ORIGINAL ARTICLE

Effect of subinhibitory concentrations of imipenem and piperacillin on *Pseudomonas aeruginosa toxA* and *exoS* transcriptional expression

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Abstract

Subinhibitory concentrations (subMIC) of antibiotics, although not able to kill bacteria, can modify their physicochemical characteristics and may interfere with some bacterial functions. This study aimed to investigate the effect of subMIC of imipenem and piperacillin on the transcriptional expression of virulence-related genes *toxA* and *exoS* in *Pseudomonas auroginosa*. Five clinical isolates of *P. aeruginosa* were screened for the presence of *toxA* and *exoS* genes and MICs of imipenem and piperacillin were determined using broth macrodilution. The expression levels of *toxA* and *exoS* at subMIC concentrations of antibiotics were measured by real-time PCR. Our results showed that the expression of *toxA* decreased at all subinhibitory concentrations of imipenem, especially at concentrations 2, 4 and 8 mg/L ($p < 0.05$). Whereas, *exoS* expression was increased 4.1- to 7-fold at subinhibitory concentrations of imipenem. The increase of *toxA* expression was measured at concentrations 16, 4, 2, 0.25 and 0.125 mg/L of piperacillin. However, piperacillin had no significant influence on *exoS* expression ($p > 0.05$). Further studies will be required to assess whether subMIC of imipenem can improve the outcomes of severe and serious infections caused by *P. aeruginosa*.

Keywords: Exoenzyme S, exotoxin A, *Pseudomonas aeruginosa*, Subinhibitory concentrations, Imipenem, Piperacillin
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Introduction

Pseudomonas aeruginosa, as an opportunistic human pathogen, can cause severe acute and chronic infections especially in immunocompromised individuals [1]. The emergence of multidrug-resistant (MDR) *P. aeruginosa* has become a serious problem in health-care settings in developing countries [2]. Treatment of infections associated with MDR *P. aeruginosa* is further complicated in Asian countries such as Japan, Taiwan, India and Iran [3]. Imipenem and piperacillin are potent, broad-spectrum penicillins with activity against β-lactamase-producing Gram-negative and Gram-positive organisms, especially against *P. aeruginosa* [4]. Some reports have demonstrated that treatment with subinhibitory concentrations (subMIC) of some antibiotics may influence bacterial virulence factors such as adherence, cell surface hydrophobicity, biofilm formation, sensitivity to oxidative stress and motility [4,5]. Previous studies have suggested that treatment with subMIC of macrolides may benefit patients with *P. aeruginosa* infections [6] However, a limited number of antibiotics are known to have beneficial effects on the expression of virulence factors at subMIC [6].

The pathogenesis of *P. aeruginosa* depends on the production of several cell-associated and extracellular virulence factors. The virulence factors play important pathological roles in colonization, the survival of the bacteria and the invasion of tissues [7,8]. Among the extracellular toxins, exotoxin A and exoenzyme S have the most important roles in pathogenesis and lead to local and systemic toxicity [9,10]. The expression of these toxins is regulated by Quorum sensing [10,11].

Exotoxin A is a type II secreted extracellular enzyme encoded by the *toxA* gene. This enzyme alone or synergistically with other hydrolases causes cell death, severe tissue damage

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and necrosis in the human host [10,12]. Exotoxin A is an ADP-ribosyl transferase that transfers an ADP-ribosyl moiety to elongation factor 2, resulting in an inhibition of protein synthesis in mammalian cells [9,10,12]. Exoenzyme S is a type III secreted bifunctional enzyme containing an N-terminal GTPase-activating protein domain and a C-terminal ADP-ribosylation domain encoded by the *exoS* gene. The 14-3-3 protein is a eukaryotic cell cofactor, required for ADP-ribosyl transferase activity of exoenzyme S [9,12]. Exoenzyme S inhibits phagocytosis by disrupting actin cytoskeletal rearrangement, focal adhesions and signal transduction cascades [9].

To the authors' knowledge, the effects of subMIC of imipenem and piperacillin on *P. aeruginosa toxA* and *exoS* expression have never been reported. To identify beneficial effects of imipenem and piperacillin on expression of the virulence factors of *P. aeruginosa* we assessed the effect of subMIC of these antibiotics on *toxA* and *exoS* transcriptional expression using real-time PCR.

