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The Production of Biofilm from Methicillin Resistant *Staphylococcus Aureus* Isolated from Post-Surgical Operation Inflammation

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ABSTRACT

Methicillin resistant *Staphylococcus aureus* (MRSA) is the most common pathogenic bacteria in the hospitals and communities, the ability to form biofilm is considered the main cause of *Staphylococcus* pathogenicity since it provides resistance to both antibiotics and host immune response, so this study was aimed to evaluate the biofilms formation and its association with antibiotic resistance in clinical isolates of MRSA, in order to achieve this aim, 237 samples were collected from different patients with wounds infections after surgeries and samples from operations galleries from varies hospitals in Baghdad ,68 isolates out of 237 were subjected to *Staphylococcus aureus* according to conventional methods Additionally, the *S. aureus* isolates were re-identified by molecular method. The results for Susceptibility of 68 *S.aureus* isolates against 22 antimicrobial agents revealed all isolates were resistant (100%) to Cefotaxime, and showed resistance to Ceftriaxone, Oxacillin, Cefipime, Cefoxitin, ampicillin, Penicillin, Nalidixicacid, erythromycin in percentage (99, 97,97,87, 87, 79, 75, 65)% respectively while other isolates showed variety in their resistant to other antimicrobial agents. while all 68 isolates were sensitive to Nitrofurantoin which is considered the most effective agent against the isolates. All 68 isolates were identified as MRSA on HiCromeMeReSa Agar Base medium, but 66 among 68 isolates were methicillin resistant in antibiotic sensitivity test, while 67 isolates had a positive reaction when detecting *mecA* gene using Polymerase Chain Reaction (PCR) technique. The finding of biofilm detection by microtiter plate method (MTPs) showed that 56 of 68 isolates produced biofilm in different degrees, while 66 (97%) among 68 isolates possessed the *icaAD* gene using PCR.

Keywords: Biofilm production, antibiotics resistance, MRSA, *icaAD*

تكوين الغشاء الحيوي من المكورات العنقودية الذهبية المقاومة للمثسلين المعزولة من اصابات العمليات الجراحية

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الخلاصه

تعد بكتريا المكورات العنقودية الذهبية المقاومه للمثسليين من الممرضات الاكثر شيوعا في المستشفيات والمجتمعات,ولها القدره على تكوين الغشاء الحيوي الذي يعد العامل الرئيسي لامراضيه المكورات العنقودية

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الذهبية لانه يوفر مقاومه لكل من المضادات الحيويه والاستجابيه المناعيه للمضيف. لذلك هدفت هذه الدراسه الى تقييم تكوين الاغشيه الحيويه وعلاقتها بالمقاومه للمضادات الحيويه في العزلات السريره لبيكتريا المكورات العنقوديه الذهبية المقاومه للميثيسيلين. لتحقيق هذا الهدف تم جمع 237 عينه من مختلف المرضى المصابين بالتهاب الجروح ما بعد العمليات الجراحية وعينات من صالات العمليات من مستشفيات مختلفه في بغداد, وظهرت النتائج ان 68 عزله من مجموع 237 تعود الى بكتريا المكورات العنقوديه الذهبية اعتمادا على الطرق الاعتيادية بالاضافه الى تشخيص العزلات باستخدام الطرق الجزيئية. اجري اختبار فحص الحساسيه لبكتريا المكورات العنقوديه الذهبية اتجاه 22 مضاد ميكروبي وظهرت النتائج ان جميع العزلات كانت مقاومه للسيفوتاكسيم بنسبه 100% , وكانت مقاومه للسفتراسكون, اوكساسيلين, سيفيبيم, سيفوكساتين, امبيسيلين, بنسيلين, نالديكسك اسيد, اريثرومايسين بنسب (99, 97, 97, 87, 87, 79, 75, 65)% على التوالي , في حين تباينت العزلات الاخرى في مقاومتها لباقي المضادات الميكروبيه. وظهرت (68) عزله حساسيتها لمضاد النترفورانتونين الذي يعد اكثر العوامل فاعليه اتجاه العزلات البكتيريه. جميع العزلات (68) شخصت على انها مقاومه للميثيسيلين على وسط الكروموكار, لكن 66 من مجموع 68 كانت مقاومه للميثيسيلين في اختبار الحساسيه للمضادات الحيويه, في حين كانت 67 عزله ذي نتيجته موجب للمورث (*mecA*) باستخدام تقنيه تفاعل البلمره المتسلسل. وظهرت نتائج الكشف عن الاغشيه الحيويه بطريقه صفيحه الزرع النسيجي قابليه 56 عزله من مجموع 68 على انتاج الغشاء الحيوي بدرجات مختلفه بينما كانت (66) (97%) من بين 68 عزله حاويه على المورث *icaAD* باستخدام تقنيه تفاعل البلمره المتسلسل.

