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Original Research Article

Antibiotic susceptibility and biofilm formation in multidrug resistant *Pseudomonas aeruginosa*

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ABSTRACT

Background: *Pseudomonas aeruginosa,* one of the Gram-Negative pathogens, serves a crucial organism in nosocomial outbreaks. The properties of this bacteria including adaptability, biofilm formation, etc. make this organism develop resistance against various antibiotic drugs. The current study aims to screen the multidrug resistant *P.aeruginosa* isolates for biofilm formation and to study the relationship of drug resistance and development of biofilm.

Materials and Methods: Clinical samples growing *Pseudomonas aeruginosa* in culture were screened for antibiotic susceptibility pattern using disc diffusion method. The multidrug resistant isolates of *P. aeruginosa* identified using culture and standard microbiological tests were included in the study and were tested for biofilm formation using micro titre plate assay**.**

Results: Out of the 224 clinical samples growing *Pseudomonas aeruginosa* in culture, 100 isolates were found to be multidrug resistant (MDR). 42% of the *Pseudomonas aeruginosa* isolates showed resistance to Cefepime followed by 26% isolates were resistant to Levofloxacin. 88% of the MDR isolates produced biofilm and among these 75% produced strong biofilm, 10% of the isolates produced weak biofilm and 3% of the isolates produced moderate biofilms.

Conclusion: The present study observed that majority of the MDR *Pseudomonas aeruginosa* isolates were found to be biofilm producers. Thus, biofilm production is said to be one of the important properties of the organism which could be attributed to their multi drug resistance.

1. Introduction

"Antimicrobial Resistance" (AMR) is a global issue threatening the management of common infections occurring in our daily life. Among the AMR, resistance developed by bacteria is the major concern and threatening worldwide.[1] The occurrence of AMR in the bacteria is mainly due to the inactivation of antibiotics by secreting enzymes, altering the target region of the bacteria where the antibiotic drugs can bind, inhibiting the efflux pump of the bacteria thus the entry of drug will get reduced.[2] These mechanisms are observed in free-floating organism. These group of organisms gets attached and adheres to the submerged surface to form biofilm. Aside from its strong inherent antibiotic resistance, the capacity of the organism to build biofilms makes therapy extremely challenging.

In the early stages of biofilm development, resistance developed by microorganism would be less, however, in the later stages the resistance developed by the microorganisms can be even more.[3] Studies showed that resistance developed by biofilm forming organism will be 1-1000-foldmore when compared to the free-floating organism. Increase in the antibiotic tolerance rate of biofilm forming organisms is multi-factorial.[4] Furthermore, many

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bacteria within biofilms go metabolically dormant, meaning that antimicrobials acting on it won't have any effect on it. The adherence of bacteria is mainly initiated by the synthesis of extracellular polymeric substances (EPS). The presence of EPS inhibited the entry of drugs inside the cells and attenuate the therapeutic property of the drug.[5] The generation of beta-lactamases and the development of biofilms work together to promote the increased resistance of Gram-negative bacilli against several drugs. They pose a severe hazard to health of the common people since they are implicated in the recurrence of infections with chronic course resulting in high rates of illness and case fatality.[6] Though many studies focused on the prevalence of resistant pattern to various antibiotics, prevalence of biofilm formation among the bacteria is less studied. In order to prevent the dissemination of multidrug-resistant (MDR) *P. aeruginosa* strains, lessen the intensity of infections, lower the mortality, and decrease the hospitalisation rates and the financial cost of treating such resistant infections, this study was conducted to observe the antibiotic sensitive pattern of the clinical isolates of *Pseudomonas aeruginosa* and the organisms' ability to form biofilm.

Pseudomonas aeruginosa is known for producing biofilms and escapes antimicrobial therapy. In the biofilm condition *Pseudomonas aeruginosa* exhibits physical, physiological and genetic changes which show tolerance and resistance to various antibiotics.[4] The current study aims to find the interrelation between antimicrobial resistance and biofilm development among multidrug resistant *Pseudomonas aeruginosa* isolates.

