



Phenotypic and Genotypic Characterization of Antibiotic Resistance in *Staphylococcus aureus* Isolated from Different Sources

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Abstract

Background: *Staphylococcus aureus* is a bacterial pathogen that can cause a wide range of nosocomial infections. Nasal colonization by *S. aureus* plays an important role both in the epidemiology and pathogenesis of infection.

Objectives: This study aimed at detecting the biofilm-forming capacity of clinical isolates and detection of *icaA* and *agr* genes.

Methods: A total of 150 clinical specimens was collected from patients in different hospitals in Baghdad. The clinical samples included wounds, abscess, sputum, and ear infections. The suspected isolates were cultured for one day at 37 °C on mannitol salt agar in an aerobic environment.

Results: The results showed that of 150 samples, 44 isolates were *S. aureus* (29.3%), of wounds samples, 22 isolates (45.83%) were *S. aureus*, 13 (37.14%) were from abscess, 7 (17.95%) from sputum, and 2 isolates (7.14%) from ear samples. This study found that most isolates formed biofilm, but the levels of biofilm were distributed across three ranges. The results also indicated that 47.7% of the isolates produced a strong biofilm, as well as 38.6 and 13.6% produced moderate and weak biofilms, respectively. The present molecular results showed that *S. aureus* from different samples were 13 (59.1%), 4 (30.77%), 3 (42.85%), 0 (0%) from wounds, abscess, sputum, ear, respectively, were positive for *agr* gene. While the results showed 18 (81.8%), 10 (76.9%), 5 (71.4%), 1 (50%), respectively, were positive for *icaA* gene.

Conclusions: Most *S. aureus* isolates isolated from wound were biofilm positive. These isolates bore *icaA* and *agr* genes in a high quantity.

Keywords: Bacterial Proteins, *Staphylococcus aureus*, Virulence Factor, *icaA* and *agr* Genes

1. Background

The *Staphylococcus aureus* is a Gram-positive bacterium that is normally found as normal flora of the body. It can be found on skin as well as in the upper respiratory system (1). *Staphylococcus aureus* is an opportunistic bacterium, which can lead to a wide range of infectious illnesses, particularly infections acquired in the community and hospitals. *Staphylococcus aureus* infection combined with influenza virus infection has been found in recent research to enhance the risk of pneumonia and eventual mortality (2). Methicillin-resistant *S. aureus* (MRSA)-related antimicrobial resistance necessitates constant efforts to develop plans to eliminate the problem. New treatments and procedures are required to avoid it (3).

Methicillin-resistant *S. aureus* can cause skin infections, urinary tract infections (UTI), bloodstream infections, food poisoning, and respiratory disorders by pro-

ducing enterotoxins and alpha pore-forming toxins, which kill host cells and tissues. There are also other factors of *S. aureus* infection pathogenicity (4). It is commonly seen in many skin disorders and various types, but not limited to a broad range of infections such as recurrent and deep wounds. It has been shown to have the ability to colonize mucosal surfaces (5). If a breach occurs in the mucous membrane or skin, bacteria can move into the tissues or bloodstream (5, 6) *S. aureus* causes a wide range of diseases, including *S. aureus*, which may induce bacteremia and endocarditis (7).

Staphylococcus aureus is a polysaccharide-like substance present in many Gram-negative bacteria, a variety of which are frequently involved in biofilm formation. The ability of MRSA to produce biofilm by tissue culture plate (TCP) was investigated, and the results indicated that MRSA isolates showed highly and strong biofilm formation (8). It is synthesized by four different polycarbon-

ate N-acetamide acetic acids (PNAG) into four polymeric polymers (9). The destruction of the *icaA* genes causes biofilm degradation (8). The gene of *icaA* is recognized to be encoded the N-acetyl-glucosamine transferase transmembrane synthesizing PNAG polymers (10). *Staphylococcus aureus* regulates virulence through the network, detects environmental cues, and responds by altering the production of virulence components required for the host to survive.

The accessory gene regulator (*agr*) is the most prominent quorum sensing QS system among the several *S. aureus* regulatory systems. It can sense the local concentration of signal molecules, allowing *S. aureus* to sense its population density and transform this information into gene expression mode (11). Each of these virulence factors activates a quorum-sensing (or titer) device (quantum system), which is referred to as the so-called bacterial sensing peptide (QS) (12).

