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Role of MexAB-OprM and MexXY-OprM efflux pumps and class 1 integrons in resistance to antibiotics in burn and Intensive Care Unit isolates of *Pseudomonas aeruginosa*

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ABSTRACT

Background: The overexpression of efflux pumps and existence of class 1 integrons are the most important mechanisms that contribute to antimicrobial resistance in *Pseudomonas aeruginosa* especially in burn and Intensive Care Units (ICUs). The present study evaluated the role of MexAB-OprM and MexXY-OprM efflux pumps and class 1 integrons in resistance to antibiotics in burn and ICU isolates of *P. aeruginosa*.
Methods: Fifteen burn and forty-two ICU isolates were obtained from four hospitals in Northwest Iran. The isolates were identified and evaluated by the disk diffusion and agar dilution methods for determining antibiotic resistances. The presence of class 1 integrons and associated resistance gene cassettes were detected by PCR and sequencing of the products. The expression levels of efflux pumps were evaluated by phenotypic and genotypic (Quantitative Real-time PCR) methods. The isolates were genotyped by Random Amplified Polymorphic DNA Typing (RAPD-PCR).
Results: All burn isolates were integron positive and Multi-drug resistant (MDR), while 78.5% and 69% of ICU isolates were found as MDR and integron positive, respectively. The *aadB* gene was the most prevalent gene cassette (63.6%) followed by *aacA4* (47.7%). Thirty-nine (68.4%) and 43 (75.4%) isolates exhibited an overexpression of MexAB-OprM and MexXY-OprM. Among burn isolates, 80% and 86.6% of them were *mexB* and *mexY* overexpressed, while 64.2% and 71.4% of ICU isolates exhibited *mexB* and *mexY* overexpression, correspondingly. The isolates were genotyped as 24 different RAPD profiles and were grouped into 15 clusters.
Conclusions: The data suggested that class 1 integron had a more significant role than efflux pumps in resistance to beta-lactams and aminoglycosides in burn and ICUs except for gentamicin in burn isolates. Based on our data, it is possible that efflux pumps were not the main cause of high-level resistance to antibiotics.

Introduction

The ubiquitous Gram-negative bacterium *Pseudomonas aeruginosa* is one of the most important nosocomial pathogens as a common cause of infections especially in burn and Intensive Care Units (ICUs) [1–3]. Thermal injuries and long duration of hospitalization along with the selective pressure of antibiotic therapy in burn patients and ICU immunosuppressive conditions are some of the causes of infection with resistant *P. aeruginosa* [4]. *P. aeruginosa* has a notable ability to possess resistance to most antimicrobial agents [5,6]. These mechanisms result in the development of multidrug resistant (MDR) *P. aeruginosa* isolates, and lead to complications in treatment especially in burn and ICU patients [7–9]. Moreover, MDR isolates of *P. aeruginosa* are

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responsible for outbreaks in ICUs and burn units [8,10]. Among the multiple resistance mechanisms, the overexpression of the Resistance Nodulation Cell Division (RND) family of efflux pumps and the acquisition of class 1 integrons are the most common cause of MDR phenotype in clinical isolates of *P. aeruginosa* [10–12]. MexAB-OprM and MexXY-OprM, the RND-type efflux pumps which expressed constitutively in wild-type cells, account for the major cause of intrinsic resistance to most antimicrobial agents in *P. aeruginosa* [3,13]. Moreover, gene cassettes carried with class 1 integrons confer resistance to aminoglycosides, β-lactams, chloramphenicol, carbapenems, and macrolides [14]. The present study aimed to investigate the frequency of class 1 integrons as well as the diversity of their internal variable regions (IVRs) and the assessment of MexAB-OprM and MexXY-OprM overexpression by phenotypic (using the efflux pump inhibitor) and genotypic (quantitative real-time PCR) methods in clinical isolates of *P. aeruginosa* in burn and ICU patients to compare the role of these mechanisms in antibiotic resistance.

Materials and methods

Bacterial isolates

Fifty-seven non-duplicated clinical isolates of *P. aeruginosa* were obtained from burn wards and ICUs of four hospitals in the city of Tabriz in Northwest of Iran from January to June 2015. Sources of our isolates included: wounds, blood, respiratory samples, peritoneal fluid, and stool. The isolates were identified by conventional microbiological methods [15] and were stored in tryptone soy broth (Merck Co., Darmstadt, Germany) with 30% glycerol (Merck) at −70 °C.

Antimicrobial susceptibility testing and MIC

The antimicrobial susceptibility testing was performed against 15 antimicrobial agents: ciprofloxacin (5 μg), levofloxacin (5 μg), ofloxacin (5 μg), gentamicin (10 μg), tobramycin (10 μg), amikacin (30 μg), ticarcillin (75 μg), piperacillin/tazobactam (100/10 μg), ceftazidime (30 μg), cefepime (30 μg), aztreonam (30 μg), imipenem (10 μg), meropenem (10 μg), doripenem (10 μg) and colistin sulfate (10 μg), (Mast Diagnostics Group Ltd., Merseyside, UK) by the Kirby-Bauer method on Mueller Hinton-agar (Merck) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [16].

