Characterization of efflux pump mediated resistance against fluoroquinolones among clinical isolates of *Pseudomonas aeruginosa* in northeast of Iran and its association with mortality of infected patients

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Summary

Pseudomonas aeruginosa is an opportunistic human pathogen which causes serious nosocomial infections with a high level of resistance. Fluoroquinolone resistance is mostly due to efflux pump over-expression and target mutations. Multidrug-efflux transporter MexB in MexAB-OprM pump is a mechanism for this resistance. The present study aimed to investigate the prevalence of resistance against fluoroquinolones among clinical isolates of *P. aeruginosa* in Mashhad, Iran.

This cross-sectional study was performed on 150 *P. aeruginosa* isolates from hospitalized patients in burn and intensive care units of the two main hospitals of Mashhad. The bacteria were identified primarily based on biochemical tests. Then antibiotic susceptibility was studied by disk diffusion method against 11 antibiotics, and minimum inhibitory concentration (MIC) was measured for ciprofloxacin, levofloxacin, and ofloxacin. Finally, the presence of *mexB* gene was evaluated using PCR method.

One hundred thirty-two isolates (88%) were multidrug resistant, 8 isolates (5.3%) were completely susceptible, and 10 isolates (6.6%) were totally drug resistant. We detected 76 (50.8) ciprofloxacin-resistant isolates and all of them were resistant to one or more other tested antibiotics. No isolate was resistant to colistin. Based on the PCR results, 42% of clinical isolates had the *mexB* gene and *mexAB-oprM* operon, and this pump was more prevalent (80%) among ciprofloxacin-resistant isolates. In Conclusion in this study, where multidrug resistant *P. aeruginosa* isolates were rampant, ciprofloxacin and multidrug resistance due to the overexpression of efflux pumps were frequent. Based on our results, there is a stronger association between *mexB* gene and the mortality of patients.

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Pseudomonas aeruginosa, Fluoroquinolone, Resistance, mexB, Efflux pump

Introduction

Healthcare-associated infections are an emerging medical problem in developing countries which cause morbidities, mortalities and health care costs.[1-3] *Pseudomonas aeruginosa* is an opportunistic human pathogen and common cause of hospital-acquired infections in patients with immunosuppression. It is considered as a serious threat for patients hospitalized with cystic fibrosis and burns.[1,2] Numerous studies have reported the occurrence of multidrug-resistant *P. aeruginosa*.[4] Recently, *P. aeruginosa* with increasing trends of drug-resistance is a significant threat to effective treatment of infections.[5,6] There are several mechanisms for drug resistance in *P. aeruginosa* including: 1) reduced antibiotic permeability due to the reduced expression or loss of OprD porin, 2) MexAB-OprM pump over-expression which increases antibiotic efflux, 3) Production of antibiotic-inactivating enzymes.[7,8]

Efflux pumps can confer high resistance to previously effective antibiotics among *P. aeruginosa*.[9] They can export one or several antimicrobial compounds such as ciprofloxacin, tetracycline, and gentamicin from the cytoplasm or periplasmic space.[10-13] Resistance-nodulation-cell division (RND) family is a major class of efflux pumps. At least five RND efflux systems have been well characterized in *P. aeruginosa*: MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM and MexJK-OprM.[9,10,13-17] MexAB-OprM is the one responsible for extrusion of quinolones and β-lactams and has an important role in fluoroquinolone resistance in *P. aeruginosa* and includes MexB, MexA, and OprM.[18,19] MexB is encoded by *mexB* gene and is a proton-driven antiporter in MexAB-OprM pump.[20,21]

Fluoroquinolones are a group of broad-spectrum antibiotics which directly inhibit bacterial DNA synthesis.[5,13,22] Since 1960, they have been used extensively for multiple clinical infections throughout the world due to their potency, oral bioavailability, spectrum of activity, and generally good safety profile. Although they are still clinically valuable, their use has become limited in some clinical settings because of an emerged bacterial resistance over the time.[23]

Quinolone-resistance during the treatment was first reported in *P. aeruginosa* and *Staphylococcus aureus*. This resistance spread to other gram-positive and gram-negative bacteria in hospitals. The subpopulations of pathogens which were drug-resistant, became prevalent two decades ago and they have remained approximately unnoticed. Recent surveillance studies showed that the resistance rate have continued to increase. This would affect the patient management and treatment guidelines.[24]

Nowadays, ciprofloxacin is used frequently in our country for treatment of many infections due to *P. aeruginosa*. Surveillance studies have reported that the resistance against fluoroquinolones is increasingly vigorously. So it is essential to know the bacterial mechanisms for resistance.[13,25]

This study tried to assess the prevalence of efflux-driven fluoroquinolone resistance in clinical isolates of *P. aeruginosa*, and investigate the relationship between the MexAB-OprM pump and the clinical characterization of infection, especially the mortality rate.

