Indian Journal of Experimental Biology
Vol. 58, August 2020, pp. 527-538

Efflux pumps and biofilm formation by both methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* strains

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Received 31 January 2020; *revised 14 March 2020*

Staphylococcus aureus is the primary cause of nosocomial infections. It produces potent toxins and causes superficial lesions, systemic diseases, and several toxaemic syndromes. In this study, we determined the role of efflux pump in conferring resistance to fluoroquinolones in the biofilm of methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin sensitive *S. aureus* (MSSA) strains. We selected five strains of *S. aureus* comprising three methicillin sensitive strains, including ATCC25923, and two strains of methicillin resistant *S. aureus*. Thioridazine, chlorpromazine, carbonyl cyanide 3-chloro-phenylhydrazone and reserpine showed activity against MSSA strains. Whereas, thioridazine and chlorpromazine were active against MRSAstrains too. Thioridazine and chlorpromazine showed a significant decrease in biofilm formation of MSSA strain 2493, and MRSA strain UC1079 compared to the control strain ATCC25923. Thioridazine showed a similar effect on MSSA strain N297214 and MRSA strain N307002. Four to eight-fold increase was found in the expression of efflux pump genes with ethidium bromide, ciprofloxacin, and moxifloxacin in these strains. However, a decrease in the expression of *norB*, *norC*, *abcA* and *mepA* was observed in MSSA, and MRSA strains with thioridazine, chlorpromazine and naringenin. This study, thus demonstrate that the major facilitator superfamily, multidrug and toxin compound extrusion, and ATP binding cassette family of efflux pumps play an essential role in developing antibiotic resistance and biofilm formation. It is the first report on existence of efflux pump genes in biofilm beside antibiotic resistance for fluoroquinolones in MRSA and MSSA strains, which need further characterization.

Keywords: Antibiofilm agent, Antimicrobial resistance, Efflux pump inhibitors, Fluoroquinolones, Gene expression, MATE family, MDR pump, Nosocomial infection

Staphylococcus aureus is an opportunistic pathogen that causes a variety of infections in human, such as skin and soft tissue infections, endocarditis, and bacteremia. Antibiotic resistance has been one of the problems in the treatment of *S. aureus* infections. Efflux of antibiotics mediated by efflux pumps led to the emergence of multidrug resistance among bacteria[1]. These efflux pumps remove the toxic substances or decrease the intracellular concentration of antibiotics in the cytoplasm[2]. Also, efflux pumps act as a defense mechanism that prevents a drug from reaching a lethal level inside the cell[3]. *S. aureus* possesses many potential multidrug resistant (MDR) efflux pump encoding genes, of which efflux pumps like *qacA* and *qacB*, belonging to the major facilitator superfamily (MFS), are encoded on plasmids.

On the other hand, the major MFS pump, *norA*, *mdeA*, *norB*, *norC*, *sdrM*, and multi-drug and toxin compound extrusion (MATE) family MDR pump, *mepA*, are located on the chromosome[4-8]. One of the major concerns is the presence of efflux genes leading to resistance to broad spectrum of biocides and fluoroquinolenes[9]. Ethidium bromide is often used to study the presence of the efflux pump and the mechanism of antibiotic resistance in bacteria[10,11]. Apart from its role in antibiotic resistance, efflux pumps had been reported playing a role in biofilm formation[12], pathogenicity, and virulence[13].

Biofilm, an aggregate of microbial cells embedded in the extracellular matrix, is one of the critical factors developing antimicrobial resistance and tolerance[14-16]. Several environmental factors, such as nutritional and metabolic cues, host-derived signals, quorum signal, and sub-inhibitory concentration of antimicrobials, contribute to biofilm formation[17]. The efflux pumps expression level, efflux harmful molecules and efflux pump substrates may influence biofilm formation[18]. Efflux pump inhibitors (EPI) alone or in combinations have been shown to either reduce or altogether abolish

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the biofilm formation and antibiotic resistance[19,20]. The EPIs have been shown to affect the expression of efflux pumps by inhibiting the drug transport, interfering with the energy metabolism to transport the drug or by blocking the efflux channel[21]. Reserpine, LY35979, phenothiazine-metal complexes, and verapamil have side effects but reported to inhibit the bacterial efflux system and restoring antibiotic sensitivity[22,23]. In this regard, certain medicinal herbs are being screened, which have shown to inhibit the efflux pump of many microorganisms[24]. It was reported that methicillin sensitive *Staphylococcus aureus* (MRSA) and methicillin sensitive *S. aureus* (MSSA) strains does not contain the same set of efflux genes when isolated from the same hospital setting[9]. There are not many studies showing the relationship between efflux pumps and biofilm formation in both MRSA and MSSA strains.

In this study, we determined the inhibitory and sub-inhibitory concentrations of ciprofloxacin, norfloxacin, chloramphenicol, penicillin, erythromycin, moxi-floxacin, and ethidium bromide in MRSA and MSSA strains. We tested the effect of inhibitors and phytochemicals on efflux pump, and biofilm formation, and correlated the results with the expression of genes encoding for multidrug resistant efflux pumps in MRSA and MSSA strains.

Materials and Methods

Bacterial isolates

A total of five strains, two each of MRSA and MSSA and ATCC 25293, characterized previously, were included in the study.

PCR assays

PCR determined the presence of efflux pump genes, namely *norB*, *norC*, *mdeA*, *lmrS*, *mepA* and *abcA*[25,26]. The amplified products were separated on 1% agarose gel, stained with ethidium bromide, and visualized in Fluoro-S-MultiImager (Bio-Rad, USA). The details of the primers are given in Table 1.

Sequencing of quinolone resistance determining region

The PCR determines the presence of *gyrA*, *gyrB*, *grlA* and *grlB* genes in quinolone resistant determining region (QRDR) as described earlier[4,27]. The amplified products were purified (ExoSAP; Affymetrix, Cleveland, USA), and both strands were sequenced using an ABI sequencer model 3500 (Life Technologies, Marsiling, Singapore) at the sequencing facility of the Institute of Life Sciences, Bhubaneswar, India.

Semi-quantitative estimation of biofilm formation

The biofilm assay was performed by employing the method of Fredheim *et al*.[28] with some modifications. Briefly, bacterial cultures were diluted to the absorbance of OD 0.05 at 595 nm. One mL of diluted culture was then added to 24-well polystyrene plates (COSTAR; Sigma) and allowed to grow for 24 h at 37°C under static condition. The planktonic cells were removed by pipette, and pallets washed once with 1X PBS and stained with 125 µL of 0.1% crystal violet for 30 min at room temperature (25°C). The bound dye was dissolved in 95% ethanol, and absorbance was recorded at 570nm (Nivo Multimode reader). The assay was performed in a single run of three wells in biological triplicates. Strains were considered biofilm positive if they had an OD of≥0.3.

