IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)
e-ISSN: 2279-0853, p-ISSN: 2279-0861.Volume 14, Issue 11 Ver. VII (Nov. 2015), PP 13-18
www.iosrjournals.org

Evaluation of resistance profile of pseudomonas aeruginosa with reference to biofilm production – An emerging challenge

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I. Introduction

Pseudomonas aeruginosa is a notoriously difficult organism to control with antibiotics or disinfectants and has become increasingly recognized as an emerging opportunistic pathogen of clinical relevance. Several different epidemiological studies track its emergence as multi-drug-resistant Pseudomonas aeruginosa (MDRPA) strains in clinical isolates. According to the CDC, the overall incidence of P. aeruginosa infections in US hospitals averages about 0.4 percent (4 per 1000 discharges), and the bacterium is the fourth most commonly-isolated nosocomial pathogen accounting for 10.1 percent of all hospital-acquired infections [1]
In Pseudomonas aeruginosa,there are three basic mechanisms by which organisms resist the action of antimicrobial agents:

1. Active efflux or impermeability resulting from porins loss (Intrinsic resistance)
2. Enzymatic or mutation-associated changes in antibiotics targets(The genetic ability to express a wide repertoire of resistance mechanisms)
3. Drug inactivation (plasmid-encoded b-lactamases/carbapenemases or aminoglycosides-modifying enzymes)
4. An additional feature which contributes to the resistance of P.aeruginosa ,in CF is its mode of growth in the lungs. Aggregates of bacteria in the lung are surrounded by a layer of alginate polysaccharide. These microcolonies or biofilms are highly resistant to eradication by antibiotics [2].

Biofilm is a distinct consortium of microbes encased in a self-produced polymer matrix consisting of polysaccharide, protein and DNA .Interestingly, cells within biofilm are clonal members that exhibit diverse gene expression.Biofilm growth is associated with increased level of mutations and with quorum sensing regulated mechanisms.The bacterial biofilm promotes virulence of bacteria by sharing of the genetic material, including genes responsible for antibiotic resistance and other virulence factors making them refractory to antibiotics. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of antibiotics to biofilm producing Pseudomonas aeruginosa may be up to 100 – 1000 fold higher compared to planktonic bacteria.It can also acts as a shield against the critical components of the immune system, including phagocytosis and the antibody/complement system. Bacterial infections by the microbe with the potential to produce biofilm are characterized by persisting inflammation and tissue damage leading to chronic infections [3].

Many antibiotics believed to be a panacea for Pseudomonas aeruginosa infections are becoming obsolete as drug-resistant strains are on the rise. In this context, up to date knowledge about the resistant profile of current strains is utmost important to target Pseudomonas aeruginosa .Classical antibiotic resistance mediated by genetic changes have been explored by several research across the globe[4,5,6]. Unlike them ,the ability to form biofilms are not readily evident in standard clinical laboratory tests and also studies indicating the role of biofilms in drug resistance of pseudomonas in clinical settings are very limited. This study was designed to evaluate the biofilm producing ability of pseudomonas aeruginosa and its resistant profile.

II. Materials And Methods

This prospective study was conducted at clinical microbiology laboratory, Department of Microbiology, Sri Muthukumaran Medical College & Research institute, Mangadu, Chennai from the period of Jan 2015 to Sep 2015.Different clinical samples received in our laboratory during the study period such as Sputum, Pus, Urine, Blood, Bronchoalveolar Lavage, Bile aspirate, Eye swab and Throat swab were processed and total of 112 isolates of pseudomonas aeruginosa were identified according to Standard Microbiological Procedure. (Gram staining, colonial morphology, catalase test, cytochrome oxidase reaction, motility, biochemical tests)[7].

These isolates were further subjected to Antimicrobial susceptibility testing by Kirby- Bauer disc diffusion method as recommended by Clinical Standard Laboratory Institute (CLSI). All isolates were tested against ten antipseudomonal antibiotics most commonly prescribed in our hospital settings[8]. Antibiotic discs

were purchased from Himedia,Mumbai. In this study, MDR P. aeruginosa was defined as resistant to one anti-microbial agent in three or more anti-pseudomonal anti-microbial classes.All isolates were subjected for biofilm production.

