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The Evaluation of Efflux Pump *Genes norA*, *norB* and *norC Related* to Fluoroquinolones Resistance in *Staphylococcus aureus* Strains Isolated from Blood Infection

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Abstract

Background & Objective: Recently, ciprofloxacin resistance in *Staphylococcus aureus* strains, due to efflux pumps, has become a significant challenge. Therefore, this study was performed to evaluate the frequency of *norA*, *norB*, and *norC* efflux pump genes and their roles in resistance to ciprofloxacin in clinical isolates of *S. aureus.*

Materials & Methods: A total of one hundred clinical blood samples were collected from patient in Qom hospitals and *S. aureus* isolates were identified by standard microbiological tests. Antimicrobial susceptibility patterns were determined by the disk diffusion method using CLSI guidelines. Subsequently, the presence of *norA*, *norB*, and *norC* efflux pump genes in ciprofloxacin isolates was detected using the PCR method.

Results: Among one hundred clinical samples, 36 *S. aureus* isolates were recovered and the results of antibiotic susceptibility tests showed that twenty of them were resistant to ciprofloxacin. 15 isolates were resistant to norfloxacin and one isolate was resistant to ofloxacin. Moreover, the *norA*, *norB*, and *norC* genes were found in 58%, 30%, and 41% of ciprofloxacin-resistant isolates, respectively.

Conclusion: Based on the results of this study, *norA*, *norB*, and *norC* efflux pumps may play a significant role in the development of resistance to ciprofloxacin in clinical isolates of *S. aureus*. Detecting these genes may prove useful in suggesting an effective treatment model for infections caused by *S. aureus*.

Keywords: *Staphylococcus aureus*, Blood infection, Bacterial drug-resistance, Efflux pump

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Introduction

Antibiotic resistance has been recognized by the efflux pumps at *Staphylococcus aureus* for the past few decades, but its clinical relevance has only recently been discovered. Five families of these pumps have now been identified as Prokaryotes, leading to resistance to many antibiotics (1). Today, active efflux mechanisms are responsible for the resistance of bacteria to a variety of non-building antibiotics and toxic compounds, and it seems that reducing the permeability of bacteria is one of the primary reasons for explaining the resistance of bacteria to drugs (2). MFS carriers are probably the largest and most diverse pumps among all the families of efflux pumps

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that have been found in all branches of biology (3). The QacA and *norA* pumps in *Staphylococcus aureus* belong to the family of MFS efflux pumps. Another family is the RND efflux pumps, which are single-component or multi-component transmission systems that contain not only an internal membrane transmitter, but also an external membrane channel and a compatible periplasmic protein (4). Efflux pumps are the most important cause of antibiotic resistance, and the most important of these pumps are the intrinsic efflux gene systems. In *Staphylococcus aureus*, *norA*, *norB*, *norC*, and *tet38* are chromosomal genes that encode efflux pumps, and the high expression of multidrug resistance (MDR) genes can be resistant to Quinolones and other compounds or tetracycline (tet 38) (5). *norA* multifunctional efflux pump systems have been widely studied in *Staphylococcus aureus*. The chromosomal gene encoding the pump is *norA*, which was first identified and collected in 1986 at a hospital in Japan in resistant bacteria to fluoroquinolones (6). After initial studies of *norA*, evidence suggests that other efflux systems are present in the *S. aureus* chromosome. *norB* is another diffusion pump similar to *norA*, belonging to the MFS family and consisting of 463 amino acids with 12 membrane sections (7). *norB* is also resistant to some *norA* substrates and causes bacterial resistance to hydrophilic fluoroquinolones (such as norfloxacin and ciprofloxacin), pesticides, and ethidium bromide dye, as well as non-norA-dependent substrates, such as hydrophobic fluoroquinolones and moxifloxacin (8). Studies show that *norB* may be involved in the response of *Staphylococcus aureus* to acidic shock and cellular oxygen deprivation, conditions that increase *norB* gene expression. It has also been shown to play an important role in bacterial pathogenesis (9). In both cases, the increase in *norB* gene expression has been proven by increasing the resistance to the substrates of this pump. One of the important substrates of *norB* is moxifloxacin (10). Evidence suggests that *norC* efflux systems are present in the *S. aureus* chromosome. *norC* is another emission pump that is similar to *norA* in the MFS family. It consists of 462 amino acids with 12 membrane parts which is 61% similar to *norB*. This pump is less resistant to hydrophobic and hydrophilic fluoroquinolones such as ciprofloxacin, moxifloxacin and garinagsin, and rhodamine dye (11). One of the ways to deal with antimicrobial resistance caused by efflux pumps is to use new antimicrobial compounds or to use an accompanying compound called an antibiotic adjuvant (12).

