Biofilm Formation and Molecular Analysis of Icaabcd Genes Among *Staphylococcus Aureus* Strains Isolated from Different Clinical Sources

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Abstract

Staphylococcus aureus is a major hospital and community pathogen having the aptitude to cause a wide variety of infections in human. The biofilm production is an important phenomenon by bacteria such as *S. aureus* that contribute to the multiple drug resistance. Moreover, biofilm formation by multidrug-resistant *S. aureus* causes evading from immune responses and is recognized as one factor contributing to chronic or persistent infections. It was demonstrated that the ica-encoded genes lead to the biosynthesis of polysaccharide adhesion (PIA) molecules, and may be involved in the accumulation phase of biofilm formation. Different studies have shown the decisive role of the ica gene as virulence factors in *staphylococcal* infections. This study was carried out to detect biofilm formation and presence of several related genes among multidrug-resistant (MDR) isolates of *S. aureus*. One hundred and fifty different clinical samples were collected from various clinical sources and healthcare workers in Baghdad City. Isolates were identified by conventional methods (cultural, microscopic and biochemical tests) in addition to the identification by the VITEK® 2 Compact and fifty isolates were recorded as *Staphylococcus aureus*. The antibiotic susceptibility profile for the isolates were tested against twelve antibiotics that belonged to different classes using disc diffusion method (Beta-lactam, lincosamide, macrolides, aminoglycoside, quinolones, ansamycins, tetracyclines, glycopeptides). The *S. aureus* isolates that showed multi-drug resistance against the tested antibiotics were further tested for their ability to produce biofilm using micro-titer plate methods. Sixteen isolates were resistant to more than six antibiotics and recorded as strong and moderate biofilm producers; these isolates were further tested using a polymerase chain reaction (PCR) technique to detect the presence of icaABCD genes using specific primers. The results revealed that all the tested S. aureus isolates were identified to have these genes.

1. Introduction

Staphylococcus aureus is a Gram-positive bacterium that represents a major public health concern. Approximately (25-30%) of healthy persons was recorded to be colonized with *S. aureus* [1, 2]. Up to 80% of people are considering with a high risk of S. aureus colonization, including (diabetic persons, health care workers, patients with weak immunity,individuals with long hospital stays, recipients with previous methicillin-resistant *S. aureus* (MRSA) infection and persons with skin infections. This pathogen is implicated in both community-acquired and nosocomial infections with considerable morbidity [3]. *S. aureus* cause several diseases that ranged from soft tissue infections to sever disease such as endocarditis, pneumonia, septicemia and catheter related infections [4]. Cases such as immunosuppression or hospitalization for long period resulted in the progression of invasive opportunistic *S. aureus* that are found in the mucous membranes and skin as they become more virulent and able to produce respiratory, skin diseases or bacteremia [5].The ability of *S. aureus* to cause infections is related to the expression of various virulence factors like surface proteins,biofilm, exoenzymes, exotoxins and exfoliative toxins [6, 7]. All these factors enabled to adherence of bacteria to the tissues causing pathogenesis and invade the immune system causing toxic effect [8]. Biofilm production is one of the important virulence factors of pathogenic bacteria such as *S. aureus* that contribute to their pathogensity and multiple drug resistance as it represent a protective way for the survival of bacteria that enabling it to adapt their surroundings [9]. This biofilm, which is an extracellular polymer matrix, surrounded a population of microbial cells and enhanced their attachment to the surfaces represent a perfect barrier against applied antibiotics and help the bacteria to evade the immune system [10, 11]. Infections with biofilm producing bacteria is of major concern in nosocomial infections because they are very difficult to eradicate due to the ability of these bacteria to evade host defenses mechanisms during their growth in a biofilms that protects microorganisms from opsonophagocytosis. In addition, they develop a tolerance to traditional antimicrobials that designated to eliminate free-floating, single-cell (planktonic) bacteria specially those associated with the inhibition of cell wall biosynthesis making them a serious health risk. According to many studies, different genes are involved in biofilm production. The intracellular adhesion (ica) genes are important for encoding of proteins mediating the synthesis of the polysaccharide intercellular adhesin (PIA), The N-acetylglucosamyltransferase encoded by icaA gene. The de-acetylation of mature PIA and the trans-membrane protein encoded by icaB (polysaccharide deacetylase), the trans-membrane protein encoded by icaC (transporter of PIA) [12].