Materials and Methods

This study included 280 isolates of *P. aeruginosa* obtained during one year from patients in Imam Reza and Ghaem university hospitals as the two main hospitals in Mashhad, northeast of Iran. Clinical data of these patients were also recorded. Based on the adequate clinical records, 150 *P. aeruginosa* isolates were selected for further evaluation. The main inclusion criterion was the contribution of isolated bacteria to clinical infection according to physician's opinion. Our isolates included 73 (48.6%) *P. aeruginosa* isolates from burn infections, 46 (30.6%) from lower respiratory tract infections and 31 (20.6%) from urinary tract infections. Initially, isolated bacteria were identified based on the standard biochemical tests.[26]

The patients ranged in age from two days to 70 years and included 84 males and 66 females.

Studied antibiotics

Antibiotic susceptibility patterns were determined using disk diffusion method for 11 common antibiotics on Mueller-Hinton Agar. All isolates were tested against imipenem (10 µg), cefepime (30 µg), ticarcillin (75 µg), levofloxacin (5 µg), tobramycin (10 µg), gentamicin (10 µg), colistin (25 µg), amikacin (30 µg), ciprofloxacin (5 µg), piperacillin (100 µg), and ofloxacin (5 µg) using MASTDISCS™ (Mast Diagnostics, Merseyside, UK). They were reported as sensitive, intermediate, and resistant according to CLSI recommendations. The minimum inhibitory concentration (MIC) of ofloxacin, levofloxacin, and ciprofloxacin (Sigma-Aldrich, UK) were determined using micro broth dilution method. Drug concentrations ranging from 0.5 to 256 mg/L were tested for these three fluoroquinolones and interpreted according to the established breakpoint values recommended by CLSI.[27]

DNA extraction and PCR

In this study, bacterial DNA was extracted by boiling method from 150 *P. aeruginosa* isolates. Boiling was performed in hot water for 15 min to lyse the cells

Tabel 1 Demographic information of patients and the related studied specimens

Infection type related to the studied isolate	Burn infection, lower respiratory tract, and urinary tract infections
Patients and specimens	66 female, 84 male
	46 children, 104 adults
	31 dead, 119 alive
	78 Imam Reza hospital, 72 Ghaem hospital
	74 Ciprofloxacin-sensitive, 76 Ciprofloxacin-resistant

and then the lysates centrifuged at 10,000 g for 10 min. Finally, the supernatant was transferred to another microtube and used as the DNA template. The extracted DNA was evaluated by agarose gel electrophoresis. Negative control strains included PAO1 (wild type)[11][10], and the positive control was obtained from Pasteur Institute, Iran.

For PCR, the *mexB* gene sequence was obtained from the NCBI, and specific primers were designed using Primer3 (htpp://Frodo.wi.mit.edu.primer3). Then the primers were re-evaluated using GeneRunner software and Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The PCR amplification was performed using the following primers: Forward 5'-CATTGATAGGCCCATTTTCG-3' and Reverse 5'-GATTGTCGATCCCGTTCATC-3'.

The PCR reaction contained 0.5 µl of 10mM dNTPs (CinnaGen, Iran), 2.5 µl of 10x PCR buffer (ParsTous, Iran), 1.5 µl of 25mM MgCl2 (ParsTous, Iran), 0.2 µl of 5U/µl Taq DNA polymerase (CinnaGen, Iran), 1µl of each 10 µM primer, 5 µL of DNA template, and PCR grade water up to the final volume of 25 µl.

PCR amplification was performed by a thermal cycler (Thouchgene, Gradicent). Cycling consisted of initial denaturation at 94º for 5 minutes, 38 cycles of denaturating at 94º for 60 sec, annealing at 52º for 45 sec and extension at 72º for 45 sec and a final extension at 72º for 10 minutes. To avoid false positive, separate areas were used for sample preparation, amplification, and analysis of the amplified products. Also, positive and negative controls were included.

