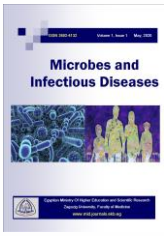
Microbes and Infectious Diseases 2022; 3(1): 112-127



Microbes and Infectious Diseases

Journal homepage: https://mid.journals.ekb.eg/

Original article

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..

ARTICLE INFO

Article history:
Received 14 September 2021
Received in revised form 31 October 2021
Accepted 1 November 2021

Keywords:
MRSA
Repurposing
Nifedipine
Antibiofilm
spa typing

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 form 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 levoflocaxin, 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

DOI: 10.21608/MID.2021.101063.1203
* *Corresponding author:* Ghada Hani Ali
E-mail address: ghada.nassef@pua.edu.eg

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, Bood 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 ≥22mm and ≤21mm, 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 37ºC. The culture was diluted to a concentration of 10^6 CFU/ml, 200 ul 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 compromising 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 cefoxitin 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, repectively. All *ica*A-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 Ca^{2+} 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 Mcule 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*,

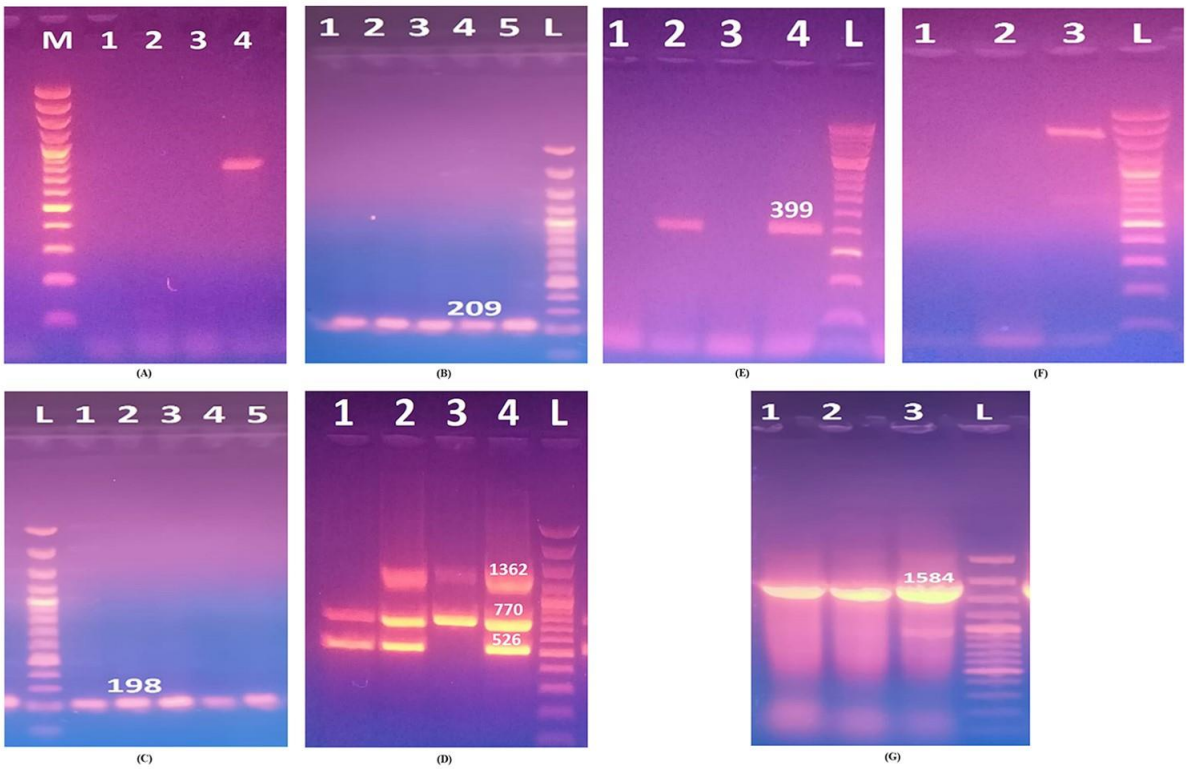


Figure 2. Effect of increasing the concentration of nifedipine on MIC of levofloxacin against *S. aureus* isolates under test.

