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Differential antibiotic resistance in biofilm forming *Pseudomonas aeruginosa* strains

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Abstract

The *Pseudomonas aeruginosa* strains have ability to form biofilm a complex community, the differential antibiotic resistance is mainly due to unique biofilm composition and its extent of biofilm formation. In this study, five isolates of *Ps. aeruginosa* have been isolated from clinical environment of Government Medical College & Hospital, Nanded on cetrimide agar plate. The identification of *Ps. aeruginosa* strains were carried out on basis of morphological features and 16S rRNA gene sequencing. The biofilm formation ability of *Ps. aeruginosa* strains were evaluated by using congo-red agar plate and micro-titer plate assay. The resistance of biofilm forming *Ps. aeruginosa* strains to 20 antibiotics from diverse class had determined. The identified *Ps. aeruginosa* strains using 16S rRNA gene sequencing showed 98-100% nucleotide homology with biofilm forming and multi drug resistance *Ps. aeruginosa* strains on NCBI nucleotide database. The *Ps. aeruginosa* strains isolated in present investigation showed strong, moderate and weak biofilm formation ability. The strong biofilm forming strain PA5 showed resistance to 13 antibiotics, moderate biofilm forming *Ps. aeruginosa* strains PA2 and PA3 showed resistance to 10 antibiotics and week biofilm forming *Ps. aeruginosa* strains PA6 and PA7 showed resistance to 7 antibiotics. The biofilm, forming *Ps. aeruginosa* strains have differential resistance to diverse class of antibiotics due to extent of biofilm formation

1. Introduction

The clinical seating areas in hospitals are a frequent source of pathogen transmission due to high contact with patients, visitors, and staff. The common pathogens that can cause healthcare-associated infections include bacteria viz. *Ps. aeruginosa, K. pneumoniae, E.faecium, A. baumannii,* and *S. aureus*[1]. The *Pseudomonas aeruginosa* is known to causing infections in immunocompromised individuals (cancer, cystic fibrosis & AIDS), hospitalized patients, and individuals with chronic conditions such as diabetes or chronic lung diseases [2]. The more common types of infections caused by *Ps. aeruginosa* include urinary tract infections, pneumonia, sepsis,

skin & tissue infections, and infections of the eye, ear and CNS or bone can also occur [3]. The commonly used antibiotics to treat *Ps. aeruginosa* infection include aminoglycosides, quinolones, polymyxins, β-lactams, aztreonam, ceftolozane/tazobactam, ciprofloxacin, colistin and imipenem-cilastatin. However, *Ps. aeruginosa* strains shows differential resistance to these antibiotics is due to mechanisms include efflux pumps, membrane impermeability, antibiotic-degrading enzymes, horizontal gene transfer, and adaptive biofilm formation [4]. The antibiotic resistance profile of pathogen varies depending on the strain and environmental conditions. These features and antibiotic inactivation mechanism of *Ps. aeruginosa* are responsible for antibiotic therapy failure, long stay of patient in hospital and enhance morbidity. The chosen antibiotic should be specific and should not allow developing antibiotic resistance. Therefore, to know the resistance and sensitivity of antibiotics to *Ps. aeruginosa* strains associated in clinical seating area of hospital, and for the effective antibiotic use to manage infection, it is essential to know the antibiotic resistance/susceptibility pattern for use of suitable and effective antibiotics. The study's aim to isolate varying ability biofilm former *Ps. aeruginosa* from a clinical seating environment and determine its antibiotic resistance pattern.

2. Materials and Methods

The study was carried out at School of Life Sciences S.R.T.M.U, Nanded during 2023-24. The Hi-grade chemicals and media were used from Himedia™ during study; molecular identification of isolates was carried out at Progenome Life Science Lab, Chhatrapti Sambhajinagar (MH). India and iMark™ micro plate absorbance redder of Bio-Era were used for biofilm detection test

2.1 Isolation of *Pseudomonas aeruginosa*

The sterile cetrimide agar plates were exposed for 5 min in clinical setting area of hospital (GMC & hospital, Nanded, MH). The plates were incubated at 37 °C overnight. Primarily Identification of *Ps. aeruginosa* was performed using morphological features.

