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Microbiological study of certain genes associated with biofilm forming capacity of methicillin resistant *Staphylococcus aureus* in Egypt: An eye on nifedipine repurposing

Ghada Hani Ali *, Nevine L. Seiffein

Department of Microbiology and Immunology, Faculty of Pharmacy, Pharos University in Alexandria, PUA, Egypt..

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ABSTRACT

Background: *Staphylococcus aureus* remains one of the most prevalent pathogens associated with several infections. **Objective:** We aim to evaluate the biofilm forming capacity along with the presence of biofilm-associated genes in methicillin resistant *Staphylococcus aureus* (MRSA) from surgical wound infections. In addition, potential antimicrobial activity of nifedipine was investigated. **Methods:** A total of 50 MRSA isolates were collected from surgical wound samples from clinical laboratories. The antimicrobial susceptibility and biofilm forming capacity were screened. Polymerase chain reaction was used to detect *icaA*, *icaD*, *hla*, *sirB*, *ebpS*, *fnbA*, *clfA*, *sdr* and *can* genes. The antimicrobial and antibiofilm effect of nifedipine, alone and combined with levofloxacin, was determined. Preliminary molecular docking was employed to predict the binding affinity between nifedipine and different target proteins. Staphylococcal protein A (*spa*) typing was performed to analyze MRSA strains. **Results:** All MRSA strains were multidrug-resistant and biofilm producers. The most abundant gene was *hla* (96%), followed by *icaA* and *sirB* with equal prevalence (88%). Biofilm formation was significantly associated with *icaA*, *icaD*, *sdrE* and *sirB* genes. In addition to the antibiofilm activity of nifedipine, there was a synergistic effect between it and levofloxacin, this finding was further given strength to by molecular docking where nifedipine had a binding affinity to HTH-type transcriptional regulator qacR. For the first time in Egypt, *spa* type t314 was reported. **Conclusion:** Nifedipine, alone and combined with levofloxacin, showed promising results as antimicrobial and antibiofilm agent. Such effect might be due to efflux inhibition activity and worth additional investigation to understand the underlying mechanism.

Introduction

Staphylococcus aureus (*S. aureus*) is one of the most prevalent causes of skin and soft tissue infections (SSTIs) [1]. Particularly, methicillin-resistant *S. aureus* (MRSA), a health problem delaying the recovery from infections and causing health deterioration. Treatment options for

staphylococcal infections, have been considerably reduced because of the spread of MRSA with multi-resistance genes, leading to poor clinical outcome [2]. *Staphylococcus aureus* biofilms seem to be specially associated with chronic wounds, and the impact of biofilms on the delay of wound healing is

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* Corresponding author: Ghada Hani Ali
E-mail address: ghada.nassef@pua.edu.eg

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of major importance, as it affects greatly the normal wound healing process in absence of infection, physical debridement and or local drugs are of little help. Staphylococcal infections appear usually among hospitalized patients and can have serious complications among which post-surgical wound infections [3].

Biofilm synthesis is a crucial virulence factor which is important for the survival as well as the persistence of MRSA in the host tissues [4]. In addition to biofilm synthesis, extracellular toxins and surface structures have a critical role in the stimulation and continuance of infection. Biofilm production is regulated by a large number of different genes, where the *icaA/D* genes (intercellular adhesion A and B) are the most commonly studied and are in charge of polysaccharide intercellular adhesion (PIA) production comprising N-acetylglucosamine as a key component of the matrix enclosing the microbial cells inside the biofilm [5]. The extracellular matrix proteins of the host have high affinity for the protein components of the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) such as fibronectin binding proteins A and B (*fnbA* and *fnbB*), serine-aspartate repeat proteins (*sdrE*), clumping factors A and B (*clfA* and *clfB*), collagen-binding protein (*cna*) and elastin binding protein (*ebpS*) [6].

Staphylococcus aureus secretes a variety of exotoxins that are capable of penetrating host cells, like hemolysins, that include four different toxins, namely alpha, beta, gamma and delta hemolysin. The alpha-hemolysin (Hla) exotoxin is one of most prominent and well-recognized virulence factors in *S. aureus* [7]. The α -toxin, which encoded by the *hla*, serves as a pore-forming cytotoxin (PFT) and has an activity against different human cells. The hemolytic, dermonecrotic and neurotoxic activity of α -toxin are responsible for this toxin's pathogenicity [8].

Another probable pathogenic feature was the presence of siderophores. The capability of iron uptake from the host through bacterial siderophores could promote the establishment of infection. *Staphylococcus aureus* has been shown to have several siderophores, including iron-uptake ABC transporters [9].

For *S. aureus* isolates, Staphylococcal protein A (*spa*) typing is a commonly used typing technique since it is an effective, inexpensive and simple technique for bacterial typing. It relies on the polymorphism of the gene encoding protein A (*spa*). The antiphagocytic protein A binds the Fc portion of immunoglobulin G (IgG) and acts as an antiplatelet, anticomplement and mitogen [10].

Drug repurposing, which involves screening existing medications for new functions, is becoming more popular in antibiotic discovery efforts, and several chemical libraries are now commercially available, different drugs are nowadays assayed for antimicrobial activity in an attempt to fight the increasingly alarming antibiotic resistance [11].

Calcium channel blockers as nifedipine, nisoldipine and felodipine, among other drugs have been tested for their antimicrobial activity and their wound healing effects such results encouraged us to assess the effectiveness of nifedipine alone and in combination with levofloxacin against staphylococcus wound isolates.

