GLOBAL JOURNAL OF BIO-SCIENCE AND BIOTECHNOLOGY

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THE ROLE AND EFFECT OF EFFLUX PUMPS AND EFFLUX PUMPS INHIBITOR ON METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* BIOFILM FORMATION

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ABSTRACT
Biofilm and efflux pumps are the leading cause for the arising of multidrug resistant *staphylococcus aureus*, especially MRSA. This bacterium relies on these mechanisms to protect itself from different toxic material and antibiotics. Exopolysaccharides, the main component of biofilm, slow down or prevent the penetration of antibiotics to the interior cells embedded in biofilm, while the efflux pumps keep toxic materials and antibiotics under the lethal level by extruding them to the outside of bacterial cells. A study conducted on *E. coli* revealed that efflux pumps involved in antibiotics resistance of biofilm, suggesting a correlation between these two mechanisms. In this study, the correlation between efflux pumps and biofilm formation in MRSA isolates was investigated by studying the effect of efflux pumps inhibitor on biofilm formation. In this study, the antibiotic susceptibility of *staphylococcus aureus* cells in biofilms and planktonic cell was investigated; 2µg/ml and 4 µg/ml of Moxifloxacin were applied to planktonic cells and cells in biofilms. The result of Moxifloxacin treatment revealed that this antibiotic has an effect on planktonic cells but not on cells in Biofilm. 4 µg/ml of Moxifloxacin was more effectiveness on planktonic cells than 2 µg/ml. while it failed to kill *S. aureus* in biofilms, resulting in the re-growing of these bacterial cells in biofilm after stopping the Moxifloxacin treatment. The effect of 2 µg/ml Moxifloxacin on planktonic cells was enhanced by the addition of efflux pumps inhibitor. Three concentration of efflux pumps inhibitor were applied 0.05mg/ml, 0.1 mg/ml and 0.25 mg/ml. 0.25 mg/ml of efflux pumps inhibitor was more efficient in reducing MRSA biofilm formation than 0.05 and 0.1 mg/ml. This outcome can suggest efflux pump inhibitors as a promising agent to reduce biofilm and to enhance its susceptibility to antibiotics.

KEYWORDS: Efflux pumps, Efflux Pumps Inhibitor, Biofilm, *norA* gene, *icaA* gene, MRSA.

INTRODUCTION

The pathogenicity of *Staphylococcus aureus* is attributed to the possession of two virulence factors, the antibiotic resistance and biofilm formation ability. Biofilm acts as a mechanism of survival by which bacteria would protect itself from the effect of antibiotics[1-3]. Pathogenic bacteria developed other protection mechanism against wide range of antibiotics and toxic materials. The emerged mechanism resulted in the appearance of multidrug resistant bacteria [4-5]. Recent studies suggested the involvement of bacterial cell transporter proteins in the appearance of multidrug resistant bacteria[6]. These proteins are now known as efflux pumps[7,8,9]. These proteins help bacteria to detoxify itself from any toxic materials by extrude them to the outside thus prevent them from reaching the lethal level inside bacterial cell [10-12]. Studies on gram negative bacteria (Salmonella, *E. coli* and *P. aeruginosa*) concluded that efflux pumps have role in biofilm formation and biofilm mediated antibiotic resistance[13-18]. In this study we investigated the correlation between efflux pumps and biofilm formation among Iraqi clinical isolates of MRSA (Methicillin Resistant *Staphylococcus aureus*). We investigated the ability of our isolates to form biofilm by Microtiter plate, test the effect of Moxifloxacin on planktonic cells and biofilm, screening the presence of *ica*A (Biofilm forming gene) and *nor*A (Quinolone resistant protein coding gene) genes by polymerase chain reaction, and the effect of efflux pump inhibitor on biofilm formation. The obtained result showed that MRSA biofilm is more resistant to Moxifloxacin than planktonic cells; supporting the idea that biofilm is a defense mechanism *S. aureus* use to protect itself against antibiotics. This study confirms the correlation between biofilm and efflux pumps since efflux pumps inhibitor reduced the biofilm formation.

