Original Article

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Molecular Identification of Quorum Sensing Genes in Clinical Strains of Pseudomonas aeruginosa and Antibiotic Resistance Profile

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ABSTRACT

BACKGROUND AND OBJECTIVE: The expression of most genes that produce Pseudomonas aeruginosa virulence factors is controlled and regulated by a gene system called quorum sensing (QS) system. Quorum sensing is a cell to cell communication system through small signaling molecules in single-celled organisms. This study aims to investigate the frequency of *Pseudomonas aeruginosa lasB, rhlR, rhlI, lasR, lasI, apr* and *rhlAB* genes isolated from clinical samples using multiplex-PCR method and determining the antibiotic resistance profile.

METHODS: In this cross-sectional study, 60 clinical isolates of *Pseudomonas aeruginosa* were collected from patients admitted to Imam Khomeini hospital in Tehran. The antibiotic susceptibility of these isolates against ceftazidime, cefotaxime, amoxicillin, ciprofloxacin, amikacin, gentamicin, imipenem, cefepime, ticarcillin and piperacillin was determined using disk diffusion method. After culturing and final confirmation using biochemical and specific tests, multiplex polymerase chain reaction (Multiplex PCR) was performed to track the intended genes.

FINDINGS: In this study, highest susceptibility was observed to be against ciprofloxacin (81.66%) and ceftazidime (65%). Multiplex PCR demonstrated that the frequency of *rhlR*, *lasR* and *lasI* genes was 5%, 48.3% and 60%, respectively, while *rhlI*, *lasB*, *apr* and *rhlAB* genes could not be identified in any of the strains.

CONCLUSION: Resistance to antibiotics is increasing in *Pseudomonas aeruginosa*, which requires continuous monitoring. QS System plays a key role in pathogenicity of *Pseudomonas aeruginosa*. Identification of these genes enables us to track and identify this bacterium quickly.

KEY WORDS: *Pseudomonas aeruginosa, Quorum sensing, Antibiotic resistance*.

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Introduction

Pseudomonas aeruginosa is a gram-negative non-fermentative bacterium found in soil, water and other humid environments and even on the surface of the human body (1).

This organism is an opportunistic pathogen that rarely causes disease in healthy host, but can be a risk factor for urinary tract infections, respiratory system, inflammation, dermatitis, soft tissue infections, bacteremia (bacteria in the blood), bone and joint infections, gastrointestinal infections and systemic infections, particularly in patients with severe burns, patients with cystic fibrosis, cancer and AIDS, whose immune systems are suppressed (2-4).

This bacterium has several pathogenic factors including exotoxin A, alginate, lipopolysaccharide, pili, lipase phospholipase C, elastase and alkaline protease. Studies show that the expression of most genes that produce *Pseudomonas aeruginosa* virulence factors is controlled and regulated by a gene system called quorum sensing (QS) system.

Quorum sensing is a cell-to-cell communication system through small molecules (SMs) in unicellular organisms, which act through secretion of communicative molecules into the medium and binding to receptor proteins and thus affect transcription and translation and exchange information directly or indirectly (5-7).

The quorum sensing system in this bacterium consists of two gene categories (*LasR-LasI* and *RhlR-RhlI*). *LasI* and *RhlI* genes express acyl-hemoserine lactone (acyl-HSL) enzymes, while *LasR* and *RhlR* genes produce transcriptional regulatory proteins that activate target genes (pathogenic) by binding to their specific signal (8, 9).

The quorum sensing system also exists in other species of *Pseudomonas*. However, the number and sequence of constituent genes are different. Several genes are controlled and expressed by quorum sensing system in *Pseudomonas aeruginosa*.

These genes, which even play a role in pathogenicity of this bacterium, include *lasI*, *lasR*, *rhlI*, *rhlR* and controlled genes of *lasB*, *apr* and *rhlAB* in clinical isolates based on multiplex polymerase chain reaction (10, 11). Biofilm formation is one of the major roles of quorum sensing system. Biofilm is an extracellular polysaccharide that intensifies chronic diseases. It also causes infection relapse, chronicity, lack of penetration of the drug into the extracellular matrix, increased duration of hospitalization, prolonged costs and treatment failure (12). Wound infections caused by biofilm-forming organisms are appropriate sources for development of resistance genes and entrance of organism into blood and causing bloodstream infection (BSI).

According to a study by GhanbarzadehCorehtash et al., of 144 studied strains, 75% contained *exoA* gene and 11.5% contained *nanl* gene. They found that high level of antibiotic resistance of multi-drug resistant *Pseudomonas aeruginosa* (MDRPA) and the ability to form biofilm is an alarm for health system (13).

