Evaluation of Biofilm Formation and Frequency of Multidrug-resistant and Extended Drug-resistant Strain in *Pseudomonas aeruginosa* Isolated from Burn Patients in Isfahan

**Abstract**

**Background:** *Pseudomonas aeruginosa* is a biofilm-forming bacterium which can result in serious health problems, particularly in burn patients. Biofilm has been assumed to protect bacteria from environmental fluctuations such as antimicrobial agent. Mucoid strains generate extensive levels of the alginate exopolysaccharide, which is an important factor of its biofilm.

**Materials and Methods:** Totally, 100 isolates of *P. aeruginosa* has been gathered from wound infections of burn patients. Polymerase chain reaction of *exolA* gene has been carried out to confirm the bacteriologic identification of isolates. The biofilm-forming capacity has been specified by capsule staining and microtiter plate test as qualitative and quantitative determination, respectively. Antimicrobial susceptibility of the isolates has been specified by disk diffusion method. **Results:** All the isolates carried the *exolA* gene. The antibiotic resistance was imipenem (90%); levofloxacin (93%); aztreonam (87%); piperacillin-tazobactam (85%); tobramycin (92%); polymyxin b (PB) (2%); and ceftazidime (CAZ) (32%). Totally, multidrug-resistant (MDR) and extended drug-resistant (XDR) isolates were 19% and 75%, respectively. Fortunately, pan drug-resistant (PDR) strain has not been observed. The assessment of biofilm formation has shown that 7% of the isolates were nonbiofilm (N), weak (W) 67%, moderate (M) 22%, and strong (S) 4%. **Conclusions:** As a result, the findings of this survey indicated that PB and CAZ were the most effective antibiotics against *P. aeruginosa*, which of course indicate a serious problem about the emergence of the PDR strains. There was no relationship between the patterns of biofilm production and antibiotic susceptibility, but high frequency of MDR/XDR and biofilm producer strains has been detected.

**Keywords:** Alginate, biofilm, burn, multidrug-resistant/extended drug-resistant, *Pseudomonas aeruginosa*

**Introduction**

*Pseudomonas aeruginosa* is a biofilm-forming opportunistic bacterium, which can make critical health problems, particularly in immunosuppressed hosts such as burn patients, patients suffering from respiratory diseases such as cystic fibrosis (CF), and cancer chemotherapy patients.[1,2] Wound infection by antibiotic-resistant organisms such as *P. aeruginosa*, *Acinetobacter*, and *Klebsiella* should be identified as a potential risk.[3] *P. aeruginosa* plays a notable role in perilous infections in burn patients. Rapid acquisition of multidrug resistance (MDR) leads to high morbidity and mortality, especially in burn centers.[4,5] *P. aeruginosa* infections are mostly difficult-to-treat because of the low antibiotic sensitivity and the high rate of the emergence of antimicrobial resistance during the process of the treatment.[6,7] Accumulation of resistance after exposure to various antibiotics and cross-resistance among them may result in MDR, extended drug-resistant (XDR), and pan drug-resistant (PDR).[6,8,9] The rapid emergence of hospital pathogens and antibiotic-resistant organisms necessitate periodic evaluation of bacterial colonization patterns and antibiogram sensitivity in burn wards.[3]

*P. aeruginosa* is a prevalent biofilm-forming bacterium which hence often used as a model organism in biofilm studies.[10] Biofilm is a complex and compressed microbial community in an exopolysaccharide matrix[11] and allows bacteria to attach to the surfaces protecting...
it from environmental fluctuations such as antimicrobial agent.[12‑16] Mucoid strains of \( \textit{P. aeruginosa} \) produce numerous amounts of the alginate exopolysaccharide which is a significant component of its biofilm.[17] Alginate is a linear heteropolysaccharide formed of D-mannuronic and L-glucuronic acid.[18] There are two different hypotheses that antibiotic resistance is different between mucoid and nonmucoid \( \textit{P. aeruginosa} \) strains; first, glycocalyx can act as a barrier to antibiotic diffusion which is related to its polyanionic properties;[19‑21] the second has revealed that some antibiotics such as tobramycin (TN) can bind to exopolysaccharide and penetrate inside bacteria.[22]

The purpose of the current study was to determine alginate/biofilm production, antibiotic susceptibility pattern, and frequency of MDR and XDR in \( \textit{P. aeruginosa} \) isolated in Imam Musa Kazem Burn Hospital in Isfahan, Iran.

