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The Evaluation of Antibiotic Resistance and *nalB* Mutants in *Pseudomonas aeruginosa* Isolated from Burnt Patients of Shohada Mehrab Yazd Hospital Burn Ward

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Abstract

Background: Due to extensive damage to the skin, burn victims may acquire life-threatening infections. Though the skin primarily protects against microbial invasions, a large number of bacteria, fungi, and viruses can be isolated from burn patients, specifically *Pseudomonas aeruginosa*, a gram-negative bacterium with both intrinsic and acquired antibiotic resistance (AR) properties. *nalB* mutations can be found on the *mexR* in the *P. aeruginosa* chromosome. This mutation can induce overexpression of the mexAB-oprMoperon, and affect the MexAB-OprM efflux pump, which removes antimicrobial agents from the bacterial cell. Identifying *nalB* mutants can be useful for monitoring factors affecting AR.

Methods: In this study, 70 *P. aeruginosa* isolates identified from burn patients and antibacterial sensitivity was evaluated using the Kirby-Bauer method. We also investigated *nalB* mutations in samples using molecular methods including Polymerase reaction chain (PCR) and Sequencing.

Results: We identified *nalB* mutations in 16 isolates. We also found that the increasing effect of *nalB* mutants induces hyper production activity of MexAB-OprM resulting in AR. Overall, these findings compliment the findings of previous reports.

Conclusions: According to the resistance patterns of the samples, both Amikacin and Ciprofloxacin showed the highest resistance (%). Further, the relationship between Ciprofloxacin resistance and *nalB* mutations was statistically significant (p= 0.016). The results confirm that the increasing effect of *nalB* mutants on hyper production activity of MexAB-OprM leads to AR.

Keywords: Antibiotic Resistance, Burn Infections, Hospital-Acquired Infections, Nosocomial Infections, *Pseudomonas aeruginosa.*

Introduction

Severe burns are among the most common types of trauma. Immediate medical attention and care is required to minimize mortality and morbidity of burn patients. In the past decade, however, survivability has improved tremendously due to advances in modern medicine (1). It is a well-known fact that the skin, the largest human organ, is an essential barrier that provides first-line defense, immunity and protection against microbial invasions. Interestingly, infections related to burns correlate to the severity of the burn injury (2). The source of microorganisms

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which colonize burn wounds can be traced back to gastrointestinal or respiratory flora, the environment (water, air, environmental surfaces), fomites, or even healthcare workers (3). Furthermore, various microorganisms such as fungi, viruses and bacteria have been isolated from burns (4-9). Here, we specifically evaluate a central microbe involved in severe burn infections, more commonly known as *Pseudomonas aeruginosa* (10-12).

Pseudomonas aeruginosa, gram-negative bacteria, is one of the root causes of nosocomial infections, and plays an essential role in morbidity and mortality amongst burn patients (13). Moreover, antimicrobial resistance caused by *P. aeruginosa* can complicate the resolution of burn infections (14, 15). Recent studies indicate that wild-type *P. aeruginosa* expresses low levels of MexAB-OprM, an efflux pump involved in exporting antimicrobials out of the cell, and promotes resistance against broad-spectrum antibiotics such as penicillin, third generation cephalosporines, monobactams, macrolides, tetracyclines and fluoroquinolones (Ciprofloxacin, Levofloxacin and Ofloxacin) (16-18). Located upstream of the mexAB-oprM operon (the operon of MexAB-OprM efflux pump), *mexR* regulates the expression of the mexAB-oprM operon (19, 20). A mutation in *mexR* was found to induce overexpression of the mexAB-oprM operon and exhibit the *nalB*-type profile of antibiotic resistance (AR) (19, 20). Furthermore, a mutation in the *nalB* locus of *mexR* in *P. aeruginosa* manifests a higher level of AR, specifically to fluoroquinolones, when compared to strains lacking the *nalB* mutant (19). Originally, the *nalB* mutation was related to nalidixic acid resistant strains and could be located on the *P. aeruginosa* chromosome. Taken together, the *nalB* mutant induces overexpression of the mexAB-oprM operon (20).

