

Regular antimicrobial susceptibility surveillance is essential. An effective national and state level area-wise monitoring of the resistance patterns antibiotic policy and draft guidelines should be introduced to preserve the effectiveness of antibiotics and for better patient management.

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