The detachment assay was performed to determine the components of biofilm matrix by the method of Fredheim *et al*.[28] using reagent and enzymes (i) 40 mM sodium periodate (NaIO4: Sigma; S1878), (ii) 0.5 mg/mL proteinase K (Sigma; P2308), and (iii) 0.5 mg/mL DNase I (Sigma; DN25). Diluted bacterial strains were seeded to 24-well polystyrene plates (COSTAR; Sigma) and were allowed to grow for 24 h at 37ºC under static

Table 1 — List of primers of efflux pump genes used in this study

Genes	Primer sequence (5'-3')	Amplicon size (bp)	Ref.
NorA-F	TTCACCAAGCCATCAAAAAG	620	29
NorA-R	CTTGCCTTTCTCCAGCAATA		
NorB-F	AGCGCGTTGTCTATCTTTCC	213	22
NorB-R	GCAGGTGGTCTTGCTGATAA		
NorC-F	AATGGGTTCTAAGCGACCAA	216	22
NorC-R	ATACCTGAAGCAACGCCAAC		
MepA-F	ATGTTGCTGCTGCTCTGTTC	718	22
MepA-R	TCAACTGTCAAACGATCACG		
MepA (RT)-F	TGCTGCTGCTCTGTTCTTTA	198	22
MepA (RT)-R	GCGAAGTTTCCATAATGTGC		
MdeA-F	AACGCGATACCAACCATTC	677	22
MdeA-R	TTAGCACCAGCTATTGGACCT		
MdeA (RT)-F	GTTTATGCGATTCGAATGGT TGGT		
MdeA (RT)-R	AATTAATGCAGCTGTTCCGA TAGA	155	27
16S-F	ACTCCTACGGGAGGCAGCAGT	180	28
16S-R	TATTACCGCGGCTGCTGGC		
AbcA-F	CAGTGGTTTGTTAGGGCGTGTC	175	This
AbcA-R	GAAATTGGTTGTACAACTGCCG		study
lmrS-F	GCAAGCTTATGGCTAAAGTT GAATTAACAAC		
lmrS-R	GCGGATCCTTAAAATTTCCTT CTATTACTTT	1441	23
lmrS (RT)-F	GCAATGGGACTAGCAGGTTTA	150	This
lmrS (RT)-R	AAATGGTACTCGCCAACTCG		study

condition. Then the above reagents were added to individual wells after 24 h. After incubation, the biofilm was disrupted by mixing, and remaining adhered cells were quantified by crystal violet staining by the method described above. The percent detachment was calculated from the average difference between the treated wells and the control wells and divided the results into three categories: (i) no detachment (<10%), (ii) intermediate detachment (10–50%), and (iii) strong detachment (>50%).

Minimum inhibitory concentration of EPI

The minimum inhibitory concentration (MIC) of all strains of *S. aureus* was tested by broth micro-dilution assay following the method described in Clinical and Laboratory Standards Institute (CLSI) manual[29]. The antibiotics that showed substrate specificity for the efflux pump in *S. aureus* were tested. These antibiotics were ciprofloxacin, norfloxacin, ethidium bromide, oxacillin, penicillin, chloramphenicol, erythromycin, and moxifloxacin. *S. aureus* strain ATCC 25923 was used as a control.

Ethidium bromide accumulation assay

The four strains of *S. aureus* and control strain ATCC 25923 were subjected to accumulation assay. Briefly, bacterial cultures were grown in 2 mL tryptic soy broth (TSB) medium at 37°C until they reach an OD600 of 0.6. The culture was centrifuged at 13000 ×g for 3 min, and the pellet was washed with 1X phosphate buffered saline (PBS). After adjusting the culture OD to 0.3 with 1X PBS, 0.4% glucose was added to the microtube containing 1.0 mL of the bacterial suspension. Then ethidium bromide (EtBr) ranging from 0.25-5 mg/L was added into a 96-well plate (Corning, Costar) containing 100 µL bacterial suspension of control strain ATCC 25923. The fluorescence was measured in the Nivo Multimode reader using 530 nm band pass and the 585 nm high pass filters as the excitation and detection wavelength, respectively. The fluorescence was captured after every 60 s for 60 min. MIC and half MIC of efflux inhibitors were determined. The concentration was as follows: 64 mg/L for thioridazine (TZ), 64 mg/mL for chlorpromazine (CPZ), 128 mg/L for reserpine, 0.5 mg/L for carbonyl cyanide 3-chloro-phenylhydrazone (CCCP), 75 mg/L for naringenin, 75 mg/L for quercetin and 50 mg/L for pyrogallol, respectively. The strains were suspended in 1X PBS at OD 600 of 0.3. The fluorescence of EtBr was measured at 530/585 nm after every 60 s for 60 min in the Nivo Multimode reader.

Efflux pump activity of ethidium bromide

All the four *S. aureus* strains were grown in 2 mL of TSB until they reached a mid-log phase. The cells were centrifuged at 13000 ×g for 3 min, then pellet washed with PBS and suspended the same at OD600 of 0.3. Ethidium bromide was added to obtain a final concentration of 0.5 mg/L and measured the fluorescence in Nivo Multimode reader at 25°C for 60 min. The TZ and CPZ that showed an effect on the accumulation of EtBr was determined under condition which optimize efflux in the presence of glucose and incubation at 25°C.

Effect of efflux pump inhibitors on biofilm formation

The inhibition of biofilm produced by four strains of *S. aureus*, two each of MRSA and MSSA, was determined by adding the efflux pump inhibitors (EPIs) to the wells containing bacteria at 0 h and after 24 h. The wells were allowed to incubate for 24 h at 37°C under static conditions. The plates were washed once with 1X PBS and then stained with 125 µL of 0.1% crystal violet for 30 min at room temperature. The bound dye was dissolved in 95% ethanol, and absorbance was recorded at 570 nm (Nivo Multimode reader).

qPCR and expression of efflux pump genes

The expression of the efflux pump genes *norB*, *norC*, *mepA*, *mdeA*, *abcA* and *lmrS* was determined by the method described earlier[24,30]. The inhibitor's effect on the biofilm was evaluated after treatment with TZ, CPZ and naringenin at their ½ MIC and MIC at 0 h and after 24 h. Briefly, the aliquots of 6 h grown culture in TSB diluted to an absorbance of 0.05 OD at 595 nm were seeded into 12-well plate (Corning). Then the inhibitors were added at 0h and after 24h and incubated at 37°C for 24 h. Cells without inhibitor was used as a negative control. The non-adherent cells were discarded, and the adherent cells scraped by ice-chilled resuspension buffer (50 mM Tris-HCl, 10 mM EDTA, 500 mM NaCl, pH 8.0). The biofilm cells were centrifuged for 5 min at 18000 ×g at 4°C. RNA was isolated by Trizol reagent (*In vitro*) using RNAiso Plus (Takara Clontech) according to the manufacturer's instructions, and synthesized cDNA using Reverse transcriptase Core kit (Eurogentec). Quantitative RT-PCR (qRT-PCR) was performed using the SYBR Green Mastermix (Promega) in the Applied Biosystem 3000™ thermal cycler. The relative gene expression was determined using the comparative threshold cycle (CT) method and compare the expression of genes in the presence or absence of EPI. The 16S rRNA gene was used as a control[31].

