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Molecular Detection of the Intracellular Adhesion Genes in Clinical Isolates of *Staphylococcus Aureus*

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KEYWORDS
Staphylococcus aureus, biofilm, 16SrRNA, icaABCD operon

ABSTRACT:

One hundred ninety (190) clinical samples were collected from different sources (wounds, burns, urine and blood) from Baquba Teaching Hospital, the Consulting Clinic and in Diyala Governorate for the period from September 15/ 2022 to December 15/ 2022. Fifty 50 (26%) *S. aureus* isolates screened by phenotypic and Biochemical examinations and genotypic by using *16SrRNA* gene. The relationship between *S. aureus* biofilm producer isolates and icaABCD operon genes was studied by using PCR. Twenty four (24) isolates of pathogenic *S. aureus* bacteria, which showed biofilm production were selected for genetic detection of the presence of biofilm related genes, including intracellular adhesion (ica) genes, which are within the biological operator icaABCD using polymerase chain reaction (PCR). The detection results showed that 20 out of 24 isolates under study 83%, were carriers of the *icaA* and *icaB* genes, and the percentage of the icaC gene in the isolates was 17 (70%), as well as the rate of *icaD* gene in the isolates under study was 19 (79%). The genetic patterns of the isolates that possess biofilm encoding genes showed that 17 (70.8%) isolates contained the type icaABCD, 2 (8.3%) isolates showed the type icaABD, one isolate by 4.2% showed the type icaA, and one isolate showed the type icaA. type icaB by 4.2%, and three isolates did not show any pattern because they did not have icaABCD operon.

Introduction

Staphylococcus aureus is a Gram-positive, catalase-positive and bacitracin-resistant coccus, which is a common colonizer of the human that considered one of the main human pathogens that is involved in the induction of a series of clinical infections (Tong *et al*., 2015). *S. aureus* infections have been sharply increased during the recent years and associated with more mortality than other bacterial agents (Naber, 2009). Biofilms are highly organized multicellular bacterial communities embedded within a complex matrix composed of polysaccharide, proteins, and/or extracellular DNA (eDNA), significantly contributes their survival in the host and has been considered as a key virulence factor responsible for serious chronic infections (Parrino *et al*., 2019). Attachment and colonization is the first step for *S. aureus* pathogenesis. Biofilm formation leads to bacterial resistance to higher concentrations of antimicrobial agents in addition to host immune responses (Verma *et al*., 2013). The *S. aureus* responsible for biofilm-associated infections can have different genetic backgrounds and, therefore, express a different spectrum of virulence factors during infection. For example, biofilm formation appears to be associated with several regulatory factors, including polysaccharide intercellular adhesin (PIA), which is produced and regulated by the intercellular adhesion (ica) ADCB operon. The icaADCB operon includes an N-acetylglucosamine transferase (*icaA*) that encodes the major enzyme, which is essential for PIA synthesis. This enzyme might require an *icaD* gene product (called IcaD) for its activity (Vanderhaeghen *et al.*, 2012; Abbasi and Zamanzad, 2015). The other genes within the ica operon are *icaB* (polysaccharide deacetylase), *icaC* (transporter of PIA), and *icaR* (the

Journal of Chemical Health Risks

www.jchr.org

JCHR (2023) 13(3), 719-726

inhibitor gene) (Cramton *et al.*, 1999; Lade *et al.*, 2019).

Methods

Isolation and identification

Fifty isolates of *S. aureus* were obtained from a collection of one hundred and ninety specimens of different clinical sources including burns, wounds swabs, urine and blood, collected from Baquba Teaching Hospital, the Consulting Clinic, and Al-Batoul Teaching Hospital in Diyala Governorate for the period from September 15, 2022 to December 15, 2023. The isolates were identified as *S. aureus* by conventional microbiological assays, including procedures such as colony morphology, Gram stain, catalase activity, growth on mannitol salt agar, blood agar and tube coagulase assay. Confirmatory genetic test was done by using 16SrRNA detection.

