مجله دنیای میکروب‌ها
سال دوازدهم شماره سوم (پیاپی ۴۰) پاییز ۱۳۹۸
صفحات ۳۰۴-۲۹۴

Original Article

شناسایی فعالیت و بیان ژن پمپ افلاکس سویه های مقاوم به سیپروفلوکساسین *استافیلوکوکوس اورئوس*

زهرا توکلی[۱]، حسن صاحب جمعی[۲]، لیلا پیشکار[۳]، زهره علی مددی[۴]، حسن نوربازرگان[۵]، امیر میرزایی*[۶]

[۱]کارشناس ارشد، گروه زیست شناسی، واحد تهران شمال، دانشگاه آزاد اسلامی، تهران، ایران. [۲]استادیار، گروه بیوشیمی و بیوفیزیک، دانشکده علوم زیستی، واحد ورامین پیشوا، دانشگاه آزاد اسلامی، ورامین، ایران. [۳]استادیار، دانشگاه آزاد اسلامی، واحد اسلامشهر، باشگاه پژهشگران جوان و نخبگان، اسلامشهر، ایران. [۴]کارشناس ارشد، گروه زیست شناسی، واحد تهران شرق، دانشگاه آزاد اسلامی، تهران، ایران.
[۵]دکتری، گروه بیوتکنولوژی، دانشکده فناوریهای نوین پزشکی، دانشگاه علوم پزشکی شهید بهشتی، تهران، ایران.
[۶]استادیار، گروه زیست شناسی، واحد رودهن، دانشگاه آزاد اسلامی، رودهن، ایران.

چکیده

سابقه و هدف: باکتری *استافیلوکوکوس اورئوس* یکی از مهمترین عوامل عفونت‌زای بیمارستانی می‌باشد. اخیرا سویه‌های *استافیلوکوکوس اورئوس* نسبت به آنتی بیوتیک سیپروفلوکساسین مقاوم شده‌اند و پمپ افلاکس در این مقاومت نقش مهمی را ایفا می‌کند. هدف از این مطالعه، بررسی وجود ژن های پمپ افلاکس (*norA* و *norB*)، بیان و فعایت آن در سویه‌های مقاوم به سیپروفلوکساسین *استافیلوکوکوس اورئوس* بود .

مواد و روش ها: در این مطالعه، تعداد ۲۵۰ نمونه بالینی به منظور جداسازی سویه های *استافیلوکوکوس اورئوس*، بررسی مقاومت آنتی بیوتیکی، وجود و بیان ژن‌های پمپ افلاکس *norA* و *norB* در آنها به ترتیب توسط روش های PCR و real-time PCR مورد مطالعه قرار گرفت. در انتها، پمپ افلاکس فعال در سویه‌های مقاوم به سیپروفلوکساسین *استافیلوکوکوس اورئوس* توسط روش اتیدیوم بروماید بررسی شد.

یافته ها: از مجموع نمونه های مورد بررسی، ۵۰ سویه *استافیلوکوکوس اورئوس* جداسازی شد که از این میان ۱۲ جدایه (۲۴٪) مقاوم به سیپروفلوکساسین بودند. میزان شیوع ژن های *norA* و *norB* در سویه‌های مقاوم به سیپروفلوکساسین به ترتیب ۱۰۰٪ و ۸۳٪ بود. همچنین تمامی سویه‌های مقاوم به سیپروفلوکساسین دارای پمپ افلاکس فعال بودند. نتایج real-time PCR نشان داد که اختلاف معنی داری در میزان بیان پمپ های افلاکس در سویه‌های مقاوم به سیپروفلوکساسین وجود دارد.

نتیجه گیری: نتایج این مطالعه نشان داد که پمپ‌های افلاکس *norA* و *norB* نقش مهمی در ایجاد مقاومت به سیپروفلوکساسین ایفا می‌کند.

واژگان کلیدی: *استافیلوکوکوس اورئوس*، پمپ افلاکس، ژن *norA*، ژن *norB*، روش real-time PCR.

