Effect of Subinhibitory concentrations of imipenem and piperacillin on transcriptional expression of *algD* and *lasB* genes in *Pseudomonas aeruginosa*

Adeleh Attar[1], Hesam Mirshahabi[1*], Fakhri Haghi[1], Habib Zeighami[1]

1. Department of Microbiology and Virology, Zanjan University of Medical Sciences, Zanjan, Iran.

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ABSTRACT

Sub Minimal Inhibitory concentrations (sub-MIC) of antibiotics, although not able to kill bacteria, can be effect on their physico- chemical characteristics and functions. This study aimed to investigate the effect of sub-MIC of imipenem and piperacillin on the transcriptional expression of virulence related genes *algD* and *lasB* in *Pseudomonas auroginosa*. Five clinical isolates of *P. aeruginosa* were screened for the presence of *algD* and *lasB* genes and minimum inhibitory concentration (MIC) of imipenem and piperacillin was determined using tube dilution method. The expression level of *algD* and *lasB* at sub-MIC concentrations of antibiotics was measured by real-time PCR. Our results showed that the expression of the *lasB* strongly decreased at all sub-MIC of piperacillin, especially at concentrations 0.125 and 2 µg/ml (*P*< 0.05). Whereas, a slight increase of the *algD* expression was measured at concentrations 16, 4, 2, 1, 0.25 and 0.125 µg/ml of piperacillin. At subMIC of imipenem, the *algD* expression was decreased 0.3 to 1.6 fold and the expression of *lasB* was decreased at concentrations of 0.25, 2, 4 and 8 µg/ml. However, imipenem had no significant influence on *algD* and *lasB* expression. Further studies will be required in order to assess whether sub-MIC of piperacillin can be improve the outcomes of severe and serious infections caused by *P. aeruginosa*.

1. Introduction

Pseudomonas aeruginosa is an important pathogen causing a wide range of acute and chronic infections especially in immunocompromized patients (Tielen et al., 2013). The emergence of multidrug resistant (MDR) *P. aeruginosa* has become a serious problem in healthcare settings in developing countries (Patzer et al., 2001). Treatment of infections associated with multidrug resistant *P. aeruginosa* is further complicated in Asian countries such as Japan, Taiwan, India and Iran (Khosravi et al., 2011). Imipenem and piperacillin are potent and broad- spectrum penicillins with activity against β-lactamase producing Gram negative and Gram positive bacteria especially against *P. aeruginosa* (Fonseca et al., 2004). Some reports have demonstrated that the treatment with subinhibitory concentrations (Sub-MIC) of some antibiotics may influence bacterial virulence

*Corresponding authors: Dr. Mirshahabi & Dr. zeighami
E-mail address: mirshahabi@zums.ac.ir
E-mail address: zeighami@zums.ac.ir

factors such as adherence, cell surface hydrophobicity, biofilm formation, sensivity to oxidative stress and motility (Fonseca et al., 2004; Kawamura-Sato et al., 2000). Beneficial effects of azithromycin in the treatment of patients with *P. aeruginosa* infections was reported in previous studies (Horii et al., 2003) However, a limited number of antibiotics are known to have beneficial effects on expression of virulence factors at subinhibitory concentrations (Horii et al., 2003).

The pathogenesis of *P. aeruginosa* depends on the production of several virulence factors. The virulence factors play important roles in the attachment and colonization and invasion of bacteria to tissues (Lanotte et al., 2004; Haj Khalifa et al., 2011). *P. aeruginosa* synthesizes an exopolysaccharide alginate in response to environmental conditions. This alginate pseudocapsule protects the bacterium from phagocytosis, dehydration and antibiotics in patients with cystic fibrosis. Moreover, it has an important role in biofilm formation (Cotton et al., 2009; Leid et al., 2005).

The main extracellular enzymes produced by *P. aeruginosa* are type I and type II-secreted hydrolases, including alkaline protease, elastase A (LasA) and B (LasB), phospholipase C and lipases. These enzymes alone or synergistically with others are causing cell death, severe tissue damage and necrosis in the human host (Tielen et al., 2013). Elastolytic metalloproteinase or elastase B (LasB) of the *P. aeruginosa* is encoded by the *lasB* gene and is a type II secretion system substrate and highly expressed during tissue colonization and infection, particularly in the lungs. The exposure of isolated human leukocytes to LasB results in an inhibition of cell chemotaxis and phagocytotic and/or microbicidal activities via proteolytic alterations of membrane immune receptors (Leduc et al., 2007).