Among *ica* genes, *icaA* and *icaD*, have been reported to play a significant role in biofilm formation [13]. hence detecting the ica locus in *S. aureus isolates*, and phenotypic detection of biofilm, is important, and it would improve the diagnostic decision for selecting the appropriate treatment. The main goal of the current study was to assess the biofilm production of *S. aureus* isolates obtained from different

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clinical sources. In addition, the possible relationship between the biofilm formation ability and ica cluster genes in clinical isolates of *S. aureus* strains were examined.

2. Material and Methods

Bacterial isolates

Fifty *S. aureus* strains were isolated from patients (skin swab, nasal swab, ear swab), health care workers (skin swab and nasal swab), and operation theater (various places of operation theater before and after sterilization) in this study. Isolates were identified morphologically and biochemically by standard laboratory methods. The coagulase was performed for the discrimination of *S. aureus* from coagulase-negative *staphylococci* (CoNS). Colonies that were coagulase positive and appeared as golden or cream on MSA plates were considered as *S. aureus* and When these bacterial isolates were grown on blood agar media, -hemolysis around their colonies was produced [14]. The primary biochemical test for these isolates is shown in table (1). These characteristics came in accordance with the cultural characteristics of *S. aureus.*After identification, the staphylococcal isolates were maintained in brain heart infusion broth (BHI), to which 15% glycerol was added, and stored at –20°C.

Table (1): Biochemical tests for staphylococcus. Aureus isolates

Test	**Result**
Mannitol fermentation	Positive with yellow colonies
Catalase	Positive
Coagulase	Positive
Oxidase	Negative

Antibiotic susceptibility test

All *S. aureus* isolates were taken and inoculated on Muller Hinton agar to examine their antibiotic resistance patterns according to Kirby-Bauer disk-diffusion technique. There after the antimicrobial discs were placed on the agar by some sterile forceps pressed firmly to ensure contact with the agar. Subsequently the plates were inverted and incubated for 24 hours at 37 °C according to the Clinical and Laboratory Standards Institute (CLSI, 2021) by measuring the inhibition zone diameter (mm).

Biofilm assay

S. aureus strains were incubated in (BHI) at 37°C for 24 h then grown colonies suspended in sterile physiological saline with turbidity adjusted to 0.5 McFarland. The 96 well microdilution plates (Cell and Tissue Culture plates, flat well bottom) were filled with 180 µl brain hear jt infusion Broth (BHI) supplemented with 1% glucose and 20 µl of bacterial suspension added to each well. After incubation at 37 C° for 24 h, broth was carefully drawn off and the plates were gently washed three times with sterile phosphate-buffered saline (PBS) and left in the room temperature for drying. In the next step 0.1% crystal violet solution was used as stain for 15 min. The wells were subsequently washed thrice with sterile PBS to wash off the excess crystal violet. crystal violet bound to the biofilm was extracted with 200 ml of 33% glacial acetic acid, Finally the absorbance was determined by ELISA reader; an OD of 630 nm >0.12 was regarded as a biofilm positive sample. Biofilm-producing isolates were selected for biofilm gene determination with molecular PCR method.

Table (2): Classification of bacterial adherence according to the tissue culture platemethod

Mean OD values	**Adherence Biofilm Formation**
<0.120	Non adherent / Weakly adherent
0.120 – 0.38	Moderately adherent
>0.38	Strongly adherent

Detection of ica operon genes by PCR

The isolates that were previously identified by morphological and biochemical characteristics as *S. aureus* and appear as MDR that had the ability to form biofilm were selected for molecular screening of biofilm icaABCD genes using PCR method. The selected colony was cultivated in 1 ml BHI for 24 h at 37°C. The bacterial genomic DNA was extracted with a ABIOpureTM Total DNA (ABIOpure, USA) as recommended by the manufacturer. Amplification of biofilm genes icaABCD was achieved by using specific primers [15] indicated in table (3). These primers were supplied by (Macrogen Company) in lyophilized form of different picomols concentration. Lyophilized Primers were dissolved in a nuclease free water to give a final concentration of 100pmol/µl as a stock solution. A working solution of these primers was prepared by adding 10µl of primer stock solution (stored at freezer -20 C) to 90µl of nuclease free water to obtain a working primer solution of 10pmol/µl.

