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# Characterization of *Pseudomonas aeruginosa* biofilm formation isolated from nosocomial infections

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**Abstract**
*Pseudomonas aeruginosa* is a gram-negative bacteria prevalent throughout nature (i.e. plants, soil, and seawater). It can withstand temperatures as high as 42°C and exist without oxygen. It is an opportunistic animal pathogen and the sixth most often isolated organism. One of the main organism that causes nosocomial infection. It was associated with numerous clinical conditions such as pneumonia, cystic fibrosis (CF), and burns or wound infections. Its capacity to form biofilms is a well-known virulence factor. This study was designed for the qualitative and quantitative assessment of biofilm production in *P. aeruginosa*. 65 samples were collected from various animal species (such as dogs, cattle, camels, horses, and buffalo) that had clinical symptoms. A total of ten (from cattle, one from sheep, one from goats, and one from dogs) *P. aeruginosa* isolates were recovered. All isolates have been evaluated for their ability to form biofilms using qualitative as well as quantitative methods. We observed that all isolates of *P. aeruginosa* recovered from hospital-acquired infections had a greater tendency to form biofilm, which may aid in their virulence.

**Keywords:** *Pseudomonas aeruginosa*, biofilm, nosocomial infection

## Introduction
Biofilms are extracellular polymeric substances (i.e., nucleic acids, proteins, and other molecules) that help attach to different surfaces. (Mann and Wozniak, 2012) [15]. Bacterial biofilms consist of up to 95% water, 3-5% microbial cells, and 4-6% extracellular polymeric substances and ions (Meliani and Bensoltane, 2015) [16]. During adverse environmental conditions, biofilms are formed by the bacterium. According to a National Institutes of Health reports, up to 60% of infections are caused by bacteria that produce biofilms (Bryers, 2008; Lewis, 2001) [2, 14]. The bacteria that produce biofilms are far more resilient to host immune responses and antimicrobial drugs. Besides acting as barriers against antimicrobial agents, the biofilm also harbors physiologically less active per sister populations, which ultimately ensure high levels of drug tolerance (Hall-Stoodley *et al.*, 2004) [12].
Unlike other bacterium, the pseudomonas may easily forms biofilms (Clutterbuck, 2007; Schaber *et al.*, 2007) [7, 20]. Initialization of the process occurs when cells secrete matrix, which eventually enables it to adhere to surfaces (Brown *et al.*, 2012) [1]. In addition to adhesion, this matrix provides 10- to 1000-fold increased resistance to antibiotics (Brown *et al.*, 2012; Williams *et al.*, 2015) [1, 27]. Alginate, Psl and Pel are the three major exopolysaccharides found in *P. aeruginosa* biofilms. (Kaplan, 2010; Wei and Ma, 2013) [13, 26]. Among them, Alginate has been most thoroughly studied in pathogenic or mucoid strains (Ramsey *et al.*, 2005) [19]. In addition to assisting in structural stability and drug resistance, it also acts as a source of nutrients and enhances the ability of the body to retain water in adverse conditions (Sutherland, 2001; Simpson *et al.*, 1988) [25, 22]. In view of these facts, this study was designed for the qualitative and quantitative assessment of biofilm production in *P. aeruginosa*.

## Materials and Methods
Isolation of *P. aeruginosa* and characterization: Based on cultural characteristics and biochemical profiling, *P. aeruginosa* was first isolated from hospitalized animals (Table 1) (Cowan and Steel, 1974; Quinn *et al.*, 1994) [8, 18]. Proteomics and molecular profiling of the bacterium were used to achieve the species-level conformation. *Pseudomonas* spp.

Were identified by a proteomic approach using MALDI-TOF-MS. brifly, from an overnight cultured (on blood agar) lopful bacterium were spreading onto a Flexi Mass DS (bioMerieux) plate, then pouring it with a 1µl CHCA solution. The calibration strain was *E. coli* ATCC 8739. In the mass range of 2-20 kDa, MALDITOF-MS analysis was carried out in the linear positive mode. The PCR amplification of 16S rRNA is also carried out for molecular-level conformation, as described by (Clarridge *et al.* 2004) [6].