Biofilm production assay

Biofilm formation was detected using a microtiter plate method (MTP) with 96 wells, according to Foster *et al*. (2020): Briefly, 20 µl of overnight cultures of the test pathogens (1%) were used to inoculate 96-well polystyrene MtPs, each well contained 1 mL of fresh brain heart infusion broth medium supplemented with 1% glucose., with three replicates for each isolate. The plate was incubated at 37°C for 24 hours, followed by washing with phosphate-buffered saline. The adherent cells were fixed by adding 95% ethanol for 20 minutes, then crystal violet stain (0.1%) was added to the wells and left for 15 minutes, then washed three times with water. Add 200 microliters of 95% ethanol for 10 min, then read the optical density (OD) using an ELISA reader at a wavelength of (630) nm. Based on the optical densities of bacterial biofilms and controls, all strains were classified into four categories: no biofilm producers, weak, moderate, or strong biofilm producers.

Molecular detection of IcaABCD operon

For extracting the genomic DNA, extraction kit (ABIOpure, USA) was used according to the manufacture instruction.. Eluted DNA was kept at -20 ° C for later analysis. The presence of the *16SrRNA* gene and biofilm-associated genes including *icaA*, *icaB*, *icaC* and *icaD* were examined via standard PCR reaction. List of the used primers are summarized in Table 1. PCR reaction was carried out in 20µl of Premix (Promega, USA). The mixture comprised of 10 µL mastermix, 1 µL of each primer, 6 µL nuclease free water, 2µL template DNA. The thermal profile included initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C (30 s) of denaturation, 55°C (30 s) of annealing (different for each gene) shown in Table 1 and 72°C (30 s) and final extension of 72°C (7 min). PCR products were electrophoresed in 2% gel agarose in 1X TBE buffer with staining of 1 µL of each loading buffer and gel red and then observed under UV emission. Finally, the sizes of the PCR products were determined by comparing them with the migration of the DNA ladder (Xu *et al.*, 2011).

Table 1: primers used in PCR amplification for genes detection

Primer Name	Sequence 5`-3`	Annealing temp. °C	Product size (pb)	Ref.
16SrRNA-F	AACTCTGTTATTAGGGAAGAACA	55	756	Ibrahim and Al-Mathkhury, 2018
16SrRNA-R	CCACCTTCCTCCGGTTTGTCACC			
icaA-F	ACACTTGCTGGCGCAGTCAA	52	188	Ghasemian *et al.*, 2015
icaA-R	TCTGGAACCAACATCCAACA			
icaB-F	AGAATCGTGAAGTATAGAAAATT	55	880	
icaB-R	TCTAATCTTTTTCATGGAATCCGT			
icaC-F	ATGGGACGGATTCCATGAAAAAGA	55	1066	

Journal of Chemical Health Risks

www.jchr.org

JCHR (2023) 13(3), 719-726

icaC-R	TAATAAGCATTAATGTTCAATT			
icaD-F	ATGGTCAAGCCCAGACAGAG	55	198	
icaD-R	AGTATTTTCAATGTTTAAAGCAA			

Results

Isolation

The bacteria was initially diagnosed on blood agar produce a clear translucent area surrounding the colonies as result for ß-haemolysin production. Mannitol salt agar is selective differential growth medium. *S. aureus* produces yellow colonies, whereas other Staphylococci produces small pink or red colonies with no color change to the medium. It encourages the growth of Staphylococcus isolates, while inhibiting the growth of others. It contains a high concentration 7.5-10.0 % of NaCl salt, making it selective for Gram-positive bacterium *Staphylococcus* and *Micrococcus*. The results of biochemical test showed positive results for catalase and coagulase.