2.2 Molecular identification of *Pseudomonas aeruginosa*

The *Ps. aeruginosa* isolates were identified by molecular PCR method by using forward primer

27_F 5'AGAGTTTGATCMTGGCTCAG 3' and reverse primer 1492_R 5'GGTTACCTTGTTA CGACTT 3' for 16s rRNA gene. The gene product sequences were analyzed by using BLAST on NCBI database. The phylogenic tree were constructed by using MEGA11 software

2.3 Evaluations of Biofilm formation potential of *Ps. aeruginosa* strains

The isolated *Ps. aeruginosa* strains were tested for ability to formation of biofilm, using the congo-red agar (CRA) plate and Micro-titer plate assay. All isolates underwent biofilm detection using the Congo Red Agar technique (CRA). Isolates were streaked on CRA plates; incubated them aerobically for 24 hours at 37ºC. The formation of biofilm is indicated by black colonies. The fresh *Ps. aeruginosa* inoculums were inoculated in Muller-Hinton (MH) broth and incubated at 37°C overnight under steady conditions. Post incubation, using sterile MH broth, the culture density was adjusted at the 0.5 McFarland standards. 100µl bacterial suspensions in respective wells and blank with uninoculated broth were added in 96-well sterile titer plate. The plate underwent 48 hours incubated at 37°C. The plancktonic growth (non adherent) was eliminated after incubation, and each well was given three PBS (pH7.2) washes. 1% of crystal violet was inserted to each well and after 20 minutes. The excess dye was rinsed with sterile distilled water for 3 to 4 times and 200µl of 95% Isopropyl alcohol in 1M HCL was added to each well and allowed to react for 5 min. Then, 100µl of colored suspension from each well was transferred to other plate and Optical density (O.D.) was read at 570 nm. The mean O.D. values were taken into consideration as the experiment was run in triplicate.

2.4 Antibiotic Resistance of *Pseudomonas aeruginosa* strains

The antibiotic resistance of all isolated of *Ps. aeruginosa* strains were performed by using Hexadiscs and Octa-discs of Himedia™ on Muller Hinton Agar (MHA). The antibiotics tested include Hexa-dics (HX032) Bacitracin (B-10), Chloramphenicol, Penicillin G, Polymyxin

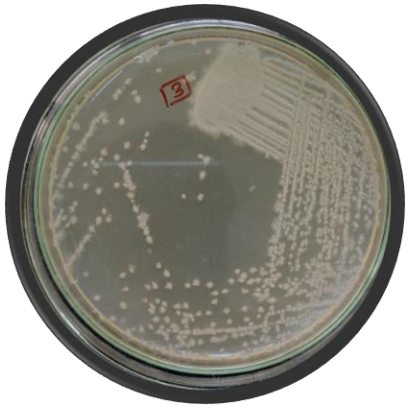
B, Gentamicin and Neomycin. Hexa-dics (HX024) Penicillin G, Methicillin, Vancomycin, Oxacillin, Erythromycin and Amphicillin. Octa-dics (OD034) Ceftriaxone, Ceftazidime, Cefotaxime, Lincomycin, Netilmicin, Ofloxacin, Vancomycin and Amikacin. Octa-dics Cefotaxime, Cephalexin, Co-Trimoxazole,

Nalidixic Acid, Furaolidone , Norfloxacin and Oxytetracyclincin In order to evaluate antibiotic susceptibility, the zone diameters generated were measured and compared to the CLSI standards[5].

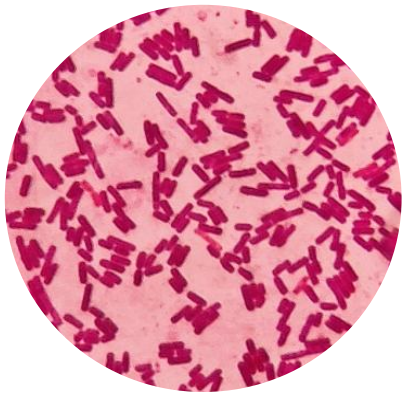
3. Results and Discussion

3.1 Isolation and Identification of *Pseudomonas aeruginosa*

The morphological features and 16S rRNA gene sequencing data of isolated *Ps. aeruginosa* on cetrimide agar confirm isolates are *Ps. aeruginosa* strains (fig.1 &fig.2). The *Ps. aeruginosa* have ability to produce water soluble pigments pyocynin, pyoverdin, pyorubin & pyomelanin. The greenish- blue color of *Ps. aeruginosa* colony is mainly due to pigment pyocyanin and pyoverdin. The presence of pyocyanin plays a role in quorum sensing, iron acquisition & virulence factor and pyoverdin function as siderophore which help the bacteria to obtain iron from iron deficient environment[6].



A



B

Fig 1: A. Isolation of *Ps. aeruginosa* on Cetrimide agar plate B. Gram's Staining of isolate

The PCR based method for identification of *Ps. aeruginosa* offer a rapid and reliable way, as these method uses specific primer to amplify target DNA sequence unique to *Ps. aeruginosa* allowing its identification however target gene found only in *Ps. aeruginosa* and toxA gene associated with *Ps. aeruginosa* pathogenicity not used during present investigation[7]. In the present study the nucleotide sequence of 16S rRNA gene showed similarity with *Ps. aeruginosa* having multi drug resistance and biofilm forming ability. The morphological features and 16SrRNA gene sequencing is an important tool for identification of *Ps. aeruginosa* from clinical seating environment.

