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Minimum Inhibitory Concentration of Ciprofloxacin against *Pseudomonas Aeruginosa* in the Presence of the Efflux Inhibitor Phenylalanine-arginine Beta-naphthylamide

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ABSTRACT

Introduction: *Pseudomonas aeruginosa* is a gram-negative, opportunistic pathogen causing infections in patients staying in the hospital and is resistant to multiple drugs. This study investigated the resistance to ciprofloxacin by the efflux system of *Pseudomonas aeruginosa*. **Materials and Methods:** For this purpose, the inhibitor of the efflux system phenylalanine-arginine beta-naphthylamide was used. In this study, 135 isolates of *Pseudomonas aeruginosa* were collected from the hospitalized patients of Imam Khomeini Hospital and outpatient clinics in Urmia during a ten-month period from June 2015 to March 2016. These isolates were re-identified by standard microbiological and biochemical methods. Finally, 51 isolates were selected for antibiotic susceptibility testing. **Results:** According to the antibiogram test, the *Pseudomonas aeruginosa* isolates exhibited highest resistance against ciprofloxacin (90.2%), tobramycin (88.2%), and gentamycin (86.3%) and the highest sensitivity towards colistin (76.4%), and imipenem (72.5%). The 51 isolates, which were selected for the minimum inhibitory concentration test, had multi-drug resistance regulators. **Conclusion:** The discovery and development of the efflux system inhibitors is an important strategy to deal with bacterial infections.

INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*) is a gram-negative opportunistic pathogen causing infection in patients with a compromised host defense system such as burn injuries, acute keratitis, and cystic fibrosis (1). The treatment of infections in hospitalized patients is difficult due to the presence of antibiotic-resistant isolates (2). *P. aeruginosa* is the leading cause of death among people with cystic fibrosis (3). *P. aeruginosa* has several virulence factors, such as pili, flagella and mucoid glycocalyx that contributes to the mucosal environment of the bacteria and also helps the bacteria against phagocytosis and immunization (1). In addition, it has secretory factors, such as proteases and enzymes, the expression of which is controlled by quorum sensing (4). High levels of intrinsic and acquired resistance to a wide range of antimicrobial agents, including most β-lactams, fluoroquinolones, macrolides, aminoglycosides, tetracycline, chloramphenicol and other antimicrobial agents are observed that is clinically important (5). The intrinsic resistance is the result of the high expression of the efflux pumps, the production of high AmpC chromosomes and the loss of purines, while the acquired resistance is due to the acquisition of resistance genes, such as an extended spectrum of β-lactams (blaSHV, blaTEM, blaVEB, blaPER, blaOXA), and carbapenemases (blaGES, blaKPC, blaIMP, blaSPM, blaVIM, blaNDM) (6). The resistance is due to a combination of the following factors; intrinsic resistance to antimicrobial agents due to the cell wall penetrability, genetic capacity to express a broad set of resistance mechanisms, mutations in chromosomal genes that regulates the expression of resistance genes, and acquisition of resistance genes from other organisms through plasmids, transposons, and bacteriophages (7). The resistance due to efflux system is a growing global problem now (8).

As a result of the activity of the efflux pumps, the concentration of many antibiotics in the bacterial cell decreases; thus resulting in structural resistance to many unconnected antibiotic classes (9). Another reason for the intrinsic resistance of *P. aeruginosa* to some antibiotics is the low

