The correlation between the presence of quorum sensing, toxin-antitoxin system genes and MIC values with ability of biofilm formation in clinical isolates of *Pseudomonas aeruginosa*

Saeed Hemati¹, Farid Azizi-Jalilian¹, Iraj Pakzad¹, Morovat Taherikalani¹, Abbas Maleki¹, Sajede Karimi¹, Azam Monjezi¹, Zahra Mahdavi¹, Mohamad Reza Fadavi¹, Kourosh Sayehmiri¹, Nourkhoda Sadeghifard⁰

¹Clinical Microbiology Research Center, Department of Microbiology, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, IR Iran. ²Prevention of Psychosocial Injuries, Research Centre, Ilam, IR Iran.

ABSTRACT

Introduction: *Pseudomonas aeruginosa* is a Gram-negative bacterium that considered as important opportunistic human pathogen. One of the mechanisms that help bacteria to tolerate survival in adverse conditions and resistance to antibiotics is biofilm formation through quorum sensing (QS) signals and toxin-antitoxin (TA) systems. QS and TA are two systems that have important roles in biofilm formation. QS is a global regulatory mechanism that enable bacteria to communicate with each other by production of auto inducers (AI) molecules in population. Because of importance biofilm formation in *P. aeruginosa* infections, here, we studied frequency of QS and TA genes among clinical isolates of *P. aeruginosa* with ability of biofilm formation.

Materials and Methods: One hundred and forty clinical isolates of *P. aeruginosa* were collected from Tehran and Ilam hospitals. The isolates were identified by biochemical tests. Biofilm formation was evaluated by microplate method. After DNA extraction by boiling method, the frequency of QS genes (*lasIR*, *rhlIR*), and TA genes (*mazEF*, *relBE*, *hipBA*, *ccdAB* and *mqsR*) were analyzed by PCR.

Results: Our results showed that maximum resistance is related to aztreonam (72.85%) antibiotic. Most of isolates were able to produce biofilm (87.15%) and the majority of them formed strong biofilm (56.42%). PCR results showed that frequency of *mazEF*, *relBE*, *hipBA*, *ccdAB*, *mqsR*, *lasIR* and *rhlIR* genes were 85.71, 100, 1.42, 100, 57.14, 93.57 and 83.57 percent, respectively.

Conclusion: Clinical isolates of *P. aeruginosa* had high ability to form biofilm, and QS and TA system genes among these isolates were very high (except *hipBA* genes). There are significaut correlation between biofilm formation and present of QS and TA system genes.

Keywords: *P. aeruginosa*, Quorum sensing, Toxin-antitoxin systems

INTRODUCTION

*Pseudomonas aeruginosa* is an opportunistic Gram-negative non-fermenting bacillus that belongs to the family Pseudomonadaceae. It was first isolated from green pus in 1882. *P. aeruginosa* has minimal nutrition requirements, which contribute to its broad ecological adaptability and distribution (1). It is one the most important nosocomial pathogens that causes infections in the respiratory tract, blood, urinary tract, ear, skin, soft tissues, eye, central nervous system, heart, bone, joint and gastrointestinal tract (2). Infection with *P. aeruginosa* is a major health problem for immune-compromised patients and
individuals with cystic fibrosis. Because it tolerates a variety of harsh physical conditions and has highly adaptable ability to survive in different environments, like hospital environments and equipments such as mechanical ventilators, intravenous lines, urinary or dialysis catheters, pacemakers, endoscopes and sinks and stability in these conditions can be potential reservoirs for contamination (3). Biofilm formation is a mechanism that helps bacteria to resist against antibiotics and immune systems and helps bacteria to survive in poor nutrient conditions (4). Several mechanisms are involved in the biofilm formation i.e. QS and TA systems (5, 6). In fact, QS is a cell signaling mechanism used by many species in response to extracellular signals (autoinducer). Genetic studies show that las (lasIR) and rhl (rhlIR) are two QS systems in P. aeruginosa and AHL or N-acylated hemoserine lactone are as messenger molecules in these systems (7). These systems have important roles in some of physiological and metabolism behaviors such as biofilm formation, virulence, antibiotic resistance and motility (8). TA systems are another systems which are effective in virulence, antibiotic resistance and motility (8). TA systems are another systems which are effective in virulence regulation, and genetic competence (14). Because of importance of biofilm formation in pathogenesis of P. aeruginosa, the correlation between lasIR and rhlIR genes and also mazEF, relBE, hipBA, ccdAB and mqsR with ability of biofilm formation in clinical isolates were determined in the present study.