According to previous studies, evaluation of subinhibitory concentrations of imipenem and piperacillin on expression of *P. aeruginosa algD* and *lasB* has never been reported. To identify beneficial effects of imipenem and piperacilin on expression of virulence factors of *P. aeruginosa,* we evaluated the effect of subinhibitory concentrations of these antibiotics on *lasB* and *algD* transcriptional expression using Real- time PCR.

2. Materials and Methods
2.1. Bacterial strains

Five strains of *P. aeruginosa* were isolated from clinical specimens. The identification of isolates was performed by routine biochemical tests. Verified isolates of *P. aeruginosa* were preserved at –70°C in Tripticase soy broth (Merck, Germany) containing 20% (v/v) glycerol for further analysis.

2.2. Detection of algD and lasB in Pseudomonas. aeruginosa isolates

All *P. aeruginosa* isolates were screened for the presence of alginate (algD) and elastase (lasB) genes using the primers listed in Table 1. Extraction of DNA was performed according to the protocol provided with the Qiagen Mini Amp kit. The PCR was performed in a reaction mixture with total volume of 25 µl, containing 2 µl template DNA; 0.2 mM of each deoxynucleoside triphosphate; 10 pmol of each primers; 10 mM Tris- HCl; 1.5 mM MgCl2; 50 mM KCl; 1.5 U of Taq DNA polymerase. PCR was performed with the Gene Atlas 322 system (ASTEC, Japan). Amplification involved at initial denaturation at 94°C, 5 minutes followed by 30 cycles of denaturation (94°C, 1 min), annealing (60°C, 1 min), and extension (72°C, 1 min), with a final extension step at 72°C for 10 minutes. The amplified DNA was separated by submarine gel electrophoresis on 1.5% agarose, stained with ethidium bromide and visualized under UV transillumination. *P. aeruginosa* reference strain PAO1 was used as positive control for amplification of algD and lasB genes.

2.3. MIC determination of imipenem and piperacilin:

Minimum Inhibitory Concentrations (MIC) of imipenem (MAST/UK) and piperacillin (Sigma) were determined using the broth macrodilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI. 2013). Concentrations below MIC were considered as subinhibitory concentrations (SIC). The range of concentrations tested for imipenem and piperacilin was 0.125–128 µg/ml. *P. aeruginosa* reference stain ATCC27853 was used as positive control for susceptibility testing.

According to the CLSI guidelines, MIC values of the imipenem and piperacillin for the reference strain were 1-4µg/ml and 1-8µg/ml, respectively.

2.4. RNA extraction and cDNA synthesis:

In order to investigate whether subinhibitory concentrations of imipenem and piperacillin can influence on algD and lasB expression, RNA was extracted from the all subMIC tubes using an RNeasy Mini kit with 1 hour on-column DNase digestion (Qiagen) according to the RNeasy Mini kit handbook. cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (ABI,UK). Reverse transcription was performed in a reaction mixture with total volume of 20 µl containing 10µl RNA, 2 µl reverse transcription buffer (10X), 0.8 µl deoxynucleoside triphosphate (25X), 2 µl RT random primers (100mM) and 1 µl reverse transcriptase (1U). The reactions were incubated at 25°C for 10 min, 37°C for 120 min, 85°C for 5 min and 4°C for 10 min.

2.5. Real Time PCR

One hundred nanograms of cDNA and 50 nM (final concentration) each primer were mixed with 10 µl 2X SYBR Green PCR Master Mix (ABI, UK). Assays were performed in duplicate with an ABI Prism model 7300 instrument. All data were normalized to the internal standard *oprL* (encoding the outer membrane protein), and melting curve analysis demonstrated that the accumulation of SYBR Green-bound DNA was target gene specific. The negative control was included in all experiments.

The threshold cycle values (Ct) were determined for each reaction. To calculate the ΔC_t values, threshold cycle (Ct) for each gene amplification was normalized to the Ct of the *oprl* gene amplified from the corresponding sample. Then ΔC_t values obtained from each sample were compared with control culture without antibiotic.