Introduction

The *Staphylococcus aureus* is a Gram-positive commensal bacterium that mostly presents as normal bacterial flora in the human skin and mucous membranes [1]. These bacteria consider one of the important pathogens in community infections and hospitals which may cause many infectious diseases, such as soft tissue infections and mild skin, infective endocarditis, osteomyelitis, bacteremia, and fatal pneumonia [2,3]. This bacterium is approximately $\sim 0.8\mu\text{m}$ in diameter, arranged in "strings of grape" under a microscope, an aerobic or anaerobic and optimally grows at 37°C , and $\text{pH}7.4$ [4]. Their colonies on blood agar plates appear thick, shiny, and round with a diameter of $1\sim 2\text{mm}$ [5,6]. Most of them are hemolytic, forming a transparent hemolytic ring around the colonies on blood agar plates [6]. Many researches had indicated that detection of *S. aureus* by using PCR amplification of *nuc* gene has a great potential to be used for the rapid diagnosis of *S. aureus* [7].

Methicillin resistance in *S. aureus* bacteria is conferred by the *mecA* gene, which is responsible for coding for penicillin-binding protein 2a (PBP2a) that causes a decrease in binding affinity for the β -lactam antibiotics and also includes the penicillinase-resistant penicillin. The *mecA* gene is located on a mobile genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*). The complex *mecA* gene contains insertion sites for other mobile genetic elements such as (plasmids and transposons) that assist in the acquisition of resistance genes to other antibiotics [8]. Epidemiological studies have shown that hospital- and community-acquired MRSA infections are increasing in many parts of the world [9]. The virulence of *S. aureus* represents by their ability to form biofilms on implanted medical devices or damaged host tissue, especially in health care settings, where antibiotics usage is high and such biofilm formation represents a survival mechanism for the bacteria [10]. Therefore, many chronic infections are associated with the biofilm formation capability of the pathogenic bacteria [11]. Depending on other studies, the mechanisms of biofilm formation is by *icaADBC* operon-which encoded for polysaccharide intercellular adhesion (PIA) or poly-N-acetylglucosamine (PNAG) by both *S. epidermidis* and *S. aureus* [12, 13]. Among *ica* genes, *icaA* and *icaD* have been reported to play a main role in the formation of biofilms [14]. The *icaA* gene encodes for N-acetylglucosamine transferase that is involved in the synthesis of N-acetylglucosamine oligomers. moreover, *icaD* has been noticed to play a

critical role in the high level expression of N-acetylglucosamine transferase, leading to the phenotypic expression of capsular polysaccharides [15]. The goal of the present study was to identify the MRSA and determine the biofilm producing ability phenotypically and genetically and it is related to MRSA isolates from wound infections after surgery and other sources.

Material and Methods

Collection of clinical specimens

One hundred forty-seven clinical samples under study were collected from different patients with (wounds infections after surgeries) after gaining approval from the College of Science Research Ethics Committee according to (CSEC/0121/0008), and ninety samples were collected from operations galleries such as patient's bed, operational instruments, walls, ground, trolley, all these samples were collected from different hospitals in Baghdad which included (Ibn-alnefees hospital, Baghdad teaching hospital, Teaching laboratories, Ghazi alharery hospital, Children protecting hospital, Specialized burning hospital). Samples that obtained from patients of different ages male and female. All these samples were identified by techniques of standard microbiological and molecular analysis characterization [16].

Bacterial isolation and identification

All swab samples that were collected by transfer media were streaked later on mannitol salt agar and blood agar and then incubated at 37°C for 24h, the isolates were identified microbiologically (morphological and biochemical) test according to Bergey's manual of systematic bacteriology [16]. Additionally, the diagnosis of *S. aureus* isolates was confirmed by using PCR technique for detection of *nuc* gene which is considered highly specific for *S. aureus* [17].

Detection of MRSA isolates phenotypically.

HiCromeMeReSa Agar Base (Hi-Media, India) was used for the detection of MRSA isolates. The medium was prepared according to manufacturer instruction and after being sterilized by autoclave was cooled to a 50°C, the MeReSa selective supplement (FD229) with five ml sterile DW into each methicillin vial (2.0 mg of methicillin) as per the direction of the supplier (HiMedia-India), was added and mixed very well, then the medium was poured into Petri plates and cooled. In the present study, detection of MRSA was determined by direct culture of each swab on HiCrome medium and the plates were incubated for 24 hour at 35°C. the results revealed all cultures showing bluish green colored growth were detected as MRSA isolates, while all others are recorded as Methicillin sensitive Staphylococcus aureus (MSSA) isolates (HiMedia Labs. Products, India)

Antimicrobial susceptibility test

This test was accomplished by using disk diffusion method according to method described in Clinical and Laboratory Standards Institute (CLSI) guidelines [18]. The antimicrobial agents that used in this study were provided by mast company\UK as follows: Nitrofurantoin (NI\300), Erythromycin (E\15), Trimethoprim/Sulfamethoxazol (TS\25), Clindamycin (CD\2), Trimethoprim (TM\5), Chloramphenicol (C\30), Chloramphenicol (C\30), Gentamycin (GM\10), Amikacin (AK\30), Doxycycline (DXT\30), Ciprofloxacin (CIP\5), Nalidixic acid (NA\30), Vancomycin (VA\30), Penicillin (P\10), Cefoxitin (FOX\30), Cefotaxime (CTX\30), Ceftriaxone (CRO\30), Cefepime (FEP\30), Oxacillin (OX\1), Rifampicin (RA\5), Ampicillin (AM\10), Meropenem (MEM\10), Azithromycin (AZM\15)

Detection of biofilm formation phenotypically

Detection of biofilm formation phenotypically was done by using the microtiter plate method according to the method described by [19].

