Original Article

The correlation between quorum sensing genes (pqsR and lasR) in antibiotic resistance of *Pseudomonas aeruginosa* isolated from burned patients

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Abstract

Background: *Pseudomonas aeruginosa*, a gram-negative bacillus, and opportunistic pathogen, is an important microorganism involved with infections in burn patients worldwide. It produces biofilms by Quorum-sensing signals and makes an antibiotic resistance. **Materials and Methods:** From April to September 2018, 100 samples of burn injuries were collected from the Central Hospital of Shahid Beheshti in Kashan. The samples were identified in terms of biochemical and phenotypic tests and a definitive diagnosis of P.aeruginosa species was examined based on a toxA gene by the PCR method. The positive samples were analyzed for antibiotics of amikacin, ciprofloxacin, norfloxacin, gentamicin, cefepime, aztreonam, meropenem, ceftazidime, colistin, and piperacillin-tazobactam. Then, samples were analyzed for lasR and pqsR (quorum-sensing genes) by PCR. **Results:** We verified eighty-five (85%) isolates as *P. aeruginosa*. According to antibiograms, 92% of the isolates were considered as multidrug-resistant (MDR), of which 85.5% were extensively drug-resistant (XDR) and none of the pan drug resistance (PDR). Also, in MDR isolates, there was one nonsense mutation. In XDR samples, two isolates had a missense mutation and nonsense mutation was seen in one strain. **Conclusion:** The results of our study show that with increasing resistance rates, more mutations occur in lasR and pqsR genes and the possibly can play a key role in antibiotic resistance. Given the mutations found in the quorum sensing genes, it can be concluded that these genes are mutagenic genes that will be effective in changing bacterial behavior and adaptability to environmental conditions.

Keywords: Quorum sensing, Antibiotic resistance, Pseudomonas aeruginosa, Burned patients

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Introduction

Pseudomonas aeruginosa is an opportunistic gram-negative non-fermenting bacillus of Pseudomonadaceae family. It was first isolated from green pus in 1882 [1]. It is one the most important nosocomial pathogens causing infections in the respiratory tract, blood, urinary tract, ear, skin, soft tissues, eye, central nervous system, heart, bone, joint and gastrointestinal tract [2]. *P. aeruginosa* is one of the most common bacteria involved in hospital infections, especially in burns [2]. This bacterium can be transmitted through flora contamination, presence at different levels of the hospital, such as hospital types of equipment, disinfectant solutions, and nurses' hands [3, 4]. Multiple drug resistance to anti-pseudomonas antibiotics is a common and growing problem in most hospitals. MDR strains of *P.*

aeruginosa cause about 4 to 60 percent of hospital-acquired infections in different parts of the world [5,6].

Multidrug-resistant (MDR) was defined as acquired nonsusceptibility to at least one agent in three or more antimicrobial categories. Extensively drug-resistant (XDR) was defined as nonsusceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e., bacterial isolates remain susceptible to only one or two antimicrobial categories). Pandrug resistance (PDR) was defined as nonsusceptibility to all agents in all antimicrobial categories [7].

The process of coordination and intercellular communication, which involves the production of extracellular and emitting molecules (self-inductors), is followed by the regulation of expression of genes, is called quorum sensing [8]. Since the quorum sensing process is involved in regulating the expression of virulence genes, it has been an appropriate target for the development of anti-quorum sensing therapy independent of the use of antibiotics [8,9].

The quorum sensing (QS) in *P. aeruginosa* is composed of rhl, pqs, and las genes, which coding for transcription regulators Rhl (RhlI, RhlR), Las (LasI, LasR), PqsR, and that self-inducing molecule of these regulators [8,9]. Among quorum sensing genes, the sensitivity of the two genes lasR and pqsR is more important, [9]. The lasR gene produces a protein called LasR that is involved in the expression of both las and rhl genes. Therefore, the gene which produces this protein (lasR gene) was selected as the most pivotal factor in regulating the expression of two quorum sensing genes [9-11].

