

Biofilm formation and methicillin resistance of *Staphylococcus aureus* isolated from clinical samples

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Abstract

Background: *Staphylococcus aureus* including methicillin resistant *S. aureus* (MRSA) is one of the most effective biofilm-forming organisms. Biofilm formation contributes in protecting the microorganism from host defenses and prevent the effective penetration of antimicrobial agents. Also, it is considered as an important contributing factor for the initiation and establishment of chronic infection by *S. aureus* and its a major obstacle in the treatment of *S. aureus* infections.

Aims: To screen clinical *Staphylococcus aureus* for their biofilm forming abilities and their association with antimicrobial resistance.

Methods: A total of 196 clinical isolates of *S. aureus* were obtained from different sample sources using standard microbiological techniques from three major hospitals in Gaza strip. Biofilm formation of these isolates was determined by tissue culture plate (TCP) method and tube adherence method (TM). Antimicrobial susceptibility test was performed using the modified Kirby-Bauer disk diffusion method as recommended by Clinical and Laboratory Standards Institute guidelines. MRSA isolates was detected using the cefoxitin disk test.

Results: Biofilm formation was observed in 174 (88.8 %) and in 145 (74.0%) isolates of *S. aureus* via TCP method and TM, respectively. The highest resistance percent was for penicillin (92.9%), followed by cefoxitin (80.6%) and oxacillin (67.9%), while the lowest resistance percent was for linezolid and ceftaroline (1%). Among the total 196 isolates, 140 (71.4%) were classified as MDR with a MAR index (≥ 0.2).

A total of 158 isolates (80.6%) were identified as MRSA distributed among hospitals as the following; 90.4%, 79.4% and 70.9% from Al-Shifa, Al-Nasser pediatrics and Al-Aqsa hospitals respectively. Large proportions (82.1%) of biofilm producers were identified among MRSA isolates. Biofilm-producing MRSA exhibited a higher percent

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(90.5%) when compared with the biofilm non-producer MRSA (9.5%). Importantly, 89.2% of biofilm-producing *S. aureus* were multidrug resistant.

Conclusions: *S. aureus* isolates possessed high biofilm-forming ability and very high tendency to exhibit antimicrobial resistance, multidrug resistance and methicillin resistance. Regular surveillance of biofilm formation by *S. aureus* and their antimicrobial resistance profile may lead more success in treating *S. aureus* infections.

Keywords

Biofilm; MDR; MRSA; TCP; TM; Gazastrip; Palestine.

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Introduction

Staphylococcus aureus is described as Gram-positive cocci, facultative anaerobic microorganisms [1]. Cells ranging in size from 0.5 to 1.7 μm in diameter. It is immotile and is not capable of forming spores. Cells are aggregated in clusters that are often described as grape-like cluster [2]. Staphylococci are widely spread colonizers of human epithelia and are opportunistic pathogens implicated with various health care associated diseases [3-4].

Several predisposing factors to *S. aureus* infection including but not limited to; immunocompromised host, invasive procedure that cause damage to the skin or mucosal surface such as indwelling medical devices, surgery, hemodialysis. In addition, homosexual men and those with close contact sports as well as those who live in close proximity, are also exposed to Staphylococcal infections [5].

A notable major obstacle in the treatment of *S. aureus* infections is their ability to develop resistance to antimicrobials. Few decades ago and up to this date, the emergence of methicillin resistance *S. aureus* (MRSA) has spread worldwide and is almost

endemic in most healthcare facilities and is increasingly becoming one of the prominent causes of death in the United States [6-7].

Biofilms form when bacteria aggregate in communities where cells are embedded in an extracellular polymeric compounds matrix attached to a surface [8]. This extracellular matrix is produced by the bacteria itself and composed of exopolysaccharides (EPSs), proteins and other macromolecules such as DNA [9]. Biofilm contribute in protecting the microorganism from host defenses and prevent the effective penetration of antimicrobial agents [10].