دریافت مقاله: فروردین ماه ۹۸ پذیرش برای چاپ: خرداد ماه ۹۸

*) آدرس برای مکاتبه: رودهن، مجتمع دانشگاهی، دانشگاه آزاد اسلامی، واحد رودهن
تلفن: ۰۲۱۷۶۵۰۵۸۹۱ پست الکترونیک: a.mirzaie@riau.ac.ir

Journal of Microbial World
Volume 12, No. 3, September 2019

Original Article

Detection of efflux pump activity and gene expression among ciprofloxacin-resistant *Staphylococcus aureus* strains

Zahra Tavakoli[1], Hassan Sahebjamee[2], Leila Pishkar[3], Zohreh Alimadadi[4], Hassan Noorbazargan[5], Amir Mirzaie[6]

[1]M.Sc., Department of Biology, Tehran North Branch, Islamic Azad University, Tehran, Iran.
[2]Assistant Professor, Department of Biochemistry and Biophysics, School of Biological Sciences, Varamin-Pishva Branch, Islamic Azad University, Varamin, Iran. [3]Assistant Professor, Young Researchers and Elite Club, Islamshahr Branch, Islamic Azad University, Islamshahr, Iran. [4]M.Sc., Department of Biology, East Tehran Branch, Islamic Azad University, Tehran, Iran.
[5]Ph.D., Department of Biotechnology, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[6]Assistant Professor, Department of Biology, Roudehen Branch, Islamic Azad University, Roudehen, Iran.

Abstract

Background & Objectives: *Staphylococcus aureus* is one of the important nosocomial infection agents. Recently, *S. aureus* strains have become resistant to ciprofloxacin and the efflux pump is considered as its contributor. Herein, we investigated the presence, expression, and activity of efflux pump genes (*norA* and *norB*) among ciprofloxacin-resistant *S. aureus* isolates.
Materials & Methods: A total of 250 clinical samples were subjected to isolation of *S. aureus* strains. The antibiotic resistance pattern was characterized and the presence and expression level of *norA* and *norB* genes was assessed using PCR test and real-time PCR test, respectively. Finally, active efflux pumps were detected in ciprofloxacin-resistant *S. aureus* strains using the ethidium bromide test.
Results: Among total clinical samples, 50 *S. aureus* strains were recovered. Of this 12 samples (24%) were resistant to ciprofloxacin. Moreover, *norA* and *norB* genes were found in 100 % and 83% of ciprofloxacin-resistant isolates, respectively. All ciprofloxacin-resistant isolates exhibited active efflux pumps. Real-time PCR results revealed that the isolates are more resistant to ciprofloxacin having a high level of efflux pump gene expression.
Conclusion: The results of this study showed that *norA* and *norB* efflux pump genes play an important role in resistance to ciprofloxacin in *S. aureus* strains.

Keywords: *Staphylococcus aureus*, Efflux pump, *norA* gene, *norB* gene, Real-time PCR.
Received: March 2019 Accepted: May 2019

Introduction

Staphylococcus aureus is one of the opportunistic agents, causing nosocomial infections worldwide (1). This bacterium is responsible for a wide variety of infections from food poisoning to life-threatening diseases such as endocarditis (2, 3).

The emergence of methicillin-resistant *S. aureus* strains (MRSA) has made staphylococcal infections a major therapeutic

Correspondence to: Amir Mirzaie
Tel: +982176505891
E-mail: a.mirzaie@riau.ac.ir
Journal of Microbial World 2019, 12(3): 294-304.

challenge (4). Much of MRSA strains are resistant to many antibacterial agents and this may limit the treatment of *S. aureus* infections (5). Fluoroquinolone antibiotics such as ciprofloxacin are used for the treatment of MRSA infections, especially oral ones. Unfortunately, after introducing ciprofloxacin for the treatment of MRSA infections, these bacteria rapidly became resistant to this drug. Ciprofloxacin and methicillin-resistant strains are increasing and several studies showed that more than 90 percent of MRSA strains are resistant to ciprofloxacin (6, 7).

There are different strategies responsible for antibiotic resistance in *S. aureus*. Efflux pumps are one of the important mechanisms of antibiotic resistance in *S. aureus* strains (8). To date, researchers have shown more than 30 efflux pumps genes in ciprofloxacin-resistant isolates (9, 10).

norA and *norB* genes encode NorA and NorB multidrug efflux proteins in *S. aureus* which is one the most characterized multidrug efflux systems in *S. aureus* (11, 12). These efflux proteins could extrude some antibiotics such as ciprofloxacin and quaternary ammonium compounds (13). Both genes are located in the chromosomal region and have been revealed to exist in ciprofloxacin-resistant *S. aureus* isolates. These efflux proteins belong to major facilitator (MF) superfamily, depend on proton motive force (PMF) and dot not use the ATP as an energy source (14, 15).

According to our knowledge, so far, a few studies have reported the efflux related-resistance and efflux pump gene expression pattern in *S. aureus* isolates. Therefore, this study was carried out to assess the presence, expression and activity of *norA* and *norB* efflux pump genes among Iranian ciprofloxacin-resistant *S. aureus* isolate.

Materials and methods

Bacterial strains

A total of 250 clinical samples (Blood, Pus, Urine and CSF) were collected from hospitalized patients of Emam Khomeini and Pars hospitals, Tehran, Iran from July to December 2015.