**Qualitative biofilm assay:** For the qualitative detection of biofilm, all isolates were cultivated on Congo Red Agar in accordance with Freidman and Kolter's (2004) [11]. Following a 24-hour inoculation on Congo red agar at 37°C, the bacteria that create biofilm form a wrinkly, dark red colony. Furthermore, pellicle forms at the interface between the liquid and air in a standing culture were also examined in accordance with Chabane *et al.* (2014) [3]. The biofilm-producing bacteria produce a thin pellicle at the air-liquid interface when incubated at 37°C for 24 hours in Luria Bertini broth supplemented with 1% glucose.

**Microtiter plate assay:** A microtiter plate test was carried out for quantitative detection using the previously described method by O'Toole (2011) [17]. 96-microtiter plates were seeded with the culture, which was grown in Luria Bertini broth following dilution in M63 medium. For the negative control, six wells were utilized. The negative control wells contained only M63 medium. Following an incubation of 24 hours at 37°C, wash each well three times with phosphate-buffered saline (PBS). Add 0.1% crystal violet solution and let it stand for five minutes before washing with PBS. 150 µl of 33% glacial acetic acid was used to resolubilize the dye that was attached to the cells. Using a microtiter plate reader, the optical density of each well stained with crystal violet was determined at 570 nm.

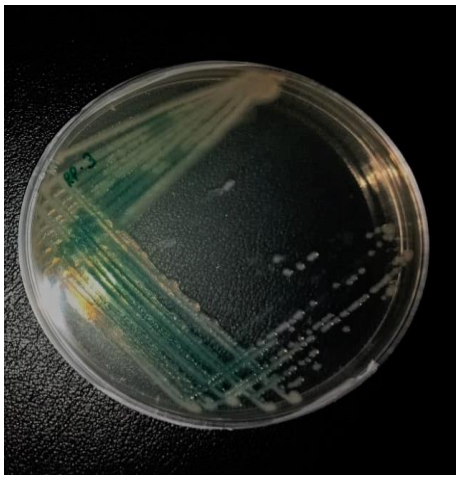
**PCR amplification of algD in *P. aeruginosa*.** PCR amplification of algD in *P. aeruginosa*. Were carried out as per Simpson *et al.*, 1998 [22].

**Results and Discussion**

From 65 clinical samples, only ten isolates were identified as P. *aeuroginas* since they produce pyocynin on cetrimide agar (Fig. 1a). These isolates were identified as *P. aeruginosa* by obtaining a high confidence score value on MALDI TOF MS (Fig 1b). Additionally, all isolates produce an identical amplicon of 16S rRNA in PCR (Fig 1c).

The biofilms let bacteria adhere to and colonize both living and nonliving surfaces. Additionally, the biofilm harbors a less active microbial population that is frequently linked to chronic or persistent infections and acts as a physiological barrier against antimicrobials (Lewis, 2001) [14]. Besides this, because biofilm has antiphagocytic properties, it shields bacteria from host immune systems. In actuality, the bacteria that may form biofilms are the source of over 60% of medical conditions (Schaudinn *et al.*, 2009) [21]. Therefore, according to Dosler and Karaaslan (2014) [9], one of the most important factors in determining a bacterial virulence is its ability to form biofilms.

(a)



(b) **Identification of P. aeruginosa isolates by MALDI-TOF MS**

| Sample no. | Probablity (%) | Pathogen detected |
|---|---|---|
| RP-1 | 84.2 | P. aeruginosa |
| RP-2 | 82.3 | P. aeruginosa |
| RP-3 | 97.2 | P. aeruginosa |
| RP-4 | 99.9 | P. aeruginosa |
| RP-5 | 99.3 | P. aeruginosa |
| RP-6 | 78.8 | P. aeruginosa |
| RP-7 | 83.1 | P. aeruginosa |
| RP-8 | 86.2 | P. aeruginosa |
| RP-9 | 97.3 | P. aeruginosa |
| RP-10 | 93.6 | P. aeruginosa |

(c)

