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Antimicrobial resistance patterns and prevalence of virulence factors among biofilm producing strains of *Pseudomonas aeruginosa*

EllappanKalaiarasan, * Harish BelgodeNarasimha

Department of Microbiology, Jawaharlal Institute of Post Graduate Medical Education and Research (JIPMER), Puducherry, India

Abstract
Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic and nosocomial pathogen which causes severe life threatening infections worldwide. The present study was carried out to analyze the antimicrobial resistance patterns, hemolysin and pigments production among biofilm forming *P. aeruginosa*. A total of 50 biofilm forming *P. aeruginosa* isolates were included in this study. During Antimicrobial susceptibility testing (AST), ceftazidime resistance was observed in 46% of biofilm forming isolates, gentamicin (42%), amikacin (34%), ciprofloxacin (26%) and meropenem (12%). Using conventional PCR assay, the studied isolates were speciated by species specific primers of *P. aeruginosa*.The biofilm assay was carried out using 96 well micro titer plates and Muller Hinton agar (MHA) plate was used to detect the pigments production. In addition, pyocyanin assay was conducted using chloroform extraction method. Hemolytic activity was accessed by using sheep blood agar plate. During PCR analysis, all the studied isolates were belongs to *P. aeruginosa*. Among biofilm forming isolates, pyoverdine, pyocyanin and pyorubin production was detected in 42%, 14% and 4% of isolates, respectively. Hemolytic activity was observed in 48% of isolates. Our study indicates that the involvement of antimicrobial resistance and virulence factors in biofilm producing *P. aeruginosa* remains a challenge for clinicians to provide treatment against such infections.

Keywords: *P. aeruginosa*, biofilm, conventional PCR, pyocyanin, pyoverdine, pyorubin, hemolysin

1. Introduction
It is well known that *P. aeruginosa* is an opportunistic virulent pathogen which causes pulmonary infections in cystic fibrosis patients and leads to respiratory failure [1].Due to intermittent colonization, the *P. aeruginosa* becomes established permanently and remains challenge for clinicians to eradicate [2].*P. aeruginosa* secret several virulence factors to combat the host defense mechanisms and enhance the morbidity and mortality rate. Biofilms are organized communities of bacteria which are encased in a matrix of extracellular polymeric substances (EPS) and thought to be determinant in 65-85% of all microbial infections [3].The mixture of biomolecules, exopolysaccharide, extracellular DNA and polypeptides exists in EPS contributes the overall structures of biofilms [4]. Pyocyanin production and biofilm formation plays an important role in establishing the *P. aeruginosa* infections on different host tissue [1]. Pyocyanin is blue redox-active phenazine pigment synthesized by *P. aeruginosa* to persuade neutrophil apoptosis and to damage neutrophil mediated defense mechanisms [5,6].The proteolytic enzymes secreted by *P. aeruginosa* help to break down the host physical barriers and hemolysin plays a vital role in lysis of cells from varied sources. [7,8]In this context, we aimed to investigate the antimicrobial resistance patterns, pigments production, hemolysin production in biofilm forming clinical *P. aeruginosa* isolates.

2.Methodology
2.1 *Pseudomonas aeruginosa* isolates
A total of 50 non-duplicate biofilm producing *P. aeruginosa* isolates were collected from wound swab, blood, sputum, pus, CSF (Table 1). According to Clinical and Laboratory Standards Institute (CLSI) guidelines (2014), antimicrobial susceptibility tests were carried out against antimicrobial agents including ceftazidime, amikacin, gentamicin, ciprofloxacin and meropenem. Biochemical tests (oxidase, catalase, urease, citrate, oxidation/fermentation glucose) were carried out to determine *P. aeruginosa* species.