**Materials and Methods**

**Bacterial isolated and identification test**

In this cross-sectional study, 100 isolates of \( \textit{P. aeruginosa} \) have been gathered from wound infections of burn patients admitted to Imam Mosa Kazem Burn Hospital in Isfahan, Iran, between March and July 2015. Each isolate has been determined due to the standard bacteriological methods including Gram-staining, growth at 42°C in cetrimide agar, oxidation-fermentation (OF), TSI, and oxidase tests. Furthermore, polymerase chain reaction (PCR) of \( \text{exoA} \) gene has been carried out to confirm the bacteriologic identification. A 397-bp fragment of the \( \text{exoA} \) gene has been selected with specified primers (forward: 5′-GACAACGCCCCTCAGCATCACCAGC-3′, reverse: 5′-CGCTGGCCATTCGTCCAGCCGT-3′).[23] Each PCR reaction was prepared in 20 µL volume include 10 µL the commercial Master Mix (containing Taq DNA polymerase, dNTPs, and MgCl₂) (Ampliqon Denmark), 1 µL DNA sample, 0.5 µL of each primer (Metabion, Germany), and 8 µL distilled. Samples were then subjected to one cycle of 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 68°C for 30 s, and 72°C for 45 s and one final cycle of 72°C for 5 min. \( \textit{P. aeruginosa} \) strain ATCC27853 (American Type Culture Collection) was included as the control.

**Determination of mucoid strain**

**Negative stain of capsule**

Mucoid strains have been determined by the use of Anthony’s capsule staining as the qualitative method.[24] Briefly, for each of 100 isolates, a thin film of skim milk suspension has been prepared and air-dried; the film has been flooded with crystal violet for 60 s. The slide has been gently rinsed with a 20% copper sulfate solution for capsule decolorization. The cell and background have been stained purple, and the capsule appears as a faint blue halo.

**Quantification of alginate/biofilm production by microtiter method**

The \( \textit{P. aeruginosa} \) isolates have been analyzed to quantify biofilm production using microtiter dish method.[25] In this method, each strain has been grown overnight at 37°C in tryptic soy broth (TSB) including 0.25% glucose. The cultures have been diluted 1:100 in TSB medium. The bacteria suspensions (125 µL) have been aliquoted into a 96-well polystyrene microtiter plate and inoculated for 24 h at 37°C without agitation. The wells have been washed three times with 300 µL distilled water; the attached bacteria have been fixed with absolute methanol for 10 min and finally stained with 125 µL of 0.1% crystal violet solution in water for about 10–15 min. After staining, the wells have been washed three times with distilled water to remove all nonadherent cells. The wells were destained with 125 µL of 30% acetic acid in water. A new sterile flat-bottomed 96-well polystyrene microtiter plate was inoculated with 125 µL destaining solution in each well. The absorbance of the destaining solution has been measured at 570 nm using an ELISA reader (Stat Fax-2100). Every experiment has been carried out in triplicate. As the control, the uninoculated medium was used. According to the optical density of each sample (ODi) and the negative control (ODc), the isolates have been categorized as strong (4× ODc < ODi), moderate (2× ODc < ODi ≤ 4× ODc), weak (ODc < ODi ≤ 2× ODc), or nonproducer of biofilm (ODi < ODc).

**Antibiotic susceptibility tests**

Agar diffusion methods (Kirby–Bauer method) have been applied to determine the antibiotic susceptibility of isolated bacteria against TN (10 µg), aztreonam (ATM, 30 µg), imipenem (IMI, 10 µg), ceftazidime (CAZ, 30 µg), levofoxacin (LEV, 5 µg), pipercillin-tazobactam (PTZ, 110 µg), and polymyxin B (PB, 300U). MDR, XDR, and PDR strains have been detected according to a new standardized international document.[26] \( \textit{P. aeruginosa} \) (ATCC 27853) was used as a control strain.

