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Relation between Expression of the las Quorum-Sensing System in Clinical Isolates of *Pseudomonas aeruginosa* and Expression of Efflux Pump and ampC

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ABSTRACT

Background: Quorum-sensing systems regulate expression of several virulence factors in *Pseudomonas aeruginosa*. This study investigated the relation between expression of the *las* quorum-sensing system and expression of *mexY* and *ampC* in 35 clinical isolates of *P. aeruginosa*.

Methods: Antibiotic susceptibility was determined by the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Expression of genes including *lasI*, *lasR*, *mexY*, *ampC* and *ampR* was assessed by real time RT-PCR.

Results: Twenty eight isolates with elevated expression of *mexY* and *ampC* (compared to *P. aeruginosa* PAO1) were resistance to ceftazidime, imipenem, ciprofloxacin and amikacin. Also these isolates demonstrated increased expression of *lasI* and *lasR*. The remaining seven isolates showed intermediate resistance to ciprofloxacin and amikacin. These seven isolates with elevated *ampR* expression demonstrated decreased expression of *mexY*, *ampC*, *lasI* and *lasR*.

Conclusion: In contrast to previous study, current study demonstrated that the expression pattern of *ampC* was identical to the *lasI* expression pattern among clinical isolates. Furthermore according to previous study we expected that AmpR positively regulated *ampC* expression but in our study, some of the isolates with elevated *ampR* expression showed decreased *ampC* expression. The expression pattern of *lasR* and *mexY* was identical in all of the isolates. It seems there was a direct or indirect relation between expression of *lasR* and *mexY* expression in *P. aeruginosa*.

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Introduction

Pseudomonas aeruginosa is a common nosocomial pathogen (1, 2) that causes infections with a high mortality rate (3). *P. aeruginosa* has intrinsic resistance to many antimicrobial agents, and only a few antimicrobial agents show potent antibacterial activity against this bacterium. The emergence of multidrug-resistant (MDR) *P. aeruginosa* strains is a serious problem (4, 5). Nosocomial outbreaks of *P. aeruginosa* infection, particularly by MDR strains, have become problematic in hospitals in various countries (6-8). Numerous studies point to a link between multidrug resistance and increased morbidity / mortality, as well as increased length of hospital stay and increased hospital costs (9, 10).

P. aeruginosa typically carries AmpC, a chromosomal β-lactamase gene, of the class C cephalosporinase. AmpC is inducible by a number of β-lactam antibiotics. Reports show that the clinical isolates of *P. aeruginosa* produce AmpC variants with improved activity against antibiotics. These antibiotics include oxyiminocephalosporins, cefepime, and carbapenems, and the isolates are referred to harbor extended-spectrum AmpC (11). In the presence of β-lactams, AmpR is the positive regulator of ampC expression in *P. aeruginosa*. AmpR is a global regulator and is involved in antibiotic resistance and the expression of many virulence factors. This regulator is a DNA-binding protein that belongs to the LysR family of regulatory proteins. AmpR, also increases the expression of *lasB* and *rhlR*, and decreases *lasA*, *lasI*, *lasR* and pyocyanin expression. AmpR may act indirectly via quorum-sensing systems (12). Balasubramanian *et al* (13) stated that AmpR is involved in regulation of MexEF-OprN and MexAB-OprM efflux pumps. The mechanisms of the above mentioned regulation have not clarified exactly with details yet.

Multidrug resistant *P. aeruginosa* clinical isolates have often been reported to be MexXY overproducers (14). The MexXY-OprM is able to eject cefepime, cefotaxime, levofloxacin, ciprofloxacin, amikacin, gentamicin, tobramycin, erythromycin, tetracycline and meropenem (15). The MexXY system is intriguing in that it is a significant determinant of aminoglycoside resistance only in *P. aeruginosa* (16).

