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Genetic Identification of Biofilm Formation using Multiplex-PCR in *Staphylococcus aureus* Isolated From Indwelling Catheter Surfaces

***Raed Ali Hussein**

Faculty of Sciences, Dept. Laboratory Investigations, Kufa University, Iraq

Abstract : Objective: *Staphylococcus aureus* is the infectious agent in the most severe catheter-related sepsis. It is also associated with biofilm-related diseases. This study was aimed to determine the biofilm producing ability through investigating the presence of *ica*ADBC operon genes in *S.aureus* isolated from in dwelling catheter surfaces as well as the relation between biofilm formation and presence of this operon.
Methods: Segments of catheters removed from 43patients were cultured on mannitol salt agar plates. *S. aureus* were identified with the API- Staph System. Isolates of *S. aureus* were studied for biofilm producing capacity. The detection of *ica*ADBC operon genes in all *S.aureus* isolate was done using Multiplex-PCR.
Results: From all the 43 samples included in this study 28 (65%) isolates were characterized as *S. aureus.* The biofilm production assay results showed that 19(67.8%) of the 28 tested *S. aureus* isolates were attached at different amount. Attachment abilities in 10 (35.7%)isolates were strong, 6(21.4%)isolates were moderate, 4 (14.2%)isolates were weak and 8(28.5%) had no attachment. The Multiplex-PCR results showed that of 28*S.aureus*isolatesrevealed the presence of *ica*ADBC operon genes in 22 (78.5%) isolates. *ica*AB gene was present in 21(75%) isolates whereas *ica* D and *ica*C genes were present in 27 (96.4%)and 26 (92.8%) of *S. aureus* isolates respectively.
Conclusion: The study of biofilm formation and genetics characteristics of biofilm genes in different isolates of *S. aureus* may allow a better understanding of the complex process of biofilm formation and infections caused by this microorganism.
Keyword : *ica*ADBC, Biofilm, and Indwelling Catheter.

1. Introduction:

Staphylococci in general are considered as the most frequent causes of biofilm-associated infections and it is among the most bacteria that infect any medical device penetrates the skin surfaces, such as indwelling catheters (1-3).*Staphylococcus aureus*is an important cause of diver nosocomial and community acquired infections (4). The most important virulence factor ability of this bacterium is the adherence and biofilm formation on host surfaces (5).

S. aureus is an important pathogen, especially in hospital settings, where it is a major source of life-threatening bloodstream infections. It has factors that enable it to adhere to specific host substrates, evade host defenses, and resist antibiotic therapy (6). One way in which bacteria become resistant to antibiotics and host defenses is through biofilm formation (7). *S. aureus* adheres proficiently to abiotic surfaces as well as biotic ones and is a problem in medical situations where implants, such as indwelling catheters and prosthetic joints,

are employed (8). Catheter infections are surprisingly ubiquitous. One group reported the observation that bacterial biofilms were present on all used catheters they inspected by electron microscopy (9). *S. aureus* is the infectious agent in the most severe and costly episodes of catheter-related sepsis (10). It is also associated with biofilm-related diseases such as infectious arthritis, endocarditis, and cystic fibrosis (11, 12).

S. aureus has the ability to attach to indwelling medical devices through direct interaction with the device's polymer surface or by establishing connections to human matrix proteins after those proteins have covered the device. Then, proliferation proceeds through the production of an extracellular matrix that contributes to intercellular aggregation; this matrix consists of several secreted polymers such as exopolysaccharide, teichoic acids, and specific proteins as well as DNA originating from lysed cells (13).

Biofilm formation is influenced by a number of factors among which, the most important is synthesis of the polysaccharide intercellular adhesion (PIA) by the organism (14). The enzymes required for PIA synthesisare encoded within the intracellular adhesion operon (*ica*operon)(15).

The *ica* operon consist of the four genes *ica*A, *ica*D, *ica*B, and *ica*C genes (*ica*ADBC)(16), these genes encodes proteins mediating the synthesis of the PIA (4). The *ica*A gene and *ica*D gene together are encoding the N-acetylglucosamyltransferase, activity together with the activity of *ica*C encodes the transmembrane protein, which is involved in to secretion and elongation of the growing polysaccharide. *ica*B is the deacetylase responsible for the de-acetylation of mature PIA and the transmembrane protein. (17). Expression of this operon increased under anaerobic internal environment of biofilm (18).

This study was aimed to determine the biofilm producing ability by molecular method through investigating the presence of *ica*ADBC operon genes in *S.aureus* isolated from in dwelling catheter surfaces as well as the relation between biofilm formation and presence of this operon.