Therefore, considering the importance of *Pseudomonas aeruginosa* strains in a clinical setting and high level of resistance in these organisms, the present study was conducted for molecular identification of quorum sensing genes in clinical strains of *Pseudomonas aeruginosa* and determination of the antibiotic resistance profile of these strains.

Methods

Bacterial strains collection: In this cross-sectional study, conducted during 6 months in 2016, 60 non-repetitive *Pseudomonas aeruginosa* samples were collected from various samples of blood, urine, respiratory secretions, trachea, bronchoalveolar lavage (BAL), phlegm and burn wound from patients admitted to Imam Khomeini hospital in Tehran and were transferred to microbiology laboratory. In order to identify the organism, standard biochemical and microbiological tests were used including gram stain, catalase, oxidase, simon citrate, production of H2S, growth at 44 °C, pigment production, Triple Sugar Iron (TSI), ornithine decarboxylase, DNase and oxidation-fermentation test (OF). *Pseudomonas aeruginosa* ATCC 27853 was used in this study as positive control.

The antibiotic sensitivity test: The antibiotic sensitivity test was run using disk diffusion method and according to The Clinical & Laboratory Standards Institute (CLSI) on Mueller Hinton culture medium (Merck, Germany).

Lawn culture of bacterial suspension equivalent to 0.5 McFarland was used on Mueller Hinton culture and antibiotic discs (produced by MAST Co., England) containing ceftazidime, cefotaxime, amoxicillin, ciprofloxacin, amikacin, gentamicin, imipenem, cefepime, ticarcillin and piperacillin. After incubation for 18-24 hours at 27 °C, the inhibition zone diameter was measured by caliper and the results were reported

as Resistant (R), Intermediate (I) and Sensitive (S) according to CLSI standards (14).

Polymerase chain reaction: DNA extraction was performed using Boiling method (15). In this method, a loop of bacteria was suspended in TE solution (10 mM tris plus 1 mM EDTA, pH=7.5) and the suspension was boiled for 10 minutes. In the next step, the bacterial cell debris was precipitated by centrifugation. The supernatant containing the DNA was transferred to another vial. DNA concentration and purity was measured by spectrophotometer ($OD_{260.280}$=1.8-2). PCR reaction was performed at a volume of 25 µl.

Each reaction consisted of 2.5 µl 10x buffer, 0.5 µl Mgcl2 at a concentration of 100 mm, 0.5 µl dNTP at a concentration of 20 mm, 1 µl of each primer at a concentration of 10 pmol (table 1), 1 unit of Taq polymerase enzyme and 1 µl genomic DNA, reaching a final volume of 25 µl with double distilled water (Nuclease Free).

Table 1. Sequence of primers used to study quorum sensing genes in *Pseudomonas aeruginosa* (Reference=10,16)

Gene	Sequences	Primer length	Bonding temperature of primer	PCR product size
lasB-F	5′ -TTCTACCCGAAGGACTGATAC- 3′	21	52°C	153
lasB-R	5′ -AACACCCATGATCGCAAC - 3′	18		
aprA-F	5′ -ACCCTGTCCTATTCGTTCC - 3′	19	52°C	140
aprA-R	5′ -GATTGCAGCGACAACTTGG - 3′	19		
rhlAB-F	5′ -TCATGGAATTGTCACAACCGC - 3′	21	52°C	151
rhlAB-R	5′ -ATACGGCAAAATCATGGCAAC - 3′	21		
lasI-F	5′ -CGTGCTCAAGTGTTCAAGG - 3′	19	52°C	607
lasI-R	5′ -TACAGTCGGAAAAGCCCAG- 3′	19		
lasR-F	5′ -AAGTGGAAAATTGGAGTGGAG - 3′	21	52°C	726
lasR-R	5′ -GTAGTTGCCGACGACGATGAAG - 3′	19		
rhlI-F	5′ -TTCATCCTCCTTTAGTCTTCCC - 3′	22	52°C	155
rhlI-R	5′ -TTCCAGCGATTCAGAGAGC - 3′	19		
rhlR-F	5′ -TGCATTTTATCGATCAGGGC - 3′	20	52°C	1661
rhlR-R	5′ -CACTTCCTTTTCCAGGACG- 3′	19		

Polymerase chain reaction was proliferated in cycler thermal device (Eppendorf, Germany) in the initial denaturation temperature (initial denaturation) (at 94°C for 5 minutes), the opening of two strings (at 94°C for 1 minute), bonding of primers at bonding temperature (at 52°C for 1 minute), polymerization (at 72 °C for 90 seconds) and the final elongation phase (at 72 °C for 10 minutes). After PCR reaction, 1.2% agarose gel was used for electrophoresis of PCR products and the gene segments were analyzed using 100 bp DNA size marker (SinaClon, Iran) (10).

