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Incidence of Methicillin Resistant *mecA* and Investigation of Biofilm Formation in some Clinical Isolates of *Staphylococcus aureus* in Baghdad

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Abstract : The most prevalent pathogenic bacteria in hospitals and communities is Methicillin resistant *Staphylococcus aureu*s (MRSA). Its capacity to form biofilms is thought to be the primary factor in its pathogenicity since it confers resistance to both human immune response and medications, so the purpose of this investigation was to assess the development of biofilms and their correlation with antibiotic resistance in MRSA clinical isolates. From 150 swabs taken from various clinical sources of patients at several hospitals in Baghdad, Iraq, 36 *S. aureus* were isolated. The study was conducted from November 2023 to March 2024, a span of five months. The diagnosis of *S. aureus* isolates was made using molecular methods, biochemical testing, and phenotypic characteristics. The molecular technique relied on PCR for gene specific detection, *16S rRNA* for staphylococcus genus diagnosis, and *mecA* for methicillin-resistant *S. aureus* diagnosis. Antibiotic susceptibility to eleven different antibiotics showed that *S. aureus* has an elevated resistance to Cefoxitin (alternative to Methicillin) (55.55%) and Vancomycin (55.55%) While the other antibiotics have varying rates of resistance: Azithromycin (38.88%), Doxycycline (30.55%), Levofloxacin and Clindamycin (16.66%), Gentamicin, Rifampin and Trimethoprime-sulfamethoxazole (11.11%), Chloramphenicol (8.33%). The results of using the microtiter plate method (MTPs) for biofilm identification revealed that all *S.aureus* isolates produced biofilm in different degrees, strong (30.55%), moderate (52.77%) and weak (16.66%). *S. aureus* had a 97.22% MRSA incidence. In summary, a high *mecA* percentage is associated with a high rate of biofilm formation and antibiotic resistance, so methicillin-resistant *S. aureus* is a developing issue, even in our neighborhood, that needs more care and attention.

Key words: Antibiotics susceptibility, MRSA, *mecA* gene, Biofilm formation.

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Introduction

The *Staphylococcus aureus* is a bacteria that gives a positive result on the gram stain. It is often present in the body's normal flora. It is present in the upper of breathing system and on the skin (1). Being an opportunistic bacteria, it can cause variety of infectious diseases, especially those that are contracted in hospitals and community. Recent studies have shown that influenza virus infection plus *S.aureus* infection increases the risk of pneumonia and death (2). Under a microscope this bacteria appears as "strings of grape" and has a diameter of about 0.8µm. It can grow either an aerobically or anaerobically, but it grows best at 37◦C, and pH7.4 (3). Their colonies have a diameter of 1∼2mm and they are spherical, glossy

and thick on blood agar plates (4). The majority of them are hemolytic, encircling the colonies on blood agar plates with a translucent hemolytic ring (5). The most prevalent pathogenic bacteria in hospitals and communities is Methicillin resistant *Staphylococcus aureus* (MRSA), whose capacity to build biofilm is thought to be the primary factor in its pathogenicity since it confers resistance to both host immune response and medications (6).

Biofilms are collections of single-celled bacteria that stick to inert surfaces to build multicellular formations. On the inert surfaces of implanted devices including joint replacements, prosthetic heart valves, and catheters, pathogenic bacteria can grow as biofilms (7). The formation of biofilms in vivo by *S. aureus* is thought to be a primary virulence factor that impacts its pathogenicity. Diabetes-related dietary and prosthetic hip infections have been linked to high rates of biofilm formation and increased resistance to antibiotics (8). Furthermore, a variety of illnesses in both human and animal hosts are caused by Staphylococcal infections, which are related to programmed cell death (9).

The aim of this work is to determine the distribution of the Methicillin Resistant -producing *S. aureus* in Iraqi patients and its possible correlation with biofilm formation and antibiotic susceptibility. This would aid in understanding the epidemiological aspects of the potentially genetic effect of *S. aureus* and their clinical implications and potential strategies for eliminating these strains.