Aliquots of amplified samples were loaded on 1.5% agarose gels and subjected to electrophoresis for 30 minutes at 80 volts. Visualization of a 210 bp DNA band was considered as positive.

Sequencing and phylogenetic analysis:

The *mexB* gene was sequenced and compared with sequences at NCBI for *P. aeruginosa* and some other gram-negative rods.

Positive PCR products of each resistogram pattern were sequenced. The *mexB* gene of 7 resistant isolates of this study as: *P. aeruginosa* 1-*mexB*, *P. aeruginosa* 2-*mexB*, *P. aeruginosa* 3-*mexB*, *P. aeruginosa* 4-*mexB, P. aeruginosa* 5-*mexB, P. aeruginosa* 6-*mexB, P. aeruginosa* 7- *mexB* and the *mexB* genes data of *Escherichia coli-mexB, Acinetobacter baumannii-mexB, Klebsiella pneumonia-mexB, Rhodopirellula baltica-mexB, P. aeruginosa-mexB* taken from GenBank were analysed. The sequences were analysed by MEGA for phylogenetic relations among these isolates and to study the possible origin of *mexB* gene among these resistant isolates. To analyze the data, we used the SPSS 16 software by chi-square test.

Results

150 strains of *P. aeruginosa* were confirmed using biochemical tests. According to disk diffusion method, 142 isolates were at least resistant to one antibiotic, and 132 (88.8%) isolates were resistant against two or more antibiotics. The highest resistance rates was observed against tetracycline (88%) and tobramycin (87.3%) and the lowest resistance rates were seen against colistin (14%). For ciprofloxacin as an important therapeutic choice for *P. aeruginosa*, we observed 50.8% resistance. It is interesting that all of ciprofloxacin-resistant *P. aeruginosa* were resistant to one or more other tested antibiotics. The pattern of resistance against these antibiotics is presented in Figure 1.

The range of MIC by micro broth dilution method was 0.5-16 mg/L for ofloxacin and levofloxacin and 0.5-164 mg/L for ciprofloxacin. The MIC obtained for ciprofloxacin showed that 54% of *P. aeruginosa* isolates should be categorized as resistant.

The *mexB* gene was detected in 60 (42%) isolates by PCR. Figure 2 shows the results for agarose gel electrophoresis of *mexB* PCR product and the related 210 bp band.

The frequency of *mexB* gene is shown in Figure 3. Accordingly, this efflux pump is more prevalent among *P. aeruginosa* isolated from patients with burn infection (55%) and then from patients with lower respiratory tract infection (33.1%).

Statistical analysis confirmed an association between *mexB* gene and resistance of bacteria against ciprofloxacin (chi-square; P value < 0.05) (Figure 4).

Statistical analysis showed a stronger association between *mexB* gene and the mortality of infected patients (chi-square; P value < 0.05) than *mexB* and resistance (Figure 5). Further analysis confirmed a significant correlation between the presence of *mexB* gene and mortality in this study (correlation 0.625).

Sequencing and phylogenetic analysis of mexB gene:

To further investigate the genetic characteristics and origin of *mexB* among these isolates, the *mexB* gene was sequenced and compared with sequences at NCBI for *P. aeruginosa* and some other gram-negative rods. Consistent with the sequence alignment data, most of these isolates showed high homology at the nucleotide level. Importantly, some nucleotide variations were observed among them in two regions (codon 148 and codon 120). Nucleotides TG in codon 148 of *mexB* gene of *P. aeruginosa* PAO1 changed to CA in strains 1, 3, 5, 7 (Figure 6).

Analysis with BioEdit software showed that strains 1, 3, 5 and 7 were 100% matched together and 94% matched with wild-type *P. aeruginosa* (Figure 7). So,

