Original Article

J Babol Univ Med Sci
Vol 18, Issu 11; Nov 2016. P:63-70

A Study of Gene Expression and Activity of NorA Efflux Pump in Clinical Isolates of Ciprofloxacin Resistant Staphylococcus Aureus

A. Mirzaie (PhD)*[1], H. Noorbazargan (MSc)[2], H. Rahmati (MSc)[3], M. Zandi (MSc)[4]

1.Young Researchers and Elite Club, Islamic Azad University, East Tehran Branch, Tehran, I.R.Iran

2.Faculty of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, I.R.Iran

3.Izaddoost Medical Laboratory, Tehran, I.R.Iran

4.Department of Nursing, Islamic Azad University, Tuyserkan Branch, Tuyserkan, I.R.Iran

J Babol Univ Med Sci; 18(10); Oct 2016; PP: 63-70

Received: Jun 25th **2016, Revised:** Jul 27th **2016, Accepted:** Sep 27th **2016.**

ABSTRACT

BACKGROUND AND OBJECTIVE: The existence of efflux pumps such as *norA* is one the mechanisms of resistance to antibiotics like ciprofloxacin in *staphylococcus aureus* bacteria. *NorA* efflux pump and its gene expression and activity in ciprofloxacin resistant *S. aureus* strains are investigated in this study.

METHODS: In this experimental study, 250 clinical samples of blood, urine, wound and trachea were collected from patients hospitalized in various hospitals of Tehran. *S. aureus* isolates were detected and thereafter, antibiotic resistance profile, gene expression of *norA* efflux pump and its existence were investigated using PCR and Real-Time PCR and the activity of *norA* efflux pump was investigated using minimum inhibitory concentration (MIC).

FINDINGS: Of 250 clinical samples, 50 *S. aureus* isolates were detected and the antibiotic susceptibility test results revealed that 68% of samples (34 samples) were resistant to methicillin and 32% of samples (16 samples) were susceptible to methicillin and 24% of all samples (12 samples) were resistant to ciprofloxacin. Real-Time PCR test results revealed that each type of strain has a different expression of *norA* gene and more resistant strains have increased expression of *norA* gene. Moreover, all ciprofloxacin resistant strains had active efflux pump.

CONCLUSION: The results of the study demonstrated that there is a significant relationship between gene expression of *norA* efflux pump, its activity and resistance to ciprofloxacin in *S. aureus* strains.

KEY WORDS: *Staphylococcus aureus, Resistance to ciprofloxacin, Efflux pump, NorA.*

Please cite this article as follows:

Mirzaie A, Noorbazargan H, Rahmati H, Zandi M. A Study of Gene Expression and Activity of NorA Efflux Pump in Clinical Isolates of Ciprofloxacin Resistant Staphylococcus Aureus. J Babol Univ Med Sci. 2016;18(11):63-70.

***Corresponding author: A. Mirzaie (PhD)**

Address: East Tehran Branch, Islamic Azad University, Ghiamdasht, Khavaran Highway, Tehran, I.R.Iran

Tel: +98 21 33594950

E-mail: Amir_mirzaie92@yahoo.com

[DOR: 20.1001.1.15614107.1395.18.11.9.3]

[DOI: 10.22088/jbums.18.11.63]

Introduction

Staphylococcus aureus is an opportunistic infective factor in hospitals worldwide and the emergence of methicillin-resistant *staphylococcus aureus* (MRSA) has become a serious issue in recent years. The cause of resistance to methicillin is the presence of *mecA* gene in these strains (1-3). Similar to ciprofloxacin, fluoroquinolones antibiotics are usually used to treat MRSA-induced infections. Unfortunately, after administration of ciprofloxacin to treat MRSA-induced infections, these bacteria became resistant to ciprofloxacin quickly (4, 5).

Several mechanisms are involved in the resistance of *staphylococcus aureus* to antibiotics and one of the major mechanisms of resistance is the presence of efflux pumps in this bacteria (6-8). Overall, bacterial efflux pumps are categorized into five main groups based on amino acid similarity: Major Facilitator Super Family (MFS), ATP-binding cassette (ABC), Resistance-Nodulation Division (RND), Small Multidrug Resistance (SMR), Multidrug and Toxic Compound Extrusion (MATE) (9-12).

