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Original Research Article

Biofilm production and its correlation with antibiotic resistance pattern among clinical isolates of *Pseudomonas aeruginosa* in a tertiary care hospital in north-east India

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ABSTRACT

Background: *Pseudomonas aeruginosa* is an ubiquitous pathogen capable of surviving in a variety of environmental conditions. It is increasingly gaining importance as a multidrug resistant nosocomial pathogen. Biofilm acts as a barrier, reducing the penetration of these drugs and consequently, preventing them from exercising their actions. The aim of this study is to isolate and identify *Pseudomonas aeruginosa* from various clinical specimen and to find out their production of biofilms and their correlation with antibiotic susceptibility pattern.
Methods: All *Pseudomonas aeruginosa* over a period of 1 year were isolated and identified from clinical specimens and antibiotic susceptibility test was done following standard operative procedures. Biofilm detection was done by Congo Red Agar method (CRA).
Results: 134 isolates of *Pseudomonas aeruginosa* was isolated. Maximum isolates were isolated from sputum samples 55 (41%) and most were from wards 68 (51%) giving a probability of increased healthcare associated infections. Biofilm production by the isolates was seen in 39 (29%). All the biofilm producing isolates shows more resistant pattern in comparison to non-biofilm producers. 69% of Imipenem and 82% of Meropenem resistant isolates produce biofilm. All the P. aeruginosa including MDR and biofilm forming strains were sensitive to Colistin.
Conclusions: Resistance to antimicrobial agents is the most important feature of biofilm infections. Ability of P. aeruginosa to form biofilms renders antibiotic treatments inefficient and therefore promotes chronic infectious diseases. As a result, infections caused by bacterial biofilms are persistent and very difficult to eradicate.

Keywords: Antibiotic susceptibility test, Biofilm, Multidrug resistant, *Pseudomonas aeruginosa*

INTRODUCTION

Pseudomonas aeruginosa is an aerobic, motile, nutritionally versatile, gram negative bacteria. It is ubiquitous, human opportunistic pathogen and has implications on morbidity, mortality and healthcare costs both in hospitals and in the community.[1] Infections caused by P. aeruginosa are frequently life-threatening and difficult to treat causing increased stay in hospital and even increased morbidity and mortality as it exhibits intrinsically high resistance to many antimicrobials and the development of multi-drug resistance in health care settings.[2,3] Biofilms are defined as microbially derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other. They are embedded in a matrix of extracellular polymeric substances (EPS) they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription.4 Within a biofilm, bacteria communicate with each other by production of chemotactic particles or

pheromones, a phenomenon called quorum sensing.[5] Availability of key nutrients, chemotaxis towards surface, motility of bacteria, surface adhesins and presence of surfactants are some factors which influence biofilm formation.[6] Biofilm are the source of persistent infections of many pathogenic microbes. They are responsible for much nosocomial infection and also associated with many medical conditions including indwelling medical device, dental plaque, upper respiratory tract infection and urogenital infection.[7,8] Multidrug-resistant organisms have been reported worldwide and are now recognized as one of the most difficult healthcare-associated infections to control and to treat.[9]

Increase in the frequency of multi-drug resistant (MDR) strains of P. aeruginosa has severely limited the availability of therapeutic options.[10]

The objectives of this study were:

1. To isolate and identify Pseudomonas aeruginosa from various clinical specimen.
2. To find out production of biofilms by the isolates.
3. To study their antibiotic susceptibility pattern and to correlate biofilm production with antibiotic resistance.

METHODS

This study was conducted in the Department of Microbiology, of a Tertiary care Teaching Hospital of North east, from November 2016 to October 2017.

Samples like blood, urine, sputum, wound swabs, catheter tips, tracheal aspirate, pus, and other body fluids obtained from patients attending in Regional Institute of Medical Sciences hospital, Imphal, Manipur submitted to Dept. of Microbiology for routine diagnostic workup between the study period were processed as per the standard Protocol. Identification and characterization was done by standard microbiological techniques.[11]

A total of 134 consecutive isolates of P. aeruginosa obtained from various clinical samples over a period of one year were included in the study. Non-repetitive clinical isolates of P. aeruginosa from the patients, were selected for further characterization.