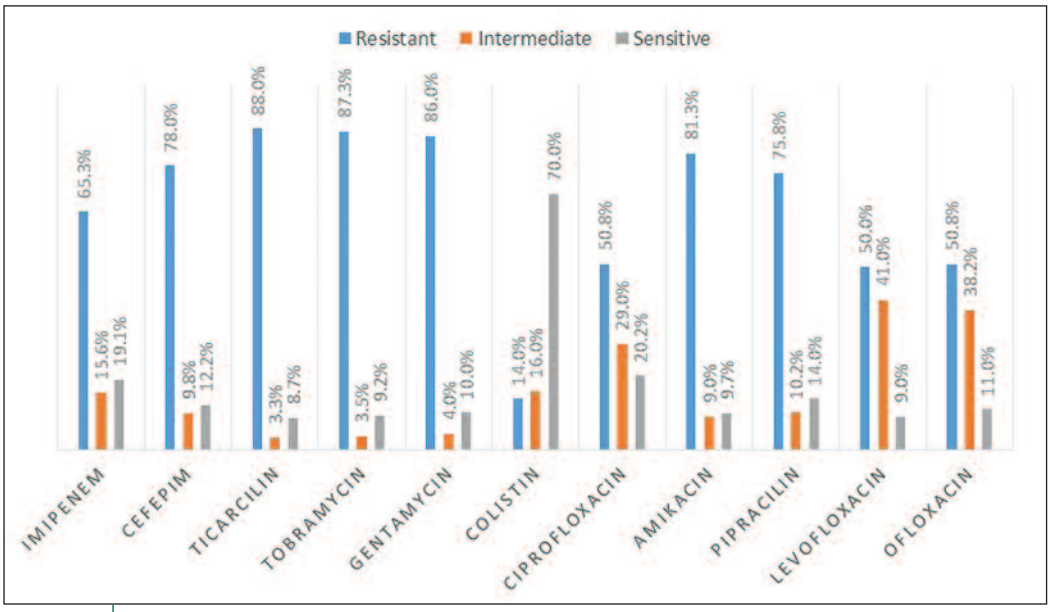

Figure 1 The resistance pattern of the isolates against 11 studied antibiotics. The percentage for sensitive, intermediate, and resistant isolates are shown.

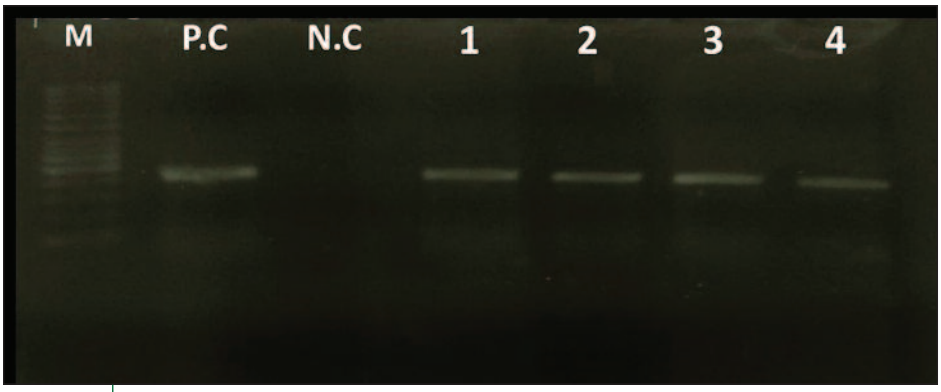

Figure 2 Agarose gel electrophoresis of *mexB* PCR product. M: 50 bp DNA size marker, P.C: Positive control, N.C: Negative control, 1 to 4: 210 bp band related to *mexB* gene

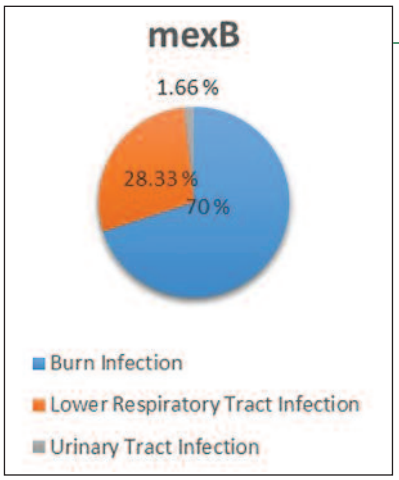

Figure 3

The frequency of *mexB* gene in different specimens. 60 (42%) of 76 fluoroquinolone-resistant isolates contained the *mexB* gene. 42 isolates (70%) were related to burn infection, 17 (28.33%) to lower respiratory tract infection and 1 (1.66%) to urinary tract infections.