Aliquot of 100µl of bacterial isolates from preserved glycerol were inoculated in (5 ml) of Brain.Heart.Infusion (BHI) broth (HiMedia\India) and incubated overnight at 37°C. The cultures were then diluted to 1:100 using B.H.I. broth with 1% glucose. Individual wells of

sterile ninety-six well U- bottom tissue culture treated plates (Sigma-Aldrich, Costar, USA) were filled with 200µl of the diluted cultures. Negative control wells contained broth only. The plates were incubated at 37°C overnight. Then discard the growth gently and washed by D.W to remove non adherent cells and left to dry. Adherent biofilm to the wells that were formed by bacteria was fixed with 2% sodium acetate for 20 minutes. then added 200µl of crystal violet (Fluka\ Switzerland), 0.1% for each well for 10min, after that the stain was removed and washed with D.W and left to dry for 1 or 2 hour at room temperature. Stained well treated with 200µl of 30% acetic acid to solubilize the stain for 10min. Finally transferred the solubilized crystal violet into a new microtiter plate and The optical density of each well was measured by micro ELISA auto reader at a wavelength of 590 nm. The experiment was performed in duplicate. The interpretation of biofilm formation was done according to the criteria as described by Stepanovic *et al.*, [20]. The criteria of production biofilm was showed in Table 1.

Table 1- the criteria for biofilm production (20)

Average OD value	Biofilm production
$OD \leq OD_c$	No biofilm formation
$OD_c < OD \leq 2 \times OD_c$	Weak
$2 \times OD_c < OD \leq 4 \times OD_c$	Moderate
$4 \times OD_c < OD$	Strong

Optical density cut-off value (OD_c) = average of negative control + 3 × standard deviation of negative control.

Detection of (*nuc, mecA, icaAD*) genes using Polymerase Chain Reaction (PCR) technique DNA Extraction

The genomic DNA of all isolates was extracted using a DNA extraction kit specific for bacteria (Geneaid / Korea), then the concentration and purity were measured and the integrity of genomic DNA was investigated using gel electrophoresis method.

Polymerase Chain Reaction amplification

The Polymerase Chain Reactions (PCR) were amplified in a thermal cycler (labnet-USA). The reaction mixture was set up as follows: 12.5 µl of Go Taq® Green Master Mix was provided by (Biolabs–England), that contained Taq DNA polymerase, $MgCl_2$, deoxynucleosides (dNTP), reaction buffer, and two dyes (green and yellow) that allow monitoring of progress during electrophoresis, 1 µl from each forward and reverse primers (10pmol), 3µl of template (DNA) and sterile distilled water was added to achieve a total volume of 25 µl. All Amplification reaction was performed going on ice under aseptic condition in a laminar airflow cabinet. Negative control reaction contained all components with no DNA template in order that any contaminating DNA in the reaction would be amplified and detected on agarose gel (Thermo, USA). To analyze the PCR products, 10 µl of PCR mixture was loaded with 2% agarose in the presence of 100 b.p DNA ladder. After performing gel electrophoresis, the gel was exposed to U.V by using U.V Transilluminator [21].

Detection of (*nuc, mecA, icaAD*) genes using PCR technique

The presence of *nuc* gene confirmed the *Staphylococcus aureus* isolates which was designed according to the method described by [17]. The primer sequence and its amplicon size were listed in Table 2. While the program that was used for *nuc* gene amplification was listed in Table 3.

Detection of methicillin resistance was performed with PCR reaction by using a specific primer for *mecA* gene of *S.aureus* isolates which were used to confirm methicillin-resistant *Staphylococcus aureus* (MRSA) isolates were designed according to [22]. The primer sequence and its amplicon size were listed in Table 2. While the program that was used for *mecA* amplification was listed in Table 3. The detection of *icaAD* gene for *S.aureus* isolates using specific primers in order to determine biofilm formation one of them was designed according to (Newly designed primer) and the other was according to [23], the primers sequences and amplicon sizes were listed in Table 2. While the program that used for *icaAD* gene amplification was listed in Table 3.

Table 2- Oligonucleotide primers sequence and amplicon sizes

Primer	Sequence	Amplicon size bp
<i>nuc</i> -F	5'-GCGATTGATGGTGATACGGTT-3'	276bp[17]
<i>nuc</i> -R	5'-AGCCAAGCCTTGACGAAGCTAAAGC-3'	
<i>mecA</i> F	5'- GTG AAG ATA TAC CAA GTG ATT -3'	147bp [22]
<i>mecA</i> -R	5'- ATG CGC TAT AGA TTG AAA GGA T-3'	
<i>icaADD</i> -F	5'-GCACTCTTTATTGATAGTCGCT-3'	450bp Study designed
<i>icaADD</i> -R	5'-CTCTCCCTCTCTGCCATTTT-3'	
<i>icaAD</i> -F	5'-TATTCAATTTACAGTCGCAC-3'	470bp [23]
<i>icaAD</i> -R	5'-GATTCTCTCCCTCTCTGCCA-3'	

Table 3- The program of *nuc*, *mecA*, and *icaAD* primers using PCR analysis.