2. Objectives

The goal of this study is to determine the biofilm-forming ability of clinical isolates from patients in Iraq, as well as the detection of the *icaA* and *agr* genes.

3. Methods

This descriptive study describes the ability of *S. aureus* to resist antibiotics through its production of biofilm.

3.1. Sample Collection

A total of 150 clinical specimens were collected from December 2020 to February 2021 from patients in different hospitals of medical city in Baghdad (Private Nursing Home Hospital and Baghdad Teaching Hospital). Patients' age ranged from 13 to 68 years. Information was additionally taken about the idea of work just as where they do live and whether they experience side effects of different infections. The clinical samples included: (1) wounds, 48 samples; (2) abscess, 35 samples; (3) sputum, 39 samples; and (4) ear infections, 28 samples. The samples were conducted by swab and then cultured on CHROM agar supplied by Condalab Company in Spain, blood agar, and mannitol salt agar manufactured by Oxoid Company (England).

3.2. Isolation and Identification of *Staphylococcus aureus*

Specifically, the bacteria were tested on a mannitol salt culture, which is unique for *S. aureus*. Three days after that, the samples were incubated for a further period of time at 37°C. The broth-saline and mannitol salt agar isolates were stored at 4°C before usage. These samples were identified using routine biochemical tests according to according to Abdolmaleki et al. (2019) (13).

3.3. Biofilm Assay

The specificity of *S. aureus* biofilms was measured. Two wells of a freshly prepared culture brain infusion broth (equivalent to 2x BHI bacteria McFarland No. 5) were added to each sterile 96-well polystyrene microtiter plate. Subsequently, all microplates were incubated at an anaerobic condition (no air) for 24 h. Each sample was replicated three times. Each well was emptied and then washed with 200 μ L purified water three times. Next, 200 μ L of methane was added, then the dishes were placed in an oven at 60°C for 15 min to remove residual moisture. Crystal violet was applied next, then they were left at room temperature for 5 min. The procedure was followed as described. After finishing the drying, the plates were incubated at 37°C for approximately 30 min afterward. Hence, additional ethanol was introduced at a rate of 200 μ L/min for the next 10 min. Finally, each well was calculated at 630 nm with a microplate reader that used visible-light spectrophotometry (14, 15). The results were divided into four categories according to their optical densities as follows: (1) strong biofilm ($4 \times \text{ODc} < \text{OD}$); (2) middle biofilm producer ($2 \times \text{ODc} < \text{OD} \leq 4 \times \text{ODc}$); (3) weak biofilm producer ($\text{ODc} < \text{OD} \leq 2 \times \text{ODc}$); (4) non-biofilm producer ($\text{OD} \leq \text{ODc}$).

3.4. DNA Extraction

Genomic DNA was extracted from the clinical isolates of *S. aureus* 44 isolates utilizing (DNA scaled-down pack that was provided by ABIO organization guidelines, USA) (16).

3.5. Purification and Concentration Measurement

After finishing the extraction process and obtaining pure DNA, the concentration and purity of DNA were measured using a Nanodrop.

3.6. Preparation of PCR Mixture

All DNA extracted from 44 isolates of *S. aureus* in the current study went through PCR procedure to assess different drug resistance genes. Each PCR reaction had a final volume of 20 μ L. Promega master mix was used, and the melted solution was homogenized using a vortex for ten min before using the PCR Master Mix (2x) (Promega company-USA). Homogenizing was done until the priming was done.

3.7. Primer Preparation

Forward and reverse primers, which were in lyophilization status, were dissolved and diluted first in free nuclease

double sterile distilled water (amount according to recommended of manufactured company) to obtain 100 picomol/ μ L and this was considered a stock solution, then it was stored in deep freeze. This stock was diluted in free nuclease double distilled water to obtain nearly 10 picomol/ μ L and was stored in deep freeze until used in PCR mixture. It was supplied by MacroGen in a lyophilized suspension. To make a lyophilized 100 pmol/mL solution of the primers, the stock solution was dissolved in nuclease-free water. A working solution of these primers (cooled to -20°C) was generated by combining 10 μ L of primer solution with 90 μ L of non-nuclease water to yield a final concentration of 10 pMol (Table 1).