2. Materials and Methods

This is a cross sectional study, which was carried out in a tertiary care facility in Chennai over a course of 6 months from July 2023 to December 2023. After obtaining Institutional ethical clearance (002/SBMC/IHEC/2019/1190), cultures of the clinical samples growing *Pseudomonas aeruginosa* were chosen and screened for antibiotic susceptibility pattern. *Pseudomonas aeruginosa* isolates showing resistance to more than one antibiotic in three or more class of antimicrobial drugs are categorized to be multidrug resistant. Only the multidrug resistant strains were included in the study and were further processed for biofilm formation.

2.1. Microbiology work

The identification of *Pseudomonas aeruginosa* was validated using standard microbiological and biochemical assays.[7] Clinical samples were obtained from different site of infection including ear swab, wound swab, pus, sputum, BAL, central line, urine, blood, ET, tissue. To identify *P. aeruginosa* isolates, standard microbiological and biochemical techniques were employed in the lab. These included Gram staining, culture, cetrimide agar growth, growth at 42°C, characteristic growth in MacConkey agar, pigment detection in nutrient agar and biochemical assays like, catalase, citrate utilization, oxidase, response on triple sugar agar, and motility tests. The samples were stored in 50% of Mueller Hinton broth (MHB) (Himedia) and 50% of glycerol at - 80°C.

2.2. Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was done using Kirby-Bauer disc diffusion method according to Clinical and Laboratory standards Institute (CLSI) 32^{nd} edition. Routine antibiotic discs from Himedia were used in this study Cefepime (30μg), Levofloxacin (5μg), Tobramycin (10μg), Ciprofloxacin (5μg), Gentamycin (10μg), Ceftazidime (30μg), Meropenem (10μg), Imipenem (10μg), Piperacillin/Tazobactam (100/10μg), Amikacin (30μg). *Pseudomonas aeruginosa* isolates showing resistant to more than one antibiotic in three or more class of antimicrobial drugs are categorized to be multidrug resistant. The multidrug resistant strains were further screened for biofilm formation.

2.3. Phenotypic screening of biofilm formation

The test to assess the formation of biofilms was carried out using microtitre plate method. The suspension of the organism was prepared in Mueller Hinton broth (MHB) medium and overnight incubation done at 37°C. Dilution of overnight culture was done in the ratio of 1:100 using MHB medium. The diluted culture sample was added in each 96 well plate, and the plate was incubated for 24 hours at 37°C. After the period of incubation, the plates were turned upside down to remove all medium. The plates were rinsed twice using Phosphate Buffer Saline (PBS) and the cells were immersed using 99% methanol for 15 minutes. In the plate, incubation was done for 15 minutes using100 μl of crystal violet. After the incubation period, the excessive crystal violet was taken out and the plates were rinsed twice before air drying. 33% acetic acid was added and the absorbance detected at 570 nm.[8] The experiment was performed in triplicates. Sterile broth was used as negative control. Biofilm forming organism was screened according to the criteria of Stepanovic et al (2007). Absorbance less than 0.17 was considered as negative, 0.17-0.34 as weak positive, 0.35-0.68 as positive and more than 0.68 as strong positive.[9]

3. Results

A total of 224 clinical samples collected from patients admitted in wards and ICU of a tertiary care teaching hospital in Chennai, grew *Pseudomonas aeruginosa* in culture. The *Pseudomonas aeruginosa* isolates were

screened for antibiotic susceptibility pattern. Out of the 224 isolates, a sum of 100 isolates of *P.aeruginosa* which were found to be resistant to more than three classes of antibiotics were included in the study and processed further. Highest prevalence of *P.aeruginosa* was observed among 60-80 years (40%) followed by 40-60 years (33%). 53.4% of the isolated were collected from men and 46.6% were collected from women. Among the 100 isolates, majority of the isolated organisms were from wound swabs (51%) followed by sputum sample (19%) (Figure 1).