S. aureus strains were identified using microbiological methods including culturing in mannitol salt agar as well as catalase, coagulase, and DNase tests. The isolates were stored at -80 0C for further studies (16).

Antimicrobial susceptibility testing

The isolates were examined for their antibiotic sensitivity by Kirby- Bauer disk diffusion test using the following disks; penicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), ciprofloxacin (10 µg), clindamycin (2 µg), amoxicillin (10 µg), trimethoprim (25 µg), amikacin(15 µg), gentamicin (10 µg), oxacillin (1 µg), vancomycin (10 µg) and cefoxitin (30 µg), colistin (10µg). MICs of ciprofloxacin in ciprofloxacin-resistant isolates were determined by the microdilution method. *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 12228 were used as positive and negative control strains, respectively, in disk diffusion and microdilution tests. All procedures were carried out in triplicate and interpreted according to CLSI guidelines (17).

Antibiotic disks were purchased from Himedia (Himedia Laboratories, Pvt. Ltd, Mumbai,

India) and ciprofloxacin powder to measure MIC was obtained from Sigma- Aldrich (USA).

Determination of methicillin resistance

Methicillin resistance was evaluated using two methods: 1- Disk diffusion test by 30 µg cefoxitin disk (≤21 mm= MRSA), 1 µg oxacillin disk (≤10 mm= MRSA), 2-Polymerase Chain Reaction (PCR) test for detection of *mecA* gene.

Minimum inhibitory concentration (MIC) of ciprofloxacin-resistant isolates

The MIC of ciprofloxacin against ciprofloxacin -resistant isolates was determined by the broth microdilution method. All procedures were carried out in triplicate and interpreted according to CLSI (Clinical and Laboratory Standards Institute) guidelines.

Briefly, Mueller Hinton broth (Merck Co., Germany) containing serial dilutions of ciprofloxacin (8-256 µg/ml) were added to 96-well microdilution plates. Selected bacterial suspension equal to 0.5 McFarland standard was further diluted and added to the plates to achieve a final inoculum of 5×10^5cfu/ml. The plates were incubated for 24 h at 37 °C in ambient air. For broth microdilution, MIC was recorded as the lowest dilution, showing no growth (17).

DNA extraction and PCR detection of mecA, norA and norB genes among ciprofloxacin-resistant isolates

Genomic DNA of ciprofloxacin-resistant isolates was extracted using the Genomic DNA Extraction Kit (Qiagen, USA). The oligonucleotide primers sequences were as following:

F 5'-TCCAGATTACAACTTCACCAGG-3' and R 5'-CCACTTCATATCTTGTAACG-3'.

PCR was performed under the following conditions: initial denaturation at 95°C for 5 min, followed by 34 cycles of 95°C for 1 min, 60 °C for 1 min and 72°C for 30 S, and a final incubation at 72°C for 5 min. The ciprofloxacin -resistant isolates were selected to detect the presence of *norA* and *norB* efflux pump genes using following primers: F norA: 5'-ATCGGTTTAGTAATACCAGTCTTGC-3' R norB: 5'GCGATATAATCATTTGAGA-TAACGC-3'.

F norB: 5'-AGCGCGTTGTCTATCTTTCC-3', R norB: 5'-GCAGGTGGTCTTGCTGATAA-3'.

PCR was performed in a 25 × µL AccuPower™ PCR PreMix (Bioneer, Korea), using 10 pmol of each primer under the following conditions: initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, (*norA*: 55°C for 1 min, *norB*: 53°C for 30S) and 72°C for 1min, and a final incubation at 72°C for 5 min. Amplified DNA fragments were separated on 1% (w/v) agarose gel, stained with RedSafe™ stain and visualized under ultraviolet light (18). To confirm the presence of efflux genes (*norA* and *norB*) in PCR products and to detect any possible mutation, all PCR products were sequenced. Genomic DNA of *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 12228 were used as a positive and negative control, respectively.

Efflux test

Carbonyl Cyanidem-Chlorophenylhydrazone (CCCP), an efflux pump inhibitor was used to determine the efflux pump activity. In brief, 0.5 McFarland overnight cultures (*norA* and *norB*

Table 1. Primers used in this study.

Primer	Sequence (5-3)	Size (bp)	Ref
norA-F	ATCGGTTTAGTAATACCAGTCTTGC	112	24
norA-R	GCGATATAATCATTTGAGATAACGC		
norB-F	AGCGCGTTGTCTATCTTTCC	213	24
norB-R	GCAGGTGGTCTTGCTGATAA		
Gmk-F	TATCAGGACCATCTGGAGTAGG	122	24
Gmk-R	CATCAACTTCACCTTCACGC		

positive ciprofloxacin-resistant isolates) were inoculated into 96 well plates containing MHB, together with Ethidium Bromide (EtBr) in serial dilution. After the preparation of serial dilution of EtBr based on its MIC, 20 µg/ml CCCP was added into the wells. The active efflux pump was considered in an isolate if MIC of EtBr added with reserpine was lower than that of EtBr alone (19).