Fifty (26%) isolates of *Staphylococcus aureus* were obtained out of 190 specimens. The results of our study near the results of Mahmood and Hussein (2022) that identified this bacteria in 29 (19.3%).The isolates were identified molecularly using the *16S rRNA* gene, using polymerase chain reaction (PCR). The results showed that all of these isolates were carriers of the gene at a rate of 100%.

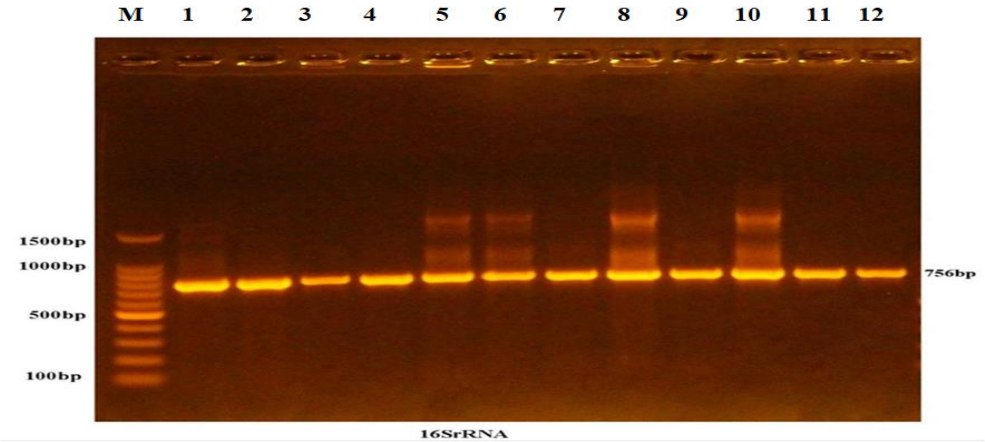

Figure 1: *16SrRNA* gene amplicon on agarose gel using the PCR technique for *S. aureus*, at a concentration of 2%. (Marker) M represents 100-1500 bp, while numbers from 1 to 22 indicate the number of isolates.

Biofilm production

The results showed that all 100% of *S. aureus* isolates were biofilm producers with different levels compared with the negative control, (Table 2). The result agree with the result of Nourbakhsh and Namvar (2016) that found that all isolates were biofilm producer.

Table 2: Biofilm producers with different degrees in different sources

Source	Urine	wounds	Burns	Blood	Total
Strong	6 (12%)	3 (6%)	7 (14%)	2 (4%)	18 (36%)
Moderate	7 (14%)	7 (14%)	7 (14%)	1 (2%)	22 (44%)
Weak	5 (10%)	5 (10%)	0 (0%)	0 (0%)	10 (20%)
Total	18 (36%)	15 (30%)	14 (28%)	3 (6%)	50 (100%)

Journal of Chemical Health Risks

www.jchr.org

JCHR (2023) 13(3), 719-726

Detection of icaABCD genes

Twenty four isolates were selected from different sources (wounds, burns, blood, and urine) of the pathogenic *S. aureus* bacteria that produce biofilm using PCR to study the presence of biofilm related genes, *icaA*, *icaB*, *icaC* and *icaD* (intercellular adhesion gene A, B, C and D). The results indicated that the DNA concentration of the extracts was variable and ranged between (23-30 ng/microliter), while the purity of the DNA extracts was good and ranged between (1.50-1.98) at a wavelength of 260/280 nm. Results showed that the *icaA* and *icaB* genes amplicons were present in 20 (83%) *S. aureus* isolates, (Table 3). The size of amplified DNA is 188 bp and 880 bp respectively, as shown in (Figure 1) and (Figure 2). The results of the current study near the result of Ghasemian et al. (2015) study in Iran, where the percentage of isolates carrying the *icaA* and *icaB* genes was 73% and 69%, respectively. The results of our study did not agree with the findings of Refaat et al. (2023), where the percentage of *icaAB* genes was 100%.