Results

The antibiotic resistance profile obtained from disk diffusion method in clinical strains of *Pseudomonas aeruginosa* demonstrated that highest level of resistance belongs to 100% amoxicillin, amikacin and cefepime, while the lowest level belongs to 13.34% ciprofloxacin, 35% ceftazidime (table 2). Investigating this antibiotic resistance profile indicated high resistance of isolated *Pseudomonas aeruginosa* strains against all antibiotics. It was also observed that of 60 studied strains, 39 strains (65%) were simultaneously resistant to more than four antibiotics. Only 8 strains (13.33%) were susceptible to all antibiotics and 7 strains (11.66%) were resistant to all antibiotics. The highest intermediate resistance was found to be against imipenem in 11 strains (18.34%) and cefepime in 8 strains (13.34%). Molecular results demonstrated that of all studied genes, only three genes of *lasI*, *rhlR* and *LasR* were positive in *Pseudomonas aeruginosa* strains. The bond length of amplified region of *lasI*, *LasR* and *rhlR* were 607 bp, 726 bp and 1661 bp, respectively (Fig 1). The highest frequency belonged to *lasI* gene, observed in 36 strains (60%). It was

subsequently observed in *LasR* in 29 strains (48.3%) and *rhlR* in 3 strains (5%). No gene was observed in other strains

Table 2. Susceptibility profile of *Pseudomonas aeruginosa* strains using disk diffusion method

Antibiotic	Resistant (R) N(%)	Intermediate(I) N(%)	Sensitive(S) N(%)
Cefotaxime	53(88.34)	-(-)	7(11.6)
Ceftazidime	21(35)	-(-)	39(65)
Amoxicillin	60(100)	-(-)	-(-)
Ciprofloxacin	8(13.34)	3(5)	49(81.66)
Amikacin	60(100)	-(-)	-(-)
Gentamicin	29(48.34)	4(6.66)	27(45)
Imipenem	43(71.66)	11(18.34)	6(10)
Cefepime	60(100)	-(-)	-(-)
Ticarcillin	54(90)	2(3.34)	5(6.66)
Piperacillin	52(86.66)	2(3.34)	6(10)

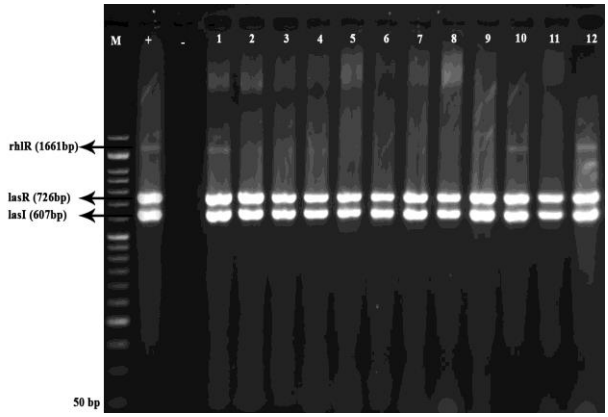

Figure 1. Results of PCR reaction for identification of quorum sensing genes, Row 1. 50 bp marker, Row 2. Positive control, Row 3. Negative control, Rows 1-12. *Pseudomonas aeruginosa* clinical samples containing *lasI* and *LasR* genes with bond length of 607 bp and 726 bp, respectively. Rows 1 to 10 and 12 containing *rhlR* gene with bond length of 1661 bp.

Discussion

According to the results of this study, the assessment of antibiotic susceptibility of 60 *Pseudomonas aeruginosa* strains isolated from the clinical samples demonstrated the level of resistance to various antibiotics: Amikacin, amoxicillin and cefepime (100%), ticarcillin (90%), cefotaxime (88.34%), piperacillin (86.66%), imipenem (71.66%), gentamicin (48.34%), ceftazidime (35%), and ciprofloxacin (13.34%). Investigating the resistance profile of *Pseudomonas aeruginosa* strains isolated from patients of the present study indicates high resistance of these strains against medical antibiotics. Several studies have been conducted so far regarding the drug resistance of this bacterium.

In this study, all *Pseudomonas aeruginosa* strains were resistant to amikacin, amoxicillin and cefepime antibiotics, which is consistent with the study of Kouchaksaraei et al. (17) regarding cefepime antibiotic and is consistent with the studies of Anitha et al. (18) and Hoque et al. (19) regarding amikacin and amoxicillin antibiotics. However, a study in Turkey reported the susceptibility of *Pseudomonas aeruginosa* strains to amikacin to be 73% (20). Studies reported the resistance to ticarcillin to be 22.3% in Saudi Arabia and 93% in Turkey (21).