The ability of bacteria to communicate across species via autoinducers has been termed quorum-sensing (QS) (17). The transcriptional regulation of many virulence genes is controlled by two *N-acyl homoserine lactone* (AHL)-dependent QS systems called LasI/R and RhlI/R (18). In the LasI/R system, LasI directs the synthesis of *N-(3-oxododecanoyl)-homoserine lactone* (3-oxo-C_{12}-HSL), which binds and activates the cognate response regulator LasR, resulting in the regulation of target gene expression. Work of the past few years showed that the quorum sensing circuitry that is operating in *P. aeruginosa* is essential for the expression of virulence factors like elastase (lasB) and exotoxinA (toxA) and it has been speculated that quorum sensing dependent regulation of pathogenic traits is a highly effective strategy to establish an infection. The las system was shown to be involved in the regulation of various virulence factors, as well as lasI itself,

thereby creating a positive regulatory feedback loop (19).

Therefore, alternative mechanisms for targeting *P. aeruginosa* have been the focus of much research. Therapeutics that target and inhibit QS in *P. aeruginosa* would attenuate the virulence of the bacterium and thus potentially assist the host immune response in clearing the infection. QS is an attractive therapeutic target because of the role that it plays in the global regulation of multiple *P. aeruginosa* factors and the importance of this role for the virulence of the organism in multiple different infections. Also inhibition or suppression of a gene or protein which is involved in regulation of QS, ampC and efflux pumps, is an appropriate therapeutic target (19).

While manipulating laboratory-derived isogenic mutants is crucial for understanding the regulatory systems in *P. aeruginosa*, it is also important to examine these systems in clinical isolates to determine the applicability of laboratory findings.

The goal of the present study was to identify relation between expressions of *lasI* and *lasR* (las quorum sensing system) and expression of *mexY* and *ampC* in clinical isolates of *P. aeruginosa*. Also the expression of ampR as a global regulator was assessed. To address this, the expression level of the above mentioned genes was evaluated by real-time reverse transcriptase PCR (RT-PCR) method.

Materials and Methods

Bacterial isolates and antimicrobial susceptibility test

A total of 35 imipenem-resistant isolates of *P. aeruginosa* were evaluated. These isolates were consecutively collected in the five months of 2012 from wound infection of burned patients hospitalized at Motahari burn hospital located in Tehran, Iran. Only a single bacterial isolate per patient was evaluated. We determined antimicrobial susceptibility for ceftazidime (30µg), imipenem (10µg), ciprofloxacin (5µg) and amikacin (30µg) (MAST, Bootle, Merseyside, UK) were determined by disc diffusion method. We interpreted the disc diffusion results according to Clinical and Laboratory Standards Institute guideline (20). *P. aeruginosa* PAO1 strain was used as a quality control strain.

Quantification of gene expression by real-time RT-PCR.

The expression level of *lasI*, *lasR*, *ampC*, *mexY* and *ampR* was determined by real-time RT-PCR according to previously described protocols (21, 22). Briefly, strains were grown in 10 ml of LB broth (Oxoid, Basingstoke, UK) at 37°C with shaking at 180 rpm to the late log phase (optical density at 600 nm [OD_{600}] of 1) and collected by centrifugation. RNA extraction was performed with the high pure RNA isolation kit (Roche, Penzberg, Germany) following the manufacturer's protocols.

Five µg of total RNA was used for cDNA synthesis by prime script RT reagent kit (Takara Bio Inc., Otsu, Shiga, Japan) with random hexamers as a primer. The cDNAs were stored at −20°C until further analysis. A Rotor gene Q real-time PCR machine (Qiagen) was used for the quantification of cDNA.