Total RNA extraction

Ciprofloxacin resistant isolates were grown overnight in MHB at 37 °C, both with and without Sub MIC doses of ciprofloxacin. Subsequently, total RNA was extracted from selected isolates using the high pure RNA isolation kit (Qiagen, USA) and according to the manufacturer's instructions. Also, reverse transcription was performed using the QuantiTect Reverse Transcription kit (Qiagen Co., USA). Finally, cDNA concentration was quantified by a nanodrop (A260/280). An A260/A280 ratio between 1.8-2 was considered as pure cDNA.

Real-time PCR

To evaluate *norA* and *norB* efflux pump gene expression, qRT-PCR was performed in triplicate using Power SYBR Green PCR Master Mix (Applied Biosystems Co., UK). Bacterial cDNA was used as the template, in 20 µl final volume containing 2 µl cDNA, 10 pmol of each primer and 10 µl Power SYBR Green PCR Master Mix (Applied Biosystems), using Bioneer Real-time PCR equipment (Korea). The real-time PCR program was carried out as follows: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 1 min at 60 °C. *gmk* (guanylate kinase) gene was used as the internal control. The primers used in this section are presented in Table 1. Relative expression of *norA* and *norB* efflux pump genes was calculated using ΔΔCT method.

Statistical Analysis

All measurements were performed in triplicate and one-way ANOVA was done to assess the level of significance. The expression level of *norA* and *norB* genes in isolates were evaluated in real-time PCR assay. P values less than 0.05 were considered significant.

Results

Isolation of S. aureus strains

In this study, a total of 50 *S. aureus* isolates were recovered from clinical samples and sent to the Microbiology Laboratory. The results of

Table 2. Antibiotic susceptibility profiles of *S. aureus* isolates by disk diffusion.

Antibiotics	Resistant		Intermediate		Susceptible	
	n	%	n	%	n	%
Cefoxitin	34	68	0	0	16	32
Oxacillin	34	68	0	0	16	32
Vancomycin	0	0	0	0	50	100
Ciprofloxacin	12	24	1	2	37	74
Penicillin	49	98	0	0	1	2
Erythromycin	28	56	9	18	13	26
Trimethoprim	43	86	3	6	4	8
Amikacin	21	42	3	6	4	8
Gentamycin	20	40	3	6	27	54
Amoxicillin	43	86	0	0	7	14
Chloramphenicol	4	8	7	14	39	78
Clindamycin	23	46	6	12	21	42
Colistin	0	0	0	0	50	100

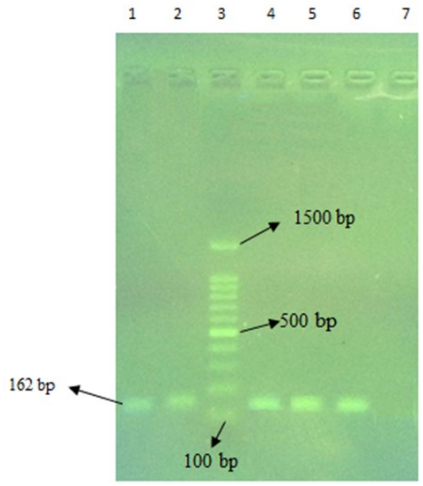

Figure 1. Amplification of *mecA* gene in MRSA isolates. Lane 1, 2, 4 and 5: MRSA isolates, 6: positive control, 7: negative control, 6: positive control, 3: 100 bp + DNA ladder (Fermentase, Lithuania).

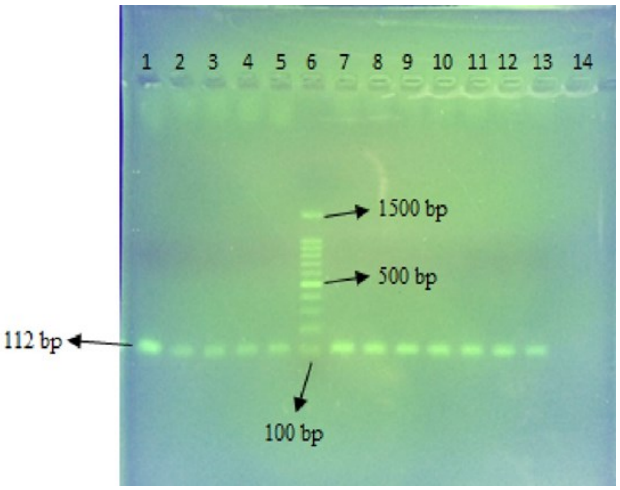

Figure 2. PCR amplification of *norA* gene in ciprofloxacin resistance strains. Lane 1-5, 7-12: ciprofloxacin resistant isolates, 13: positive control, 14: negative control, 6: 100 bp+ DNA ladder.