A wide range of bacterial pathogens produce and persist in biofilms. *S. aureus* is one of the most effective biofilm-forming organisms [11]. The ability of biofilm formation is considered as an important contributing factor for the initiation and establishment of chronic infection by *S. aureus* [12]. Biofilm formation by *S. aureus* may lead to the delay of re-epithelialization of the infected tissues, leading to increased healing time [13].

It is not fully known whether *S. aureus* biofilm forming ability qualify it to colonize/infect certain

sites or tissues. In addition, correlating biofilm forming ability to antimicrobial resistance is necessary in the battle of combating *S. aureus* and its drug resistance.

Materials and Methods

Sample Collection and Processing

A total of 196 isolates were collected from three major hospitals (Al-Shifa, Al-Aqsa and Al-Nasser Pediatrics hospitals) from different departments and different sites of infection. Isolates were obtained from the microbiology department along with patient's data.

The sampling period started from April 2018 and continued for eight months. All isolates were transferred to Islamic University of Gaza (IUG) Microbiology Research laboratory. Each isolate was then streaked onto the surface of Blood Agar and Mannitol Salt Agar plates and incubated for 24 hours at 37°C for purity check and identification purposes. Glycerol with Brain Heart Infusion Broth (BHIB) were used for long-term storage of isolates.

S. aureus identification

After the incubation period, colonies were identified based on colony color and morphology in addition to Gram stain and the final identification was performed using conventional biochemical tests (e.g, catalase, coagulase, and DNase test). Only confirmed *S. aureus* isolates were further tested.

Antimicrobial susceptibility testing

The susceptibilities of the isolates to ceftaroline, ceftazidime, cefoxitin, chloramphenicol, ciprofloxacin, clarithromycin, clindamycin, gentamicin, linezolid, oxacillin, penicillin, rifampicin, tetracycline and trimethoprim-sulfamethoxazole, were determined using the **disk diffusion method** according to the guidelines and interpretive criteria of the Clinical and Laboratory Standards Institute (CLSI) [14]. A

0.5 McFarland standardized inoculum was spread using cotton swab onto the surface of two Muller Hinton (MH) agar plates. The appropriate antimicrobial disks were applied and plates were transferred to a refrigerator for 30 minutes and then was incubated for 24 hours at 37°C. The zone of inhibition was measured and interpreted as sensitive, resistant or intermediate according to the CLSI.

Tissue culture plate method

A single colony of *S. aureus* was picked from an overnight-incubated Blood agar plate and inoculated into 2 mL of BHIB supplemented with 0.25% (wt/vol) glucose (15). The broth was incubated overnight at 37 °C. The culture was then diluted 1:100 with fresh medium. A sterile individual well in 96 flat-bottom polystyrene wells was filled with 200 µL of the diluted culture. Negative control contained broth only and a Positive control (A control strain of biofilm forming *S. aureus* (ATCC: 6538) was used. The control strain was also processed in a similar manner. The plate was incubated at 37 °C for 72 hours. After incubation, the content of each well was removed. Each well was washed three times with 250 µl of DW. After 15 min, plates were stained for 5 min with 0.2 ml of 2% crystal violet per well. Excess stain was removed and rinsed off by placing the plates under running tap water. The plates were air-dried.

The adherent cells were re-solubilized with 160 µl pure methanol per well [16]. The optical density of each was measured at 570 nm. Results interpreted according to the followings criteria; OD <0.5 (negative), OD ≥ 0.5 (positive) (17). Wells containing only sterile BHIB served as a background control; their average absorbance value was subtracted from all experimental readings [15].

Tube adherence method

An aliquot of 0.1 mL of bacterial culture (obtained by adjusting turbidity to 0.5 McFarland standards)

was transferred to glass test tube containing 10 mL BHIB which was incubated at 37°C for 72 hours.

The medium was then removed and the tubes were washed with distilled water, air dried and bio-film formation were assayed by 2% crystal violet [16]. Visible lining of the wall and bottom of the tube by presence of a film were considered as positive.