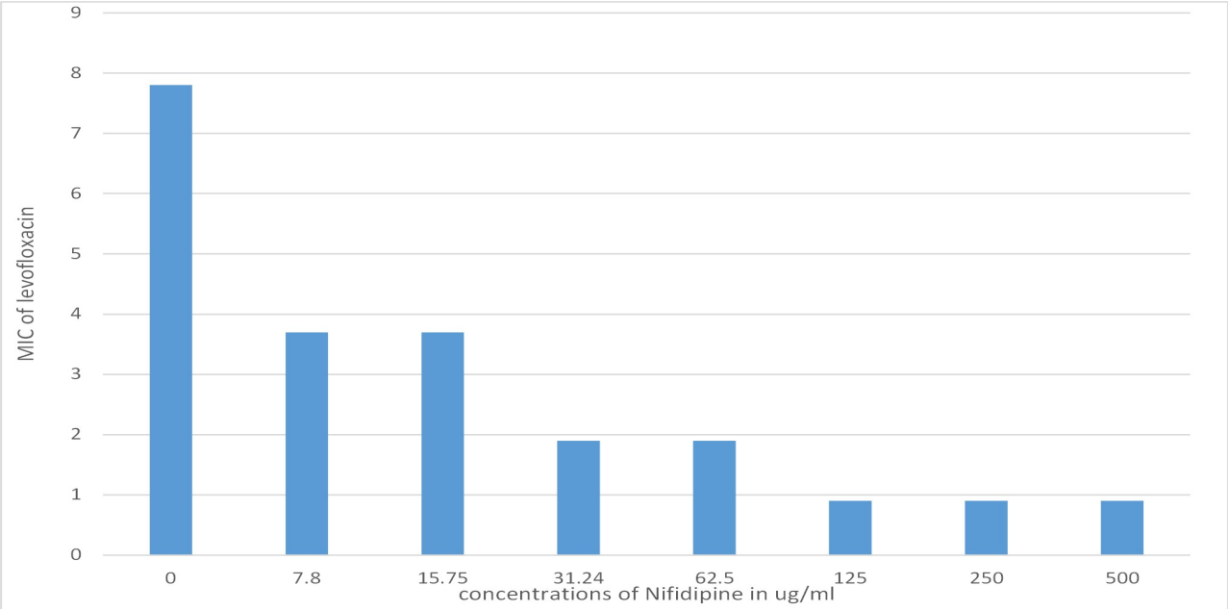
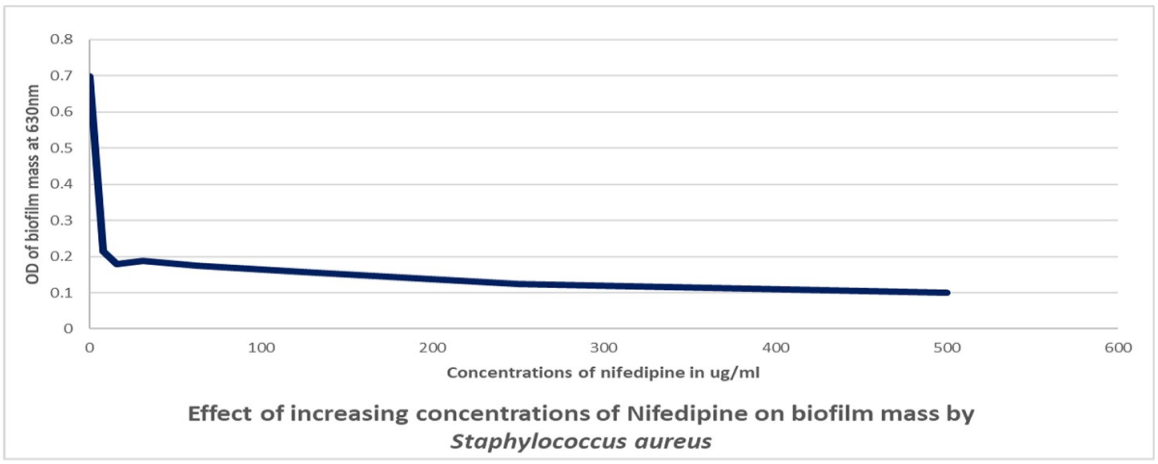