Real-time RT-PCR experiments were performed using the SYBR Premix Ex Taq

II (Tli RNaseH Plus) kit (Takara Bio Inc., Otsu, Shiga, Japan) according to manufacturer's instructions. The final volume of the reaction mixture was adjusted to 25 µL. Two microliters of water was used as a substitute for template DNA to serve as a nontemplate control. Samples were run in triplicate; virtually all individual results were within 0.5 Ct units of the averaged triplicate value. The housekeeping gene *rpoD* was used as the normalizing gene. Primers were used for the amplification of *lasI*, *lasR*, *ampC*, *mexY*, *ampR* and *rpoD* (*rpoD* was used as a reference gene to normalize the relative amount of mRNA) were showed in Table I. Expression level of *lasI*, *lasR*, *ampC*, *mexY* and *ampR* genes for clinical isolates of *P. aeruginosa* were compared with *P. aeruginosa* PAO1.

Reverse transcription was performed at 37°C for 15 min. The cycling conditions for the Real-time PCR were: 95°C for 30 sec (holding) and 40 cycles of 95°C for 5 sec, 60°C for 45 sec (cycling). After 40 cycles, a melting curve using SYBR Green was determined with a ramp speed of 1°C per 5 second at 65°C-95°C. Primer concentrations were adjusted to provide amplification efficiencies of ~90-110% for all experiments. Primer dimers and other artifacts were evaluated by melting curve analysis and eventually only dimer- and artifact-free reactions were considered valid. A melt curve was run at the end of 40 cycles to test for the presence of a unique PCR reaction product.

The threshold cycle of each sample, which identified the cycle number during PCR when fluorescence exceeded a threshold value, was determined by the software, and the relative expression of target genes was assessed using the relative quantification method as described previously (23). The results represented relative expression levels (fold change) for target genes in the tested isolates compared to the PAO1 wild-type strain *(Figures 1-4).*

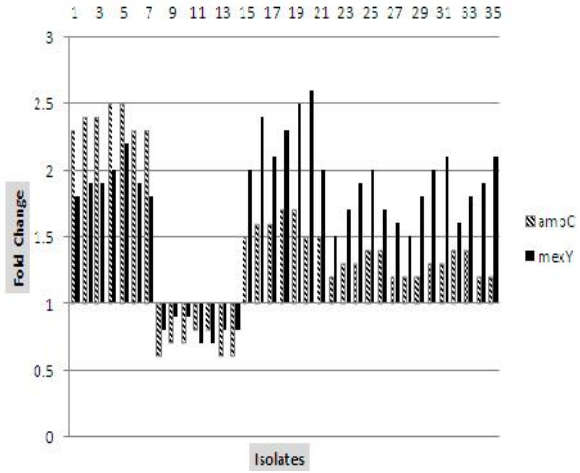

Figure 1. *mexY* and *ampC* expression in clinical isolates. Twenty eight isolates had increased *mexY* and *ampC* expression and seven isolates had decreased *mexY* and *ampC* expression in comparison with *P. aeruginosa* PAO1

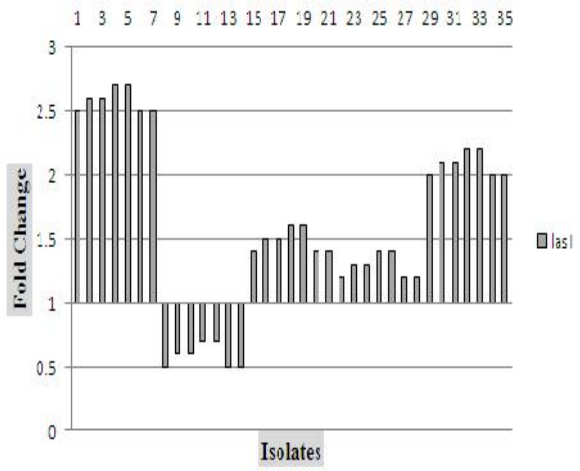

Figure 2. *lasI* expression in clinical isolates. Twenty eight isolates had increased *lasI* expression and seven isolates had decreased *lasI* expression in comparison with *P. aeruginosa* PAO1.