Material and methods

Bacterial isolates and identification of *Staphylococcus aureus*

Between Novembers 2023to March 2024, one hundred fifty samples from several clinical sources, diagnoses made from patients visiting Baghdad hospitals (Al-Kindy teaching hospital, Imam al-kazemin city teaching hospital, Ghazi al-harery hospital, specialized burning hospital and Al-Karkh general hospital). These samples were Wound 50 (33.33%), Urine 60 (40%), Burns 6 (4%), Sputum 11(7.33%), Nasal swap 10 (6.66%), Throat swap 8(5.33%) and Cerebro Spinal Fluid (CSF) 5(3.33%), which were selected between the ages of 13 and 80 from both genders. All specimens were directly injected into blood agar and left to incubate for twenty-four hours at 37°C. By using the Gram stain response, catalase test, tube coagulase test, and growth on Mannitol salt agar (MSA) as a selective medium, colonies on culture plates identified and verified as *S. aureus*, according to Bergey's manual (10). Then, we were confirmed the identification by genotypic detection using PCR technique.

Phenotypic methicillin resistance detection

The cefoxitin disk diffusion test, which uses a 30µg disk on Mueller Hinton agar as an alternative to methicillin, was used to screen all detected *S. aureus* isolates for methicillin resistance. It was reported that inhibition zone diameters of ≤ 21mm were resistant to cefoxitin (methicillin) while >21 mm were sensitive to it. (11)

Molecular identification by PCR assay

Table (1): Primer used in this study

Primer Name	Primer's sequence (5′⟶3′)	Product size (bp)	Reference
***16S rRNA* PCR Primer**	AACCTACCTATAAGACTGGG	578	(12)
	CATTTCACCGCTACACATGG		
mecA	ACTGCTATCCACCCTCAAAC	163	(13)
	CTGGTGAAGTTGTAATCTGG		

Table (2): PCR Components

Components	Volume (µl)
Master Mix	12.5
Forward-primer (10 pmol/µl)	1
Reverse-primer (10 pmol/µl)	1
Nuclease Free Water	6.5
DNA	4
Total volume	25

Gel electrophoresis protocol

Agarose gel electrophoresis of amplified PCR product for the detection of genes run on 1.5 % agarose (70 volt for 80 min.) stained with ethidium bromide.

Table (3): PCR steps of *16SrRNA*

primer	Steps	Temperature (°C)	Duration	cycles
16SrRNA	Initial denaturation	94	5 min	1
	Denaturation	94	20 sec	35
	Annealing	55	45 sec	
	Elongation	72	45 sec	
	Final extension	72	10 min	1

Table (4): PCR steps of *mecA*

Primer	Steps	Temperature (°C)	Duration	Cycles
mecA	Initial denaturation	94	5 min	1
	Denaturation	94	2 min	35
	Annealing	57	2 min	
	Elongation	72	1 min	
	Final extension	72	7 min	1

Antibiotic susceptibility test

The Kirby-Bauer method was used on MHA (Hi-media) to evaluate *Staphylococcus aureus* isolates for antibiotic susceptibility (14). For 18 hours, plates were incubated at 37°C. After the incubation period, the diameter of the inhibitory zone was measured using the Clinical and Laboratory Standards Institute (CLSI 2023) (15) established standards. The following antibiotics were examined in this study: Cefoxitin (FOX:30 µg), Gentamycin (GEN: 10 µg), Azithromycin (AZM: 15µg), Doxycycline (DOX: 30 µg), Levofloxacin (LVX: 5 µg), Nitrofuration (NIT: 300 µg), Clindamycin (CLI: 2 µg),

Chloramphenicol (CHL: 30 µg), Rifampin (RIF: 5 µg), Trimethoprime-sulfamethoxazole (SXT: 1.25/23.75 µg) and Vancomycin according to the criteria recommended by Clinical and Laboratory Standards Institute (CLSI 2018) (16).

