



THE RELATIONSHIP BETWEEN BIOFILM PRODUCTION AND ANTIBIOTIC SENSITIVITY OF (MRSA) *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Because the pathogenicity importance of Methicillin Resistant *Staphylococcus aureus* (MRSA) and what it cause of nosocomial infection to surgical wounds, the production of biofilm and its resistance against many antibiotics was studied. From a total of 45 swabs taken from different surgical infected skin wounds, there were 10 MRSA isolates that were identified by conventional and confirmatory methods (API Staph kit), Slime layer production tested by Congo red medium showed only 3 positive strains of MRSA, whereas, biofilm production tested by Crystal violet staining in a polystyrene plate showed that 5 strains of MRSA were moderate producers of biofilm. The relationship between MRSA strains production of biofilm and antibiotics sensitivity was compared and there were 5 strains producing biofilm were resistant to more than 7 antibiotics, whereas the non-biofilm producers of MRSA strains were resistant to only 4 antibiotics. In conclusion MRSA strains producing biofilm show more resistance to more number of antibiotics in skin wounds.

KEY WORDS

Pathogenicity, *Staphylococcus aureus*

INTRODUCTION

Staphylococcus aureus is a Gram-positive, coccal bacterium, aerobic or microaerophilic, non-motile, non-spore former, and is frequently found in the nose, respiratory tract, and on the skin. It is often positive for catalase and nitrate reduction [1]. *Staphylococcus aureus* is not always pathogenic it is considered as normal flora and opportunistic microorganism. *Staphylococcus* was first identified in 1880 in Aberdeen, Scotland, by the surgeon Sir Alexander Ogston in pus from a surgical abscess in a knee joint [2]. Methicillin-resistant *Staphylococcus aureus*, particularly (MRSA), is associated with a wide range of diseases from superficial skin infections to life-threatening conditions such as bacteremia, endocarditis, pneumonia or toxic shock syndrome [3], it is consistently one of the top four causes of nosocomial infections [4].

The capacity of this clinically essential bacterial species to effectively hold on inside the hosts, is generally because of the outflow of a battery of virulence factors, which advance attachment, securing of nutrients, and avoid host immunologic responses [5]. Moreover, sensitivity of methicillin resistant strains to gentamycin could imply that there is a probability of sensitivity to all aminoglycosides for example, streptomycin and kanamycin. However, this is not absolutely sure as each of the aminoglycosides have a slightly extraordinary mechanism of resistance due to their diverse aminoglycoside modifying enzymes chromosomal mutation [6]. The relative danger of gaining antibiotic resistant *S. aureus* in the surgical wound was high. This is owing to wound conditions which support the spread of nosocomial diseases [7].

Biofilms are depicted as a microbial population joined to a substrate, surrounded by a self-derived extracellular

matrix. This method of life presents important clinical repercussions since it is assessed that over half of all hospital infections are originated from these microbial communities [8]. Biofilms are the inhabitants of bacteria developing on biotic and abiotic surfaces and insert themselves in a self-derived extracellular framework of exopolysaccharide (EPS), proteins and some micro molecules such as DNA [9]. Biofilms play an important role in nature, in which under non-ideal conditions they can survive in, medical devices utilized inside and outside the body are usually covered with biofilm producing bacterium which can promote and maintain disease, they are not easily killed by antibiotics [10]. Several studies have demonstrated that biofilms produced by bacteria are in charge of persistent infections in humans for example dental caries periodontitis, and many others [11]. Biofilm formation in *S. aureus* is regulated by expression of Polysaccharide Intracellular Adhesion (PIA) which mediates cell to cell adhesion and is the gene product of *ica* ABDC [12]. Having the ability of biofilm-formation decrease their susceptibility to antibiotics. *Staphylococcus aureus* is known to form biofilms on different surfaces [13]. In fact, biofilms can resist antibiotic concentration 10-10,000 folds higher than those required to inhibit the growth of free floating bacteria [14].

The chronic infections caused by *S. aureus*, persist and increase the rate of morbidity and mortality in human population due to the development of biofilm [15]. Biofilms have an enormous impact on healthcare, and was showed to be associated with 65% of nosocomial infections [16].

The aim of this study was to assess the relationship between antibiotic sensitivity and biofilm production of (MRSA) bacterium isolated from surgical infected wounds.

MATERIALS AND METHODS:

A. Bacterial Isolation and Identification: -

Swab specimens were collected from different surgical wound infections of patients attending different hospitals in Baghdad, each swab was inoculated on Blood agar and Mannitol Salt agar, incubated at 37°C for 24 hr. for primary isolation. The colonies were purified by sub-culturing on Mannitol Salt agar, the isolates were identified according to the morphological characteristics on culture medium, microscopically on

slide and biochemically [17,18] with the use of API system kit [19].