Results

PCR assays

All the strains of *S. aureus*, except MSSA strain N297214 negative for *norA* gene, were positive by PCR for *norA, norB, norC, mdeA* and *abcA* genes (Table 2). However, both MSSA strains were positive for *lmrS* and *mepA* genes. *S. aureus* strain ATCC 25923 was negative for the *lmrS* gene (Table 2).

Mutations in QRDR of fluoroquinolones

MRSA and MSSA isolates showing mutations in DNA gyrase genes, *gyrA*, *gyrB*and topoisomerase IV genes, *grlA* and *grlB*of QRDR are shown in Table 3. All the four strains were resistant to gatifloxacin, ofloxacin, ciprofloxacin and moxifloxacin, respectively. MRSA and MSSA strains showed mutation at position Val378Phe in the *gyrB* gene. However, MRSA strain N307002 and MSSA strain 2493 showed additional mutation at position Phe493Leu in the *gyrB* gene. Strain 1079 carried mutation at Ser85Pro in the *gyrA* gene, Asp424Glu and Pro453Ser in *grlB* gene, but no variation in the *grlA* gene (Table 3). MSSA strain N297214 showed mutation at Ser84Ala in *gyrA*, Ile45Met, Phe80Tyr in *grlA*, and Gln385His, Glu386Pro in *grlB*, respectively. However, MSSA 2493 had no variation at *gyrA* and *grlA* but carried mutation at Asp509Gly in the *grlB*gene (Table 3). No mutation was found in the *gyrA*, *gyrB* and *grlA* genes of ATCC 25923.

Biofilm formation and detachment assay

All the four strains, two each of MRSA and MSSA, formed biofilm on polystyrene microtiter plates. Both MRSA strains formed healthy biofilm, but MSSA N297214 formed moderate to weak, and MSSA2493 formed moderate biofilm. Strain ATCC 25923 formed a moderate to weak biofilm. Results of detachment assay performed with NaIO4, proteinase K and DNase I on MRSA and MSSA is shown in Fig. 1A. We found that all strains showed strong detachment after treatment with proteinase K, moderate detachment with DNase I, and low level detachment with $NaIO_4$, respectively. The control strain *S. aureus* ATCC 25923 displayed strong detachment upon proteinase K treatment. Therefore, these strains had protein as their principal component in the biofilm matrix and were used for further study (Fig. 1B).

Table 2 — Presence or absence of efflux pump genes in MRSA and MSSA strains

S. aureus strains	Presence of efflux pump genes encoding for						
	norA	*norB*	*norC*	*mdeA*	*lmrS*	*mepA*	*AbcA*
MRSA N307002	+	+	+	+	-	-	+
MRSA 1079	+	+	+	+	-	+	+
MSSA N297214	-	+	+	+	+	+	+
MSSA 2493	+	+	+	+	+	+	+
ATCC 25923	+	+	+	+	-	+	+

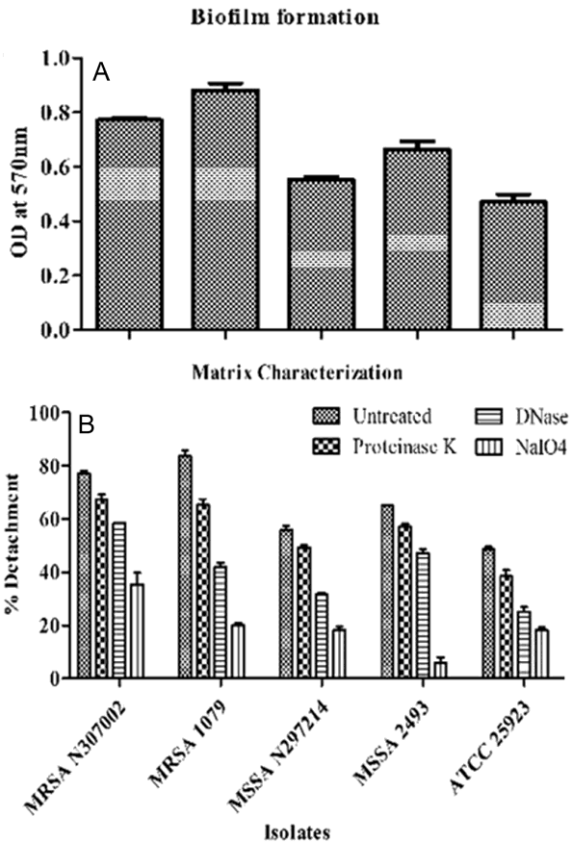

Fig. 1 — (A) Quantification of biofilm formed by *S. aureus* strains in 24 h by crystal violet assay; and (B) Percent detachment of preformed biofilms obtained with MRSA and MSSA after treatment with NaIO4, proteinase K and DNase I.

Table 3 — Fluoroquinolone susceptibility and mutations found in QRDR genes in MRSA and MSSA strains

Isolate No.	Mutation(s) in QRDR genes encoding for				MIC (mg/L) for antibiotics			
	gyrA	*gyrB*	*grlA*	*grlB*	Gati-floxacin	Moxi-floxacin	Oflox-acin	Cipro-floxacin
MRSA N307002	-	Val378Phe, Phe493Leu	Ser81Pro	Asp424Glu	8 (R)	8 (R)	>64 (R)	>64 (R)
MRSA 1079	Ser85Pro	Val378Phe	-	Asp424Glu, Pro453Ser	16 (R)	16 (R)	>64 (R)	>64 (R)
MSSA N297214	Ser84Ala Leu84Ala	Val378Phe	Ile45Met, Phe80Tyr	Gln385His, Gln386Pro	>64 (R)	>64 (R)	>64 (R)	>64 (R)
MSSA 2493	-	Val378Phe, Phe493Leu	-	Asp509Gly	8 (R)	8 (R)	>64 (R)	8 (R)
ATCC 25923	-	-	-	-	1 (S)	2 (S)	1 (S)	<1 (S)

Effect of EPI on MIC

MIC wasdetermined for antibiotics among MRSA and MSSA strains that ranged between <1 and >512 µg/mL. TZ, CPZ, CCCP, reserpine, naringenin, quercetin and pyrogallol showed a reduction in MIC of antibiotics in *S. aureus*. TZ and CPZ showed a significant decrease in the MIC of antibiotics. However, CCCP, naringenin, quercetin and pyrogallol showed less effect on MIC. Reserpine had no or less inhibitory effect on MIC (Table 4).

Accumulation of ethidium bromide

The EtBr accumulation assay showed that the concentration of EtBr required for detection varied from strain to strain. One strain of MRSA and both of MSSA needed 0.5 mg/L EtBr, but MRSA strain N307002 showed accumulation atone mg/L of EtBr(Fig. 2). *S. aureus* ATCC 25923 showed an accumulation of EtBr at 0.25mg/L. CPZ showed inhibition of accumulation of EtBr in all strains.