Detection of Biofilm formation : 96 well microtiter plate (Himedia) based method was adopted as per O'Toole [9].10 ml of trypticase soy broth with 1% glucose were inoculated with the isolates and incubated for 24 hrs. 1 in 100 dilution of isolates were prepared using fresh medium,from which 100µL of bacterial suspension was added to each well. After 24 hrs of incubation planktonic cells ate removed by rinsing the wells. In this assay, the extent of biofilm formation is measured using the dye crystal violet (CV).Well with 100µL sterile TSB broth without isolates was considered as Negative Control (NC). Each isolate was tested for biofilm production in 3 replicate wells Optical density (OD) of stained adherent biofilm was obtained by spectrophotometry at wavelength 570 nm.

Data recording:

1. OD of the negative controls taken as the cut off value.
2. OD of the negative control ≥ OD of the isolate– No biofilm production
3. OD of the negative control < OD of the isolate – Biofilm producer.

Statistical analysis: Data were analysed using statistical methods Fischer's exact two-sided test was used to compare categorical data (biofilm producer and non producer) among two groups. All p values < 0.05 were considered as statistically significant.

III. Results

A total of 112 pseudomonas aeruginosa isolates were identified during the study period..Hence the isolation rate was found to be 4.66%(112/2401).Distribution of Pseudomonas aeruginosa isolates with respect to age and gender was summarised in Fig 1.out of 112 isolates 68 were from male patients and 44 from female patients. Irrespective of difference in gender, maximum strains were isolated in the age group of 21-40 yrs.(male –51.47% & female -43.18%).Data analysis also revealed high percentage of occurrence of Pseudomonas aeruginosa in male patients in all age groups. Thus male patients are more prone to Pseudomonas aeruginosa infections.

Table 1 shows the isolation rate of Pseudomonas aeruginosa from various clinical sources in relation to biofilm production. The highest isolation rate was observed from pus(47.32%), ,followed by sputum(25%),Urine(15.17%)Throat swab(4.46%),Blood(2.68%) and Miscellaneous samples(5.36%) including Ear swab, Cervical swab, Bile aspirate Etc Wound infection was observed to be the most common infection by biofilm producing Pseudomonas aeruginosa as maximum isolates(25) were obtained from pus/wound swab which accounts for 52% of total. Respiratory infections was the next common infection as 16(33.33%) isolates were identified from Sputum .Another interesting observation was that all isolates from blood samples was found to be biofilm non producer Pseudomonas aeruginosa.

Fig 2 summarizes the overall antimicrobial resistance profile of pseudomonas aeurginosa isolated.Imipenam was the most effective drug against which none of the isolates were observed to be resistant(0%).Least resistance was observed to Piperacillin and Tazobactam (PIT) (11.6%) , Amikacin (19.6%),Levofloxacin(18.75%),Ofloxacin(16.07%) in comparison with the combination drug Piperacillin and Tazobactam (PIT), 23.21% of isolates were resistant to Piperacillin alone. Analysis of resistant pattern revealed high level of resistance to Ciprofloxacin50.89% and all the Cephalosporins tested(28% - 38%).

All isolates were subjected for biofilm production and resistant profile of them was correlated with the biofilm status in Table 2.We found there was a statistically significant difference between the two groups biofilm producer and non biofilm producer. There was high occurrence of resistance to most of the antipseudomonal antibiotics in biofilm producer as compared to non biofilm producer.

Among 112 isolates, 17 were found to be MDR (resistant to more than 3 antimicrobial categories) which accounts for 15.17%. As far as MDR was concerned, out of 17 isolates,12 were found to be associated with biofilm production. when the two groups were compared, it was observed that among 48 biofilm producer,12 (25%) were MDR and out of 64 non producer 5(7.8%) isolates were MDR.A statistically significant association was observed.Thus, the biofilm production was significantly higher in isolates that were MDR ($P<0.0001$) as shown in Table 3.

