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Original Article

Expression of *norA*, *norB* and *norC* efflux pump genes mediating fluoroquinolones resistance in MRSA isolates

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Abstract

Introduction: Although fluoroquinolones are used to treat methicillin-resistant *Staphylococcus aureus* (MRSA)-induced infections, acquisition of antibiotic resistance by bacteria has impaired their clinical relevance. We aimed to evaluate the frequency of *norA*, n*orB*, and *norC* efflux pump genes-mediating fluoroquinolones resistance and measure their expression levels in MRSA isolates.

Methodology: 126 *S. aureus* isolates were collected from different clinical samples of adult hospitalized patients and identified by conventional microbiological methods. MRSA was diagnosed by cefoxitin disc diffusion method and minimum inhibitory concentration (MIC) of ciprofloxacin by broth microdilution method. The expression levels of efflux pump genes were measured by quantitative real-time polymerase chain reaction (qRT-PCR).

Results: 80 (63.5%) MRSA isolates were identified and showed high level of resistance to erythromycin (80%), gentamicin (75%), clindamycin (65%) and ciprofloxacin (60 %). *norA*, *norB*, and *norC* were detected in 75%, 35% and 55% of the MRSA isolates respectively. *norC* was the most commonly overexpressed gene measured by qRT-PCR, occurring in 40% of MRSA isolates, followed by *norA* (35%) and *norB* (30%). The expression of these genes was significantly higher in ciprofloxacin-resistant than quantitative real-time PCR ciprofloxacin-sensitive MRSA isolates.

Conclusions: This study showed high prevalence and overexpression of efflux pump genes among MRSA isolates which indicates the significant role of these genes in the development of multidrug resistance against antibiotics including fluoroquinolones.

Key words: efflux pump genes; fluoroquinolones; MRSA; *norA*; *norB*; *norC*.

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Introduction

Staphylococcus aureus, a Gram-positive bacterium, poses a serious concern in the nosocomial and the community settings due to its ability to induce fatal conditions and life-threatening infections [1]. Its potential virulence is markedly associated with a wide diversity of its resistance mechanisms to various antimicrobial compounds [2].

The most serious and challenging type is the methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA causes major outbreaks in nosocomial environments with a significant therapy challenge particularly in the intensive care units (ICUs) that urgently necessitates finding effective therapeutic methods to lessen its threat to the health care systems [3]. MRSA strains generate a substitute penicillin-binding protein (PBP), PBP2a, which is encoded by the *mecA* gene. The PBP2a protein has reduced affinity to multiple beta-lactam antibiotics making MRSA strains extensively resistant to almost all these antibiotics [4] and warrants searching for other effective antibiotics.

Although vancomycin is the drug of choice for MRSA infections, it is expensive, and needs careful monitoring of the intravenous injection. It also has various serious adverse effects, including nephrotoxicity and ototoxicity, limiting its routine use

and reducing patient compliance. Moreover, previous investigations showed that vancomycin could develop low grade to absolute resistance [5,6]. Therefore, fluoroquinolone antibiotics have been introduced as a potential alternative therapeutic option to parenteral vancomycin treatment based on in vitro and in vivo animal model data [6,7].

However, multiple fluoroquinolone resistance mechanisms have unfortunately emerged in *S. aureus*. Both mutations, in the quinolone-resistance determining region (QRDR) of topoisomerase IV encoded by *grlA/grlB* genes and DNA gyrase encoded by *gyrA/gyrB* genes, are examples of this resistance mechanism. Furthermore, there is an emerging drug efflux resistance mechanism that *S. aureus* strains use to resist fluoroquinolone therapy. This mechanism is less well-differentiated and remains to be identified [8].

Efflux-mediated resistance has gained more attention in comparison with the other known resistance mechanisms. Of note, bacteria have several efflux pumps (EPs) that could expel many unrelated groups of antimicrobial agents outside the cell, inducing multidrug resistance phenotypes [9].