MATERIALS AND METHODS

Collection of bacterial isolates. A total of 140 non-duplicate, clinical isolates of P. aeruginosa were collected from a nationwide distribution of several hospitals in two cities in Iran (Tehran and Ilam) between October 2012 and June 2013 and identified using conventional biochemical tests. All isolates stored at −80°C in TSB containing 20% glycerol.

MIC detection. MIC of antibiotics ceftazidime, piperacillin, ticarcillin, carbencillin, aztreonam, meropenem, gentamicin and amikacin were done according o CLSI protocol with microdilution method (15).

Polymerase chain reaction amplification. Polymerase chain reaction (PCR) was done to screen all 140 isolates for the presence of the 11 genes, lasI, lasR, rhlI, rhlR, ccdA, ccdB, relA, relB, mqsR, mazE and mazF (Table 1). PCR reaction was done by C1000TM Thermal Cycler (BIO RAD, USA). For DNA extraction, one colony of each isolate cultured in LB broth medium for overnight and the DNA was extracted by boiling method. PCR were performed in a total volume of 25 μl containing 1 μl PCR buffer, 2 mM MgCl2, 2 mM dNTPs, 10 pmol of primers, 0.25 U Taq DNA polymerase (CinnaGen Co, Iran) and 5 μl of template DNA. PCR products were analyzed by electrophoresis on 1% (w/v) agarose gel (Merck, Germany) containing DNA safe. Agarose gels visualized by gel documentation (Gel Doc™ XR+, USA) (16).

Microtiter plate biofilm assay. For biofilm formation; (1) isolates were incubated overnight at 37°C in LB broth, (2) Optical density (OD) of bacterial suspension was adjusted between 0.4-0.6 at 600 nm by spectrophotometer, (3) one hundred and ninety μl from LB broth medium was added to wells of polyvinylchloride 96-well microtiter plates and then, (4) 10 μl from bacterial suspension was added to each wells. The isolates were continuously incubated with shaking at 30 rpm at 37°C for overnight. Biofilm assay was performed as triplicate for each isolate, and LB broth medium used as negative control.

Estimation of bacterial biofilm. After incubation, to estimate quantity of biofilm in each well: (1) micro plates were washed with distilled water. (2) The wells stained with 0.1% crystal violet and left at room temperature for 10 min and then washed with distilled water for three times (4). In final step, 200 μl of 95% ethanol was added and OD at 492 nm was measured with an ELISA reader. These OD values were considered as an index of bacteria adhering to surface
and forming biofilms. For quantitative analysis of the biofilm production, the average absorbance from the control wells (Ac) was subtracted from the A492 nm of all test wells. Averages and standard deviations were calculated for all experiments. Isolates were classified as follows: A≤Ac=no biofilm producer, Ac<A≤(2×Ac)=weak biofilm producer, (2×Ac)<A≤(4×Ac)=moderate biofilm producer and (4×Ac)<A=strong biofilm producer (17).

Statistical analysis. Data expressed by percentage, mean and standard deviation (SD). To find correlation between biofilm formation and frequency QS and TA genes, X² and Fisher's exact test was used. Monte Carlo method with 10.000 tables with starting seed 200.000 was used when Chi-square was not value. Adjustment was done by using logistic regression. A P values <0.05 were considered to indicate statistical significance.

RESULTS

MIC results. Our findings showed that the resistance of these isolates to the antibiotic that used is high. These results are summarized in Table 2.

Biofilm formation results. Microplate method showed that most isolates about 87.15% tend to form biofilm, 12.85% not producing any biofilm. Among biofilm producing strains, 56.42% formed strong biofilm (Table 3).