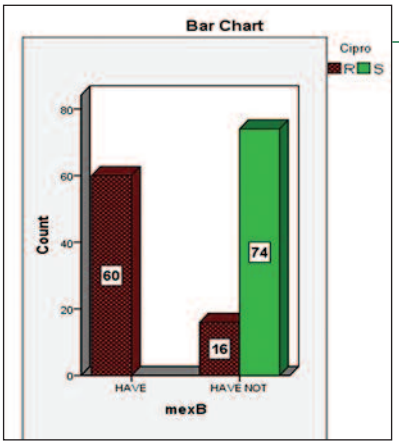

Figure 4

Association of ciprofloxacin resistance with the presence of *mexB* gene. Among 150 studied isolates, 60 isolates were *mexB*-positive and all of them were ciprofloxacin-resistant. 90 remaining isolates were *mexB*-negative and they included 74 ciprofloxacin-sensitive and 16 ciprofloxacin-resistant isolates.

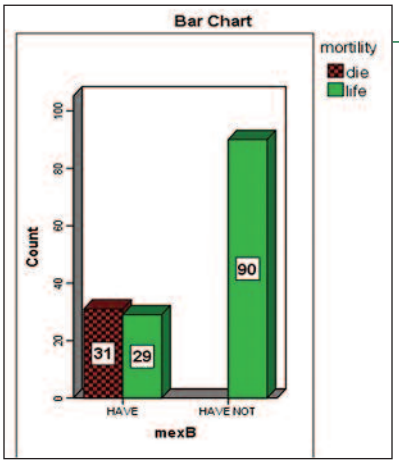

Figure 5

Association of mortality with the presence of *mexB* gene. Among 150 studied patients, 31 died and *mexB* gene was detected in all 31 related isolates. 119 remaining patients were alive and 29 of them were related to *mexB*-positive isolates and 90 to *mexB*-negative isolates.

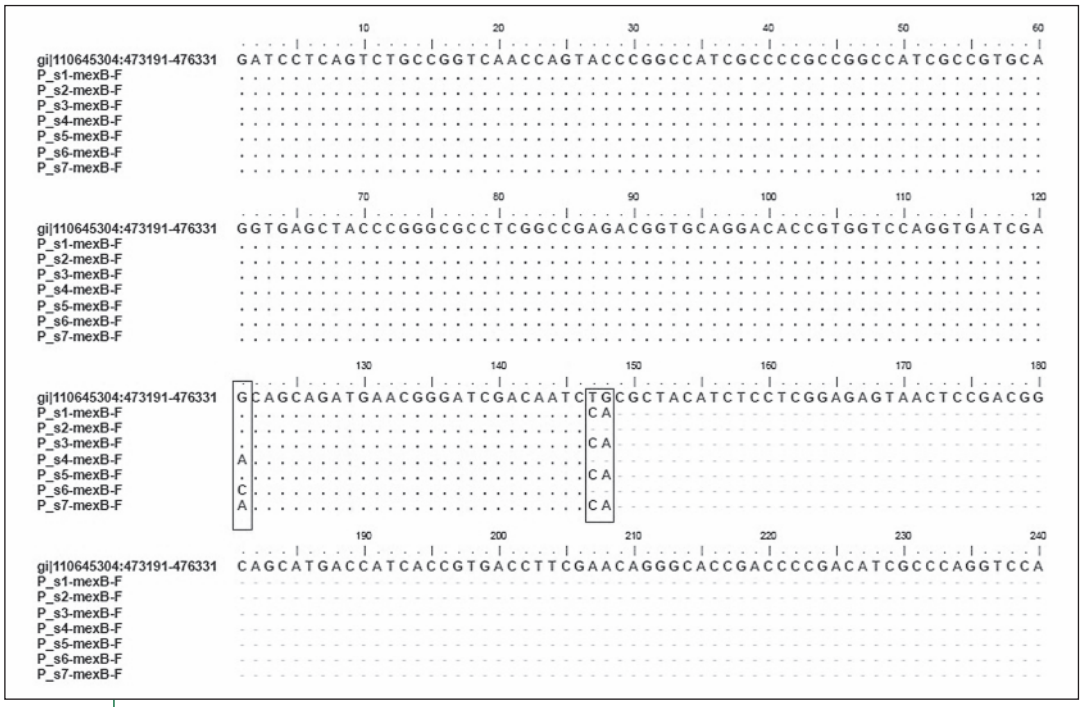

Figure 6 The *mexB* sequencing and alignment results. Alignment of *mexB* genes of our isolates and wild strain *P. aeruginosa* PAO1.

mexB sequence analysis and evaluation of their similarity with one another and with the wild-type strain proposed absence of drastic changes.