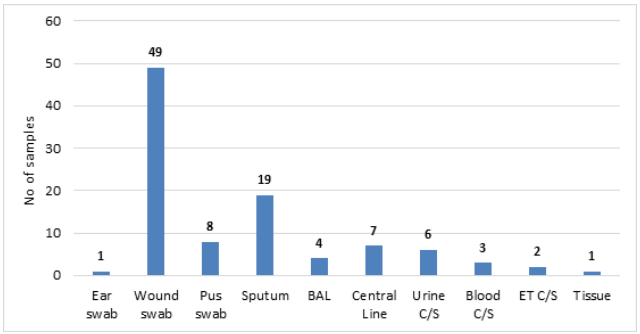

Figure 1: Distribution of multidrug resistant *Pseudomonas aeruginosa* among the clinical samples

3.1. Antimicrobial resistance profile

P.aeruginosa isolates were screened for antibiotic susceptibility using different classes of antibiotics listed in. *P.aeruginosa* shows resistant to more than three class of antimicrobial drug were selected for this study. The highest resistance was observed to 4^{th} generation Cephalosporin, Cefepime (42%) followed by Fluoroquinolones class of drug LE (26%). Moderate resistance was observed to the Carbapenem class of drugs including Meropenem (16%) and Imipenem (13%). 24% of the strains demonstrated resistance to aminoglycoside group of drugs namely Tobramycin. 23% of the isolates exhibited resistance to Gentamycin. Least resistance was detected for Piperacillin -Tazobactam (7%) (Figure 2).

4. Discussion

P.aeruginosa, one of the invasive pathogens, is now emerging as an important organism involved in hospital acquired infections. It's remarkable endurance in clinical settings is due to its ability to generate antibiotic-resistant biofilms.This opportunistic pathogen is playing a major role in nosocomial infections including septicemia, infections in the intensive care unit, ventilator-associated infections, surgical site infections, otitis media, burns infections, keratitis and urinary tract infections.[7] These infections result in the increase in hospital stay which link with the economic burden for the patient when compared with the drug-susceptible counterparts. Presence of various genes in

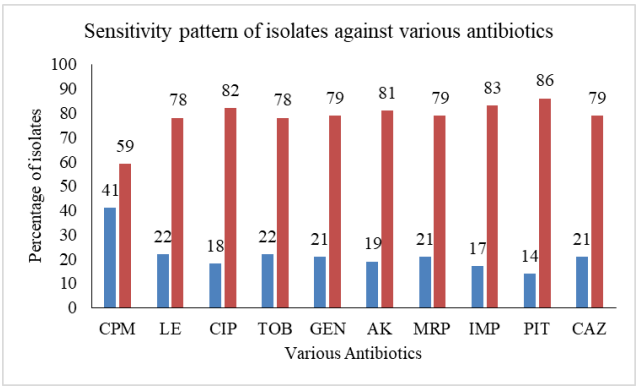

Figure 2: Graph showing the resistance pattern of the organism to various antibiotics Abbreviation: CPM: Cefepime, LE: Levofloxacin, CIP: Ciprofloxacin, TOB: Tobramycin, GEN: Gentamycin, AK: Amikacin, MRP: Meropenem, IPM: lmipenem, PIT: Piperacillin-tazobactam, CAZ: Ceftazidime

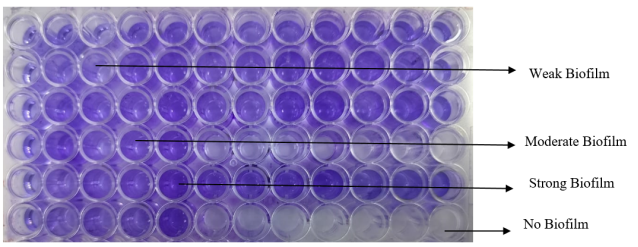

Figure 3: Graph showing the resistance pattern of the organism to various antibiotics