The purpose of this study was to test the prevalence of genes which are encoding adherence factors in MRSA isolates and their correlation to extent of biofilm formation, spa typing to detect molecular epidemiology was performed. Nifedipine, alone and combined with levofloxacin, was tested for synergism antimicrobial and antibiofilm activity, preliminary molecular docking was performed to shed a light on the probable mechanism of action of nifedipine

Materials and Methods

Collection and identification of clinical isolates

Fifty MRSA isolates were obtained from post-operative wound samples provided from Microbiology Laboratory at Alexandria Main University Hospital and Medical Research Institute. Sample inoculation was performed into Mannitol Salt Agar, Boord Agar and Mac Conkey agar and the samples were then incubated for 24 hours at 37°C. They were classically identified by colony morphology; coagulase test, Gram staining and catalase test [12]. All tested strains were finally stored at -20°C in Luria Bertani glycerol.

Antibiotic sensitivity pattern of *S. aureus*

The antibiotic sensitivity testing of the tested *S. aureus* isolates was carried out by Kirby-Bauer disc diffusion method [13] according to the Clinical and Laboratory Standards Institute (CLSI) recommendations [14]. The following antibiotic discs were used: cefotaxime (CTX 30µg), cefoxitin (FOX 30µg), ciprofloxacin (CIP 10µg), erythromycin (E 15µg), clindamycin (DA 10µg), rifampicin (RD 30µg), tetracycline (TE 30µg), linezolid (LZD 30µg), oxacillin (OX 5µg), cotrimoxazole (SXT 25µg), gentamicins (CN 30µg), nitrofurantoin (F 50µg) and vancomycin (VA 30µg). Cefoxitin was used for the identification of MRSA strains, where inhibition zone diameter ≥ 22 mm and ≤ 21 mm, was interpreted as sensitive and resistant, respectively. Besides,

isolates showing resistance to 3 or more antibiotic classes were recognized as Multi-drug resistant (MDR).

Determination of biofilm forming capacity of all isolates

The experiment was done as mentioned previously, briefly, the organisms under test were cultured in nutrient broth for 24 hours at 37°C. The culture was diluted to a concentration of 10⁶ CFU/ml, 200 µl of each culture were distributed in triplicate in a flat bottom micro titer plate with lid, incubated at 37 for 24 hours, At the end, the cells were removed, microtiter plates were washed three times with 200 µl saline and let to air dry. Attached biofilm mass was fixed using 95% ethanol stained with 100µl of 1% (wt/vol) crystal violet for 5 min. Then, the wells were emptied and washed three times with 300 µl of sterile distilled water at the end plate was air dried for 2 h. The optical density (OD) of each well was measured at 590 nm using ELISA reader [15].

Polymerase chain reaction (PCR) assay on MSCRAMM, biofilm-related and other virulence genes

For the PCR, DNA templates were provided by preparing bacterial suspension of five colonies in 500 µl DNase and RNase-free water. The suspension was incubated in a boiling water bath for 10 minutes and centrifuged for 5 minutes at 15,000 rpm. The supernatant containing the DNA was employed as DNA template for PCR and kept at -20°C for subsequent use [16].

Simplex and multiplex PCRs were both performed to investigate the presence of the following genes: *icaA*, *icaD*, *hla*, *sirB* and some MSCRAMM genes (*ebpS*, *fnbA*, *clfA*, *sdr* and *cna*). **Table 1** lists all primers utilised in this study. A reaction mixture of 25 µl total volume comprising 12.5 µl 2X MyTaq HS Red Mix, 0.5 µl DNA extract and ten picomoles each of the primers, was used for PCR. The DNA thermal cycler (Tpersonal Thermocycler biometra, Applied Biosystem (USA) was utilized for DNA amplification. For separation of PCR products, 2 percent agarose gel was employed in TBE buffer. Gels were run for 1 hour at 100 V, stained in 2 µg/ml ethidium bromide and visualization was performed under UV transilluminator (BIORAD, Italy).

Detection of antimicrobial activity of nifedipine and its effect on levofloxacin

Stock solutions of both levofloxacin and nifedipine were prepared by dissolving 400 mg of each of levofloxacin and nifedipine in 100 ml sterile distilled water and 100 ml slightly acidified sterile distilled water respectively. The experiment was done in checkerboard arrangement as previously mentioned [17] using both levofloxacin and

nifedipine in 2 fold serial dilutions to determine MIC alone and in combination.

The plates were covered and incubated at 35- 37°C for 14 hours.

The MIC was considered as the least concentration showing no turbidity and was calculated for levofloxacin alone and in combination with nifedipine [17]

Determination of antibiofilm activity nifedipine alone and in combination with levofloxacin antibiotic

The same checkerboard plate pattern above was used, where after 24 hrs of incubation the plates were emptied washed 3 times with saline and fixed with 200 µl of 99 % methanol, wells were emptied, left to air dry and the formed biofilms mass were detected by adding 200 µl 2%crystal violet for 5 min then plates were washed and dried. Each well was eluted before reading by 160 µl 33% glacial acetic acid and OD was measured at 630nm using ELISA reader [18].

Preliminary molecular docking

We have used mcule.com online platform to perform a preliminary survey of possible target protein. For our screening, we used free default database. Nifedipine structure was obtained from chemweb and tested against a set of *S. aureus* target proteins present on the site.

Statistical analysis

Data were supplied to the computer and analysed with the version 20.0 edition of IBM SPSS programme (Armonk, NY: IBM Corp). Qualitative data with number and percentage were described. Significance of the results was assessed at the 5% level by using the chi-square test and Fisher's Exact or Monte Carlo correction.

Spa typing

The variable repeat region of *spa* gene in *S. aureus* strains was amplified by PCR primers as described before [19]. After purification and sequencing of the PCR products, *spa* database website (<http://www.ridom.de/spaserver>) was utilized to designate the sequences to specific spa types.

Results

Identification of MRSA strains and antibiotic sensitivity

The fifty collected *S. aureus* isolates were all catalase and coagulase positive, while 98% and 80% were hemolysin and protease producers, respectively. The isolates were obtained as swabs from post-surgical wounds. The samples were phenotypically recognized as MRSA isolates by both the ceftioxin and oxacillin disc-diffusion test.