IV. Discussion

In the recent past, Pseudomonas aeruginosa considered as an epitome of opportunistic infections, being increasingly implicated in community acquired infections [10].The increasing frequency of MDR Pseudomonas aeruginosa is a serious concern as they are not only difficult to eradicate but often associated with increased mortality [11].Production of biofilm by Pseudomonas aeruginosa is an important survival strategy which is primarily responsible for antibiotic resistance. The present study evaluated the biofilm forming potential of

Pseudomonas aeruginosa using quantitative technique. Finally, a relationship between biofilm formation and their resistant profile was also examined.

In this study, isolation rate was observed to be 4.46% which was consistent with the findings of various studies conducted in India and abroad. Jamshaid et al.,6.67%,Srinivas et al 9.28%,Chander et al.,17.05% and Ahmed Bakr Mahmoud et al.,19% [12,13,14,15].

Demographic data such as age and sex of patients revealed the occurrence of Pseudomonas aeruginosa to be higher in male patients in all age groups and most of them belonged to the age group of 21 -40 yrs. Similar findings was reported by Srinivas et al.,[13] that Pseudomonas aeruginosa isolation rate was higher in male patients. Ahmed et al.,[15] had also shown similar observation in his recent study on MDR pathogen. In contrast to our findings, chander et al.,[14] showed high occurrence rate among female patients in his similar study. Pseudomonas aeruginosa infections were most common in wound infections. In accordance with our result Tarana sarwat et al.,[10] who founded that maximum strains were isolated from pus / swab. An another study done in Gujarat had shown higher isolation rate from urine[16].This could be explained by the fact that distribution of Pseudomonas aeruginosa with respect to age, gender and specimens may differ with geographical location, study period and sample size.

On further evaluation 48 isolates were positive for biofilm production. Among them, majority was isolated from sputum (52%) followed by pus 33.3%. A similar study in Kerala also reported the association of biofilm formers with sputum. This may be due to enhanced ability of isolates from sputum to form biofilm and regulatory protein that controls the conversion of susceptible strain to resistant was also identified.[17] On the otherhand, Afreenish Hassan et al.,[18] found high percentage of biofilm producer associated with urinary catheters. This wide variation might be due to the fact that biofilm associated Pseudomonas aeruginosa associated infections are on the rise.

Pseudomonas aeruginosa, being a stubborn MDR pathogen has been frequently associated with life threatening infections in hospital. Rapid evolution of genome due to continuous selective pressure of antibiotics leads to development of resistance [19]. This was observed in our study in which isolates exhibited high level of resistance to all drugs that are commonly prescribed.In our study Pseudomonas aeruginosa showed resistance to ciprofloxacin(50.89%),ceftazidime(38.39%), ceftriaxone(34.82%) and cefepime(28.57%).This may be explained by the fact that Fluoroquinolones are concentration dependent antibiotics,routine use of ciprofloxacin for P.aeruginosa infections can lead to clinically significant resistance. Similar high resistance was observed by Carlos J et al.,[20] who reported 75% to ciprofloxacin,67% to ceftazidime,100% to ceftriaxone. One remarkable finding is that all our isolates irrespective of their biofilm status were found to be susceptible to Imipenam. Imipenam(0%) and Piperacillin Tazobactem (PIT)(11.6%) was the most effective drugs against Pseudomonas aeruginosa infections. Similar is the finding of Tarana sarwat et al., who reported highest sensitivity to Imipenam. This was quite similar to the findings of Shaikh et al.,(100%) and Mohan et al., (94.3%)[21,22].

On comparison with planktonic bacteria,MIC and MBC are found to be 100 -1000 fold higher among the biofilm producer. This can be attributed due to biofilm mode of growth of Pseudomonas aeruginosa.As it confers increased tolerance to antibiotics and horizontal transfer of resistant genes. We observed that there was significant difference in resistant profile between biofilm producer and Non producer.Comparative analysis between these two groups revealed resistance rate of 68.75% against 37.5% to ciprofloxacin ($p<0.001$),75% against 10.94% to ceftazidime($p< 0.001$),56.25% against 18.75% to ceftriaxone($p<0.001$).

