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Molecular characteristic of *Staphylococcus aureus* isolated from patients with nasal carriage infections

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Staphylococcus aureus is a pathogenic microorganism that leads to a range of infections in both humans and animals due to its ability to form biofilms and develop antibiotic resistance. A total of 150 nasal swabs were collected from outpatients, from which 52 (34.7%) isolates were identified as *S. aureus*. Identification of *S. aureus* isolates was conducted by biochemical analysis (catalase and coagulase assays). Biofilm formation was evaluated using Congo-red agar, and the presence of *icaA* and *icaD* genes was confirmed through molecular analysis. Antibiotic sensitivity patterns were assessed by regular antimicrobial testing. All the samples were catalase- and coagulase-positive. Biofilm production was detected in 15 (28.8%) specimens. All the samples were *icaA* and *icaD* gene-positive. Antibiotic resistance tests confirmed that the highest resistance was exerted against Oxacillin (86.5%), Tetracycline (67.3%), Ciprofloxacin (65.4%), and Erythromycin (63.5%). Vancomycin and Gentamicin demonstrated a strong efficacy. A moderate efficacy was achieved with Rifampin (96.2%), Cotrimoxazole (94.2%), and Augmentin (90.4%). The present study highlighted the rate of antibiotic resistance among nasal *S. aureus* isolates, including resistance to Oxacillin, which is the gold standard for methicillin-resistant *S. aureus* (MRSA). These outcomes were further elucidated by positive biofilm genes (*icaA* and *icaD*), suggesting the pathogenic potential of these isolates. These outcomes warrant further study, and effective antibiotic criteria are essential to manage the increasing resistance of *S. aureus* to ensure the effectiveness of available therapy.

Keywords: *Staphylococcus aureus*; *icaA* gene; *icaD* gene; antibiotic sensitivity test.

Introduction

Staphylococcus aureus is one of the most paradoxical microbial organisms linked to human life. This Gram-positive coccus, well-known for forming grape-like clusters under the microscope and its golden-yellow colonies on agar plates, possesses significant virulence factors that contrast with its microscopic size. As a catalase- and coagulase-positive organism, it can neutralise hydrogen peroxide and can cause plasma to clot. However, it is part of the normal flora of the skin, vagina, and nostrils of nearly a third of the human population. This dualistic behaviour makes it an opportunistic microorganism, waiting for any skin breach due to burn, injury, or surgery, to cause infection (Ravi, 2011).

Approximately 20–40% of healthy individuals are asymptomatic carriers of *S. aureus* (Ryan & Ray, 2004; Sivaraman et al., 2009; Ravi, 2011). In clinical environments, the rate of nasal carriers that disseminate the infectious agent reaches approximately 16.8% to 56.1%. Moreover, in healthcare settings, potential reservoirs for *S. aureus* can be personnel's uniform.

Direct contact transmission or aerosol transmission are the usual transmission routes. Regardless of colonization, *S. aureus* is the main human microorganism, causing a broad range of infections, including postsurgical wound infections, cellulitis, impetigo, abscesses, and life-threatening conditions in immunocompromised patients (Cole et al., 2001; Gebremariam & Zelelow, 2014). The bacteria acquire different virulence factors, including coagulase, hyaluronidase, leukocidin, lipase, proteases, protein A, and capsule formation, which contribute to their pathogenicity (Nester et al., 2009). Biofilm production plays a special role in augmenting *S. aureus* survival and resilience, providing a fight against the host immune responses and antibiotic therapy. Biofilms enable chronic infections and are a main concern in both community and healthcare-associated infections. The elimination of *S. aureus* infections has become progressively challenging due to the rapid rise of antibiotic resistance, particularly against β-lactam antibiotics, hindering effective therapeutic options (Kumar et al., 2011). The extensive misuse and overuse of antibiotics have broadly worsened the resistance development, complicating infection control attempts (Assafi et al., 2015). This study aimed to investigates and identify *S. aureus* isolates from nasal swabs of patients and assess their antibiotic resistance patterns.