Rifampicin, vancomycin and linezolid were the most active drugs while beta lactams were the least effective antimicrobial agents against tested isolates of *S. aureus*. Ten (20%) and 12 isolates (24%) were resistant to clindamycin and erythromycin, respectively. The resistance rate to ciprofloxacin (42%) and doxycycline (44%) was less than 50%, where it was above 80% against cotrimoxazole (82%) and gentamicin (88%). Surprisingly, the 50 MRSA isolates were all MDR.

Analysis of biofilm formation

Phenotypic biofilm formation was evaluated by the microtiter plate test. All MRSA isolates tested were found to be biofilm producers with varying degrees: 8 (16%) and 40 (80%) isolates were strong biofilm and moderate producers, respectively, while only 2 strains (4%) were defined as weak producers.

Polymerase chain reaction assay of MSCRAMM, biofilm-related and other virulence genes

Out of the 50 studied strains of MRSA, 44 (88%) and 42 (84%) possessed the *icaA* gene and *icaD* gene, respectively. All *icaA*-positive strains harbored the *icaD* gene except two isolates which were *icaD*-negative. The prevalence of *clfA*, *fnbA*, *sdrE*, *ebpS* and *cna* genes were 12, 44, 60, 84 and 28%, respectively. Forty-eight (96%) and 44 (88%) isolates possessed *hla* and *sirB* genes, respectively (Figure 1 A-G).

The co-incidence of the examined virulence genes was explored among the 50 MRSA clinical isolates. Two isolates had only the *hla* gene. The other 48 strains possessed at least two virulence genes. The most prevalent pattern was found among 8 isolates (16%) which harbored 6 genes (*icaA*, *icaD*, *sdrE*, *ebpS*, *sirB* and *hla* genes), followed by another one containing 7 genes (*icaA*, *icaD*, *sdrE*, *fnbA*, *ebpS*, *sirB*, *hla*) among 6 isolates. Only two (4%) of tested strains all the genes investigated (Table 2).

All tested genes were detected in weak, moderate and strong biofilm producers, with the exception of *clfA* which was found only in moderate biofilm producers and *fnbA* and *sdrE* which were absent in weak biofilm producing strains.

Detection of antimicrobial activity of nifedipine and its effect on levofloxacin

The Ca²⁺ channel blocker nifedipine was assayed against the isolates where, it was found that its minimum inhibitory concentration (MIC) alone was 125 ug/ml.

When nifedipine was combined with different concentration of levofloxacin antibiotic a significant decrease in the MIC of levofloxacin by the increasing concentration of nifedipine between 7.8 ug/ml and 500 ug/ml was detected with a plateau at above 125 ug/ml (MIC of nifedipine) (Figure 2).

Determination of antibiofilm activity nifedipine alone and combined with levofloxacin antibiotic

When prevention of biofilm formation was detected a drastic decrease in the biofilm mass was observed by increasing concentrations of Ca channel blocker under test as shown in figure (3A).

When the nifedipine was combined with the levofloxacin an additive effect was noticed as it assisted the ability of the antibiotic to prevent adhesion and/or biofilm mass growth of the bacteria under test where A, B, C, D, E and F are lines showing the different concentrations of nifedipine (Figure 3B).

Preliminary molecular docking

Molecular analysis using Mcdue database showed that nifedipine had a binding affinity to a translational regulator protein responsible of efflux activity namely HTH-type transcriptional regulator *qacR* with a value -8.4 (Figure 4).

Statistical analysis

Statistical analysis revealed that both *icaA* and *icaD* genes have shown a significant relationship to phenotypic biofilm synthesis. Interestingly, they were more associated to moderate and weak biofilm producers than strong biofilm producing MRSA isolates. In addition, a significant correlation was observed between *sdrE* genes and strong and moderate biofilm producing MRSA strains. Appealingly, *sirB* was also significantly associated with biofilm production, especially moderate and weak biofilm producers (Table 3).

Spa typing

Spa typing was performed in randomly selected 12 MRSA isolates. Three *spa* types were identified; *spa* type t127, t134 and t223. *Spa*-type t127 was found to be the most common as it was assigned to 8 (66.6%) isolates, while both *spa* types t134 and t223 were equally distributed with prevalence of 16.7% for each one. All types had different biofilm-related genes combination but *icaA*, *icaD*, *sirB* and *hla* genes were all present among the typable isolates.

Figure 1A-G. A Ethidium bromide-stained agarose gel showing the band of amplified PCR product 767 bp *sdrE* gene at lane 4. L: DNA ladder, Lane 1-3 are *sdrE* negative, **B** Lane L, 100-bp DNA ladder; lanes 1-5, the 209-bp PCR product of *hla* gene, **C** Lane M, 50-bp DNA ladder; lanes 1-5, the 198-bp PCR product of *icaD*, **D** The electrophoresis result of amplified multiplex PCR product of *icaA*, *fnbpA* and *ebpS* genes at 770 bp, 1362 bp and 526 bp, respectively. Lane (L): marked 100-bp DNA ladder, **E** Lane L, 50-bp DNA ladder; lanes 2 and 4, the 399-bp PCR product of *sirB*, **F** Ethidium bromide-stained agarose gel showing the band of amplified PCR product 1722 bp *cna* gene at lane 3. L: DNA ladder, Lane 1 and 2 are *cna* negative, **G** Lane L, 100-bp DNA ladder; lanes 1-3, the 1584-bp PCR product of *clfA* gene,