Previous studies which contains the sequence of *mexR* obtained from isolated *nalB*-type *P. aeruginosa* found that most strains carried the *nalB* mutation (16, 19). Thus, both the presence of the *nalB* mutation and the relationship between Ciprofloxacin (fluoroquinolone) resistance was investigated in isolated *P. aeruginosa* strains from several adult burn patients.

Materials and methods

Sample collection and study design

This study was conducted at the Microbiology Laboratory of the Department of Microbiology, Shahid Sadooghi University of Medical Science, Yazd, Iran from April 5, 2018 to December 20, 2018 within eight months from sample collection. Samples were collected from burn wounds of patients at the time of admission to Shohada Mehrab Yazd Hospital Burn Ward and prior to starting the treatment courses. Samples collected from burn wounds involved the use of sterile swabs. Swabs were then cultured on blood agar and eosin methylene blue agar (EMB) followed by a 24 h incubation at 37 °C. The strains identified using biochemical testing (Table 1) were stored at -70 °C. Data analysis was performed using SPSS 10 (SPSS Inc., Chicago, IL) and approved by the Ethical Committee of the Sahid Sadooghi University of Medical Sciences, Yazd, Iran.

Evaluation of antibiotic resistance

Antimicrobial susceptibility was performed by disc diffusion using the Kirby-Bauer method using Commercially available antibiotics (MAST, UK) (21). The antibiotics used in the study included: Ciprofloxacin (5 µg), Levofloxacin (5 µg), Norfloxacin (10 µg), Ofloxacin (5 µg), Gentamicin (10 µg), Amikacin (30 µg), Ceftazidime (30 µg) and Imipenem (10 µg). Minimum inhibitory concentrations (MIC) of Ciprofloxacin as a Fluoroquinolone antibiotic (for detection of *mexR* defective strains (19)), measured by broth microdilution method between concentrations from 512 µg/ml to 0.25 µg/ml. *P. aeruginosa* ATCC 27853 was used for quality control for all antimicrobial susceptibility assays. The results were analyzed according to the CLSI 2018 guidelines (22).

DNA extraction

DNA was extracted from the strains using a boiling method: Colonies grown overnight (48-72h) were suspended in 300 µl of double distilled water (DDW). Suspensions were boiled for ten minutes and centrifuged at 19000 × G for five minutes at room temperature (RT). The

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supernatant was subsequently transferred to a new microtube and stored at 20 ºC (23).

Primer design

The primer3 (http://frodo.wi.mit.edu/) tool was used to design the forward and reverse primers for *mexR*. The following forward and reverse primer sequences were used in the study: Forward primer: ACATTAGGTTTACTCGGCCAA; Reverse primer: GCCAGTAAGCGGATACCTGA.

Polymerase Chain Reaction (PCR) cycles

The PCR reaction was carried out in an Applied Biosystems thermocycler. The initial PCR contained 0.6 µM of each forward and reverse primers, 12.5 µl Taq DNA polymerase, 2X Master Mix Red (Amplicon, Denmark) and sterile distilled water to a final volume of 25 µl. The following conditions were used during DNA amplification: 95 °C for five minutes (initial denaturation), 94 °C for 45 S (denaturation), 55 °C for 30 S (annealing) and 72 °C for 45 S (extension) as three steps of 40 PCR cycles and 72 °C for five mins (final extension).

Electrophoresis

PCR amplicons (4 µl per amplicon) were separated by 1% (w/v) agarose gel electrophoresis (80 V, 45 min) and stained with GeleStain (Pishtaz Teb Co.) at a concentration of 20 mg/100 ml in water.

Sequencing

PCR products were sequenced by the Macrogen Company in Korea.

BLAST and detection of nalB mutants

The results were analyzed using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/). To locate mutations, sequences were compared to the *mexR* gene sequence (Accession number: 877857).

Statistical analysis

The study investigated the relationship between *nalB* mutations and Ciprofloxacin resistance. Statistical analysis was performed using a chi-square test, where a p-value <0.05 was considered significant.