More than ten multidrug resistance EPs have been identified for *S. aureus*, including the chromosomally encoded NorA, NorB, NorC, MdeA, MepA, SepA and SdrM, as well as the plasmid-encoded QacA/B, QacG, QacH, QacJ and Smr. The well-studied EP in *S. aureus* is NorA, a 388 amino acid protein with twelve transmembrane segments, and is a major facilitator superfamily (MFS) [2].

Japanese researchers were the first to identify the NorA encoding gene in the chromosome of a fluoroquinolone-resistant *S. aureus* from an isolate collected in 1986 [10]. The *norA* gene possesses some genetic diversity, with three *norA* alleles (*norAI*, *norAII*, and *norAIII*) which had up to 10% difference in the nucleotide sequence [11].

Several studies have demonstrated the ability of *NorA* to extrude a group of chemically and structurally diverse agents, hydrophilic fluoroquinolones including norfloxacin and ciprofloxacin, dyes such as ethidium bromide, and biocides including quaternary ammonium compounds. A study demonstrated that the upregulation of expression of the *norA* gene inducing NorA-mediated efflux resistance effect towards fluoroquinolones, biocides and dyes [12]. However, the frequency of *norA*, *norB*, and *norC* efflux pump genes in Egyptian patients and their role in mediating the resistance to fluoroquinolones remain to be identified.

The aim of this study was to evaluate the frequency of *norA*, *norB*, and *norC* efflux pump genes mediating fluoroquinolones resistance and measure their expression levels in clinical isolates of MRSA at Suez Canal University Hospitals (SCUHs), Egypt.

Methodology

Sample collection

This cross-sectional study was performed at the SCUH in Ismailia, Egypt during the period from March to December 2022. SCUHs serve the Eastern zone of Egypt which includes 5 governorates (Ismailia, Port Said, Suez, North Sinai and South Sinai). A total of 126 *S. aureus* isolates were collected from different clinical samples taken from adult patients with several active infections and admitted to different wards at the SCUHs. Both genders and different age groups of patients were included. The study was conducted and authorized by the Research Ethics Committee of the Faculty of Medicine, Suez Canal University (SCU), Egypt (approval number: Research 5071#). In addition, a written informed consent was taken from patients whose samples were included in this study.

Bacterial isolation and identification

Specimens were collected under controlled non-septic conditions and transported immediately to the Medical Microbiology and Immunology department, Faculty of Medicine, Suez Canal University for processing. The samples were inoculated on blood agar plates (Oxoid, Hampshire, UK) and incubated aerobically at 35 °C for 24 hours. The standard conventional microbiological methods including Gram-positive cocci on Gram staining, golden yellow colonies on mannitol salt agar (Oxoid, Hampshire, UK) due to mannitol fermentation, positive catalase and coagulase tests, and complete hemolysis on blood agar were utilized to identify and recognize *S. aureus* in the isolates.

All isolates were kept in tryptic soy broth (TSB) supplemented with 15% glycerol and stored at -20 °C until further processing.

Detection of methicillin resistance in S. aureus

S. aureus isolates were confirmed as MRSA by cefoxitin disk diffusion test using 30 µg cefoxitin disk (Oxoid, Hampshire, UK) on Mueller-Hinton agar plates. Subsequently, the plates were incubated for 16–18 hours at 35 °C. The zone diameter was interpreted as consistent with the Clinical and Laboratory Standard Institute (CLSI) guidelines [13]. *S. aureus* ATCC 25923 was used as a control strain.

Antimicrobial susceptibility testing by disk diffusion method

All the MRSA isolates were exposed to antibiotic susceptibility testing using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar plates (Oxoid, Hampshire, UK) incubated for 16–18 hours at 35 °C. The antibiotic disks (Oxoid, Hampshire, UK) used were doxycycline (30 µg), tetracycline (30 µg), chloramphenicol (30 µg), clindamycin (2 µg), erythromycin (15 µg), gentamicin (10 µg), linezolid (30 µg), trimethoprim/sulfamethoxazole (1.25 µg/23.75 µg), ciprofloxacin (5 µg) and levofloxacin (5 µg). Zones of inhibition were determined in accordance with the CLSI guidelines [13].