1. Microscopical examination: -

The bacterial strains were stained by Gram stain to detect their response to the stain to reveal their color, shapes and arrangements.

2. Analytical Profile Index (API): -

The API **Staph** kit was used for the identification of *Staphylococcus spp.* each strip consists of 20 microtubes containing dehydrated substrates which were inoculated with saline bacterial suspension, after incubation the microtube colors changed as a result of metabolism reactions or revealed by the addition of reagents. The results were read according to a reading table obtained with the kit from the manufacture company.

B. Antibiotic sensitivity test: - (Disk diffusion method)

Antibiogram of MRSA strains were based on the susceptibility patterns for selected antibiotics representing various classes of antimicrobial agents, as shown in Table (1). Kirby-Baure method was used according to [20, 21] to carry out antimicrobial susceptibility. The isolates were interpreted as susceptible, intermediate or resistant to a particular antimicrobial agent by comparison with standard inhibition zones as mentioned according to (CLSI,2011). With the identification of Methicillin resistant *S. aureus* (MRSA), and detection of Cefoxitin antibiotic sensitivity against *S. aureus* strains as a confirmatory test for (MRSA) bacterium which is superior and a surrogate test for the Oxacillin test due to its higher sensitivity, ≥ 22 considered sensitive and ≤ 21 considered as resistant.

1. Preparation of Inoculum: -

Bacterial strains were prepared in inoculum normal saline to adjust its turbidity, 3-4 colonies were taken from an 18-hr. fresh culture by sterilized loop and suspended in 5 ml normal saline to get a culture with 1.5×10^8 CFU/ml and compared with turbidity standard of McFarland tube No. 0.5.

2. Inoculation and Applying antibiotic discs: -

By a sterile cotton swab a portion of bacterial culture (swab dipped into inoculum tube) was transferred and carefully evenly spread on Mueller Hinton agar medium and left for 10 min. Subsequently, the anti-microbial discs were placed on the agar medium with some sterile forceps and pressed firmly to ensure contact with the agar surface. The plates containing the antibiotic discs were then inverted and incubated at 37°C for 18 hr.

3. Reading the inhibition zone: -

Inhibition zone for each antibiotic was measured by a metric ruler in millimeters (mm) according to the clinical laboratories standards institute (CLSI,2013). Many factors effect diameter of inhibition zone that should be considered such as thickness of medium, humidity and the age of medium [22,23].

C. Ability of bacterial strains to produce Slim layer: -

The test was achieved by **Congo red agar** method according to [24], briefly,

Brain heart infusion agar (BHA) was supplemented with: 5gm/100ml (sucrose) + 0.08gm /100ml (Congo red powder). Prepared and sterilized in autoclave for 15 minutes (only). The MRSA strains (previously inoculated in Nutrient broth) streaked on BHA plates and incubate aerobically for 24-48 hr. at 37C°.

Note: (+ve) positive results were indicated by black colonies with dry crystalline appearance. **(-ve)** negative results were indicated by white colonies while weak producers remain pink.

D. Detection of Biofilm production by Crystal Violet staining: -

The ability of MRSA isolates to produce biofilm were evaluated by using crystal violet staining technique in polystyrene microtiter plats and then O.D was determined at 490 nm. The O.D represents the degree of the biofilm thickness [24].

Overnight cultures of trypticase soy broth (TSB) supplemented with (1% glucose) were diluted until 10⁸ CFU/ml in TSB. Individual wells of polystyrene, flat-bottomed 96 well plates were filled with 200 micro liter aliquots of the cultures which were further incubated for 24 hr. at 37C°, next the wells were washed 3 times with 200 micro liter of sterile phosphate buffer saline (pH: 7.2). Biofilms were fixed with heating at 60 C° during 15 min. 200 micro liter of crystal violet solution (0.1% wt. /vol.) was added to all wells and left for (15 min). Excess crystal violet was rinsed with distilled water and air dried overnight. Bounded crystal violet was released by adding 200 microliters of 96% ethanol.

Absorbance was measured spectrophotometrically at 490 nm (A₄₉₀) and was proportional to biofilm biomass. The test was performed in triplicates and negative control wells contained TBS. The results were calculated according to [25] as in Table (2).

RESULTS AND DISCUSSION:

A. Bacterial Isolation and Identification - :

Clinical swab specimens (50) collected from different patients with surgical infected wound showed growth of 20 (40%) isolates that were identified as *S.aureus*, which showed positive growth on Mannitol salt agar, the isolates fermented the mannitol sugar and appeared as golden colonies and under microscope as aggregated coccoid cells with purple color. Isolates were further identified by API Staph Kit for the confirmation of *S.aureus* identification.