Biofilm formation assay

According to (Haney et al., 2021) (17), A sterile 96-well polystyrene microtiter plate with a flat bottom was filled with wells containing 180 µl of B.H.I. broth with 1% glucose and 20 microliters of bacterial suspension that were grown in brain heart infusion broth at 37 °C for 18-24 hours and it was adjusted to McFarland standard 0.5 (1.5×108CFU/ml). In brief; an aliquot each microtiter plate was covered and allowed to incubate aerobically for a full day at 37°C. Every isolate biofilm underwent three assays. BHI broth wells devoid of bacteria were used as a negative control and bacterial suspension only as Positive control. Each well's contents were aspirated, and the wells were then three times cleaned with 200 µl of distilled water to visualize biofilms. The 200 µl of methanol were then added and left for 15 minutes. After allowing each microtiter plate to air dry, 200 µl of 0.1% crystal violet solution was applied and allowed to sit at room temperature for five minutes. The previously mentioned wash procedure was repeated. After that, the plates were incubated for almost 30 minutes at 37°C to reach completely dry.

After that, for about ten minutes, 200µl of 100% ethanol was added and transferred into another microtiter plate. Finally, the optical density (OD) of each well was measured at 600 nm via an ELISA microtiter plate reader. Cut off value was calculated as the mean of OD600 of control plus 3 standard deviation. Biofilm intensity was categorized in accordance to criteria listed in Table (5):

Table(5): Biofilm intensity of *Staphylococcus aureus* isolates.

Mean OD600	Biofilm intensity
OD ≤ ODc	Non –adherent
ODc < OD ≤ 2*ODc	Weak
*ODc < OD ≤ 4*ODc	Moderate
4* ODc < OD	Strong

OD= optical density, ODc= cut off value (mean of OD600 of control plus 3 standard deviation).

Statistical analysis

To find the impact of different components in research parameters, the statistical analysis system, or SAS (2018) application, was run. The study employed the Chi-square test to compare percentages (0.05 and 0.01 probability) with statistical significance.

Result and discussion

Isolation and identification of *Staphylococcus aureus*

Total of 150 clinical samples were collected from three hospitals in Baghdad, Samples grown directly on mannitol salt agar and blood agar are identified by the presence of beta-hemolytic colonies on blood agar and yellow (golden) colonies that result from the fermentation of mannitol sugar, which turns phenol red golden. These samples also show resistance to high salt concentrations of MSA in the form of a selective medium. These provided *S. aureus*-specific morphological traits that were typical as shown in Figure (1).

Figure (1): Colonies of S. aureus growing for 24 hours at 37 °C on mannitol salt agar.

The specimens subjected to the standard biochemical tests. These samples have positive catalase and coagulase reactions. While, the oxidase tests yielded a negative result. According to the findings, only 36 samples (or 24%) received the standard biochemical testing and Staphylococcus aureus-specific morphological features. The distribution of sample proportions was as follows: Wound 10 (27%), Urine 16 (41%), Burns 1 (2%), Sputum 4 (11%), Nasal swab 2 (5%), Throat swab 1 (2%), CSF 2 (5%).These result agree with (Ahmed and Al-Daraghi, 2018) (19), who reported that *S.aureus* isolates in UTI was 15 and in wound was 9.

Molecular identification of *Staphylococcus aureus*

The amplifying of DNAs from phylogenetically divergent bacteria by targeting conserved regions of the *16SrRNA* gene have become a powerful tool in detection and identification of bacteria (20). The bacterial DNA amplified for this gene used PCR technique in a monoplex pattern by used specific primers, and the optimum condition. The results of the PCR reaction by gel agarose electrophoresis showed that 36 (100%) *S. aureus* isolates were positive for the *16S rRNA* gene (578 bp), as shown in Figure (2). These results are in agreement with (Shamkhi,; Saadedin, and Jassim , 2019) (21), which was the rate of clinical *S. aureus* isolates that gave a positive result for the*16SrRNA* gene is 100% as well.