Table 3: Percentage of biofilm genes in relation to sources of isolation

Source	*icaA*	*icaB*	*icaC*	*icaD*
Burns	5 (21%)	5 (21%)	5 (21%)	5 (21%)
Wounds	5 (21%)	6 (25%)	4 (17%)	5 (21%)
Urine	7 (29%)	7 (29%)	6 (25%)	7 (29%)
Blood	3 (12%)	2 (8%)	2 (8%)	2 (8%)
Total	**20 (83%)**	**20 (83%)**	**17 (70%)**	**19 (79%)**

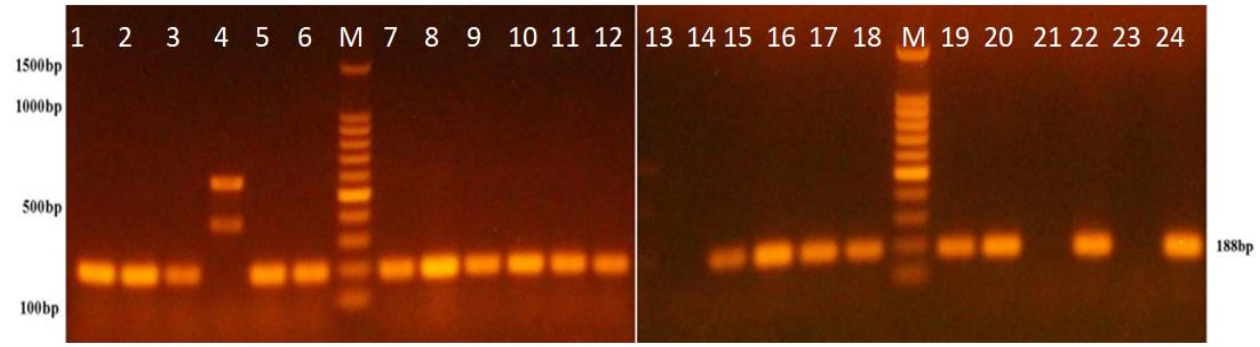

Figure 2: *icaA* gene amplicon on agarose gel using the PCR technique for *S. aureus*, at a concentration of 2%, a voltage difference of 100 volts, a current of 100 mA, a time of 60 min, and using the Ladder DNA 1500 - 100 bp after staining with Ethidium Bromide and exposed to UV light. (Marker) M represents 100-1500 bp, while numbers from 1 to 24 indicate the number of isolates.

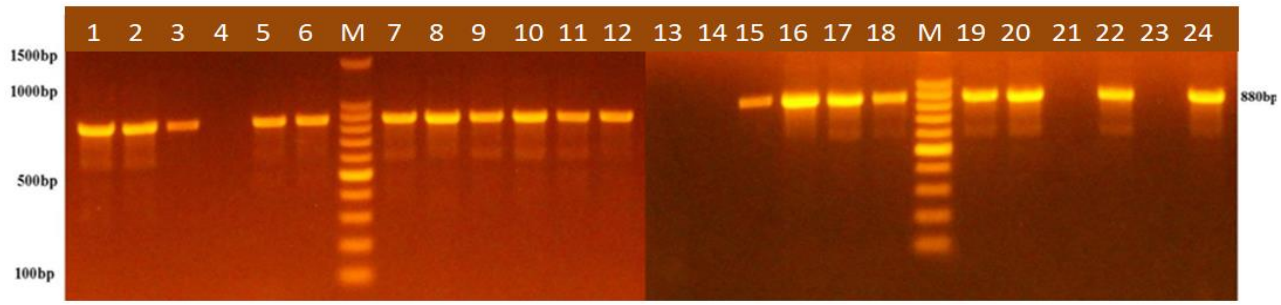

Figure 3: *icaB* gene amplicon on agarose gel using the PCR technique for *S. aureus*, at a concentration of 2%, a voltage difference of 100 volts, a current of 100 mA, a time of 60 min, and using the Ladder DNA 1500 - 100 bp after staining with Ethidium Bromide and exposed to UV light. (Marker) M represents 100-1500 bp, while numbers from 1 to 24 indicate the number of isolates.