MATERIALS & METHODS

Bacterial isolates

Fourteen strains of *Staphylococcus aureus* were isolated from Iraqi people. Most of these isolates were obtained from noise and some from blood and wounds. *Staphylococcus aureus* isolates were identified by different biochemical tests oxidase, coagulase, and catalase[19]. Additionally, the Staphylococcus aureus isolates were confirmed by PCR for the presence of nuc and mec genes presence.

Assay for biofilm formation by Microtiter plate

Overnight cultures of staphylococcus isolates grown on nutrient broth media were diluted 1:100 into brain heart infusion broth supplemented with 1% glucose for biofilm assay**.** 200 µl of the diluted cultures were transferred into 96 Microtiter plate, a duplicate was used for each isolates.

The diluted cultures were incubated for 24 hours at 37 ºC. After the 24 hours incubation period, the bacterial growths were poured out and the Microtiter plate was washed with distilled water to remove any non-adherent cell, then the plate was left to dry. Each well was stained with 200 µl of 0.1% crystal violet to stain the layer of cells that had attached to the wells bottom. The excess stain was poured out and washed with distilled water and the plate was left to dry for approximately two hours[20]. For biofilm quantification, each stained well was treated with 200 µl of 30% acetic acid for 10 minute to solubilize the dye. The solubilized crystal violet was transferred into a new Microtiter plate. The optical density of each well was measured by micro ELISA auto reader at wavelength 590 nm, 200 µl of 30% acetic acid was used as a blank and to determine the background[21]. A duplicate was performed and the average optical density and standard deviation were calculated. OD590< 0.2 a non- biofilm forming cell, 0.2 < OD590 > 0.9 a moderate biofilm forming cell, OD590 ≥ 0.9 a strong biofilm forming cell.

Biofilm and planktonic assay for Moxifloxacin resistance

In case of biofilm [22], 2 µl of 0.1 O. D_{600} bacterial isolates were inoculated into 96 wells Microtiter plate containing 200 µl of Brain Heart infusion broth supplemented with 1% glucose. The bacterial isolates were allowed to form biofilm at 37 Cº for 24 hours. After the 24 hours of incubation, the formed biofilms were exposed to two Moxifloxacin concentrations below its 8µg/µl MIC concentration[23] 2 µg/µl and 4 µg/µl for 24 hours. The next day, 200 µl of a new fresh nutrient broth medium was added to each well after pouring out the antibiotic-containing medium, so the bacteria that survived the antibiotic treatment due to the biofilm would reproduce for the next 24 hours of incubation at 37Cº. 1 µl of each bacterial isolates were transferred onto antibiotic free nutrient agar to test the effect of these two Moxifloxacin concentrations on the biofilm and to compare the obtained result with the planktonic assay. In planktonic assay[24], 1:100 diluted bacterial isolates, on 96 well plate, were directly exposed to 25% (2 µg/µl) and 50% (4 µg/µl) of Moxifloxacin MIC concentration for 24 hours at 37Cº. Next day, 1µl from each antibiotic treated bacterial isolates were transferred onto antibiotic free nutrient agar to test the effect of two concentrations of antibiotic on planktonic cells.

Detection of *ica*A and norA loci

DNA isolation

DNA was extracted from 1ml of overnight culture using Promega DNA extraction kit supplemented with 30 µg/ml lysozyme enzymes.

PCR for detection of icaA loci and norA gene.

1µl of 100ng DNA was used as template for PCR. *ica*A and *norA* presence among the extracted DNA of isolates was determined by polymerase chain reaction. The *icaA* forward and reverse primers were (5'-AAACTT GGTGCGGTTACAGG-3') (5'-TCTGGGCTTGACCAT GTTG-3'), respectively [25] that will yield 750 bp fragment. While the *norA* forward and reverse primers were (5'-GGCGGTATATTTGGG GCA CT-3') (5'-ACGCAC CTG CGATTAAAGGA-3') respectively, which will yield 310pb fragment.