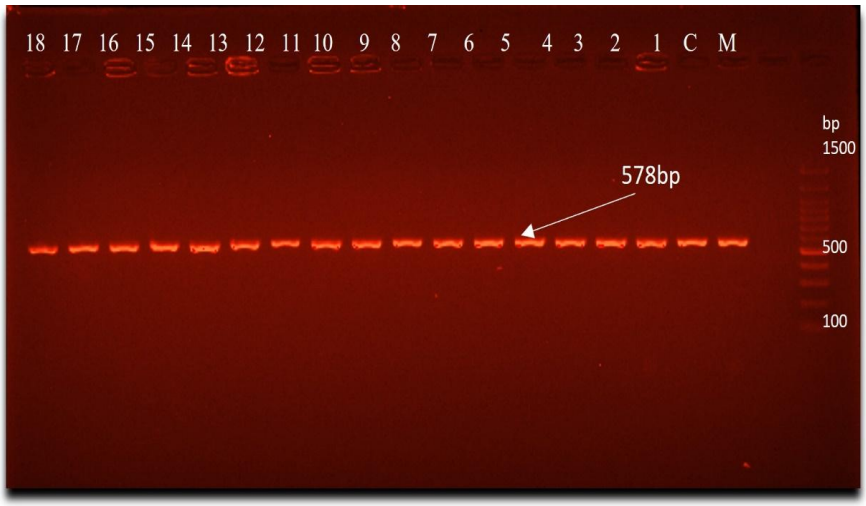


Figure (2): Lanes 1–18 of the ethidium bromide-stained agarose gel electrophoresis of the amplified PCR product for the identification of the *16SrRNA* gene (578 bp) done on 1.5% agarose (80 min at 70 volts); M: Marker DNA ladder (100 bp) and C: Negative control.

Antibiotics susceptibility test

The results in detailing of antibiotic susceptibility tests for *S.aureus* isolates each isolating source had been elucidated in table (6):

Table (6): Percentages of antibiotic susceptibility rate of 36 S. *aureus* isolates against 11 antibiotic agents.

Antibiotic	S	I	R	
	N (%) n=36			P-value
Cefoxitin	16 (44.44)	0	20 (55.55)	0.0001 **
Gentamicin	32 (88.88)	0	4 (11.11)	0.0001 **
Azithromycin	22 (61.11)	0	14 (38.88)	0.0001 **
Doxycycline	24(66.66)	1 (2.77)	11 (30.55)	0.0001 **
Levofloxacin	29 (80.55)	1 (2.77)	6 (16.66)	0.0001 **
Nitrofuration	36 (100)	0	0	0.0001 **
Clindamycin	27 (75)	3 (8.33)	6 (16.66)	0.0003 **
vancomycin	16 (44.44)	0	20 (55.55)	0.0001 **
Chloramphenicol	33 (91.66)	0	3 (8.33)	0.0001 **
Rifampin	32 (88.88)	0	4 (11.11)	0.0001 **
Trimethoprime-sulfamethoxazole	31 (86.11)	1 (2.77)	4 (11.11)	0.0001 **
P-value	0.0001 **	0.208 NS	0.0001 **	---
** ($P \leq 0.01$).				