Moreover, TZ and CPZ showed an accumulation of EtBr at almost the same level in MRSA. Reserpine showed virtually a similar level of accumulation as TZ in MSSA 2493. Naringenin was the most effective inhibitor compared to quercetin and pyrogallol in all strains (Fig. 3). The most effective inhibitor for ATCC 25923 was CPZ and TZ, followed by reserpine and naringenin.

Intrinsic efflux activity of the MRSA and MSSA

The conditions established by accumulation assays were used to load cells with EtBr and perform efflux assays by real-time fluorimeter. CPZ displayed unusual efflux pump activity compared to TZ. MRSA showed 50% efflux activity, while MSSA showed 60-70%

Table 4 —MICs of antibiotics obtained before and after treatment with efflux pump inhibitors among MRSA and MSSA*s* strains

Antibiotics and EPIs	MIC of antibiotics after treatment of EPIs and shown by *S. aureus* isolates					Antibiotics and EPIs	MIC of antibiotics after treatment of EPIs and shown by *S. aureus* isolates				
	MRSA N307002	MRSA 1079	MSSA N297214	MSSA 2493	ATCC 25923		MRSA N307002	MRSA 1079	MSSA N297214	MSSA 2493	ATCC 25923
Ethidium bromide	32	64	64	32	8	Erythromycin	>512	>512	1	>512	2
TZ	4	2	2	<1	4	TZ	32	<1	<1	<1	<1
CPZ	<1	<1	4	4	2	CPZ	32	>512	<1	<1	2
Res	4	32	64	32	32	Res	>512	>512	2	>512	2
CCCP	8	8	8	4	8	CCCP	64	>512	<1	<1	<1
Naringenin	32	64	64	32	32	Naringenin	>512	>512	16	256	256
Quercetin	32	32	32	32	32	Quercetin	>512	>512	16	32	32
Pyrogallol	32	64	64	64	64	Pyrogallol	>512	>512	32	>512	>512
Ciprofloxacin	128	128	8	8	<1	Oxacillin	512	128	<1	<1	1
TZ	<1	<1	<1	<1	<1	TZ	8	4	<1	<1	<1
CPZ	<1	<1	<1	<1	<1	CPZ	128	<1	<1	<1	<1
Res	64	128	<1	8	2	Res	512	>512	<1	128	1
CCCP	16	16	8	16	<1	CCCP	256	>512	2	<1	1
Naringenin	128	256	128	64	64	Naringenin	>512	>512	>512	>512	>512
Quercetin	256	256	64	64	64	Quercetin	>512	>512	>512	>512	>512
Pyrogallol	256	256	64	64	64	Pyrogallol	>512	>512	>512	>512	>512
Norfloxacin	512	512	16	32	<1	Penicillin	512	32	128	512	128
TZ	<1	128	8	8	<1	TZ	512	512	512	512	512
CPZ	2	<1	<1	<1	<1	CPZ	512	512	512	512	512
Res	256	256	8	16	64	Res	>512	>512	>512	>512	>512
CCCP	256	256	8	2	32	CCCP	512	512	512	>512	>512
Naringenin	16	16	8	8	8	Naringenin	>512	>512	>512	>512	>512
Quercetin	16	8	8	8	2	Quercetin	>512	>512	>512	>512	>512
Pyrogallol	16	16	8	8	8	Pyrogallol	>512	>512	>512	>512	>512
Moxifloxacin	8	16	>64	8	1	Chloramphenicol	32	32	32	32	16
TZ	<0.125	<0.125	<0.125	<0.125	<0.125	TZ	16	16	16	16	16
CPZ	8	<0.125	<0.125	<0.125	<0.125	CPZ	16	16	8	16	8
Res	8	16	2	8	4	Res	16	16	32	16	8
CCCP	<1	<1	<1	<1	<1	CCCP	16	8	16	8	2
Naringenin	0.25	16	2	8	8	Naringenin	128	128	64	128	128
Quercetin	8	16	8	16	16	Quercetin	128	256	128	128	128
Pyrogallol	16	8	8	8	8	Pyrogallol	128	128	64	8	8

[TZ, Thioridazine; CPZ, Chlorpromazine; Res, Reserpine; CCCP, Carbonyl cyanide 3-chlorophenylhydrazone]

