Original Article

Role of PA3574 (*nalD*) gene in development of ciprofloxacin resistance in *Pseudomonas aeruginosa* isolates

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Abstract

***Background & Aims*:** *Pseudomonas aeruginosa* (*P. aeroginosa*) is a gram-negative opportunistic pathogen and one of the mortality causes of nosocomial infections. One of the drug resistance mechanisms in *P. aeruginosa* is mutations in genes negatively regulated the expression of mexAB-oprM efflux pump system such as *nalD*. The aim of this study was to investigate the role of *nalD* mutations in *P. aeruginosa* isolates of Guilan province in ciprofloxacin resistant development.

***Materials & Methods*:** In this study, 45 *P. aeruginosa* isolates were obtained from different clinical samples of Rasht and Lahijan hospitals and laboratories between 2014 to 2016, which were identified by biochemical tests. Resistance pattern of ciprofloxacin against tothe strains was determined by disc difusion method and MIC. PCR-sequencing was performed to assess *nalD* gene mutations in ciprofloxacin resistant strains.

***Results*:** Seventeen from 45 isolates of *P.aeruginosa* were ciprofloxacin resistant (MIC≥1024 µg/ml). PCR-sequencing analysis showed that one resistant isolate had one- C nucleotide deletion in codon 193 (p.Leu193CysfsX, in gene level: c.577delC)) (c.577delC) and three resistant isolates had L153Q missense mutations in *nalD* gene.

***Conclusion*:** It appears that mutations of *nalD* gene lead to overexpression of mexAB-oprM and subsequently ciprofloxacin resistance in *P.aeruginosa* isolates in Guilan province.

***Keywords*:** Ciprofloxacin, mutation, *nalD*, *Pseudomonas aeruginosa*, Sequencing.

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Introduction

P. aeruginosa is one of the most common causes of nosocomial infections, which are capable of resistance to antibiotics innatly and adaptivly (1). The bacteria can cause opportunistic nosocomial infections such as wound infection, urinary tract infection, respiratory tract infection (2) and burns infection (3). Fluoroquinolones are an important group of antibiotics in the treatment of infection with *P. aeruginosa* which are used for the treatment of nosocomial infection (4). Resistance to a wide range of antibiotics in *Pseudomonas aeruginosa* can be as a result of several mechanisms, such as altering the structure of antibiotics enzymatic targets, low permeability of the outer membrane and increased expression of efflux pumps genes (5). MexAB-oprM pump is one of the most important efflux pumps in *P.*-

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aeruginosa that has an important role in the intrinsic resistance to antibiotics (6). Increased expression of these pumps in clinical isolates of *P.aeruginosa* results in considerable resistance to different antibiotics in bacteria (7). *nalD* is one of the genes regulating the expression of the efflux pumps in which mutation can lead to increased expression of e MexAB-oprM efflux pump. Increase of MexAB-oprM efflux pump leads to the withdrawal of the drug from the cell and in result development of antibiotic resistance in *P. aeruginosa* (8). NalD is one of the family members of TetR / AcrR that binds to the second promoter of *MexAB-oprM* operon and similar to MexR repressor inhibits the expression of this operon. Increaseof MexAB-oprM efflux pumps leads to the resistance to quinolones in clinical isolates of *P. aeruginosa.* Different studies have shown the mutations in each of these two genes (*nalD* and *mexR*) plays role in increaseof MexAB-oprM efflux pump (9).

Today, researchers are looking for better ways to treat a variety of bacterial infections with resistant to antibiotics. Occurrence of mutations in some genes or obtaining drug resistance genes in *P. aeruginosa* increases drugs resistance in the hospital samples. *P. aeruginosa* is a life-threatening cause in people with severe burns (10), respiratory problems such as cystic fibrosis (11) and patients with cancer (1).Thus, further information about the frequency of mutations causing resistance to antibiotics in the strains is needed to establish new therapeutic strategies in each province . Ciprofloxacin is one of the drugs with the inhibiting effect on the growth of *P. aeruginosa*. But since the bacteria with long-term treatment, for example in patients with cystic fibrosis, easily becomes resistant to drugs (12), it is necessary to utilize appropriate treatment strategies to treat the infection effectively. Due to the high cost of genetic researches in medical centers, there is no possibility to study the genetic causes of resistance for each bacterial isolate. Hence, in this study, the role of one of the genes involved in resistance to ciprofloxacin (*nalD*) in *P. aeruginosa* isolates in Guilan province has been investigated.