PCR results. PCR results showed that frequency of mazEF, relBE, hipBA, ccdAB, mqsR, lasIR and rhlIR

| Target gene | Primer sequences (5’ to 3’) | Amplicon size(bp) | Denaturation (1min) | Annealing (45sec) | Extension (1min) | Final extension (10min) |
|-------------|----------------------------|-------------------|---------------------|-------------------|-----------------|------------------------|
| lasI        | F: GTTTCAGGCGGCAAAAGG     | 238               | 94                  | 61.9              | 72              | 72                     |
| lasR        | F: TCGAACATCCGGTAGAAAAA   | 128               | 94                  | 61.9              | 72              | 72                     |
| rhlI        | F: CCGTGCGAAGAATTACGCG    | 308               | 94                  | 61.9              | 72              | 72                     |
| rhlR        | F: TCGTTCAGGACACATTCCTC   | 284               | 94                  | 61.9              | 72              | 72                     |
| ccdA        | F: GAGAGGCGGCTATACGCG     | 199               | 94                  | 58.8              | 72              | 72                     |
| ccdB        | F: GAGACGAGGGGCACTAAAGGAAT | 272              | 94                  | 58.7              | 72              | 72                     |
| relE        | F: GACGAGGCGGCTATACGCG    | 267               | 94                  | 58.7              | 72              | 72                     |
| relB        | F: ATGGGTAGATACACTGCG     | 240               | 94                  | 58.7              | 72              | 72                     |
| mqsR        | F: AGCCACACACATACAGTT     | 194               | 94                  | 58.7              | 72              | 72                     |
| mazE        | F: ATGTACCAGGCTAGTTAAGGCG | 249               | 94                  | 58.7              | 72              | 72                     |
| mazF        | F: ATGTACCAGGCTAGTTAAGGCG | 288               | 94                  | 58.7              | 72              | 72                     |
| hipA        | F: CTGTCCTTGGGATGAAAGCAGGC | 1314              | 94                  | 58.7              | 72              | 72                     |
| hipB        | F: AGCCAAACGCAAATTGGGCAGA | 225               | 94                  | 58.7              | 72              | 72                     |
genes were 85.71, 100, 1.42, 100, 57.14, 93.57 and 83.57 percent, respectively. ccdAB and relBE genes had highest frequency, and in contrast, hipBA genes had the lowest frequency (Figs 1-3).

**Correlation between the presence of QS system genes with biofilm formation.** Monte Carlo method with 10,000 tables with starting seed 200,000 was used to finding correlation between the presence of QS system genes and biofilm formation. Results showed that there are significant correlation between lasIR genes (P=0.012) and rhlIR genes (P=0.030) with biofilm formation.

**Correlation between the presence of TA system genes with biofilm formation.** Monte Carlo method with 10,000 tables with starting seed 200,000 was used to finding correlation between the presence of TA system genes and biofilm formation. Our finding showed that there are significant correlation between mazEF genes (P=0.002) and mqsR genes (P=0.001) with biofilm formation.

**Correlation between the MIC values with biofilm formation.** According to our finding of statistical analysis that mentioned above, the results showed that there are significant correlation between MIC values of ceftazidime (P=0.003), meropenem (P=0.002) and amikacin (P=0.001) with biofilm formation.

**Correlation between the presence of TA system genes with MIC values.** There are significant correlation between the frequency of mazEF genes with resistance to gentamicin (P=0.027), meropenem (P=0.022), piperacillin (P=0.011) and amikacin (P=0.004).

**Correlation between the presence of QS with TA system genes.** Significant correlations were found between the frequency of mqsR genes and the frequency of lasIR genes (P=0.005) as well as rhlIR genes (P=0.032).

**DISCUSSION**

*Pseudomonas aeruginosa* is an opportunistic pathogen that causes urinary tract infection, respiratory tract infection and burn infections. Cancer, AIDS, immunocompromised statute and patients suffering from cystic fibrosis are more susceptible to these bacteria (18). The organism persists in hospital environment because of its strong resistance to antimicrobial agents and causes nosocomial infections (19). In the environment, bacteria expose to numerous stresses, and response to stress and ensure survival in the population by different mechanisms including biofilm formation, QS and TA systems (5, 6).

### Table 2. MIC results of clinical isolates of *P. aeruginosa*.

| Antibiotic   | S       | I   | R  |
|--------------|---------|-----|----|
| Ceftazidime  | 34 (24.28%) | 11 (7.85%) | 95 (67.85%) |
| Piperacillin | 44 (31.42%) | 12 (8.57%) | 83 (59.28%) |
| Ticarcillin  | 42 (30%)  | 12 (8.57%) | 86 (61.42%) |
| Carbenicillin| 48 (34.28%) | 10 (7.14%) | 82 (58.57%) |
| Aztreonam    | 35 (25%)  | 3 (2.14%) | 102 (72.85%) |
| Meropenem    | 48 (34.28%) | 8 (5.71%) | 84 (60%) |
| Gentamicin   | 40 (28.57%) | 9 (6.42%) | 91 (65%) |
| Amikacin     | 49 (35%)  | 18 (12.85%) | 73 (52.14%) |
| Ciprofloxacin| 47 (33.57%) | 3 (2.14%) | 88 (62.85%) |

S: sensitive, I: intermediate, R: resistance.