2. Material and Methods

2.1.ClinicalIsolates:

Forty three patients were included in this study; all of them were received indwelling catheters in Al-Sader medical city and AL-Hakim general hospital in Al-Najaf, Iraq. Segments of catheters removed from the patients were cut understerile conditions and cultured on mannitol salt agar plates. After incubation at 37°C, for up to two days, the colonies with yellow color and had yellow zone which suspected to be *S. aureus* were identified with the API-Staph System (bioMe´rieux, Marcy l'Etoile, France) for conformation detection.

2.2.Biofilm Formation Assay:

Isolates of *S. aureus* were studied for biofilm producing capacity by microtiter plate method described by Christensen *et al.* (19) modified by Wojtyczka*et al.* (20).Bacteria were suspended in Muller-Hinton Broth (HIMEDIA, India)in density equivalent to 0.5 McFarland standards and 100 µL from each bacterial suspension was inoculated onto 96-well tissue microtiter plates. The plates were incubated at 37°C for 24 h. Then the medium was removed and the wells were washed three times with phosphate buffer saline. Then 150 µL of 1%crystal violet was added to each well and incubated for 30 min at room temperature. The dye was removed by four times wash with sterile deionized water. The samples were incubated with 200 µL of95% isopropanol in 1 M HCl for 5 min. Finally, 100 µL of colored isopropanol from each well was transferred to a fresh microtiter plate. The optical density (OD) of suspension was measured at wavelength of 490 nm (A_{490}) with ELISA microplate reader. The negative control comprised all reagents but without bacterial inoculums.

According to Wojtyczka *et al.* (20), the samples with the OD > 0.11 considered positive, bacterial strains were considered non-adherent when the OD was equal or lower than 0.11; weakly adherent when the OD was higher than 0.11 or equal or lower than 0.17 and strongly adherent when the OD was higher than 0.17 (table 1).

Table 1: Classification of biofilm formation abilities

The optical density (OD) at A_{490}	Biofilm formation abilities
OD $\leq$ 0.11	non-adherent
0.11 <OD $\leq$ 0.17	weakly adherent
OD > 0.17	strongly adherent

2.3. DNA isolation:

Several colonies of each *S. aureus* isolate were suspended in 1.5 ml microfuge tube containing 1ml of phosphate buffer saline (pH 7.5). Genomic DNA was extracted with the Wizard® Genomic DNA Purification Kit (Promega, USA) for isolating Genomic DNA from Gram positive bacteria. According to the kit manual each sample tube were centrifuged at 13,000–16,000 × g for 2minutes to pellet the cells and the supernatant was removed, then 10mg/ml lysozyme (Sigma) were added to the resuspended cell pellet, and gently pipeted to mix. The purpose of this pretreatment is to weaken the cell wall so that efficient cell lysis can take place, and then proceeding with the kit protocol.

2.4. Detection of *ica*ADBC operon genes using Multiplex-PCR assay:

The detection of *ica*ADBC operon genes in all *S.aureus* isolate was done using the primer pairs listed in table (2). The primers synthesized by AccuOligo® Bioneer Corporation USA. The amplification was done according to the Experimental Protocol of AccuPower® TLA PCR PreMix tube, the PCR reaction mixture was performed using 5µl of the template DNA, 4µl of each primer (10pmole/µl, 2µl forward and 2µl reverse), were added to each AccuPower® TLA PCR PreMix tube. Distilled water was added to the tubes to a final volume of 20 µl.

Table 2: List of primers used in this study

Gene	Primer	Product Size	Reference
*ica*A B	F 5'-AAACTT GGT GCG GTT ACA GG-3' R 5'-TCT GGG CCT GAC CAT GTT G-3'	750(bp)	(25)
*ica*C	F 5-ATGGGACGGATTCCATGAAAAAGA-3 R5-TAATAAGCATTAATGTTCAATT-3	1100 (bp)	(21)
*ica*D	F 5-ATGGTCAAGCCCAGACAGAG-3 R 5-AGTATTTTCAATGTTTAAAGCAA-3	198 (bp)	(22)

The PCR program consisted of initial denaturation at 94 ºC for 5 min, and 30 cycles involving denaturation at 94 ºC for 1 min, and annealing at 55 ºC for 1 minand extension at 72 ºC for 1 min followed by a final extension at 72 ºC for 10 min. All reaction mixtures were held at 4°C (23,24).

3. Results:

3.1. Bacterial Strains:

According to colony phenotype on mannitol salt agar plates and the API Staph system results, from all the 43 samples included in this study 28 (65%) isolates were characterized as *S. aureus.*

3.2. The biofilm production assay:

The biofilm production assay by microtiterplate method results showed that 19(67.8%) of the 28 tested *S. aureus* isolates were attached at different amount (Table 3). Attachment abilities in 10 (35.7%) strains were strong(OD at A_{490}> 0.17), 6(21.4%) strains were moderate(OD at A_{490} ranging from0.11 to 0.16), 4 (14.2%)strains were weak and 8(28.5%)had no attachment (table 3).

