

Original Research Article

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Qualitative and Quantitative Biofilm Assay of *Pseudomonas aeruginosa* Isolates from Clinical Samples

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ABSTRACT

Pseudomonas aeruginosa is a Gram-negative bacterium found in multiple habitats, including soil, marine habitats and plants, can survive in harsh conditions such as high temperatures 42 °C and is capable of growing without oxygen. It is the fifth most commonly isolated organism and an opportunistic pathogen of animals. It is considered to be one of the major causes of nosocomial infections responsible for various clinical conditions such as pneumonia, urinary tract infections, and complications in clinical burns, wounds, and cystic fibrosis (CF). *P. aeruginosa* biofilm production is a well-known virulence factor and in the biofilm mode of growth it is involved in chronic as well as in acute infections. In present investigation 65 clinical sample of nosocomial infection taken from different animal species, namely dog, cattle camel, horse and buffalo. Total ten samples were found positive for *Pseudomonas aeruginosa*, which include four from Dog, one from Sheep, one from Goat, and four from Cattle whereas no samples were found positive for horse and buffalo. The aim of this study was to detect biofilm formation by study isolates of *Pseudomonas aeruginosa* qualitatively and quantitatively. Ten isolates were taken from diseased animal in veterinary clinics of RAJUVAS Bikaner. Qualitative biofilm formation assays (Pellicle formation assay and Congo red agar method) and quantitative assay by Microtitre plate assay used for biofilm production.

Keywords

Pseudomonas aeruginosa,
Biofilm,
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Introduction

Biofilms are highly-structured microbial communities that attach to different surfaces and are enclosed in a matrix of extracellular polymeric substance (EPS). Biofilms contain nucleic acids, protein, and other molecules (Mann and Wozniak 2012). These dynamic

biofilms contain open water channels for transport of nutrients into the biofilm and waste products out of them (Stoodley *et al.*, 2002). Bacterial biofilm consist of up to 97% of water, 2-5% of microbial cells and 3-6% of EPS and ions (Meliani and Bensoltane, 2015). Biofilms are formed by the bacteria in the environment and as well as during human and

animal infections, whenever bacteria face any adverse condition. In *P. aeruginosa* biofuel production is a well-known virulence factor and in the biofilm mode of growth it is involved in chronic as well as in acute infections (Schaber *et al.*, 2007). *Pseudomonas* organism can readily form biofilms as compared to other bacteria that require specific conditions such as temperature and pH (Clutterbuck, 2007). The process is initiated through secretion of matrix by *P. aeruginosa* cells and such cells bind to surfaces (Brown *et al.*, 2012). This matrix provides a protective boundary that allows it to adhere to an environmental substrate. The coating of matrix confers 10- to 1000-fold more protection to the organism against antibiotic treatment (Brown *et al.*, 2012; Williams *et al.*, 2015).

According to report of National Institute of Health, bacteria in biofilms are responsible for more than 60% of all bacterial infections (Bryers, 2008; Lewis, 2001). Bacteria in biofilms are more resistant to antimicrobial agents and host of immune defences as biofilms have barriers in the form of extracellular matrix and also there is presence of subpopulations of persisters that are physiological by less active and thus have high-level drug tolerance (Hall-Stoodley *et al.*, 2004).

In *P. aeruginosa*, three exopolysaccharides (*Psl*, *Pel* and alginate) have been characterized for their role in the formation of biofilms (Kaplan, 2010; Wei and Ma, 2013). Alginate is the one of the most extensively characterized exopolysaccharides from mucoid strains, which forms highly viscous bacterial colonies on agar (Ramsey *et al.*, 2005). It is responsible for providing nutrients and also helps in water retention in biofilms. It plays an important role in protection and structural stability of biofilms (Sutherland, 2001). Overproduction of

alginate provides resistance to antibiotics also (Simpson *et al.*, 1988). Taking view of the fact that, biofilms are responsible for the maximum resistance in *P. aeruginosa*.

Materials and Methods

Isolation and species level conformation

The preliminary isolation of ten *Pseudomonas aeruginosa* was carried out as per cultural characteristics and biochemical profiling from different clinical conditions (Cowan and Steel, 1974; Quinn *et al.*, 1994). The clinical data regarding the distribution of isolates, type of specimens and type of infection are presented in table 1.