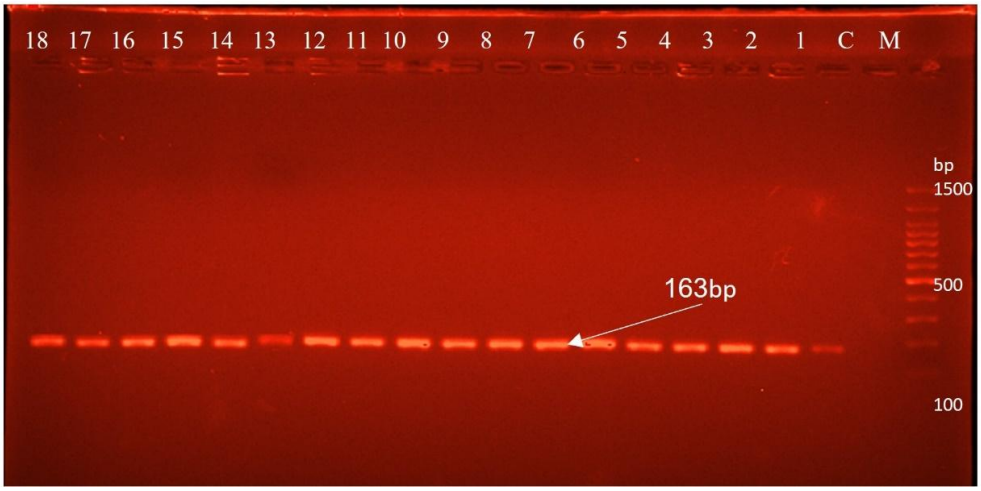
The results showed highest resistance of *S. aureus* isolates against to Cefoxitin (55.55%), Vancomycin (55.55%), then isolates began gradually to decline resistance with Azithromycin (38.88%), Doxycycline (30.55%), Levofloxacin and Clindamycin (16.66%), Gentamicin, Rifampin and Trimethoprime-sulfamethoxazole (11.11%), Chloramphenicol (8.33). While most of isolates were highly sensitive to Nitrofuration (100%), Chloramphenicol (91.66%), Gentamicin and Rifampin (88.88%), Trimethoprime-sulfamethoxazole (86.11%), Levofloxacin (80.55%), Clindamycin (75%), then isolates began gradually to decline sensitive to Doxycycline (66.66%), Azithromycin (61.11%), Cefoxitin (44.44%) and Vancomycin (44.44%). There were a highly significant differences in percentage isolates resistance from all isolating sources against assortment antibiotics and among different classes and sub-classes at ($P<0.01$).The results agreed with antibiotic susceptibility results of (Hamad, 2023) (22) who reported that most of *S. aureus* strains resistant to Gentamicin (9.4%), Levofloxacin (12.5%), while result disagree with Azithromycin (56.2%), Clindamycin (40.6%), Trimethoprime-sulfamethoxazole (21.9%), Vancomycin (0). Also result agreed with (Jabur and Kandala, 2022) (23), who reached to (6%) resist to Chloramphenicol, (0) resist to Nitrofuration and (43%) resist

to Azithromycin ,while disagree with Vancomycin (19%) , Doxycyclin (10%) and Cefoxitin (87%) ,but result agreed with (Maharjan, *et al*. 2021) (24),who reached to (60.8%) resist to Cefoxitin , while disagree with Chloramphenicol (56.8%) , also result agreed with (Awayid and Mohammad, 2022) (25), who reached to (5.8%) resist to Rifampin , while disagree with Gentamicin (85.2%) and Cefoxitin (100%), also result disagree with (Hantoosh, 2022) (26), who reached to (25%) resist to Rifampin and (26%) resist to Nitrofuration.

In addition to the above result agreed with (Hamad, 2023) (22),who reported that *S.aureus* isolates sensitive to Gentamicin (90.6%),Levofloxacin (87.5%) and Trimethoprime-sulfamethoxazole (78.1%), while result disagree with Azithromycin (43.7%), Clindamycin (59.4%) and Vancomycin (100%),also result agreed with (Jabur and Kandala, 2022) (23), who reached to (100%) sensitive to Nitrofuration, (94%) sensitive to Chloramphenicol and (57%) sensitive to Azithromycin , while disagree with Vancomycin (81%) , Doxycyclin (90%) and Cefoxitin (13%),but result agreed with (Maharjan *et al*. 2021) (24),who reached to (39.2%) resist to Cefoxitin , while disagree with Chloramphenicol (32.4%) , also result agreed with (Awayid, and Mohammad, 2022) (25), who reached to (94.2%) sensitive to Rifampin, while disagree with Gentamicin (17%) and Cefoxitin (0), also result disagree with (Hantoosh, 2022) (26),who reached to (75%) sensitive to Rifampin and (74%) sensitive to Nitrofuration. Antibiotic-resistant *S. aureus* is becoming a more serious issue, and treatment failures come with high financial and medical expenses. Numerous mobile genetic factors contribute to the spread of antibiotic resistance. Antibiotic resistance emerges through a variety of processes, including modified drug targets, enzymatic drug inactivation, enhanced efflux of antimicrobial chemicals, and changed drug accessibility (27). This bacterium has the ability to develop resistance to all clinically prescribed antibiotic classes. Resistance can arise via horizontally transferred resistance determinants or from de novo alterations in chromosomal gene (28).