MDR Pseudomonas aeruginosa develops resistance by different mechanisms like betalactamases production; Aminoglycosides modifying enzymes, Active efflux pump and altered outer membrane permeability. In addition, there existing an important mode of survival by biofilm production. We identified 17 out of 112 isolates were resistant to more than 3 antibiotic class tested ,thus MDR rate was 15.17%.A similar finding was observed in another study at North India which reported 31.3%. As consistent with our findings Zahra et al.,[24] also observed 30%.In contrast, high rate of MDR has been reported elsewhere in the world. Such as 52%in Egypt and 60% in Turkey [25].we observed that most of the MDR isolates(14/17) are associated with biofilm production.this is in accordance with the findings of previous studies which identified strong association between biofilm production and MDR[26]

V. Tables & Figures

Fig 1: Distribution of Pseudomonas aeruginosa isolates with respect to age and gender

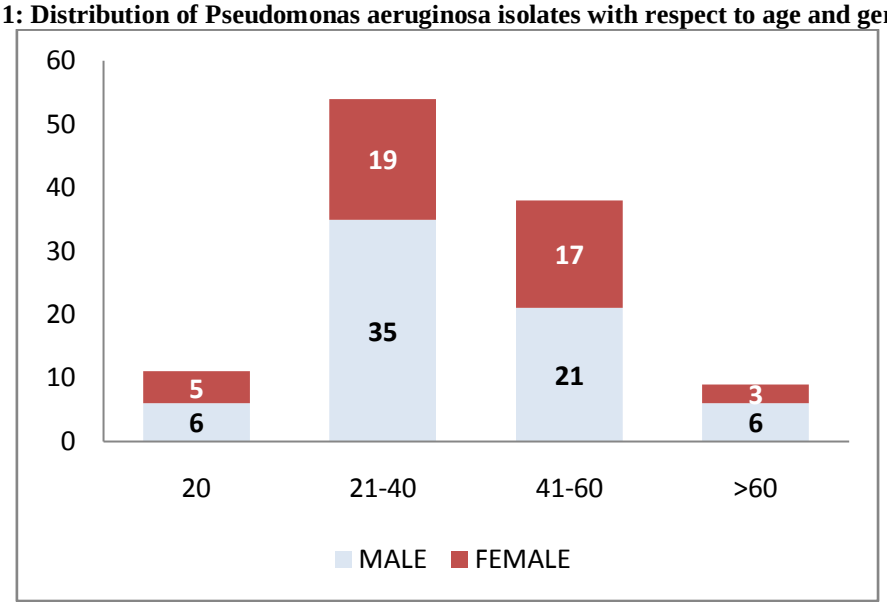

Table 1: Isolation rate of biofilm producing Pseudomonas aeruginosa from various clinical sources

Type of specimen	Distribution of Pseudomonas aeruginosa n=112(%)	Total no of isolates producing biofilm n=48 (%)
Pus	53 (47.32%)	16(33.33%)
Sputum	28(25%)	25(52%)
Urine	17(15.17%)	6(12.5%)
Throat swab	5(4.46%)	1(2.08%)
Blood	3(2.68%)	0
Miscellaneous	6(5.36%)	0
Total	112(100%)	48(100%)

Fig 2: Overall Antimicrobial resistance profile of Pseudomonas aeurginosa isolated