Materials and methods

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 trypticase soy broth (Merck, Darmstadt, Germany) containing 20% (volume/volume) glycerol for further analysis.

Detection of toxA and exoS in *P. aeruginosa* isolates

All *P. aeruginosa* isolates were screened for the presence of exotoxin A (*toxA*) and exoenzyme S (*exoS*) genes using the primers listed in Table 1. Extraction of DNA was performed according to the protocol provided with the Qiagen Mini Amp kit (QIAGEN Inc., Valencia, CA). 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 primer; 10 mM Tris–HCl; 1.5 mM $MgCl_2$; 50 mM KCl; 1.5 U of *Taq* DNA polymerase. PCR was performed with the Gene Atlas 322 system (ASTEC, Fukouka, Japan). Amplification involved an initial denaturation at 94°C for 5 min 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 min. The amplified DNA was separated by submarine gel electrophoresis on 1.5% agarose, stained with ethidium bromide and visualized under UV transillumination. The *P. aeruginosa* reference strain PAO1 was used as a positive control for amplification of *toxA* and *exoS* genes.

TABLE 1. Primers used in this study

Gene	Primer sequence	Amplicon size (bp)	Ref.
toxA-F	5′-ACA TCA AGG TGT TCA TCC -3′	125	[23]
toxA -R	5′-GAC GAA GAA GGT GGC ATC -3′		
exoS-F	5′-GGC GGA TGC GGA AAA GTA C -3′	121	[11]
exoS-R	5′-CTG ACG CAG AGC GCG ATT -3′		
oprL-F	5′-AAC AGC GGT GCC GTT GAC -3′	87	[11]
oprL-F	5′-GTC GGA GCT GTC GTA CTC GAA -3′		

MIC determination of imipenem and piperacillin

The MIC of imipenem (MAST, Merseyside, UK) and piperacillin (Sigma, St Louis, MO, USA) were determined using the broth macrodilution method according to the CLSI guidelines [13]. Concentrations below MIC were considered subinhibitory (subMIC). The range of concentrations tested for imipenem and piperacillin was 0.125–128 mg/L. The *P. aeruginosa* reference stain ATCC27853 was used as positive control for susceptibility testing. According to the CLSI guidelines, MIC values of imipenem and piperacillin for the reference strain were 1–4 mg/L and 1–8 mg/L, respectively.

RNA extraction and cDNA synthesis

To investigate whether subMIC of imipenem and piperacillin can influence *toxA* and *exoS* expression, RNA was extracted from the all subMIC tubes using an RNeasy Mini kit with 1 h on-column DNase digestion (Qiagen) according to the RNeasy Mini kit handbook. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Reverse transcription was performed in a reaction mixture with a total volume of 20 µL containing 10 µL RNA, 2 µL reverse transcription buffer (10×), 0.8 µL deoxynucleoside triphosphate (25×), 2 µL RT random primers (100 mM) and 1 µL reverse transcriptase (1 U). 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.

Real-time PCR

One hundred nanograms of cDNA and 50 nM (final concentration) of each primer were mixed with 10 µL 2× 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, the 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

Statistical analysis

The data were analysed with SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA) 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.

Results

MIC determination of imipenem and piperacillin

The MIC values of imipenem and piperacillin for five clinical isolates were in the range 0.5–16 mg/L and 1–64 mg/L, respectively.

Effect of subMIC of imipenem on the toxA expression

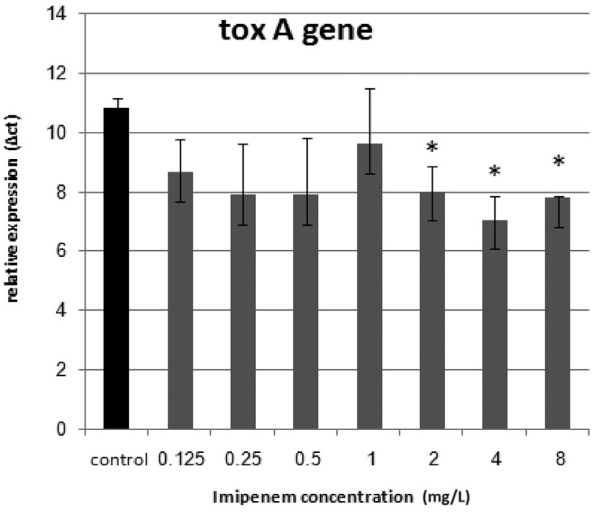
Imipenem was applied in subMIC ranging from 0.125 to 8 mg/L. The expression level of *toxA* at all subMIC of imipenem was decreased in comparison with the control culture without antibiotic (Fig. 1a). Decrease in expression level of *toxA* at subMIC of 2, 4 and 8 mg/L of imipenem was significant ($p < 0.05$).