20 µl of PCR reaction contains 1x master mix buffer, 10 pmol/µl forward and reverse primers, 100 ng/µl DNA and water. The PCR reaction was carried out with the following parameters: initial denaturation at 94°C for 10min, second denaturation at 94°C for 1 min, annealing at 54°C for 1min for *icaA* and 56°C for 1 min for *norA* gene, extension at 72°C for 50 sec. 30 cycle of amplification was applied. To analyze the PCR products, 10 µl of PCR mixture was loaded to 1.5 % agarose in the presence of 100 b.p DNA ladder. After performing gel electrophoresis, the gel was exposed to U.V by using U.V Tran's illuminator.

Effects of efflux pumps inhibitor, Fluphenazine, on Biofilm formation of *MRSA*

200µl of brain heart infusion broth supplemented with 1% glucose were dispensed into 96 wells Microtiter plate. 0.05 mg/ml, 0.1 mg/ml, and 0.25 mg/ml of Fluphenazine were pipetted into separate wells containing media for each investigated strain. The added media was inoculated with 2 µl of O. D_{600} = 0.1 bacterial growth. The Microtiter plat was incubated in 37 ºC for 24 hours. The reading of result was done by using ELIZA reader at wavelength 590 nm [22].

Effects of efflux pumps inhibitor, Fluphenazine, on MRSA susceptibility to Moxifloxacin at planktonic state

200 µl of brain heart infusion broth was pipetted into 96 wells Microtiter plate and inoculated with 2 µl of 0.1 O. D_{600} bacterial growths. The inoculated bacteria were treated directly with 4 µg/ml and 0.25 mg/ml Moxifloxacin and Fluphenazine, respectively and incubated for 24 hours at 37Cº. 1 µl from each antibiotic treated bacterial isolates were transferred onto antibiotic free nutrient agar to test the role of efflux pumps inhibitor in enhancing the antibiotic effects on planktonic cells [24].

RESULTS

Biofilm mediates *Staphylococcus aureus* resistance to Moxifloxacin

Among the 14 clinical isolates of MRSA that were screened for their ability to produce biofilm by using Microtitter plate technique, three were biofilm non - forming isolates (O. D_{590} < 0.2), one was moderate biofilm forming isolate (0.2< O. D_{590}> 0.9), and ten were strong biofilm forming isolates (O.D > 0.9) (Fig.1 and Table 1). The major and most understandable biofilm mechanism within *S. aureus* is the extracellular polysaccharide intercellular adhesion (PIA) which is coded by the *icaADBC* operon. The 14 MRSA isolates were screened for the presence of *icaA* gene. Only three out of fourteen isolates were *icaA* negative (two were non-Biofilm forming bacteria and one was moderate as detected by Microtiter plate) (Table1), and the eleven remaining isolates were *icaA* positive as detected by PCR (Fig. 2 and Table 2). The effect of Moxifloxacin on planktonic cells of MRSA was examined and compared with its effect on biofilm of the same strains grown in the same media and conditions. All MRSA isolates that form biofilms were resistant to Moxifloxacin (2 and 4 µg/ml), while sensitive in planktonic (Fig. 3). Indicating that Moxifloxacin was more active against planktonic cells than biofilms.

TABLE1: The ability of MRSA isolates to form Biofilm in the absence and presence of Efflux Pumps Inhibitor, and the effect of Moxifloxacin on planktonic cells and cells in biofilm

Isolate	O.D 590 EI -	O.D 590 EI + (0.25mg/ml)	Planktonic Moxifloxacin		Biofilm Moxifloxacin	
			2 µg/ml	4 µg/ml	2 µg/ml	4 µg/ml
2	0.971	0.101	3 colonies	2 colonies	++	++
6	1.8	0.3	++	+	+++	+++
3N	1.5	0.202	3 colonies	++	++	++
2N	1.58	0.21	14 colonies	5 colonies	+++	+++
7N	0.95	0.09	2 colony	1 colonies	+	++
5N	0.18	0.015	–	–	–	–
19	1.27	0.098	9 colonies	6 colonies	++	+++
18	1.24	0.045	4 colony	1 colonies	+++	+++
15	0.95	0.11	2 colonies	–	+	++
47	0.19	0.023	–	–	–	–
35	0.87	0.078	–	–	+	–
54	1.95	0.45	++	15 colonies	+++	+++
11	1.13	0.02	5 colonies	1 colony	+	++
17	0.15	0.010	–	–	–	–

EI- absence of Fluphenazine, EI+ presence of Fluphenazine, Non biofilm forming isolate, + weak biofilm forming isolate. ++ / +++ strong biofilm forming isolate.