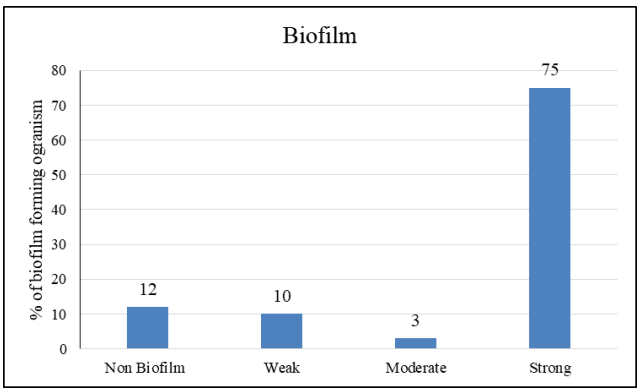

Figure 4: Percentage of biofilm forming organism under various categories. 12% of the isolates produced no biofilm and 75% of the isolates produced strong biofilm

P.aeruginosa including inducible *ampC* is one of the major reasons in establishing drug resistance.[10]

The pattern antibiotic resistance in *P.aeruginosa* exhibited increased resistance to Cefepime 42% followed by Levofloxacin 24%, Tobramycin 24%and Gentamycin 23%. The susceptibility of the organism to Ciprofloxacin is found to be reduced in the present study. Ciprofloxacin and levofloxacin are the two most often utilised fluoroquinolones (FQs) in managing infections due to *P. aeruginosa*. FQs are a key class of antibiotics that have favourable pharmacokinetic and pharmacodynamic features. The resistant pattern to the FQs is mainly by altering the quinolones-resistant determining regions.[11] 24% of the strains showed resistance to tobramycin an aminoglycoside class of antibiotics. Many studies showed *P. aeruginosa* is susceptible to tobramycin and showed good improvement in the cystic fibrosis patients.[12,13] Increased resistance to tobramycin could be caused by the biofilm forming ability of the organism.[14]

In the current study *P. aeruginosa* isolates showed resistance to Cefepime was 42% which is higher than the previous study conducted in SBMCH on 2020 showed *P. aeruginosa* were resistant to Cefepime was 24.3%.[15] This demonstrates that the organism acquired resistance to Cefepime over the course of two years highlighting the adaptability of pathogens and potential challenges in treatment efficacy. Previous study, performed in Iran showed *P. aeruginosa* was resistant to Carbapenem drugs like Meropenem and Imipenem drugs up to 45%[16] but the present study showed, 13% of the isolates exhibited resistance to Imipenem and 16% resistant to Meropenem. The study performed in USA also showed 23.7% of the isolates demonstrated resistance to Meropenem[17] but interestingly in the present study, the rate of resistance to Carbapenem drug was less.

Different antimicrobial resistance levels reported in numerous researches are most probably attributable to variations in the use of antibiotics in various geographic locations. Additionally, the significant occurrence of multidrug resistance in *P.aeruginosa* strains may be due to the use of multiple antibiotics to treat community and hospital acquired infections, due to a mutation in the genome of the organism. Therefore, depending on the bacterial isolation site, appropriate therapeutic regimen for *P.aeruginosa* infections must be used.

Resistance to antibiotics is also mainly due to the formation of biofilm. Adherence of the organism to the surface is the first step in the creation of a biofilm. Proteins and other organic compounds are adsorbed onto material surfaces when they are in a fluid environment. It has been proven that these conditioning films, or organic coatings, change the characteristics of the material's surface and have an impact on microbial attachment.[18] Biofilm helps to escape the organism from immune system mainly by escaping from the phagocytosis process and persist in the region for long time. Thus, in chronic infections, biofilm is an important mechanism to protect the organism which results in the treatment failure.[19] *P. aeruginosa* is commonly known for the formation of biofilm and it relies on a durable biofilm to strive, exist, and persist in the cystic fibrosis lung polymicrobial surroundings.[20] This organism possesses a variety of factors of virulence including, elastase, exotoxin A and exoenzymes which plays a crucial role in the treatment failure and results in higher mortality rate in the burns patients.[16,21] The important virulence factor in *P. aeruginosa* is biofilm formation which helps the organism to develop antibiotic resistance and increases the flow of horizontal gene transfer from resistant to susceptible organisms.[22]