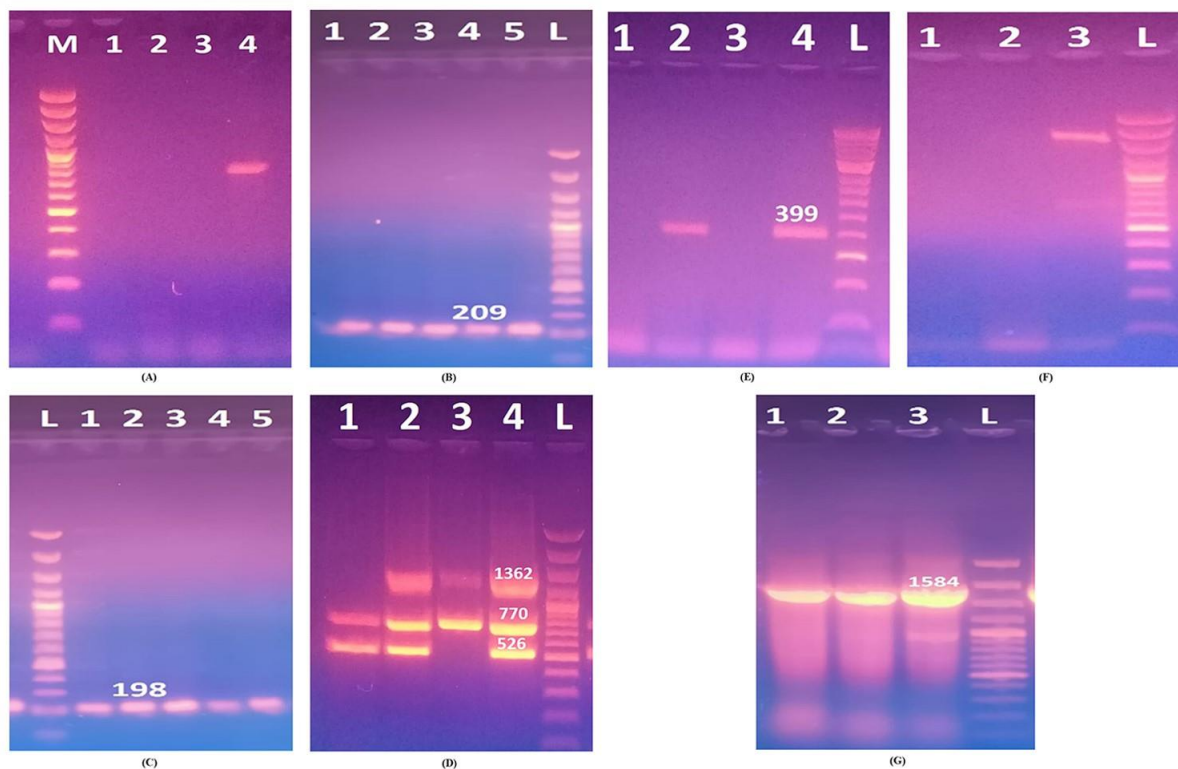


Figure 2. Effect of increasing the concentration of nifedipine on MIC of levofloxacin against *S. aureus* isolates under test.