3.8. PCR Amplification

The *icaA* and *agr* genes were amplified using primers (Table 1). The PCR amplification was conducted in a total volume of 20 μ L containing 3 μ L DNA, 10 μ L Master Mix PCR (Promega Company, USA), 10 pmol of each primer (forward and reverse), and then nuclease-free water was added to a tube to a total volume from 20 μ L. Thermo cycling conditions were as follows: (1) initial denaturation at 5 min at 94°C followed by 30 cycles of denaturation 94°C for 30 sec; (2) annealing at 58°C for 30 sec; (3) extension at 72°C for 30 sec and a final extension of 72°C for 5 min.

3.9. Agarose Preparation

Agarose (1 g of agar per 100 mL of water previously mixed with 1X TBE buffer and boiled and allowed to cool at 50°C) was applied to the 100 mL solution. A 3 μ L of ethidium bromide solution was previously prepared and added to agar. Placing the comb in agarose solution permitted to polymerize (soaked in around 30 min at room temperature), and it was then withdrawn after the agarose solution hardened. The gel was put into the 1X TBE buffer-filled gel tube. It was completely flushed out with buffer (19).

3.10. Sample Preparation

Seven microliters of the PCR product were added to each well of the gel to be used for gel electrophoresis using a DNA ladder (100-bp DNA) (Intron company - Korea). The DNA samples were recorded by ultraviolet transilluminator documentation method (20, 21).

3.11. Statistical Analysis

To recognize the impact of various parts in research parameters, the Statistical Analysis System- SAS (2012) application was used. In this analysis, the chi-square test was utilized to make a significant correlation between rates.

4. Results

4.1. Isolation

The current results showed that of 150 samples, 44 isolates were *S. aureus* (29.3%), of wounds samples 45.83% were *S. aureus*, 37.14% were from abscess, 17.95% from sputum, 7.14% from ear samples (Table 2). All isolates were identified to species morphologically and biochemically based on Bergey's manual of systematic bacteriology (22). Al-Zoubi et al. (23) showed that most of *S. aureus* were isolated from wound specimens and abscess. Also, *S. aureus* has been found to be a common etiology of pneumonia and wounds, resulting from surgical operations that occur often in healthcare facilities, which is the second most prevalent source of bloodstream infections in hospitals (24).

4.2. Biofilm Formation

The results indicated that 47.7% of the isolates produced a strong biofilm, especially in patients between 40 to 68 years, 38.6% produced moderate biofilms, and 13.6% had weak biofilm (Figure 1 and Table 3). This coincides with the study conducted by Muhammad, who addressed biofilm formation by MRSA (25). He presented that 100% of the *S. aureus* isolates were capable to produce a biofilm. In 2017, Saleh and Khalaf conducted another local investigation. They found that 15% of *S. aureus* isolates formed weak biofilms, 15% formed moderate biofilms, and 70% formed strong biofilms (26).

4.3. Extraction of DNA

The DNA of *S. aureus* was extracted using an extraction kit provided by the ABIO Company. In the beginning, all 44 isolates isolated from different sources were grown on mannitol salt agar medium to activate bacteria, which was reserved on the maintenance medium and then transferred to the nutrient broth (Figure 2).

4.4. DNA Concentration and Purity Measurements

The concentration and purity of DNA were assessed by Nanodrop at the end of the DNA extraction process. The authors found a concentration of (75 - 290 ng/L) and purity of (1.65 - 2.2 nm).

4.5. Detection of *icaA* and *agr* Genes

The present molecular results showed that *S. aureus* from different samples were 13 (59.1%), 4 (30.77%), 3 (42.85%), 0 (0%) from wounds, abscess, sputum, ear, respectively, were positive for *agr* gene (Table 4 and Figure 3). The results showed that 18 (81.8%), 10 (76.9%), 5 (71.4%), 1 (50%) were from wounds, abscess, sputum, ear, respectively, were positive for *icaA* gene (Table 4 and Figure 4).

Table 1. The Primers Used in the Current Study

Genes	Gene Sequences	Product Size (bp)	References
<i>agr</i>		164	(17)
F	5'-TGATAATCCTTATGAGGTGCTT-3'		
R	5'-CAC TGT GAC TCG TAA CGA AAA-3'		
<i>icaA</i>		188	(18)
F	5'-ACACTTGCTGGCGCAGTCAA-3'		
R	5'-TCTGGAACCAACATCCAACA-3'		

Table 2. Number and Percentages of *Staphylococcus aureus* Isolates from Different Samples

Sample Sources	No. of Samples	No. of <i>S. aureus</i> Isolates	Percentage
Wounds	48	22	45.83
Abscess	35	13	37.14
Sputum	39	7	17.95
Ear	28	2	7.14
Total	150	44	29.3
Chi-square (χ^2)	-	-	11.483 ^a

^a (P ≤ 0.01).

