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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*

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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 for mation 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

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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 biofilm formation and they are present in almost all prokaryotes (9). A typical TA system consists of two genes (they are coded by the chromosomal or plasmid or both of them), one for a stable toxin and another for an unstable antitoxin. *mazEF* (*mazF* toxin and *mazE* anti-toxin), *relBE* (*relB* toxin and *relE* anti-toxin), *hipBA* (*hipA* toxin and *hipB* anti-toxin), *ccdAB* (*ccdB* toxin and *ccdA* anti-toxin) and *mqsRA* (*mqsR* toxin and *mqsA* anti-toxin) are TA systems that have been identified in bacteria (5, 10-13). TA systems are related to some bacterial behaviors such as biofilm formation, Plasmid maintenance, phase variation, 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, carbenicillin, aztreonam, meropenem, gentamicin and amikacin were done aaccording 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^{TM} XR^{+}, USA) (16).

Microtiter plate biofilm assay. For biofilm formation; (1) isolates were incubated overnight at 37ºC 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 37ºC 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

Table1. Primers used for detection of target genes and PCR programs for amplification

Target gene	Primer sequences (5' to 3')	Amplicon size(bp)	Cycle: 33			
			Denaturation (1min)	Annealing (45sec)	Extension (1min)	Final extension (10min)
lasI	F: GTGTTCAAGGAGCGCAAAGG R: AACGGCTGAGTTCCCAGATG	238	94	61.9	72	72
lasR	F: TCGAACATCCGGTCAGCAAA R: GTTCACATTGGCTTCCGAGC	128	94	61.9	72	72
rhlI	F: CCGTTGCGAACGAAATAGCG R:CAGTTCGACCATCCGCAAAC	308	94	61.9	72	72
rhlR	F:TCGCTCCAGACCACCATTTC R: GACGGAGGCTTTTTGCTGTG	284	94	61.9	72	72
ccdA	F: GACAGTTGACAGCGACAGCT R: TCACCAGTCCCTGTTCTCGTC	199	94	58.8	72	72
ccdB	F: GAGAGAGCCGTTATCGTCTGTT R: TCCCCAGAACATCAGGTTAATG	272	94	58.7	72	72
relE	F: GACGAGCGGGCACTAAAGGAAT R: TCAGAGAATGCGTTTGACCG	267	94	58.6	72	72
relB	F: ATGGGTAGCATTAACCTGCGT R: TCAGAGTTCATCCAGCGT	240	94	58.8	72	72
mqsR	F: ACGCACACCACATACACGTT R: GCCTGGGTCTGTAAACATCCT	194	94	58.7	72	72
mazE	F: ATGATCCACAGTAGCGTAAAGCGT R: TTACCAGACTTCCTTATCTTTCGG	249	94	58.7	72	72
mazF	F: ATGGTAAGCCGATACGTACCC R: TGGGGCAACTGTTCCTTT3	288	94	58.5	72	72
hipA	F: CTTGTCACTTGGATGAACAACCAG R: TCACTTACTACCGTATTCTCGGC	1314	94	58.8	72	72
hipB	F: AGCCCAACGCAATTGGCGAATGCA R: CTGTTCTGTTGATTCTGGCGAGGC	225	94	58.7	72	72

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, X^2 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*

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

Antibiotic	N (%)		
	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.

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 QS 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 aeuroginosa 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,

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%)

20). 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 ceftazidime (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. aeuroginosa* (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).

Here, 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. aeuroginosa* and

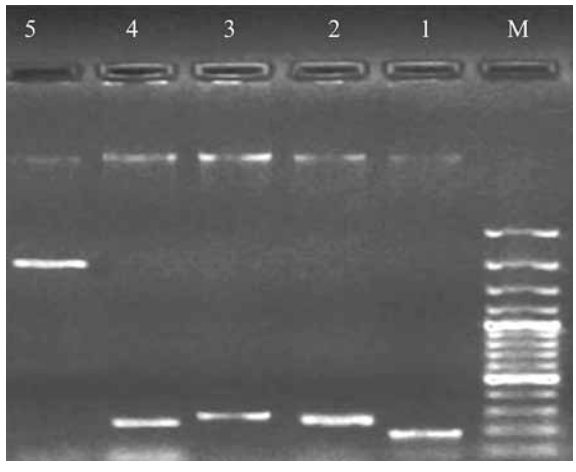

Fig 1. Electrophoresis of PCR product of TA systems genes on the agarose gel 1%.
M: marker 100 bp, 1: *mqsR* gene 194 bp, 2: *ccdB* gene272 bp, 3: *mazF* gene 249 bp, 4: *relE* gene267 bp, 5: *hipA* gene 1314 bp

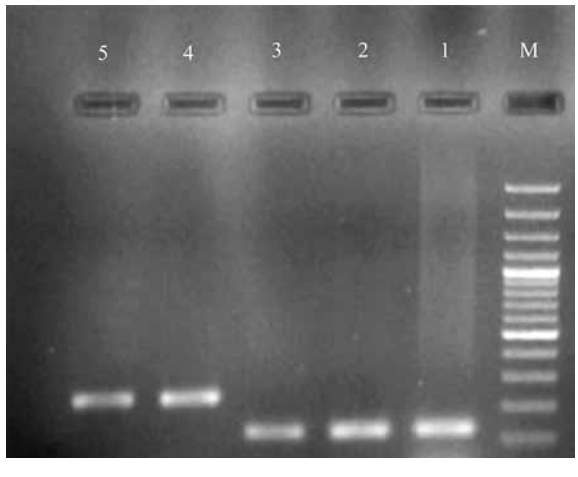

Fig 2. Electrophoresis of PCR products of *lasR* and *lasI* genes on the agarose gel 1%.
M: marker 100 bp, 1: *lasR* gene 128 bp, 2: *lasI* gene238 bp

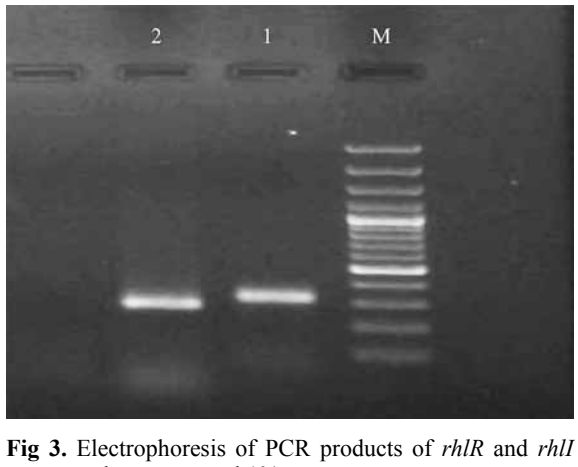

Fig 3. Electrophoresis of PCR products of *rhlR* and *rhlI* genes on the agarose gel 1%.
M: marker 100 bp, 1: *rhlI* gene 308 bp, 2: *rhlR* gene284 bp

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. aeuroginosa* 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 candidutes 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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