ΔC_t sample $_=$ Ct sample $-$ Ct *oprL* sample
ΔC_t control $_=$ Ct control $-$ Ct *oprL* control

2.6. Statistical analysis

The data were analyzed with SSPS version 17.0 software (SPSS, Inc., Chicago, IL) and expressed as means and standard deviations of ΔC_t values. The chi-square test was used to determine the statistical significance of the data. A *P* value of < 0.05 was considered significant.

Table1. Primers used in this study

Gene	Primer sequence	Amplicon size (bp)	Ref.
lasB-F	5'-AGACCGAGAATGACAAAGTGGAA-3'	81	(Najafimosleh et al., 2013)
lasB-R	5´-GGTAGGAGACGTTGTAGACCAGTTG-3'		
algD-F	5´-ACGAAGTGGTGGCGAGTTC-3'	105	(Rashno Taee et al., 2014)
algD-R	5´-TGGTGTGCGGCATGAAGC-3'		
oprL-F	5´- TGCGATCACCACCTTCTACTTC-3'	105	(Rashno Taee et al., 2014)
oprL-R	5´-CGCTGACCGCTGCCTTTC-3'		

3. Results
3.1. MIC determination of imipenem and piperacilin

The MIC values of imipenem and piperacillin for five clinical isolates were in the range 0.5-16 µg/ml and 1-64 µg/ml, respectively.

3.2. Effect of subinhibitory concentrations of imipenem on the algD expression

Imipenem was applied in subinhibitory concentrations ranging from 0.125 to 8 µg per ml. The expression level of *algD* at all

subinhibitory concentrations of imipenem was decreased in comparison with the control culture without antibiotic (Fig 1a). However, decrease in expression level of *algD* at any subinhibitory concentrations of imipenem was not significant ($P > 0.05$). As shown in Fig 1(a), at a concentration of 1 µg per ml of imipenem, the expression level of *algD* was lower than others.

3.3. Effect of subinhibitory concentrations of imipenem on the lasB expression

The expression level of *lasB* at subinhibitory concentrations of 0.25, 2, 4 and 8 µg/ml of imipenem was decrease in comparison with the control culture without antibiotic. Whereas, at concentrations of 0.125, 0.5 and 1 µg/ml of imipenem, the expression level of *lasB* was increased in comparison with control (Fig 1b). These differences in expression level of *lasB* were not statistically significant ($P > 0.05$).
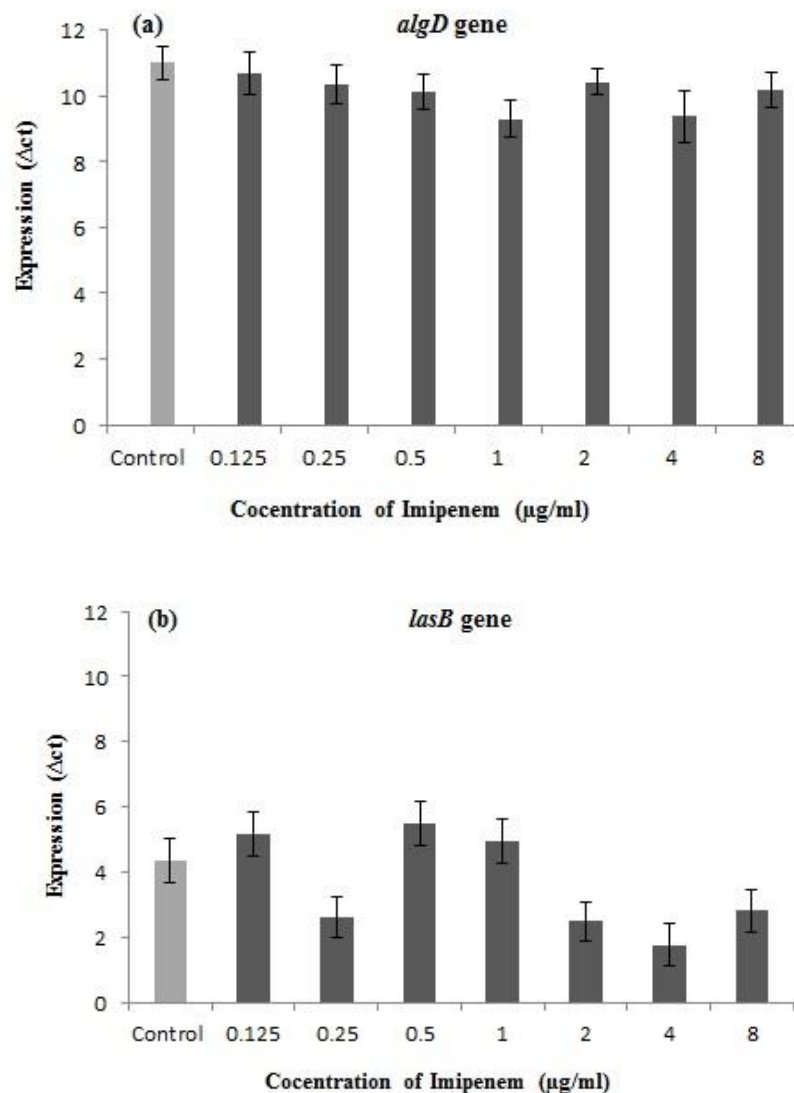