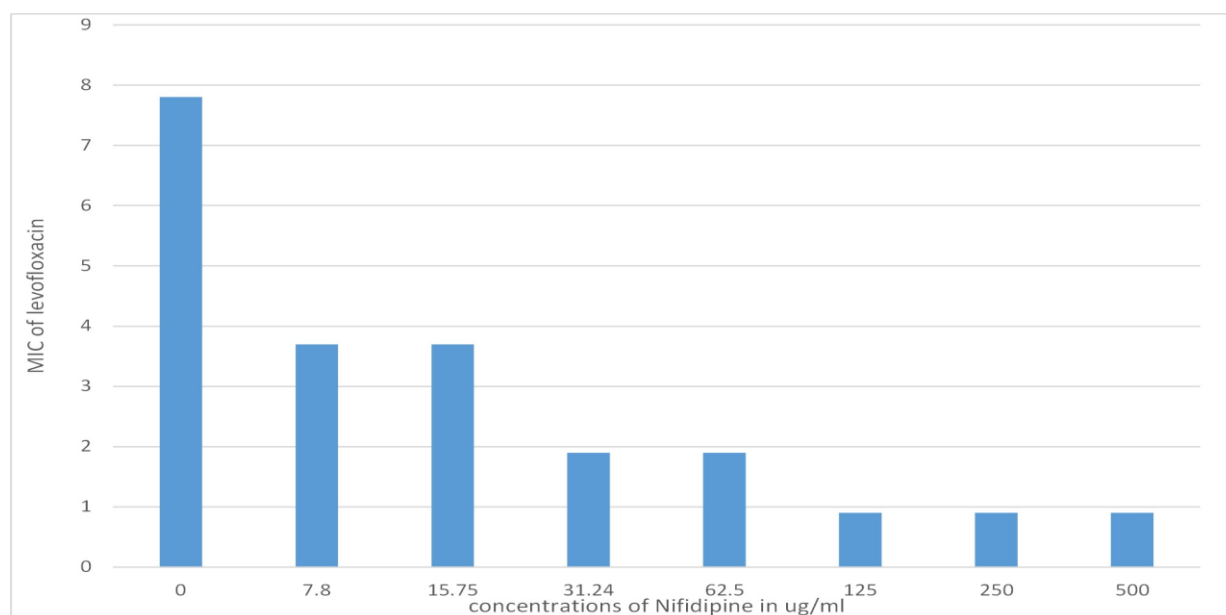
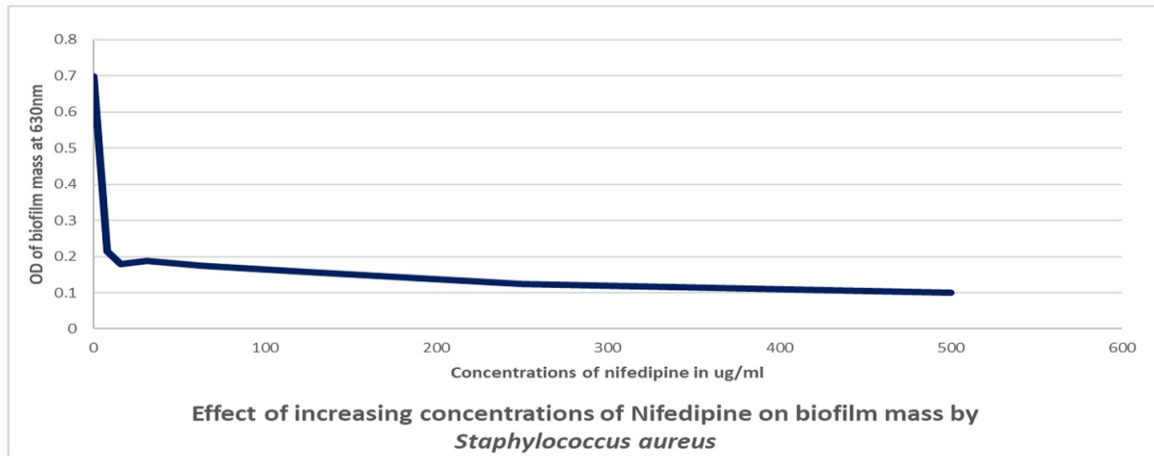
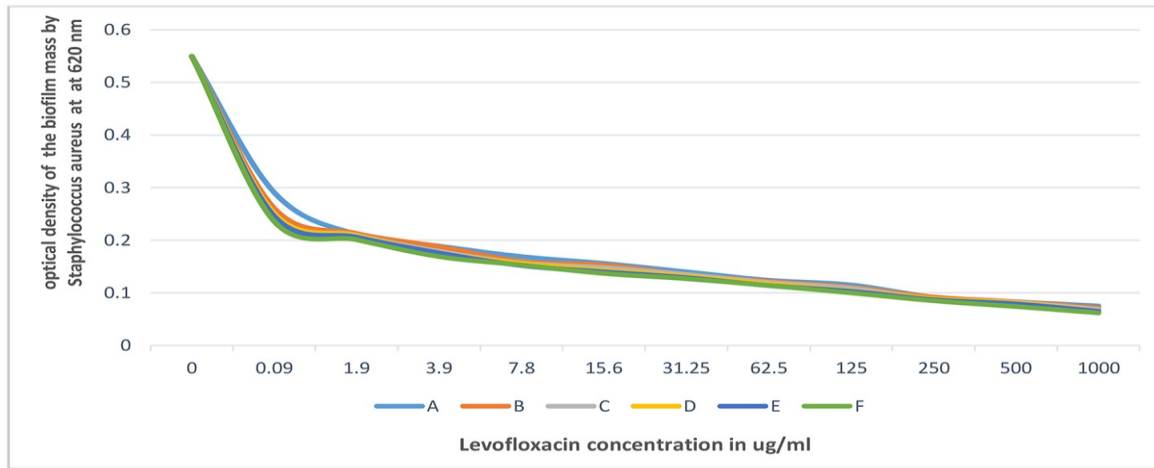


Figure 3. A Effect of nifedipine increasing concentrations on biofilm formed mass by *S. aureus* **B** Effect of combination of nifedipine and levofloxacin on biofilm forming capacity of *S. aureus*.



(A)



(B)

Figure 4. Pose for binding of nifedipine to the HTH-type transcriptional regulator qacR.

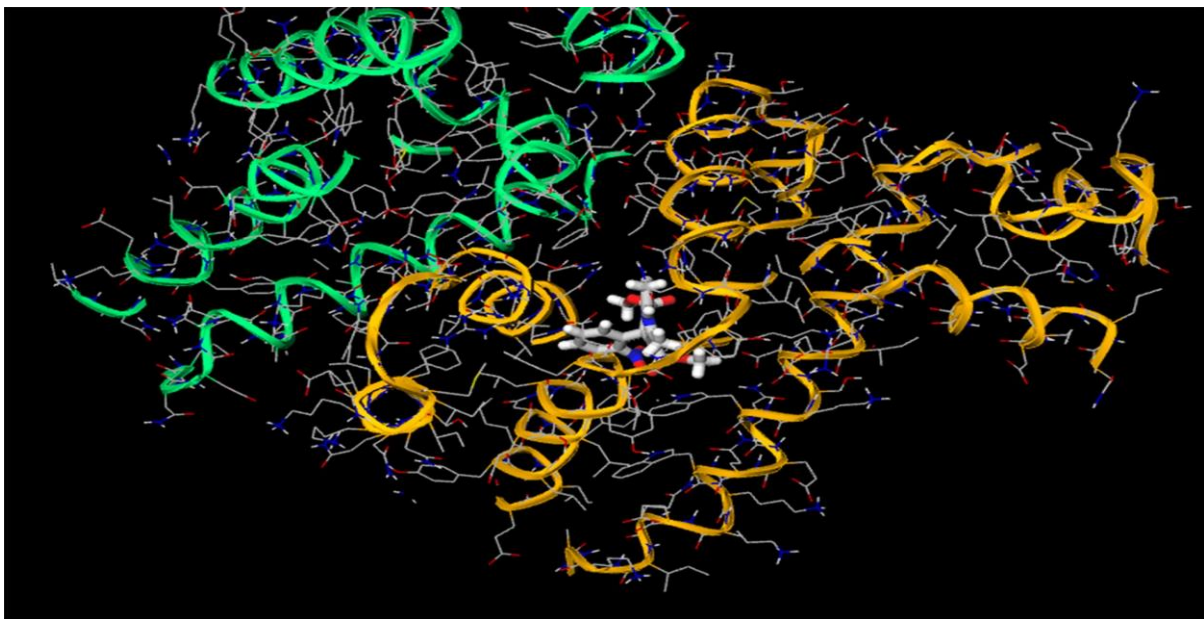


Table 1. Primers used and the annealing temperatures for the amplified genes.