Materials and Methods

2.1 bacteria identification

In this study, 45 strains of *P. aeruginosa* were collected from various clinical specimens of burns, respiratory, urine and tissue necrosis of some hospitals of Rasht (Velayat, Aria, Qa'em and Razi) and two laboratories of Lahijan (Mehr and Razi) from October 2014 to June2016. The identification of isolates of *P. aeruginosa* were performed based on Gram staining, oxidase test, pigment production and growth in Cetrimideagar (Quelab, Canada) at 42°C (13).

2.2 Antibiotic susceptibility assay

Determination of antibiotic sensitivity in the isolates was performed with standard disk diffusion method and according to CLSI 2013 (14). The antibiotic ciprofloxacin disk (5µg) was purchased from HiMedia, India. After 18-24 hours of incubation at 37°C, the diameter of inhibition zone around each disc was measured and results were recorded.

2.3 Determination of minimum inhibitory concentration (MIC)

Ciprofloxacin (Ronak Daru, Iran) by consecutive dilution in liquid medium based on CLSI 2013 standards was used to determine the minimal inhibitory concentration Minimum Inhibitory Concentration (MIC). For this purpose, the strains were incubated in Mueller Hinton Broth medium (Quelab, Canada) in the presence of different concentrations of the drug for 24 hours at 37 ° C. The lowest concentration of antibiotic that no growth was observed in the presence of it had been considered as the minimum inhibitory concentration of (MIC) (15).

2.4 *nalD gene amplification*

Ciprofloxacin resistant isolates of *P. aeruginosa* were incubated for 24 hours at 37 ° C in Mueller Hinton Broth medium. TOP General Genomic DNA Purification Kit (Topazjen, Iran) was used for DNA extraction of isolates. The samples were analyzed with agarose gel (1.5%) electrophoresis. PCR reaction was carried out using AccuPower® PCR PreMix kit (Bioneer, South Korea) in a final volume of 25 µl; by adding genomic DNA, primers *nalD* (20µM) and deionized water to the PreMix solution(containing the enzyme, cofactor and buffer). Primers were synthesized by Bioneer Co. (South Korea). PCR reaction has been performed using the thermocycler Ananlytik Jena (Germany) according to the following schedule: initial denaturing at 94 ° C for 5 min, 30 cycles at 94 ° C for 30 s, 60 ° C for 30 s, 72 ° C for 1 min and a final extension at 72 ° C for 5 minutes. Primer sequences (16) were as follows: F primer: 5'-GCGGCTAAAATCGGTACACT-3' and R primer: 5'-ACGTCCAGGTGGATCTTGG-3'. After ensuring the production of PCR products (639 bp) and their single band on agarose gel 2%, the samples were sent to Bioneer (South Korea). After of PCR products purification , the samples were sequenced. The results of sequencing were analyzed using CLC main workbench software v3. 5 and online software BLAST (BLAST) in terms of the presence or absence of mutations resistant to standard reference samples (PAO1) available at NCBI (Accession number: AE004091.2).

2.5 Statistical analysis

$\chi 2$ test was used to evaluate the significance of the results. $P < 0.05$ was considered as the significance of the results.

Results

In this study, 45 isolates of *P. aeruginosa* from 120 samples of suspected patients infected with the bacteria was isolated from hospitals and laboratories of Guilan province. Disk diffusion and MIC results showed that about 38% of strains were resistant to ciprofloxacin. Frequency of resistant isolates had significant differences ($P < 0.05$) with frequency of susceptible, and intermediate ones.. The most ciprofloxacin sensitive isolates were observed in the urine samples. In addition, none of the urine samples were resistant to the drug. The mostciprofloxacin resistant strains were isolated from burns (17). However, no significant difference was observed between infection position detected and ciprofloxacin susceptibility. The highest rates of resistance to ciprofloxacin was determined as MIC = 1024 µg / ml in 30% of resistant isolates.

nalD gene were amplified in 17 isolates by PCR to determine molecular reason of resistance to ciprofloxacin (Fig. 1).