Effect of subMIC of imipenem on the exoS expression

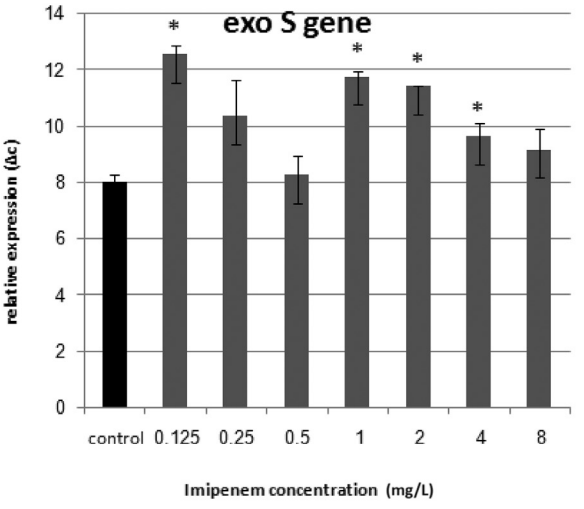
The expression level of *exoS* at all subMIC of imipenem was increased in comparison with the control culture without antibiotic (Fig. 1b). Increase in expression level of *exoS* at subMIC of 0.125, 1, 2 and 4 mg/L of imipenem was statistically significant ($p < 0.05$).

Effect of subMIC of piperacillin on the toxA expression

Piperacillin was applied in subMIC ranging from 0.125 to 32 mg/L. The expression level of *toxA* at subMIC of 0.125, 0.25, 2, 4 and 16 mg/L of piperacillin was increased in comparison with the control culture without antibiotic. Whereas at other concentrations of piperacillin, the expression level of *toxA* was decreased in comparison with control (Fig. 2a). These differences in expression level of *toxA* were statistically significant only at concentrations of 0.125 and 0.25 mg/L of piperacillin ($p < 0.05$).



(a)



(b)

FIG. 1. Effect of subinhibitory concentrations of imipenem on (a) the *toxA* and (b) the *exoS* expression in *Pseudomonas aeruginosa* isolates. ΔCt values obtained for each subinhibitory concentrations of imipenem were compared with the control culture without antibiotic. *$p < 0.05$.

Effect of subMIC of piperacillin on the exoS expression

The expression level of *exoS* at concentrations of 2, 4 and 32 mg/L of piperacillin was increased in comparison with the control culture without antibiotic (Fig. 2b). At other concentrations of piperacillin, the expression level was decreased in comparison with control. These differences in expression level of *exoS* were not significant ($p > 0.05$).