Determination of minimum inhibitory concentrations (MIC) of ciprofloxacin by broth micro-dilution method

Consistent with the CLSI standard guidelines, MIC of ciprofloxacin (Sigma-Aldrich, Massachusetts, USA) was measured using broth microdilution assay in 96-well microtiter plates. In brief, a cation-adjusted Mueller–Hinton broth (CAMHB) was used to prepare two-fold serial dilutions to achieve a suitable range of antibiotic concentration (0.5-512 µg/mL). Two hundred microliters were used as a total volume in each well and bacterial inocula were added to the wells to achieve a final density equivalent to 0.5 McFarland. The plates were then incubated at 35 °C for 24 hours. MIC was defined as the lowest concentration of antibiotics that produced no growth as evidenced by the absence of turbidity in the wells after incubation at 35 °C for 16-20 hours. MIC breakpoints for ciprofloxacin were determined according to the CLSI guidelines as follows: resistant: ≥ 4 µg/mL, intermediate: 2 µg/mL and susceptible: ≤ 1 µg/mL. *S. aureus* ATCC 29213 was used as a quality control organism [13].

DNA extraction and detection of efflux pump genes using the polymerase chain reaction (PCR) method

The DNeasy extraction kit (Qiagen, Hilden, Germany) was used to extract genomic DNA as per the manufacturer's manual. A Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA) was utilized to determine the quality and quantity of the extracted DNA [14].

All MRSA isolates were screened for the presence of efflux pump genes *norA*, *norB* and *norC* using their specific primers sequences (Table 1). PCR reaction was performed at a final volume of 25 µL containing 3 µL (150 ng) of extracted DNA as a template, 1 µL forward primer (15 pmol), 1 µL reverse primer (15 pmol), 12.5 µl of 2× ABT Master Mix (Applied Biotechnology Company, Cairo, Egypt) (including 1.5× PCR buffer, 0.5 mmol/L of dNTPs, 4 mmol/L of $MgCl_2$, and 0.08 IU of *Taq* DNA polymerase), and 7.5 µL nuclease free water.

Amplification was accomplished on Biometra thermal cyclers (Biometra, Göttingen, Germany) using the following cycling conditions: primary denaturation for 5 min at 95 °C, 30 cycles of amplification composed of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s for all genes, and extension at 72 °C for 30 s, followed by a step of final extension at 72 °C for 5 minutes. Amplified products were electrophoresed using 2.5% agarose gel and visualized with ultraviolet irradiation [15].

Analyzing the expression level of norA, norB, and norC genes using quantitative real-time PCR (qRT-PCR)

All MRSA isolates were used to determine the expression level of *norA*, *norB*, and *norC* efflux pump genes. MRSA strains were grown on brain heart infusion broth (BHI) overnight at 37 °C. RNA extraction was performed using RNeasy Plus Mini Kit (Qiagen, Hilden, USA). The Nanodrop ND-1000 spectrophotometer at the absorbance ratio of 260/280 nm (NanoDrop Technologies, Wilmington, DE, USA) was utilized to assess the integrity and purity of RNA. Then, cDNA synthesis was executed using the COSMO cDNA synthesis kits (WF-1020500X, Willowfort, UK) based on the manufacturer's manual.

The quantitative reverse transcriptase PCR (qRT-PCR) was executed with a final volume of 20 µL containing (10 µL of SYBR Green qPCR Master Mix (WF1030800X, Willowfort, UK), 2 µL forward primer, 2 µL reverse primer (Table 1), 2 µL cDNA and 4 µL

Table 1. Primers used for detection of efflux pump genes by PCR and qRT-PCR.