NorA gene is a member of (efflux pumps) MFS family. It is a protein with 388 amino acids and consists of 12 components that move across the plasma membrane, which is 24% similar to efflux pumps Tet (A) in *escherichia coli* bacteria (13-15).

Jo et al. demonstrated that the gene expression of *norA* efflux pump is different in various strains (16). Since no previous study has been dedicated to gene expression of efflux pump and their function in ciprofloxacin-resistant *staphylococcus aureus* isolates in Iran, the present study aims to analyze the antibiotic resistance pattern and presence of efflux pump and to investigate the function and gene expression of *norA* efflux pumps in clinical strains of ciprofloxacin-resistant *staphylococcus aureus*.

Methods

Sampling, culture and detection of *staphylococcus aureus* isolates: In this experimental study, 250 clinical samples of blood, urine, wound and trachea were collected from patients with active infection hospitalized in several hospitals of Tehran (Imam Hussein, Sarem and Bu Ali hospitals) during 6 months (2015-2016). *Staphylococcus aureus* isolates were detected using Gram staining test, Mannitol salt agar, Brad Parker environment, Catalase test, Coagulase test, Mannitol fermentation and DNase.

Investigating the antibiotic susceptibility of the separated isolates: After *staphylococcus aureus* isolates were detected and confirmed, susceptibility of strains to different antibiotics was evaluated using disk diffusion method based on CLSI standard (Clinical and Laboratory Standards Institute, 2014) (17). Susceptibility of *staphylococcus aureus* isolates to antibiotic cefoxitin discs (10 µg), vancomycin (10 µg), ciprofloxacin (5 µg), penicillin (10 units), erythromycin (15 µg), trimethoprim (25 µg), amikacin (15 µg), ampicillin (10 µg), gentamicin (10 µg), amoxicillin (10 µg), chloramphenicol (30 µg) and clindamycin (2 µg) (MAST, UK) was evaluated in Mueller Hinton agar (Merck, Germany). In all tests, standard strains of *staphylococcus aureus* (ATCC 33591), *staphylococcus aureus* (ATCC 25923) and *staphylococcus aureus* (ATCC 12228) were used as methicillin-resistant positive control (containing *mecA* gene), ciprofloxacin-resistant positive control and negative control, respectively.

DNA extraction and detection of *mecA* and *norA* genes using PCR method: DNA extraction was done manually (phenol-chloroform) and 1% agarose gel was used to confirm the extraction of genome from electrophoresis. PCR reaction was done at a final volume of 25 µl including 1 µl of the extracted DNA as template, 0.5 µl forward primer, 0.5 µl reverse primer (10 Pmol), 12.5 µl Master Mix (Cinnagen, Iran) and 10.5 µl double-distilled water. Then, PCR reaction was done for *mecA* gene using forward primers of 5'-TCCAGATTACAACTTCACCAGG-3' and reverse primers of 5'-CCACTTCATATCTTGTAACG-3' and *norA* gene from primers of 5'-ATCGGTTTAGTAATACCAGTCTTGC-3' including 5'-GCGATATAATCATTTGAGATAACGC-3' sequence according to the determined time and temperature plan (table 1). In all PCR reactions, water was used as negative control and standard bacterial DNA (ATCC) was used as positive control.

[DOR: 20.1001.1.15614107.1395.18.11.9.3]
[DOI: 10.22088/jbums.18.11.63]

RNA extraction, cDNA synthesis and analyzing gene expression of *norA* using Real Time PCR method: For RNA extraction, ciprofloxacin-resistant strains were cultured in Mueller Hinton Broth medium at 37°C for 24 hours in sub-MIC concentrations of ciprofloxacin. Then, RNA extraction was performed using RNA extraction kit (Qiagen, USA) based on the instructions. After that, cDNA synthesis was done using Quanti Tect Reverse Transcription kit (Qiagen, USA). Finally, concentration of the extracted cDNA was determined using Nanodrop.