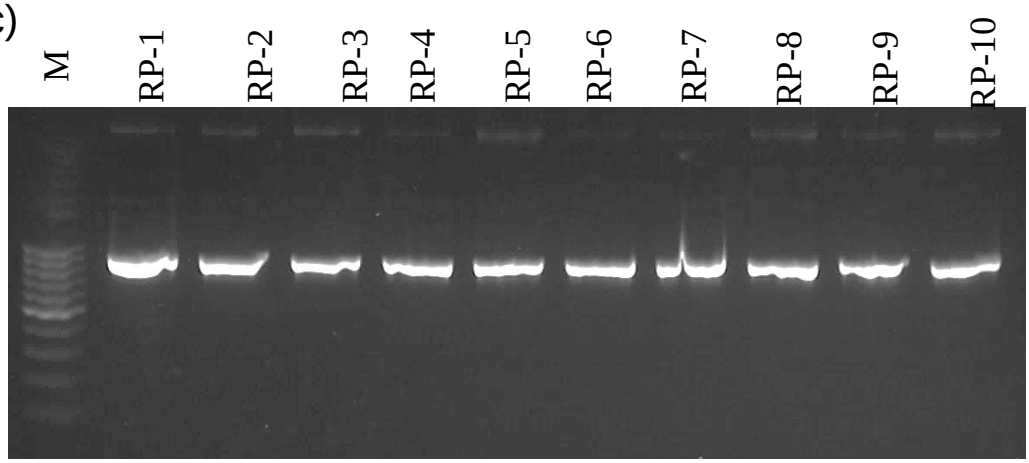



**Fig 1:** Isolation and identification of *P. aeruginosa*: (a) pigment production on cetrimide agar (b) identification of *P. aeruginosa* isolates by MAKDU-TOF MD (c) rRNA based identification of *P. aeruginosa*.

**Table 1:** Details of *P. aeruginosa* isolates

| S. No. | Type of specimen | Type of infection | Study isolates no (%) |
|---|---|---|---|
| 1 | Ear swab | Otitis media | 2 (10%) |
| 2 | Wound swab | Burn | 2 (13.33%) |
| 3 | Wound swab | Surgical wound | 2 (20%) |
| 4 | Pus swab | Pus | 1(10%) |
| 5 | Urine | UTI infection | 3 (30%) |