Steps	Temperature	Time
Initial Denaturation	95°C	5 min
Denaturation	94°C	30 sec
Annealing	56°C *,50°C**,52°C***	2min
Extension	72°C	1min
Final extension	72°C	5 min

* Annealing Temperature for *nuc* gene, No. of cycles = 25 cycle

** Annealing Temperature for *mec* gene, No. of cycles = 25 cycle

*** Annealing Temperature for *icaAD*, No. of cycles = 30 cycle

Results and discussion

Identification of *Staphylococcus aureus*

A total of two hundred thirty-seven clinical isolates that were collected from different hospitals in Baghdad were distributed as follows: wound swabs (95), Blood samples (52), and other swabs (90) that were collected from operations galleries, All these samples(two hundred thirty-seven). Firstly cultured on blood agar media and Mannitol Salt Agar to investigate only isolates that gave positive results from tolerating the high concentration of salt (7.5%) NaCl and fermenting the mannitol by convert the phenol red in the agar to yellow [24]. The results showed that only 68 (28.69%) of isolates gave positive results from 237 samples including 38(55.8%)from wounds, 22(32.3%) from Blood and 8(11.7%) from operation galleries. Surgical site infection (SSI) is the most common problem for patients who undergo operative procedures and represents the second most common type of healthcare-associated infection (HAI). [25,26] the *S.aureus* considered the most common causes of SSIs causing as many as 37% of cases of SSIs in community hospitals with MRSA of particular concern[27]. The nasal carriage of *S.aureus* is epidemiologically linked to the development of *S. aureus* SSI

notably in orthopedic surgery [28]. The Kaljimer *et al.* [29] reported, that nasal carriage of *S. aureus* was the only independent risk factor for *S. aureus* SSI after orthopedic implant surgery. In general, patients who are carriers *S. aureus* are two to nine times more likely to develop an SSI, and it has been shown that 85% of SSIs can be traced to endogenous colonization of the patients [30].

Characterization of *Staphylococcus aureus* isolates

After examining the samples by Gram stain, coagulase, oxidase, catalase tests, and cultured on mannitol salt agar. The isolates which were appeared as Gram-positive, coagulase positive, oxidase negative, catalase positive and mannitol positive were detected as *S. aureus*, all 68 isolates of *S. aureus* were previously detected morphologically and biochemical tests were further identified by the production of thermostable nuclease that is the main product for *nuc* gene [31], therefore only *S. aureus* isolates were recognized by the presence of *nuc* gene using specific primer. So the outcomes of PCR products for 68 isolates of *S. aureus* appeared positive results for specific gene sequences (*nuc* gene) that confirm all isolates were *S. aureus* (Figure 1). These results suggested that the thermostable nuclease-encoding *nuc* gene is highly specific for *S. aureus* and found in all *S. aureus* isolates in this study.



Figure 1- Agarose gelectrophoresis for PCR amplification products of (276bp)for *nuc* gene among *S. aureus* isolates in (2% agarose, 70 volts for 90min). Lane 1: Marker(100bp) DNA ladder .Lane(2-14):represents *S. aureus*, Lane NC: represents negative control.

Antibiotic susceptibility test of *S. aureus*

The sixty-eight isolates of *S. aureus* bacteria were examined towards [22] antibiotic discs for its susceptibility using the disk diffusion (Kirby-Bauer) method. The results as shown in Figure 2 that revealed the sixty-eight isolates were 100% resistant to Cefotaxime while 97% of isolates were resistant to Oxacillin which considers the alternative antibiotic of methicillin and in detection of MRSA isolates according to the recommendation of [18].The resistance of isolates for remaining antibiotics were; Ceftriaxone 99%, Cefipime 97%, Cefoxitin and ampicillin 87% , Penicillin79%, Nalidixicacid 75%, erythromycin 65 , azithromycin 43% , ciprofloxacin 26% ,trimethoprim 25% , vancomycin and gentamycin 19% , Meropenem 18% , Rifampin 15% ,Trimethoprim-sulfamethoxazole 13% , doxycycline 10% , clindamycin 9%, chloramphenicol 6% , amikacin 4% ,while all 68 isolates were sensitive to Nitrofurantoin. The results of antibiotic sensitivity showed high resistance of isolates to β -

lactam antibiotics this resistance may due to the presence of β -lactamase enzymes (that are involved in bacterial resistance to beta-lactam antibiotics) that hydrolyzed the β -lactam ring and inactivating the antibiotic [32] or due to the expression of *mecA* gene or *blaZ* gene that produced by *S.aureus* isolates or other alternative mechanisms [33]. Results of the current study showed that all *S.aureus* isolates have resistance to at least 8 antibiotics of different groups(MDR) that corresponds with the study of [34] and [35] were approved the multiple drug resistance of clinical isolates of *S.aureus* in Baghdad in percentage 100%, while [36] reported that 61% of clinical isolates were multiple drug resistance (MDR). These bacteria overcome the world [37], and the spread of this MDR *S.aureus* in the hospitals causes very dangerous infections for patients especially those with immunocompromised in intensive care units [38] The development of resistance for *S. aureus* to many antibiotics has been involved in the acquisition of determinants by horizontal gene transfer of mobile genetic elements [39]. Resistance can also emerge by the acquisition of mutations that alter the drug binding sites on molecular targets and by increasing the expression of endogenous efflux pumps.