Primer	Primers sequence (5'-3')	Band Size	Annealing Temperature	Reference
<i>clfA-F</i>	GTAGGTACGTTAATCGGTT	1584	45	[7]
<i>clfA-R</i>	CTCATCAGGTTGTTTCAGG			
<i>cna-F</i>	AGTGGTTACTAATCATG	1722	45	[7]
<i>cna-F</i>	CAGGATAGATTGGTTTA			
<i>ebpS-F</i>	CAATCGATAGACACAAATTC	526	50	[7]
<i>ebpS-R</i>	CAGTTACATCATCATGTTTA			
<i>fnbA-F</i>	CACAACCAGCAAATATAG	1362	50	[7]
<i>fnbA-R</i>	CTGTGTGGTAATCAATGTC			
<i>hla-F</i>	CTGATTACTATCCAAGAAATTCGATTG	209	55	[7]
<i>hla-R</i>	CTTCCAGCCTACTTTTTTATCAGT			
<i>icaA-F</i>	GATTATGTAATGTGCTTGGA	770	50	[67]
<i>icaA-R</i>	ACTACTGCTGCGTTAATAAT			
<i>icaD-F</i>	ATGGTCAAGCCCAGACAGAG	198	55	[26]
<i>icaD-R</i>	CGTGTTTTCAACATTTAATGCAA			
<i>sdrE-F</i>	CAGTAAATGTGTCAAAGA	767	45	[67]
<i>sdrE-R</i>	TTGACTACCAGCTATATC			
<i>sirB-F</i>	CAGCTACGGCTACCGAAATA	399	61	[7]
<i>sirB-R</i>	CATTTTTGGGGCTATTGTTGT			

Table 2. Percentage of coexistence of genes under test among tested isolates.

Coexistence of tested genes	Percentage (%)
<i>hla</i>	4
<i>ebpS, hla</i>	4
<i>ebpS, sirB, hla</i>	4
<i>icaA, sdrE, ebpS, sirB</i>	4
<i>icaA, icaD, ebpS, hla</i>	4
<i>icaA, icaD, ebpS, sirB, hla</i>	4
<i>icaA, icaD, ebpS, sirB, hla</i>	4
<i>icaA, icaD, fnbA, can, sirB, hla</i>	4
<i>icaA, icaD, sdrE, can, sirB, hla</i>	4
<i>icaA, icaD, ebpS, can, sirB, hla</i>	4
<i>icaA, icaD, sdrE, ebpS, sirB, hla</i>	16
<i>icaA, icaD, sdrE, ebpS, sirB, hla</i>	4
<i>icaA, icaD, fnbA, ebpS, sirB, hla</i>	4
<i>icaA, icaD, sdrE, fnbA, ebpS, sirB, hla</i>	12
<i>icaA, icaD, sdrE, ebpS, can, sirB, hla</i>	4
<i>icaA, icaD, fnbA, ebpS, can, sirB, hla</i>	4
<i>icaA, icaD, sdrE, fnbA, ebpS, clfA, sirB, hla</i>	8
<i>icaA, icaD, sdrE, fnbA, ebpS, can, sirB, hla</i>	4
<i>icaA, icaD, sdrE, fnbA, ebpS, can, clfA, sirB, hla</i>	4

Table 3. Relation between biofilm producer and tested biofilm-related genes.

Biofilm producer	N	<i>icaA</i>		<i>icaD</i>		<i>sdrE</i>		<i>hla</i>		<i>ebpS</i>		<i>sirB</i>		<i>fnbA</i>		<i>cna</i>		<i>clfA</i>	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Strong	8	4	50.0	4	9.5	2	6.7	8	100.0	6	75.0	4	50.0	2	25.0	2	25.0	0	0.0
Moderate	40	38	95.0	36	85.7	28	93.3	38	95.0	34	85.0	38	95.0	20	50.0	10	25.0	6	15.0
Weak	2	2	100.0	2	4.8	0	0.0	2	100.0	2	100.0	2	100.0	0	0.0	2	100.0	0	0.0
χ^2		9.618*		6.648*		8.045*		1.197		1.006		9.618*		2.847		4.331		1.112	
MC p		0.006*		0.041*		0.013*		1.000		0.717		0.006*		0.220		0.123		0.668	

 χ^2 : Chi square test

MC: Monte Carlo

p: p value for comparing between different categories

*: Statistically significant at $p \leq 0.05$

Discussion

Staphylococcus aureus, a prevalent pathogen linked with severe infections, is a common and widespread pathogen in human. MRSA has developed as a significant human pathogen showing multidrug resistance and therefore posing a worldwide concern. The frequency of methicillin resistance has risen dramatically in recent years [20]. It seems that indiscriminate use of antibiotics in addition to the health care personnel's lack of awareness have contributed to the high prevalence of MRSA.

In this study the resistance to ciprofloxacin, erythromycin and gentamicin was 42, 22 and 88%, respectively. Similar results were presented in another study [21]. As a result of the misuse and overuse of certain antibiotics, resistance to them appears to be widespread and the use of these antibiotics appears to be ineffective. For this reason, preventing treatment failures requires suitable measures.

Like our result, Azmi et al. [21] revealed that all isolates tested showed decreased sensitivity to nitrofurantoin, linezolid and vancomycin. According to several researches, vancomycin is the most efficient antibiotic against MRSA, yet reduced susceptibility to this antibiotic has been noticed in different reports [22]. Vancomycin and other glycopeptides still are the last line of defense against *S. aureus* infections. The results of the current study revealed that all the biofilm producing strains were susceptible to vancomycin. The results provided in this study are consistent with susceptibility rates in several countries [23].