Primer	Sequences (5' to 3')	Amplicon (bp)	Reference
***nor*A**	*nor*A-F: GACATTTCACCAAGCCATCAA *nor*A-R: TGCCATAAATCCACCAATCC	102	[11]
***nor*B**	*nor*B-F: ATGTTTGTCGTTGGAGCAGG *nor*B-R: AATACACGCTGCTGATACGC	168	[11]
***nor*C**	*nor*C-F: ATGAATGAAACGTATCGCGG *nor*C-R: GTCTGCACCAAAACTTTGTTGTAAA	129	[11]
***gm*K**	*gm*K-F: TCAGGACCATCTGGAGTAGGTAAAG *gm*K-R: TTCACGCATTTGACGTGTTG	108	[11]

nuclease free water). The PCR cycling conditions were performed using StepOne™ real time PCR analyzer (Applied Biosystems, California, USA) as follows: 3 min denaturation at 95 °C, followed by 35 cycles of 15 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C. Negative controls were utilized to exclude presence of contamination. The expression of *norA*, *norB*, and *norC* genes were normalized to the expression of *gmK* housekeeping gene (internal control) [15] and evaluated based on 2 (-ΔΔCT) relative expression quantification method [16,17].

Statistical analysis

SPSS program, version 22.0 software (SPSS, Chicago, IL, USA) was utilized for statistical analysis of the data. Categorical values were represented using numbers and percentages. The normality of distribution was examined by the Kolmogorov–Smirnov test. While, numerical values were represented using mean, standard deviation, and standard error of the mean. Pearson's Chi-square test or Fisher's exact test was performed to analyze categorical data. The association of ciprofloxacin resistance and the presence of efflux pump genes was investigated using an unpaired student-t test. *A p* value less than 0.05 was statistically significant.

Results

Bacterial isolation and identification

A total of 126 non-duplicate clinical isolates of *S. aureus* were collected from adult patients admitted to different wards at SCUHs from March to December 2022. These isolates were obtained from 69 males (54.8 %) and 57 females (45.2%) with a mean age of 52.7 ± 12.82 years. The isolates were mostly collected from wound pus (n = 46; 36.5 %), followed by blood (n = 37; 29.4%), urine (n = 29; 23 %) and sputum (n = 14; 11.1%).

Detection of methicillin resistance in S. aureus

Eighty MRSA strains were identified from the total 126 *S. aureus* isolates (63.5%) using cefoxitin disk diffusion test on Mueller-Hinton agar plates.

Antimicrobial susceptibility testing

The MRSA isolates showed high resistance rate to erythromycin (80%) followed by gentamicin (75%) and clindamycin (65%). Sixteen isolates (20%) were resistant to doxycycline while 15 isolates (18.8%) were resistant to tetracycline. All MRSA isolates showed high sensitivity to linezolid (100%).

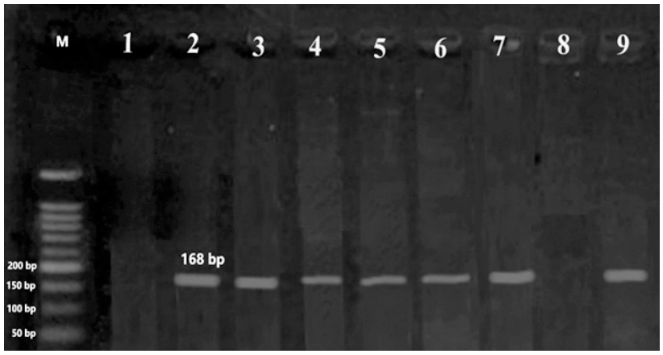
Determination of MIC of ciprofloxacin among MRSA isolates by broth micro-dilution method

MIC in 33.8% (27/80) of the MRSA isolates was 0.5-1 µg/mL (susceptible); 6.2% (5/80) isolates had MIC values of 2 µg/mL (intermediate), and 60% (48/80) had MIC values of ≥ 4 µg/mL (resistant). MIC50 and MIC90 for ciprofloxacin were 32 and 128 µg/mL, respectively.