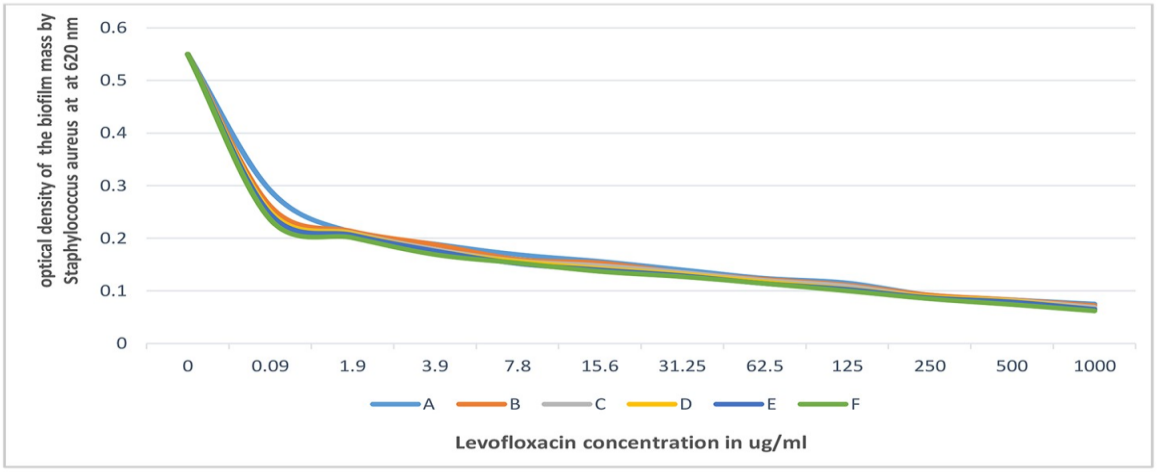