The aim of this study was to evaluate efflux pump genes *norA*, *norB*, and *norC* related to fluoroquinolone resistance in *Staphylococcus aureus* strains isolated from blood infection.

Materials and Methods

One hundred blood samples (13) were obtained from hospitalized patients in Qom hospitals. Samples were collected from patients hospitalized in different departments of Kamkar and Shahid Beheshti hospitals in 2019. Patients were selected for sampling who experienced continuous fever during the last 24 hours.

In order to identify the collected strains, gram staining, mannitol fermentation test, DNase, catalase, and coagulase were used. Then, sensitivity of isolates to Ciprofloxacin, Norfloxacin, and Ofloxacin antibiotics was determined using the Kirby-Bauer method. The results of the antibiogram were interpreted using the CLSI 2019 break-points (14).

DNA of confirmed isolates was purified by using a genomic DNA extraction kit (Sinaclon, Iran) as recommended by the manufacturer. 36 isolates were screened for the presence of *norA*, *norB*, and *norC* genes by PCR, using *norA* forward: GTGGTATGAGTGCTGGTATGG and reverse: GAAACTTCTGCCATAAATCCACC, norB forward: CAAACACTCGGATGCAAGAAAC and reverse: GACGCCAAATGCTCCACC, *norC* forward: TGGGTTGGAGATGGATTTTC and reverse: ACAATTAGCCCTGCAACGTC previously described primers (primers were designed in this study). For each reaction,

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a sample containing distilled water was used as a negative control and a confirmed isolate with the target gene (*norA*, *norB*, and *norC*) was used as a positive control.

PCR was down 33 cycles denaturation 95ºC 4min, second denaturation 94ºC 35 sec annealing 53ºC 35 sec, extension 72ºC 35 sec and final extension 72ºC 5min. After purification, the OD of each of the positive samples was read and then the PCR product was sent to the Sinaclon Company to determine the sequence. The submitted sequences were analyzed with CLC Sequence Viewer software, then compared with standard gene-related sequences in the NCBI database and Finch Tv software (version 1.4.0).

Chi-square statistical test and SPSS-26 software were used for statistical analysis of the data of this research.

Results

Out of a total of one hundred samples collected, 36 strains of *Staphylococcus aureus* were isolated from 100 samples, and the morphological and biochemical features were the criteria for confirming the identity of the isolates. Antibiotic susceptibility of 36 isolates of *Staphylococcus aureus* was determined by the agar disk diffusion method to the desired antibiotics, which showed that out of 36 isolates of *Staphylococcus aureus* only 20 to ciprofloxacin disk (55%), 15 cases were resistant to norfloxacin (42%) and 1 case to ofloxacin (3%) (Chart 1).

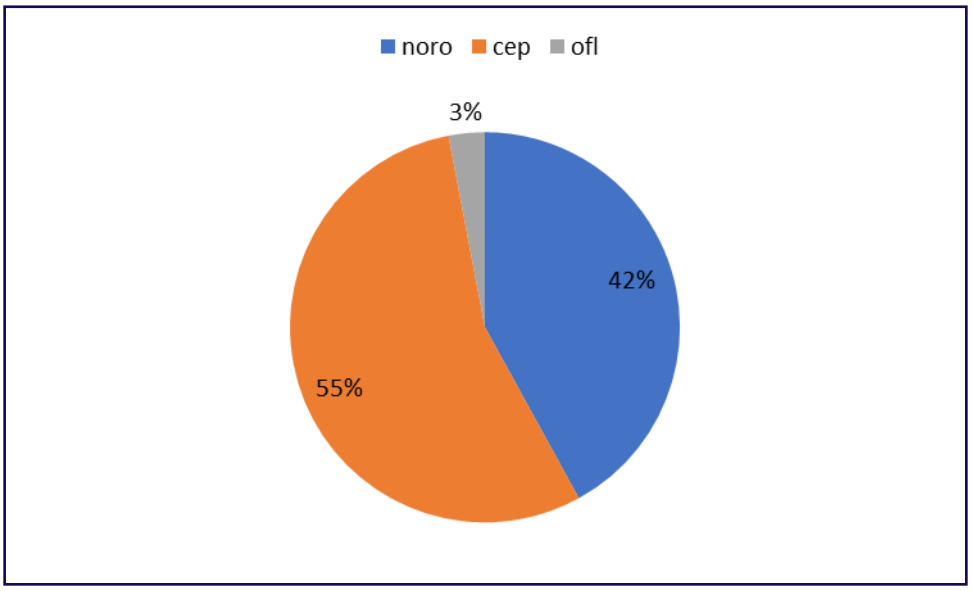

Chart 1. the percentage of resistance to each antibiotic separately,noro; norfloxacin, cep: ciprofloxacin, ofl: ofloxacin