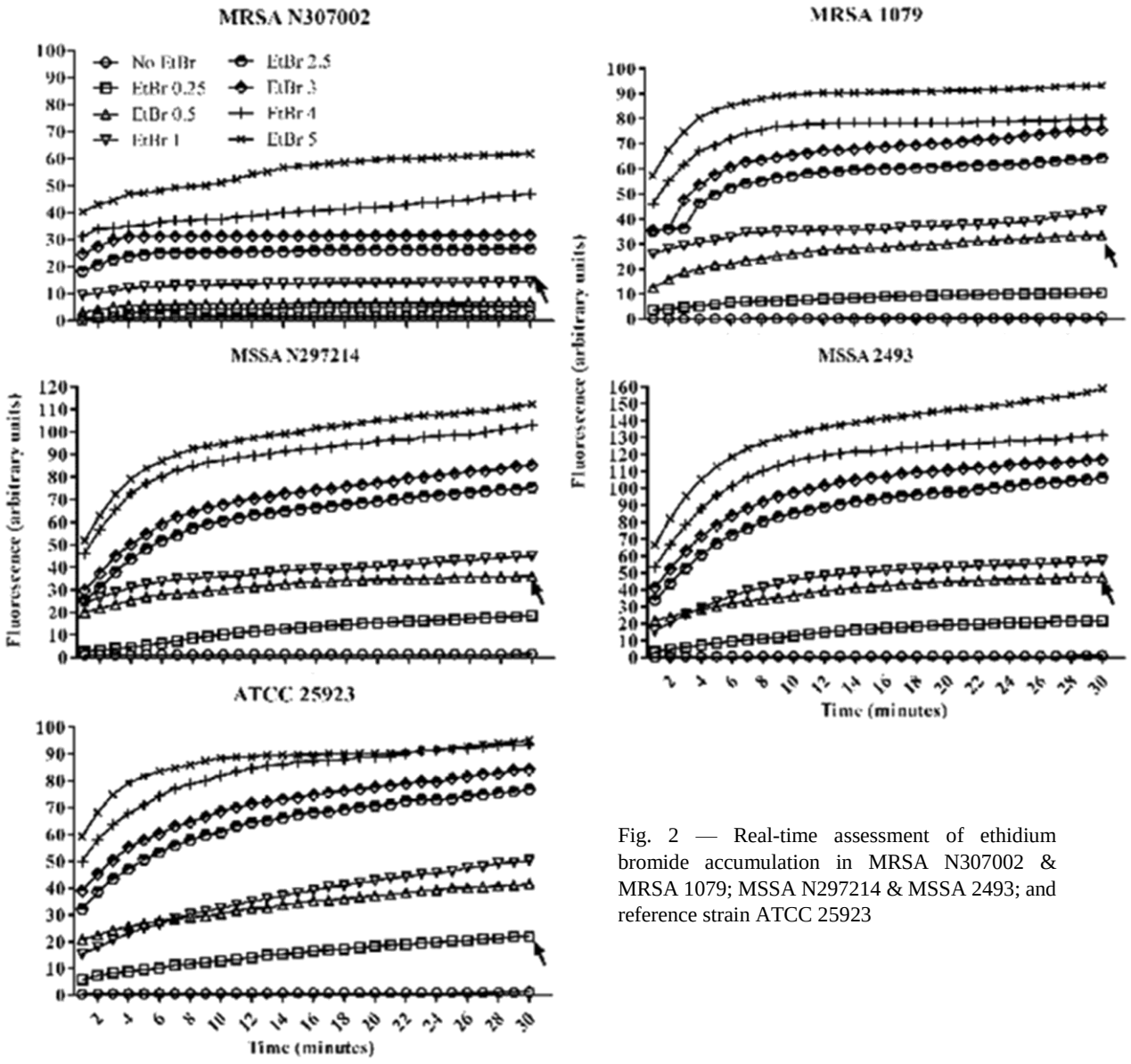

Fig. 2 — Real-time assessment of ethidium bromide accumulation in MRSA N307002 & MRSA 1079; MSSA N297214 & MSSA 2493; and reference strain ATCC 25923

efflux activity in the presence of CPZ (Fig. 4A). When TZ was used, efflux activity was 35-40% in MRSA and 40-50% efflux activity in MSSA (Fig. 4B). *S. aureus* ATCC 25923 showed 50% efflux activity in the presence of CPZ and 30% efflux activity with TZ. When EtBr efflux was assessed in the presence of reserpine and naringenin, there was no basal level of efflux activity (data not shown).

Effect of inhibitors on biofilm formation

TZ, CPZ, reserpine, and naringenin efficacy at sub-inhibitory concentrations were assessed on biofilm formation. At 0 h, MRSA N307002 with TZ and CPZ didn't show any effect on biofilm formation while reserpine and naringenin were able to reduce the biofilm by 50-60%. Although TZ did not affect MRSA 1079, however, CPZ and reserpine and naringenin reduced biofilm by 20 - 80% (Fig. 5). On the other hand, there was no effect of inhibitors on biofilm formation in MSSA N297214. However, naringenin showed 40% reduction in biofilm formation. Similarly, CPZ had no impact on MSSA 2493. However, TZ and reserpine showed 30% reduction in biofilm while naringenin had 60% reduction in biofilm. No effect of the inhibitors was observed in decreasing the biofilm formation of control strain ATCC 25923 (Fig. 5).

After 24 h, MRSA strains showed no significant reduction in biofilm formation; however, MSSA N297214 showed 30% reduction with CPZ and 40%

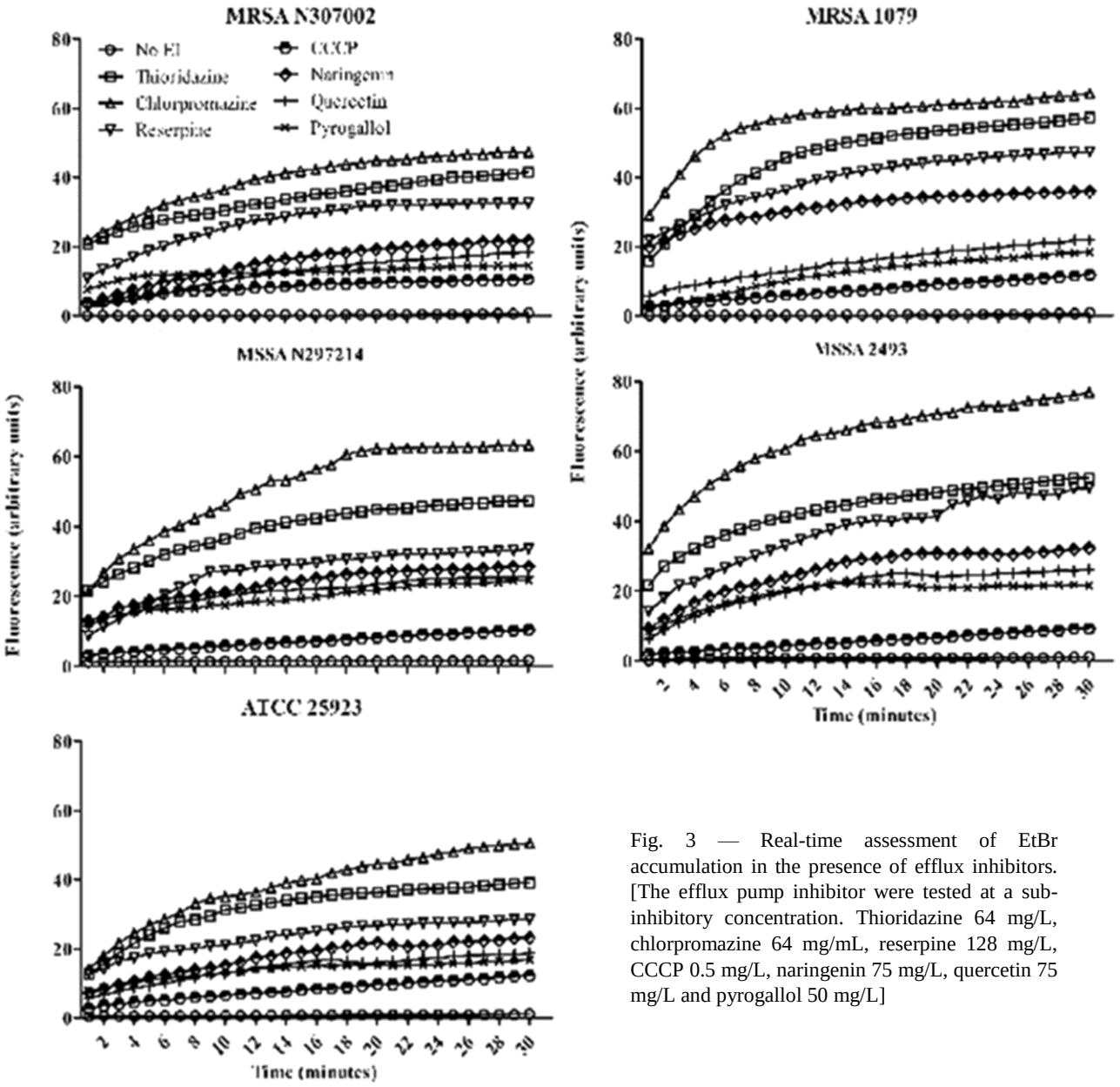

Fig. 3 — Real-time assessment of EtBr accumulation in the presence of efflux inhibitors. [The efflux pump inhibitor were tested at a sub-inhibitory concentration. Thioridazine 64 mg/L, chlorpromazine 64 mg/mL, reserpine 128 mg/L, CCCP 0.5 mg/L, naringenin 75 mg/L, quercetin 75 mg/L and pyrogallol 50 mg/L]

with naringenin, but no effect was observed in MSSA 2493 (Fig. 5). The biofilm formation in control strain ATCC 25923 was reduced by 10% with TZ and 20% with CPZ but no effect was observed with other inhibitors (Fig. 5).