Detection of efflux pump genes and coexistence of different genes in MRSA isolates using the PCR method

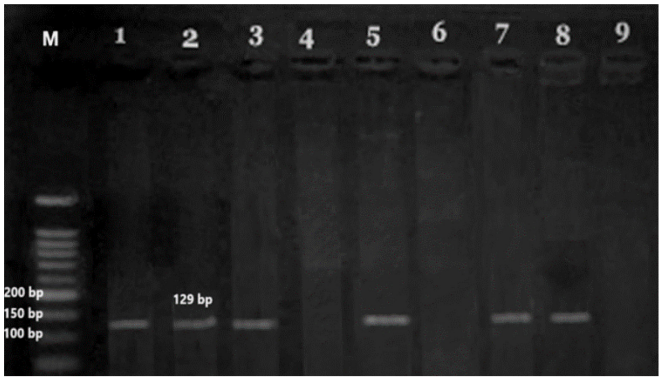
The *norA* gene was detected using PCR in 75% of the MRSA isolates, *norB* in 35% of the isolates and *norC* in 55% of the isolates (Figures 1 and 2). The coexistence of *norA/norB* genes, *norA/norC* genes, *norB/norC* genes and *norA/norB/norC* genes among MRSA isolates was 30%, 40%, 25% and 20%, respectively.

Figure 1. Agarose gel electrophoresis of norB gene PCR amplified products (168 bp).



Lane M: 50 bp molecular size standard DNA ladder. Lanes 2-7 and lane 9: methicillin resistant *Staphylococcus aureus* (MRSA) isolates containing norB gene.

Figure 2. Agarose gel electrophoresis of norC gene amplicons (129 bp).



Lane M: 50 bp molecular size standard DNA ladder. Lanes 1-3, lane 5 and lanes 7-8: methicillin resistant *Staphylococcus aureus* (MRSA) isolates containing norC gene.

Table 2. Frequency and coexistence of efflux pump genes and ciprofloxacin susceptibility among the 80 methicillin resistant *Staphylococcus aureus* (MRSA) strains.

Genes	Ciprofloxacin susceptibility		Total N (%)	*p* value
	Sensitive N=32 (%)	Resistant N=48 (%)		
*nor*A	16 (50 %)	44 (91.7 %)	60 (75%)	**0.035***
*nor*B	12 (37.5 %)	16 (33.3 %)	28 (35%)	**0.037***
*nor*C	12 (37.5 %)	32 (66.7 %)	44 (55%)	0.199
*nor*A + *nor*B	8 (25 %)	16 (33.3 %)	24 (30%)	0.15
*nor*A + *nor*C	4 (12.5 %)	28 (58.3 %)	32 (40%)	**0.04***
*nor*B + *nor*C	8 (25 %)	12 (25 %)	20 (25%)	1.00
*nor*A + *nor*B + *nor*C	4 (12.5 %)	12 (25 %)	16 (20%)	0.49

*Statistically significant difference.

All the ciprofloxacin-resistant MRSA isolates had at least one of the efflux pump genes. The efflux pump genes were significantly more common in ciprofloxacin resistant than ciprofloxacin-susceptible MRSA isolates. The frequency of efflux pump genes and ciprofloxacin susceptibility among the 80 MRSA isolates are presented in Table 2.