antimicrobial susceptibility testing showed that among 13 selected antibiotics, the highest resistance was shown to a penicillin (98%), ampicillin (90%) and the lowest resistance was shown to vancomycin and colistin (0%). Also, 68% and 24% of the strains were MRSA and ciprofloxacin-resistant, respectively (Table 2).

PCR amplification of mecA, norA and norB and sequencing

Figures 1, 2 and 3 showed PCR amplification of *mecA* and efflux genes. While *mecA* and *norA* genes were found in all ciprofloxacin-resistant strains, *norB* was detected in 83% (10 isolates) of the strains. Also, all MRSA isolates were ciprofloxacin-resistant and efflux genes were not detected in ciprofloxacin sensitive and negative control strains.

PCR amplification of genes yielded amplicons

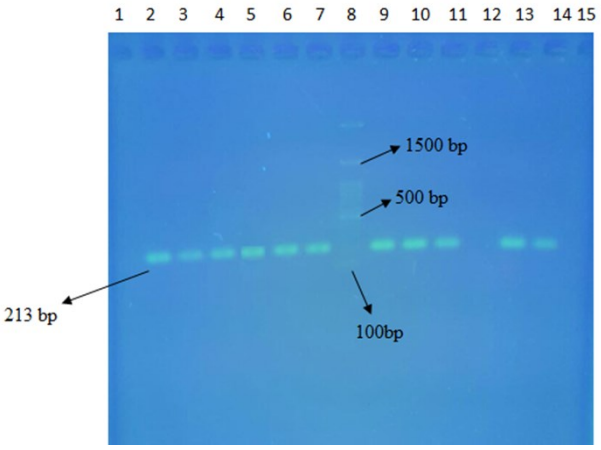

Figure 3. PCR detection of *norB* gene among ciprofloxacin resistant isolates. Lane1: negative control, 3-7, 9-11: *norB* positive isolates, 8: 100 bp $^{+}$ DNA ladder, 12, 15: negative *norB* isolates.

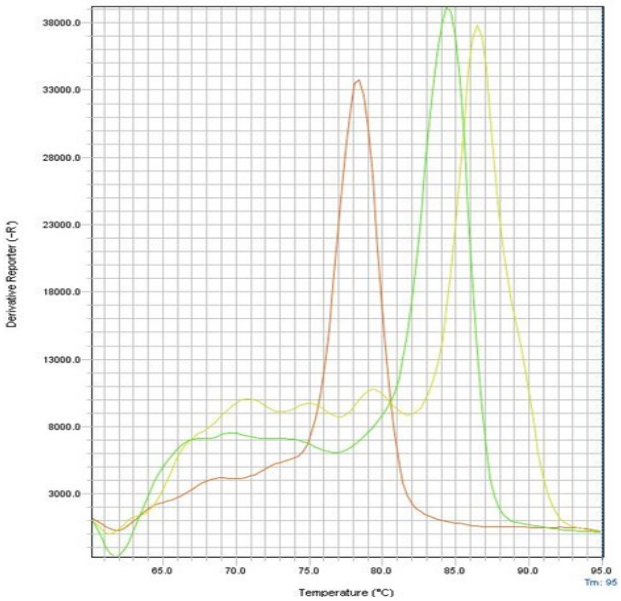

Figure 4. Melting curve of *norA* and *norB* genes. Red line: *norA*, Green line: *norB* and yellow line: *gmk* gene.

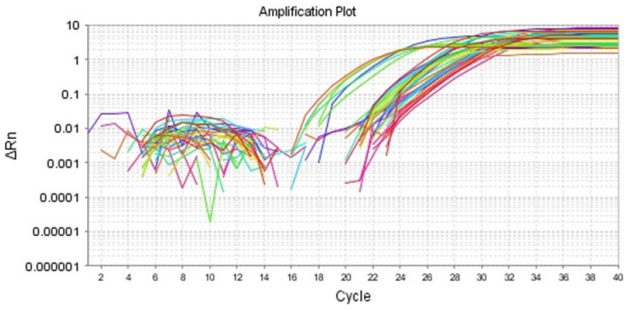

Figure 5. Amplification plot of *norA*, *norB* and *gmk* genes.

of 162 bp for *mecA* gene, 112bp for *norA* gene and 213 bp for *norB* gene. Sequencing efflux genes showed 100% homology with the published DNA sequence of *norA* and *norB*, where no mutation was detected.