Figure 3. A Effect of nifedipine increasing concentrations on biofilm formed mass by *S. aureus* **B** Effect of combination of nifedipine and levofloxacin on biofilm forming capacity of *S. aureus.*



(A)



(B)

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

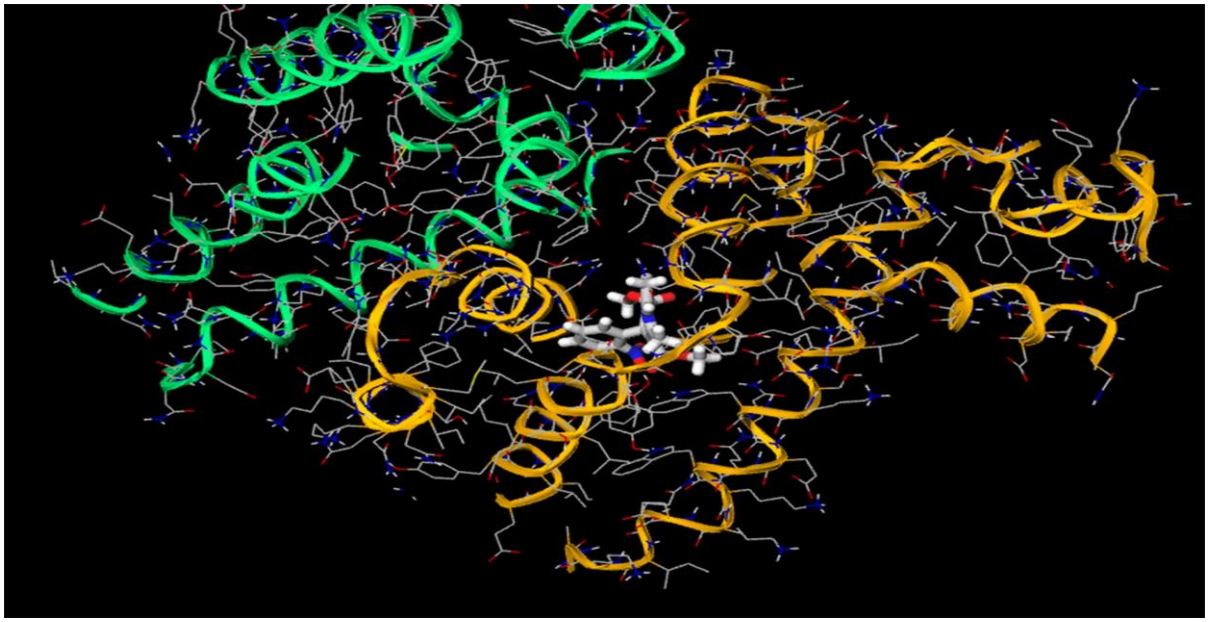


Table 1. Primers used and the annealing temperatures for the amplified genes.

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

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

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

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

Biofilm producer	N	*icaA*		*icaD*		*sdrE*		*hla*		*ebpS*		*sirB*		*fnbA*		*cna*		*clfA*	
		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

Stapylococcus 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 *ica*AD gene detection and biofilm production, where 100 percent of the strains carrying *ica*D 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 **Alseqely 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 Golf 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 levoflocaxin, 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.

Acknowledgment

The authors express their gratitude to the drug research center in Faculty of pharmacy, Pharos University in Alexandria, for their continuous support.

Conflict of interest: None.

Financial disclosure: None.

References

1-**Lacey KA, Geoghegan JA, McLoughlin RM.** The Role of *Staphylococcus aureus* Virulence Factors in Skin Infection and Their Potential as Vaccine Antigens. Pathogens 2016; 5(1):22.

2-**Rahimi F.** Characterization of Resistance to Aminoglycosides in Methicillin-Resistant *Staphylococcus aureus* Strains Isolated From a Tertiary Care Hospital in Tehran, Iran. Jundishapur journal of microbiology 2016; 9(1):e29237.

3-**World Health Organization [WHO].** Global guidelines for the prevention of surgical site infection. 2nd ed. Geneva, Switzerland: WHO; 2018.

4-**Lister JL, Horswill AR.** *Staphylococcus aureus* biofilms: recent developments in biofilm dispersal. Frontiers in cellular and infection microbiology 2014; 4:178.

5-**Rohde H, Burandt EC, Siemssen N, Frommelt L, Burdelski C, Wurster S, et al.** Polysaccharide intercellular adhesin or protein factors in biofilm accumulation of Staphylococcus epidermidis and *Staphylococcus aureus* isolated from prosthetic hip and knee joint infections. Biomaterials 2007; 28(9):1711-20.

6-**Seo YS, Lee DY, Rayamahji N, Kang ML, Yoo HS.** Biofilm-forming associated genotypic and phenotypic characteristics of Staphylococcus spp. isolated from animals and air. Research in veterinary science 2008; 85(3):433-8.

7-**Zhang L, Gao J, Barkema HW, Ali T, Liu G, Deng Y, et al.** Virulence gene profiles: alpha-hemolysin and clonal diversity in *Staphylococcus aureus* isolates from bovine clinical mastitis in China. BMC veterinary research 2018; 14(1):63.

8-**Nasaj M, Saeidi Z, Asghari B, Roshanaei G, Arabestani MR.** Identification of hemolysin encoding genes and their association with antimicrobial resistance pattern among clinical isolates of coagulase-negative Staphylococci. BMC research notes 2020; 13(1):68.

9-**Delgado S, García P, Fernández L, Jiménez E, Rodríguez-Baños M, del Campo R, et al.** Characterization of *Staphylococcus aureus* strains involved in human and bovine mastitis. FEMS immunology and medical microbiology 2011; 62(2):225-35.