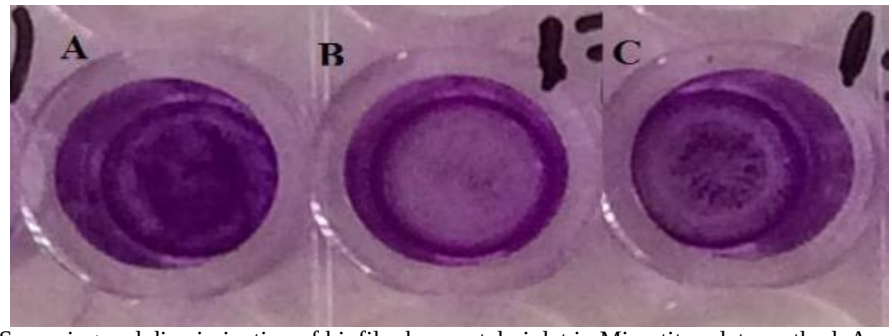

FIGURE 1: Screening and discrimination of biofilm by crystal violet in Microtiter plate method. A: strong biofilm forming isolates. B: biofilm non-forming isolates. C: moderate biofilm forming isolates

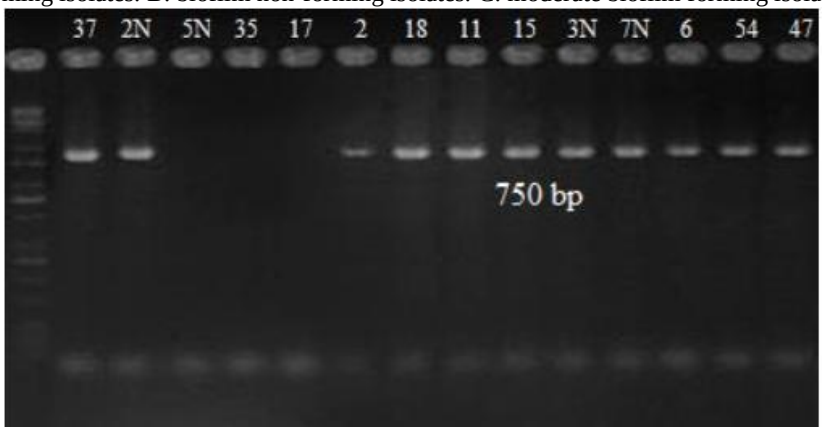

FIGURE 2: PCR based detection of *icaA* in Iraqi clinical isolates of methicillin resistant *S. aureus* first lane on the left: DNA ladder (100 bp); lane 2, 3, and 7-14 positive samples; lane 4,5, and 6 negative sample

TABLE 2: Detection of *icaA* and *norA* genes among investigated MRSA isolates by PCR.

isolate	*icaA* gene	*norA* gene
7N	positive	Positive
5N	Negative	Positive
2	Positive	Positive
47	Positive	Positive
35	Negative	Positive
37	Positive	Negative
17	Negative	Negative
11	Positive	Positive
2N	Positive	Positive
3N	Positive	Positive
18	Positive	Negative
6	Positive	Positive
15	Positive	Positive
54	Positive	Negative