After identifying the role of quorum sensing in various vital processes, including expression of virulence factors and the development of resistant bacterial infections, and the spread of antibiotic resistance, the idea is to use this system to design new targeted drugs [12,13].

This study sought to determine the possible changes of lasR and pqsR genes in resistant and sensitive strains of *P. aeruginosa* to the standard strain. The significance of this study is to determine the molecular relationship between quorum sensing and antibiotic resistance in helping designing new methods to defeat antibiotic resistance.

Methods

Identification of P. aeruginosa. Our study protocol was permitted by the Ethics Committee of the Kashan University of Medical Sciences. In this cross-sectional study, 100 suspected cases of *P. aeruginosa* belonging to burn patients from the microbiological department of Shahid Beheshti hospital in Kashan were received in 6 months. They were re-identified after being transferred to the lab. At first, diagnostic tests including hot dyeing, oxidase, Triple Sugar Iron Agar (TSI), SIM (Sulfide, Indole, Motility), Oxidative fermentative (OF) test, Gelatinase, as well as a culture on citrated citrate and incubation at 42 ° C were used [14].

DNA extraction and Molecular identification. The DNA from a clinical isolated and standard strain of *P. aeruginosa* ATCC27853 was extracted by the boiling method. The purity and concentration of extracted DNAs were checked by UV spectrophotometric method, the concentration and purity of the DNA sample were measured by optical absorption at wavelengths of 260 nm and 280 are obtained. For final confirmation, all samples were examined by toxA primers with the expected product length of this gene was 297 bp as a specific gene for P.aeruginosa (Table 1).

Table1. PCR primer used in this study

Pd (II) complex (µM)	Sequences (5´-3´)	Amplicon Size (bp)	References
toxA	F: TTCCCAGGTATCGTCGAGGT R: GTAACCAGCTCAGCCACAT	297	This study
lasR	F: CTATGGCCTTGGTTGACGGT R: CAGGACCGACTCCATGAAA	447	This study
pqsR	F: TGGTTTTCTACGACGTGCGA R: CAATGGATGTCCCGTCTCA	433	This study

In this experiment, the standard strain of *P. aeruginosa* ATCC27853 was used as a positive control and sterile distilled water for negative control.

Antibiotic susceptibility testing. The antibiotic susceptibility test was done in terms of the Kirby-Bauer disc diffusion technique at turbidity of 0.5 McFarland standards on Mueller-Hinton agar (Himedia Co., India) plates regarding the Clinical and Laboratory Standards Institute guidelines (CLSI 2018 edition) (15). The antibiotic disks used (Himedia Co., India) include ciprofloxacin (5 µg), amikacin (30 µg), norfloxacin (5 µg), gentamicin (10 µg), cefepime (10 µg), aztreonam (30 µg), meropenem (10 µg), ceftazidime (30 µg), colistin (10 µg), and piperacillin/tazobactam (36 µg).

Polymerase chain reaction for quorum sensing genes. The sequences of the lasR and pqsR genes was extracted from the National Center for Biotechnology Information (NCBI) database and primer were designed for each gene. The expected length product of the lasR gene was 447bp, and the expected length product of the pqsR was 433 bp (Table 1). The designed primers were synthesized by Metabion Company, Germany. For doing of PCR reaction, each reaction was made in a volume of 20 µL, which contained Master Mix 10 µL (Danish Amplicon), 1 µL Forward Primer (10 pmol / µL), 1 µL Turner Primer (10 pmol / µL), 2 µL DNA Sample and 6 µL distilled water. After preparing the samples, the PCR reaction was performed. In this experiment, the standard strain of P.aeruginosa ATCC27853 was used as a positive control and sterile distilled water for blank control. Finally, the PCR products were examined using the 2% agarose gel electrophoresis and stained with SYBR Safe DNA and visualized and pictured through a transilluminator (UVITEC Alliance 4.7, Bio-Active., Ltd., Bangkok, Thailand).