TZ, CPZ, reserpine and naringenin efficacy was further assessed at inhibitory concentrationon biofilm formation. At 0 h, MRSA N307002 showed a significant reduction in biofilm formation with all inhibitors; however, MRSA 1079 and MSSA ATCC 25923, except reserpine, also showed a decrease in biofilm formation (Fig. 5).

MRSA strains did not affect biofilm formation when TZ and reserpine were added after 24 h; however, MRSA N307002 showed a reduction in biofilm formation with CPZ and naringenin. On the other hand, MRSA 1079 showed a decrease in biofilm formation with CPZ, but no effect was observed with naringenin (Fig. 5). MSSA N297214 showed a reduction inbiofilm with all inhibitors; however, MSSA 2493 showed a decrease with TZ but not with other inhibitors. ATCC 25923 had an inhibitory effect on biofilm formation with TZ and CPZ but not with reserpine and naringenin (Fig. 5).

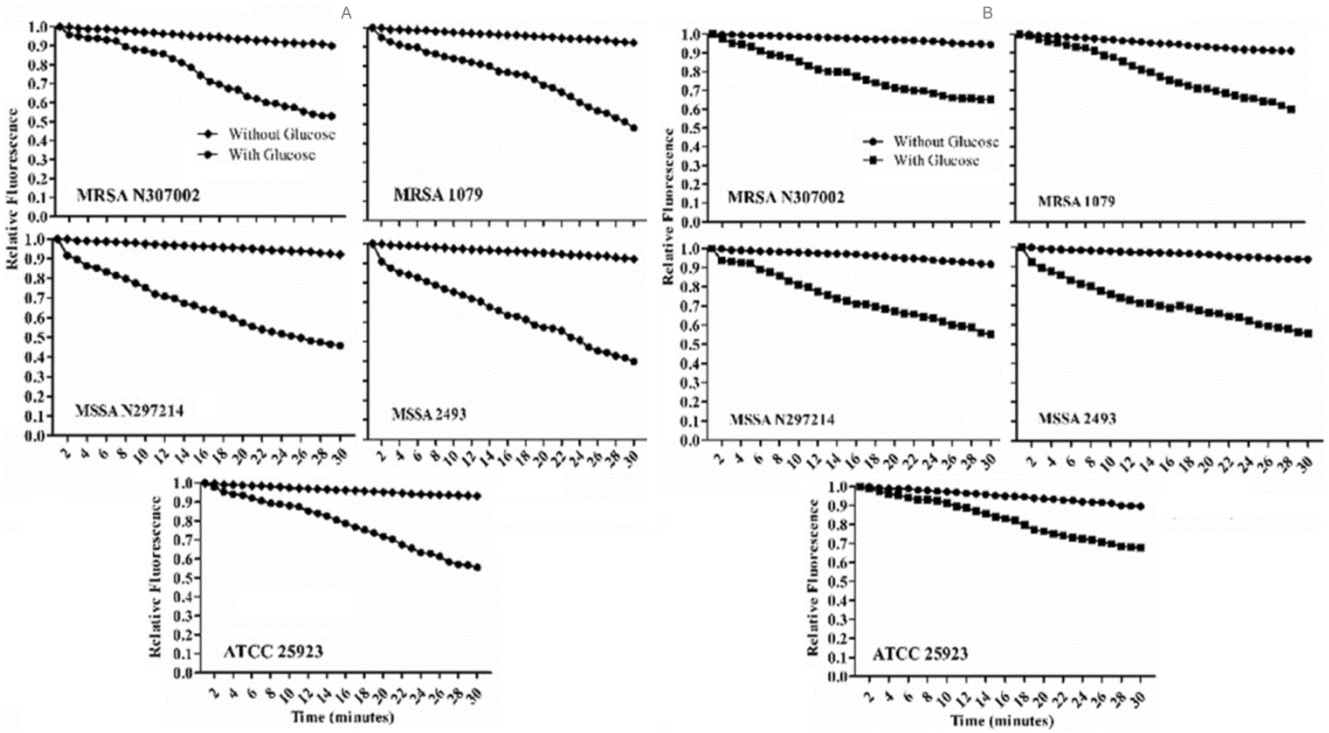

Fig. 4 — Ethidium bromide efflux assay was done in the presence/absence of 0.4% glucose, with or without the efflux inhibitor at a sub-inhibitory concentration of 64 mg/L in MRSA and MSSA strains. (A) Chlorpromazine; and (B) Thioridazine