Real-time PCR results

Relative expression of *norA* and *norB* efflux pump genes in ciprofloxacin-resistant isolates was evaluated using real-time PCR. It was shown that *norA* and *norB* gene expression is variable among different strains. Specific amplification, non-pairing of primers, and lack of non-specific amplification were obvious in the melting curve (Figure 4) and gene amplification plots (Figure 5).

Moreover, the isolates with a high level (over-expression) of *norA* and *norB* gene expression showed greater resistance to ciprofloxacin ($P<0.05$). The results of the efflux pump genes expression level in ciprofloxacin-resistant isolates are shown in Table 3.

Table 3. Fold change in mRNA levels of efflux genes.

Isolates No.	Gmk	norA	norB
7	1.0	180.65	140.22
9	1.0	14.57	-
31	1.0	35.77	25.55
32	1.0	18.44	-
33	1.0	114.91	95.56
34	1.0	400.31	320.77
43	1.0	14.57	5.6
45	1.0	20.32	15.33
46	1.0	15.23	15.11
47	1.0	40.08	33.99
50	1.0	200.15	190.22
ATCC 25923	1.0	11.52	10.15
ATCC 12228	1.0	-	-

-:Not detected

MIC and efflux assay:

As shown in Table 3, the MIC of isolates was between 15.62 to 250 µg/ml. The presence of active efflux pumps in isolates was evaluated based on their ability to accumulate EtBr and ciprofloxacin.

For efflux assay, 12 ciprofloxacin-resistant isolates showed active efflux activity by reducing EtBr and ciprofloxacin MIC values by 2 to 4 folds, in the presence of CCCP. Ciprofloxacin sensitive strains (together with negative control) showed no efflux activity. The results of MIC and efflux activity tests are given in Table 4.

Discussion

S. aureus is a common human pathogen and nosocomial infection agent. One of the serious problems in treatment and prevention of *S. aureus* infections is the antibiotic resistance issue which is mediated by the efflux pump and recently has been one of the interesting issues

Table 4. Determination of MICs of EtBr, ciprofloxacin and its combination with CCCP against ciprofloxacin resistant isolates.

Isolates	MIC (µg/ml)			
	CIP	EtBr	EtBr +	CIP
7	125	62.5	31.25	62.5
9	15.62	7.81	3.9	7.81
31	31.25	7.81	7.81	31.25
32	62.5	15.6	3.9	31.25
33	125	62.5	15.62	62.5
34	250	125	31.25	62.5
43	31.25	15.62	7.8	15.62
45	31.25	7.81	1.95	15.62
46	15.62	7.81	3.9	3.9
47	62.5	15.62	7.81	15.6
48	31.25	7.81	7.81	7.81
50	250	125	125	125
ATCC 25923	62.5	31.25	7.81	31.25

CIP: Ciprofloxacin

for most of the researchers (20-22).

In this study, out of 50 isolates, 34 isolates (68%) were MRSA. The rate of antibiotic resistance to ciprofloxacin was 24% which was partly similar to that of other reports (23). Moreover, vancomycin and colistin antibiotics were the most active agents against ciprofloxacin-resistant strains.

The heterogeneity of MRSA percentage in different studies is probably due to antibiotic administration, study design and different laboratory tests used to determine the methicillin resistance pattern. In this study, MRSA strains were detected by the disk diffusion test and PCR method.

To study ciprofloxacin resistance pattern and its correlation to efflux pumps, screening of efflux pumps was carried out by determination of ciprofloxacin and EtBr MIC value, both in the presence and absence of efflux pump inhibitor, CCCP. EtBr is a common efflux substrate that has been used in most studies as the positive control to detect bacterial active efflux pumps. Furthermore, the combination of EtBr-CCCP has been used as a standard positive control in efflux activity assays (24).

CCCP decreased the MICs by 2-4 folds, a result which was reported by other studies (25). MICs reduction of isolates in the presence of CCCP showed the presence of a proton gradient dependent efflux pump in our strains. Besides, the detection of efflux pump genes was done by PCR showing efflux pump genes (*norA* and *norB*) in ciprofloxacin-resistant strains. *norA* gene is located on the bacterial chromosome. It is highly conserved and can be observed in ciprofloxacin-resistant isolates (26), as it was detected in all ciprofloxacin-resistant isolates of this study the structure of the efflux pump norB gene is similar to that of *norA* and is categorized into MFS proton drove efflux pump. It can confer resistance to hydrophilic fluoroquinolones and biocides such as ciprofloxacin and dye ethidium bromide (27). Over-expression of *norB*, like *norA*, was correlated with increased resistance to ciprofloxacin. In our study, *norB* gene was detected in 83% of ciprofloxacin-resistant isolates and overexpression was seen in more resistant isolates. It seems that the over-expression of efflux pumps genes in ciprofloxacin-resistant isolates plays a critical role in resistance to ciprofloxacin and so the study of gene regulators is quite necessary.