Sequencing of the lasR and pqsR genes. Data were expressed as mean standard deviation (S.D). To compare variables between diabetic patients, we used paired t-test analysis. A $P<0.05$ was considered statistically significant.

Results

In this study, out of 100 samples, we verified eighty-five (85%) isolates as *P. aeruginosa*. To confirm 85 P.aeruginosa strains, all samples were tested for the presence of toxA gene. This gene was present in all strains. (Figure1).

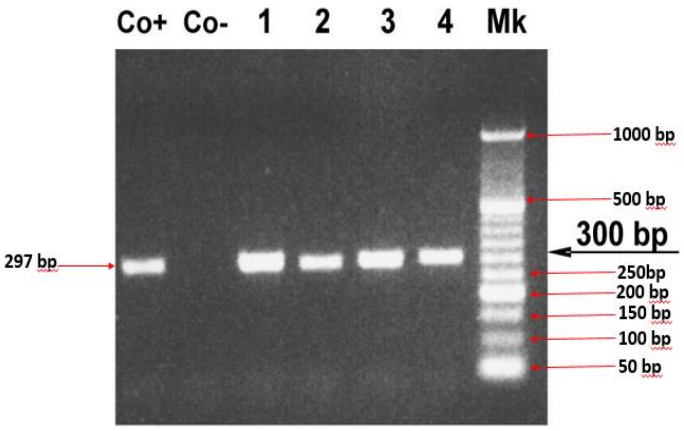

Figure 1: The agarose gel electrophoresis showing the amplified PCR product of b *toxA* gene (297 bp) in S1-S4 indicating the intact DNA of the corresponding specimens. CO-: negative control; Co+: Positive control,MK: marker.

The highest resistance was observed for amikacin (90.8%) and norfloxacin (90%) and the least resistance to colistin (5%). Resistance to other antibiotics included ciprofloxacin 89%, aztreonam 86.5%, meropenem 87.5%, ceftazidime 60.5%, gentamicin 80.3%, cefepime 80%, and piperacillin-tazobactam 79.2%. According to antibiograms, seventy-seven (92 %) of the isolates were considered as MDR, of which sixty-eight (85.5%) isolates were XDR and there were no PDR isolates in our report (Table 2).

Table 2. The used antibiotics in our report in clinical isolates of *Pseudomonas aeruginosa*

Antibiotic (content µg)	Sequences (5´-3´)	Amplicon Size (bp)
Amikacin (30 µg)	Aminoglycosides	90.8
Gentamicin (10 µg)	Aminoglycosides	80.3
Aztreonam (30 µg)	Monobactams	86.5
Ceftazidime (30 µg)	Third-generation cephalosporins	60.5
Piperacillin/tazobactam (36 µg)	Beta lactam - beta lactamase inhibitor (anti-pseudomonal)	79.2
Meropenem (10 µg)	Carbapenems	87.5
Cefepime (10 µg)	Fourth-generation cephalosporins	80
Ciprofloxacin (5 µg)	Fluoroquinolones	89
Norfloxacin (5 µg)	Fluoroquinolones	90
Colistin (10 µg)	Polymixin	5

*WHO releases the 2019 AWaRe Classification Antibiotics

The PCR products of lasR and pqsR genes are displayed in Figure 2.