Materials and methods

A total of 150 nasal swab samples were collected from patients between the first of December 2021 to first of January 2022, at the outpatient clinics of Al-Karama Teaching Hospital (Baghdad, Iraq). Each swab was numbered for identification code. Swabs were preconditioned with sterile 0.85% NaCl saline solution to reduce the nasal cavity soreness, then inserted into the anterior nares, rotated against the inner surface, and placed in transport media. The specimens were streaked onto mannitol salt agar and blood agar (Mast Group Ltd., UK; Biomark Lab., India) and incubated aerobically at 37 °C for 24 hours before examination. Isolation and identification of the *Staphylococcus aureus* were conducted based on standard laboratory protocols, including:

– Gram staining: Gram-positive, grape-like clusters;

– Biochemical tests: Catalase-positive and coagulase-positive;

– Colony morphology: Beta-hemolytic colonies on blood agar and golden-yellow, mannitol-fermenting colonies on mannitol salt agar (Jain et al., 2008; Biswas et al., 2015);

– Biofilm production test: The biofilm-forming ability of the *S. aureus* isolates was assessed using Congo-red agar (CRA), prepared by adding 0.8 g Congo red and 36 g sucrose to 1 L of brain-heart infusion (BHI) agar. After 24-hour incubation at 37 °C, the biofilm producers were identified based on the colony color:

– Black colonies: Biofilm producers (slime-positive); red colonies: non-biofilm producers (slime-negative) (Freeman et al., 1989; Hassan et al., 2011);

– Molecular characterization: To confirm the *S. aureus* identification and detect biofilm-associated genes (*icaA* and *icaD*), PCR was

used. DNA Extraction: DNA was extracted from the overnight cultures of *S. aureus* on blood agar using the ABIO DNA extraction kit, following the manufacturer's instructions. Concentration of DNA was measured using a Quintus fluorimeter by mixing 200 µL of Quintus Fluor Dye with 1 µL of DNA and incubating for 5 minutes at room temperature.

PCR analysis for detection of *icaA* and *icaD* biofilm genes:

1. Extraction of DNA: The extraction of genomic DNA from the bacterial swabs was conducted according to DNA extraction protocol.

2. Primers and expected product size: Specific primers targeting the *icaA* and *icaD* genes were used to confirm *S aureus* as the source of biofilm formation. These primers, supplied in a lyophilized form by Macrogen Company (Table 1), were prepared as follows:

– Lyophilized primers were prepared in a stock solution containing no nuclease, achieving a total end concentration of approximately 100 pmol/µL;

– A working primer solution (10 pmol/µL) was prepared by diluting 10 µL of primer stock solution with 90 µL of nuclease-free water;

– The primers amplified the *icaA* and *icaD* gene segments, producing fragments of 188 bp and 211 bp, respectively (Kot et al., 2018).

Table 1
Oligonucleotide primers used for *icaA* and *icaD* gene amplification in the *S. aureus* isolates

Primer Name		Sequence 5`-3`	Annealing temperature, °C	Product size, bp
icaA	F	ACACTTGCTGGCGCAGTCAA	55	188
icaA	R	TCTGGAACCAACATCCAACA		
icaD	F	ACCCAACGCTAAAATCATCG	56	211
icaD	R	GCGAAAATGCCCATAGTTTC		

3. PCR conditions: Amplification of PCR was performed following the program outlined in Table 2. The reaction conditions ensured optimal amplification of the target genes.

Table 2
PCR cycling conditions for amplification of *icaA* and *icaD* genes in the *S. aureus* isolates

Steps	°C	m:s	Cycle
Initial denaturation	95	05:00	1
Denaturation	95	00:30	
Annealing	55.56	00:30	30
Extension	72	00:30	
Final extension	72	07:00	1
Hold	10	10:00	

4. Gel electrophoresis analysis: The PCR products were analyzed using gel electrophoresis to confirm the presence of *icaA* and *icaD* genes. A positive control strain, *S aureus* ATCC 25923, was included in the experiment for validation.

The antibiotic susceptibility was evaluated using the Kirby-Bauer disk diffusion method (Baer et al., 1966) on Mueller-Hinton agar (Oxoid, UK), following the CLSI guidelines (CLSI, 2010). The bacterial suspension was optimised to the 0.5 McFarland standard and incubated at 37 °C for 15 minutes before inoculation. The following antibiotic discs were tested: Augmentin (30 µg), Ciprofloxacin (10 µg), Oxacillin (1 µg), Cotrimoxazole(1.25/23.75 µg), Erythromycin (15 µg), Tetracycline (30 µg), Rifampicin (5 µg), Vancomycin (30 µg), and Gentamicin (10 µg). The zones of inhibition were measured to determine susceptibility or resistance.

Minimum inhibitory concentration (MIC) for antibiotics was measured using the Vitek 2 system (bioMérieux, France) according to the manufacturer's instructions. The results were interpreted based on the CLSI breakpoints.

The Fisher's exact test was used to summarize the association between the biofilm production and the presence of *icaA/icaD* genes in the *S aureus* isolates. A $P < 0.0001$ was considered statistically significant, supporting the hypothesis that *icaA/icaD* genes are strongly associated with the biofilm-producing phenotype. Also, the Chi-square test was applied to assess the antibiotic resistance profile. A P-value < 0.00001 was considered very significant. This suggests that *S. aureus* does not respond uniformly to all antibiotics; some are significantly more effective than others (Fisher, 1922; McHugh, 2013).