Gene expression analysis of norA, norB and norC using qRT-PCR

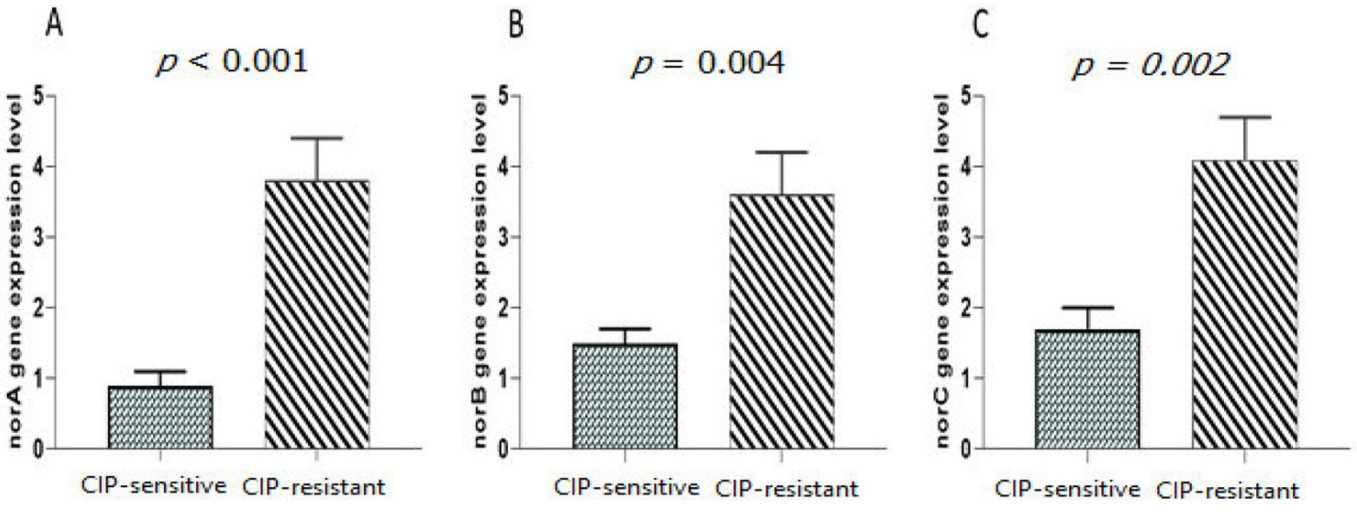
Among the 80 MRSA isolates, the *norC* was the most commonly overexpressed gene occurring in 32 (40%) isolates, followed by *norA* (n = 28, 35%) and *norB* (n = 24, 30%). The expression of the efflux pump genes was significantly higher among ciprofloxacin-resistant than ciprofloxacin-sensitive MRSA isolates. The expressions of *norA*, *norB* and *norC* genes in ciprofloxacin-resistant MRSA isolates increased significantly by 2.9, 2 and 2.4 folds, respectively, in comparison to their expressions in ciprofloxacin-sensitive MRSA isolates (*p* values < 0.001, 0.004 and 0.002, respectively) as demonstrated in Figure 3.

Discussion

Methicillin-resistant *S. aureus* is a major causative agent of many infections in health-care and community settings. In the last two decades, the proportion of MRSA has exponentially increased worldwide. Our study showed that 80 MRSA isolates were identified from a total of 126 *S. aureus* isolates (63.5%) when tested by cefoxitin disk diffusion test. Rehman *et al*. [18] and Garoy *et al*. [19] reported a higher prevalence of MRSA at 78.3% and 72.0%, respectively. Lower frequencies of MRSA detected by Tsige *et al.* [20], Bostanmaneshrad *et al*. [15] and Hassanzadeh *et al.* [21] were 28.3%, 45% and 50%, respectively by using cefoxitin disc diffusion method and PCR of the *mecA* gene. MRSA prevalence varies widely between countries and is attributed to different infection control policies that are followed by these countries.

MRSA are considered MDR pathogens due to their increasing resistance to several known non-□-lactam antibiotics including aminoglycosides and tetracycline, in addition to macrolides, and fluoroquinolones. In our study, MRSA isolates showed high resistance to

Figure 3. The expression levels of norA, norB, and norC genes in CIP sensitive and resistant MRSA isolates.