B. Antibiotic sensitivity test: - (Disk diffusion method)

Disc diffusion method was used to detect the antibiotic sensitivity of *S. aureus* against defferent kinds of antibiotics previously mentioned in Table (1), only 10 (50%) isolates were identified as MRSA. *Staphylococcus aureus* infections are very common, and MRSA continues to be a serious challenge as their prevalence is reported to be increasing. Various studies worldwide have reported the prevalence rate of MRSA strains to be 68% (27), 57% (28), 40% (29) and 32% (30).

The varying in the prevalence of MRSA strains could be due to factors like poor healthcare facilities and uncontrolled antibiotic usage that vary from one country to another. Both Ampicillin (AM) and Methicillin (ME) showed complete resistance against all MRSA strains, Fig (1). Whereas, complete susceptibility was shown against all MRSA strains to Amikacin (AK), Azithromycin (AZM), Ciprofloxacin (CIP), and Imipenem (IPM) as shown in Table (3).

C. Detection of bacterial production ability of Slim layer: -

The capsular polysaccharide has been reported to be a component of the cell surface and the biofilm, that contribute cell adherence to biomaterials and to protect the bacterium from host defense. Hence, it is referred to as capsular polysaccharide/adhesin (PS/A). Some studies proposed that PS/A was a high-molecular weight polymer of b (1–6)-linked glucosaminyl residues substituted on the amino group with succinate and acetate groups referred to as poly acetyl glucosamine (PNAG) referred to as slime. PS/A and PNAG are structurally and immunologically similar to polysaccharide intercellular adhesin (PIA) [26].

Results of our study showed growth of 3 (30%) MRSA strains on Brain heart infusion agar with Congo red which displayed mostly purple to black colonies. Other studies showed that 47% of MRSA strains were positive

on CRA [27], whereas, others reported a positive CRA assay result in only 4-5% of the *S. aureus* strains tested [28], as shown in Fig (2).

Table (1): Antimicrobial discs used in this study.

Antibiotics (Antimicrobial discs)	Disc potency (µg/disc)	Code
Amikacin	10	AK
Amoxillin 20µg / Clavulanic acid 10µg	30	AMC
Ampicillin	25	AM
Azithromycin	15	AZM
Cefixime	5	CFM
Cefotaxime	10	CTX
Cephalothin	30	KF
Chloramphenicol	30	C
Clarithromycin	15	CLR
Cloxacillin	10	CX
Ciprofloxacin	5	CIP
Imipenem	10	IPM
Meropenem	10	MEM
Penicillin	10	P

Table (2): Values of biofilm formation by bacterial isolates.

OD values	Biofilm Formation
< OD _c	Non
OD _c < OD _t ≤ 2*OD _c	Weak
2*OD _c < OD _t ≤ 4*OD _c	Moderate
4*OD _c < OD _t	High

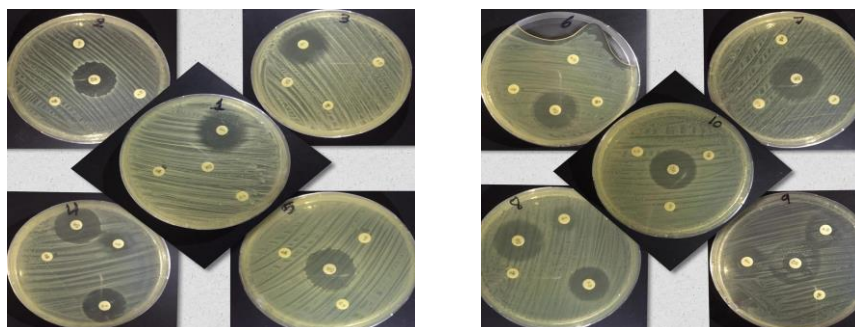


Figure (1): antibiotic sensitivity of *S. aureus* against Methicillin, Cloxacillin, Ampicillin and Chloramphenicol.



Figure (2): Slim layer production of MRSA strains on Congo red medium.

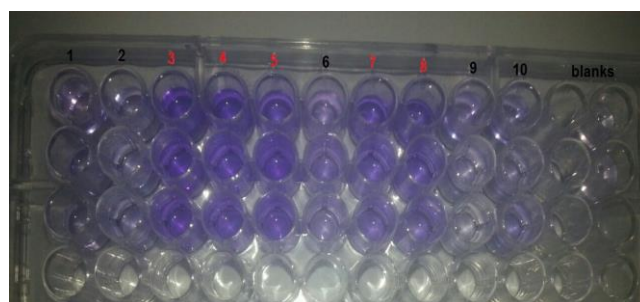


Figure (3): Crystal violet microtiter plate test for biofilm production of MRSA strains.

Table (3): Antibiotic sensitivity of MRSA strains against antibiotics and its relation with biofilm production.