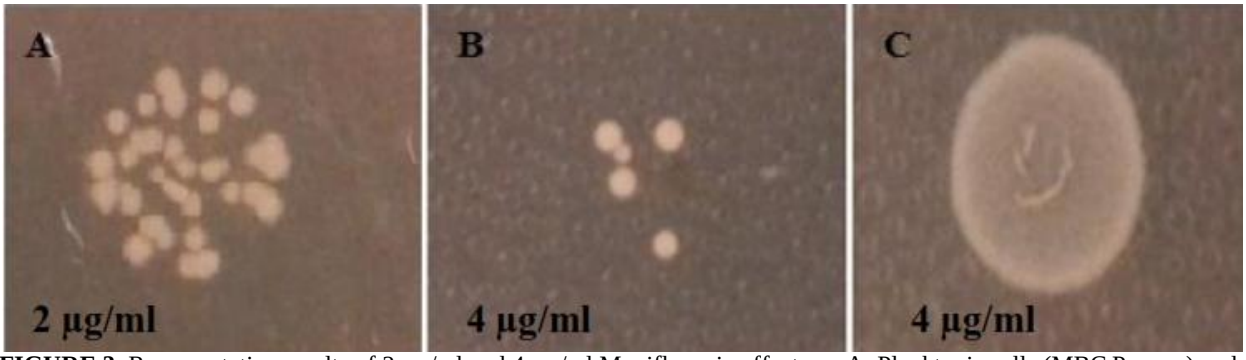

FIGURE 3: Representative results of 2 µg/ml and 4 µg/ml Moxifloxacin effects on A: Planktonic cells (MBC-P assay) and B: Biofilm (MBC-B assay).

FIGURE 4: PCR based detection of *norA* gene in Iraqi clinical isolates of methicillin resistant *S. aureus*: first lane on the left: DNA ladder (100 bp); lane 2-6, 9-11 and 13-14 positive samples; lane 7,8,12, and 14 negative samples

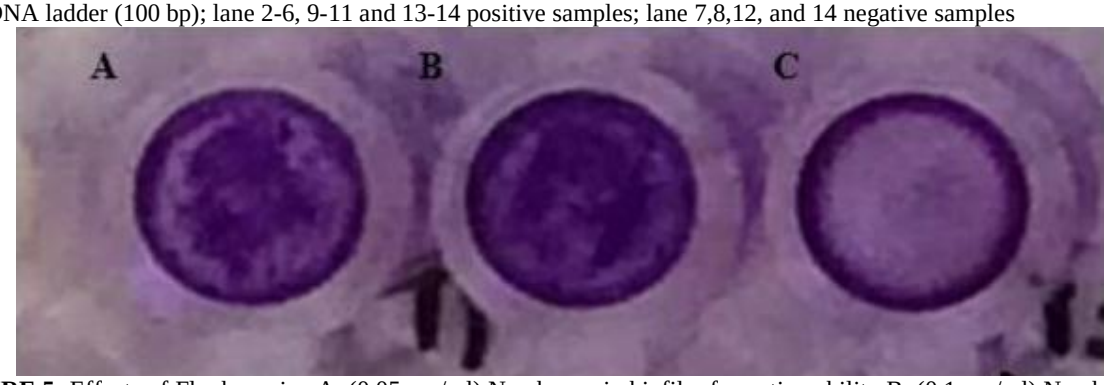

FIGURE 5: Effects of Fluphenazine A: (0.05 mg/ml) No change in biofilm formation ability B: (0.1 mg/ml) No change in biofilm formation ability c: (0.25 mg/ml) reduced the biofilm forming ability of the investigated MRSA isolates.

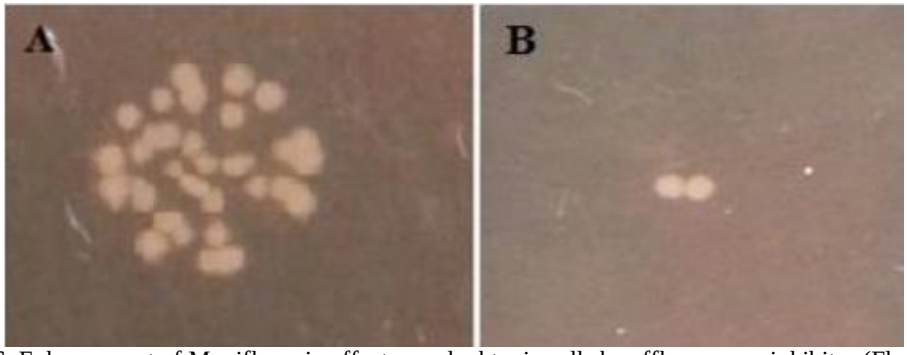

FIGURE 6: Enhancement of Moxifloxacin effects on planktonic cells by efflux pumps inhibitor (Fluphenazine) A: planktonic cells treated with 4 µg/ml B: planktonic treated with 4 µg/ml and 0.25 mg/ml efflux pumps inhibitor.