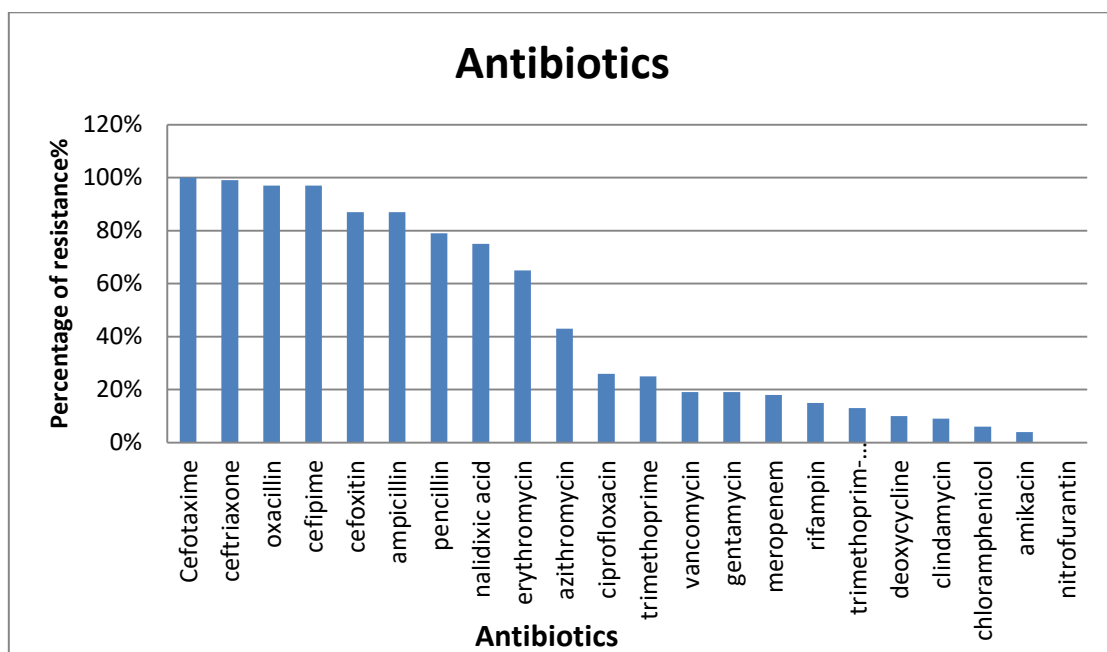


Figure 2-The antimicrobial susceptibility test for *S.aureus* isolates

Determination of methicillin resistant *S.aureus*

In order to detect MRSA isolates phenotypically using HiCrome MeReSa Agar Base, the findings as shown in Figure 3 revealed positive for all 68(100) isolates by giving bluish green colored growth on this media. Using this method in the detection of MRSA isolates because it is cost-effective; saves time and supplies powerful outcomes that mimic PCR method results [40]. Also, the authors mentioned that the concentration of 4 mg of (cefoxitin /liter) promotes the inhibition of all MSSA isolates and the growth of all MRSA isolates [41]. Alzaidi, [42] identified MRSA by using chromogenic agar and noticed that among 192 isolates of *S. aureus*, 26 (61.9%) from environment and 100 (66.6%) from the patients, while Nasser *et al* [43] showed that 77.9% of *S. aureus* isolated from Indian hospitals were identified as MRSA. Most studies showed the use of chromogenic media for identification of MRSA may generally provide acceptable diagnosis performance, although specificity and sensitivity of chromogenic media vary amongst suppliers [44].

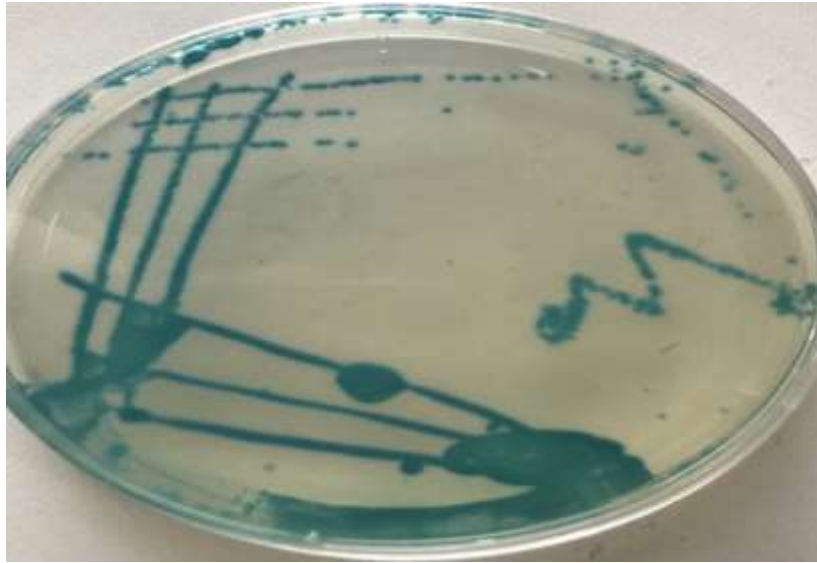


Figure 3-The MRSA isolate on HiCromeMeReSa Agar Base medium after incubation at 37°C for 24 hours.