Primer Name	Seq.	Annealing Temp. (°C)	Product Size (bp)
icaA-F	5`-GAGGTAAAGCCAACGCACTC-3`	60	151
icaA-R	5`-CCTGTAACCGCACCAAGTTT-3`		
icaB-F	5`-ATACCGGCGACTGGGTTTAT-3`		141
icaB-R	5`-TTGCAAATCGTGGGTATGTGT-3`		
icaC-F	5`-CTTGGGTATTTGCACGCATT-3`		209
icaC-R	5`-GCAATATCATGCCGACACCT-3`		
icaD-F	5`-ACCCAACGCTAAAATCATCG-3`		211
icaD-R	5`-GCGAAAATGCCCATAGTTTC-3`		

Table (3): PCR amplification program of icaABCD genes.

Table (3): Oligonucleotide primers used for the amplification of biofilm formation genes icaABCD

Steps	**°C**	**m: s**	**Cycle**
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	35
Annealing	60	00:30	
Extension	72	00:30	
Final extension	72	07:00	1
Hold	10	10:00	

3. Results and Discussion

One hundred and fifty different clinical samples that were collected from various clinical sources and healthcare workers in Baghdad Hospitals. Fifty isolates (33%) were characterized as *S. aureus* depending on the conventional cultural, biochemical and microscopic examination in addition to a confirmatory test by the VITEK® 2 Compact system. The rest of the clinical samples which represent (67%) were found to be related to different genus of pathogenic bacteria. The antibiotic susceptibility profile for the *S. aureus* isolates against twelve types of the antibiotics (Cefoxitin, Ceftazidime, Penicillin G, Clindamycin, Erythromycin, Azithromycin, Gentamicin,

Vancomycin, Ciprofloxacin, Rifampin, Tetracycline and TrimethoprimSulfamethoxazole) that act to inhibit the bacteria through different mechanisms was tested and the results are listed in figure (1) compared with the published data update by the CLSI (2021).

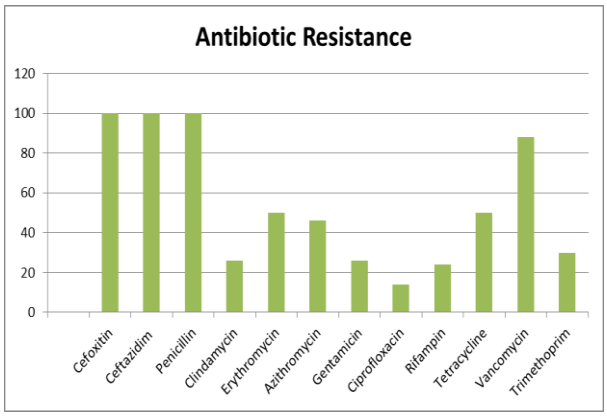

Figure (1): Percentage of resistance among S. aureus isolate.

In our study fifty isolates of *S. aureus* presented different degrees of resistance against diverse antibiotics. Generally, cefoxitin, Penicillin and Ceftazidim showed resistance to all isolates 100% (50/50), as well as Vancomycin resistant that reach 88% (44/50). Moderate resistance was recorded to Azithromycin 46% (23/50), Erythromycin and tetracycline 50% (25/50). On the other hand, high sensitivity rate was recorded for *S. aureus* against to Ciprofloxacin 80% (40), Clindamycin and Rifampin 72% (36/50), gentamycin 37 (74%), and trimethoprim-sulfamethoxazole 70% (35/50) which represent the effective antibiotics against *S. aureus* isolates.Biofilm formation was observed in 44 (88%) of them. Strong biofilm was detected in 16 (32%) of the tested isolates, while 16 (32%) of them were able to form moderate biofilms with an OD of 630 nm and mean values of ELISA reader was in ranged between (0.2 – 0.38). On the other hand, 12 (24%) were recognized as weak biofilm and 6 (12%) isolate was considered as a non-producer as shown in table (4). Among these biofilm producing isolates we selected sixten that showed MDR, as they considered being more pathogenic.