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permeability of the outer membrane (one hundredth of the outer membrane of Escherichia coli (*E. coli*)) (10). Indeed, the intrinsic multi-drug resistance of this organism is due to the synergism of efflux pumps and a low permeability of the outer membrane (11). Biofilm formation protects this bacterium from host immune responses, and results in survival and increased resistance to antimicrobial agents (12). Expression of the efflux pumps is one of the reasons for the resistance of biofilm bacteria to various antimicrobial agents (13). Recently, Zhang et al. reported a new efflux pump PA1877-1818 in *P. aeruginosa*, whose expression was higher in biofilms compared to planktonic growth and appears to be involved in the antibiotic resistance of biofilms (14). The main efflux system involved in multiple-drug resistance in *P. aeruginosa* belong the resistance-nodulation-division (RND) family, which pull out a wide range of drugs and other substrates to the outside of the cell. Among the RND efflux systems, four efflux pumps MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM have the highest clinical significance in *P. aeruginosa*, which are different in terms of their expression pattern and substrate specificity (15). Therefore, using inhibitors of the efflux system can reduce the activity of these pumps and increase the activity of various pharmaceutical agents (substrates of these pumps) against pathogenic bacteria (16).

In this study, phenylalanine-arginine beta-naphthylamide (PAβN) was used as an inhibitor of the efflux system to evaluate its effect on the minimum inhibitory concentration of ciprofloxacin antibiotic as a pump substrate and its effect on *P. aeruginosa* resistance against ciprofloxacin was investigated.

MATERIALS AND METHODS

Data collection and *P. aeruginosa* isolates; A number of *P. aeruginosa* isolates from different clinical specimens, including urine, wound secretions, tracheotomy, blood samples, and bronchus of patients referred to the Imam Khomeini Hospital of Urmia were collected from June 2015 to March 2016 and transferred to the medicine faculty. The collected samples were cultured on the Muller Hinton agar, and suspected colonies were purified and re-identified. Identification of the isolates was carried out through standard microbiology methods, including hot staining, colony morphology, movement evaluation, indole methyle red voges proskauer and citrate (IMViC) test and specific biochemical test, such as oxidase test. Finally, 51 isolates of *P. aeruginosa* were selected for the antibiotic susceptibility test.

Antibiotic susceptibility test and determination of minimum inhibitory concentration (MIC) of ciprofloxacin; An antibiotic susceptibility test for *P. aeruginosa* isolates was performed on Muller Hinton agar based on Clinical & Laboratory Standards Institute (CLSI) (17) using diffusion disk (Kirby-Bauer) method. The antibiotic disks (MAST, made by U.K.) were imipenem (10 µg), aztreonam (10 µg), cefepime (30 µg), ceftazidime (30 µg), gentamicin (10 µg), ciprofloxacin (30 µg), tobramycin (10 µg), colistin (10 µg), amikacin (30 µg), piperacillin-tazobactam (10/100 µg). In this method, bacterial suspensions were prepared as 0.5 McFarland and spread on the Muller Hinton agar. Then, antibiotic disks were placed at appropriate intervals on the agar and incubated at 35°C for 16 h. After this time, the diameter of the halo around each disc was measured and the results were recorded. Positive control and negative control strains for the antibiogram included *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922, respectively. In the next step, the minimum inhibitory concentration (MIC) of ciprofloxacin against these isolates was determined by the broth microdilution method. Serial dilutions (0.225-125 µg/ml) of the antibiotic were prepared for the MIC test.

The antibiotic ciprofloxacin was purchased in the form of a lyophilized powder (SIGMA ALDRICH) and was dissolved in its appropriate solvent (sterile water) and an antibiotic stock (ten times more concentration) was prepared ((ESCMID) -2003- Clinical_Microbiology_and_Infection). The MIC test was carried out in 96-well microplates, each row had a specific isolate, along with a positive control well (a mixture of a Muller Hinton broth and bacterial suspension) and a negative control well (a mixture of Muller Hinton broth and antibiotics), and incubated for 18 h at 35°C and the results were recorded and analyzed according to the standards set by CLSI.

Determination of MIC of ciprofloxacin in presence of PAβN: As already known, efflux systems are one of the main reasons for the resistance to antibiotics (18). To demonstrate the role of these systems of *P. aeruginosa* in ciprofloxacin resistance, the MIC test for the isolates of ciprofloxacin-resistant bacteria were performed in the presence of the PAβN inhibitor. The purified isolates of *P. aeruginosa* were exposed to 50 µg/ml of PAβN (15, 19) and then cultured on the Muller Hinton Agar and incubated for 24 h at 35°C. The MIC tests were performed using serial dilutions of ciprofloxacin (0.125-0.256 µg/ml). The result was recorded according to CLSI.