For analysis, gene expression of *norA* efflux pump was assessed using quantitative Real Time PCR (qRT-PCR) by SYBR Green Master Mix (Applied Biosystem, England). The materials used at a 20 µl Master Mix volume included 2 µl cDNA, 10 Pmol forward and reverse primers and 10 µl SYBR Green Master Mix in Bioneer machine (Korea). The temperature plan used in qPCR included 90°C for 10 minutes, 95°C for 15 seconds, 60°C for 1 minute, which was performed in 40 cycles (table 2). Moreover, *gmk* (guanylate kinase) gene was used as internal control. Finally, the relative gene expression of *norA* was calculated using ΔΔCт method.

Determination of antibiotic susceptibility and the minimum inhibitory concentration (MIC) of ciprofloxacin: MIC test was preformed based on CLSI using microplate dilution method for ciprofloxacin and ethidium bromide. The MIC test was repeated three times using microdilution method in 96-well plates. The concentration of ethidium bromide solution (2-250 µg/ml) and the concentration of ciprofloxacin (0.5-128 µg/ml) were used to study MIC. It is worth mentioning that the well containing ciprofloxacin and ethidium bromide-free bacteria was used as negative control and the well containing standard bacteria, ciprofloxacin and ethidium bromide was used as positive control.

Phenotypic assessment of active efflux pump: In this test, a compound named CCCP (Carbonyl cyanide 3-chlorophenylhydrazone) was used as efflux pump inhibitor. This compound destroys oxidative phosphorylation and concentration gradient of cell membranes and inhibits the activity of efflux pump in bacteria and the procedure of this method is similar to MIC test. In this method, after determining the minimum inhibitory concentration of ethidium bromide, CCCP compound is added to the wells as efflux pump inhibitor at a concentration of 20 µg/ml and active efflux pump is detected when the minimum inhibitory concentration of ethidium bromide with CCCP is lower than the minimum inhibitory concentration of ethidium bromide without CCCP.

Statistical analysis: The statistical analysis of this study was done using SPSS V.21 and the results were assessed by One-Way ANOVA and $p<0.05$ was considered significant.

Table 1. Time and temperature conditions of PCR reaction for mecA and norA genes

Primary denaturation (Minute/Centigrade)	Secondary denaturation (Second/Centigrade)	Attachment of primers (Second/Centigrade)	Polymerization (Second/Centigrade)	Final Polymerization (Minute/Centigrade)	Number of Cycles
5/94	50/94	50/60	50/72	5/72	30
4/94	50/95	30/60	60/72	4/72	30

Table 2. Primers used in qRT-PCR

Primer	Sequence (5-3)	Size (bp)	Ref.
norA-F	ATCGGTTTAGTAATACCAGTCTTGC	112	21
norA-R	GCGATATAATCATTTGAGATAACGC	112	
Gmk-F	TATCAGGACCATCTGGAGTAGG	122	21
Gmk-R	CATCAACTTCACCTTCACGC	122	

[DOR: 20.1001.1.15614107.1395.18.11.9.3]
[DOI: 10.22088/jbums.18.11.63]

Results

Antimicrobial susceptibility test results: The antibiogram results demonstrated that of 50 studied isolates, 34 isolates were resistant to methicillin (68%) and were considered as MRSA strains, while the level of resistance to ciprofloxacin was 24% (table 3).

Table 3. Resistance and susceptibility of *staphylococcus aureus* strains to various antibiotics

Strains / Antibiotic	Susceptible N(%)	Moderate N(%)	Resistant N(%)
Methicillin (cefoxitin)	16(32)	0(0)	34(68)
Vancomycin	50(100)	0(0)	0(0)
Ciprofloxacin	37(74)	1(2)	12(24)
Penicillin	1(2)	0(0)	49(98)
Erythromycin	13(26)	9(18)	28(56)
Trimethoprim	4(8)	3(6)	43(86)
Amikacin	4(8)	3(6)	21(42)
Ampicillin	5(10)	0(0)	45(90)
Gentamicin	27(54)	3(6)	20(40)
Amoxicillin	7(14)	0(0)	43(86)
Chloramphenicol	39(78)	7(14)	4(8)
Clindamycin	21(42)	6(12)	23(46)
Colistin	100(100)	0(0)	0(0)

Results of amplification of *mecA* gene: Amplification of *mecA* gene was used for molecular study of methicillin-resistant gene and a 162 bp band was expected considering the design of primers (Fig 1). Results showed the amplification of *mecA* gene in 68% of staphylococcal samples (34 samples). The prevalence of *mecA* gene in staphylococcus aureus samples isolated from wound samples was more than other samples; of 30 samples, 15 samples belonged to wound samples (p<0.032).