Table 1: Biofilm Producing *P. aeruginosa* isolates collected from different clinical samples

Wound Swab	60%
Sputum	22%
Pus	12%
Blood	4%
CSF	2%

2.2 Biofilm assay
The biofilm forming ability isolates were tested using 96 well microtitre plates with little modifications [1]. The overnight cutlers of *P. aeruginosa* isolates were inoculated in Muller Hinton broth (MHB) supplemented with 0.5% glucose for 2 days at 37°C. After removing the planktonic bacteria, the wells were stained with 0.2% of crystal violet (10 minutes, at room temperature) and rinsed with distilled water. After air drying, ethanol (95%) was added and incubated for 15 minutes. The absorbance was measured spectrophotometrically at 570 nm. The experiments were carried out in triplicates.

2.3Identification of *P. aeruginosa* using Polymerase Chain Reaction (PCR)
In this study, DNA extraction was carried out using boiling lysis method. The 16srRNA gene was amplified from all the

studied isolates using the following specific primers of *Pseudomonas* genus Forward (5'-GACGGGTGAGTAATGCCTA-3') and Reverse (5'-CACTGGTGTTCCTTCCTATA -3') [9]. The PCR condition were as follows: Initial denaturation at 95ºC for 10 minutes followed by 35 cycles of denaturation at 94ºC for 1 minute, annealing at 53ºC for 30 seconds, extension at 72ºCfor 2 minutes, with final extensionat 72ºC for 10 minutes. All reactions were performed in a thermal cycler (Eppendorf, Germany).

2.4Species specificity screening of *Pseudomonas* isolates

After confirming that all the isolates were belongs to *Pseudomonas* genus, we further screened for the specific *P. aeruginosa* species using gyrase B gene primers such as forward 5'-GGCGTGGGTGTGGAAGTC-3'and reverse 5'-TGGTGGCGATCTTGAACTTCTT-3'[9]. The PCR condition were as follows: Initial denaturation at 95ºC for 10 minutes followed by 35 cycles of denaturation at 94ºC for 1 minute, annealing at 55ºC for 30 seconds, extension at 72ºCfor 2 minutes, with final extensionat 72ºC for 10 minutes. All reactions were performed in a thermal cycler (Eppendorf, Germany).

2.5Pigment production

The strains were streaked on the Muller Hinton agar (MHA) plates and incubated for 37ºC for 24 hours for visual analysis of pigment production. The colonies with blue-green pigment were interpreted as pyocyanin, yellow-green pigment as pyoverdin and brown-red pigment as pyorubin[10].

2.6Pyocyanin assay

Pyocyanin was extracted from the overnight cultures of *P. aeruginosa* grown in LB broth for 24 hours. The cells were removed after the centrifugation (10000 rpm, 10 min) and pyocyanin in supernatant was extracted into chloroform (supernatant, 7.5ml and chloroform, 4.5ml). Pyocyanin was again re-extracted into 1.5 ml of 0.2M HCL and centrifuge for 10000 rpm, 10 min [1]. The supernatant with pyocyanin was measured at 520 nm. The experiment was performed in triplicates.

2.7Hemolysin production

In order to determine the hemolysin production, the strains were streaked on the sheep blood agar and incubated at 37 ºC for 24 h. The clear holo formation around the colony was indicative of hemolysin production [10].

3. Results and Discussion

Antimicrobial resistance patterns, hemolysin and pigment production among biofilm producing *P. aeruginosa* was evaluated. In this study, a total of 50 biofilm producing *P. aeruginosa* clinical isolates were characterized. *P. aeruginosa* is a nosocomial pathogen and notorious which causes severe life threatening infections in immunocompromised and cystic fibrosis patients. Biofilms are bacterial communities exist in polysaccharide matrix which are associated with antibiotic resistance and resist phagocytosis. Biofilms displayed more tolerance to antimicrobial agents as compared as planktonic logarithmic phase cells. Production of biofilms and antimicrobial resistance plays a vital role in bacterial defense mechanisms during infectious process which allow bacteria to invade the host tissue. These both remain a challenge for clinicians to provide therapeutic approach against such infections. In our study, the antibiograms of biofilms forming *P. aeruginosa* were analyzed (table 2). Ceftazidime resistance was observed in 46% of isolates, gentamicin (42%), amikacin (34%), ciprofloxacin (26%) and meropenem (12%). Several studies have been carried out on determining the relationship between antimicrobial resistance and biofilms formation [11,12].