### Table 3. Results of biofilm formation by microplate method.

| Biofilm formation producer | No producer | Weak producer | Moderate producer | Strong producer | Total |
|----------------------------|-------------|---------------|-------------------|----------------|-------|
| N (%)                      | 18 (12.85%) | 13 (19.28%)   | 30 (21.42%)       | 79 (56.42%)    | 140 (100%) |
Bioinformatic analysis of published prokaryotic genomes has been demonstrated the position of TA and QS loci. However, little effort has been made to survey large collections of clinical bacterial isolates for the presence and functionality of these systems (21).

Here, the results of antimicrobial susceptibility showed that resistance of these isolates to antibiotics that used in this study were high. Maximum and minimum resistances were related to aztreonam (72.85%) and amikacin (52.14%). One of the reasons for this high resistance is widespread using of antibiotics in Iran. Antibiotic resistance of strains and their strong ability to form biofilm hinder the eradication of infections with this organism (22).

It has been estimated that antibiotic concentration required to kill bacteria in the biofilm is 100 to 1000 fold more than their planktonic form (23).

In this study, the majority of isolates were able to form biofilm (87.15%) and strong biofilm formation was observed in 56.42% of isolates. To find correlation between the MIC values and biofilm formation, we observed significant correlation between MIC values of cefazidime (P=0.003), meropenem (P=0.002) and amikacin (P=0.001) with biofilm formation. The more biofilm formation, the higher MIC values.

QS systems are mechanisms that regulate biofilm formation. These systems influence on the initiation of biofilm formation and also in process of biofilm maturation (21). In our study, PCR results showed that frequency of QS genes among these isolates was high. In the study by Cabrol et al. (2003) frequency of lasR gene was 100% on sixty six isolates of P. aeruginosa (24).

According to results, there was a significant correlation between the lasIR genes (P=0.012) and rhlIR genes (P=0.030) with biofilm formation. It suggests that the frequency of these genes among the biofilm producing isolates was high.

Concerning TA systems, early studies reported that these systems did not play any role in biofilm formation. For example, in Streptococcus mutans which lacking homologues of the mazF and relE toxin genes had no effect on biofilm formation to compare with parental strains (25). But, recent studies showed that TA systems are involved in biofilm formation (17).

In our study, frequency of TA system genes for ccdAB, relBE, mazEF, mqsR and hipBA were 100, 100, 85.71, and 1.42, respectively. In other study; Williams and et al. (2011) reported that frequency of relBE and mazEF among clinical isolates of P. aeruginosa and...
MRSA were 100%; also Moritz et al. (2007) reported that frequency of relBE and mazEF among clinical isolates of vancomycin-resistant enterococci (VRE) were 13% and 93%, respectively (21, 26). However in our study, hipBA genes had minimum frequency. It seems that these genes had no effect on the biofilm formation. But, hipBA genes are involved in biofilm formation in some species such as E. coli (27).

For finding correlation between the presence of TA system genes with biofilm formation, there was a significant correlation between mazEF genes (P = 0.002) and mqsR genes (P = 0.001) with biofilm formation. It could be concluded that among the isolates that formed biofilm, the frequency of these genes was high.

TA systems are also related to antibiotic resistance (28). In this study, we also observed a significant correlation between the MIC values of gentamicin (P = 0.027), meropenem (P = 0.022), piperacillin (P = 0.011) and amikacin (P = 0.004) with mazEF genes. It means that among the isolates that had high frequency of mazEF genes, the MIC values these antibiotics against the isolates were high.

Because both QS and TA systems are involved in biofilm formation, the relation of QS and TA systems with each other was investigated. In previous studies, the relation of MqsR with motility, biofilm formation and produce of autoinducer-2 quorum sensing system, and also the relation of MazF toxin with activity of death factor (EDF) of QS were cleared (29, 30). Here, we also observed significant correlation between mqsR genes with QS system genes and among the isolates that had high frequency of mqsR genes, the frequency of lasIR genes (P = 0.005) and rhlIR genes (P = 0.032) were high.