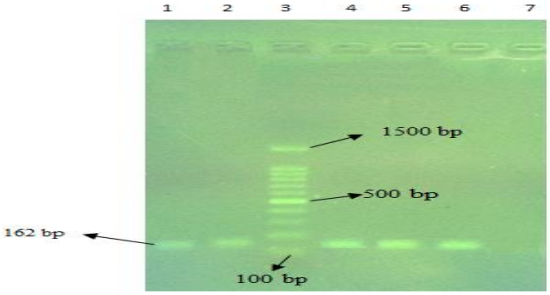

Figure 1. Results of amplification of mecA gene in methicillin-resistant strains. 1-2, 4, 5: methicillin-resistant samples, 7: negative control, 6: positive control, 3: DNA 100bp+ marker

Results of amplification of *norA* gene: To examine the existence of efflux pump *norA* in the isolated *staphylococcus aureus* strains, the specific primers of this gene were used and a 112 bp band was expected. *norA* gene was resistant to ciprofloxacin in all 12 strains (Fig 2).

Assessment of *norA* gene expression in ciprofloxacin-resistant strains: Results demonstrated that various strains with different levels of resistance reveal different gene expression of *norA* and the more resistant strains revealed higher gene expression of *norA* and their difference with gene expression of *gmk* was statistically significant (p<0.05) (Fig 3).

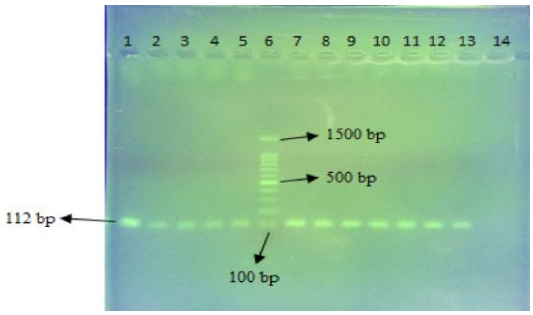

Figure 2. Results of amplification of *norA* gene in ciprofloxacin-resistant strains. 1-5 and 7-12: ciprofloxacin-resistant samples, 13: negative control, 14: positive control, 6: DNA 100bp+ marker

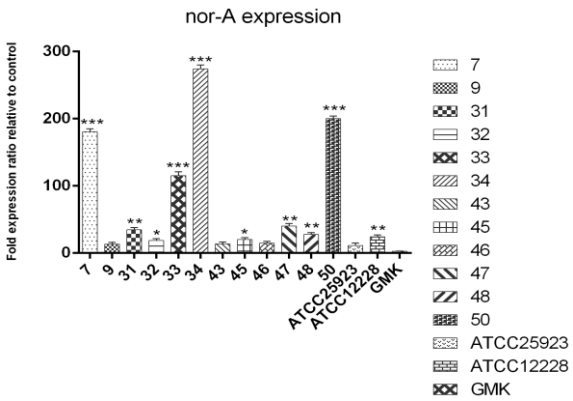

Figure 3. Gene expression of *norA* in various ciprofloxacin-resistant strains. Figures of *norA* gene expression show Fold change in gene expression. Results showed Fold change in gene expression compared with the reported control samples (n=3: p<0.001 ***, p<0.01: ** p<0.05=*)

Assessment of minimum inhibitory concentration (MIC) and phenotypic study of efflux pump: Results of MIC and CCCP activity are presented in

[DOR: 20.1001.1.15614107.1395.18.11.9.3]
[DOI: 10.22088/jbums.18.11.63]

Table 4. It is obvious that minimum inhibitory concentration of ciprofloxacin in strains was in the range of 15.62-250 and in proximity to efflux pump inhibitor (CCCP), the level of MIC in ciprofloxacin and ethidium bromide decreased, which indicates the activity of efflux pump in ciprofloxacin-resistant strains (table 4).

