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Detection of Biofilm Operon, Some Virulence Factors, and Antibiotics Susceptibility of *S. aureus* Isolated from Patients in Holly Karbala City

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Abstract: The formation of biofilms in *Staphylococcus aureus* has the potential to impede the effectiveness of antibiotics, leading to challenges in infection treatment. The aim of this research is to determine the prevalence rate of some virulence genes in *S. aureus* isolate and learn more about the virulence components of *S. aureus.* The *S. aureus* was clinically isolated from different 250 samples in Karbala City (al-Husain Medical City and al-Hassan al Mujtaba Teaching Hospital). Isolate biofilm formation was examined using a microtiter plate assay. The biofilm operon and some of the MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) genes were detected by PCR assay. The disk methods and the VITEK 2 compact were utilized to determine antibiotic susceptibility. The sixty *S. aureus* isolates were isolated and identified. The microtiter plate method divided the isolates into (25%) strong, (66.6%) moderate, and (8.3%) weak biofilm. PCR assay results showed that 100% of isolates have *ica* operon, 100% have clumping factors genes, and 0% of (*bap)*. This research proved the association between the *icaADBC* operon and biofilm production in *S. aureus*.

Keywords: *S. aureus,* biofilm operon, virulence factors, MDR.

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Introduction

Staphylococcus aureus a gram-positive coccus may be found in several various body parts. These include the axilla, rectum, GI tract, and vagina. The front of the nose, however, is where this bacterium is most commonly found. *S. aureus* has the ability to migrate from a commensal site on the skin to the nasal mucosa, where it can engage with specific ligands found on the epithelial cells, including cytokeratin 10 (K10) and loricrin (1). Long-term colonization and intermittent invasive infections characterize the interaction between *S. aureus* and humans, which is reflected in the wide variety of immune evasion strategies discovered in this bacterium. It is well established that *S. aureus* produces a wide variety of virulence and immune evasion components to suppress the host immune response. (2).

The *S. aureus* exists in two distinct states: the planktonic state and the biofilm state, each characterized by unique attributes (3). In summary, biofilms are formed when bacterial cells adhere to a surface by VWF, and then further adhere via a hydrophobic-hydrophilic contact between the bacteria and the surface. The bacteria then establish microcolonies by adhering to the host matrix via MSCRAMMs (microbial surface components

recognizing adhesive matrix molecules). Polysaccharide intercellular adhesion (PIA) is the primary biofilm component in *Staphylococcus aureus*. PIA is mostly made of poly- (1-6)-N-acetylglucosamine (PNAG), which enhances bacterial attachment to exterior surfaces and promotes intercellular adhesion between bacterial cells. PIA is synthesized by *S. aureus* via the *icaADBC* biosynthetic genes, which are located in the ica locus(4).

Under conditions, the production of biofilms has a significant role in the increase of infections caused by *S. aureus*, leading to heightened resistance against antimicrobial agents and the emergence of antibiotic resistance patterns. The presence of biofilms on medical equipment and devices, including implants, contributes to the proliferation and severity of nosocomial infections. Consequently, it is imperative to make concerted efforts to eliminate these factors of antibiotic resistance (5). The phenomenon of the production of biofilm may be classified into three basic phases: adhesion, maturation/ proliferation, and separation(6).

Adherence to surfaces is the first phase in biofilm development, and several different (MSCRAMMs) are expressed to aid in this process. Extracellular matrix components including elastin, fibronectin A and B, laminin, collagen, fibrinogen, and clumping factors may be bindable to these MSCRAMMs. Common signal sequences among proteins may aid in their ability to bind to the membrane of cells and other surfaces. (7). During the second phase, a biofilm matrix is synthesized, facilitating cellular connections. In the case of *S. aureus*, this matrix primarily comprises the PIA/PNAG exopolysaccharide and teichoic acids (8). The biofilm matrix is thought to consist not only of actively produced molecules but also of polymeric compounds released by dead cells. The most noteworthy is DNA, which becomes "extracellular DNA" (eDNA)when it is released from dead cells. Substantial biofilm-structuring factor activity promotes cell cluster separation from the biofilm. (9).