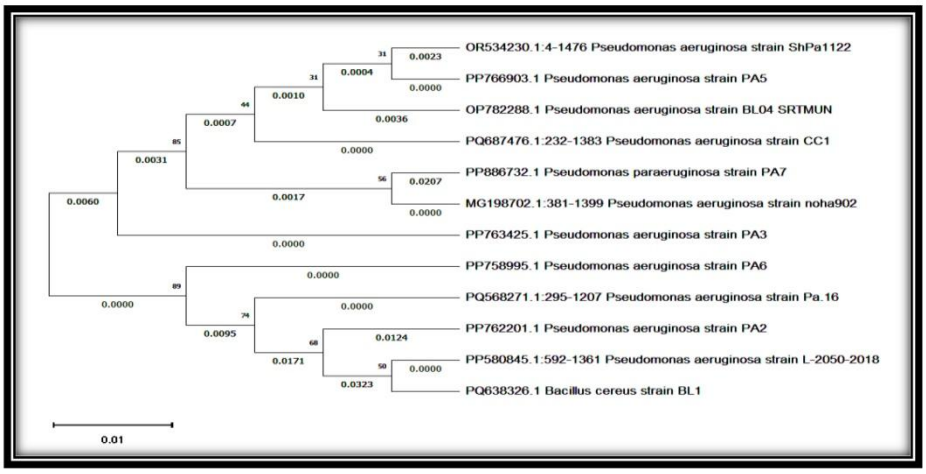


Fig. 2: Phylogenetic tree analysis of *Ps. aeruginosa* constructed by using MEGA 11

3.2 Evaluations of Biofilm formation potential of *Pseudomonas aeruginosa* strains

The out of five *Ps. aeruginosa* strains PA5 was strong biofilm former (PA5), moderate and weak biofilm formation ability was shown by PA2, PA3 and PA6, PA7 respectively. The *Ps. aeruginosa* strain exhibits a range of abilities to from biofilm as strong, moderate and weak biofilm producer. Biofilm formation is influenced by various factors viz. presence of gene algD, psID and peIF involved in biofilm matrix production, over production of exopolysaccharide like alginate, which are more resistance to antibiotics and immune response, environmental conditions like nutrient availabilities and surface properties also influence formation of biofilm [8]. The biofilm former strains of *Ps. aeruginosa* were involved in chronic infections by creating a habitat that protects bacteria and increases their resistance to immune system

and antibiotics[9]. The treatment failure, recurrent infection and medical device related infection is common with infection of biofilm producer strains.

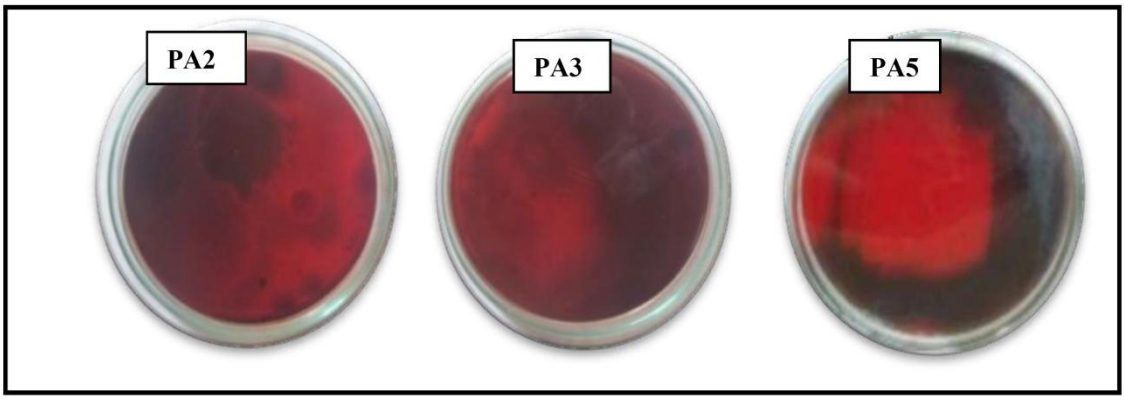
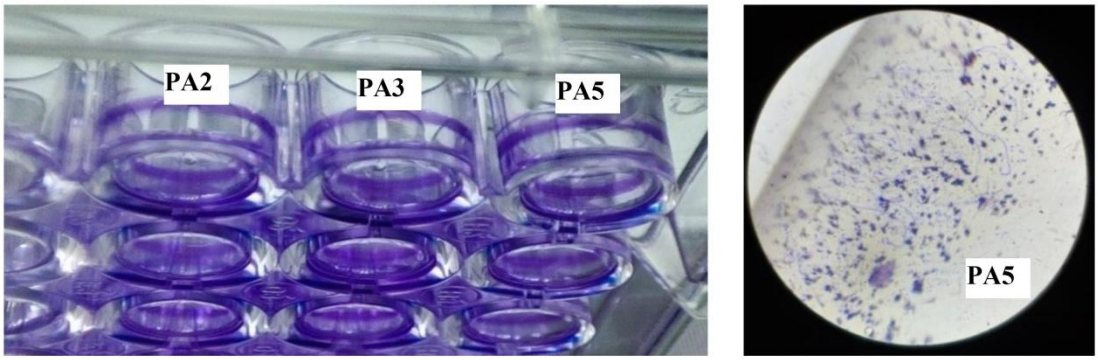