Table 2: Resistance patterns of biofilm Producing *P. aeruginosa* isolates

Ceftazidime	46%
Gentamicin	42%
Amikacin	34%
Ciprofloxacin	26%
Meropenem	12%

3.1 Molecular and biochemical identification of Isolates

During biochemical assays (oxidase, catalase, urease, citrate, oxidation/fermentation glucose and growth at 42ºC), all the studied isolates were confirmed as *P. aeruginosa*. *Pseudomonads* identification is hampered by biochemical assays, when typically rely on phenotypic expressions. In order to overcome this situation, the molecular identification of *P. aeruginosa* was carried out using genus specific and species specific primers.All the 50 biofilm producing isolates were belongs to *P. aeruginosa*. No biofilms were found to be other species such as *P. fluorescens* and *P. putida*. Studies have been reported that, the persistence of chronic *P. aeruginosa* lung infections in cystic fibrosis patients may be due to biofilm producing isolates. The speciation of *Pseudomonads* using biochemical and molecular level is a customary practice in microbiology laboratory [9].*Pseudomonads* speciation studies were carried out using molecular level in order to avoid the variation in phenotypic expression of such strains during different environment.

3.2 Pigments production in biofilm producing isolates

Pigments such as pyocyanin (green or blue-green), pyorubin (red-brown) and pyoveridin (yellow-green) play a crucial role in determining the *P. aeruginosa*. Pyocyanin contributes acute as well as chronic infections and has various effects by decreasing the host-response and inducing the apoptosis in neutrophils. Pyocyanin plays an important role in stimulating the IL-8 release. Pyoverdine plays a significant role in secreting theexotoxin A, which was found to be an important virulent markers of*P. aeruginosa*. Pyorubin, helps in protecting the organism against oxidative stress. Pyorubin has been reported to have antimicrobial activity, which would give the strain producing it a selective advantage over other microbes in its natural environment[13]. The results of pigment production study depict that the pyocyanin production was observed in 14% of isolates, pyorubin was detected in 4% of isolates and pyoverdine was detected in 42% of biofilm isolates (table 3). In our study, we observed that about 40% of *P. aeruginosa* isolates were non-pigmented.

Table 3: Pigment producing biofilm Producing *P. aeruginosa* isolates

Pyoverdine	42%
Pyocyanin	14%
Pyorubin	4%
No Pigment	40%

3.3 Hemolysin production in biofilm producing isolates

Haemolysins lyse red blood cells and contributes the host invasion through their cytotoxic effects on eukaryotic cells [14].*P. aeruginosa* produces two main haemolysins such as phospholipase and lecithinase, which are involved in cell invasion. Other types of haemolysins have been described such as heat-stable haemolyticglycopeptide which consists two molecules each of L-rhamnose and 1-b-hydroxydecenoic acid. It was found to be not toxic to human cells but toxic to alveolar macrophages. In our study, Hemolytic activity was detected in 48% of isolates. All pyocyanin (14%) and pyorubin (4%) producing isolates were found to be harboring with hemolytic activity. Among 21 (42%) of pyoverdine producing isolates, only seven (14%) of isolates were found to be harboring with hemolytic activity and five (10%) non-pigmented biofilms showed the hemolysin activity. Similar to previous study, our results displayed that *P. aeruginosa* strains showed the β-haemolytic on blood agar plates[15].There are three haemolysis outcomes on blood agar. The first is α-haemolysis and it can be identified by a green envelope which surrounds intact cells. The second is β-haemolysis and it can be identified from the clear colourless zones that surround the cells where the blood cells have been completely lysed. The third is γ-haemolysis and it defines a negative result for haemolysis where there has been no action on the red blood cells [16].

4. Conclusion

The results of our study highlighted that the presence of multidrug resistance and virulence factors production among biofilm forming *P. aeruginosa* represent a current therapeutic challenge worldwide.

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