Data were expressed as mean and standard error of means. Unpaired student *t*- test was used as a test of significance and p < 0.05 was considered as significant. CIP: ciprofloxacin; MRSA: methicillin resistant *Staphylococcus aureus*.

erythromycin (80%), gentamicin (75%), clindamycin (65%) and ciprofloxacin (60%); lower resistance rate against doxycycline (20%) and tetracycline (18.8%); and high sensitivity to linezolid (100%). Our results were similar to the report by Hassanzadeh *et al.* [21] who found that MRSA isolates showed high resistance to ciprofloxacin (70%), clindamycin (63.3%), erythromycin (58.3%) and moderate resistance to doxycycline (28.3%). Similarly, Rossato *et al*. [22] in Brasil reported that MRSA isolates were highly resistant to erythromycin (74.2%), ciprofloxacin (64.5%), clindamycin (46.1%) and tetracycline (14.3%); and highly susceptible to linezolid (100 %). Moreover, Tsige *et al.* found that the isolated MRSA showed high resistance to erythromycin (61.5%), ciprofloxacin (61.5%) and gentamicin (53.8%) [20]. However, compared to our results, higher resistance rates of MRSA isolates against erythromycin (91.1%), ciprofloxacin (84.4%) and clindamycin (80%), were detected by Bostanmaneshrad *et al.* [15] These variations in the antimicrobial drug resistance rates among different countries might be attributed to the availability and indiscriminate use of these antibiotics in certain areas which indicates that the antibiotic resistance patterns vary according to regional and geographical factors.

Fluoroquinolones are broad spectrum antibiotics that are effective against MRSA. However, these antibiotics have also acquired resistance and this has impaired their clinical relevance. In our study, the resistance rate to ciprofloxacin was 60% when performing MICs by broth micro-dilution method which was similar to that observed in Egypt (58%), Ethiopia (61.5%) and Brasil (64.5%) [20,22,23]. However, our finding was lower than other studies conducted in Egypt (96%) [24], Brasil (79%) [25] and Iran (84.4%, 70%) [15,21].

Resistance to fluoroquinolones in *S. aureus* is usually accompanied by mutations in the QRDR of the target genes, *grlA/B* and *gyrA/B*, that code for the topoisomerase IV (*GrlA/B*) and DNA gyrase (*GyrA/B*) proteins, respectively. Another mechanism involved in quinolone resistance in *S. aureus* is the overexpression of the chromosomally encoded MDR efflux pump genes, namely *norA*, *norB*, and *norC* that encode a multidrug efflux protein (NorA, NorB, NorC) capable of exporting fluoroquinolones outside the bacteria [8].

Our study demonstrated that *norA*, *norB*, and *norC* genes were present in 75%, 35% and 55% of the MRSA isolates, respectively. Moreover, our results also demonstrated that *norA* and *norC* efflux pump genes were significantly more common in ciprofloxacin resistant (91.7% and 66.7%, respectively) than ciprofloxacin-susceptible (50% and 37.5%, respectively) MRSA isolates. However, *norB* gene was present in only 33.3% of the ciprofloxacin resistant MRSA isolates, indicating that other mechanisms of resistance are involved in addition to the activity of efflux pumps (Table 2).

Our findings are in accordance with Hassanzadeh *et al.* who reported that the frequency of *norA*, *norB* and *norC* genes among MRSA isolates was 41.7% (for all) and that efflux pump genes were significantly more common in ciprofloxacin resistant versus ciprofloxacin-susceptible MRSA isolates and found significant correlation between the presence of *norA* and *norB* genes and ciprofloxacin resistance [21]. In addition, Shamkhi *et al.* reported that the frequencies of *norA*, *norB* and *norC* genes among the MRSA isolates were 80.21%, 56.25% and 17.81%, respectively [26]. Bostanmaneshrad *et al*. reported higher frequencies of *norA*, *norB* and *norC* genes among the MRSA isolates that were 100%, 100% and 95%, respectively [15]. Similarly, Saiful *et al*. and Conceição *et al*. demonstrated that 84.2% and 86.4% of MRSA isolates, respectively harbored *norA* [27,28].