Molecular analysis of *mecA* gene

Identifying methicillin resistant gene (*mecA*) in *S.aureus* is the best detection method for the presence of the gene that is responsible for the resistance or not, by using PCR for accurate identification with specific primer as mentioned in Table 3. The results showed that 67 isolates were possessed this gene (*mecA*) and absent in only one isolate (SAW8). Molecular size of this gene (*mecA*) was about (147bp) by comparing with ladder (100bp) as in Figure 4. These results are corresponding with [45] who mentioned that 98.7% of MRSA were possessing *mecA* gene. Therefore the isolates that contain *mecA* gene indicate that were MRSA, and this gene was responsible for the resistance towards β -lactam groups according to [46] who mentioned that all MRSA contain a copy of exogenous *mec* gene that codes for PBPs with low affinity for β -lactams (*mecA*, *mecB*, *mecC*, *mecD*) which causes resistance.

By comparing with the cefoxitin and oxacillin disc diffusion method, 65(96%) of isolates were resistant to oxacillin and 59(87%) of isolates were resistant to cefoxitin that means high prevalence of methicillin resistant genes in these isolates such as *mecA* gene as detected by a genetical method that 67(99%) of these isolates contain this gene therefore considered as MRSA.



Figure 4- Gel electrophoresis in agarose for products amplified using PCR (147bp) of *mecA* gene among *S.aureus* isolates in (2% agarose, 70 volts for 90min). Lane M: 100bp DNA ladder, Lane (2-13): represents *S.aureus* isolates. Lane NC: represents negative control.

Detection the production of biofilm by *S.aureus*

This method is most widely used and considered the standard test for detecting the formation of biofilms according to [47,48]. In order to detect the ability of sixty-eight isolates to form biofilm by using the MTP method and dependent on quantitatively measuring the absorbance for the stained (biofilms) at 590nm. The results showed that among 68 isolates, 36 (53%) isolates formed strong biofilm, 12(17.6%) of isolates formed moderate biofilm and 8(11.8%) of isolates formed weak biofilm while 12(17.6%) of isolates have not produced biofilm as shown in Table 4. Microtiter plate (MTPs) method has been intensively used in clinical research for screening of antimicrobial compounds [49, 50] in addition studying the formation of biofilm [51, 52]. The crystal violet (CV) is considered the most common method used for the formation of biofilm in MTPs, the staining with crystal violet which derived from the original method by Christensen *et al.* [53], which measured the biomass of biofilm in the bottom of the well. CV is considered the main dye, which stains the cells both dead and living by linking to the surface molecules that are negatively charged with polysaccharides in extracellular matrix for biofilms [54]. This is considered a bulk method which revealed information for the total amount of biofilms produced without revealing any information.

Table 4- The number and percentage of *S.aureus* isolates to biofilm formation using microtiter plate method.

Biofilm formation	Number of isolates	Percentage
Weak biofilm formation	8	(11.8%)
Moderate biofilm formation	12	(17.6%)
Strong biofilm formation	36	(53%)
Non biofilm formation	12	(17.6%)
Total number of isolates	68	(100%)

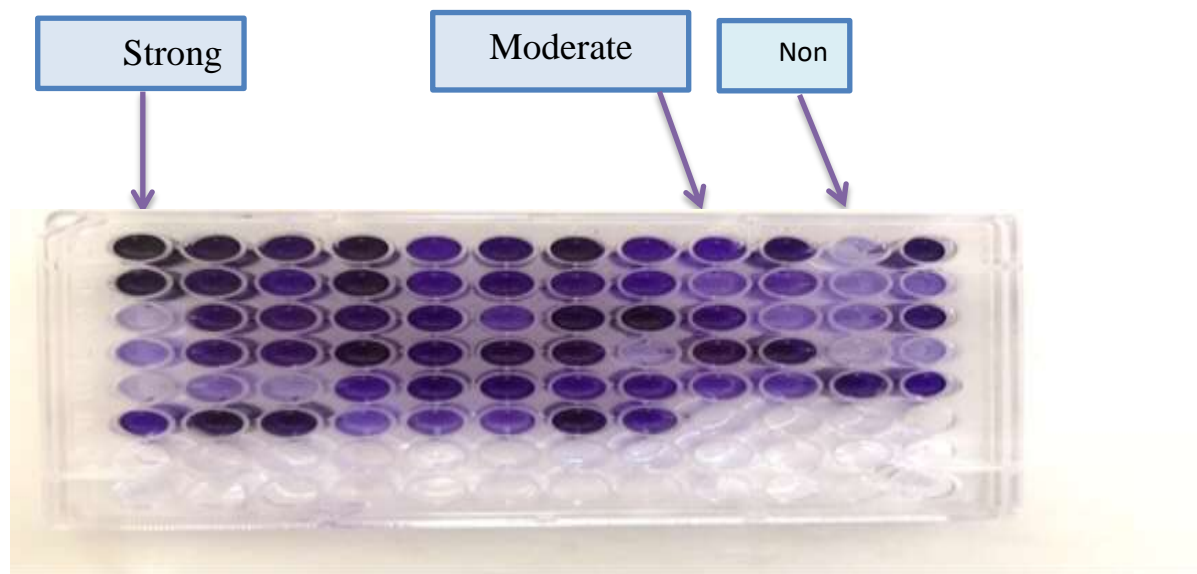


Figure 5-Microtiter plate method for biofilm production with different intensities (Strong, moderate, Non)