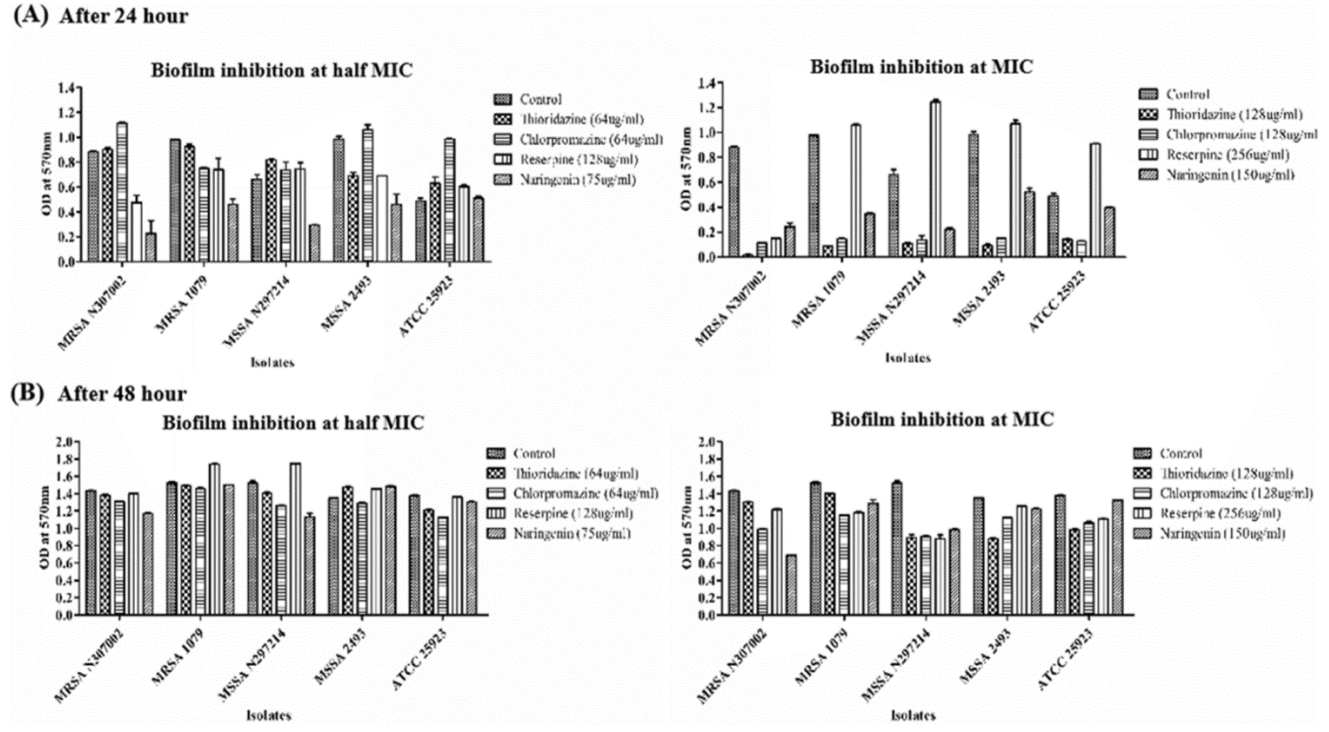

Fig. 5 — Effects of thioridazine, chlorpromazine, reserpine and naringenin at half MIC and MIC on biofilm formation of MRSA and MSSA strains. (A) Inhibitors were added initially (0 h) at half MIC and MIC, and the biofilm was determined after 24 h; and (B) Inhibitors were added after 24 h of biofilm at half MIC and MIC, and the biofilm inhibition was determined after 48 h

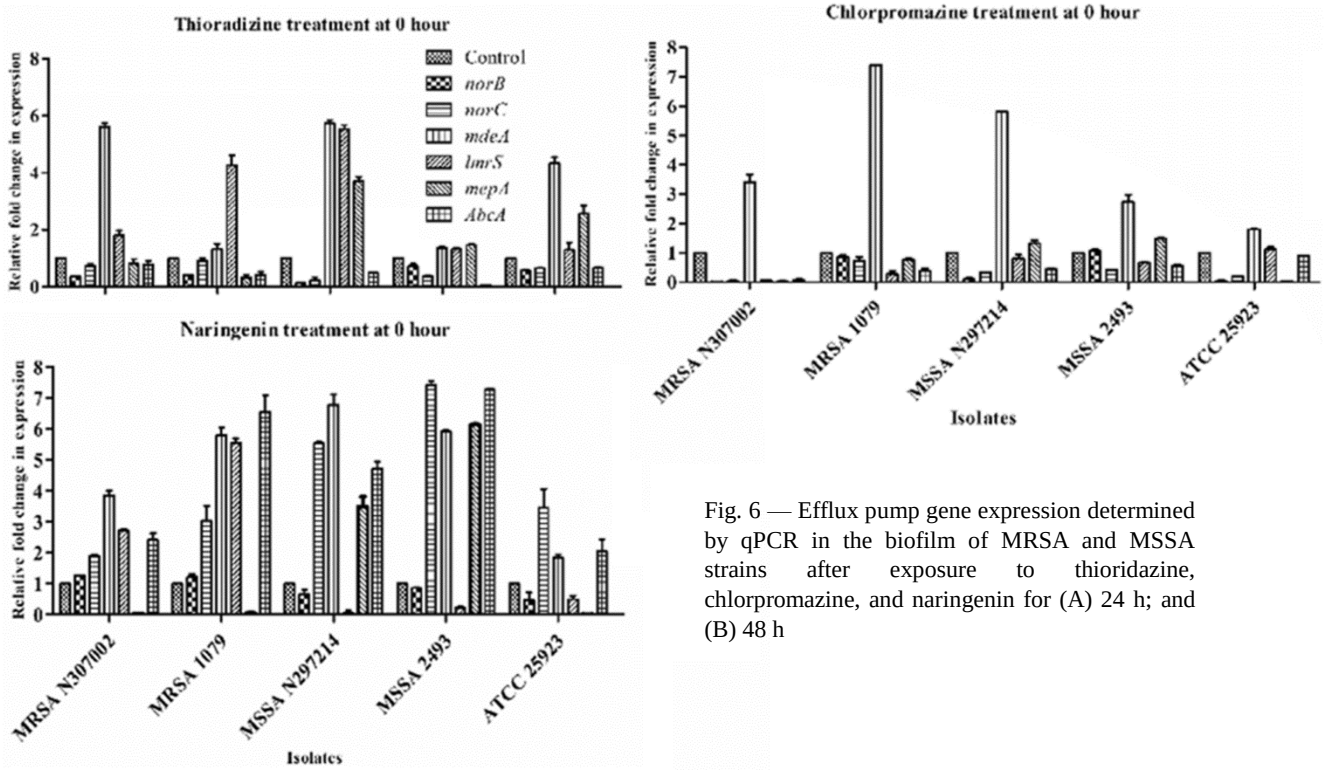

Fig. 6 — Efflux pump gene expression determined by qPCR in the biofilm of MRSA and MSSA strains after exposure to thioridazine, chlorpromazine, and naringenin for (A) 24 h; and (B) 48 h

Efflux pump gene expression in biofilm

The gene expression of *norB*, *norC*, *mdeA*, *lmrS* (MFS family), *mepA* (MATE family) and *abcA* (ABC family) in biofilm was determined in the presence of efflux inhibitors TZ, CPZ and naringenin at 0 h (Fig. 6A). TZ downregulated the *norB*, *norC*, *mepA* and *abcA* genes in MRSA; however, MSSA showed a reduction in the expression of *norB*, *norC* and *abcA* genes. The expression pattern of ATCC 25923 was similar to that of MRSA strains. CPZ upregulated the expression of the *mdeA* gene but downregulated other genes in MRSA isolates (Fig. 6A). Whereas *norB*, *norC*, *lmrS* and *abcA*genes were downregulated by CPZ in MSSA N297214, MSSA 2493 showed downregulation of *norC*, *lmrS* and *abcA*genes. *S. aureus* ATCC 25923 downregulation of *norB*, *norC*, *mepA* and *abcA* genes with chlorpromazine. On the other hand, naringenin showed reduced expression of the *mepA* gene in MSSA but downregulation of *norB* and *lmrS*genes in MRSA and *norB*, *lmrS* and *mepA*genes in ATCC 25923 (Fig. 6A). Thus, TZ, CPZ and naringenin affect the genes belonging to MFS, MATE and ABC family.