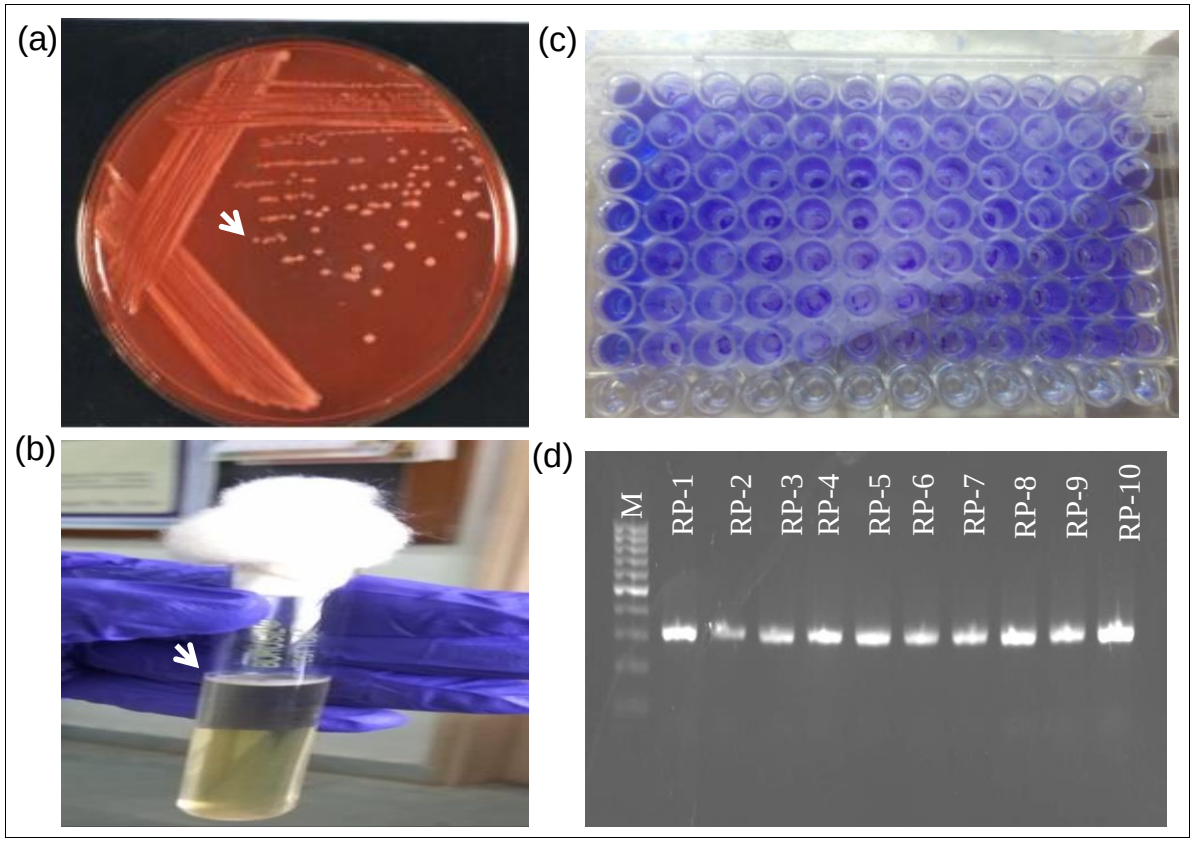



**Fig 2:** Qualitative and Quantitative assay for biofilm detection: (a) Production of dark, red and wrinkled colony by R aeruginosa on Congo red agar (b): Pellicle formation by R aeruginosa (c) Microtiter plate assay for biofilm characterization (d) PCR amplification of algD in R aeruginosa.

Unlike other bacteria, the pseudomonas may easily form biofilms (Clutterbuck, 2007; Schaber *et al.*, 2007) [7, 20]. Initialization of the process occurs when cells secrete matrix, which eventually enables it to adhere to surfaces (Brown *et al.*, 2012) [1]. In addition to adhesion, this matrix provides 10- to 1000-fold increased resistance to antibiotics (Chen *et al.*, 2018; Brown *et al.*, 2012; Williams *et al.*, 2015) [4, 1, 27]. In the current study, all 10 isolates were tested for biofilm production. For the qualitative detection of biofilm, all isolates were cultivated on Congo Red Agar in accordance with Freidman and Kolter's (2004) [11]. Following a 24-hour inoculation on Congo red agar at 37°C, all 10 isolates produced a wrinkly, dark red colony (Fig. 2a). Furthermore, pellicle forms at the interface between the liquid and air in a standing culture were also examined in accordance with Chabane *et al.*'s (2014) [3]. All 10 isolates produce a thin pellicle at the air-liquid interface when incubated at 37°C for 24 hours in Luria Bertini broth supplemented with 1% glucose (Fig 2b).

The method that is most commonly used and is often considered the gold standard for biofilm identification is the microtiter plate test (Stepnovic *et al.*, 2000) [28]. Christensen's criteria, which were as follows: non-biofilm producers less than 0.125, weak biofilm producers between 0.125 and 0.25, and strong biofilm producers more than 0.25, were used to categorize the isolates based on their formation of biofilm. Strong biofilm production was produced by all 10 *Pseudomonas aeruginosa* isolates having an OD greater than 0.25 (Fig 2c). Alginate, Psl and Pel are the three major exopolysaccharides found in *P. aeruginosa* biofilms. (Kaplan, 2010; Wei and Ma, 2013) [13, 26]. Among them, Alginate has been most thoroughly studied in pathogenic or mucoid strains (Ramsey *et al.*, 2005) [19]. In addition to assisting in structural stability and drug resistance, it also acts as a source of nutrients and enhances the ability of the body to retain water in adverse conditions (Sutherland, 2001; Simpson *et al.*, 1988) [25, 22]. Therefore, we also performed PCR to detect the presence of the algD gene. As shown in Fig. 2d, all isolates were produces an identical band, therefore considerd as positive for the presence of the algD gene. In conclusion, we observed that all isolates of *P. aeruginosa* recovered from hospital-acquired infections had a greater tendency to form biofilm, which may aid in their virulence.

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**Conclusions**

Pseudomonas aeruginosa isolates from nosocomial infections exhibit heightened biofilm-forming capacity, emphasizing its potential role in virulence and highlighting the need for

targeted interventions in healthcare settings

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