10-**Shakeri F, Shojai A, Golalipour M, Rahimi Alang S, Vaez H, Ghaemi EA.** Spa Diversity among MRSA and MSSA Strains of *Staphylococcus aureus* in North of Iran. International journal of microbiology 2010; 2010.

11-**Truong M, Monahan LG, Carter DA, Charles IG.** Repurposing drugs to fast-track

therapeutic agents for the treatment of cryptococcosis. PeerJ 2018; 6:e4761.

12-**Cheesbrough M.** District Laboratory Practice in Tropical Countries. New York: Cambridge University Press; 2006.

13-**Bauer AW, Kirby WM, Sherris JC, Turck M.** Antibiotic susceptibility testing by a standardized single disk method. Technical bulletin of the Registry of Medical Technologists American Society of Clinical Pathologists Registry of Medical Technologists 1966; 36(3):49-52.

14-**Patel JB, Cockerill F, Bradford PA.** Performance standards for antimicrobial susceptibility testing: twenty-fifth informational supplement. Clinical & Laboratory Standards Institute 2015; 35(3):29-50.

15-**Zmantar T, Kouidhi B, Miladi H, Mahdouani K, Bakhrouf A**. A microtiter plate assay for *Staphylococcus aureus* biofilm quantification at various pH levels and hydrogen peroxide supplementation. The new microbiologica 2010; 33(2):137-45.

16-**Oufrid S, Ghazlane Z, Jamali L, El Otmani F, Talmi M, Elmdaghri N, et al.** Correlation between staphylococcal biofilm formation in vitro and potential for catheter-related infections. Journal of infection in developing countries 2015; 9(4):368-72.

17-**Pillai K, Moellering R, Eliopoulos G.** Antimicrobial combinations. In: Lorian V, ed. Antibiotics in laboratory medicine. 5th ed. Philadelphia: Lippincott Williams & Wilkins; 2005: 365–440.

18-**Abdi-Ali A, Mohammadi-Mehr M, Agha Alaei Y.** Bactericidal activity of various antibiotics against biofilm-producing *Pseudomonas aeruginosa*. International journal of antimicrobial agents 2006; 27(3):196-200.

19-**Adame-Gómez R, Castro-Alarcón N, Vences-Velázquez A, Toribio-Jiménez J, Pérez-Valdespino A, Leyva-Vázquez MA, et al.** Genetic Diversity and Virulence Factors of S. aureus Isolated from Food, Humans, and Animals. International journal of microbiology 2020; 2020:1048097.

20-**Yousefi M, Pourmand MR, Fallah F, Hashemi A, Mashhadi R, Nazari-Alam A.** Characterization of *Staphylococcus aureus* Biofilm Formation in Urinary Tract Infection. Iranian journal of public health 2016; 45(4):485-93.

21-**Azmi K, Qrei W, Abdeen Z.** Screening of genes encoding adhesion factors and biofilm production in methicillin resistant strains of *Staphylococcus aureus* isolated from Palestinian patients. BMC genomics 2019; 20(1):578.

22-**Shekarabi M, Hajikhani B, Salimi Chirani A, Fazeli M, Goudarzi M.** Molecular characterization of vancomycin-resistant *Staphylococcus aureus* strains isolated from clinical samples: A three year study in Tehran, Iran. PloS one 2017; 12(8):e0183607.

23-**Askari E, Soleymani F, Arianpoor A, Tabatabai SM, Amini A, Naderinasab M.** Epidemiology of mecA-Methicillin Resistant *Staphylococcus aureus* (MRSA) in Iran: A Systematic Review and Meta-analysis. Iranian journal of basic medical sciences 2012; 15(5):1010-9.

24-**Edwards AM, Bowden MG, Brown EL, Laabei M, Massey RC.** *Staphylococcus aureus* extracellular adherence protein triggers TNFα release, promoting attachment to endothelial cells via protein A. PloS one 2012; 7(8):e43046.