RESULTS

Out of the 135 isolates, 51 were selected for antibiotic susceptibility testing. Antibiogram test showed highest resistance of the *P. aeruginosa* isolates against ciprofloxacin (90.2%), tobramycin (88.2%), gentamycin (86.3%), and the highest sensitivity against colistin (76.4%) and imipenem (72.5%). All the 51 isolates had multidrug resistance regulators (MDRs) (Table 1).

The MIC results were compared with the standards set by CLSI. Based on the comparison, 45 isolates (88.2%) showed resistance and 2 isolates (3.9%) exhibited intermediate resistance to ciprofloxacin. Four isolates (7.8%) showed sensitivity to ciprofloxacin. To evaluate the effect of efflux inhibitor PAβN on the minimum inhibitory concentration of this antibiotic, antibiotic susceptibility test in the presence of this efflux inhibitor was performed with 47 isolates that were resistant to ciprofloxacin. The results were classified as sensitive, intermediate and resistant. In the presence of the efflux inhibitor, the resistance to ciprofloxacin decreased by 12.8%. The results of are presented in Table 2.

DISCUSSION

For many years, *Pseudomonas aeruginosa* has been recognized as the leading cause of ulcer and surgical infections. It is an opportunistic and invasive secondary pathogenic bacterium that does not cause infection in healthy tissues. It is mainly found in patients with immunodeficiency and in patients with cystic fibrosis (20). This organism shows a high level of intrinsic resistance and only a limited number of antimicrobial agents are active against it. In addition, *P. aeruginosa* is able to withstand the existing anti-*Pseudomonas* agents (21).

Efflux pumps are one of the important causes of multiple drug resistance in bacteria (22). However, the high prevalence of resistance in bacteria is not due to the presence of the multi-drug efflux system; nevertheless, the expression of efflux system genes cannot be ignored in the MDR isolates (23). Since, inhibition of the efflux system can increase the intracellular concentration of drugs and thus increase their effectiveness, the discovery and development of the efflux system inhibitors is an important strategy to deal with bacterial infections (15).

In this study, when the MIC test was performed with the 51 isolates using the microdilution method, 45 isolates (88.23%) were resistant and 2 isolates (3.92%) showed an intermediate resistance to ciprofloxacin. The results of this study were consistent with the study of Adabi et al. (21), where sensitivity test using dilution agar method showed resistance to this antibiotic in 127 isolates (82.5%), although the volume of the isolates in the two studies were different. However, the resistance rate for ciprofloxacin was higher in our study compared to the study of Sonnet et al., where, approximately 25% of the isolates were resistant (19). Gilli et al., during two years (2014-2015) in India, determined the susceptibility pattern of *P. aeruginosa* isolates by the MIC method using the Vitek2 system and showed that 96% of the isolates were resistant to ciprofloxacin and 44% of the studied isolates had MDR phenotype (22).

In the presence of PAβN, the efflux system inhibitor, 61.7% of the isolates showed 2-2.56 times reduction in MIC of ciprofloxacin, of which 44.7% exhibited two times reduction of the MIC. This result was consistent with the study carried out by Fernando and Kumar, who reported that fluoroquinolones are the major substrates for the efflux pumps in *P. aeruginosa* isolates (24).

On the other hand, 38.8% of the isolates did not show any reduction in the MIC levels of ciprofloxacin in the presence of inhibitors. Mechanisms, such as mutations in the target enzyme (DNA gyrase) were likely to be involved in the resistance of these isolates against ciprofloxacin (20).