The minimum inhibitory concentration (MIC) of imipenem, gentamicin, colistin, ciprofloxacin, and levofloxacin, (Sigma-Aldrich Co, St. Louis, USA) were determined using the agar dilution method on cation-adjusted Mueller-Hinton agar (Merck) according to the guidelines of the CLSI. The antimicrobial agents were incorporated into the agar medium, with each plate containing a different concentration of the agents and allowed the agar to solidify at room temperature. The final inoculum required is 10^4 CFU per spot of 5–8 mm in diameter. Thus, we diluted the 0.5 McFarland suspension 1:10 in sterile broth or saline to obtain a concentration of 10^7 CFU/ml. The final inoculum on the agar will then be approximately 10^4 CFU per spot. *P. aeruginosa* ATCC 27853 and wild-type strain PAO1 (IROST, Iranian Research Organization for Science and Technology, Tehran, Iran) were used as the control strains in susceptibility testing.

DNA extraction and PCR assay for class 1 integron

Genomic DNA extraction for identification of class 1 integron was performed as described previously [17]. The presence of the variable regions of class 1 integrons (gene cassettes) was assessed using the primers as described previously [17]. The PCR was performed in a final reaction volume of 25 μl for a total of 35 cycles. The amplification was attained in an Eppendorf Mastercycler® Gradient Thermal Cycler (Eppendorf, Hamburg, Germany) as follows: initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 60 s, primer annealing at 57 °C for 60 s, extension at 72 °C for 2 min and final extension at 72 °C for 10 min.

DNA sequencing and analysis of sequences

After extraction of the PCR products from the gel by the AccuPrep Gel Purification Kit (Bioneer Co., Daejeon, Korea), the product strands purified using the AccuPrep PCR Purification Kit (Bioneer). Applied Biosystems 3730/3730xl DNA Analyzers Sequencing (ABI, Bioneer) was used for sequencing of the purified amplicons. The nucleotide sequences were compared to the GenBank databases on the Website of http://www.ncbi.nlm.nih.gov/BLAST/.

Efflux pump inhibition

To investigate the interaction between imipenem, gentamicin, colistin, ciprofloxacin, levofloxacin and phenylalanine arginine β-naphthylamide (PAβN), the MICs of the antibiotics were determined in the absence and presence of PAβN (Takara Bio Inc., Otsu, Shiga, Japan) at a concentration of 50 μg/ml based on previous studies [18,19]. The isolates were confirmed as efflux pumps overexpressed when the MICs in the presence of PAβN were determined to be at least four-fold lower than the MICs in the absence of PAβN [19,20]. The PAO1 wild-type strain of *P. aeruginosa* was used as the reference strain.

Total RNA extraction and cDNA synthesis

The extraction of total RNA from *P. aeruginosa* isolates was performed using the total RNA extraction kit (SinaClon Co., Tehran, Iran) according to the manufacturer's instructions. The RNAs were then treated with RNase-free DNaseI (SinaClon), and the concentration and purity of the products were determined by NanoDrop spectrophotometer (ND-1000, Wilmington, USA) [13]. Synthesis of cDNA was performed using the M-MuL V reverse transcriptase (SinaClon) by adding 5 μg DNA-free RNA and random hexamer as a primer according to the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR)

The amplification of *rpsL*, *mexB*, and *mexY* genes were carried out using the SYBR Premix EX TaqII (Tli RNaseH Plus) (Takara Bio Inc.) in duplicate runs by the primers as described previously for the *rpsL* [21], *mexB* [22] and *mexY* [23]. A Rotor-Gene Real-Time PCR machine (Corbett Research, Sydney, Australia; Model RG 3000) was used for the quantification of cDNAs by using the house-keeping gene (*rpsL*) as the normalizing gene. The cycling conditions for the amplification were as follows for the *rpsL* gene: 95 °C for 5 min and 40 cycles of 20 s at 95 °C, 20 s at 60 °C and 30 s at 72 °C, for the *mexB* gene: 95 °C for 5 min and 45 cycles of 20 s at 95 °C, 10 s at 68 °C and 15 s at 72 °C with 3 Mm in $MgCl_2$ concentration and for the *mexY* gene: 95 °C for 5 min and 45 cycles of 15 s at 95 °C, 10 s at 60 °C and 10 s at 72 °C.

P. aeruginosa (ATCC 27853) was chosen as control for preparing standard cDNAs. Moreover, the PAO1 wild-type strain was included in each set of the reactions for assessing the validation of reactions. A control reaction without cDNA was included each run as a no-template control. The ratios of gene expression between the target gene and the reference gene (*rpsL*) in isolates compared to the PAO1

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