Among the virulence factors expressed by *S. aureus* are proteins that bind fibrin(ogen), collagen, elastin, von Wille- brand factor, vitronectin, and bone sialoprotein; the MSCRAMMs, which include the binding proteins Clumping factors A (ClfA) and. B (ClfB), mediate these interactions between the bacteria and the host. (ClfA) binds fibrin(ogen) at its carboxy-terminal domain, which is located on the chain. This interaction promotes bacterial clumping in host plasma and permits bacterial adherence to fibrin (ogen)-coated surfaces. (10). The identification of the ClfB has emerged as a significant factor in nasal colonization, as evidenced by recent studies. The binding of ClfB to (CK10), which is found in epithelial cells, has been observed. Furthermore, previous research has demonstrated that antibodies targeting ClfB could provide both active and passive protection against staphylococcal colonization in the nasal cavity of mice(11).

Materials and methods

Bacterial culture conditions

Two hundred fifty patient samples representing a wide range of conditions were obtained from two hospitals in Holly Karbala City. (Al-Husain Medical City and Al-Hassan al Mujtaba Teaching Hospital).These specimens included nares swabs (n= 55), abscesses (n=60), ulcers (n=5), blood (n=30), midstream urine (n= 40), and wound swabs (n= 60). Then An examination of the colonies was

performed utilizing methods for determining the species of Staphylococcus, morphological characteristics, production of catalase and coagulase, and growing on mannitol salt agar (12). The VITEK 2 compact (bio- Mérieux, America) was then employed for conclusive verification.

Antibiotics susceptibility test

The examination of antibiotic resistance was conducted using the disk diffusion method on Mueller Hinton agar. The S. aureus isolates had tested with 11 antibiotics (Liofilchem, Italy) including: Oxacillin (30µg) penicillin P (10µg), Tetracycline (30µg), Azithromycin (15µg), Doxycycline (30µg), Gentamycin (10µg) Levofloxacin (5µg) Ciprofloxacin (5 µg) Trimethoprim (5 µg), clindamycin (2µg), and Chloramphenicol (30µg) as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2021) using the Kirby- Bauer disk diffusion method (13).

Methods for quantifying biofilm production

Biofilm production by bacterial isolates may be evaluated using the Microtiter-plate technique, which is a phenotypic and quantitative approach defined by (14). In summary, Utilizing sterile 96-well U-shaped bottom polystyrene microplates. The well was filled with 200 µl of Tryptic Soy Broth (TSB) solution added 0.75 percent glucose (for a total of 1 percent glucose in TSB) with the bacteria concentration adjusted to match the McFarland standard no. 0.5. The plates were then covered and subjected to aerobic incubation at a temperature of $37C^{o}$ for a duration of 24 hours. The experiment involved conducting three replicates for each bacterium. Control wells were prepared by introducing sterile Tryptic Soy Broth (TSB) without bacteria. The wells were subjected to aspiration and subsequently washed 3 times using 200 µl of sterile (PBS). The bacteria that remained attached to the wells were then fixed by treating them with 200 µl of methanol for a duration of 15 minutes. Following the process of air drying, the wells were subjected to staining with a 200 µl solution of crystal violet at a concentration of 0.1%. This staining process was carried out for a duration of 15 minutes, maintaining the ambient room temperature. Placing the plate under running water helped remove any remaining discoloration. After that, we dried the plates. After 15 minutes, 200µl of glacial acetic acid (33% concentration) was used to resolubilize the adhering cells. Finally, an ELISA reader (Biotek, UK) was used to measure the amount of biofilm in each well at 600 nm. The cut_off value (ODc) was determined by calculating the mean optical density (OD) of the control wells plus three standard deviations. The mean optical density (OD) of the negative control in this study was determined to be 0.092. The standard deviation of the OD value was determined to be 0.00173. Consequently, the cut-off value was computed as 0.0971. The biofilm gradation in clinical isolates was then assessed based on the criteria outlined in (Table 1) (15).