Results

Of one hundred fifty sterile cotton swab specimens collected from patients' nasal sites, 52 (34.7%) cases were positive for *S. aureus*. Residual results, such as other organisms, mixed cultures, and negative cultures, were excluded from the study. The Gram staining showed Gram-positive grape-like clusters. The colony morphology on blood agar was beta-hemolytic; on mannitol salt agar, the colonies exhibited mannitol fermentation with golden-yellow colonies; and on MacConkey agar plates, the colonies appeared pink, indicating lactose fermentation. (Jain et al., 2008; Biswas et al., 2015) (Fig. 1).

Fig. 1. Mannitol salt agar showing mannitol fermentation by the *S. aureus* isolates, indicated by golden yellow colonies

Biochemical tests, such as catalase and coagulase (catalase and coagulase positive test), were important phenotypic identifying markers of *S aureus* (Murray et al., 2003). The study found that 100% isolates were positive for catalase and coagulase.

Observing the biofilm formation rate, 15 (28.8%) isolates out of 52 swab specimens formed black colonies on CRA and were considered as slime producers. All the colonies that looked black with dry crystallized density (Fig. 2) indicated positive results, while the colonies that remained pink (Fig. 3) indicated negative results (Mathur et al., 2006).

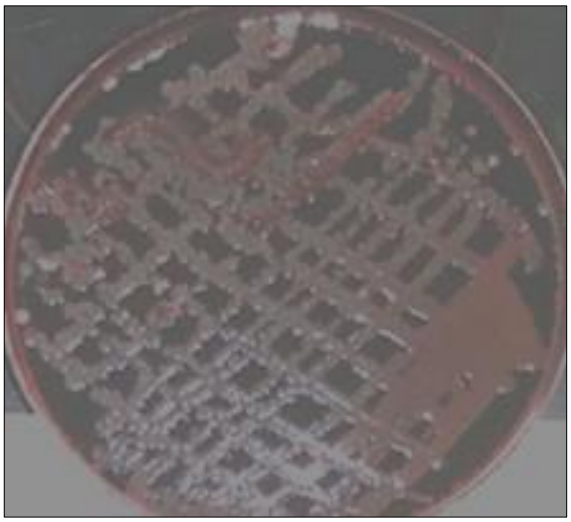

Fig. 2. Positive biofilm formation by the *S. aureus* isolates on Congo red agar, indicated by black colonies with dry crystalline density

The PCR technique was applied to the staphylococcal strains. As shown in Figures 4 and 5, those positive for *icaA*, were also positive for *icaD* (intercellular adhesion gene *A* and *D*), giving a 188 bp

band for the *icaA* gene and a 211 bp band for the *icaD* gene. Both bands were obtained from the slime-producing clinical isolates of *S. aureus*.

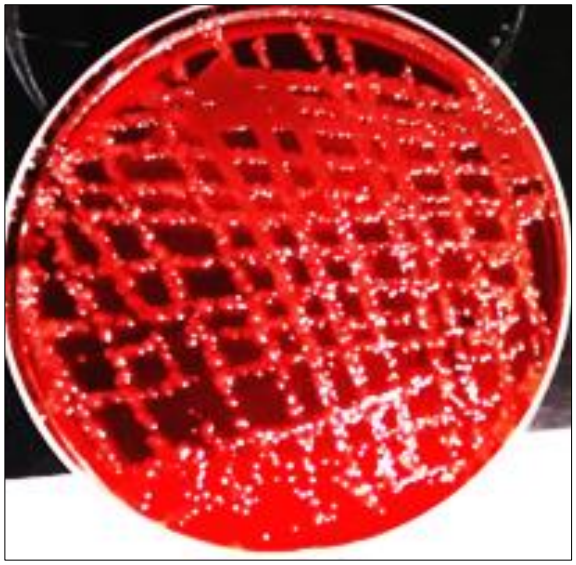

Fig. 3. Negative biofilm formation by the *S. aureus* isolates, indicated by pink colonies