The resistance to ceftazidime was reported to be 9% in France, 12.3% in Brazil, 26% in Turkey, 4.6% in Japan, 35% in Russia, 12% in Canada, 11.1% in US and 15% in Spain (22-26). The resistance to ticarcillin and ceftazidime was reported to be 90% and 50%, respectively in Kermanshah (27), which is consistent with the present study.

In a study by Nahaei et al., the level of resistance to ceftazidime, gentamicin, ciprofloxacin, amikacin and imipenem was reported to be 69%, 51%, 22%, 15% and 2%, respectively (28). Taghvaee et al. reported the resistance of 108 *Pseudomonas aeruginosa* isolates to ceftazidime (33.3%), imipenem (22.2%), amikacin (20.3%), ciprofloxacin (15.7%) and gentamicin (19.4%) (29). In a similar study, Ranjbar et al. reported the resistance to several antibiotics including ceftazidime (57.5%), amikacin (90%), ciprofloxacin (65%), gentamicin (67.5%) and imipenem(97.5%) (30).

Moreover, a study by Kianpoor et al. demonstrated the resistance to ceftriaxone, amikacin, ceftazidime, ciprofloxacin and imipenem antibiotics to be 82.14%, 57.14%, 53.57%, 42.85% and 14.28% (31). Najafi Moghadam et al. reported the resistance to piperacillin to be 43.75% (32), which was not consistent with the present study. Doosti et al. reported the resistance to piperacillin and cefotaxime to be 44.9% and 91.7%, respectively (33).

Considering the results of previous studies, one can conclude that the resistance of *Pseudomonas aeruginosa* strains to various antibiotics is relatively high; the results of these studies differs according to the time and location of isolation. On the other hand, it is necessary to consider that these resistance patterns are constantly changing. In addition, the frequency of quorum sensing genes (*lasI*, *lasR*, *rhlI*, *rhlR*, *lasB*, *apr* and *rhlAB*) in *Pseudomonas aeruginosa* strains was

analyzed in the present study. Virulence factors of *Pseudomonas aeruginosa* are controlled by a gene system called quorum sensing (QS) system. This system consists of two categories of genes; *LasR-LasI* and *RhlR-RhlI*. *LasI* and *RhlI* genes express acyl-hemoserine lactone (acyl-HSL) enzymes, while *LasR* and *RhlR* genes produce transcriptional regulatory proteins that activate target genes (pathogenic) by binding to their specific signal.

Results of the present study demonstrated that the highest and lowest frequency of the gene belonged to *lasI* in 36 strains (60%) and *rhlR* in 3 strains (5%), respectively. The frequency of *rhlR*, *lasR* and *lasI* genes was 5%, 48.3% and 60%, respectively in all of the studied strains. Of 39 *Pseudomonas aeruginosa* strains with simultaneous resistance to three antibiotics, 36 strains contained *lasI* quorum sensing gene. There was a statistically significant relationship between strains resistant to three antibiotics and *lasI* gene expression. In a study, the frequency of *lasR*, *lasI*, *rhlR* and *laslI* quorum sensing genes was reported to be 5%, 78.3%, 65% and 43.3%, respectively (34). Aghamollaie et al. reported the frequency of *lasI* quorum sensing gene to be 48.5% (35).

In a study by Senturk et al., 4 isolates contained *lasR*, *lasI*, *rhlR* and *rhlI* genes. One of the isolates lacked *lasR* gene and one isolate was reported negative regarding three genes of *lasR*, *lasI* and *rhlR* (36). Deficiency in the production of quorum sensing genes may be due to production of extracellular enzymes, which degrades quorum sensing coding genes (36). Another reason for difference in expression of quorum sensing genes may be mutation events in quorum sensing coding genes. Bjarnsholt et al. found that increased mutation in quorum sensing genes impairs the function of *lasR* and *rhlR* genes (37). The presence of several *Pseudomonas aeruginosa* strains in the site of infection may impair the expression of quorum sensing genes (36). The cause of this difference in the expression of quorum sensing genes and its relationship with drug resistance of *Pseudomonas aeruginosa* strains requires further researches in the future. Due to presence of various pathogenic genes in *Pseudomonas aeruginosa* and considering the key role of quorum sensing genes, the frequency of these genes has not been specified properly and different studies in different countries reveal diverse results. According to the results of the present study, the highest frequency belongs to *lasI* (60%) and *lasR* (48.3%). We can conclude that QS system plays a significant role in pathogenicity of *Pseudomonas aeruginosa*. On the other hand, these genes were mostly observed in clinical isolates that are simultaneously resistant to three antibiotics. Determining a relationship between these genes and drug resistance requires further investigations.

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