Table 4. Determination of minimum inhibitory concentration of ciprofloxacin, ethidium bromide, CCCP and their combination in ciprofloxacin-resistant strains

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

Discussion

Of the 50 isolated *staphylococcus aureus* strains in this study, 34 strains (68%) were resistant to methicillin (MRSA) and 12 strains (24%) were resistant to ciprofloxacin.

The antibiotic resistance caused by efflux pump is one the major mechanisms in staphylococcus aureus and has become a very important topic for researchers in recent years (18, 19).

In this study, MRSA of strains was studied using disk diffusion method and PCR. The study of Moradi et al. using 104 *staphylococcus aureus* samples demonstrated that highest levels of susceptibility of strains was to vancomycin (96.2%), chloramphenicol (88.2) and rifampin (81.7) and resistance of strains to cefoxitin was 40.4% (MRSA) (20). To assess the resistance to ciprofloxacin and its relationship with efflux pump in this study, screening of strains was done to examine the existence and function of *norA* gene using PCR method and minimum inhibitory concentration of ethidium bromide in proximity to efflux pump inhibitor (CCCP). *norA* gene was present in all ciprofloxacin-resistant strains. *norA* gene is located inside chromosome and is highly protected among strains. Similar results have been reported in other studies.

Results of a study by Pourmand et al. revealed that *norA* gene is present in all ciprofloxacin-resistant strains and its gene expression increases in proximity to biocide hexahydroquinolone (21). According to a study by Saiful et al., of 19 isolated MRSA strains, 16 strains contained *norA* gene and all strains had active efflux pump (22).

A comparison between our study and previous studies shows similar results, confirming the presence of efflux pump genes in ciprofloxacin-resistant strains. To examine the activity of efflux pump, CCCP decreased by 2-4 times the level of MIC in strains compared with ethidium bromide and ciprofloxacin, which was noted in most of the similar studies. Ethidium bromide is a typical substrate of efflux pump that is used as a positive control in most studies to measure the activity of efflux pump.

Costa et al. demonstrated that efflux pump plays a key role in decreasing resistance to antibiotics and biocides (23). As mentioned in "Results" section, level of MIC in ethidium bromide decreases in the presence of CCCP, which is in line with other studies and confirms that efflux pump induces resistance to antibiotic. In this study, gene expression of *norA* in ciprofloxacin-resistant strains was assessed.

Our results demonstrated that ciprofloxacin-resistant strains have different expression of *norA* gene and the more resistant strains revealed higher relative gene expression of *norA*, which was in line with similar studies. Results of a study by Huet et al. demonstrated that *norA* efflux pumps were present in all strains and their expression increased in proximity

[DOR: 20.1001.1.15614107.1395.18.11.9.3]
[DOI: 10.22088/jbums.18.11.63]

to biocides (24). Results of the present study showed that *norA* efflux pump is directly related to ciprofloxacin-resistance and therefore, developing efflux pump inhibitors can help to control the risk of efflux pump-resistant strains.

Acknowledgments

Hereby, we express our deepest sense of gratitude to our colleagues in Islamic Azad University of East Tehran, Mr. Arian Rahimi and all those who participated in this study.

[DOR: 20.1001.1.15614107.1395.18.11.9.3]
[DOI: 10.22088/jbums.18.11.63]

References

1.Phillips CJ, Wells NA, Martinello M, Smith S, Woodman RJ, Gordon DL. Optimizing the detection of methicillin-resistant staphylococcus aureus with elevated vancomycin minimum inhibitory concentrations within the susceptible range. Infect Drug Resist. 2016;31(9):87-92.

2.Liu Y, Zhang J, Ji Y. PCR-based approaches for the detection of clinical methicillin-resistant staphylococcus aureus. Open Microbiol J. 2016;14(10):45-56.

3.Sganga G, Tascini C, Sozio E, Carlini M, Chirletti P, Cortese F, et al. Focus on the prophylaxis, epidemiology and therapy of methicillin-resistant Staphylococcus aureus surgical site infections and a position paper on associated risk factors: the perspective of an Italian group of surgeons. World J Emerg Surg. 2016;14(11):26.

4.Zarei KR, Mahmoodzadeh H, Mehdizadeh A, Ghorbani T, Imani Fooladi AA. Distribution of tsst-1 and mecA Genes in Staphylococcus aureus isolated from clinical specimens. Jundishapur J Microbiol. 2016;9(3): 29057.