Biofilm formation by *S. aureus* has been recognized as the most effective way of defense against host immune responses. In addition to allowing bacteria to colonize host tissues, it also prevents antimicrobial chemicals and host immune responses from clearing the bacteria, resulting into

increased morbidity and death rates due to abscess spread [24].

The biofilm-forming capacity among strains of MRSA isolated from post-operative wound infections in MRSA-infected patients has been examined, in conjunction with their clinical molecular biological characteristics, and its relationship to antibiotics has been determined. Variations in antibiotic resistance patterns were observed among MRSA strains.

The biofilm has a protective role for the bacteria growing in it and makes it inherently resistant to a wide variety of antibiotics. In this study, all MRSA isolates were MDR. This is in contrast to numerous studies, whereas lower rates of MDR were shown [25, 26]. Multidrug resistance is thought to be caused by intimate cell contact in the biofilm, which facilitates the transmission of plasmids harboring MDR genes, limiting treatment choices and burdening the healthcare system economically and socially.

Biofilm is an ideal medium for the transfer of resistance plasmids [27] leading to dramatic increase in antibiotic resistance among bacteria growing in biofilm [28]. The explanation for this may be difficulty of antibiotic to penetrate the biofilm layer and the existence of antibiotic breakdown mechanisms. Besides, biofilm production provides a horizontal gene transfer platform among bacteria, resulting in an increase in the bacterial virulence and drug resistance.

In the current research, biofilm formation was evaluated by using the microtiter plate method, and it was noticed that about all *S. aureus* isolates produced biofilms with variable biomass; out of which 8 isolates (16%) were categorized as "Strong biofilm producers". A study in Egypt revealed that 69.8% of *S. aureus* clinical isolates were biofilm producers; however, in opposition to our results, most (16/43, 37.2%) of the samples demonstrated weak biofilm production [29].

Different interpretations of findings might explain the inconsistencies in the categorization of biofilm phenotypes. As a result, standardization of biofilm development methodologies and interpretation is essential.

In addition to the *icaA/icaD* genes, the incidence of six chosen genes implicated in biofilm development was determined in order to improve the understanding of the molecular process of biofilm production by MRSA strains.

Numerous studies have demonstrated the importance of the *ica* locus in biofilm formation [26, 30]. Because the *icaA* and *icaD* genes are involved in the production of PIA, the *ica* locus might be used as therapeutic target for treatment of implant-associated *S. aureus* infections.

In this study, *icaA* and *icaD* genes were detected among 88% and 84% of MRSA isolates, respectively, among which the *icaA* gene was the most frequent (49, 63.6%). Our data are similar to that of **Gowishankar et al.** who observed the *ica* genes in 84.13% of *S. aureus* isolates in India [31], while **Avila-Novoa** detected the genes among 52.3% of isolates in Brazil [32].

In several studies the genotypes and phenotypes of *S. aureus* strains were found to be completely congruent in certain investigations, where all investigated strains harboring *icaD/icaA* were biofilm producers, showing that *S. aureus* strains lacking the *icaD* gene are incapable of forming biofilms [21, 33].

Interestingly, the distribution of *icaA* genes in strong biofilm producers was less than in weak and moderate biofilm producing MRSA. In contrast to our finding, another study showed no variation in *ica* genes distribution in strongly and weakly virulent strains [5,34]. The PIA is involved in intercellular adhesion and multilayer biofilm formation. In our study all strains which expressed *icaA/D* genes were biofilm producers but with different biofilm mass. The bacteria may potentially have formed biofilms via alternative mechanisms, such as fibronectin binding proteins. According to other published research, certain strains do not form biofilm despite the presence of the *ica* locus [35]. In this study, six (12%) and eight (16%) isolates that produced biofilms phenotypically lacked the *icaA* and *icaD* gene, respectively, which may be a result of point mutation. As has recently been discovered, many strains of MRSA do not require the presence of PIA to produce biofilm [36]. Among MRSA strains, as well as among other *S. aureus* genotypes, a growing number of adhesion molecules have been identified to affect biofilm production.

In this study, a significant correlation was shown between *icaAD* gene detection and biofilm production, where 100 percent of the strains carrying *icaD* produced biofilms, which is in accordance with Liberto et al findings [37]. Like our results, **Rohde et al.** [34] found that there was no variation in the distribution of the *icaD* gene in variable virulent strains. In our study, we found a biofilm-forming potential of all MRSA isolates, indicating that hospital conditions, particularly post-surgical wounds, may be more conducive to biofilm formation.

Our data indicated that all MRSA isolates were capable of forming biofilms, indicating that hospital conditions, especially surgical wounds, may be more conducive to biofilm development. The existence of *ica* genes may explain the function of different adherence mechanisms in the pathogenesis of infection. However, in certain investigations biofilm development was not necessarily associated with the identification of *icaA/icaD* genes [38].

There are several proteins of MSCRAMMs-family expressed on the surface of the MRSA strains that particularly identify the host's extracellular matrix components and bind to it. The MSCRAMM proteins, which are encoded by various genes, are an essential category of virulence factors that initiate these processes. In this study, 5 MSCRAMM genes were examined in all 50 *S. aureus* strains. Based on the findings, *cna*, *clfA*, *fnbA*, *ebp*, and *sdrE* genes were found in 28%, 12%, 44%, 84% and 60%, respectively. Several studies have examined the prevalence of these genes, with varying results [39, 40]. In certain cases, disputes have arisen owing to differences in *S. aureus* genetic composition and gene regulator systems, environmental circumstances, or isolate type (animal and human).