Fig1. (a) Effect of sub-MIC concentrations of imipenem on the *algD* and (b) *lasB* expression in *P. aeruginosa* isolates. Δct values obtained for each subMIC concentration of imipenem were compared with the Positive control culture without antibiotic.

3.4. Effect of subinhibitory concentrations of piperacillin on the algD expression

Piperacillin was applied in subinhibitory concentrations ranging from 0.125 to 32 μg per ml. The expression level of *algD* at subinhibitory concentrations of 0.125, 0.25, 1, 2, 4, and 16 μg/ml of piperacillin was increased in comparison with the control culture without antibiotic. Whereas, at concentrations of 0.5, 8 and 32 μg/ml of piperacillin, the expression level of *algD* was decreased in comparison with control (Fig 2a). These differences in expression level of *algD* were not statistically significant ($P> 0.05$).

3.5. Effect of subinhibitory concentrations of piperacillin on the lasB expression

The expression level of *lasB* at all subinhibitory concentrations of piperacillin was decreased in comparison with the control culture without antibiotic (Fig 2b). Decrease in expression level of *lasB* at all subinhibitory concentrations of piperacillin was significant ($P< 0.05$). As shown in Fig 2(b), at a concentration of 0.125 μg per ml of piperacillin, the expression level of *lasB* was lower than others.
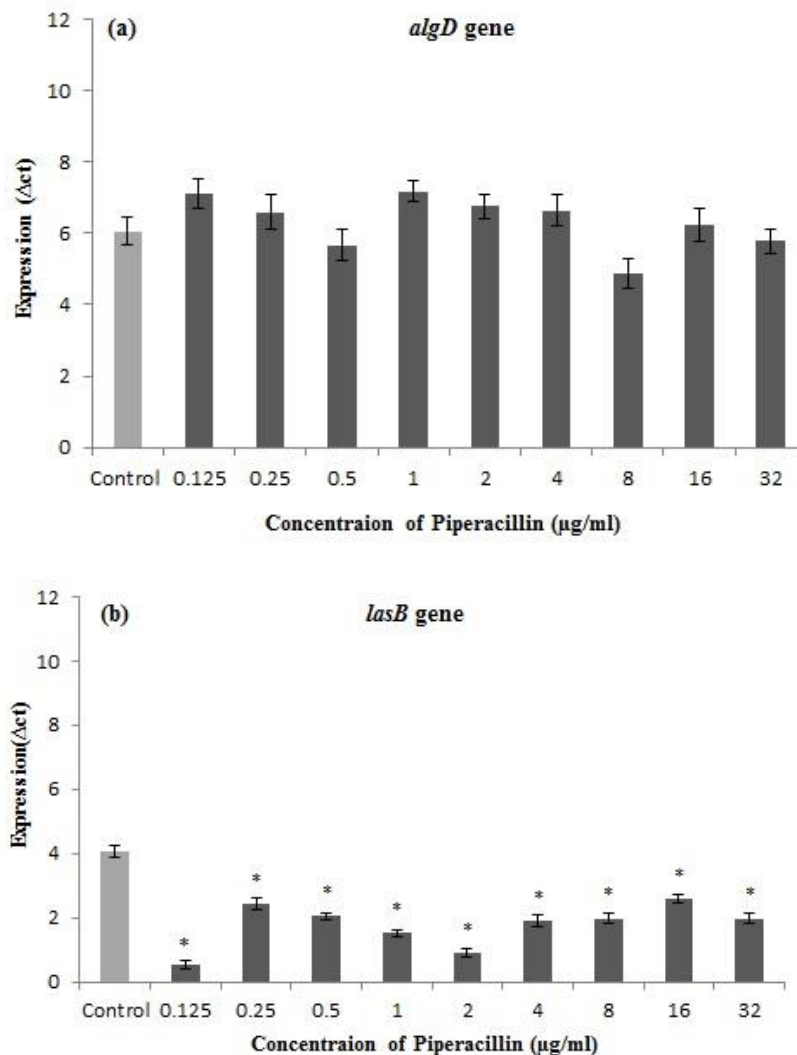