Polymerase chain reaction

DNA from *S. aureus* isolates was extracted following the manufacturer's instructions for a Gram-positive DNA extraction kit (Norgen Canada). The isolated genomic DNA was analyzed for both quality and quantity with the use of 2% agarose gel electrophoresis and a QubitTM dsDNA HS assay kit (ThermoFisher® USA). The DNA samples were ultimately placed in a 1 µL microtube. Then stored

at a temperature of -20C^{o} for subsequent investigations.

Molecular detection of *ica* genes encoding (*icaA, icaB, icaC, and icaD*), clumping factor genes (*clfA, clfB*), and biofilm-associated protein (*bap*). The primers utilized in this investigation were designed with reference to the *Staphylococcus aureus* genome database available on the (NCBI). The primers were provided in a lyophilized state by Macrogen Company (Korea). The size of the genes under investigation and their corresponding genetic sequences were specifically determined for the purposes of this study, as presented in (Table 2). The PCR technique was employed using primers specific to the target gene to validate the identification of *S. aureus* isolates. The PCR reaction mixture, with a final volume of 20 µL, comprised of various components. These included 2 µL of a 10X PCR buffer, 1.5 mL of a 25 mM MgCl2 solution, 2 µL of a 10 mM dNTPs solution, 1 µL of each primer, 2.5 µL of the DNA sample, and 1.25U of Taq DNA polymerase from NEB® (England). The final volume was then adjusted to 20 µL using 10.5 µL of nuclease-free water. The amplifications were conducted using a DNA Thermal Cycler (SimpliAmp, Germany). The PCR product (4 µL) was subjected to analysis using 1.5% agarose gel electrophoresis in Tris Acetic EDTA (TAE 1X) buffer, stained with ethidium bromide, visualized under ultraviolet transillumination, and documented using Gel Doc imaging.

Table (1): Classification of biofilm generated by *Staphylococcus aureus* strains

Optical density values OD	Criteria outlined
OD ≤ 0.097	Non biofilm formation
0.0971 < OD ≤ 0.194	Weak biofilm formation
0.194 < OD ≤ 0.388	Moderate biofilm formation
0.388 < OD	Strong biofilm formation

Table (2): The name, sequence, and product size of primers used in this study

Gene	Function	Nucleotide sequences 5'- 3'	Amplicon size (bp)	Annealing temperature C^{o}
icaA	intracellular adhesion A	F: CGACGTTGGCTACTGGATAC R: ACACATGGAAGCGGTTCATA	106	56
icaB	intracellular adhesion B	F: CACAGGTCATGTTGGGGAAGAA R: TGCAAATCGTGGGTATGTGTITC	118	53
icaC	intracellular adhesion C	F: GCGTTAGCAAATGGAGACTATTGG R: TGCGTGCAAATACCCAAGATAACA	101	53
icaD	intracellular adhesion D	F: TCGCTATATCGTGTGTCTTTTGGA R: CGCGAAAATGCCCATAGTTTCA	164	53
clfA	clumping factor A	F: ACGGATCAGGTTCTGGTGACG R: TCGCTGCCAGAATCTGAACCA	136	56
clfB	clumping factor B	F: TCACTTGCCGTAACTTGACCA R: ACAAACGATTTCCAATGCGCA	141	53
bap	biofilm associated protein	F: CGAACGTCAAGTATCGCTTTCA R: ACCTTCTTTTAGCGGCACTGA	175	52

Results and discussion

Isolation and identification of *S. aureus* isolates

Two hundred fifty clinical specimens were obtained from patients in Holly Karbala City, ranging in age from one year to eighty years. Sixty different isolates were found to be S. aureus belonged to the 21 wounds (35%), 14 nasal cavity (23.33%), 5 blood (8.33%), 10 urine (16.66%), 9 abscesses (15%), and 1 ulcer (1.66%). *S. aureus* detection by VITEK 2 compact. The bacterium *S. aureus* was adaptable enough to thrive on a wide variety of media, including blood agar and brain heart infusion. *S. aureus* colonies on blood agar appear mucoid and have a characteristic yellow color with a clear zone surrounding the colonies, showing hemolysis, *S. aureus* also has a positive catalase, and a positive coagulase.