Data analysis

Collected data were summarized, tabulated and analyzed using Statistical package for Social Sciences (SPSS) software. Chi square test was used to detect significant differences among hospitals and or samples. The results are presented as tables and figures.

Results

A total of 196 *S. aureus* clinical isolates were collected from Al-Shifa (N=83), Al-Nasser pediatrics (N=34) and Al-Aqsa (N=79) hospitals. One hundred forty-four isolates (73.5%) were obtained from male patients and the remaining 52 (25.5%) were from female patients.

All the isolates were obtained from clinical samples, collected from different sources The pus group includes pus from different sources, wound and ear charge samples (N=171), while the body fluids group contains urine, blood, plural and cerebrospinal fluid (N=10). The group labelled as "Others" contain sputum, bone, skin and soft tissue samples (N=15).

Antimicrobial susceptibility test

As shown in **Table 1**, the highest resistance rate was against penicillin G (92.9%), followed by cefoxitin (80.6%) and oxacillin (67.9%); both of which are used to detect methicillin resistance, while the lowest resistance rate was against linezolid and ceftaroline (1%)

Out of 13 antimicrobials used in this study, penicillin G had the lowest activity in all three hospitals

Table 1. Antimicrobial susceptibility profile of 196 *S. aureus* against 13 antimicrobials.

Antimicrobial	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Ceftaroline	184	93.9	10	5.1	2	1.0
Cefoxitin	38	19.4	0	0.0	158	80.6
Chloramphenicol	169	86.2	3	1.5	24	12.2
Ciprofloxacin	142	72.4	13	6.6	41	20.9
Clarithromycin	126	64.3	8	4.1	62	31.6
Clindamycin	168	85.7	5	2.6	23	11.7
Gentamicin	145	74.0	1	0.5	50	25.5
Linezolid	194	99.0	0	0.0	2	1.0
Oxacillin	40	20.4	23	11.7	133	67.9
PenicillinG	14	7.1	0	0.0	182	92.9
Rifampicin	175	89.3	5	2.6	16	8.2
Tetracycline	122	62.2	5	2.6	69	35.2
Trimethoprim-sulfamethoxazole	143	73.0	12	6.1	41	20.9

in the study as shown in **Table 2**. Chi square statistical analysis showed significant differences among hospitals with regard to resistance against several antibiotics (P value ≤ 0.05) with Al-shifa hospital showing the highest resistance rate for most of the tested antimicrobials.

Multiple Antibiotic Resistance index (MARI) and multidrug resistance

MAR index was calculated for each isolates by dividing the number of antibiotics that the isolate was resistance to by the number of all antibiotics which the isolates was tested for. Any isolate with an index of more than 0.2 is considered as MDR. Among the 196 isolates, 71.4% (N=140) were classified as MDR with a MAR index (≥ 0.2).

MRSA and MSSA

To identify MRSA, cefoxitin antibiotic disks were used, 80.6% of the isolates were resistant to cefoxitin. The highest resistance to cefoxitin was found at Al-Shiffa hospital 90.4% followed by Al-Nasser pediatrics hospital (79.4%) and Al-Aqsa hospital (70.9%)

Table 2. Antimicrobial resistance percent among hospitals.

Antimicrobial	Al-Aqsa hospital	Al-Shifa hospital	Al-Nasser hospital	P- value
	%(N=79)	%(N=83)	%(N=34)	
Ceftaroline	0.0	2.4	0.0	0.012*
Cefoxitin	70.9	90.4	79.4	0.007*
Chloramphenicol	11.4	16.9	2.9	0.228
Ciprofloxacin	13.9	33.7	5.9	0.002*
Clarithromycin	39.2	31.3	14.7	0.051*
Clindamycin	11.4	15.7	2.9	0.310
Gentamicin	11.4	43.4	14.7	0.000*
Linezolid	2.5	0.0	0.0	0.224
Oxacillin	59.5	77.1	64.7	0.025*
Penicillin G	93.7	94.0	88.2	0.514
Rifampicin	7.6	8.4	8.8	0.131
Tetracycline	29.1	45.8	23.5	0.009*
Trimethoprim-sulfamethoxazole	29.1	13.3	20.6	0.125

*: Statistically significant.