Results

Identification and evaluation of antibiotic resistance

Seventy strains of *P. aeruginosa* were identified following conventional biochemical testing in the microbiology laboratories which mentioned in (Table 1). Both antibiotic susceptibility patterns and MIC profiles of the strains are listed in (Table 2). The mean age of the burn patients was 41.1 ± 31.9 yrs. (< 12- 73 yrs.). There were 53 males (75.8 %) and 17 female participants (24.2 %) resulting in a 3.11:1 male to female ratio.

Mutation investigation

Following PCR and gel electrophoresis (Fig. 1), 70 *mexR* positive strains were identified. All positive *mexR* strains were sequenced to confirm the presence of *mexR*. To investigate the presence of possible mutations, the sequences were compared to *mexR* in *P. aeruginosa* PAO1 (reference sequence) using an online software (ClustalW2: https://www.ebi.ac.uk/Tools/msa/clustalw2/). In total, 16 samples contained *nalB* mutations (Table 3).

Table 1. *P. aeruginosa* identification tests.

Identification tests	Results
TSI medium	alkaline/ alkaline: non fermentative
Oxidase	Positive
Growth in 42 °C	Positive
Citrate	Positive
Urease	Negative

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Table 2. Antibiotic susceptibility patterns of *P. aeruginosa isolates.*

Antibiotic	Antibiotic susceptibility assay					
	Agar dick diffusion (number and percentage of isolates)			**Broth microdilution** (number and percentage of isolates)		
	Resistant	**Intermediate**	**Susceptible**	**Resistant**	**Intermediate**	**Susceptible**
Ciprofloxacin	51 (72.9%)	15 (21.4%)	4 (5.7%)	51 (72.9%)	15 (21.4%)	4 (5.7%)
Levofloxacin	57 (81.4%)	7 (10.0%)	6 (8.6%)			
Norfloxacin	52 (74.3%)	4 (5.7%)	14 (20.0%)			
Ofloxacin	54 (77.1%)	7 (10.0%)	9 (12.9%)			
Amikacin	61 (87.1%)	3 (4.3%)	6 (8.6%)			
Gentamicin	51 (74.3%)	8 (12.9%)	11 (12.9%)			
Ceftazidime	54 (75.7%)	7 (11.4%)	9 (12.9%)			
Imipenem	60 (85.7%)	3 (4.3%)	7 (10.0%)			

Table 3. *nalB* mutations and susceptibility to Ciprofloxacin in 16 isolates of *P. aeruginosa*.

Sample	Nucleotide Mutation	Susceptibility to Ciprofloxacin
1	Transversion in nucleotide number 377, A → T/	Resistant
2	Transition in nucleotide number 264 and 168, T → C and in nucleotide number 327, A → G /	Resistant
3	Transversion in nucleotide number 370, C → A /	Resistant
4	Transition in nucleotide number 327, A → G /	Resistant
5	Transversion in nucleotide number 377, A → T /	Resistant
6	Transition in nucleotide number 168, T → C /	Resistant
7	Transition in nucleotide number 168, T → C /	Resistant
8	Transition in nucleotide number 264 and 168, T → C and in nucleotide number 327, A → G /	Resistant
9	Transition in nucleotide number 327, A → G /	Susceptible
10	Transition in nucleotide number 168, T → C /	Resistant
11	Transition in nucleotide number 327, A → G and Transversion in nucleotide number 377, A → T /	Resistant
12	Transition in nucleotide number 264 and 168, T → C and in nucleotide number 327, A → G /	Resistant
13	Transition in nucleotide number 223 T → C and in nucleotide number 327, A → G /	Resistant
14	Transition in nucleotide number 327, A → G and Transversion in nucleotide number 377, A → T /	Susceptible
15	Transition in nucleotide number 168, T → C and in nucleotide number 264, T → C and in nucleotide number 329, A → G /	Resistant
16	Transition in nucleotide number 166 and 262, T → C and in nucleotide number 325, A→ G /	Susceptible