Molecular test results

Out of one hundred samples collected, 36 samples had antibiotic resistance, and DNA extraction and PCR reaction were performed on them with specific primers after extracting the genomic DNA of bacteria. Electrophoresis of the samples in 2% agarose gel in the initial PCR reaction showed a band of 160 bp *norA*, 260 bp *norB* and 130 bp *norC* which indicated the amplification of the genes (Figure 1). Out of 36 resistant samples, 58% , 30% and 41% of the samples showed a positive result based on primers and had an antibiotic resistance genes

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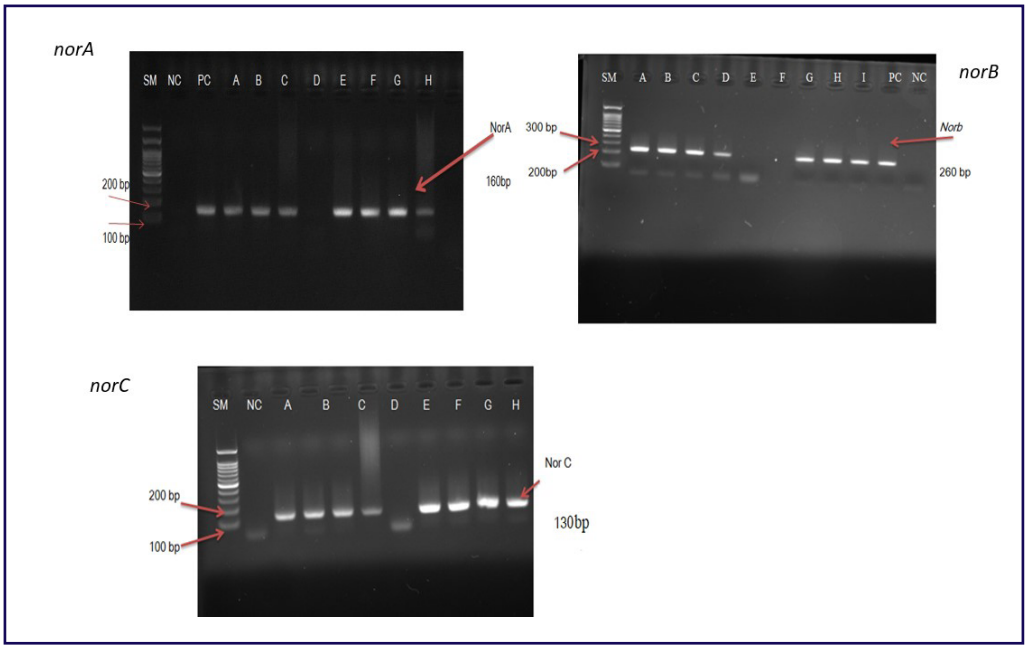

Figure1. *norA,norB and norC* gel elechtrophoresis. SM: DNA ladder, NC: Negative control, PC: Positive control

Molecular analysis for the presence of *norA*, *norB*, and *norC* genes based on primers available for each gene showed that 58% of the isolates had *norA* gene. *norB* gene was present in 30% of the isolates and 41% of the isolated strains had *norC* gene. Based on the obtained molecular results, 4% are positive for two genes and 11% of bacteria are positive for three genes. About 38% of the bacteria did not have any of the genes (Chart 2).

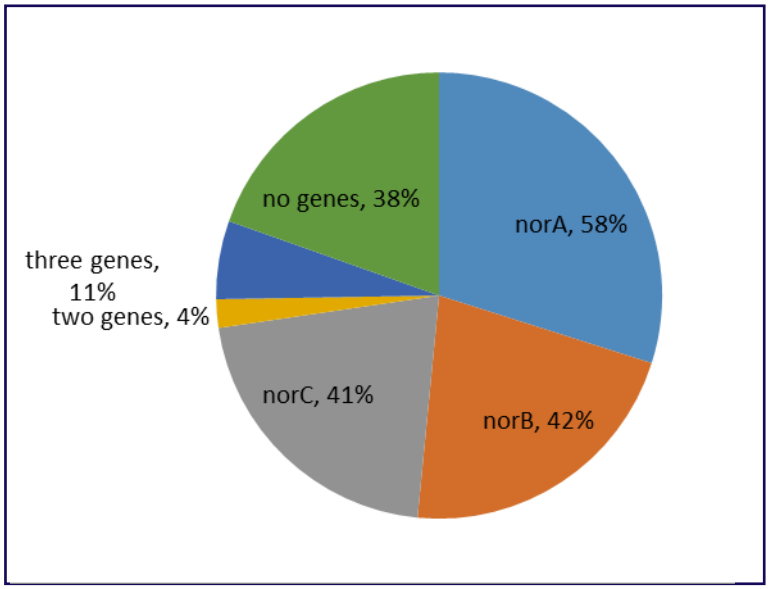

Chart 2. Frequency distribution of each gene

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Results of the sequencing

The result of sequencing for strain 9 for *norA*, strain 14 for *norB* and 21 indicates a 100% similarity of these genes in *Staphylococcus aureus.*

Discussion

The resistance of efflux pumps to fluoroquinolones, such as ciprofloxacin, norfloxacin, and Sparfloxacin, in clinical isolates of *Staphylococcus aureus* has been described in the last two decades (15-16).