Biofilm results

Among 196 isolates, 88.8 % (N=174) were biofilm producers, while only 11.2% (N=22) did not show biofilm formation by the **Tissue Culture Plate method**. The biofilm-producing isolates percentage in Al-Shiffa hospital was 94.0%, while the percentage was 82.3% and 91.2% in Al-Aqsa and Al-Nasser Pediatrics hospitals respectively. Biofilm formation was 100% positive in all specimen type except pus, the percent was (87.1%).

About 74.0% (N=145) of the isolates were biofilm producers by the **Tube Method** distributed among hospitals (79.5%),(67.1%), (76.5%) for Al-Shiffa, Al-Nasser pediatrics and Al-Aqsa hospitals respectively. The biofilm formation was 100% in the specimens that comes from body fluids while it was (71.9%) in the pus group and (80.0%) in the other specimen types as presented in **Table 3**.

Biofilm and antimicrobial resistance

Table 4 shows the biofilm forming ability versus antimicrobial resistance. For all the antimicrobialsu-

Table 3. Biofilm production using Tissue Culture Plate Method and Tube Method according to hospitals, specimen source and departments .

	Tissue Culture Plate Method				P- value	Tube Method				P- value
	Positive		Negative			Positive		Negative		
	No.	%	No.	%		No.	%	No.	%	
Hospital										
Al-Aqsa	65	82.3	14	17.7	0.055*	53	67.1	26	32.9	0.184
Al-Shiffa	78	94.0	5	6.0		66	79.5	17	20.5	
Al-Nasser	31	91.2	3	8.8		26	76.5	8	23.5	
Specimen source										
Pus	149	87.1	22	12.9	0.163	123	71.9	48	28.1	0.124
Body fluids	10	100	0	0.0		10	100	0	0.0	
Other	15	100	0	0.0		12	80.0	3	20	
Department										
Pediatrics	14	87.5	2	12.5	0.994	12	75.0	4	25.0	0.691
Out patients	54	88.5	7	11.5		45	73.8	16	26.2	
Nursury	30	90.9	3	9.1		27	81.8	6	18.2	
Surgery	59	88.1	8	11.9		49	73.1	18	26.9	
Other	17	89.5	2	10.5		12	63.2	7	36.8	

Table 4. Biofilm forming ability versus antimicrobial resistance.

Antimicrobial		Positive		Negative		Total	P-value
		No.	%	No.	%		
Ceftaroline	R	2	100	0	0.0	2	0.446
	S	162	88.0	22	12.0	184	
	I	10	100	0	0	10	
Cefoxitin	R	143	90.5	15	9.5	158	0.007*
	S	31	81.6	7	18.4	38	
Chloramphenicol	R	21	87.5	3	12.5	24	0.811
	S	150	88.8	19	11.2	169	
	I	3	100	0	0.0	3	
Ciprofloxacin	R	37	90.2	4	9.8	41	0.847
	S	125	88.0	17	12.0	142	
	I	12	92.3	1	7.7	13	
Clarithromycin	R	54	87.1	8	12.9	62	0.552
	S	112	88.9	14	11.1	126	
	I	8	100.0	0	0.0	8	
Clindamycin	R	18	78.3	5	21.7	23	0.184
	S	151	89.9	17	10.1	168	
	I	5	100.0	0	0.0	5	
Gentamicin	R	45	90.0	5	10	50	0.888
	S	128	88.3	17	11.7	145	
	I	1	100.0	0	0.0	1	
Linezolid	R	2	100	0	0.0	2	0.613
	S	172	88.7	22	11.3	194	
	R	119	89.5	14	10.5	133	
Oxacillin	S	34	85.0	6	15.0	40	0.675
	I	21	91.3	2	8.7	23	
Penicillin G	R	160	87.9	22	12.1	182	0.167
	S	14	100	0	0.0	14	
Rifampicin	R	15	93.8	1	6.3	16	0.567
	S	154	88.0	21	12.0	175	
	I	5	100	0	0.0	5	
Tetracycline	R	62	89.9	7	10.1	69	0.118
	S	109	89.3	13	10.7	122	
	I	3	60.0	2	40.0	5	
Trimethoprim-sulfamethoxazole	R	34	82.9	7	17.1	41	0.406
	S	129	90.2	14	9.8	143	
	I	11	91.7	1	8.3	12	

sed, the majority of the resistant strains was biofilm producers but the results don't reach statistically significant level.