Studies showed that different agents are involved in biofilm formation among P. aeruginosa isolates and QS and TA system are important systems. Thus, knock out essential genes (such as QS and TA system genes) in biofilm formation in the planktonic state can be considered as potential targets to prevention of biofilm formation (31). Understanding the molecular basis of biofilm formation and disturbing these mechanisms can provide a novel approach to prevent biofilm formation and eradication of infections.

Finally, our finding showed that these isolates have high ability to biofilm formation (especially strong biofilm) and QS and TA system genes that influence biofilm formation and antibiotic resistance had high frequency in these isolates (except hipBA genes).

Thus, because of important physiologic roles of these systems in bacterial, they can be candidates as novel targets for treating infections of multidrug-resistant bacteria specially by using drugs that activate silent toxins of TA systems or drugs that disturbing QS systems (18, 32-35).

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REFERENCES

1. El Solh AA, Alhajhusain A. Update on the treatment of Pseudomonas aeruginosa pneumonia. J Antimicrob Chemother 2009; 64:201.
2. Neidig A, Yeung AT, Rosay T, Tettmann B, Strempel N, Rueger M, et al. Typ A is involved in virulence, antimicrobial resistance and biofilm formation in Pseudomonas aeruginosa. BMC Microbiology 2013; 13:77.
3. Belishe RB, Edwards KM, Vesikari T, Black SV, Walker RE, Hultquist M, et al. Live attenuated versus inactivated influenza vaccine in infants and young children. N Engl J Med 2007; 356: 685-696.
4. Klausen M, Heydorn A, Ragas P, Lambertsen L, Aaes-Jørgensen A, Molin S, et al. Biofilm formation by Pseudomonas aeruginosa wild type, flagella and type IV pili mutants. Mol Microbiol 2003; 48: 1511-1524.
5. Wang X, Wood TK. Toxin-antitoxin systems influence biofilm and persister cell formation and the general stress response. Appl Environ Microbiol 2011; 77: 5577-5583.
6. O’Loughlin CT, Miller LC, Siryaporn A, Drescher K, Semmelhack MF, Bassler BL. A quorum-sensing inhibitor blocks Pseudomonas aeruginosa virulence and biofilm formation. PNAS 2013; 110: 17981-17986.
7. Hartmann A, Schikora A. Quorum sensing of bacteria and trans-kingdom interactions of N-acetyl homoserine lactones with eukaryotes. J Chem Ecol 2012; 38: 704-713.
8. Szabó MÁ, Varga GZ, Hohmann I, Schelz Z, Szegedi E, Amaral L, et al. Inhibition of quorum-sensing signals by essential oils. Phytother Res 2010; 24: 782-786.
9. Mutschler H, Gebhardt M, Shoeman RL, Meinhart A. A novel mechanism of programmed cell death in bacteria by toxin-antitoxin systems corrupts peptidoglycan synthesis. PLoS Biology 2011; 9: e1001033.
10. Schuster CF, Bertram R. Toxin-antitoxin systems are ubiquitous and versatile modulators of prokaryotic cell fate. FEMS Microbiol Lett 2013; 340: 73-85.
11. Yamaguchi Y, Park J-H, Inouye M. Toxin-antitoxin systems in bacteria and archaea. Annu Rev Genet 2011;
The page contains a list of references, primarily in English, discussing various aspects of bacterial biofilms, toxin–antitoxin systems, and antimicrobial strategies. Here are some highlighted points:

1. The mechanisms of biofilm resistance to antimicrobial agents are reviewed.
2. The structural overview of toxin–antitoxin systems is provided, including their role in developing antimicrobial agents.
3. Antibiogram of biofilm infections and their impact on clinical isolates is discussed.
4. The development of quorum-sensing inhibitors as a strategy for controlling bacterial biofilms is highlighted.
5. The use of the quorum-sensing regulator MqsR in bacterial biofilms is discussed.
6. The role of toxin–antitoxin systems as a target for antimicrobial therapy is examined.
7. The potential of toxin–antitoxin systems as an antibacterial strategy is explored.

These references are from various journals, including "Nature Microbiology," "Trends in Microbiology," and "Staphylococcus aureus." The content covers a range of topics from structural overviews to practical applications of these systems in combating bacterial infections.