In the current study, biofilm production was seen in 88% of the strains which was almost similar to previous studies in which 71%,[23] 77.5%[24] and 100%[16] was detected in the clinical specimens of P.aeruginosa. In the present study, 75% of the isolates were observed to be strong biofilm producers. In a previous investigation done by Ghasemian et al., 42.5% were strong biofilm producers.[16] Previous studies showed all the strains of *P. aeruginosa* were biofilm producers, but the current study showed 12% of the strains were non biofilm producers.[16] Majority of the biofilm producing organisms were almost resistant to three or more than three antibiotics. Biofilm formation inhibits the diffusion of antibiotics thus develops the resistant for the antibiotics.[25] This synergistic relationship is one of the important factors in the failure of treatment because biofilms protect the bacterial cell from antibiotic exposure.[26] Additionally, biofilm forming strains have distinct Minimal Inhibitory Concentration (MIC) levels compared to planktonic cells, and hence, combination therapy may help to eliminate biofilm producing isolates.[27] This investigation showed that, there was a substantial association between MDR and biofilm generation, with 88% of isolates forming biofilm exhibiting MDR.Thus, the present study showed that there is a possibility of association between antimicrobial resistance and biofilm development in *P. aeruginosa*.

Treating biofilm-forming MDR *Pseudomonas aeruginosa* infection often calls for a combination of antibiotics, higher dosages, or novel therapies. Because biofilms are robust and enduring, it is challenging to entirely eradicate the infection, needing prolonged or intensive therapeutic protocols that could burden treating patients and enhance the likelihood of adverse effects.[28]

P. aeruginosa is extremely difficult to eliminate when it develops biofilm components. This leads to failing treatment for diseases such as cystic fibrosis, recurrent urinary tract infections, or prolonged infections of the wound.[29] MDR and biofilm formation together may result in longer hospital stays, more frequent doctor visits, and a requirement

for more sophisticated treatment procedures. This puts a pressure on healthcare personnel and resources in addition to increasing healthcare expenses.[30]

Studying biofilms is crucial for shaping effective antibiotic policies in hospitals. Biofilms, which protect bacteria from both the immune system and antibiotic treatments, complicates infection management by making bacteria more resistant to standard therapies. Research on biofilm formation reveals that traditional antibiotics often fail to penetrate the biofilm matrix, necessitating the use of combination therapies or novel agents to achieve efficacy. For instance, understanding these mechanisms informs clinicians and hospitals on the selection of appropriate antibiotics and the implementation of more stringent infection control measures. Enhanced knowledge from biofilm studies also aids in developing targeted treatment strategies and informs surveillance practices to monitor resistant strains. This integrated approach helps optimize antibiotic use, improve infection control, and reduce the spread of resistant infections.

5. Conclusion

Pseudomonas aeruginosa is an opportunistic pathogen isolated in the patients admitted in hospitals. Formation of biofilm in *Pseudomonas aeruginosa*, which is often related with antibiotic resistance is a great concern. Our findings showed that there is a possibility of connection between drug resistance and biofilm development. Since the isolates are antibiotic resistant, there is often shifting in choice of antibiotics which further increases the problem of global Antimicrobial resistance. Since, most of the drug-resistant isolates are shown to be biofilm formers, the combination therapy based on antibiotic treatment along with antibiofilm agents can be used in treating the biofilm associated Pseudomonal infections. There should be effective execution of infection control practices in hospitals to control the spread of nosocomial pathogens like *P.aeruginosa*. Prudent antibiotic usage, detection of biofilm formation and high standards of hospital infection control practices aid in combating the *Pseudomonas aeruginosa* infection as well as preventing the development of resistant strains.

6. Ethical Approval

This study was conducted by Institute Ethical committee prior to the publication with Ref. No. 002/SBMC/IHEC/2022/1843.

7. Source of Funding

None.

8. Conflict of Interest

The author declares no conflict of interest.

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