Similar results were obtained by Saiful et al, in 2008 in a study to determine the efflux genes and their correlation with resistance to antibiotics; where it was shown that most of the isolates harbored norA gene in MRSA strains and efflux pumps are active among isolates using EtBr method (28). our results showed a significant correlation between antibiotic susceptibility pattern and efflux pump genes in isolates. It can be concluded that efflux pumps lead to ciprofloxacin resistance or other antibiotic resistance in bacterial strains. A study by Costa SS et al in 2013demonstrated the efflux pump as an important agent for fluoroquinolone resistance in *S. aureus* and suggested it as a major mechanism in the early stages of resistance development (29).

Conclusions

In conclusion, this study is the first to detect efflux pump genes and activity in ciprofloxacin -resistant S. aureus isolates in Iran.

Antibiotic-resistant strains especially MRSA and ciprofloxacin-resistant strains of *S. aureus* have emerged as important nosocomial pathogens among hospitalized patients. The results of this study showed a significant correlation between ciprofloxacin resistance and *norA*, *norB* efflux pump genes in resistant *S. aureus* isolates.

The level of *norA* and *norB* gene expression was different in resistant isolates, so that the isolates which were more resistant to ciprofloxacin, showed a high level of efflux pump gene expression. Overall, the characterization of resistance mechanisms among resistant *S. aureus* strains and the application of new drugs are needed to control antibiotic resistance and avoid the spreading of multi-drug resistant isolates.

Ethical Consideration

Authors of all ethics including non-plagiarism, Dual publishing has complied with data distortions and data making in this article.

Acknowledgment

The authors would like to acknowledge the valuable contributions of Soheil Salehi.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

1. Corredor A, Luligo E, Moncayo O, Santacruz I, Álvarez A. Relationship between super antigenicity, antimicrobial resistance and origin of *Staphylococcus aureus* isolated. Colomb Med (Cali). 2016; 47: 15-20.

2. Hefzy EM, Hassan GM, Abd E, Reheem F. Detection of panton-valentine leukocidin-positive methicillin-resistant *Staphylococcus aureus* nasal carriage among Egyptian health care workers. Surg Infect (Larchmt). 2016; 17: 369-375.

3. Jeremić LP, Kapulica NK, Ristanović E, Josić D, Lepsanović Z. Prevalence of panton-valentine leukocidin genes in community-associated methicillin-resistant *Staphylococcus aureus* in the District of Pomoravlje. Vojnosanit Pregl. 2016; 73: 256-260.

4. Osman KM, Amer AM, Badr JM, Helmy NM, Elhelw RA, Orabi A, Bakry M, Saad AS. Antimicrobial resistance, biofilm formation and *mecA* characterization of methicillin-susceptible *S. aureus* and Non-*S. aureus* of beef meat origin in Egypt. Front Microbiol. 2016; 29: 222-230.

5. Mohamed NA, Ramli S, Amin NN, Sulaiman WS, Isahak I, Jamaluddin TZ, Salleh NM. *Staphylococcus aureus* carriage in selected kindergartens in Klang Valley. Med J Malaysia. 2016; 71: 62-65.

6. Firsov AA, Smirnova MV, Strukova EN, Vostrov SN, Portnoy YA, Zinner SH. Enrichment of resistant *Staphylococcus aureus* at ciprofloxacin concentrations simulated within the mutant selection window: bolus versus continuous infusion. Int J Antimicrob Agents. 2008; 32:

488-493.

7. Mustapha M, Bukar-Kolo YM, Geidam YA, Gulani IA. Phenotypic and genotypic detection of methicillin-resistant *Staphylococcus aureus* in hunting dogs in Maiduguri metropolitan, Borno State, Nigeria. Vet World 2016; 9: 501-506.

8. Poole K. Efflux pumps as antimicrobial resistance mechanisms. Ann Med. 2007; 39: 162-176.

9. Li XZ, Nikaido H. Efflux-mediated drug resistance in bacteria. Drugs. 2004; 64; 159-204.

10. Kosmidis C. B.D, Schindler P.L, Jacinto D, Patel K, Bains S.M, Seo G.W, Kaatz A. Expression of multidrug resistance efflux pump genes in clinical and environmental isolates of *Staphylococcus aureus*. Int J Antimicrob Agents. 2012; 40: 204-209.