25-**Belbase A, Pant ND, Nepal K, Neupane B, Baidhya R, Baidya R, et al**. Antibiotic resistance and biofilm production among the

strains of *Staphylococcus aureus* isolated from pus/wound swab samples in a tertiary care hospital in Nepal. Annals of clinical microbiology and antimicrobials 2017; 16(1):15.

26-**Karki S, Sah AK, Lamichhane J, Maharjan A, Sharma L, Rajbhandari R, et al.** Biofilm Formation and Detection of icaD Gene in *Staphylococcus aureus* Isolated from Clinical Specimens. The Open Microbiology Journal 2019; 13(1):230-5.

27-**Serray B, Oufrid S, Hannaoui I, Bourjilate F, Soraa N, Mliji M, et al.** Genes encoding adhesion factors and biofilm formation in methicillin-resistant *Staphylococcus aureus* in Morocco. Journal of infection in developing countries 2016; 10(8):863-9.

28-**Neupane S, Pant ND, Khatiwada S, Chaudhary R, Banjara MR.** Correlation between biofilm formation and resistance toward different commonly used antibiotics along with extended spectrum beta lactamase production in uropathogenic Escherichia coli isolated from the patients suspected of urinary tract infections visiting Shree Birendra Hospital, Chhauni, Kathmandu, Nepal. Antimicrobial resistance and infection control 2016; 5:5.

29-**Neopane P, Nepal HP, Shrestha R, Uehara O, Abiko Y.** In vitro biofilm formation by Staphylococcus aureus isolated from wounds of hospital-admitted patients and their association with antimicrobial resistance. International journal of general medicine 2018; 11:25-32.

30-**Bereket W, Hemalatha K, Getenet B, Wondwossen T, Solomon A, Zeynudin A, et al.** Update on bacterial nosocomial infections. European review for medical and pharmacological sciences 2012; 16(8):1039-44.

31-**Gowrishankar S, Kamaladevi A, Balamurugan K, Pandian SK.** In Vitro and In Vivo Biofilm Characterization of Methicillin-Resistant Staphylococcus aureus from Patients Associated with Pharyngitis Infection. BioMed research international 2016; 2016:1289157.

32-**Avila-Novoa M-G, Iñíguez-Moreno M, Solís-Velázquez O-A, González-Gomez J-P, Guerrero-Medina P-J, Gutiérrez-Lomelí M.** Biofilm formation by *Staphylococcus aureus* isolated from food contact surfaces in the dairy industry of Jalisco, Mexico. Journal of Food Quality 2018; 2018:1746139.

33-**Namvar AE, Asghari B, Ezzatifar F, Azizi G, Lari AR.** Detection of the intercellular adhesion gene cluster (ica) in clinical *Staphylococcus aureus* isolates. GMS hygiene and infection control 2013; 8(1):Doc03.

34-**Rohde H, Knobloch JK, Horstkotte MA, Mack D.** Correlation of *Staphylococcus aureus* icaADBC genotype and biofilm expression phenotype. Journal of clinical microbiology 2001; 39(12):4595-6.

35-**Cramton SE, Gerke C, Schnell NF, Nichols WW, Götz F.** The intercellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation. Infection and immunity 1999; 67(10):5427-33.

36-**O'Neill E, Pozzi C, Houston P, Humphreys H, Robinson DA, Loughman A, et al.** A novel Staphylococcus aureus biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. Journal of bacteriology 2008; 190(11):3835-50.

37-**Liberto MC, Matera G, Quirino A, Lamberti AG, Capicotto R, Puccio R, et al.** Phenotypic and genotypic evaluation of slime production by conventional and molecular microbiological techniques. Microbiological research 2009; 164(5):522-8.

38-**Vancraeynest D, Hermans K, Haesebrouck F.** Genotypic and phenotypic screening of high and low virulence *Staphylococcus aureus* isolates from rabbits for biofilm formation and MSCRAMMs. Veterinary microbiology 2004; 103(3-4):241-7.