**Statistical analyses**

Statistical Package for Social Sciences software (SPSS Inc. No. 23, Version 23.0. Armonk, NY: IBM Corp.) was used for statistical analyses. Fisher’s exact test or Chi-square test was used for the categorical data analysis. \( P < 0.05 \) was considered statistically significant.

**Results**

One hundred \( \textit{P. aeruginosa} \) have been isolated from burn wounds. The isolates were Gram-negative, growing at 42°C, OF, TSI and oxidase-positive. The identification of the isolates has been confirmed by amplification of \( \text{exoA} \) gene which particularly belongs to \( \textit{P. aeruginosa} \) [Figure 1]. All the isolates carried the \( \text{exoA} \) gene.

The antibiotic susceptibility patterns of the \( \textit{P. aeruginosa} \) isolates are shown in Table 1. A high rate of resistance has...
been observed against IMI, TN, and LEV (approximately 90%), ATM (87%), PTZ (85%). The lowest and medium resistances have been observed to PB (2%) and CAZ (32%), respectively. Totally, MDR and XDR isolates were 19% and 75%, respectively. PDR strain has not been observed.

Mucoid strains were determined in all isolates as an alginate producer by capsule stain method (qualitative methods). Ninety-three percent of the isolates indicated biofilm formation. In addition, microtiter method was conducted as a quantitative assessment of biofilm production. The results have been shown that 7% of isolates were nonbiofilm (N), weak (W) 67%, moderate (M) 22%, and strong (S) 4%. The relation between the biofilm formation and the antibiotic susceptibility patterns is shown in Table 2 (P < 0.05).

**Discussion**

In *P. aeruginosa* infections, alginate/biofilm production has been measured as an important determinant of pathogenicity.[27] Mucoid strains of *P. aeruginosa* produce vast levels of the alginate considered the main component of its biofilm.[17] In the present study including *P. aeruginosa* isolated from burn patients, we assess presence and rate of the alginate as a virulence marker and determine the frequency of MDR and XDR strain.

In this study, 93% of *P. aeruginosa* isolates have been specified as mucoid type. Frequency of mucoid isolates of *P. aeruginosa* in our study is consistent with results of some studies that are mentioned below in detail; in Ghanbarzadeh et al.’s study in Iran, 144 isolated from burn patient 92.4% were mucoid; further, in Jabalameli et al.’s study in Iran with 96 sample, the mucoid strains were 96%; further, in India, in a study conducted by Ugargol et al. with overall theme as the characterization of virulence factors, such as alginate in a tertiary care hospital, 96.9% of 250 *P. aeruginosa* isolates identified as mucoid phenotype; similar proportion has been reported in burns isolates.[2,28,29]

In some surveys, mucoid strains were lower than our result. According to the findings of Kádár et al. in 2010, 23.3% of sixty clinical samples of *P. aeruginosa* were positive for biofilm formation.[30] In another study performed by Ghadaksaz et al. in Iran, the frequency of biofilm formation was 50.1% among 104 clinical isolates of *P. aeruginosa*.[31] In a study conducted at Manipal, 68% of *P. aeruginosa* strains produced alginate.[32] The results mentioned above have shown that mucoid strains have lately increased in comparison with previous studies in Iran and another country.

The differences of mucoid shift between our result and another study may be related to the site variation of clinical samples, such as ocular infections (no biofilm production), urine (34.2%), chronic rhinosinusitis (28.6%), and CF (33.3%).[32,33-35]

Quantitative biofilm determination by the use of the microtiter test showed that 93 strains produced biofilm, which 67 samples were weak biofilm producers, 22 samples were moderate, and four samples were strong. In a study performed by Jabalameli et al., biofilm production has been observed in more than 96% of the isolates which 22.9% were weak biofilm formers, 26% were moderate, and 47% were strong.[29]