After identification by phenotypic methods, antibiotic susceptibility test of all 134 clinical isolates of P. aeruginosa were performed for each isolate by the Kirby-Bauer disc diffusion method on Mueller-Hinton Agar using 0.5 MacFarland Turbidity standard and comparing zone sizes with Control strain *Pseudomonas aeruginosa* ATCC 27853.[12] The antibiotics tested were Ceftazidime (30µg), Cefepime (30 µg), Ceftazidime and clavulinic acid, Piperacillin-tazobactam (100µg)/10 µg), Imipenem (10 µg), Meropenem (10 µg),Gentamicin (10 µg), Amikacin (30 µg), Ciprofloxacin (5 µg), Levofloxacin, Colistin (10 µg) and Fosfomycin (50 µg), Norfloxacin (30 µg), Nitrofurantoin (300 µg) for urinary isolates. Antibiotic susceptibility results were interpreted by measuring the zone diameters produced and correlating them with the CLSI standards.[13]

Biofilm detection was carried out for all 134 isolates by the Congo Red Agar method (CRA). CRA medium was prepared with brain heart infusion broth 37 g/L, sucrose 50 g/L, agar 10 g/L and Congo Red indicator 8 g/L. Congo Red stain was prepared as a concentrated aqueous solution and autoclaved separately from the other medium constituents. Then it was added to the autoclaved brain heart infusion agar with sucrose. Inoculate CRA plates with test organisms and incubate at 37ºC for 24h aerobically. Black colonies indicate biofilm production.[11] All the analysis was performed using simple percentage method.

RESULTS

During the study period 134 isolates of *Pseudomonas aeruginosa* from various clinical samples was studied as described in (Table 1) which shows maximum isolates were isolated from sputum samples 55 (41%).

Table 1: Isolation from different clinical samples.

Clinical samples	No. of isolates (%)
Sputum	55 (41%)
Urine	32 (25.3%)
Pus	22 (16.4%)
Tracheal aspirate	12 (8.9%)
Swab	8 (5.9%)
Catheter tip	2 (1.4%)
Drain	2 (1.4%)
Blood	1 (0.7%)

In present study authors found that Pseudomonas infection mainly occurred in population aging between 41-60 years (Figure 1).

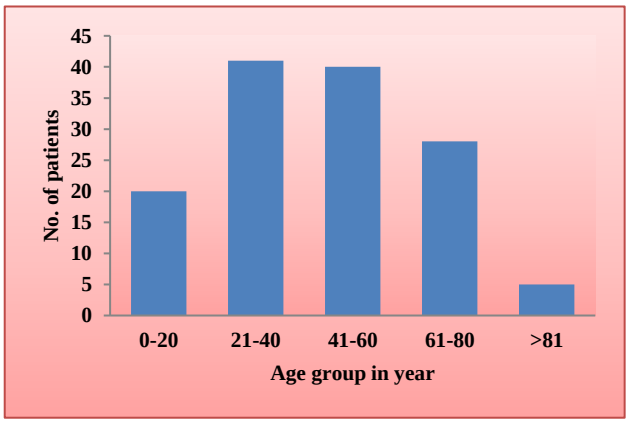

Figure 1: Age-wise distribution of *Pseudomonas aeruginosa* isolates.

Gender ratio was 1.48:1 (male: female) thus, a male preponderance was observed in present study. Most of the isolates were from wards 68 (51%) followed by OPD and ICU as shown in (Figure 2) giving a probability of increased healthcare associated infections.

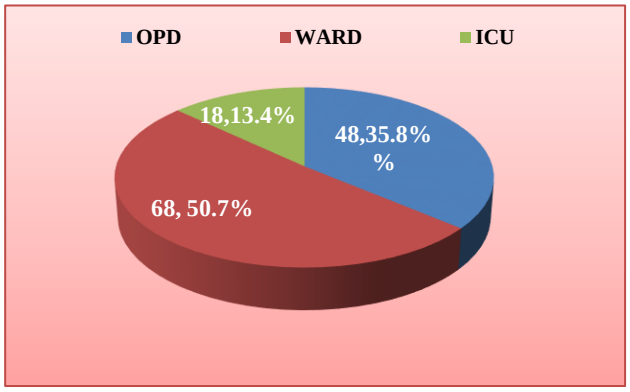

Figure 2: Distribution of isolates from wards, ICU, OPD.