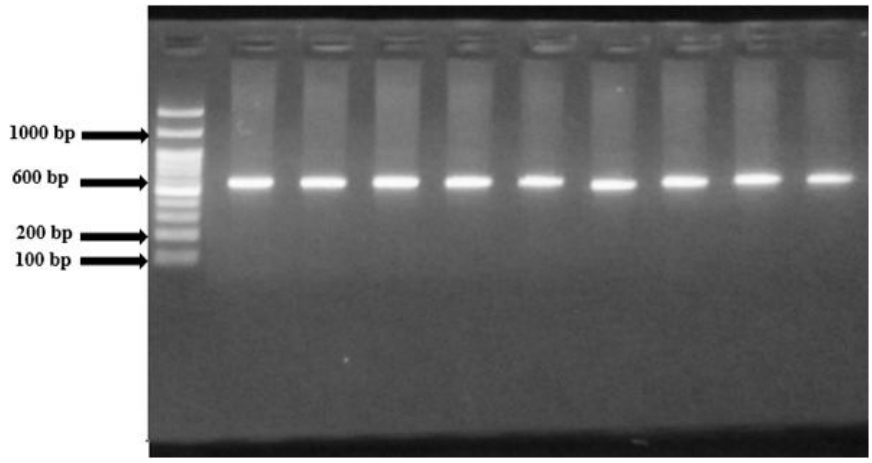

Figure 1. PCR products' electrophoresis of *nalD* gene on agarose gel 2%. DNA Ladder 100 bp and nine *P. aeruginosa* resistant isolates, respectively, from left to right. The length of PCR of each product is 639 bp.

Sequencing analysis showed that four of the isolates had missense mutations or one nucleotide deletion. Deletion of a nucleotide C (CAC**C**TGCTG> CAC-TGCTG) in position 577 in *nalD* gene (p.Leu193CysfsX, c.577delC) was found in a resistant isolate that altered the frame reading from codon 193 to the end of the gene (figure 2). In three isolates, the missense mutation L153Q was observed (Figure 3), which amino acid leucine was replaced to glutamine in codon 153 due to the change of base T to base A. Also, several silent mutations have been observed in isloates along with missense mutations (Table 1). Totaly, mutations listed were reported in all ten isolates.

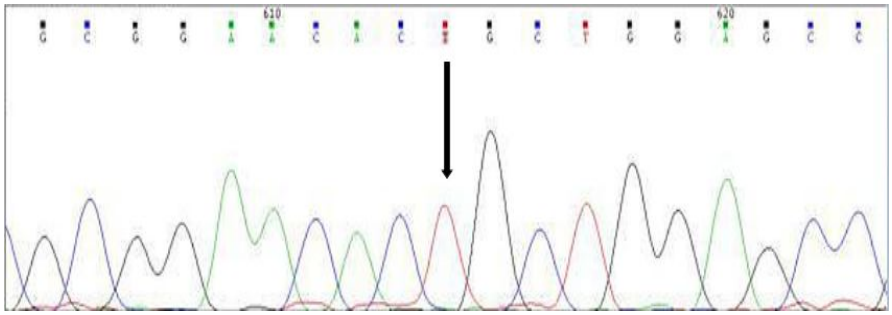

Figure 2. Electropherogram analysis showed anucleotide deletion (C) at position 577 (arrow) in *nalD* gene in a isolates (CAC<u>C</u>TGCTG> CACTGCTG (p.Leu193CysfsX,c.577Delc).

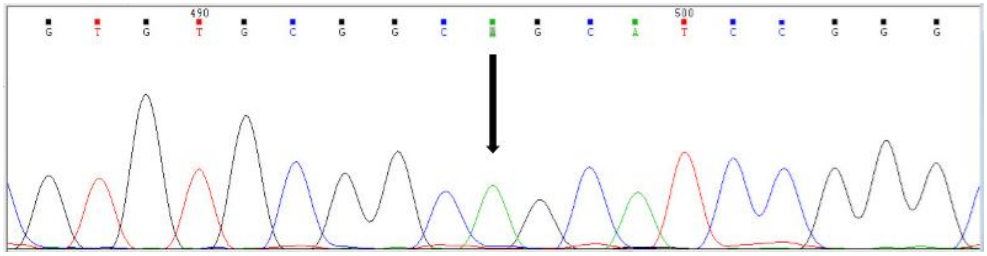

Figure 3. Electropherogram for replacement of T with A in position 494 (CTG>CAG) which led to muationL153Q