Table (4): Biofilm-forming ability by *Staphylococcus aureus* using microtiter plate method.

Type of biofilm formation	Number (No.)	Percentage (%)
Strong	16	(32%)
Moderate	16	(32%)
Weak	12	(24%)
Non-producers	6	(12%)
*(measured at wavelength of 630 nm)		

Depending on the results of the PCR, sixteen out of the fifty selected *Staph. aureus* isolates that previously recorded as multidrug resistant. PCR technique was used to detect the presence of (*icaA, icaB, icaC*, and *icaD*) genes. These genes were detected by presence of single band with a molecular weight of (151 bp, 141 bp, 209 bp and 211 bp) for (*icaA, icaB, icaC*, and *icaD* genes respectively) compared with marker used as described in figure (2).

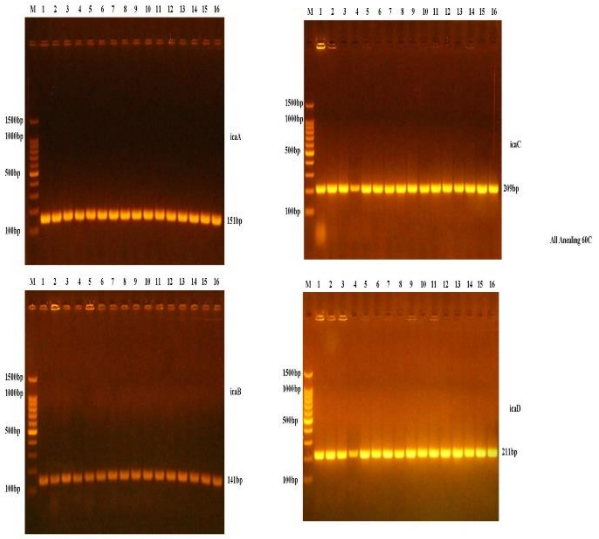

Figure (2): PCR profiles for the amplified genes (icaA, icaB, icaC, and icaD) of Staphylococcus aureus samples were fractionated on agarose (1.5%) at 100v/m Amp for 90 min.

S. aureus is an important cause of different diseases in humans and animals [16]. In *S. aureus* the *ica* is an important genes in production of slime and usually the *S. aureus* strains that harbor *ica*ADBC cluster of genes are capable to form biofilm [17]. The biofilm matrix maturation is initiated by polysaccharide intercellular adhesin (PIA), which is synthesis by the intercellular adhesin (*ica*) *icaA, icaB, icaC*, *icaD*, and regulatory gene (*icaR*) (18). These genes encode (*ICAA, ICAB*, *ICAC* and *ICAD*) proteins. The slime production is stimulated by *icaA* and *icaD* genes [18] (19). And *icaC* performs as polysaccharides receptor. While, *icaB* role is not completely elucidated [19].

Our results revealed that all the tested *S. aureus* isolates were identified to have these genes. The current result came in accordance with [20] as they reported the presence of *icaA, icaB* and *icaD* genes in all the tested isolates. In addition, [21] found that (84%) of the *S. aureus* strains carry (*icaADBC*) genes. We found a significant relationship between biofilm formations of *S. aureus* isolates and antibiotic resistance when we compared our results for antimicrobial resistance pattern and genotypic biofilm formation of *S. aureus* isolates; a result that improves the specificity of the molecular method (PCR) for detection of genes involved in biofilm formation. Suggesting that these isolates are more capable of PIA formation and therefore strong biofilm producers so they considered causing chronic and persistent infections.

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