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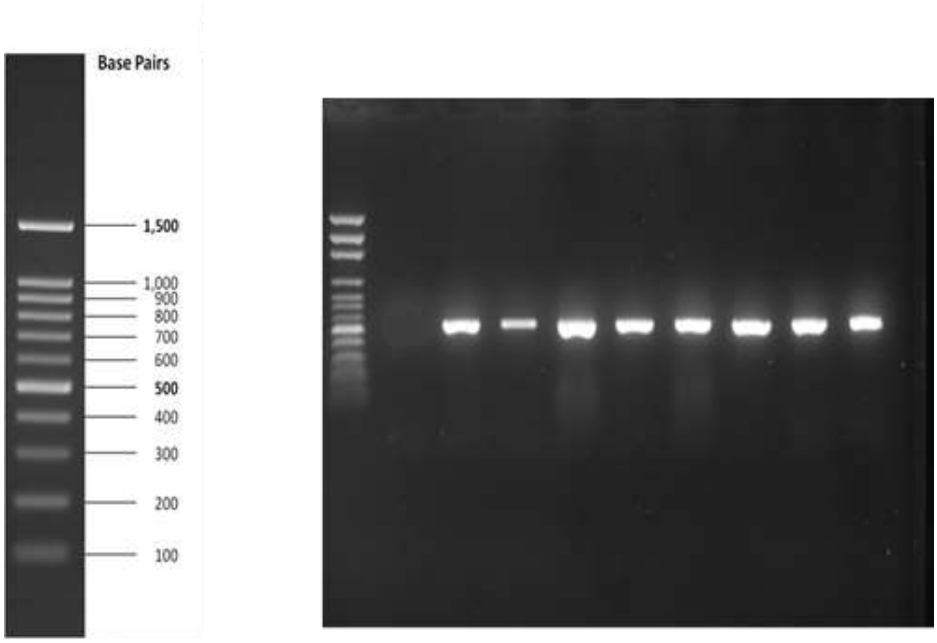

Fig. 1. Amplification of *mexR* gene (545bp). From left to right respectively: Ladder 100 bp; Control Negative; Samples 1-8.

Discussion

Pseudomonas aeruginosa is a central pathogen involved in nosocomial infections, specifically the development of AR in burn patients. Thus, identifying factors affecting AR could play a key role in improving current interventions and infection management (24). *P. aeruginosa* is resistant to a wide range of antibiotics intrinsically and can transfer resistance genes to other bacteria (24, 25). Importantly, *nalB* mutations in *mexR* leads to the overexpression of the mexAB-oprM operon and can result in multidrug resistance (26). Fluoroquinolones, carbapenems and aminoglycosides are among the most critical antibiotics used to combat *P. aeruginosa* infections (13, 25, 27). This study reveals that the identification of *nalB* mutants should be considered in order to prevent the development of AR. Similar to previous reports, the relationship between *nalB* mutants and Ciprofloxacin resistance was found to be significant. Finally, *nalB* mutants can increase MexAB-OprM hyper production activity, thereby leading to AR, specifically fluoroquinolone resistance (16-19, 25, 28-33).

Seventy *P. aeruginosa* strains isolated from burn patients showed the highest resistance to Amikacin (87.1%) compared to Ciprofloxacin (72.9%). There were 16 *nalB* mutants in 70 *mexR* defective strains and 13 *nalB* mutants (81.25%) were found to be resistant to Ciprofloxacin. The relationship between the Ciprofloxacin resistance and *nalB* mutations were significant (p= 0.016). In conclusion, these findings align with previous observations and emphasize the overall importance of *nalB* mutants and AR.

The researchers had no pre-existing knowledge of the medical history of burn patients such as the correlation between the duration of hospitalization and antibiotic susceptibility patterns, type of burn, need for ICU or a ventilator and number of surgeries. It is suggested that the association between these factors and antibiotic susceptibility patterns of the strains could be a critical subject for future investigations. Further research is also required to investigate other potential mutations affecting efflux pumps. Lastly, during our relentless fight against antibiotic resistance, this study further highlights the benefits of gaining a deeper understanding of resistance mechanisms caused by *nalB* mutations.

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