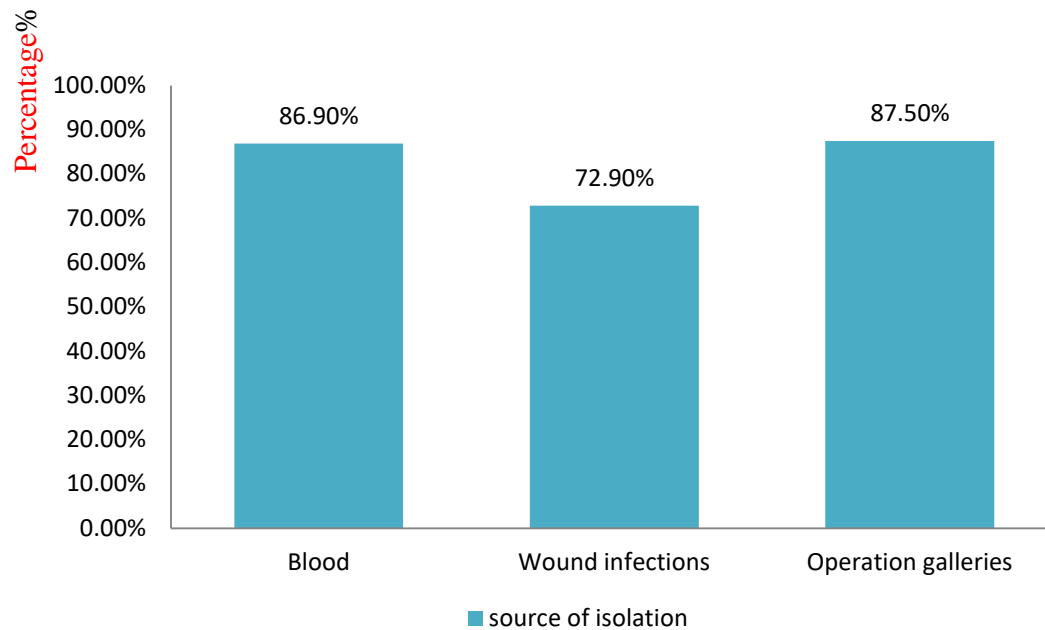


Figure 6- Biofilm production in *S.aureus* isolates from different sources by Microtiter plate (MTPs)

the ability to produce biofilm from *S.aureus* isolates was explained in Figure 6 according to its isolation source which includes three different sources (Wound infection after surgeries, blood samples, and operation galleries) the results revealed that *S.aureus* isolates in operation galleries formed biofilm higher than blood samples and Wound infection after surgeries and in percentage (87.5,86.9,72.9)% respectively, therefore this higher percentage in production of biofilm leads to many chronic infections that are associated with the ability of pathogenic bacteria to produce biofilm as recommended by [11].

Molecular analysis of *icaAD* gene

The molecular detection of *icaAD* gene was performed with specific primers using PCR technique as mentioned in Table 3. This technique was applied to all 68 isolates and the results showed that 66 (97%) isolates possess *icaAD* gene and only 2 (2.9%) isolates (SAB7, SAO7) do not have this gene. The molecular size of the PCR products was *icaAD* (470bp) and *icaADD* (450bp) compared with the (100bp) ladder as in Figure 7. The two primers gave the same results therefore preferred the last primer (newly designed primer) *icaADD* because gave very perfect results and consider modern.

These results corresponded with [55] who reported that high prevalence of *ica* genes among *S. aureus* bacteria, also this study indicates that high distribution of the *icaAD* gene among *S. aureus* isolates, but not related always to biofilms or slime layer formation in vitro.