A total of 125 (89.2%) MDR isolates were biofilm producers while the remaining 15 isolates (10.7%) were non-biofilm producers as presented in **Table 5**. (No statistical significant difference was found).

Among the 158 (80.6%) isolates identified as MRSA, 143 (90.5%) isolates were biofilm producers as shown in **Table 6**. (No statistically significance difference was found).

Table 5. MAR index and Tissue Culture Plate results.

MAR index	Positive		Negative		Total	P-value
	No.	%	No.	%		
0.07	11	73.3	4	26.7	15	0.113
0.1	38	92.7	3	7.3	41	
0.2	40	83.3	8	16.7	48	
0.28	39	97.5	1	2.5	40	
0.3	21	87.5	3	12.5	24	
0.4	13	100.0	0	0.0	13	
0.5	2	100.0	0	0.0	2	
0.57	6	75.0	2	25.0	8	
0.6	1	100.0	0	0.0	1	
0.7	2	100.0	0	0.0	2	
0.78	1	50.0	1	50.0	2	

Table 6. MSSA and MRSA and Tissue Culture Plate results.

<i>S. aureus</i>	Positive		Negative		P-value
	No.	%	No.	%	
MSSA	31	81.6	7	18.4	0.0.118
MRSA	143	90.5	15	9.5	

Discussion

All *S. aureus* isolates examined in this study were at least resistance to one of the 13 antimicrobials tested. The lowest antimicrobial resistance was 1.0% for both ceftaroline and linezolid, however, these two antimicrobials are not used in treatment protocols

in Gaza strip hospitals and are not available in the local drug market.

Linezolid is a worldwide effective and well tolerated antimicrobial in patients with *S. aureus* infections. In a study done in Nepal, linezolid showed the highest rate of susceptibility (100%) [18]. In other study done in Cleveland showed that there is an 10.4% emergence of linezolid-resistant *S. aureus* after prolonged treatment of cystic fibrosis [19]. Thus, linezolid should be considered in treating MRSA infections.

The highest resistance rate was for penicillin (92.9%), this rate is higher than the percents conducted in two studies in 2016 and 2017 that was 65.9% and 62% respectively, in Gaza Strip [20-21], and was lower than Elbayoumi study that done in 2011, the resistance percent for penicillin was 100% [22] and Hujir study in 2006 (94.0%) [23].

Also oxacillin resistance percent in this study was higher than the result obtained in tow previous studies done in 2006 and one study in 2017 [21-23]. These variations could be explained by the variation of sample size, types of samples tested and of course by the specific area covered by these studies.

The rate of resistance for trimethoprim-sulfamethoxazole, ciprofloxacin and chloramphenicol was higher than the percent in Elmanama study in 2016 [20]. On the other hand, the resistance to rifampicin and clindamycin was lower than that in 2006 and 2016 (20-23). This increase in the susceptibility for these antimicrobial may be due to the limited prescription and use in the treatment procedure during the recent years.

The increase in the antibiotics resistance rates in this study could be due to the excessive use, misuse and the absence of restriction on the prescription of these antibiotics in Gaza strip [24].

For most antimicrobials tested in the study, Al-Shiffa hospital demonstrated the highest rate of resistance, the differences between hospitals may

be due to the differences in the treatment protocols between them, the varied level of commitment of infection control measures and procedures and the high number of patients.