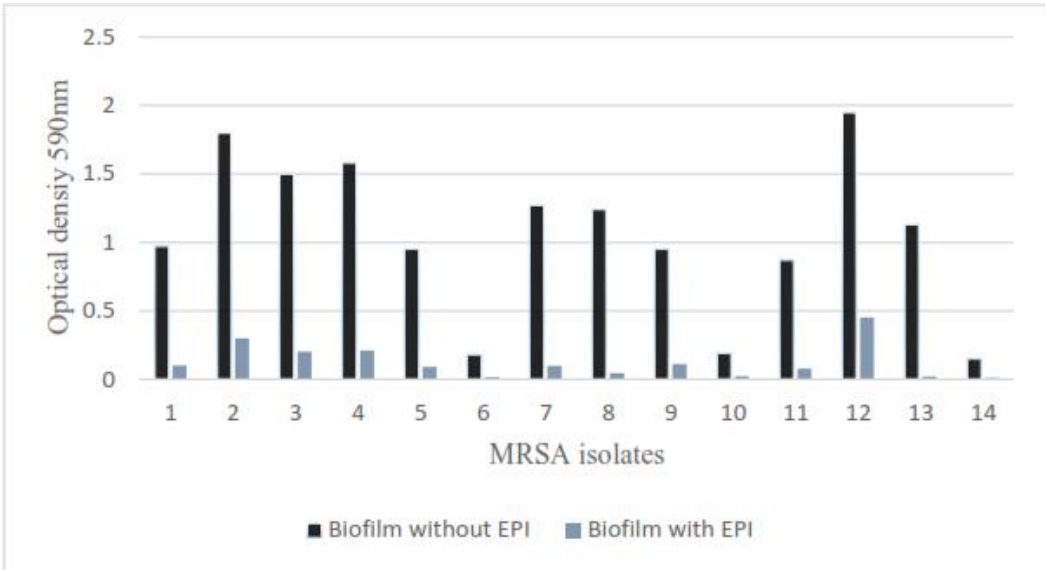

FIGURE 7: comparison between MRSA isolates biofilm formation in the absence of Fluphenazine (EPI) and biofilm formation in the presence of Fluphenazine. 0.25 mg/ml of EPI significantly reduced the ability of MRSA isolates to form biofilm

Fluphenazine efflux pumps inhibitor reduced MRSA biofilm formation and enhanced the effect of Moxifloxacin against planktonic cells

First, the fourteen MRSA isolates were screened for the presence of *norA* gene that codes for quinolone resistance protein *NorA*. From the fourteen isolates, ten isolates have *norA* gene (Fig. 4 and Table 2). All the *norA* negative isolates, except one, were *icaA* positive. The only isolate that was both *norA* and *icaA* negative showed the lowest biofilm formation ability (O.D 590 < 0.2) which considered as a non-biofilm forming isolate (Table1). Biofilm forming ability and efflux pumps possession

caused MRSA to be a multidrug resistant bacterium. A conducted study suggested a correlation between biofilm formation and efflux pumps activity enhancement, and some revealed that efflux pump genes are upregulated upon biofilm formation. So the question was if the biofilm formation ability of MRSA could be controlled by efflux pump inhibitors. The ability of Fluphenazine efflux pumps inhibitor to reduce the ability of MRSA to form biofilm was tested and examined. Fluphenazine successfully reduced the ability of the investigated MRSA isolates to form biofilm (Figs 5 and 7 and Table 2) and enhanced the effect of Moxifloxacin on planktonic cells (Fig. 6).