Detection of *mecA* gene

The *mecA* gene was the private genetic marker for detection of Methicillin Resistance *S. aureus* (MRSA) (29). This gene's bacterial DNA was amplified using the PCR method in a monoplex pattern using particular primers and the ideal conditions. The amplification of the *mecA* gene produced a product of 163 bp, which was verified by agarose gel electrophoresis and captured on camera using an ultraviolet trans illuminator as shown in Figure (3). (97.22%) of clinical S. aureus isolates tested positive for the *mecA* gene. These findings are consistent with a study by (Kadhum and Abood, 2022) (30), which found that 100% of clinical *S. aureus* isolates tested positive for the *mecA* gene.



Figure(3): Lanes 1–18 of the ethidium bromide-stained agarose gel electrophoresis of the amplified PCR product for the identification of the *mecA* gene (163 bp) done on 1.5% agarose (90 min at 70 volts); M: Marker DNA ladder (100 bp) and C: Negative control.

Although *S. aureus's mecA* gene promotes methicillin resistance, sixteen phenotypic MRSA isolates from the study group lacked the *mecA gene*, suggesting that other resistant genes may be in charge of the isolates' resistance (31). These differences could be explained by the features of the population being studied. The prevalence of *S. aureus* may be significantly lower in a population receiving antibiotics at the time of sampling than in a sample from a hospital setting, where the high incidence of infectious individuals may result in a significantly higher prevalence. Techniques related to sampling and culture may also result in variances. The current study's *S. aureus* isolates seemed to have a high level of methicillin (Cefoxitin) resistance. These results are less than findings of (Al-Geobory, 2011) (32), who found a resistant percentage of 90.9%.

Misuse of B-lactam antibiotics by individuals may be the cause of the ongoing rise in resistance to these drugs. Different classes of antibiotics, including the more recent fluoroquinolones, Quinupristin/Dalfopristin (Streptogramin), Linezolid, and Vancomycin, are used to treat severe MRSA infections occurred by multidrug-resistant strains (33).

Antibiotic use in the recent year without a prescription, and use of antibiotics to treat colds were all substantially linked to MRSA colonization in the study population. This result is consistent with a prior study's finding that MRSA was substantially correlated with both recent and past antibiotic use (34). These findings demonstrated that one aspect to take into account in MRSA colonization is antibiotic misuse.

The prevalence of antibiotic susceptibility according to source of isolates

The percentages of Multidrug sensitive (MDS) and Multidrug resistance (MDR) isolates according to isolating sources had been elucidated in table (7):

Table (7): Percentages of prevalence of antibiotic susceptibility according to source of isolates.

Isolate sources	Total	Multidrug sensitive (MDS) isolates		Multidrug resistance (MDR) isolates		
	NO.	No.	%	No.	%	P-value
Wound	10	6	66.66	4	44.44	0.502 NS
Burns	1	0	0	1	100	0.647 NS
Urine	16	7	43.75	9	56.25	0.617 NS
Sputum	4	3	75	1	25	0.441 NS
CSF	2	2	100	0	0	0.316 NS
Nasal swab	2	2	100	0	0	0.327 NS
Throat swab	1	1	100	0	0	0.316 NS
P-value	--	--	0.0051 **	--	0.0045 **	---
	** (P≤0.01) , NS: Non-Significant.					