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Table 1. base and amino acid changes in *nalD* in ciprofloxacin resistant isolates of *Pseudomonas aeruginosa*

Sample number	Base change(amino acid change)	Name of mutation	Mutation frequency	Type of mutation
47	TGC(Cys)>TGT(Cys)	C92C	14%	Silent
	TTG(Leu)>CTG(Leu)	L99L	19%	Silent
	ATC(Ile)>ATT(Ile)	I111I	10%	Silent
	GAC(Asp)>GAT(Asp)	D180D	10%	Silent
	CTG(Leu) >TGC (Cys)	p.Leu193CysfsX	4.5%	Single nucleotide deletion
45	CTG(Leu)>CAG(Gln)	L153Q	14%	missense
	GAT(Asp)>GAC(Asp)	D185D	19%	Silent
44	CTG(Leu) >CAG (Gln)	L153Q	14%	missense
	GAT(Asp)>GAC(Asp)	D185D	19%	Silent
43	CTG(Leu)>CAG(Gln)	L153Q	14%	missense
	GAT(Asp)>GAC(Asp)	D185D	19%	Silent
16	TGC(Cys)>TGT(Cys)	C92C	14%	Silent
	TTG(Leu)>CTG(Leu)	L99L	19%	Silent
	ATC(Ile)>ATT(Ile)	I111I	10%	Silent
	GAC(Asp)>GAT(Asp)	D180D	10%	Silent
49	CTG(Leu)>TTG(Leu)	L57L	4.5%	Silent
	TTG(Leu)>CTG(Leu)	L99L	19%	Silent
23	AAG(Lys)>AAA(Lys)	K26K	10%	Silent
1	AAG(Lys)>AAA(Lys)	K26K	10%	Silent
48	GAT(Asp)>GAC(Asp)	D185D	19%	Silent
38	TGC(Cys)>TGT(Cys)	C92C	14%	Silent
	TTG(Leu)>CTG(Leu)	L99L	19%	Silent

Discussion

One of the problems in the treatment of infectious diseases is their resistance to different antibiotics. *Pseudomonas aeruginosa* is a Gram-negative pathogenic and opportunistic nosocomial bacteria that causes a wide range of infections in humans and may be associated with high resistance to antibiotics (18). *P. aeruginosa* has a native resistance to different antibiotic due to low permeability outer membrane, producing a variety of antibiotics inhibiting enzymes and the expression of multiple efflux pumps. Also, this pathogen has different the resistance pattern to various antibiotics in different geographic regions. Beta-lactams, aminoglycosides, and fluoroquinolones are the most common antibiotics used to treat *Pseudomonas* infections which can be observed as multi-drug resistance in some bacterial strains simultaneously (19).

In this study, resistance to ciprofloxacin was reported in 38% of the 45 isolates of *P.aeruginosa* obtained from infected patients referred to hospitals and laboratories in Guilan province. In the study of Fazeli *et al* in 2014 in Tehran, resistance to ciprofloxacin was reported a 40% of isolates(20). Negi *et al* in India in 2014 showed that about 57% of *P. aeruginosa* isolates

were resistant to ciprofloxacin (21). In the study of Hemmati *et al* on 120 isolates of *P. aeruginosa* during 2011-2012 in Zanjan province, the resistance to ciprofloxacin was reported in 32.5% of isolates (22).

In 2014, Goli et al reported 65% of *P. aeruginosa* isolates (among the 100 isolates) were resistance to ciprofloxacin in Tabriz hospitals (23). The results of this study were similar to the results of study of Fazeli *et al*. On the other hand, the prevalence of ciprofloxacin resistance in this study was lower than study of Negi and colleagues in India and this result showed that ciprofluxacin resistance in P. aeroginaso strains are high. Also compared with the study of Hemmati in Zanjan province in previouse years, the resistance was higher in Gilan and it seems that a higher resistance to the drug is happened in Zanjan in recent years. According to various studies, it is expected that the indiscriminate use of antibiotics in Guilan province and the acquisition of new mutations can increase the resistance in the province.