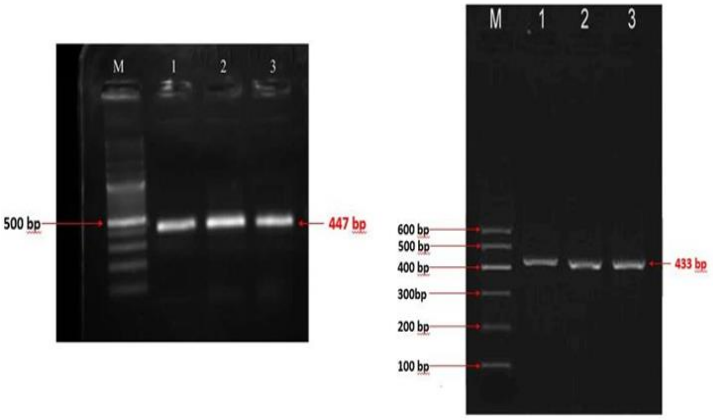

Figure 2: The agarose gel electrophoresis showing the amplified PCR product of the b *lasR* gene (447 bp) in S1-S3 in the left side *pqsR* gene (433 bp) in S1-S3 in the right side of figure.

The data from the sequencing were extracted by Chromas software. The studies on the quality of the sequence were then. Finally, the data were aligned with the NCBI database and the Clustal Omega site with reference strain, and mutations in each gene were determined. The amino acid sequences of the strains were then compared with the reference strain and the amino acids altered in the mutated sites were determined. The mutations found are shown in Table 3.

Table 3. The types of genetic mutations in the study and their status of antibiotic resistance category

Positive sample number	Antibiotic resistance category	lasR Mutation	pqsR Mutation
Sample 1	XDR	Without Mutation	Silent, Nonsense
Sample 2	XDR	Silent, Missense	Without Mutation
Sample 3	XDR	Without Mutation	Silent, Missense
Sample 6	XDR	Without Mutation	Silent, Nonsense
Sample 4	MDR	Without Mutation	Silent
Sample 5	MDR	Silent	Silent
Sample 7	MDR	Without Mutation	Without Mutation

Discussion

The antibiotics used to treat *P. aeruginosa* infection include ciprofloxacin, tobramycin, ceftazidime, gentamicin, and imipenem [16]. *P. aeruginosa* bacteria present a high degree of resistance to these antibiotics [16]. Interestingly, the response to ciprofloxacin is very effective at the beginning of the treatment, but high-level resistance is rapidly acquired by *P. aeruginosa*, making the treatment ineffective in the 30% of strains obtained from clinical isolates [16, 17].

The mechanisms that could increase *P. aeruginosa* antibiotic susceptibility still remain unclear [16]. The occurrence of MDR bacteria has motivated studies for weakening virulence differently through quorum sensing inhibition procedures instead of bactericidal and bacteriostatic strategies [17]. *P. aeruginosa* is considered one of the most predominant colonizers of burns [18]. It is recognized among the most critical respiratory bacterial pathogens causing substantial morbidity and mortality; to combat this MDR organism unique therapeutic approaches are required [19, 20]. *P. aeruginosa* possesses at least two well-defined, interrelated quorum sensing systems, las , and rhl, that control the production of different virulence factors, including elastases (LasB and LasA), alkaline protease, hydrogen cyanide, exotoxin A, pyocyanin, lectins, rhamnolipids, and superoxide dismutase. Each quorum sensing system consists of two components, the autoinducer synthases (LasI and RhlI, respectively) and their cognate transcriptional regulators (LasR and RhlR, respectively) [21-25].

There is a confidential record that mutations in quorum sensing systems can intrude with the ability of *P. aeruginosa* to make well known and localized harm in wound infections of burn patients [26]. This contamination can lead to graft loss, prolonged clinic stays, systemic sepsis, or even multiplied mortality in burn units [28]; Also, prolonged antibiotic therapy suppressed normal flora [29]. Now, mutations in quorum sensing genes of *P. aeruginosa* caused it to lose its pathogenic potential in comparison to wild type strains [27]. The lasR gene encodes a protein that