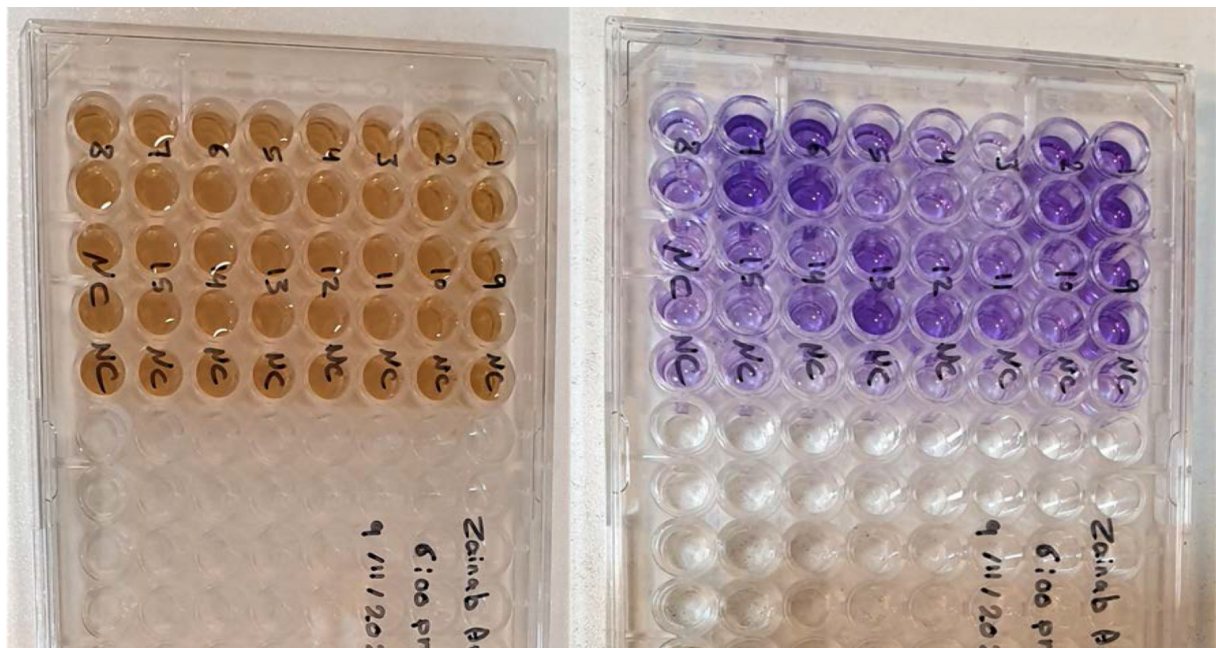


Figure 1. Biofilm formation by *Staphylococcus aureus* isolates

5. Discussion

Bacterial infection is a critical issue in hospitals and healthcare facilities. Infections caused by MRSA isolates have increased recently, and these infections are more

commonly linked to mortality than infections caused by other bacteria (27). Bacterial biofilms are more resistant to antimicrobial treatment and immunological factors from the host (28). In the present study, the *agr* and *icaA* genes of *S. aureus* and their biofilm-forming capacity were studied.