5.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(5):501-6.

6.Poole K. Efflux pumps as antimicrobial resistance mechanisms. Ann Med. 2007;39(3):162-76.

7.X.-Z. Li, H. Nikaido. Efflux-mediated drug resistance in bacteria. Drugs. 2004;64(2);159-204.

8.Kosmidis C, Schindler BD, Jacinto PL, Patel D, Bains K, Seo SM, et al. Expression of multidrug resistance efflux pump genes in clinical and environmental isolates of staphylococcus aureus. Int J Antimicrob Agents. 2012;40(3):204-09.

9.Paulsen I.T, Lewis K. Microbial Multidrug Efflux. Horizon Sci. 2002;3(2):143-4.

10.Li XZ, Nikaido H. Efflux-mediated drug resistance in bacteria: an update. Drugs. 2009;69(12):1555-623.

11.Soto SM. Role of efflux pumps in the antibiotic resistance of bacteria embedded in a biofilm. Virulence. 2013;4(3):223-9.

12.De Kievit TR, Parkins MD, Gillis RJ, Srikumar R, Ceri H, Poole K, et al. Multidrug efflux pumps: expression patterns and contribution to antibiotic resistance in pseudomonas aeruginosa biofilms. Antimic Age Chemother. 2001;45(6):1761-70.

13.Yoshida H, Bogaki M, Nakamura S, Ubukata K, Konno M. Nucleotide sequence and characterization of the Staphylococcus aureus norA gene, which confers resistance to quinolones. J Bacteriol. 1990;172(12):6942-9.

14.Noguchi N, Okada H, Narui K, Narui K, Sasatsu M. Comparison of the nucleotide sequence and expression of norA genes and microbial susceptibility in 21 strains of staphylococcus aureus. Microb Drug Resist. 2004;10(3):197-203.

15.Motallebi M, Jabalameli F, Asadollahi K, Taherikalani M, Emaneini M. Spreading of genes encoding enterotoxins, haemolysins, adhesin and biofilm among methicillin resistant Staphylococcus aureus strains with staphylococcal cassette chromosome mec type IIIA isolated from burn patients. Microb Pathog. 2016;97:34-7.

16.Jo A, Ahn J. Phenotypic and genotypic characterisation of multiple antibiotic-resistant Staphylococcus aureus exposed to subinhibitory levels of oxacillin and levofloxacin. BMC Microbiol. 2016;29;16(1):170.

17.Clinical and laboratory standards institute (CLSI). Performance standards for antimicrobial susceptibility testing; 16th informational supplement. CLSI, Wayne, Pa. 2014;53(3):1-23.

18.Costa SS, Falcão C, Viveiros M, Machado D, Martins M, Melo-Cristino J, Amaral L, Couto I. Exploring the contribution of efflux on the resistance to fluoroquinolones in clinical isolates of staphylococcus aureus. BMC Microbiol. 2011;27(11):241.

19.Ding Y, Onodera Y, Lee JC, David C. Hooper. NorB, an efflux pump in Staphylococcus aureus strain MW2, contributes to bacterial fitness in abscesses. J Bacteriol. 2008;190(21):7123-9.

20. Moradi N, Javadpour S, Karmostaji A. Reduced sensitivity of staphylococcus aureus to vancomycin. Hormozgan Uni of Med Sciences. 2011;15(3):169-77.[In Persian].

[DOR: 20.1001.1.15614107.1395.18.11.9.3]
[DOI: 10.22088/jbums.18.11.63]

21.Pourmand MR, Yousefi M, Salami SA, Amini M. Evaluation of expression of NorA efflux pump in ciprofloxacin resistant Staphylococcus aureus against hexahydroquinoline derivative by real-time PCR. Acta Med Iran. 2014; 52(6):424-9.

22.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(4):245-51.

23.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;13:2(1):83-99.

24.Huet AA, Raygada JL, Mendiratta K, Seo SM, Kaatz GW. Multidrug efflux pump overexpression in Staphylococcus aureus after single and multiple in vitro exposures to biocides and dyes. Microbiology. 2008; 154(10):3144-53.

[DOR: 20.1001.1.15614107.1395.18.11.9.3]

[DOI: 10.22088/jbums.18.11.63]