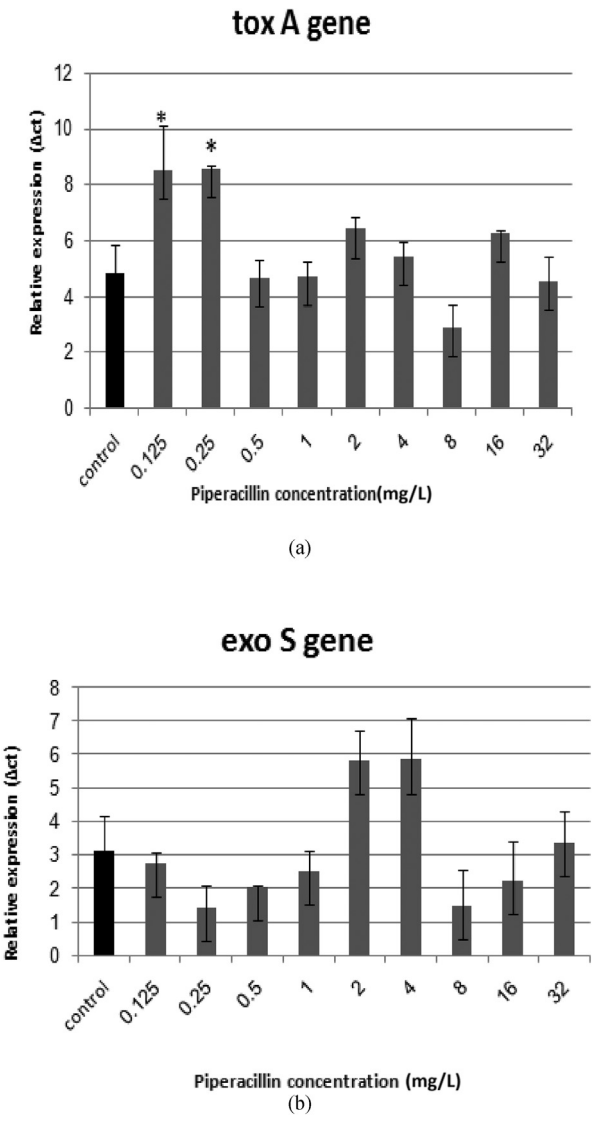


FIG. 2. Effect of subinhibitory concentrations of piperacillin on (a) the *toxA* and (b) the *exoS* expression in *Pseudomonas aeruginosa* isolates. ΔCt values obtained for each subinhibitory concentration of piperacillin were compared with the control culture without antibiotic. *p < 0.05.

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 [14,15]. Several studies have now shown that subMIC of antibiotics can transcriptionally modulate a large number of genes [16]. In this study, we have therefore analysed the effect of subMIC of imipenem and piperacillin on the expression of *toxA* and *exoS* genes. Our results showed that the expression of the *toxA* decreased at all subMIC of imipenem, especially at concentrations 2, 4 and 8 mg/L ($p < 0.05$). Whereas, the *exoS* expression was increased 4.1- to 7-fold at subMIC of imipenem. The increase of *toxA* expression was measured at concentrations 16, 4, 2, 0.25 and 0.125 mg/L of piperacillin. However, piperacillin had no significant influence on *exoS* expression ($p > 0.05$). The effect of subMIC of various antibiotics has been studied on morphology and biochemical properties [6], the expression of resistance-related genes [17], biofilm formation [18], and motility and flagella formation [4,6] in *P. aeruginosa*. According to Shen et al., the expression of some virulence factors in *P. aeruginosa* was increased at subMIC of vancomycin, tetracycline, ampicillin and azithromycin [19].

Treatment with subMIC of some antibiotics suppresses the expression of virulence factors in various Gram-negative bacteria. Recent studies showed that subMIC of macrolides and clindamycin inhibit the biofilm formation in *P. aeruginosa* and macrolides suppress the flagellin expression in *P. aeruginosa* and *Proteus mirabilis* [5,6]. Horii et al. showed that subMIC of mupirocin decreased the flagella formation in *P. aeruginosa* [6]. In a study carried out by Fonseca et al., subMIC of piperacillin and tazobactam interfered with the pathogenic potential of *P. aeruginosa* as adhesiveness, cell-surface hydrophobicity, motility, biofilm formation and sensitivity to oxidative stress [4].

Previous studies demonstrated that subMIC of azithromycin interfere with the synthesis of autoinducers such as 3-oxo-C12-homoserine lactone (HSL) and C4-HSL in the quorum-sensing cell-to-cell signalling system, leading to a decrease in expression of virulence factors [20–22]. In fact, subMIC of azithromycin were shown by microarray analysis to repress a large number of genes that are quorum-sensing-regulated, and similar observations were made with other antibiotics [16]. Babic et al. showed that tobramycin at subMIC inhibits the RhlI/R quorum-sensing system in *P. aeruginosa* [16].

In conclusion, we have shown that subMIC of imipenem can reduce *toxA* expression in *P. aeruginosa*. Further studies will be required to assess whether subMIC of imipenem can improve the outcomes of severe and serious infections caused by *P. aeruginosa*.

Conflicts of interest

The authors declare that they have no competing interests.

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