Journal of Chemical Health Risks

www.jchr.org

JCHR (2023) 13(3), 719-726

The results also showed that the percentage of the *icaC* gene in *S. aureus* isolates was 17 (70%), The size of amplified DNA is 1066 bp when compared with DNA Ladder size, as shown in (Figure 3). The results of our study agreed with the results of Ghasemian et al. (2015), where the percentage of isolates carrying the *icaC* gene was 73%. The results of our study did not agree with the results of Goudarzi et al. (2019) that was 50.7%.

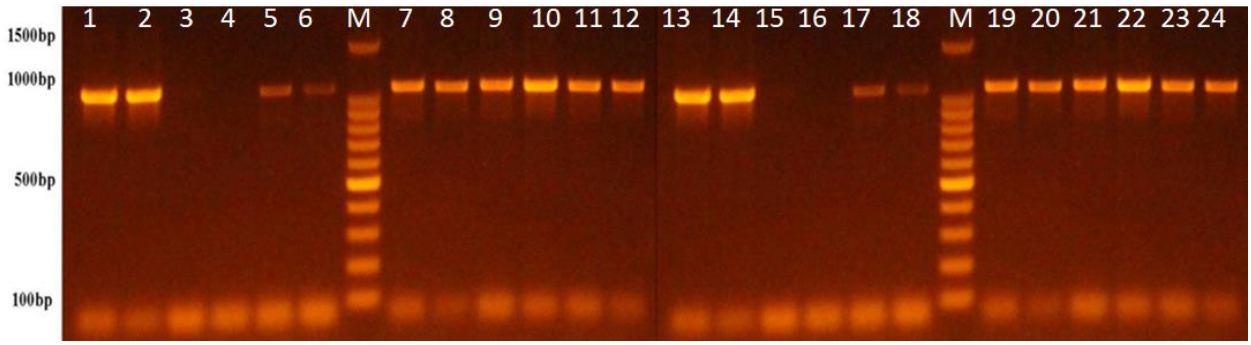

Figure 4: *icaC* gene amplicon on agarose gel using the PCR technique for *S. aureus*, at a concentration of 2%, a voltage difference of 100 volts, a current of 100 mA, a time of 60 min, and using the Ladder DNA 1500 - 100 bp after staining with Ethidium Bromide and exposed to UV light. (Marker) M represents 100-1500 bp, while numbers from 1 to 24 indicate the number of isolates.

While 19 (79%) biofilm producing *S. aureus* isolates showed positive results for *icaD*. The size of amplified DNA is 198 bp (Figure 4). The results of our study agreed with the results of Goudarzi et al. (2019) by 77.3%, and disagree with the findings of Mahmood and Hussein (2022) study in Kirkuk, that the percentage of isolates carrying the *icaD* gene was 88.9%.