The phylogenetic tree was constructed using the neighbor-joining method with Mega6 software (Bootstrapping 1000 and Cutoff 95%; Kimura 2-parameter). This phylogenetic tree confirmed high similarity for *mexB* gene among studied *P. aeruginosa* strains which were resistant against fluoroquinolones and suggest a common origin for them with *P. aeruginosa* PAO1 (wild-type strain) (Figure 8). It could be supposed that mexB gene is from common origin among *P. aeruginosa* and some bacteria of *Enterobacteriaceae* family.

Discussion

In our study, the presence of *mexB* efflux pumps was observed in 42% of fluoroquinolone-resistant isolates which were much lower than rates previously reported for the *mexB* pump in the south of Iran (87.87%).[28]

Sequence Identity Matrix

Seq	gi|110645304	P_s1-mexB-F	P_s2-mexB-F	P_s3-mexB-F	P_s4-mexB-F	P_s5-mexB-F	P_s6-mexB-F	P_s7-mexB-F
gi|110645304	ID	0.941	0.941	0.941	0.935	0.941	0.935	0.935
P_s1-mexB-F	0.941	ID	0.986	1	0.979	1	0.979	0.993
P_s2-mexB-F	0.941	0.986	ID	0.986	0.993	0.986	0.993	0.979
P_s3-mexB-F	0.941	1	0.986	ID	0.979	1	0.979	0.993
P_s4-mexB-F	0.935	0.979	0.993	0.979	ID	0.979	0.993	0.986
P_s5-mexB-F	0.941	1	0.986	1	0.979	ID	0.979	0.993
P_s6-mexB-F	0.935	0.979	0.993	0.979	0.993	0.979	ID	0.979
P_s7-mexB-F	0.935	0.993	0.979	0.993	0.986	0.993	0.979	ID

Figure 7 The *mexB* genes identity (ID) of our 7 analyzed sequences (resistant isolates) to each other and to the wild-type strain gene: P-s1, P-s3, and P-s5 are 100% identical with each other and 94% identical to the *mexB* gene of wild-type strain. This sequence identity confirmed the absence of drastic nucleotide changes.
(Provided by sequence identity matrix, BioEdit software)