Walkty et al. examined the drug resistance pattern of 2906 clinical isolates of *P. aeruginosa* by microdilution method and found that 23.5% of the isolates exhibited resistance or intermediate resistance. In 421 isolates with MDR phenotype, the resistance to ciprofloxacin was 77% in this study (23). In a similar study, Sader et al. reported that 66.5% of the MDR isolates of *P. aeruginosa* were resistant to ciprofloxacin (24).

Other studies have also shown a 23% to 57% resistance of the *P. aeruginosa* isolates against ciprofloxacin (25-27). Negi et al. reported that 18% of the *P. aeruginosa* isolates were resistant to more than 3 classes of antibiotics (27).

Many studies have been conducted on the resistance patterns of *P. aeruginosa* in Iran and other countries, and the results are different. The results are dependent on the type of sample and the differences in the origin of the isolates, time and place, the method and technique of studying and the use of antibiotics. *P. aeruginosa* has become resistant to more number of antibiotics in the recent years (28- 30), and this resistance is increasing rapidly.

Pseudomonas aeruginosa is resistant to all the existing anti-*Pseudomonas* agents (21). The efflux system, or the active transfer of antibiotics outside the cell, is also a major cause of multiple drug resistance in bacteria, including *P. aeruginosa* (31). However, the high prevalence of resistance in bacteria is not due to the presence of a multi-drug efflux system alone. The expression of the efflux system in the MDR isolates cannot be ignored (32). Since inhibition of the efflux system can increase the intracellular concentration of drugs and increase their effectiveness, the discovery and development of the inhibitors of the efflux system is an important strategy for coping with bacterial infections (15).

In the presence of an inhibitor, 47 isolates were found to be resistant to ciprofloxacin, 29 isolates showed a 2 fold or more reduction in MIC, while in a previous study by Sonnet et al., 27 isolates were resistant to ciprofloxacin, and 25 isolates

Table 1. Antibiotic susceptibility pattern of *P. aeruginosa* isolates using disk diffusion method

Antibiotic	Resistant N (%)	Intermediate N (%)	Susceptible N (%)
Imipenem	10 (19.6%)	4 (7.8%)	37 (72.5%)
Ceftazidime	31 (60.8%)	5 (9.8%)	15 (29.4%)
Aztreonam	32 (62.7%)	12 (23.5%)	7 (13.7%)
Cefepime	34 (66.7%)	4 (7.8%)	13 (25.5%)
Colistin	12 (23.5%)	-	39 (76.5%)
Ciprofloxacin	46 (90.2%)	3 (5.9%)	2 (3.9%)
Amikacin	20 (39.2%)	2 (3.9%)	29 (56.9%)
Gentamicin	44 (86.3%)	1 (2%)	6 (11.7%)
Tobramycin	45 (88.2%)	1 (2%)	5 (9.8%)
Piperacillin/ Tazobactam	19 (37.2%)	18 (35.3%)	14 (27.5%)

Table 2. Minimum inhibitory concentration of ciprofloxacin for MDR isolates of *P. aeruginosa*

Antibiotic	In the absence of inhibitor	In the presence of inhibitor	
	Resistant N (%)	Susceptible N (%)	Resistant N (%)
Ciprofloxacin	47 (100)	6 (12.8)	41 (87.2)

showed a 4 fold or more reduction in MIC (19). The comparison of these two studies suggests that MIC reduction in the presence of inhibitor in our study was twice as low as in the study of Sonnet et al. One of the most probable reasons is that in our isolates, mechanisms other than the activity of the efflux system have been implicated in resistance to ciprofloxacin, and therefore, the inhibitor of the above-mentioned efflux system has little effect on the reduction of MIC. Lomovskaya et al. believed that the use of an inhibitor of the early efflux system could prevent resistance to fluoroquinolones (33).

CONCLUSION

The discovery and development of the efflux system inhibitors is an important strategy to deal with bacterial infections and the inhibition of the efflux system has little effect on the reduction of MIC.

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AUTHER CONTRIBUTIONS

All authors contributed equally in this study.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

ETHICAL STANDARDS

This study was approved by the ethics committee of Urmia University of Medical Sciences.

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