According to the earlier studies, it has been thought that antibiotic susceptibility is different between mucoid and nonmucoid *P. aeruginosa* strains.[1,16,37] In many literature,
there are challenges about whether the mucoid phenotype leads to increased resistance. One hypothesis suggests that the glycocalyx usually acts as a polyanionic barrier to antibiotic penetration.[19-21] This was refuted by the fact that although some antibiotics such as TN bind to the exopolysaccharide, the resulting reduction in the diffusion coefficient of antibiotic in a biofilm would not be enough to prevent the entry of antibiotics.[38]

According to Table 1 in this study, there was no relationship between the patterns of biofilm production and antibiotic susceptibility. The results of the study performed by Ahangarzadeh et al. showed that the mucoid strains \((n = 43)\) were statistically more resistant to some antibiotic than the nonmucoid strains \((n = 90)\).[18] In two separate studies performed by Ghanbarzadeh et al. and Abidi et al., the statistical analysis showed that biofilm formation in the MDR \(P. aeruginosa\) (MDRPA) isolates was higher than that in the non-MDRPA isolates.[28,39]

In contrast, several studies suggest that mucoid strains are more sensitive to some antibiotic than nonmucoid strain.[36-40] For example, in Shawar et al.'s study of inhaled-TN therapy examined the susceptibility of 1240 CF isolates, it found that for all seven antibiotics tested, mucoid strains were more susceptible in comparison with nonmucoid strains.[37] Furthermore, an important finding in Burns et al.'s study was that overall and for each drug tested, mucoid isolates were more susceptible.[41]

\(P. aeruginosa\) remains one of the most significant opportunistic causes of nosocomial infections, and it has increased resistance to a range of antimicrobial agents in burn centers.[42] In this study, we also carried out antibiogram test to determine the antibiotic susceptibility pattern. Our results showed that \(P. aeruginosa\) isolates were almost resistant to all tested antibiotic, except PB (2%) and CAZ (32%). As a result, MDR and XDR isolates were 19% and 75%, respectively. Because of the XDR isolates are a subset of the MDR isolates; their frequency can be reported as 94% MDR and 85% XDR. Over the recent years, various articles have confirmed an increasing MDR among \(P. aeruginosa\) isolated from burn wound infections in Iranian hospitals.[43,44]

According to the results of the study performed by Jabalameli et al., there is a high frequency (>80%) of resistance against all tested antibiotics in our study, except for PB.[29] In a study conducted by Ghanbarzadeh et al. entitled biofilm formation and virulence factors among \(P. aeruginosa\) isolated from burn patients, a high rate of resistance has been observed against ATM (86.8%), ciprofloxacin (93.7%), piperacillin (85.4%), amikacin (82%), CAZ (82.6%), and IMI (79.2%). Totally, 93.1% of the isolates were characterized as MDRPA.[28] Ghazi et al. in 2012 investigated antibiotic resistance pattern in clinical isolates of \(P. aeruginosa\) and showed that all clinical isolates were resistant to CAZ, PTZ, and cefepime followed by ATM, ticarcillin, amikacin, and TN (96.5%).[45] In a study in 2013 at the Burn Centre of Guilan in the north of Iran, the percentage of resistance to tested antibiotics was as follows: CAZ 57.5%, ciprofloxacin 65%, gentamicin 67.5%, piperacillin 87.5%, amikacin 90%, and IMI 97.5%. Totally, 45.3% were MDR.[46] Yousefi et al. and Shahcheraghi et al. found that 30.1% and 5.46% of the isolates were MDR, respectively.[47,48]

### Conclusions

As a result, it can be concluded that the rate of MDR in Iran is higher than other countries. The results of antibiogram showed that PB and CAZ were the most effective drugs against \(P. aeruginosa\) in vitro, but the high speed of increased resistance might lead to the emergence of the PDR strains, which is a serious warning to our country. The increased rate of MDR/XDR \(P. aeruginosa\) isolates can cause limitations in antibiotic therapy as a final strategy to treat the infections. Therefore, it is important to investigate the antibiotic susceptibility pattern of \(P. aeruginosa\) isolates. There was no relationship between the patterns of biofilm production and antibiotic susceptibility, but high frequency of MDR/XDR and biofilm producer strains has been detected.

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### Conflicts of interest

There are no conflicts of interest.

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