is essential for the initiation of quorum sensing response involved in biofilm formation [22,23]. However, *P. aeruginosa* strains with mutations in lasR have been predominately isolated from infections and emerged in the course of in vitro evolution [22-25]. In comparison, we identified five silent and one missense mutations in the lasR gene sequence, however, 5 of the medical isolates have been devoid of any mutation, signifying that other mechanisms are possibly concerned. It appears that the loss of quorum sensing regulation because of mutations in the key quorum-sensing regulator, lasR, is especially common among patients with cystic fibrosis [30]. Basically, *P. aeruginosa* acute virulence in diverse version hosts may be reduced as a result of the inactivation of lasR, whereas the loss of lasR characteristic may constitute a marker of early-stage chronic contamination of the cystic fibrosis airway with clinical implications for antibiotic resistance and disease progression [31]. In the present study, silent mutations prevailed over the other types of mutations. On the other hand, it's far strongly meant that the inactivation of lasR might be associated with conferring resistance to antibiotics [32], even as we located the best one missense mutation with a detrimental effect on the protein sequence. The sequences assessed here have been absolute proof against antibiotics so that 3 of them have been MDR and the others had been XDR. López-Causapé et al. stated the principle antibiotic resistance mutations among which, P117G become dominant [33], however, it should be noted that silent mutations in role L36 occurred in two medical isolates. Bottomley et al. stated the atomic interactions between protein LasR and its autoinducer. The amino acid residues concerned in these interactions blanketed Tyrosine-56, Arginine-61, Aspartate-73, Threonine-115, and Serine-129. These residues can simultaneously cause protein folding, leading to the dimerization of LasR, and therefore allowing for DNA binding to the promoter and consequent transcriptional activation of QS-managed genes. Furthermore, Bjarnsholt et al. reported that mutations in Tyrosine-56 and Threonine-75 within the lasR protein would impair autoinducer binding due to the fact they strongly have interaction with the autoinducer [34]. In comparison, we did no longer find any mutation in this location, but we found the missense mutation in location Arginine 180 (R180Q). Compared to this missense mutation, silent mutations had been determined in positions L36, A121, N209, and N214. It needs to be cited that several transcriptional regulators, which belong to QS, were expressed at a higher stage in PAO1 than in ATCC 27853[35]. That is why many studies make comparisons with reference strain PAO1. In a study, the importance of Gln194 or Tyr258 for pqsR feature turned into assessed by way of building full-duration pqsR mutants with mutations at either mentioned positions. The outcomes of this research indicated that even though Q194E retained at the least 88% capability, this mutant became definitely inactive. On the other hand, a hydrophobic amino acid at position 258 is needed for pqsR functionality because Y258A mutation renders pqsR inactive [36]. No mutations at positions 194 and 258 were visible in our study. As indicated by using previous research, the mutation of pqsR ends up within the termination of phnAB and pqsABCDE expression, implying pqsR is critical for *P. aeruginosa* quorum-sensing sign transduction. Apart from the missense mutation, there has been a nonsense mutation at position 259(E259-), which was repeated in isolates. Since the PROVEAN net server cannot determine the effect of nonsense mutations, there is a consensus about nonsense mutations that have an extra dramatic change than missense mutations and result in a premature forestall codon, produce truncated and generally nonfunctional proteins [35]. Furthermore, these nonsense mutations are much more likely to have pathogenic consequences due to disrupting protein structure [37].

Conclusion

The results of our study show that with increasing resistance rates, more mutations occur in the quorum sensing genes. Three of the XDR samples and one of the MDR specimens were effective in mutation. Also, the rate of mutation in the pqsR gene was greater than the lasR gene.

Given the mutations found in quorum sensing genes, it can be concluded that these genes are mutagenic genes that will be effective in changing bacterial behavior and adaptability to environmental

conditions. Also, the higher mutation rates in XDR strains indicate the effect of antibiotics on mutation in quorum sensing genes. Our study aimed to better understand the relationship between quorum sensing and antibiotic resistance, and to explore a new path to this problem, and to pave the way for designing more appropriate strategies in this regard.

Conflicts of Interest

It is declared by the authors that there are no competing interests.

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