Strain no.	CTX _c	AMC	IPE	MEM	AM	CIP	AK	CFM	KF	CLR	AZM	CTX	CX	ME	P	C	No. of antibiotic resistance
	Inhibition zone in (mm)																
1	21	17	23	28	R	14	25	30	16	22	15	25	R	R	R	25	4
2	26	19	26	41	R	35	25	34	19	21	29	30	18	R	R	R	4
3	R	R	23	R	R	11	29	R	R	19	16	R	R	R	R	26	10
4	R	R	34	37	R	37	25	22	R	R	26	R	17	R	23	15	7
5	R	14	31	35	R	39	29	15	R	R	16	R	19	R	R	R	8
6	29	21	30	31	R	35	24	12	21	20	20	23	20	R	R	R	4
7	R	R	30	31	R	38	28	16	R	R	32	R	16	R	R	R	9
8	R	R	24	20	R	22	20	R	R	R	15	R	15	R	21	14	8
9	24	R	29	36	R	36	26	28	R	18	25	34	20	R	18	15	4
10	30	24	26	40	R	38	26	23	15	19	23	30	21	R	R	R	4

Green Square : R : resistant ,non biofilm producer; Blue Square : R: resistant, biofilm producer. White Square : sensitive ,non biofilm producer; Yellow Square : sensitive biofilm producer. Red Square : Number of antibiotic resistance. c: conformatory .

D. Detection of Biofilm production with Crystal Violet staining: -

Biofilm formation is the most important factor in the development of chronic infection and allows for immune evasion as well as resistance to antimicrobial agents [29], so that the only method of successful treatment is the removal of the injured tissues or devices [30].

From 10 (MRSA) isolates only 5 isolates were biofilm producers, isolate number 3,4,5,7 and 8 were biofilm producers while isolates 1,2,6,9, and 10 were biofilm non-producer, as shown in Fig (3). A percentage of 50% of the MRSA strains were biofilm producers, this result was very close to the percentage of Mirani,Z.A. *etal*, 2013 who found that the percentage of biofilm producers of MRSA strains was 57% [31]. Whereas, John,N.P. and Murugan,S. 2014 showed that only 16.8% of MRSA strains were strong biofilm producers [32]. It has been suggested that biofilm formation in (MRSA) is usually regulated by surface adhesins, which are repressed under *agr* expression [33].

The molecular basis of biofilm formation by *Staphylococci* has been showed that the initiation of

biofilm production needs the adherence of cells to a surface, then the formation of microcolonies, which develop into a mature biofilm structure [34]. The extracellular polysaccharide adhesion is regulated by intercellular adhesion (*ica*) gene [40]. PS/A mediate initial adherence to solid surfaces and PIA mediate accumulation of cells on biofilms [35].

E. Correlation between slime formation and development of biofilm biomass:

In order to investigate whether slime production is indicative for strong biofilm formation, the correlation between these two characteristics was tested. Phenotypic detection of slime production on CRA was not related to the quantitative detection of strong biofilms, measured by crystal violet staining in our study, which was used as a gold standard. This result was confirmed by Croes, S. *et al* study which found no correlation between slime producing MRSA and an enhanced tendency to form large amounts of biomass [36]. These studies strongly suggest that CRA screening forms no alternative for crystal violet staining to detect biofilm production [37]. Jain, A. *et al*, 2009 reported differences between commensal and blood stream

isolates *S. aureus* isolates according to positive CRA screening, 20% and 75%, respectively. The variations could be due to differences in genetic backgrounds of the strains used, or to differences in interpretation of the colonies [38].

F. Correlation between biofilm biomass and development of antibiotic resistance:

The antibiotic resistance pattern of the biofilm producing MRSA strains and non-producing biofilm strains was observed towards AMC (80% versus 20%), CFM (40% versus 0%), CLR (80% versus 0%), KF (100% versus 20%) and MEM (20% versus 0%).

These results show that these antibiotics are ineffective against MRSA biofilm producers, results also show that MRSA biofilm producers referred to as **3,4,5,7 and 8** were resistant to more than 7 antibiotics, whereas, MRSA non-biofilm producers were less resistant to antibiotics as shown in Table (3). These results indicate the importance of biofilm biomass in the resistance to antibiotics and as a result increase the bacterial verulence which make the treatment of such bacterial infection a big problem.

The potential to produce biofilm highlight the high prevalence of resistant bacteria in this region. Also, MRSA biofilm producers showed high resistance to most antibiotics mentioned in this study when compared to MRSA non-biofilm producers, this observation was supported by other studies [39]. The main reason for antibiotic resistance is due to the decreased diffusion of antibiotics through the biofilm matrix and decreased metabolic activity of bacteria [40]. Researches have studied the strategies employed by bacteria to produce biofilm and they have concluded that biofilm producing bacteria secrete certain chemicals that protect them from disinfectants, antimicrobial and immunologic systems [41].

CONCLUSION:

The bacterial strains of (MRSA) that produce biofilm resist more number of antibiotics which may cause dangerous infections to patients after surgery.

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