Table 3:Biofilm formation by microtiter tissue culture plates method

Adhesion formation	*S. aureus* isolates (n=28)	Percentage (%)
Biofilm forming	20	(71.4%)
Strong	10	(35.7%)
Moderate	6	(21.4%)
Weak	4	(14.2%)
None	8	(28.5%)

3.3. Identification of *ica*ADBCgenes:

All the primers used in the study showed specificity with a single band (Figure 1). Genetic analysis of 28*S.aureus*isolatesrevealed the presence of *ica*ADBC operon genes in22 (78.5%)isolates. *ica*AB gene was present in 21(75%) isolates whereas *ica*D and *ica*C genes were present in 27 (96.4%) and26 (92.8%) of *S. aureus* isolates respectively (Table 4).

Table 4: Distribution of *ica*Operon Genes among *S. aureus* isolates

*ica*Operon Genes	Number of *S. aureus* isolates
*ica*ADBC	22 (78.5%)
*ica*AB	21(75%)
*ica*D	27 (96.4%)
*ica*C	26 (92.8%)

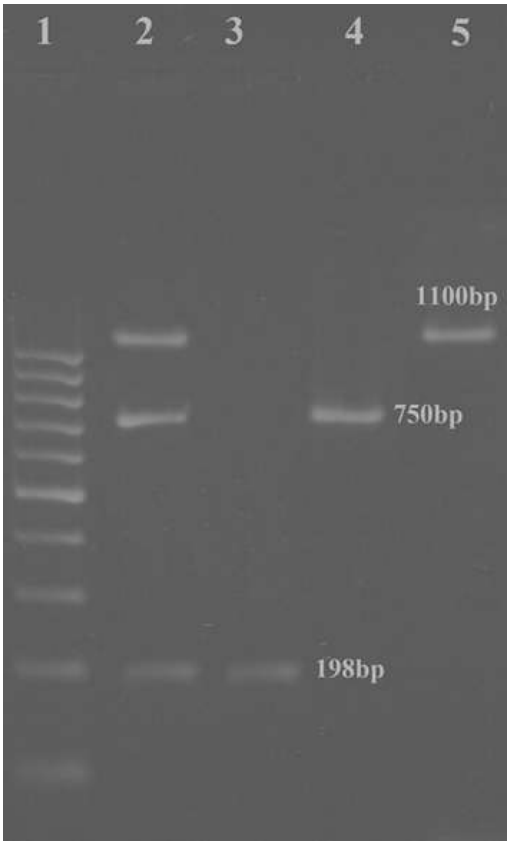

Figure 1:Gel Electrophoresis for the molecular identification of *ica*ADBCoperon genes in *S. aureus* isolates.Lane1:100 bp DNA ladder;Lane2:198 (bp), 750(bp) and 1100(bp)Multiplex-PCR products for *ica*ADBC operon gene;Lane3:198 (bp) product for *ica*D gene; Lanes 3: 750(bp) product for *ica*AB gene.Lanes3: 1100(bp) product for *ica*C gene.

4. Discussion:

With the increasing use of indwelling medical devices, infections caused by *staphylococcus* spp., have become more prevalent as a cause of hospital-acquired infection. The major pathogenic factor is the ability to produce biofilm which making the clinical treatment difficult (26).Early detection and management of biofilm-forming staphylococci can be one of the essential steps towards the prevention and management of device-associated nosocomial infections (27).

S. aureus strains that are able to form biofilm cause chronic polymer-associated infections (28). Biofilm support the adhesion and colonization of *S. aureus* on surfaces, causing difficult to treat infections (29). Infections associated with the use of catheters, are mainly due to *S. aureus*, particularly those biofilm producing strains. The biofilm formation needs polysaccharide intercellular adhesion, which is synthesized by the enzymes encoded by *ica*gene cluster (30).

This study was showed that 71.4% of *S. aureus* isolates were able to produce biofilm and that was agreed with Namvar et al (30), of these isolates 78.5% have *ica*ADBCoperon genes as shown by the genetic analysis. These results confirmed that *S. aureus* isolates had no ability to produce biofilm unless they have *ica*ADBCoperon genes.

In Conclusion the detection of the *ica*ADBCoperon genes in *S.aureus* isolates could be helpful to improve the clinical decision for the treatment of *S. aureus* infections in patients receiving indwelling catheter.

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