39-**Chen Q, Xie S, Lou X, Cheng S, Liu X, Zheng W, et al.** Biofilm formation and prevalence of adhesion genes among *Staphylococcus aureus* isolates from different food sources. MicrobiologyOpen 2020; 9(1):e00946.

40-**Rahimi F, Katouli M, Karimi S.** Biofilm production among methicillin resistant *Staphylococcus aureus* strains isolated from catheterized patients with urinary tract infection. Microbial pathogenesis 2016; 98:69-76.

41-**Kalligeros M, Shehadeh F, Karageorgos SA, Zacharioudakis IM, Mylonakis E.** MRSA colonization and acquisition in the burn unit: A systematic review and meta-analysis. Burns: journal of the International Society for Burn Injuries 2019; 45(7):1528-36.

42-**Anderson MJ, Lin YC, Gillman AN, Parks PJ, Schlievert PM, Peterson ML.** Alpha-toxin promotes Staphylococcus aureus mucosal biofilm formation. Frontiers in cellular and infection microbiology 2012; 2:64.

43-**Ortines RV, Liu H, Cheng LI, Cohen TS, Lawlor H, Gami A, et al.** Neutralizing Alpha-Toxin Accelerates Healing of *Staphylococcus aureus*-Infected Wounds in Nondiabetic and Diabetic Mice. Antimicrobial agents and chemotherapy 2018; 62(3):e02288-17.

44-**Yu F, Liu Y, Lv J, Qi X, Lu C, Ding Y, et al.** Antimicrobial susceptibility, virulence determinant carriage and molecular characteristics of *Staphylococcus aureus* isolates associated with skin and soft tissue infections. The Brazilian journal of infectious diseases 2015; 19(6):614-22.

45-**Lin MH, Shu JC, Huang HY, Cheng YC.** Involvement of iron in biofilm formation by Staphylococcus aureus. PloS one 2012; 7(3):e34388.

46-**Marchetti M, De Bei O, Bettati S, Campanini B, Kovachka S, Gianquinto E, et al**. Iron metabolism at the interface between host and pathogen: From nutritional immunity to antibacterial development. International journal of molecular sciences 2020; 21(6):2145.

47-**Tristan A, Ying L, Bes M, Etienne J, Vandenesch F, Lina G.** Use of multiplex PCR to identify *Staphylococcus aureus* adhesins involved in human hematogenous infections. Journal of clinical microbiology 2003; 41(9):4465-7.

48-**Fasihi Y, Fooladi S, Mohammadi MA, Emaneini M, Kalantar-Neyestanaki D.** The spa typing of methicillin-resistant *Staphylococcus aureus* isolates by High Resolution Melting (HRM) analysis. Journal of medical microbiology 2017; 66(9):1335-7.

49-**Abou Shady HM, Bakr AE, Hashad ME, Alzohairy MA.** Staphylococcus aureus nasal carriage among outpatients attending primary health care centers: a comparative study of two cities in Saudi Arabia and Egypt. The Brazilian journal of infectious diseases 2015; 19(1):68-76.

50-**Alseqely M, Newton-Foot M, Khalil A, El-Nakeeb M, Whitelaw A, Abouelfetouh A.** Association between fluoroquinolone resistance and MRSA genotype in Alexandria, Egypt. Scientific reports 2021; 11(1):4253.

51-**Hashemizadeh Z, Hadi N, Mohebi S, Kalantar-Neyestanaki D, Bazargani A.** Characterization of SCCmec, spa types and Multi Drug Resistant of methicillin-resistant

Staphylococcus aureus isolates among inpatients and outpatients in a referral hospital in Shiraz, Iran. BMC research notes 2019; 12(1):614.

52-**Boswihi SS, Udo EE.** Emerging variants of methicillin-resistant *Staphylococcus aureus* genotypes in Kuwait hospitals. PloS one 2018; 13(4):e0195933.