Eight of the 13 antibiotics tested in the study showed the highest rate of resistance in the surgery department, with a statistically significant level for ciprofloxacin ($P=0.01$), gentamycin ($P=0.030$) and tetracyclin ($P=0.048$). This may be due to the excessive use of these antimicrobial agents in the surgery departments in Gaza Strip hospitals with the absence of clear guidelines. Therefore, it's very important to focus on the choice and duration of antimicrobials agents that used either as pre or post operative prophylaxis or post infection [6].

For detecting MRSA, cefoxitin disk was used rather than methicillin and oxacillin because cefoxitin would overcome the failure of routine oxacillin disks to detect heterogeneous MRSA, it is a good indicator of penicillin binding protein 2a in *S. aureus* isolates that carry *mecA* gene [21]. Therefore, testing of cefoxitin give more reproducible and accurate results than testing with methicillin or oxacillin. This fact was evident by the results of this study where oxacillin resistance rate was 67.9% while cefoxitin was higher (80.6%).

The overall MRSA percent in this study was 80.6% and this is higher than the results of study conducted in 2006 in Gaza strip which was only 22% [23], and higher than another study done in 2015 (56.3%) [25]. In 2017, MRSA percent in southern "Israel" was 40% [26], while in other neighboring countries like Egypt, Jordan and Lebanon MRSA percent was 52%, 56% and 30% respectively [27].

This increase in MRSA percent in Gaza strip in this study over the last decade maybe due to the presence of high percent of health care worker carrying MRSA according to study done in 2017 [14], the weak infection control procedure and the absence of protocols for diagnosis, detection and quarantine for MRSA patients in Gaza Strip hospitals.

High percent of MRSA was found in pediatric departments (93.8%) and surgery departments (85.1%). In a study done in Gaza Strip hospitals in 2017 for detecting nasal carriage MRSA among health care worker the highest percent of MRSA was in surgery department (35%) [21]. This high resistance rate in these departments may be due the crowdeness, high work load, weak infection control procedure and shortage of stuff.

The prevalence of biofilm formation varied between the two testing methods (88.8% and 74.0% (TCP and TM). Also, there was a little variation between the two methods used by a study in Nepal, where biofilm formation was (69.8%) and (65.1%) for TCP and TM, respectively [28].

Tube method is an easy and low cost test, but it's less accurate than TCP method because it depends on the personal observation (visual inspection) as a qualitaive methode, it can be used as screening test for biofilm formation. While TCP method is a qantitative method that can detect even the weak biofilm producer strains [28].

The present study, shows that the biofilm-producing isolates were associated with higher incidence of antimicrobial resistance when compared with non-producer isolates. In addition, the major rate of the MDR isolates were biofilm producer (89.2%), particularly among the isolates that were identified as MRSA (90.5%). However, theses differences did not reach a statistically significant level.

These results is concordant with a study done in Nepal (2018) concluded that higher rate of antimicrobial resistance is demonstrated among biofilm producers than in biofilm nonproducers. The biofilm-positive strains have a higher tendency to exhibit multidrug resistance and methicillin resistance compared to biofilm-negative strains [28]. Another study showed that 97.5% of biofilm producers from burn wound samples were MRSA [29]. A study done in Spain, found that all the staphylococcal isolates that produced biofilm except one harbor horizontal transfer of antibiotic resistance genes [30].

In addition, our results is concordant with Be-lbase study in Nepal that showed higher rates of multidrug resistance and methicillin resistance were found among biofilm-producing isolates in comparison to biofilm non-producing isolates [18]. Also, a recent study from Jordan, has demonstrated that most MRSA strains can produce biofilm, and one methicillin-sensitive *S. aureus* (MSSA) isolate typed as spa t955 showed biofilm formation potential through protein deposition [31]. Therefore, we suggest that biofilm forming capacity of MRSA strains are important factor in developing infection.

Conclusion

The findings in this study demonstrated that higher rates of antimicrobial resistance are exhibited by biofilm producers than by nonproducers biofilm isolates. The biofilm-positive strains have a higher tendency to exhibit multidrug resistance than by nonproducers biofilm isolates. In addition, among MRSA isolates, the majority were biofilm producer.

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