The reasons for multi-drug resistant *P aeruginosa* include increased expression of genes involved in efflux pump such as mexA, *mexB* and *oprM* and inhibition of negative regulator genes such as *nalD*, *nalB* (*mexR*) and *nalC* have been identified in various studies (24, 25). MexAB-OprM is responsible for resistance to drugs such as tetracycline, chloramphenicol, quinolones and beta-lactams except imipenem (26). NalD similar to NalC and NalB binds to the promoter region of the operon MexAB-OprM as an inhibitor and reduces its expression (27). Mutations in each inhibitor genes including nalD lead to increased expression of efflux pump system (28), resulting in increased resistance to drugs and reduce the effectiveness of antibiotics. In a study, Llanes and colleagues (2004) observed upregulation of *MexAB* in multidrug resistant strains and identified seven mutations *nalC* in the stains (29).

Sobel and colleagues in their study in 2005 reported increased expression of MexAB-OprM in *P. aeruginosa* isolates with multidrug resistance that *nalD* gene had a mutation such as Ser32Asn and a deletion of 24 bp (30). Quale *et al*in 2006 observed several mutations in *nalC* and *nalD* with an increased expression of mexA in hospital isolates with multidrug resistances. In their study, nine mutations, including mutations of *nalD* Pro196Gln, Cys149Arg, Thr188Ala and Asp147Asn had been reported (31). Tomas and colleagues in 2010 on 25 *P. aeruginosa* strains resistance to antibiotics isolated from patients with cystic fibrosis identified Asp187His, Leu201Pro, Ala145Thr and Thr188Ala mutations in *nalD* gene (32).

Of 17 ciprofloxacin resistant isolates in this study, deletion of a single nucleotide C at codon 193 in an isolate (p.Leu193CysfsX,c.577delC) and missense mutation Leu153Gln in three isolates in *nalD* gene were observed which is reported for the first time in the world (as novel mutations). Since the gene had 639 base pairs length,single-nucleotide deletion in a strain occurred in the first one-third of the gene led to produce a truncated protein NalD with premature stop codon in translational level and resulting presence of a mis-function NalD in this strain. . Since the expression of *mexA*/*B* and*oprM* genes is under the the control of two promoters and *NalD* and *MexR* as negative regulators through binding to the first and second promoter inhibits the expression of these genes (33),thus in the strain due to deletion in *nalD* gene and in result lack of NalD function, repression of the efflux pump genes does not occurred by this repressor and increased expression of these genes in this strainmay be partly due to mutations occured in *nalD*. it seems that that this NalD protein with lose of function can be one of the important reasons of ciprofloxacin resistance in this strain.

On the other hand, missense mutation Leu153Gln may be involved in misfunction of NalD and increased expression of MexAB-oprM efflux pumps in three studied strains. Silent mutations observed in resistant strains are ineffective on the structure or function of the

protein and subsequently in resistance pattern of antibiotics. Since missense mutations and deletion have been observed only in four strains in this study, resistance observed in other strains can be due to mutations in other genes involved in resistance such as subunits of topoisomerase II and IV, negative regulators of efflux pumps MexAB and MexXY such as *MexR* and *NalC*. So, in previous study by thisgroup, all samples resistant to ciprofloxacin had *gyrA* gene mutation (34). Also, some strains had mutations in one or more genes such as *parC*, *mexZ* (18), *mexR* (data not shown) and *nalC* (1). Thus the occurrence of a single mutation in the *nalD* gene or other genes is not enough for resistance to the drug.Association of mutation in several genes and the acquisition of several resistance genes by bacteria can be the cause ofdrugs resistance development.

Given the high prevalence of antibiotics resistance such as ciprofloxacin in strains isolated from Guilan province as well as resistance to high doses of antibiotics in this study, the risk of antibiotic resistance is evident in this province and probably in the whole country. In addition, occurrence of two new mutations in the *nalD* gene, for the first time in the worldwide, indicates the importance of this gene in the resistance to quinolone family of antibiotics in *P. aeruginosa*. Given that few studies have been conducted on mutations of *nalD* gene, bioinformatics and laboratory studies is required to identify the most important parts of the this inhibitor and its role in expression of MexAB-oprM efflux systems and subsequently develop resistance to a variety of quinolones.

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