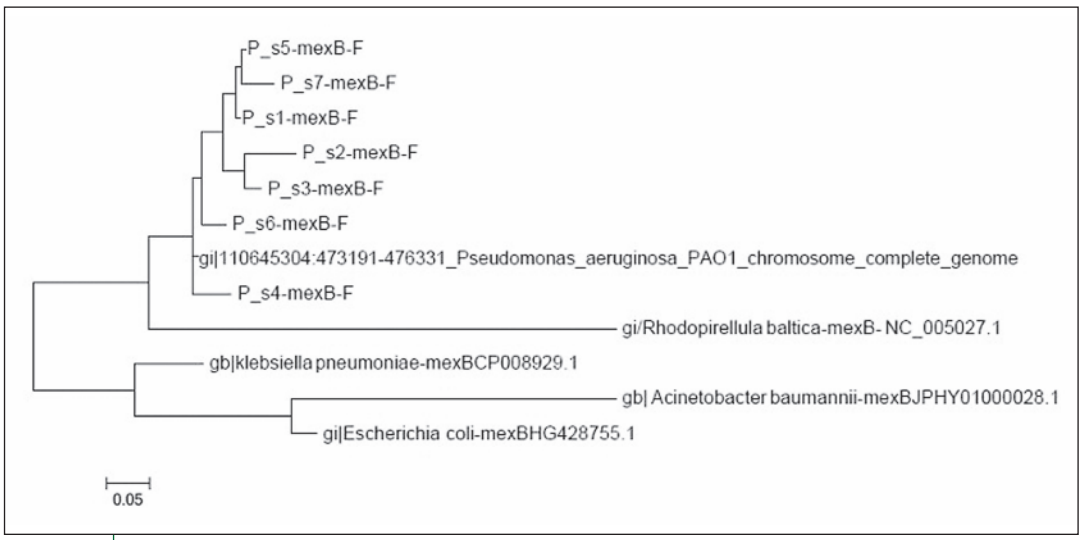

Figure 8 **The phylogenetic tree for mexB gene of 7 resistant isolates in this study.** *P. aeruginosa* 1-*mexB, P. aeruginosa* 2-*mexB, P. aeruginosa* 3-*mexB, P. aeruginosa* 4-*mexB, P. aeruginosa* 5-*mexB, P. aeruginosa* 6-*mexB, P. aeruginosa* 7- m*exB* and the *mexB* genes data of *Escherichia coli-mexB, Acinetobacter baumannii-mexB, Klebsiella pneumoniae-mexB, Rhodopirellula baltica-mexB, P. aeruginosa-mex*B taken from GenBank were analysed. The phylogenetic tree was constructed using the neighbor-joining method. The scale bar 0.05 indicates 2% of nucleotide sequence substitution.

This lower resistance in our study is perhaps due to different types of infections and multicentric design of our study.

P. aeruginosa as an opportunistic pathogen is one of the main causes of nosocomial infections.[29] The findings of this study clearly showed very high resistance rate among *P. aeruginosa* strains against most of the antibiotics in our region. Most of the isolated *P. aeruginosa* were resistant against at least two or more antibiotics. The resistance rate was especially high in *P. aeruginosa* isolated from burned patients. There was a significant correlation between the presence of *mexB* gene and mortality which was more significant than the association of this gene with resistance against ciprofloxacin. Although further research is needed for explaining this association, it could be due to the role of this pump in the pathogenesis of *P. aeruginosa* and resistance to other existing antimicrobial agents through overexpression of efflux pump in addition to fluoroquinolone resistant. This hypothesis has been strengthened as these efflux pumps have broad substrate specificity, and extrude many classes of antibiotic including β-lactams and quinolones.[30]

Ciprofloxacin is frequently used as an effective treatment against Pseudomonas but due to increasing resistance to ciprofloxacin and increased mortality, it is important to choose the antibiotics more carefully according to susceptibility tests. The least resistance was observed against colistin, tetracyclines, and aminoglycosides. The results of this study propose that these antibiotics should be considered as effective therapeutic choices.

In a study in Tehran, 170 strains of *P. aeruginosa* were isolated from burn patients. Among isolated bacteria, resistance against amikacin and imipenem were reported in 81.70% and 52.90% of isolates.[31] By comparing the results of this study, it can be concluded that there is an increasing trend of resistance to antibiotics, although colistin still shows low resistance rate.

In another study, 250 patients with high-degree burn were studied in the Ghotbodin Shirazi hospital in Shiraz. 26.40% (66 cases) of patients with burn wounds were infected with *P. aeruginosa* which were resistant to all antibiotics tested except colistin that emphasized the dangerous trend of drug resistance.[28] In this study, 66.66% of the isolates (44 cases) had an efflux pump, among them 42.92% and 87.87% carried *mexA* and *mexB* genes, respectively.[32] In another study on 53 isolates of *P. aeruginosa* from patients with cancer, lymphoma, burns, and bacteremia, 100% of isolates were positive for *mexAB-oprM* genes of operon and explained high resistance to antibiotics among the isolates.[17]

In a study on 104 *P. aeruginosa* isolated from burn patients in a hospital in Tehran, a very high resistance rate to nalidixic acid (86.54%) and ofloxacin (81.87%) and much lower resistance to imipenem (40.91%) and piperacillin (44.9%) were reported. In this study, the gene coding MexAB-OprM efflux pump was present in 27% of clinical isolates of *P. aeruginosa* which were resistant to quinolones.[32] This rate was lower than our results perhaps due to different types of infections.

In these studies, in contrast to former studies, there was a significant increase in resistance against new antibiotics in comparison to older ones, and there was a significant increase in the detection of genes coding the efflux pump among isolated bacteria. According to this study and other reports, the emergence of antibiotic-resistant strains of *P. aeruginosa* is on the rise.

Based on statistical analysis of the results of this study, a significant association was shown between the drug resistance and mortality of the patients. Also for the first time, a very stronger correlation (0.625) was observed between the gene coding an efflux pump (MexAB-OprM) and the mortality of the patients. The results of this study indicate that a significant association was present between the *mexB* and antibiotic resistance in *P. aeruginosa* and also mortality among patients admitted to these two hospitals. This relationship necessitates further research to find the reason in addition to needs for special attention to trace the epidemiology and transmission of *mexB* genes in nosocomial infections.