The results of the antimicrobial susceptibility testing by the disk diffusion method showed that all isolates were susceptible to Colistin, whereas other antibiotics exhibited various susceptibility rates as shown in (Table 2). Higher resistance rate was seen in antibiotics Cefepime 85.8% and Ceftazidime 64.2%. Lower resistance was observed to Fosfomycin 6.2% Gentamicin 14.9%.

Table 2: Antibiotic resistance pattern of the isolates (n=134).

Antibiotics	Sensitive isolates (%sensitivity)	Resistant isolates (%resistance)
Imipenem	116 (86.5%)	18 (13.5%)
Meropenem	105 (78.4%)	29 (21.6%)
Piperacillin/ tazobactam	100 (74.6%)	34 (25.4%)
Ceftazidime and clavulanic acid	88 (65.7%)	46 (34.3%)
Ceftazidime	48(35.8%)	86(64.2%)
Cefepime	19 (14.2%)	115 (85.8%)
Ciprofloxacin	98 (73.1%)	36 (26.9%)
Levofloxacin	90 (67.2%)	44 (32.8%)
Norfloxacin (n=32)	13 (40.6%)	19 (59.4%)
Gentamicin	114 (85.1%)	20 (14.9%)
Amikacin	88 (65.7%)	46 (34.3%)
Nitrofurantoin (n=32)	10 (31.3%)	22 (68.7%)
Fosfomycin(n=32)	30 (93.8%)	2 (6.2%)
Colistin	134 (100%)	0 (0%)

The antibiotics combined with a β-lactam inhibitor, mainly Piperacillin/ tazobactam and Ceftazidime and clavulanic acid showed resistance rates of 25.4% and 34.3% respectively. The antibiotics Levofloxacin, Ciprofloxacin and Norfloxacin showed resistance rates of 32.8%, 26.9%, and 59.4% respectively. Resistance pattern for other drugs was Amikacin 34.3%, Nitrofurantoin 68.7%. Carbapenem class of antibiotics Imipenem and Meropenem presented with resistant rates of 13.5%, and 21.6% respectively. Almost all the isolates showed in-vitro resistance to one or more of the antibiotics mentioned earlier.

Among 134 *Pseudomonas aeruginosa isolates* 39 (29.1%) isolates showed positive for biofilm formation by Congo red agar method (Table 3). P. aeruginosa isolates was divided into strong biofilm producers (11.9%), moderate biofilm producers (17.2%) and negative biofilm producers (70.9%). In present study authors consider both strong and moderate biofilm producer as positive biofilm producer.

Table 3: Biofilm production of the isolates by Congo red agar method.

Total no of isolates: 134	
Strong	16 (11.9%)
Moderate	23 (17.2%)
None	95 (70.9%)

In present study authors found that maximum isolates from the ICU produce biofilm followed by ward and OPD (Table 4).

Table 4: Biofilm formation in isolates from ICU, ward and OPD.

	ICU (%)	Ward (%)	OPD (%)
Biofilm producer (n=39)	15 (83.3%)	20 (29.4%)	4 (8.3%)
Non-biofilm producer (n=95)	3 (16.7%)	48 (70.6%)	44 (91.7%)

The biofilm, produced by P. aeruginosa from different clinical specimens, was shown in (Table 5). All the isolates from catheter tip showed positive biofilm formation.

Table 5: Biofilm formation in different clinical isolates.

Clinical samples	Biofilm producer (n=39) (%)	Non-biofilm producer (n=95) (%)
Sputum (n=55)	6 (10.9%)	49 (89.1%)
Urine (n=32)	15 (46.9%)	17 (53.1%)
Pus (n=22)	8 (36.4%)	14 (63.6%)
Tracheal aspirate (n=12)	7 (58.3%)	5 (41.7%)
Swab (n=8)	0 (0%)	8 (100%)
Catheter tip (n=2)	2 (100%)	0 (0%)
Drain (n=2)	1 (50%)	1 (50%)
Blood (n=1)	0 (0%)	1 (100%)

All antibiotic resistant strains except to colistin, produced the biofilm at rates of more than 50% (50% to 82.8%).