In current study (MDR) and (MDS) isolates had been existed in table (3-2) exhibited that there were a highly significant differences in percentages of MDR isolates and MDS isolates at $p<0.01$ in each isolating source and among seven sources. The distribution of MDR varied in respect to the site of infections, with highest number and percentage founded in burns (100%) and urine (56.25%) isolates, burn isolate were more resistant to Azithromycin, Gentamicin , Levofloxacin and Vancomycin with 100%, urine isolates were more resistance to Cefoxitin (56.25%) and Azithromycin (50%) followed with Doxycyclin and Vancomycin (43.75%).The decreasing in the percentage of susceptibility in wound isolates were (44.44%) with high resistant to Doxycyclin and Vancomycin with (50%), but Cefoxitin with (40%), While (25%) resistance percentage of sputum isolates with high resistant to Cefoxitin (75%), but Vancomycin with (50%).

These results were analyzed according to (Falagas and Karageorgopoulos, 2008) (35), who considered that the expressions “pan drug resistance” that mean a pathogen resists towards entire antibiotics, while, it only resists one or two antibiotic, which known as “extensive drug resistance”, but the resistance to more or equal three classes of antibiotics that indicates to “multidrug resistance”, these definitions are utilized in most places of the world.

These variances could be the result of variations in the geographic region, clinical specimen sources, genetic background, and isolate collection site (36). Prolonged surgery, intensive care, and the continual use of antibiotics that can choose a resistant bacteria population.

Biofilm formation of *S. aureus*

Table (8): Relationship between Biofilm formation and Total of *S. aureus* isolates had been elucidated.

Biofilm formation	Total of *S. aureus* isolates N (%) n=36
Strong	11 (30.55)
Moderate	19 (52.77)
Weak	6 (16.66)
Chi-Square (P-value)	7.242* (0.0267)
* ($P \leq 0.05$).	

According to the microtiter plate method, 36 isolates (100%) were able to adhere and form a thin layer that varied significantly in thickness (strong, moderate, and weak). It is possible that the isolates' varying capacities to form biofilm contributed to the variation in biofilm thickness. Findings regarding biofilm formation showed that Strong biofilm was formed by the 11 (30.55%) isolates, moderate biofilm by the 19 (52.77%) isolates, and weak biofilm by the 6 (16.66%) isolates. These outcomes concur with (Hamad, 2023) (22), who determined that all isolates of *S. aureus* were 100% biofilm-producing and percentage was distributed as (31.25%) strong, (53.12%) moderate and (15.6%) weak.

Because they form biofilms to protect themselves from harm, bacteria are more resistant to medications. Several biofilm constituents contribute through diverse antibiotic resistance routes (37). Antibiotic resistance is supported by the situation where a colony grows exponentially but then becomes sluggish or lacks growth/persisters. By using the efflux system and enzymes, the glycocalyx matrix renders antimicrobial agents inactive and protects the edge of the biofilm. Interestingly, a specific nutrient is absent from the cells in the midst of a biofilm, which inhibits their growth (38).

However, isolates showed different levels of biofilm formation: weak, moderate, and strong biofilm producers, with a high percentage for strong biofilm producers followed by moderate and weak biofilm producers. Various variables influence *S. aureus* adhesion, including the existence of Clumping factor, CNA (Collagen binding protein),EbpS (Elastin-binding protein),Eap/Map (Extracellular adherence protein/MHC analogous protein) ,FnBPs(proteins that bind to fibronectin) and Rich proteins with Ser-Asp (SDr) (39). So, different distribution of these adhesion factors affect the initial number of cells that succeeded in adhesion and biofilm formation.

Conclusion

This research found that a high *mecA* percentage is associated with a high rate of biofilm formation and antibiotic resistance, indicating a strong potential for biofilm formation. Therefore, the *mecA* percent indicates the pathogenicity of multidrug-resistant MRSA.

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