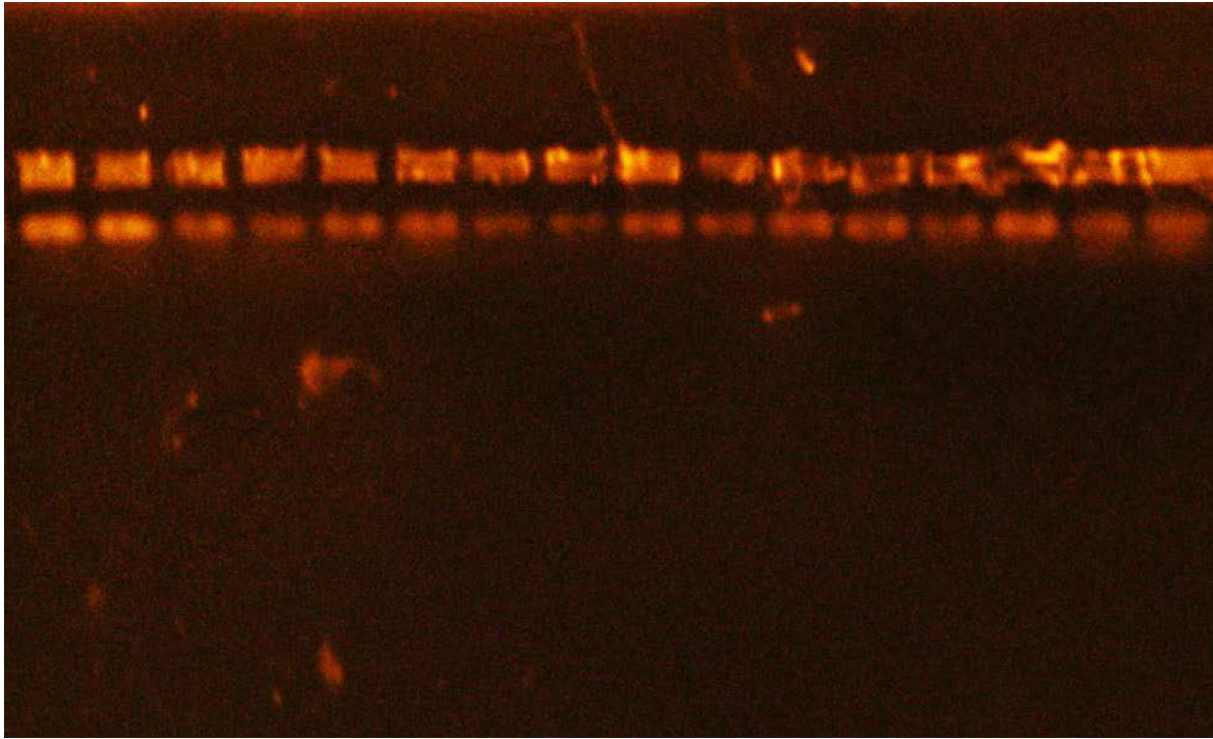


Figure 2. Gel electrophoresis of genomic DNA extraction from *Staphylococcus aureus* 1% agarose gel at 6 vol/cm for 1:00 hours.

Table 3. *Staphylococcus aureus* Isolates with Variable Biofilm-Forming Abilities ^a

Variables	No.	Percentage
Weak	6	13.6
Moderate	17	38.6
Strong	21	47.7

^a Chi-square (χ^2) = 9.477 **, ($P \leq 0.01$).

The production of biofilm in *S. aureus* is controlled by *icaA* biofilm operon and *agr* locus, the highly dynamic biofilm composition prompting the exchange and removal of nutrient, gas, waste, and solution through channels (29). In an alternative study in 2018, it was confirmed that, in comparison to the planktonic condition for the same MRSA expression, *icaA* gene expression was significantly increased under biofilm conditions (1). The biofilm-forming processes of *S. aureus* are determined by the *icaA* gene cluster that is responsible for the synthesis of polysaccharide intracellular adhesion and capsular polysaccharide adhesion (30). Moreover, *S. aureus* isolates that were screened from wound infections were positive for *icaA* gene (31). It was also found that the *agr* gene was the most frequent biofilm-forming gene among MRSA strains (32).

Biofilm formation was assessed using a tissue culture plate approach in our investigation, and the results showed that 47.7% of the 21 MRSA isolates produced strong biofilms, whereas only 38.6% of isolates formed moderate biofilms. This contradicted the findings of Dakheel et al. (2016) who investigated 25 MRSA strains and found that 88% of isolates produced intermediate biofilms, while 11% produced weak biofilms (33). Another study found that 47.9% of MRSA isolates were moderate biofilm-forming, 39.7% were strong biofilm-forming, and 11% were weak biofilm-forming (34). As a result, it appears that the strains in our investigation are more pathogenic, producing more strong biofilms. This may be attributable to the specimen type or the use of antibiotics in Iraq. Furthermore, the use of inadequately sterilized medical devices resulted in environmental selection in our strains that favored strong biofilm formation. Multiple genes, including *SarA* and *agr* genes, regulate the *ica* genes. They can interact and regulate biofilm creation by interacting with each other. The *SarA* gene appears to be a master controller of biofilm formation, increasing the synthesis of fibronectin and fibrinogen-binding proteins, as well as toxins for tissue dissemination, while suppressing the expression of protein A (35).