Molecular detection of *ica* operon and MSCRAMMs genes

The *ica* operon PCR technique was employed to confirm the presence *of S. aureus* in all 60 isolates, resulting in a 100% confirmation rate. All isolates exhibited the presence of two MSCRAMMs and four PIA genes at the genotypic level, as determined by the use of particular primers (Table 2). The observed prevalence rates of the genes in question were as follows: *icaA* (100%), *icaB* (100%), *icaC* (100%), *icaD* (100%), *clfA* (100%), and *clfB* (100%) (Figures 1, 2, 3, 4, 5 and 6). Similarly, Atshan *et al*. (16) reported that *icaADBC* locus was present in all of the 60 MSSA and MRSA tested clones (16). According to Rodhe et al. PCR testing, all 80 *S. aureus* isolates were positive for *icaADBC* by using PCR method (17).

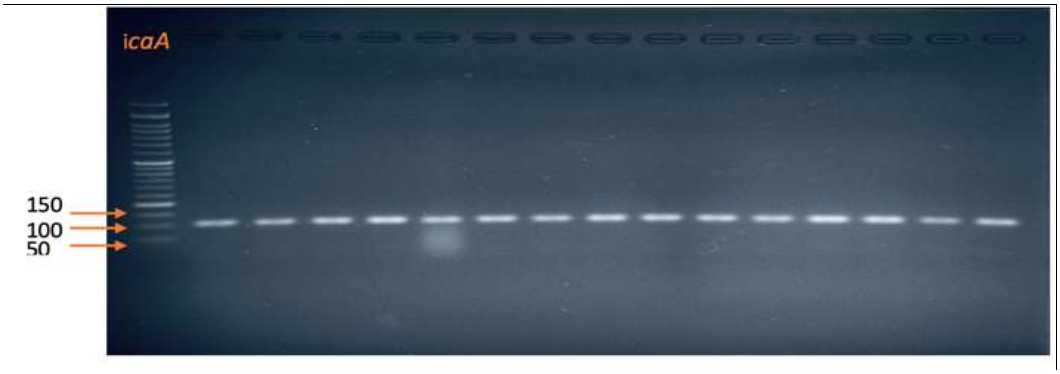

Figure (1): Amplification result of *icaA* gene.

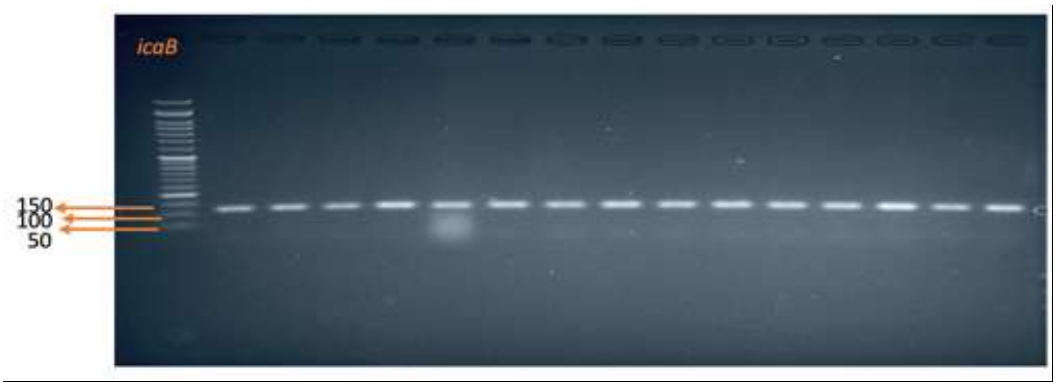

Figure (2): Amplification result of *icaB* gene.