DISCUSSION

The ability of MRSA to tolerate different agents like antibiotics and biocides is attributed to two virulent factors, the ability to form biofilm and the possession of efflux pumps. Each one of these two defense mechanisms has its way of action. Although the mechanisms by which bacteria in Biofilms resist different antibacterial agents and antibiotics are not well understood, it is presumably that the exopolysaccharides act as a protecting shelled that slow down or prevent the penetration of antibiotic into the interior, and that is why cells in biofilms are better than planktonic cell in resisting bactericides and antibiotics [2, and26]. While the efflux pumps reduce the concentration and accumulation of toxic material inside bacterial cells. Both exopolysaccharides and efflux pumps enhance the resistance of biofilm to antibiotics. Beside toxic materials and antibiotics extrusion, Efflux pumps system is responsible on extruding cells metabolites including primary polysaccharide which is the main component of Biofilm [18, and 27]. Therefore, this study was conducted to investigate the correlation between efflux pumps and Biofilm formation among MRSA isolates by studying the effect of efflux pump inhibitor on the ability of MRSA to form biofilm. To approach this aim, 14 clinical isolates of MRSA were tested for their ability to form biofilm in the absence and presence of efflux pump inhibitor. In this study Fluphenazine was used as efflux pumps inhibitor. At the same time the presence of *icaA* and *norA* gene within these tested isolates were also investigated. And to improve that cells in biofilm is far better than cell in planktonic in the concept of antibiotic resistance, both were subjected to Moxifloxacin.

The MBC-Biofilm and MBC-planktonic assay revealed that Methicillin Resistance Staphylococcus aureus biofilm is more resistant to Moxifloxacin than planktonic cells (Fig. 3). 4µg/ml of Moxifloxacin was sufficient to increase the susceptibility of MRSA planktonic cells, but on the other hand this concentration failed to decrease the MRSA biofilm antibiotic resistance. All isolates that possesses *icaA* and *norA* (except isolate 47) were biofilm forming bacteria in Microtiter plate method and all were resistant to Moxifloxacin (Figs 2 and 4 and Table1 and 2). although isolate number 47 was positive for both *icaA* and *norA* as detected by PCR, it failed to form biofilm as detected by Microtiter plate and was sensitive to Moxifloxacin, suggesting a defect in *icaA* and *norA* gene expression.

Two isolates (5N and 35) were *icaA* negative and *norA* positive (Figs 2 and 4 and Table 2). Isolate 5N was non biofilm forming bacteria while isolate 35 was moderate and both were sensitive to Moxifloxacin. The obtained results from these two isolates indicate that presence of efflux pump *norA* gene alone along with the absence of biofilm forming capacity is not enough to offer resistance to Moxifloxacin comparing with result obtained from *icaA* and *norA* positive isolates and *icaA* positive/ *norA* negative isolates which all were Moxifloxacin resistant. The comparison of biofilm resistance to Moxifloxacin between *icaA* +/ *norA* + isolates and *icaA* +/ *norA* - isolates revealed no differences, suggesting that efflux pump *norA* gene has a minor contribution to biofilm resistance of MRSA isolates to Moxifloxacin, or this gene needs to be overexpressed within *norA*+ isolates in order to enhance the biofilm antibiotic resistance, or the *norA* – isolates have other active efflux pumps genes and proteins rather than *norA*. This result is consistent with a previous study conducted on *Pseudomonas aeruginosa* in which the presence or absence of MexAB-OprM pump showed no difference in biofilm antibiotic resistance [28], while other study also conducted on *Pseudomonas aeruginosa* revealed that biofilm resistance to antibiotic was enhanced by the overexpression of MexAB-OprM efflux pumps [29]. Treating planktonic MRSA cells that were *norA*+ or *norA*- with efflux pump inhibitor enhanced the potency of Moxifloxacin against them (Fig. 6).

Or study was conducted to study the role of efflux pumps in biofilm formation. If there is any correlation between them, the addition of efflux pumps inhibitor, that blocks the efflux pumps activity, would effect MRSA biofilm forming ability. The addition of Fluphenazine reduced biofilm formation of MRSA by inhibiting the activity of efflux pumps. The inactivation of efflux pumps would result in blocking the extrusion of polysaccharide and allow the antibiotics to accumulate inside bacterial cells until reaching lethal level. Or finding is consistence with a study conducted on Enterobacteriaceae (*E. coli and Klebsiella* strains) in which addition of efflux pumps inhibitor reduced biofilm formation [30, and 31]. This outcome can suggest efflux pump inhibitors as a promising anti biofilm agents and as agent to enhance biofilm su*sceptibil*ity to antibiotics since the used efflux pumps inhibitor targeted the Moxifloxacin resistance of our MRSA isolates.

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