The study shows that the biofilm producing isolates are more resistant to antibiotics in comparison to non-biofilm producing isolates as described in (Table 6).

Table 6: Correlation between antibiotic resistance pattern of biofilm producers and non-biofilm producers.

Resistant isolates	Biofilm producer (%)	Non-biofilm producer (%)
Imipenem	13 (72.2%)	5 (27.8%)
Meropenem	24 (82,8%)	5 (17.2%)
Piperacillin/ tazobactam	28 (82.4%)	6 (17.6%)
Ceftazidime and clavulanic acid	32 (69.6%)	14 (30.4%)
Ceftazidime	40 (46.5%)	46 (53.5%)
Cefepime	41 (35.6%)	74 (64.4%)
Ciprofloxacin	22 (61.1%)	14 (38.9%)
Levofloxacin	31 (70.5%)	13 (29.5%)
Norfloxacin (n=32)	13 (68.4%)	6 (31.6%)
Gentamicin	15 (75%)	5 (25%)
Amikacin	34 (73.9%)	12 (26.1%)
Nitrofurantoin	13 (59.1%)	9 (40.1%)
Fosfomycin	1 (50%)	1 (50%)

DISCUSSION

Biofilms not only contribute to the resistance mechanisms against broad spectrum antibiotics but also against host immune systems. The antibiotic susceptibility of biofilm-producing bacteria is reduced because of a restricted antibiotic penetration, an adaptive response and the presence of persisting cells.[15] The structure of biofilms is increasingly recognized as a crucial factor in the persistence of several infections. Chronic infections have been remarkably demonstrated to involve biofilm production, especially those infections associated with indwelling devices such as catheters and prostheses.[16] Previous studies have shown that the antibiotic resistance of bacteria due to biofilm formation contributes to the persistence of bacterial cells and causes problems in the complete eradication of infection.[17]

In present study authors have seen that all the biofilm producing isolates are more resistant to antibiotics in comparison to non-biofilm producers except in case of two drugs Cefepime and Ceftazidime where non-biofilm producers are more resistant than biofilm producers. Here the explanation is that the antibacterial resistance may not be due to biofilm but may be because of some other factors.

The formation of a biofilm is a multi-stage process that is initiated by the surface attachment of planktonic bacteria to form a monolayer, followed by aggregation leading to the formation of microcolonies, maturation to form mushroom-shaped structures and dispersal.[16] The formation of microcolonies in P. aeruginosa has been attributed to many factors. These include: type IV pili, flagella, free DNA, alginate and Pel and Psl polysaccharides. Even if one of the factors is not functioning, the biofilm is still able to perform well.[18]

The conventional antibiotic susceptibility test cannot predict the bacteria involved in biofilm production. In this technique, the concentration of antibiotics used is aimed at inhibiting the planktonic cell which differs from cells in the biofilm state. The bacterial biofilm is 10-1,000 times more resistant to antimicrobial agents than the planktonic cell.[19] The minimum biofilm eradication concentration (MBEC), the concentration of an antimicrobial agent required to kill a bacterial biofilm, should be tested in the laboratory to select the appropriate type and concentration of antibiotics needed to eliminate bacterial biofilms. This may improve the success rate of treating infectious diseases. Moreover, the ability of bacteria to form biofilms has been enhancing the spread of antibiotic resistance and the accumulation of virulence genes. New therapeutic strategies should be aimed at a co-treatment approach that combines traditional antibiotics with a substance that interferes with biofilms, and this may render the biofilms more susceptible to treatment.

CONCLUSION

Our study shows that 39% of the Pseudomonas isolates is capable of producing biofilms. Bacteria that have the ability to form biofilms, coupled with the emergence of multidrug resistant strains, are a significantly increasing concern in healthcare. Data from biofilm-producing strains, which are resistant to antibiotic or empirical treatment in individual areas, should be used to elucidate the appropriate therapies, especially in cases related to implanted devices and chronic infections.

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