Fig2. (a) Effect of sub-MIC concentrations of piperacillin on the *algD* and (b) *lasB* expression in *P. aeruginosa* isolates. Δct values obtained for each subMIC concentration of piperacillin were compared with the Positive control culture without antibiotic.

 * significant at *P< 0.05*.

4. Discussion

Subinhibitory antibiotic concentrations are known to exhibit effects on the cell structure and the expression of important bacterial virulence factors such as adhesins or toxins (Rachid et al., 2000; Gomes et al., 2013). Several studies have now shown that subinhibitory concentrations of several antibiotics can transcriptionally modulate a large number of genes (Babic et al., 2010). In this study, we have analyzed the effect of subinhibitory concentrations of imipenem and piperacillin on the expression of *lasB* and *algD* genes. Our results showed that the expression of the *lasB* strongly decreased at all subinhibitory concentrations of piperacillin, especially at concentrations 2 and 0.125 µg/ml ($P< 0.05$). The *algD* expression was decreased 0.3 to 1.6 fold at subinhibitory concentrations of imipenem. Whereas, a slight increase of the *algD* expression was measured at concentrations 16, 4, 2, 1, 0.25 and 0.125 µg/ml of piperacillin. However, imipenem and piperacillin had no significant influence on *algD* expression. The effect of subinhibitory concentrations of various antibiotics has been studied on morphology and biochemical properties (Horii et al., 2003), the expression of resistance related genes (Kolayli et al., 2004), biofilm formation (Bagge et al., 2004) and motility and flagella formation (Fonseca et al., 2004; Horii et al., 2003) in *P. aeruginosa*. According to Shen *et al*, the expression of some virulence factors such as *lasB* in *P. auroginosa* was increased at subinhibitory concentrations of vancomycin, tetracycline, ampicillin and azithromycin. However, similar to our results, these antibiotics had no significant effect on *algD* expression (Shen et al., 2008).

Treatment with subinhibitory concentrations of some antibiotics suppresses the expression of virulence factors in various Gram-negative bacteria. Recent studies showed that subinhibitory concentrations of macrolides and clindamycin inhibit the biofilm formation in *P. aeruginosa* and macrolides suppress the flagellin expression in *P. aeruginosa* and *Proteus mirabilis* (Kawamura-Sato et al., 2000; Horii et al., 2003). Horii *et al* showed that subinhibitory concentrations of mupirocin decreased the flagella formation in *P. aeruginosa* (Horii et al., 2003). In a study carried out using Fonseca *et al*, subinhibitory concentrations of piperacillin and tazobactam interfered with pathogenic potential of *P. aeruginosa* as adhesiveness, cell-surface hydrophobicity, motility, biofilm formation and sensitivity to oxidative stress (Fonseca et al., 2004).

Previous studies demonstrated that subinhibitory concentrations of azithromycin interfere with synthesis of autoinducers such as 3-oxo-C12-homoserine lactone (HSL) and C4-HSL in the quorum-sensing (QS) cell-to-cell signaling system, leading to decrease in virulence factors expression (Skindersoe et al., 2008; Rathinam and Viswanathan, 2014; Zhanjun et al., 2014). In fact, subinhibitory concentration of azithromycin were shown by microarray analysis to repress a large number of genes which are QS regulated and similar observations were made with other antibiotics (Babic et al., 2010). Babic *et al* showed that tobramycin at subinhibitory concentration inhibits the RhlI/R quorum sensing system in a *P. aeruginosa* (Babic et al., 2010).

Conclusion

In conclusion, we have shown that subinhibitory concentrations of piperacillin can reduce *lasB* expression in *P. aeruginosa*. Although, further studies will be required in order to assess whether subinhibitory concentrations of piperacillin can be improve the outcomes of severe and serious infections caused by *P. aeruginosa*.

Acknowledgements

Refereces

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