Figure (3): Amplification result of *icaC* gene.

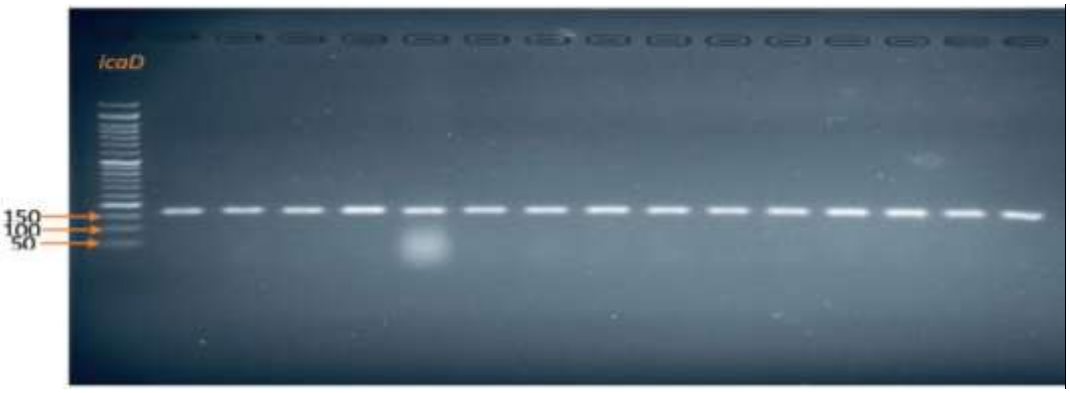

Figure (4): Amplification results of *icaD* gene.

Figure (5): Amplification result of *clfA* gene.

Figure (6): Amplification result of clfB gene.

The involvement of the *icaADBC* locus in the process of biofilm development highlights the significance of cell wall proteins, specifically MSCRAMMs, in the pathogenicity of *Staphylococcus aureus* through the formation of biofilms. In this investigation, it was shown that the occurrence rate of *icaADBC* genes was comparable to that of MSCRAMMs genes, with the exception of *bap* gene, which was found in nearly all of the *S. aureus* isolates. All *S. aureus* samples tested negative for the presence of the bap gene, which aligns with previous findings indicating its absence in human strains of *S. aureus* (18). Among the 75 strains studied by Goudarzi *et al.* from Iranian patients with UTIs, the frequency of the *clfA* and *clfB* genes was similarly high at 94.7% and 92%, respectively. respectively. Consistent with the findings of Smith *et al*., the present study found that wounds, nares, and blood had the highest rates of robust biofilm (19).

Biofilm formation assessed quantitatively

The Microtiter plate assay is extensively utilized and has been regarded as the established method for detecting biofilm development. The method has been demonstrated to be an accurate, sensitive, and consistent screening methodology for evaluating biofilm production by *S. aureus* clinical isolates. In addition, it may be used as a quantitative tool to evaluate and contrast the adhesion properties of distinct strains. The phenotypic characteristics showed that 15 (25%) isolates were strong, 40 (66.6%) isolates were moderate, and 5 (8.3%) isolates were weak (figure 7). The maximum rate of strong biofilm also belonged to the wound (1.23433), nares (0.5136), and blood (0.438) (figure 8). The current study's findings confirmed the existence of a substantial link between antibiotic resistance and biofilm development. (Table 3). Mohammed *et al* 14% of their local *S. aureus* isolates were high biofilm producers, 43% were moderate biofilm producers, and 43% were low biofilm (20) have seen and reported similar tendencies. Based on their data, Mathur *et al*. similarly conclude from their data that about 14.47% and 39.4% of *S. aureus* isolates exhibited high and moderate biofilm formation, respectively; while 46% were weak isolates (16) arrive at the same conclusion. Quantitatively, the data revealed that biofilm production was greater in MDR strains. The production of biofilms and the emergence of (MDR) strains have been identified as significant factors contributing to the pathogenicity of chronic infections caused by *Staphylococcus aureus*, posing a potential hazard to public health. Furthermore, it should be noted that host tissues, as well as abiotic surfaces such as implant devices and catheters, have the potential to facilitate the initial adhesion of bacterial cells and subsequent biofilm development. The existence of persister cells inside the biofilm matrix provides a mechanism for evading the host defense system and resisting the effects of antimicrobial agents. To better understand how to prevent and treat *S. aureus* infections, it may be useful to characterize the genotypes and phenotypes of isolates with known biofilm-forming and antibiotic-resistance potential (21).