11. Paulsen I.T, Lewis K. Microbial multidrug efflux. Horizon Scientific 2002; 3: 143-144.

12. Li XZ, Nikaido H. Efflux-mediated drug resistance in bacteria: an update. Drugs. 2009; 69: 1555-1623.

13. Soto SM. Role of efflux pumps in the antibiotic resistance of bacteria embedded in a biofilm. Virulence. 2013; 1: 223-229.

14. De Kievit TR, Parkins MD, Gillis RJ, Srikumar R, Ceri H, Poole K, Iglewski BH, Storey DG. Multidrug efflux pumps: expression patterns and contribution to antibiotic resistance in *Pseudomonas aeruginosa* biofilms. Antimicrob Agents Chemother. 2001; 45: 1761-1770.

15. de Araújo RS, Barbosa-Filho JM, Scotti MT, Scotti L, da Cruz RM, Falcão-Silva Vdos S, de Siqueira-Júnior JP, Mendonça-Junior FJ. Modulation of drug resistance in *Staphylococcus aureus* with Coumarin derivatives. Scientifica (Cairo*)*. 2016; 2016: 6894758.

16. Baba T, Takeuchi F, Kuroda M, Yuzawa H, Aoki K, Oguchi A, Nagai Y, Iwama N, Asano K, Naimi T, Kuroda H, Cui L, Yamamoto K, Hiramatsu K. Genomic and virulence determinants of high virulence community-acquired MRSA. Lancet. 2002; 359(9320): 1819-1827.

17. Clinical and laboratory standards institute (CLSI), 2006. Performance standards for antimicrobial susceptibility testing; 16th informational supplement. CLSI, Wayne, Pa. M100-S16, 26, no. 3. 2006.

18. Costa SS, Viveiros M, Amaral L, Couto I. Multidrug Efflux Pumps in *Staphylococcus aureus*: an Update. Open Microbiol J. 2013; 7: 59-71.

19. Patel D, Kosmidis C, Seo SM, Kaatz GW. Ethidium bromide MIC screening for enhanced efflux pump gene expression or efflux activity in *Staphylococcus aureus*. Antimicrob Agents Chemother. 2010; 54(12): 5070-5073.

20. Xia J, Gao J, Tang W. Nosocomial infection and its molecular mechanisms of antibiotic resistance. Biosci Trends. 2016; 10: 14-21.

21. Santos Costa S, Viveiros M, Rosato AE, Melo-Cristino J, Couto I. Impact of efflux in the development of multidrug resistance phenotypes in *Staphylococcus aureus*. BMC Microbiol. 2015; 15: 232-230.

22. Liger F, Bouhours P, Ganem-Elbaz C, Jolivalt C, Pellet-Rostaing S, Popowycz F, Paris JM, Lemaire M. C2 arylated benzo thiophene derivatives as *Staphylococcus aureus* NorA efflux

pump inhibitors. Chem Med Chem. 2016; 11: 320-330.

23. Sudhanthiramani S, Swetha CS, Bharathy S. Prevalence of antibiotic resistant *Staphylococcus aureus* from raw milk samples collected from the local vendors in the region of Tirupathi, India. Vet World. 2015; 8: 478-481.

24. Patel D, Kosmidis C, Seo SM, Kaatz GW. Ethidium bromide MIC screening for enhanced efflux pump gene expression or efflux activity in *Staphylococcus aureus*. Antimicrob Agents Chemother. 2010; 54: 5070-5073.

25. Truong-Bolduc, Strahilevitz J, Hooper D.C. NorC, a new efflux pump regulated by MgrA of *Staphylococcus aureus*. Antimicrob Agents Chemother. 2006; 50: 1104-1107.

26. Sierra JM, Ruiz J, Jimenez De Anta MT, Vila J. Prevalence of two different genes encoding NorA in 23 clinical strains of *Staphylococcus aureus*. J Antimicrob Chemother. 2000; 46: 145-146.

27. Ding Y, Onodera Y, Lee JC, Hooper DC. NorB, an efflux pump in *Staphylococcus aureus* strain MW2, contributes to bacterial fitness in abscesses. J Bacteriol. 2008; 190: 7123-7129.

28. Saiful AJ, Mastura M, Zarizal S, Mazurah MI, Shuhaimi M, Ali AM. Efflux genes and active efflux activity detection in Malaysian clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA). J Basic Microbiol. 2008; 48: 245-251.

29. Costa SS, Junqueira E, Palma C, Viveiros M, Melo-Cristino J, Amaral L, Couto I. Resistance to antimicrobials mediated by efflux pumps in *Staphylococcus aureus.* Antibiotics (Basel). 2013; 2: 83-99.