Table 4. *icaA* and *agr* Genes for *Staphylococcus aureus* Isolated from Different Clinical Samples ^a

Samples	Total Isolates of <i>S. aureus</i>	No. of <i>S. aureus</i> with <i>agr</i>	No. of <i>S. aureus</i> with <i>icaA</i>	No. of <i>S. aureus</i> with <i>agr</i> and <i>icaA</i>
Wound	22	13 (59.1)	18 (81.8)	8 (36.3)
Abscess	13	4 (30.77)	10 (76.9)	2 (15.4)
Sputum	7	3 (42.85)	5 (71.4)	1 (14.3)
Ear	2	0 (0)	1 (50)	0 (0)
Total	44	20	34	11
Chi-square (χ^2)	-	12.408 ^b	14.266 ^b	-

^a Values are expressed as No. (%) unless otherwise indicated.

^b ($P \leq 0.01$).

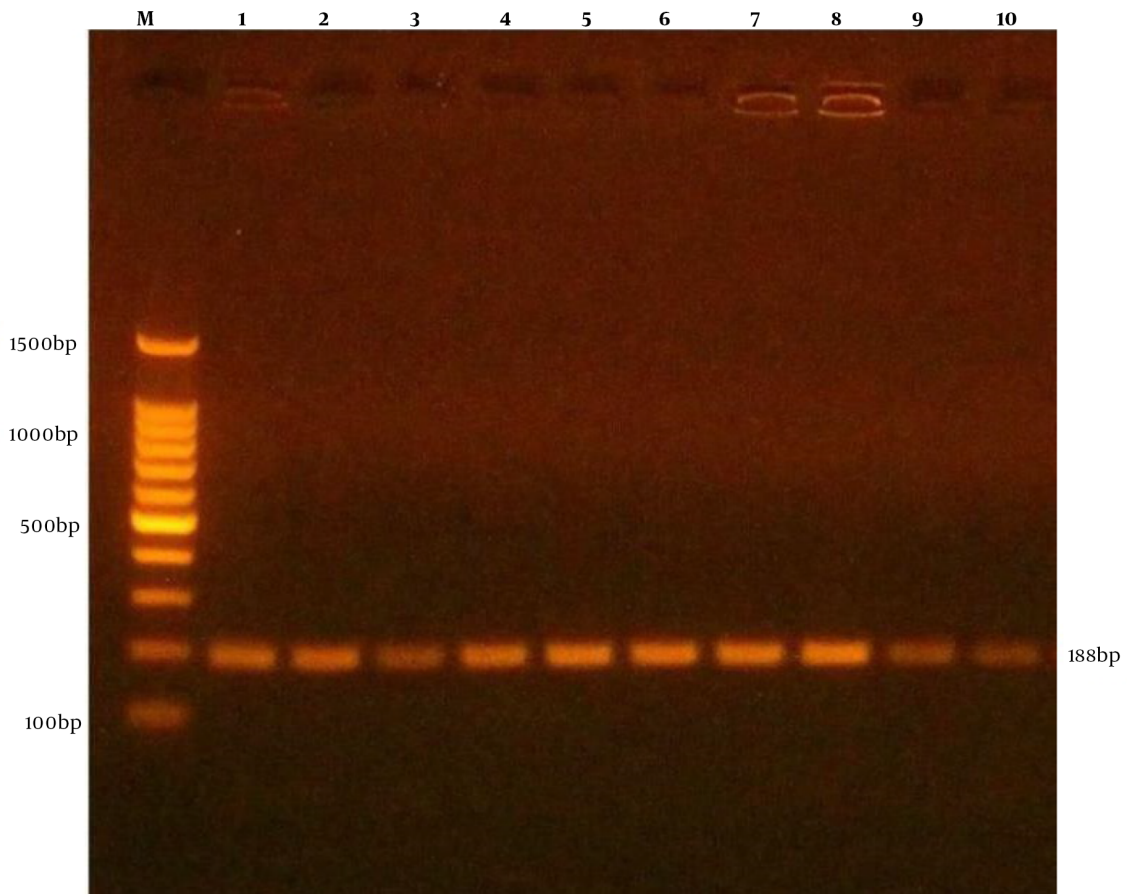


Figure 3. The results of the amplification of *icaA* gene of *Staphylococcus aureus* samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100 bp ladder marker. Lanes 1-10 represent 188 bp PCR products.