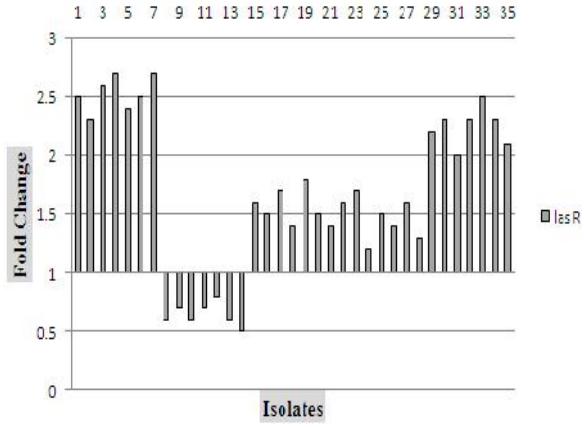

Figure 3. *lasR* expression in clinical isolates. Twenty eight isolates had increased *lasR* expression and seven isolates had decreased *lasR* expression in comparison with *P. aeruginosa* PAO1

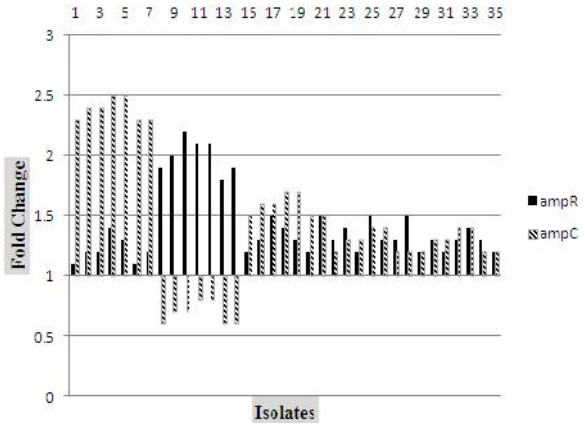

Figure 4. *ampR* and *ampC* expression in clinical isolates. Twenty eight isolates had increased *ampR* and *ampC* expression in comparison with *P. aeruginosa* PAO1. The rest of the isolates had increased *ampR* expression while they showed decreased *ampC* expression

Results

The research was performed on 35 imipenem-resistant isolates of *P. aeruginosa* were collected from burn wound infections during the five months of 2012 in Motahari hospital in Tehran, Iran.

The susceptibility data and relative expression of *lasI*, *lasR*, *ampC*, *mexY* and *ampR* of the 35 clinical isolates are presented in Table 2 and Figures 1-4.

28 isolates (80%) had increased expression of *mexY* and *ampC* (compared to *P. aeruginosa* PAO1) and they were resistance to all examined antibiotics including ceftazidime, imipenem, ciprofloxacin and amikacin. These 28 isolates demonstrated increased *lasI* and *lasR* expression in comparison with *P. aeruginosa* PAO1. The remaining seven isolates with intermediate resistance to ciprofloxacin and amikacin showed decreased *mexY* and *ampC* expression. Also these seven isolates had decreased *lasI* and *lasR* expression. In these seven isolates, the mean expression level of *lasI*, *lasR*, *ampC* and *mexY* was 58%, 64%, 68% and 80% of *P. aeruginosa* PAO1 respectively. The expression level of *lasI* was similar to the expression level of *lasR* in all of the isolates. Most of the isolates demonstrated low level increase in the expression of *ampR* in comparison with *P. aeruginosa* PAO1.

Discussion

In the last decades, the emergence of multi-drug resistant *P. aeruginosa* has been observed worldwide. Some of the antimicrobial agents have become less effective against these organisms reducing the available therapeutic options for treatment of these infections. Therefore, alternative mechanisms for targeting *P. aeruginosa* have been the focus of much research. Therapeutics that target and inhibit quorum sensing in *P. aeruginosa* would attenuate the virulence of the bacterium and

thus potentially assist the host immune response in clearing the infection.