Although the incidence of biofilm-encoding genes is not necessarily correlated with biofilm synthesis, numerous studies have identified several variables which contribute to biofilm formation and its progression in *S. aureus* isolates, including surface adhesion properties [41]. In the present study, among the different tested genes encoding MSCRAMM proteins, *sdrE* was significantly associated with biofilm production.

It was revealed in a previous study that alpha-toxin, expressed by *hla* gene, stimulates biofilm development in *S. aureus* [42]. A recent study showed that neutralizing alpha-toxin enhances healing of *S. aureus*-infected wounds [43]. Therefore, the prevalence of *hla* gene among was examined in the current study and the gene was found in 96% of the isolates. Similarly, Yu and

colleagues [44] demonstrated that 95.3% of the isolates possessed the gene.

Concerning biofilm formation, it was shown that iron can be implicated in biofilm formation [45]. Moreover, it was revealed that using certain iron chelators had antibiofilm activity [46]. In this regard, it was thought that *sirB* gene could have an indirect effect on biofilm forming capacity of the organism. Interestingly, the gene was observed in 88% of the isolates in the present study and it was significantly correlated to biofilm formation.

The coexistence of biofilm-related genes was studied in this study, where 4% of the strains harbored all examined genes. Relatively similar investigations were shown in other studies [21, 47]. The most prevalent combination of biofilm genes was that of *icaA*, *icaD*, *sdrE*, *ebpS*, *sirB* and *hla* genes. The incidence of such gene combination may give the strains a selective advantage, such as better ability for adhesion and colonization of the host.

It was shown by different research that *spa* typing is both faster and easier to execute and understand than other molecular methods. *Spa* typing appears to be very reproducible, and the resultant sequences may be examined using a commercially available software programme. Consequently, *spa* typing appears to be an attractive choice for infection-control [48].

In the present study, *spa* typing was performed in 12 randomly selected MRSA isolates. Three *spa* types were identified; *spa* type t127 (66.6%), t134 (16.7%) and t223 (16.7%). *Spa* type t127 and t223 have been recognized in recent studies in Egypt [49, 50]. **Abou Shady et al.** [49] detected *spa* type t223 in 10% of the isolates, where in the study of **Alsequey et al.** [50] the prevalence of *spa* type t127 and t223 was 12.5% and 4%, respectively.

Notably, *spa* type t314 is the first to be reported in Egypt with prevalence of 16.7%. Recently, this type has been also first recognized in Iran [51]. It is worth to mention that *spa* type t314 was detected in several studies in Kuwait [52, 53]. Apart from the Gulf region, many reports have revealed *spa* t314 with variable prevalence rates [54, 55].

The nifedipine was chosen as a repurposed drug in this study as the isolates were wound isolated biofilm forming *S.aureus*. Our results showed that nifedipine was active against staphylococcal wound isolates with MIC ranging between 62.5 and 250 ug/ml under test conditions.

In addition, increasing concentration of the nifedipine alone led a great decrease in biofilm mass as shown in figure (concentrations bet 7.8 and 500

ug/ml) drug also assisted the power of the antibiotic in preventing biofilm formation at different concentrations.

The results of the antimicrobial activity of nifedipine were in accordance with **Pal et al.** [56] in 2006 that stated the presence of antimicrobial activity attributed to the nifedipine against both Gram positive and Gram negative bacteria with an MIC ranging between 25–200µg/ml against most tested bacteria.

In addition, in the current study, it was shown that the drug affected the MIC of the levofloxacin at different concentrations causing a 6-fold decrease in MIC of the antibiotic 3.9 to less than 0.9 ug/ml. This is in accordance with another study where nifedipine and amlodipine showed synergistic effect with different antibiotic classes against *E. coli* and *S. aureus*, respectively [57], amlodipine and bepridil were synergic with levofloxacin against pseudomonas biofilm [58].

Such activity was noticed as nifedipine increases host resistance to intracellular microorganisms by limiting the availability of iron, when tested against *Salmonella typhi* serovar muruim [59] and inhibits *E coli* chemotaxis [60].

Combining these ion channel blockers with tuberculosis chemotherapy due to their antimicrobial effect and enhancing macrophage killing activity may improve anti-mycobacterial killing, avoid resistance and decrease the time of treatment, thereby offering a new approach for tuberculosis treatment [61].

Different studies have been carried out to investigate the mechanism of activity of calcium channel blockers showing that fluconazole plus amlodipine caused down-regulating of CNA1, CNB1 (encoding calcineurin) and YVC1 (encoding calcium channel protein in vacuole membrane) [62]. It was also found that_nifedipine improves iron extrusion from the cytoplasm by enhancing ferroportin 1 (Fp1) expression, therefore decreasing tissue colonization and death due to in vivo infections [63].

The drug in the current study had an effect on the antimicrobial activity of levofloxacin (synergism). On the contrary to that stated by Elkhatib in 2013 that nifedipine and nicardipine had no effect on levofloxacin MIC [64]. On the other hand, **Asok et al.** [65] detected synergism between streptomycin and amlodipine when combined against bacterial isolates. An asset for the use of the nifedipine in wound infections is its ability to accelerate and promote healing in wounds of different origin [66].

Remarkably, Preliminary Molecular docking was performed and showed that the drug had a binding affinity to a translational regulator protein responsible of efflux activity namely HTH - type transcriptional regulator *qacR*. This finding could explain the synergistic effect between nifedipine and levofloxacin in this study.

Conclusion

The present study showed that biofilm forming capacity of MRSA isolates obtained from hospitalized patients with post-surgical wounds was variable, and assisted by presence of an array of virulence factors and genes. The antimicrobial and antibiofilm capacity of nifedipine, either alone or combined with levofloxacin, was promising, especially if formulate into a local gel or cream to be put on incisions as a prophylaxis against infections specially that the drug is reported as a wound healing enhancer. Among the three *spa* types of MRSA isolates *spa* type t314 was reported for the first time in Egypt.

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