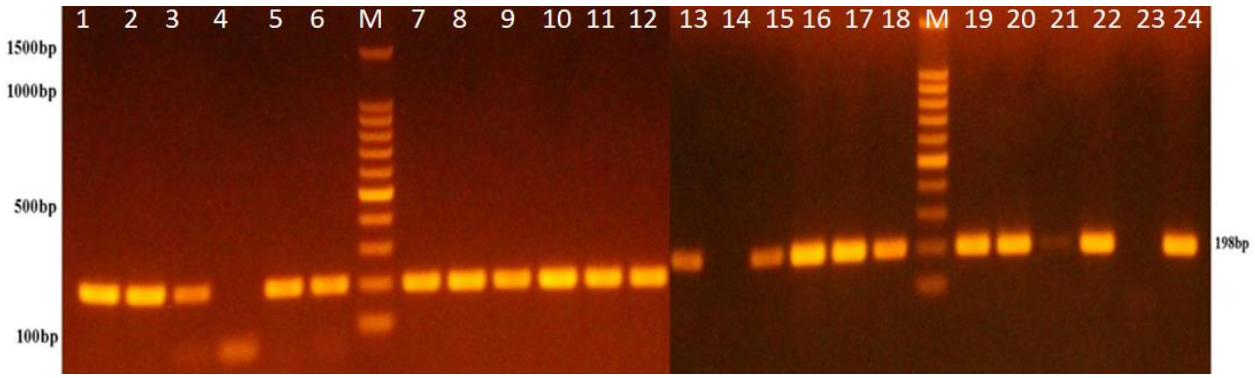

Figure 5: *icaD* gene amplicon on agarose gel using the PCR technique for *S. aureus*, at a concentration of 2%, a voltage difference of 100 volts, a current of 100 mA, a time of 60 min, and using the Ladder DNA 1500 - 100 bp after staining with Ethidium Bromide and exposed to UV light. (Marker) M represents 100-1500 bp, while numbers from 1 to 24 indicate the number of isolates.

Genetic pattern for biofilm associated genes

Table 4 shows the patterns of the isolates under study that possess the genes encoding biofilm formation. The results show that 17 isolates contained the icaABCD pattern at a rate of 70.8%, two isolates showed the icaABD pattern at a rate of 8.3%, and one isolate showed the icaA pattern at a rate of 4.2%. One isolate showed the icaB pattern at a rate of 4.2%, while three isolates did not show any pattern because they did not possess the operon icaABCD.

Journal of Chemical Health Risks

www.jchr.org

JCHR (2023) 13(3), 719-726

Table 4: genetic patterns for S. aureus that have ica genes

Patterns	No. of isolates	%
icaABCD	17	70.8
icaABD	2	8.3
icaA	1	4.2
icaB	1	4.2
No operon	3	12.5

Discussion

The formation of biofilm in *S. aureus* is a critical step in the development of chronic infections. Accordingly, in order to control and manage these infections understanding the biofilm formation mechanism is thought to be important (Lister and Horswill, 2014). Current research was performed to investigate the capability of production of biofilms and the presence of operon icaABCD and adherence genes in *S. aureus* isolates from clinical samples of hospitalized patients.

It is believed that the development of Staphylococcus biofilm is a two-stage process. The initial step involves the attachment of bacterial cells to a substrate's surface while in the second step, biofilm production involves the aggregation of bacteria using PIA (Mack *et al*., 1996; Mahmood and Hussein, 2022) through adhesion of cells. The PIA is found to be regulated by gene locus consisting of four intercellular adhesion (icaADBC) genes arranged in the structure of an operon. There are various genes which contribute to the formation and preservation of staphylococci-formed biofilms. The most investigated of these genes are the *icaA* and *B* genes which are required in production of PIA, which contains N-acetylglucosamine used for the primary constituent of the matrix of exopolysaccharides within biofilm (Rohde *et al*., 2007; Mahmood and Hussein, 2022).

The *icaA* is needed for co-expression of *icaD* and N-acetylglucosamine which can enhance the capsular polysaccharide characteristics (Satorres *et al.,* 2007) and signaling the essential part the icaD locus which has been identified as a virulence factor in *S. aureus* pathogenesis (O'Gara, 2007). The ica expression has been found associated with environmental factors (Cramton et al., 2001; Mahmood and Hussein, 2022). Numerous factors such as low doses of antibiotics, osmolality and anaerobic environments are recognized as expression enhancers for biofilm formation. Furthermore, the operon expression can be activated and deactivated by inserting and excising the insertion sequence (IS) (Cho *et al.*, 2002).

Conclusion

According to our findings, biofilm formation was present in *S. aureus* isolates with frequencies of the adhesion ica encoding genes. These results revealed the importance of ica operon in biofilm production.

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