53-**Alfouzan W, Udo EE, Modhaffer A, Alosaimi A.** Molecular Characterization of Methicillin- Resistant *Staphylococcus aureus* in a Tertiary Care hospital in Kuwait. Scientific reports 2019; 9(1):18527.

54-**Khan AA, Ali A, Tharmalingam N, Mylonakis E, Zahra R.** First report of mecC gene in clinical methicillin resistant *S. aureus* (MRSA) from tertiary care hospital Islamabad, Pakistan. Journal of infection and public health 2020; 13(10):1501-7.

55-**Rao Q, Shang W, Hu X, Rao X.** *Staphylococcus aureus* ST121: a globally disseminated hypervirulent clone. Journal of medical microbiology 2015; 64(12):1462-73.

56-**Pal T, Dutta NK, Mazumdar K, Dasgupta A, Dastidar SG.** Assessment of antibacterial activity of the cardiovascular drug nifedipine. Oriental Pharmacy and Experimental Medicine 2006; 6(2):126-33.

57-**Gunics G, Farkas S, Motohashi N, Shah A, Harsukh G, Kawase M, et al.** Interaction between 3,5-diacetyl-1,4-dihydropyridines and ampicillin, and erythromycin on different E. coli strains. International journal of antimicrobial agents 2002; 20(3):227-9.

58-**Elkhatib WF, Haynes VL, Noreddin AM.** Microbiological appraisal of levofloxacin activity against *Pseudomonas aeruginosa* biofilm in combination with different calcium chanel blockers in vitro. Journal of chemotherapy (Florence, Italy) 2009; 21(2):135-43.

59-**Mair SM, Nairz M, Bellmann-Weiler R, Muehlbacher T, Schroll A, Theurl I, et al.** Nifedipine affects the course of *Salmonella enterica* serovar Typhimurium infection by modulating macrophage iron homeostasis. The Journal of infectious diseases 2011; 204(5):685-94.

60-**Tisa LS, Sekelsky JJ, Adler J.** Effects of organic antagonists of Ca(2+), Na(+), and K(+) on chemotaxis and motility of *Escherichia coli.* Journal of bacteriology 2000; 182(17):4856-61.

61-**Machado D, Pires D, Perdigão J, Couto I, Portugal I, Martins M, et al.** Ion Channel Blockers as Antimicrobial Agents, Efflux Inhibitors, and Enhancers of Macrophage Killing Activity against Drug Resistant *Mycobacterium tuberculosis*. PloS one 2016; 11(2):e0149326.

62-**Liu S, Yue L, Gu W, Li X, Zhang L, Sun S.** Synergistic Effect of Fluconazole and Calcium Channel Blockers against Resistant Candida albicans. PloS one 2016; 11(3):e0150859.

63-**Mitchell C, Skomurski JF, Vary JC.** Effect of ion channel blockers on germination of *Bacillus megaterium* spores. FEMS Microbiology Letters 1986; 34(2):211-4.

64-**Elkhatib WF.** New Paradigm in treatment of biofilm-associated infections: Applications to *Pseudomonas aeruginosa* as a biofilm model organism: International Congress on Bacteriology & Infectious Diseases. Journal of Bacteriology and Parasitology 2013; 4(4):105.

65-**Asok Kumar K, Mazumdar K, Dutta NK, Karak P, Dastidar SG, Ray R.** Evaluation of synergism between the aminoglycoside antibiotic streptomycin and the cardiovascular agent amlodipine. Biological & pharmaceutical bulletin 2004; 27(7):1116-20.

66-**Mojiri-Forushani H.** The role of calcium channel blockers in wound healing. Iranian journal of basic medical sciences 2018; 21(12):1198-9.

67-**Peacock SJ, Moore CE, Justice A, Kantzanou M, Story L, Mackie K, et al.** Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus.* Infection and immunity 2002; 70(9):4987-96.

Ali GH, Seiffein NL. Microbiological study of certain genes associated with biofilm forming capacity of methicillin resistant *Staphylococcus aureus* in Egypt: An eye on nifedipine repurposing. Microbes Infect Dis 2022; 3(1): 112-127.