Moreover, *norA* was present in all (100%) of the ciprofloxacin-resistant MRSA strains as reported by studies conducted by Mirzaie *et al.*, Pourmand *et al*. and Zahmatkesh *et al* [29–31]; while the *norB* was found in 83% of ciprofloxacin resistant MRSA isolates as reported by Zahmatkesh *et al* [31]. Geographical diversity, type of clinical samples, specificity and sensitivity of the techniques used and other factors could be responsible for the difference in the prevalence of genes between these studies.

One of the mechanisms by which *S. aureus* could resist fluoroquinolones is the upregulation of the *norA* and *norB* by increasing its transcription and consequent increase in its protein levels in the cytoplasmic membrane as demonstrated by Kaatz *et al.* This upregulation could be induced by a mutation(s) or occurring naturally in response to some inducers or natural substrates. In addition, this induction of some variants of *norA*, and probably other efflux pump genes of *S. aureus,* could result in higher MIC values in the presence of their substrates and could explain the diversity in MIC values and expression levels [32].

In our study, we analysed the expression of *norA*, *norB*, and *norC* efflux system in MRSA isolates by qRT-PCR and found that *norC* was the most commonly overexpressed gene occurring in 40% of MRSA isolates, followed by *norA* (35%) and *norB* (30%). The expression of the efflux pump genes was significantly

higher among ciprofloxacin-resistant than ciprofloxacin-sensitive MRSA isolates. The expressions of *norA* and *norC* in ciprofloxacin-resistant MRSA isolates increased significantly by 2.9, 2, and 2.4 folds, respectively in comparison to their expressions in ciprofloxacin-sensitive MRSA isolates ($p < 0.001$, 0.004, and 0.002, respectively).

In agreement with our results, Mirzaie *et al.* reported that ciprofloxacin-resistant MRSA isolates had different expression of *norA* and the more resistant strains had higher relative expression of *norA* [29]. Bostanmaneshrad *et al.* stated that *norA* was the most commonly overexpressed gene occurring in 63.6% of the ciprofloxacin-resistant MRSA isolates. Only 18.2% of the isolates showed overexpressed *norB* and *norC,* which was different from our findings [15].

A similar study conducted by Rajabi *et al.* demonstrated that in ciprofloxacin-resistant *S. aureus* isolates, the expression of *norA*, *norB* and *norC* were significantly increased by 6.8, 7.1 and 2.8-fold, respectively; in comparison to the expression of the 16S rRNA gene as internal control [33].

In contrast to our results, several studies found *norB* as the most commonly overexpressed gene. For example, Kwak *et al.* demonstrated that the *norB* was the most commonly overexpressed gene, occurring in 42.6% of the *S. aureus* strains, *norA* was overexpressed in only 1.7% of the isolates and none overexpressed *norC*. Moreover, they found that 60.9% of the ciprofloxacin-resistant MRSA were significantly associated with *norB* overexpression compared to ciprofloxacin susceptible MRSA (20%), which is similar to our finding [34]. DeMarco *et al.* revealed that 25.4% of *S. aureus* isolates, taken from blood samples, showed *norB* overexpression, followed by 22.8% and 16.7% overexpression in *norA* and *norC*, respectively [35]. Moreover, Kosmidis *et al.* reported that *norB* is predominately overexpressed in the isolated strains from patients in San Francisco, USA, higher than any other place [36].

Conclusions

Our results demonstrated high prevalence and overexpression of efflux pump genes among MRSA isolates and the significant role of these genes in the development of multi-drug resistance against antibiotics, including fluoroquinolones. However, the role of other factors and mechanisms involved in resistance should not be ignored.

Authors' contributions

All authors designed, performed the experiments, and wrote the article. All authors revised and approved the published version of the manuscript.

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