Overall, there was a relation between *mexY* and *ampC* expression and antibiotic susceptibility in studied clinical isolates. Also there was a relation between expression of *lasI and lasR* (*las* quorum sensing system) and expression of *mexY* and *ampC*. In addition, there was no relation between *ampR* expression and expression of *lasR*, *mexY* and *ampC* in some of the isolates *(Table 2 and Figures 1-4).*

The MexXY-OprM efflux system and the AmpC chromosomal cephalosporinase are the important contributor to intrinsic antimicrobial resistance in *P. aeruginosa* (11, 16). The MexXY-OprM is able to eject ciprofloxacin and amikacin (15).

According to our results, Of the 35 isolates, 28 with elevated *mexY* expression (from 1.5- to 2.6- fold compared to *P. aeruginosa* PAO1) showed resistance to ciprofloxacin and amikacin but seven isolates with decreased *mexY* expression (from 0.7- to 0.9- fold compared to *P. aeruginosa* PAO1) demonstrated intermediate resistance to ciprofloxacin and amikacin. Thus the expression level of *mexY* had impact on antibiotic susceptibility among clinical isolates in this study. Also expression pattern of *ampC* gene was similar to *mexY* expression pattern among all of the clinical isolates *(Figure 1)*. Therefore the expression of *mexY* and *ampC* associated with antibiotic susceptibility.

Swada *et al* (24) showed that *rhl* quorum-sensing system has been associated with increased expression of MexAB-OprM efflux system in laboratory-derived strains. However Bratu *et al* (25) stated that no association was found between the expression of two quorum-sensing systems and mexAB-oprM expression (or antimicrobial resistance) among clinical strains. Thus, it has not been clearly established a correlation between expression of an efflux-encoding gene and quorum-sensing systems. Also it has not been researched the relation between quorum-sensing and MexXY-OprM efflux pump.

According to results of present study, the expression pattern of *lasI*, *lasR* and *mexY* was identical for each isolate. 28 of isolates showed increased *lasI*. *lasR* and *mexY* expression and 7 isolates demonstrated decreased *lasI*, *lasR* and *mexY* expression *(Figures 1-3)*. Suggesting that, there is a relation between expression of *lasI* and *lasR* (*las* quorum-sensing system) and *mexY* expression.

AmpR is a global regulator of β-lactamase, quorum-sensing and some virulence factors. Thus, AmpR plays a dual role, positively regulating the *ampC* expression levels and negatively regulating the *lasI* expression levels. Therefore in *P. aeruginosa*, the *ampC* and *lasI* genes are inversely transcribed in laboratory-derived strains (12). In contrast, our results demonstrated that the expression pattern of *ampC* was identical to the *lasI* expression pattern among clinical isolates. For each isolate, the expression level of both of these genes had identically increased or decreased *(Figures 1, 2)*. In the present study, the results showed increased low level expression of *ampR* in isolates which had increased *ampC* expression (80% of isolates). Also the remaining isolates (20%) with increased *ampR* expression, showed decreased *ampC* expression *(Figure 4)*.

suggesting, in addition to AmpR as a global regulator, other factors were involved in regulation of these genes.

Conclusion

In conclusion, the expression pattern of *mexY* and *ampC* was identical and associated with antibiotic susceptibility. Also our results showed relation between expression of *lasI* and *lasR* (*las* quorum-sensing system) and expression of *mexY*. In contrast to previous study, current study demonstrated that the expression of *ampC* was identical to the *lasI* expression for each isolate. In other words *ampC* and *lasI* genes were not inversely transcribed. Also according to previous study we had expected that AmpR positively regulated *ampC* expression but some of the isolates with elevated *ampR* expression showed decreased *ampC* expression. Therefore further studies on clinical isolates should be performed to understand the complex regulatory network linking all these critical factors which are important in resistance to antibiotics and producing virulence factors.

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Conflict of Interest

None declared conflicts of interest.

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