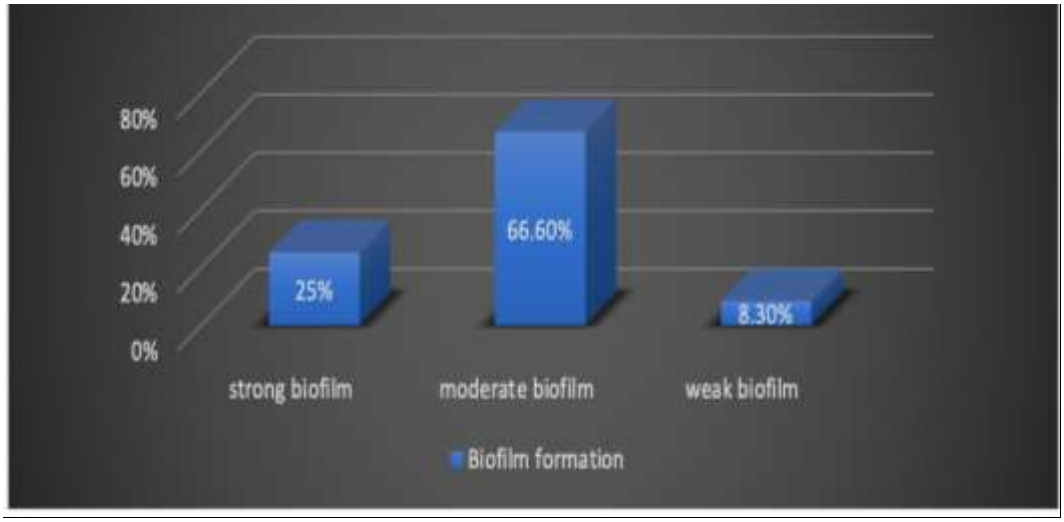

Figure (7): Classification of biofilm formation among *S. aureus* isolates.

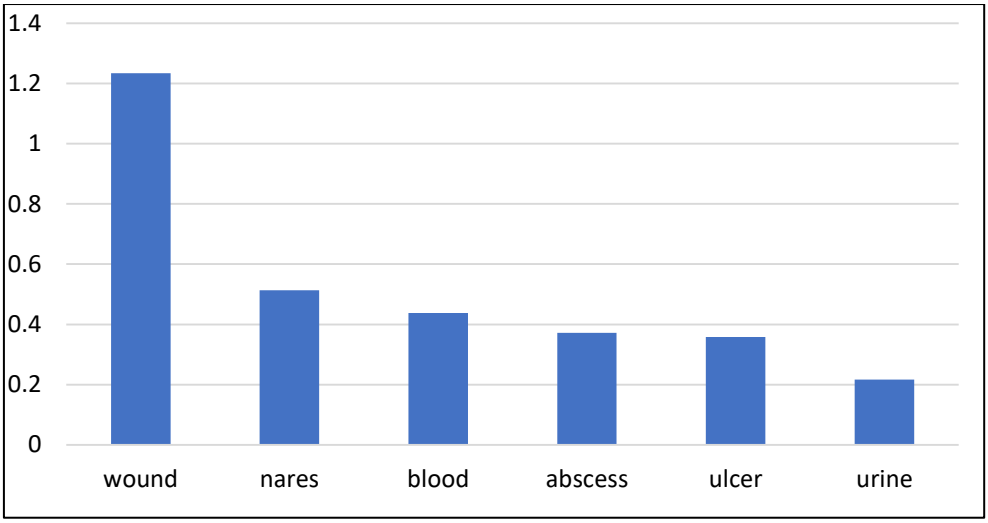

Figure (8): Frequency of *S. aureus* isolates by site of infection.