In this study, resistance to norfloxacin was 42%, resistance to ciprofloxacin was 55% and resistance to ofloxacin was 3%. Molecular studies for the presence of *norA*, *norB* and *norC* genes were considered to have *norA* gene in 21 samples (58%) of isolated bacteria and in 11 samples (30%) of bacteria with primers of *norB* gene showed positive results. Also, in isolates for *norC* gene in 15 samples (41%) this gene was included. Based on the molecular results obtained in 4 bacteria for the two genes *norA*, *norC* and *norB* in 11 bacteria (11%) are positive for all three genes (30%).

In a study conducted by Gade et al., resistance to ciprofloxacin and ofloxacin in *S. aureus* isolated from blood infection was reported as 92.5% and 80.4%, respectively (17-18). Another study by Afzal et al. showed 56% of *S. aureus* isolated from eye infection were phenotypically resistant to ciprofloxacin. Also, 21.42% of the resistant isolates have high experssion in *norB gene* (19). The results are slightly different from our research, which is probably due to the different criteria of patients in sample collection.

Hadadi's study showed that among the strains with activated pumps, almost half of them (48%) had increased expression. 57% showed increased expression in the gene of a single pump, usually *norA*, while the remaining 43% isolates showed increased expression in 2 or more efflux pumps, most of which were *norB /C*. A recent study of 52 ciprofloxacin-resistant *S. aureus* bacterial isolates showed that the activity of efflux pumps increased in 23% of cases, which is associated with increased bacterial resistance to fluoroquinolones (20). The results of this research are consistent with the level of gene expression obtained in our research. A plausible explanation for this could be that these isolates had a sufficient number of pumps in their cell wall before the bacterium was exposed to the drug so that there was no need to increase the expression of the relevant genes. Although fluoroquinolones are not used to treat staphylococcal infections, their widespread use in the treatment of nosocomial infections of this bacterium in the hospital has been suggested as the most important factor in the development and spread of *S. aureus* resistance to fluoroquinolones (20).

In a study conducted in 2016 by Haddadi Zahmatkesh et al. 68% were methicillin and 24% ciprofloxacin-resistant, and the gene frequencies for *norA* and *norB* in resistant strains were 100% and 83%, respectively, which showed that the efflux pump in Ciprofloxacin resistance plays a key role and the presence of these genes can be important in suggesting a therapeutic pattern (20). As in our study, increased expression of light genes was observed in fluoroquinolone-resistant isolates.

Also, Costa et al confirmed the main role of efflux pump as the first agent for antimicrobial resistance in *S. aureus* (5). In 2015, another study showed that all strains of *S. aureus* resistant to ciprofloxacin have the *norA* gene and 83% of the isolates have the *norB* gene (20).

A study in Boston on the expression of *norB* gene and *norC* gene in ciprofloxacin-resistant staphylococci was performed on 115 isolates from 2009 and 2011 isolated from Korea. *norB* was present in 42% of the samples

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and *norA* and *norC* genes were present in 33.62% and 53.2% of the samples, respectively (21-22). The difference in the expression of resistance genes in different studies can be due to the difference in the distribution of strains in different geographical locations, depending on the infection control of the location and the amount of resistant isolates.

Based on the role of efflux pumps in bacterial resistance to some antibiotics, studies are being conducted to prevent the expression of each of the efflux pump genes and to evaluate antibiotic resistance. Accordingly, studies by Pourmand et.al in 2014 showed the increased expression of *norA* in methicillin and ciprofloxacin resistant *S. aureus* strain (23).

In our study, the determination of the correlation between the expersion of *nor genes* with fluoroquinolone resistance phenotype is one of the strengths of the study.

Conclusion

The results of this study showed that pump efflux is one of the important mechanisms of resistance to fluoroquinolone antibiotics such as ciprofloxacin, while the role of other factors involved in resistance should not be ignored. Finally, it is suggested that further studies be performed to produce and extend the molecules of efflux pump inhibitors. It is also recommended that the expression of pump efflux genes in ciprofloxacin-resistant strains be studied by molecular methods such as real-time PCR.

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Conflict of interests

The authors declare that they have no competing interests.

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