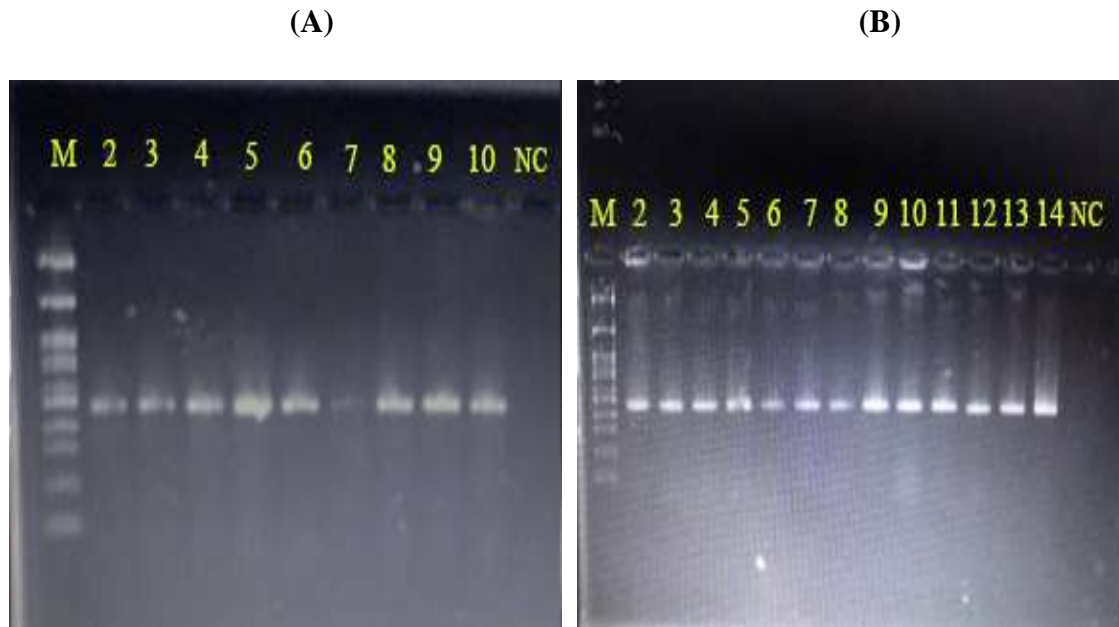


Figure 7-Agarose gel electrophoresis for PCR amplification products of (A:470, B: 450bp) *icaAD* gene among *S.aureus* isolates (2% agarose, 70 volts for 90min).Lane M: 100bp DNA ladder. Lane (A:2-10; B:2-41): represents *S.aureus* isolates. Lane NC: represents negative control.

Table 5- β-lactams antibiotic resistance pattern and biofilm formation in *S.aureus* isolates.

β –Lactam antibiotics	Co. µg/disk	MTPs (N=56)	<i>icaAD</i> (N=66)
Penicillin	10	46 (82.1%)	53 (80.3%)
Ceftriaxone	30	55 (98.2%)	65 (98.4%)
Cefoxitin	30	50 (89.2%)	57 (86.3%)
Cefotaxime	30	56 (100%)	66 (100%)
Cefepime	30	54 (96.4%)	65 (98.4%)
Meropenem	10	11 (19.6%)	12 (18.1%)
Oxacillin	1	55 (98.2%)	64 (96.9%)
Ampicillin	10	50 (89.2%)	58 (87.8%)

All isolates that formed biofilm detected by phenotypic and genotypic methods were tested for β-lactam antibiotics resistance as described above. The number and percentage refer to the biofilm producing resistant strains against the respective antibiotic, some variation showed between the phenotypical method (MTPs) and the presence of *icaAD* gene in isolates in the formation of biofilm. So, heterogeneity in the origins of genetics is considered a causative agent for variation between genotypic and phenotypic characterization but not because of the absence and presence of genes required for the biofilm as recommended by [56].

Comparison between MRSA and MSSA in their ability to produce biofilm and antibiotic resistance

The productions of biofilm in MRSA and MSSA is related to the presence of antibiotic resistance gene *mecA*, the *ica* operon, which is needed for the formation of biofilm polysaccharide, has been found to be repressed by high-level expression of the *mecA* gene [57]. MRSA strains tend to produce PIA-independent biofilms, whereas MSSA strains tend to produce PIA-dependent biofilms [58,59]. The absence of PIA is the most notable feature of PIA-independent biofilms. Their biofilms rely entirely on extracellular proteins and

extracellular DNA (eDNA) for structural stability, as shown by electron microscopy [57, 58]. While PIA-dependent their extracellular matrix is predominantly made up of PIA, which is made up of the polysaccharide poly- β (1-6)-N-acetylglucosamine, but it can also contain proteins, eDNA, and amyloid fibrils [60]. As a result, PIA-dependent biofilms seen in MSSA strains appear to be thicker and less penetrable than PIA-independent biofilms found in MRSA strains. The result of the present study revealed that only one isolate is considered as MSSA because of the absence of *mecA* gene (SAW8 unless it is positive for phenotypic method), this isolate forming strong biofilm in microtiter plate method and possess *icaAD* gene by detection in PCR. Therefore this isolate was resistant to 14(63.6%) antibiotics and sensitive to 7(31.8) antibiotics while intermediate only for 1(4.5) antibiotic among 22 antibiotics, this high percentage of resistance against antibiotics belongs to the production of PIA-dependent biofilms that being thicker and less penetrable than in MRSA as recommended by [58]. While another two isolates that do not possess *icaAD* and possess *mecA* gene (SAB7, SAO7) that are considered as MRSA showed resistance towards 8(36.3%) antibiotics only among 22 antibiotics this low percentage of resistance belongs to PIA-independent biofilms that depend only on extracellular proteins and extracellular DNA for their structural integrity [57,58].

Conclusion

In the present study demonstrates a high prevalence of MRSA isolates production biofilms from infections after surgeries and from operations galleries staphylococcal samples. The presence of *icaAD* gene and *mecA* gene in *S.aureus* bacteria leads to resistance to several antibiotics that leads to convert the infections from acute to chronic and that is difficult to treat later. To overcome recurrent infections and chronic, the biofilm detection of microorganisms is important, in order to detect the activity of anti-biofilm agents towards biofilm, and detection the agents for antibacterial activity against biofilm-embedded microorganisms.

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