Fig 3: Biofilm forming *Ps. aeruginosa* strains (PA2, PA3 and PA5) on Congo-red agar plate



A **B**

Fig 4: A. Shows Micro-titer plate assay showing biofilm formation of *Ps. aeruginosa* strains (PA2, PA3 and PA5) **B**. Microscopic view of the Microtiter platewellof *Ps. aeruginosa* strain PA5

Table 1: Evaluation of Biofilm formation potential by congo red agar plate (CRA) and Micro-titer plate assay (MTP) of five isolated *Ps. aeruginosa* strains

Sr. No.	*Ps. aeruginosa* isolated strains	Biofilm Formation Strength	CRA Assay (Black Colony)	MTP Assay (OD at 570nm)
1	**PA2**	Moderate	++	0.112
2	**PA3**	Moderate	++	0.116
3	**PA5**	**Strong**	**+++**	**0.126**

4	**PA6**	Weak	+	0.094
5	**PA7**	Weak	+	0.095

3.3 Antibiotic resistance of *Ps. aeruginosa* Strains

The strong biofilm producer *Ps. aeruginosa* strain (PA5) showed resistance to 13 antibiotics (Table. 2), moderate biofilm former *Ps. aeruginosa* strains (PA2 & PA3) showed resistance to 10 antibiotics whereas 7 antibiotics inhibited the growth of weak biofilm producer *Ps. aeruginosa* strains (PA6 & PA7).

Table: 2 Antibiotic resistance of biofilm forming *Ps. aeruginosa* strains

Sr. No	Antibiotics (Conc. in mcg)	*Ps. aeruginosa* strains
1.	Bacitracin (B-10)	**5 (PA2, PA3 ,PA5, PA6 & PA7)**
2.	Methicillin (MET-5)	**5 (PA2, PA3 ,PA5, PA6 & PA7)**
3.	Oxacillin (OX-1)	**5 (PA2, PA3 ,PA5, PA6 & PA7)**
4.	Amphicillin (AMP-10)	**5 (PA2, PA3 ,PA5, PA6 & PA7)**
5.	Ceftazidime (CAZ-30)	**5 (PA2, PA3 ,PA5, PA6 & PA7)**
6.	Cefotaxime (CTX-30)	**5 (PA2, PA3 ,PA5, PA6 & PA7)**
7.	Cephalexin (CN-30)	**5 (PA2, PA3 ,PA5, PA6 & PA7)**
8.	Co-Trimoxazole (COT-25)	**3 (PA2, PA3 & PA5)**
9.	Furaolidone (FR-30)	**3 (PA2, PA3 & PA5)**
10.	Oxytetracyclincin (O-30)	**3 (PA2, PA3 & PA5)**
11.	Chloramphenicol (C-30)	**1 (PA5)**
12.	Nalidixic Acid (NA-30)	**1 (PA5)**
13.	Norfloxacin (NX-10)	**1 (PA5)**

The strong biofilm producer bacteria have increased resistance as dense mass of self-produced polymer hinders antibiotic penetration and reduce the concentration of the drug reaching bacterial cell, the slow growth rate of bacteria within the biofilm, along with reduced nutrient availability and oxygen, contribute to tolerance and resistance. In addition to these biofilms associated bacteria can effectively

exchange resistance gene and express enzyme that inactivates antibiotics or efflux them out of cell [10]. In present investigation though *Ps. aeruginosa* (PA5) showed resistance to 13 antibiotics from class polypeptide, β-lactams, sulfonamides, chloramphenicol and fluoroquinolones, however for treating biofilm producing *Ps. aeruginosa* strain infection, antibiotic combination is more effective than single antibiotics. The combination of Gentamicin/Ciprofloxacin and

Tobramycin/Clarithromycin are promising[11] . In severe cases imipenem and meropenem from carbapenem might be considered if other antibiotics have failed [12].

Conflict of interest

The authors declare that do not have any conflict of interest.

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