Antibiotics susceptibility test

The antibiotics Susceptibility results showed antibiotics showed all isolates resistant to Oxacillin. The highest resistance rate of the isolates was to Penicillin PG (98%). Resistance rate to other antibiotics was observed (63%) resistance to Tetracycline, (51%) resistance to Azithromycin, (43%) resistance to Doxycycline, (15%) resistance to Gentamycin, (13.3%) resistance to Levofloxacin, (13.3%) resistance to Ciprofloxacin, (11.6%) resistance to Trimethoprim, (10%) resistance to clindamycin, (3.33%) resistance to Chloramphenicol. According to the findings of this investigation, *S. aureus* has a significant level of resistance to antibiotics. Consistent with recent research by Bimanand *et al.* (100% PG resistance) and Goudarzi *et al.* (87% PG resistance) (22,23), The results of this investigation show a correlation between antibiotic resistance and biofilm development in *S. aureus* isolates (Table 3).

Table (3): Antimicrobial resistance and biofilm profile of MDR *S. aureus* isolates.

MDR Isolates	Resistance Phenotype	Biofilm Production	*icaA* gene	*icaB* gene	*icaC* gene	*icaD* gene	*clfA* gene	*clfB* gene	*bap* gene
1	**OX-TE-DO-AZM-PG**	strong	+	+	+	+	+	+	-
2	**OX-CIP-LEV-PG**	strong	+	+	+	+	+	+	-
3	**OX-TE-DO-AZM-PG-CN**	strong	+	+	+	+	+	+	-
4	**OX-TE-AZM-PG**	strong	+	+	+	+	+	+	-
5	**OX-TE-DO-AZM-PG**	strong	+	+	+	+	+	+	-
6	**OX-TE- DO-TM- PG**	strong	+	+	+	+	+	+	-
7	**OX-TE-DO-AZM-PG**	strong	+	+	+	+	+	+	-
8	**OX-TE-DO-AZM-CIP-TM-PG**	moderate	+	+	+	+	+	+	-
9	**OX- TE-DO-PG-CN**	moderate	+	+	+	+	+	+	-
10	**OX-TE-AZM-PG**	strong	+	+	+	+	+	+	-
11	**OX-CIP-LEV-PG**	moderate	+	+	+	+	+	+	-
12	**OX-TE-DO-PG-CN**	strong	+	+	+	+	+	+	-
13	**OX-TE-DO-AZM-PG**	moderate	+	+	+	+	+	+	-
14	**OX-TE-DO-AZM-PG**	strong	+	+	+	+	+	+	-
15	**OX-TE-DO-AZM-PG-CIP-TM-LEV-CN**	strong	+	+	+	+	+	+	-
16	**OX-TE- DO-CIP-PG- TM**	moderate	+	+	+	+	+	+	-
17	**OX-CIP-PG**	moderate	+	+	+	+	+	+	-

Conclusion

The results of our investigation indicate the *icaADBC* operon and clumping factors genes were identified in *S. aureus* strains obtained from patients, however, the *bap* gene was not observed in the samples collected from patients, this might be a sign of therapy failure in persistent *S. aureus* infections. The identification of these genes in isolates that produce biofilms suggests their significance in the production of biofilms in *S. aureus*, hence conferring antibiotic resistance to the bacterium. It is imperative to direct our attention towards the correlation between multidrug resistance (MDR) and the process of biofilm development. Thus, one of the key virulence aspects of the *S. aureus* isolates is its ability to create biofilms, particularly in wounds and in patients hospitalized in intensive care units (ICUs).

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