Proteomic based identification of *Pseudomonas* spp. by MALDI-TOF-MS (VITEK MS RUO)

Microbial identification with VITEK MS RUO (Research Use only) software and database was performed according to manufacturer's instructions by directly smearing pure and isolated colony from overnight cultured specimen (on blood agar) on to 48 positioned Flexi Mass DS disposable target plate (bioMerieux) and loaded with 1µl matrix (CHCA) solution. *E. coli* ATCC 8739 was used as the calibration strain. MALDI-TOF-MS analysis was performed in the linear positive mode in the mass range of 2-20 kDa.

Maintenance of isolates

The part of the pure culture stock was maintained on slants for short-term storage at 4°C, while another part was stored in buffered glycerol (1:1) at -80°C.

Characterization of biofilm production trait

For the qualitative detection of biofilm all isolates grown on Congo Red Agar as per

described by Freidman and Kolter (2004), then Pellicle formation at air-liquid interference assay was performed as per described by Chabane *et al.*, (2014). For quantitative detection Microtiter plate assay was performed as per described previously by the method of O'Toole (2011).

Qualitative biofilm assay

Slime production assay

Slime production in *P. aeruginosa* strains were determined by cultivation on Congo red agar (CRA). Inoculated CRA plates were incubated at 37°C for 24hr. The production of dark red and wrinkled colony morphology indicated the production of an extracellular matrix, which is involved in biofilm production.

Pellicle formation assay

A pellicle is a biofilm that assembles at the air-liquid interface of a standing liquid culture. Strains were inoculated in Luria Bertini broth supplemented with 1% glucose and incubated at 37°C for 24hr. After incubation, a thin pellicle was formed at the air-liquid interface which is visible to the necked eye.

Microtiter plate assay

Biofilm production in microtiter plate was done as per the method of O'Toole, 2011. The bacterial culture was grown overnight at 37°C in 5 ml Luria Bertini broth and overnight culture was then diluted with M63 medium. Two hundred µl of diluted cultures was added to each well of a 96 Microtiter plate. Each sample was inoculated in 3 wells in order to minimize errors. Six wells were used for negative control. The negative control wells contained only 200 µl of M63 medium. The microtitre plate was incubated at 37°C for 24

hr. After incubation, the contents of the wells were decanted into a discard container and each well was washed three times with 300 µl of sterile phosphate buffered saline. The plates were then drained in an inverted position and allowed to air dry at room temperature. Later 150 µl of 0.1% crystal violet solution was added into wells and the excess stain was rinsed off by placing the microtiter plate under running tap water. The microtiter plate was allowed to air dry in an inverted position. After drying, the dye bound to the cells was resolubilized with 150 µl of 33% glacial acetic acid. The plate was incubated at room temperature for 10-15 min. The optical density of each well stained with crystal violet was measured at 570 nm using a microtiter plate reader. The average OD values were calculated for all the tested strains and negative controls.

Results and Discussion

In the opportunistic pathogen *Pseudomonas aeruginosa* (*P. aeruginosa*) biofilm is responsible for high level of drug resistance and requires attention (Chen *et al.*, 2018). The ability to form biofilm is one of the main factors affecting pathogenicity of *P. aeruginosa*. All the isolates were tested for biofilm production phenotypically by three different methods viz., cultivation on modified Congo red agar, pellicle formation and microtiter plate assay.

Freeman *et al.*, 1989 used a simple qualitative method to detect biofilm production by using Congo Red Agar (CRA) medium. In case of *P. aeruginosa* production of dark red and wrinkled colonies indicated the production of an extracellular matrix, which is involved in biofilm formation (Freidman and Kolter, 2004).

In present study, all the ten *P. aeruginosa* isolates produced dark red and wrinkled

colonies on modified Congo red agar, indicating the production of extracellular matrix.

P. aeruginosa is able to form biofilms not only on mucosal or other solid surfaces but also at the air-liquid interface of standing cultures and such structures are called as pellicles (Friedman and Kolter, 2004). Complete coverage of the surface of the culture by an opaque layer of cells and matrix material is considered as pellicle. In the present study all the ten isolates produced a pellicle at the air-liquid interface.