New strategies for combating *mexB* gene such as siRNA targeting *mexB* gene and blocking the activity of this pump can be used as a new tool for the treatment of infections due to *P. aeruginosa*.

Conclusion

The high rate of resistance against an important group of antibiotics in our study is a serious alarm for the healthcare providing system in our region. So antibiotic stewardship programs specially selecting appropriate medications are highly recommended and should be followed vigorously.

Acknowledgement

We are grateful to our colleagues in Faradid biotechnology laboratory for their cooperation in this work.

Conflict of interests

The authors declare no conflict of interests.

Περίληψη

Χαρακτηρισμός της οφειλόμενης σε αντλίες εκροής αντοχή στις φλουοροκινολόνες μεταξύ κλινικών στελεχών *Pseudomonas aeruginosa* που απομονώθηκαν στο ΒΑ Ιράν και συσχέτισή τους με τη θνητότητα μολυσμένων με τα στελέχη αυτά ασθενών

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Το μικρόβιο *Pseudomonas aeruginosa* είναι ευκαιριακό παθογόνο το οποίο προκαλεί σοβαρές νοσοκομειακές λοιμώξεις και παρουσιάζει υψηλά επίπεδα αντοχής στα αντιβιοτικά. Η αντοχή στις φλουοροκινολόνες οφείλεται κυρίως στην υπερέκφραση αντλιών εκροής, καθώς και σε συγκεκριμένες σε αυτές μεταλλάξεις. Ο μεταφορέας εκροής πολλών φαρμακευτικών ουσιών MexB στην αντλία MexAB-OprM είναι ένας από τους κυριότερους μηχανισμούς αντοχής. Η παρούσα μελέτη σκοπό είχε τη μελέτη συχνότητας αντοχής στις φλουοροκινολόνες κλινικών στελεχών *P. aeruginosa* στην πόλη Mashhad του Iran. Μελετήθηκαν 150 στελέχη *P. aeruginosa* που απομονώθηκαν από νοσηλευόμενους ασθενείς στις Μονάδες Εγκαυμάτων και Εντατικής Θεραπείας δύο κύριων νοσοκομείων στην πόλη Mashhad. Η αρχική ταυτοποίηση των βακτηρίων έγινε με τις κλασικές βιοχημικές δοκιμασίες και ακολούθησε έλεγχος αντοχής έναντι 11 αντιβιοτικών και μέτρηση της Ελάχιστης Ανασταλτικής Συγκέντρωσης (MIC) σε σιπροφλοξασίνη, λεβοφλοξασίνη και οφλοξασίνη. Η παρουσία, τέλος του *mexB* γονιδίου μελετήθηκε με PCR μεθοδολογία.

Σύμφωνα με τα αποτελέσματα της μελέτης, 132 στελέχη (88%) παρουσίαζαν πολυαντοχή, 8 (5.3%) ήταν πλήρως ευαίσθητα και 10 (6.6%) παρουσίαζαν πλήρη αντοχή. Ανιχνεύθηκαν 76 (50.8) σιπροφλοξασίνη-ανθεκτικά στελέχη και ταυτόχρονα παρουσίαζαν αντοχή σε ένα ή περισσότερα από τα υπόλοιπα υπό έλεγχο αντιβιοτικά. Κανένα στέλεχος δεν παρουσίαζε αντοχή στην κολιστίνη. Σύμφωνα με την PCR, 42% των υπό μελέτη στελεχών είχαν το *mexB* γονίδιο και *mexABoprM* οπερόνιο, και το φαινόμενο αυτό ήταν πιο συχνό (80%) μεταξύ των σιπροφλοξασίνη-ανθεκτικών στελεχών.

Συμπερασματικά, στην παρούσα μελέτη απομονώθηκε μεγάλος αριθμός πολυανθεκτικών στελεχών *P. aeruginosa* και, τόσο η αντοχή στην σιπροφλοξασίνη, όσο και η πολυαντοχή, οφειλόταν πολύ συχνά σε υπερέκφραση αντλιών εκροής. Σύμφωνα, τέλος, με τα αποτελέσματα, υπήρχε στενή συσχέτιση μεταξύ του *mexB* γονιδίου και της θνητότητας των ασθενών.

Λέξεις κλειδιά
Pseudomonas aeruginosa, φλουοροκινολόνες, αντοχή, mexB γονίδιο, αντλία εκροής

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