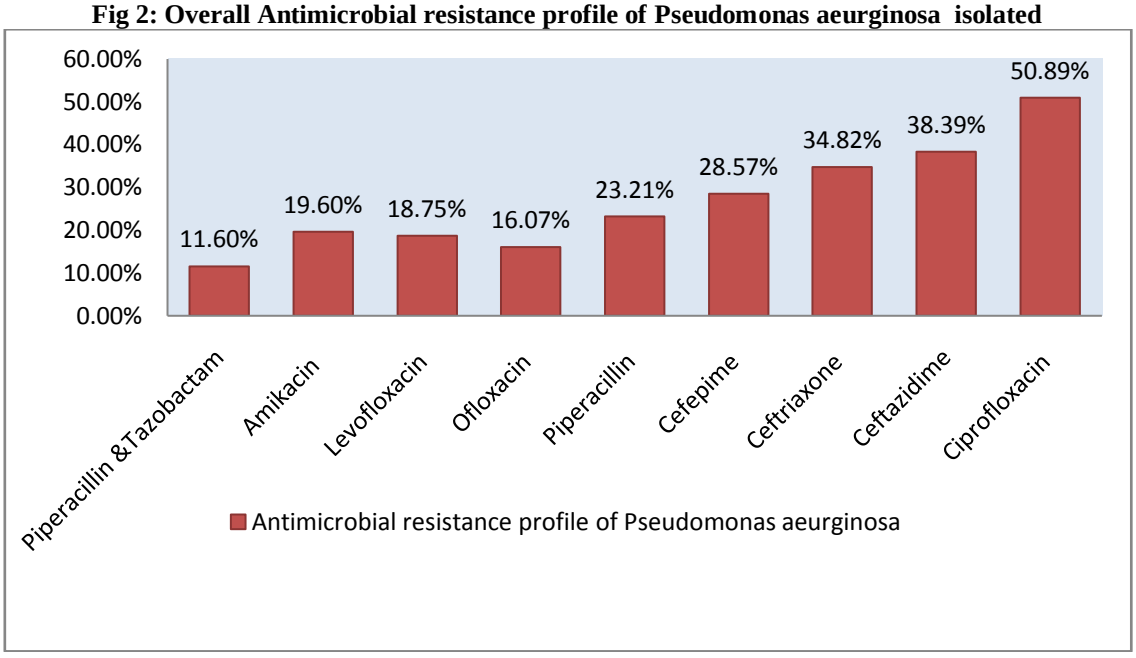

Table 2: Resistance Profile of Biofilm Producing And Non Producing Pseudomonas aeurginosa Isolates

Antibiotics tested	No of resistant isolates among Biofilm producer n =48 (%)	No of resistant isolates among Non Biofilm producer n =64 (%)	P VALUE	Significance
Imipenam	0 (%)	0 (%)	NA	
Piperacillin &Tazobactam	11(22.91%)	2(3.12%)	0.0019	HS
Amikacin	08(16.66%)	14(21.87%)	0.632	NS
Levofloxacin	14(29.16%)	07(14.58%)	0.0026	HS
Ofloxacin	09(18.75%)	9(14.06%)	0.6054	NS
Piperacillin	12(25%)	14(21.87%)	.8216	NS
Cefepime	19(39.58%)	13(20.31%)	0.0345	S
Ceftriaxone	27(56.25%)	12(18.75%)	0.0001	ES
Ceftazidime	36(75%)	7(10.94%)	0.0001	ES
Ciprofloxacin	33(68.75%)	24(37.5%)	0.0012	HS

S – significant; HS – Highly significant; ES – Extremely significant; NS – Not significant; NA – Not applicable.

Table 3: Distribution of MDR isolates according to biofilm status

Total MDR Isolates n =112(%)	Biofilm Positive n =48(%)	Biofilm Negative n =64(%)	P value
17 (15.17%)	**14 (29.16%)**	**3 (4.68%)**	**.0004 Extremely statiscally significant**

V. Conclusion

To conclude, our study highlighted the existence of Pseudomonas aeruginosa with the potential to form biofilm. It is also evident that judicious use of antibiotics at the early stage can significantly reduce the mortality and morbidity. Appropriate antibiotics at the right time are the only precious resource we have at hand now to contain the menace of Multi drug resistance.This can be accomplished by continuous monitoring of Pseudomonas aeruginosa resistant trends and we strongly recommend that biofilm detection can be included as routine diagnostic procedure to predict the emergence of resistant isolates at the earliest.

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