In the quantitative biofilm formation assay, results showed that all 10 isolates of *Pseudomonas aeruginosa* were produced biofilm strongly (OD was more than 0.25) in tissue culture plates when grown in M63 minimal medium. Biofilm production on tissue culture plates is considered the golden standard method for biofilm detection. The isolates were classified according to biofilm production according to the criteria laid down by Christensen as following: nonbiofilm producers less than 0.125, weak biofilm producer between 0.125- 0.25 and strong biofilm producers more than 0.25.

Table.1 Distribution of clinical isolates *Pseudomonas* spp. according to the type of specimens and infection

Study isolates no (%)	Type of infection	Type of specimen
2(10%)	Otitis media	Ear swab
2(13.33%)	Burn	Wound swab
2(20%)	Surgical wound	Wound swab
1(10%)	Pus	Pus swab
3(30%)	UTI infection	Urine
Total=10(15.38%)		

Bacterial communities in biofilms attach to surfaces, either living or abiotic and are surrounded by an extracellular matrix consisting of bacterium-derived DNA, proteins and exopolysaccharides. In nature, bacteria are considered to be mainly found in the form of biofilms. There is a realization that majority of the chronic or persistent infections are caused by bacteria in biofilms. In fact, 80 percent of medical bacterial infections are also caused by organisms growing in biofilms (Schaudinn *et al.*, 2009). Bacteria in biofilm are highly resistant to immune system and antibiotics, however, most of the research on characterizing the physiology of bacteria and discovery of all current antibiotics and other antimicrobial products has been based on planktonic

bacteria (Dosler and Karaaslan, 2014) forgetting the fact that established biofilms can be up to 1,000-fold more resistant to antibiotic treatment than planktonic bacteria, making them very resistant to current treatments (Lewis, 2001). In the opportunistic pathogen *Pseudomonas aeruginosa* (*P. aeruginosa*) biofilm is responsible for high level of drug resistance and requires attention (Chen *et al.*, 2018).

Under the field of biofilm production, particularly qualitative biofilm assay by pellicle formation assay and congo red agar, the results showed that out of 10 (15 %) isolates of *Pseudomonas aeruginosa* formed biofilm as pellicle at air liquid interface. Further, in the other qualitative assay (congo

red agar) our study revealed that out of 10 (15 %) isolates of *Pseudomonas aeruginosa*, were produced red pigmented colony on congo red agar.

In order to enable easier study of bacterial attachment and colonization, a variety of experimental, direct and indirect, observation methods have been developed. Microtiter plate assay is the most frequently used techniques for quantifying biofilm formation Stepnovic *et al.*, (2000). Microtiter plate procedure is an indirect method for estimation of bacteria in situ, it has the advantage of enabling researchers to rapidly analyze adhesion of multiple bacterial strains or growth conditions within each experiment, easy technique and used widely for antimicrobial agents susceptibility of biofilm. Our results showed that all pseudomonal isolates which were submitted to this study were produced biofilm strongly at 570 nm optical density

In conclusion, *P.aeruginosa* is an increasing global problem and a public health threat, posing many therapeutic challenges. Currently, it is the second most important opportunistic pathogen accounting for 10-11% of total nosocomial infections. Biofilm formation in *P. aeruginosa* infections is one of the main virulence determinants, which significantly increases bacterial resistance to antibiotics and innate host defenses.

Out of 65 samples processed a total of 10 isolates producing pyocynin were obtained on Cetrimide agar. These isolates were further tested preliminarily through Gram's staining and other primary biochemical tests and were confirmed as *P. aeruginosa*, as high confidence score value on MALDI TOF MS (VITEK MS RUO) was obtained.

All these 10 isolates were tested for biofilm production phenotypically by three different

methods viz., cultivation on modified Congo red agar, pellicle formation and microtiter plate assay. All the *P. aeruginosa* isolates produced dark red and wrinkled colonies indicating the production of an extracellular matrix, which is involved in biofilm production. These isolates also produced pellicle at the air-liquid interface. All the isolates were able to produce biofilm in tissue culture plate assay.

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