When TZ added after 24 h in biofilm, MRSA N307002 showed downregulation of *lmrS* gene; however, MRSA 1079 showed downregulation of *lmrS* and *abcA* genes. On the other hand, MSSA N292714 showed reduced expression of *norB* and *mepA* genes, but MSSA 2493 showed downregulation of *norB*, *mdeA* and *lmrS* genes (Fig. 6B). CPZ showed reduced expression of *lmrS* and *mepA* genes in MRSA but *norB*, *norC* and *abcA*genes in MSSA N297214 and *norB*, *norC*, *lmrS*, *mepA* and *abcA* genes in MSSA 2493. The downregulation of *norB*, *norC*, *mepA* was observed in ATCC 25923 (Fig. 6B). Naringenin, downregulated the expression of *norB*, *norC*, *lmrS* and *mepA* genes in MRSA N307002 but *norB*, *norC*, *lmrS* and *abcA* genes in MRSA 1079. In contrast, MSSA N297214 showed downregulation of *norB*, *lmrS* and *mepA* genes, but MSSA 2493 downregulated all genes except for the *mdeA* and *lmrS* genes. The expression of *norB*, *norC* and *lmrS* was affected by naringenin in *S. aureus* ATCC 25923 (Fig. 6B). Therefore, TZ, CPZ, and naringenin inhibited genes belonging to either MFS or MATE and/or ABC family in MRSA and MSSA strains.

Discussion

Several workers reported the occurrence of antibiotic resistance among *S. aureus*strains, of which MRSA acquires additional antibiotic resistance genes[32,33]. This phenomenon is not only limited to planktonic bacteria but also extends to biofilm. MRSA

showed the presence of efflux pump genes *norA, norB, norC, mdeA, mepA* and *abcA* gene, which is similar to those workers who also reported the existence of efflux pumps genes in MRSA[34]. Besides, MSSA strains is also shown to carry genes, namely *norA, norB, norC, mdeA, lmrS, mepA* and *abcA* which was not reported earlier among *S. aureus* strains.

Several mutations have been reported in *S. aureus* showing resistance to fluoroquinolones. These mutations are mainly attributed by QRDRs of the DNA gyrase and the topoisomerase IV genes encoding for *gyrA, gyrB, grlA* and *grlB*[3,35]. MRSA and MSSA strains were resistant to gatifloxacin, moxifloxacin, ciprofloxacin and ofloxacin. They showed MIC in range of from 8 to >64 mg/L. For the first time, we found additional mutations in the QRDRs of MRSA and MSSA strains. These mutations in *gyrA, gyrB, grlA* and *grlB* among *S. aureus* lead to a higher level of MIC for fluoroquinolones. These observations, thus suggest that mutations are continuously occurring in *S. aureus* strains, which should be monitored in real-time to track the changes in antibiotic resistance.

Staphylococcus aureus strains were resistant to fluoroquinolones and showed high MIC values. We found the role of efflux inhibitors TZ, CPZ and CCCP in reducing the MIC for fluoroquinolones, ethidium bromide, and other antibiotics indicating their role as efflux pump inhibitors among *S. aureus* strains. Our results corroborate with the findings of other workers who have reported a reduction in MIC of antibiotics after exposure to TZ and CPZ[36]. Similarly, reserpine was shown to reduce the MIC of antibiotics among *S. aureus*[10]. Phenothiazine was shown to alter the susceptibility of oxacillin in MSSA and MRSA strains[35]. Although this study showed the reduction of MIC for oxacillin in MRSA strains with TZ and CPZ but no effect was found with reserpine.

In contrast, reserpine reduced the MIC of ciprofloxacin and moxifloxacin in MRSA and MSSA strains. This finding is similar to those who have shown a reduction in the MIC of norfloxacin and ciprofloxacin[37]. It shows that TZ, CPZ and resperine can be used effectively in reducing the MIC of antibiotics, thus facilitating the action of these drugs in the treatment of infection.

Flavonoids, quercetin, naringenin and pyrogallol have antimicrobial activities[38]. In this study, we found naringenin at 75 mg/L (275.5µM) concentration showing a reduction in MIC of norfloxacin and moxifloxacin in MRSA and MSSA strains. This observation, thus indicates the role of this compound as an effective efflux pump inhibitor. Naringenin was found to be an effective efflux pump inhibitor after reserpine, as demonstrated by ethidium bromide accumulation assay. In the past, EtBr-reserpine combination has been used as a standard control to study the efflux activity in *S. aureus*[39,40]. Also, CCCP found to increase EtBr fluorescence[41]. In contrast, CPZ was found most effective inhibitor followed by TZ that leads to ethidium bromide accumulation. These findings thus suggests that flavonoid may be used as effective efflux pump inhibitors.

We evaluated the ability of TZ, CPZ and reserpine at two different time points of biofilm formation. At the initial time point and sub-inhibitory concentration, TZ did not affect, but CPZ had some effect on biofilm formation in MRSA. In contrast, TZ reduced biofilm formation in MSSA, but CPZ did not show any effect on biofilm formation. These observations thus indicate that TZ and CPZ at their MIC, showed significant effect on biofilm formation among MRSA and MSSA strains, suggesting their role in biofilm formation. Earlier workers have also demonstrated the role of CCCP and CPZ in biofilm formation in *S. aureus*[42]. Naringenin was reported to be an effective inhibitor in the biofilm, suggesting a self defense function for this compound in its natural niche. In this study, naringenin showed a significant reduction in biofilm formation in MRSA and MSSA strains. However, in 24 h grown biofilm, CPZ and naringenin was found effective against MRSA but TZ was effective against MSSA. This observation thus suggests that TZ, CPZ and naringenin act differently among MRSA and MSSA strainsdepending on duration of growth of biofilm.

Staphylococcus aureus has been reported to upregulate the expression of efflux pump genes during biofilm growth[43]. Several workers have identified the use of efflux pump inhibitors as antibiofilm agents[17]. In this study, we found a differential gene expression pattern in the biofilm after treatment with inhibitors. CPZ and TZ had shown reduced expression of *norB, norC, mepA, abcA* and to a certain extent *lmrS* genes in the biofilm formed by MRSA but *norB, norC* and *abcA* genes in MSSA thereby reducing the biofilm formation. This finding thus indicates the role of these inhibitors in biofilm formation among MRSA and MSSA strains. Similar to TZ and CPZ, naringenin also showed downregulation of MFS, MATE, and ABC genes leading to the reduction of biofilm. This finding

thus suggests their role in the inhibition of biofilm formation.

The methodologies applied in this study showed that efflux activity is an essential component of the resistance to fluoroquinolones and other antibiotics that are substrates of different efflux pumps in MRSA and MSSA strains. TZ, CPZ, reserpine and naringeninwere found to inhibit the efflux pump system that may be used as an antibiofilm agent. There is a need for further study to combine broad spectrum efflux pump inhibitors with antibiotics that could help in the treatment of *S. aureus* infections.

Conclusion

The reduction in MIC of antibiotics in the presence of efflux inhibitors showed that efflux activity is an essential component of the resistance to fluoro-quinolones and other antibiotics that are substrates of different efflux pumps in MRSA and MSSA strains. The expression study on efflux pump genes indicated that TZ, CPZ, reserpine and naringenin were found to inhibit the efflux pump system that may be used as an antibiofilm agent.

Acknowledgment

This study was supported by the Department of Biotechnology, New Delhi. Author SA acknowledges Senior Research Fellowship provided by the Institute of Life Sciences, Bhubaneswar.

Conflict of Interest

Authors declare no conflict of interests.

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