5.1. Conclusions

Most *S. aureus* isolates that were isolated from wound were biofilm-positive as it was less for the rest of the isolates, and the biofilm formation capacity was weaker. These isolates were bearing *icaA* and *agr* genes in a high quantity.

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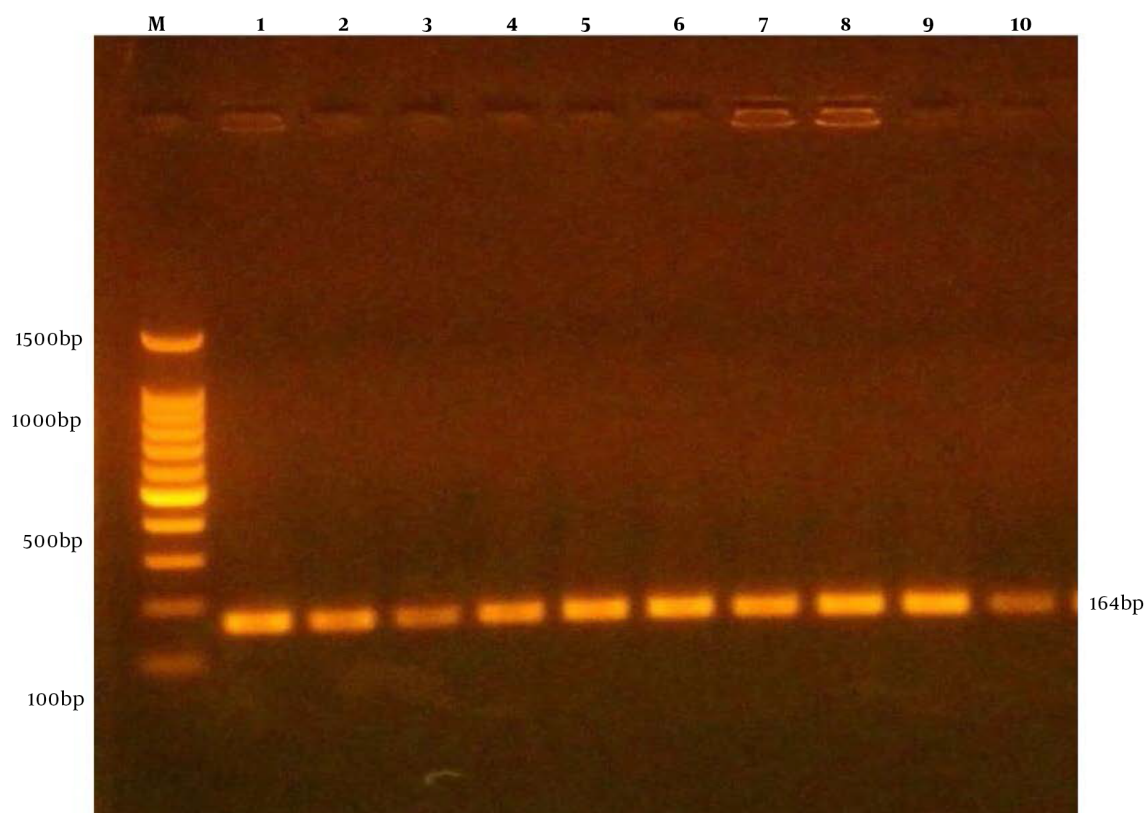


Figure 4. The results of the amplification of *agr* gene of *Staphylococcus aureus* samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100 bp ladder marker. Lanes 1 - 10 represent 164 bp PCR products.

Footnotes

Authors' Contribution: Saade Abdalkareem Jasim wrote the manuscript and analyzed the data. Zainab Amer Hatem performed the PCR assays and collected the data. The critical revision of the manuscript was done by Zainab Hussein Mahdi.

Conflict of Interests: There was no conflict of interest.

Ethical Approval: The Iraqi Ministry of Health has approved the process of conducting samples (administrative order 256020 on 21 October 2020). All the patients signed informed consent forms before the study.

Funding/Support: This project was not financially supported.

Informed Consent: Samples were obtained through communication with patients and according to the ethics of scientific research

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