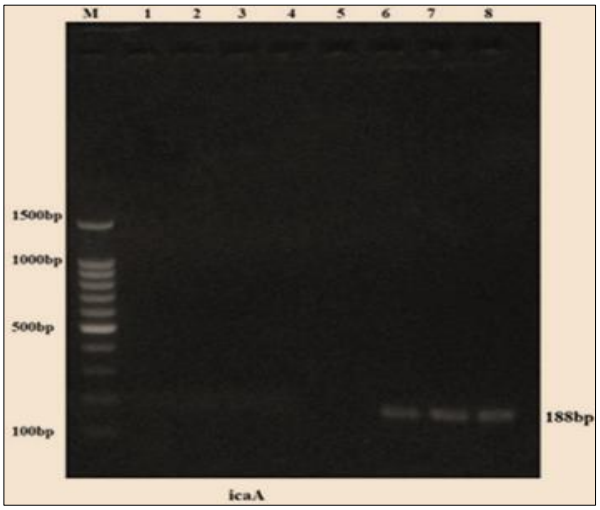

Fig. 4. Agarose gel electrophoresis of *icaA* gene amplification (188 bp): lanes 1–5 – negative samples, M – 100 bp DNA ladder

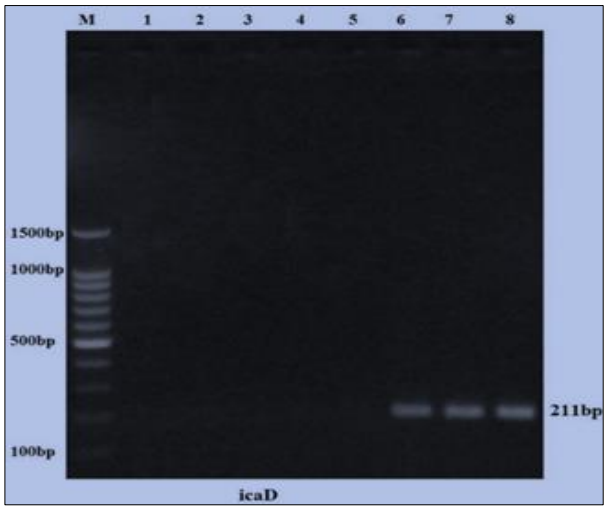

Fig. 5. Agarose gel electrophoresis of *icaD* gene amplification (211 bp): lanes 1–5 – negative samples, M – 100 bp DNA ladder

In this study, *S. aureus* showed the frequency of resistance ranging 3.8% to 86.5%. In particular, the resistance of the *S. aureus* isolates measured 9.6% to Augmentin, 65.4% to Ciprofloxacin, 86.5% to Oxacillin, 5.8% to Cotrimoxazole, 63.5% to Erythromycin, 67.3% to Tetracycline, and 3.8% to Rifampicin. The most active antibiotic against all the isolates of *S. aureus* were Vancomycin and Gentamicin (100%), followed by Rifampicin (96.2%), Cotrimoxazole (94.2%), and Augmentin (90.4%), while the least active antibiotics were Erythromycin (36.5%), Ciprofloxacin (34.6%), Tetracycline (32.7%), and Oxacillin (13.7%).

Discussion

The genus *Staphylococcus*, particularly *S aureus*, is responsible for a wide range of infections in humans. While it is a natural component of the human and animal microbiota, its opportunistic nature allows it to cause severe conditions such as bacteremia, endocarditis, osteomyelitis, and toxic shock syndrome. The findings of this study align with previous research, reinforcing the global concern surrounding *S. aureus* and its clinical implications (Tong et al., 2015). The results of the current study are in agreement with the findings of Karimi et al. (2017), who isolated 65 (19%) *S. aureus* strains from 340 nasal swab samples. Also, Karmakar et al. (2016) found that among 165 samples, 100 strains (60.6%) were *S aureus*. Azmi et al. (2019) separated 25 (10.1%) of *S. aureus* strains from nasal secretions. In Australia, a comparable result (28.9%) was yielded by Munckhof et al. (2009) for the adult population, and Rohde et al. (2009) in the USA demonstrated a 29.6% *S. aureus* carriage rate in 203 patients.

The findings of this study regarding *S. aureus* nasal carriage rates verify the results from previous studies. The variation in the prevalence of colonization rates – 10.1% to 60.6% – may be due to geographical factors, sample size, detection methods, and host immune responses. Interpretation of these carriage rates is essential because nasal colonization is a fundamental reservoir for infection, especially in clinical settings where transmission to immunocompromized individuals can lead to severe outcomes.

The biofilm formation test demonstrated that 15 (28.8%) of the 52 isolates formed biofilms, which is consistent with prior study, perhaps because biofilm production increases the pathogenicity of *S. aureus* by affording resistance to antibiotics and evasion from the host immune system. This adaptive mechanism confounds therapy and eradication attempts, often leading to continuous and recurrent infections (Mathur et al., 2006). The present study reinforces the evidence that biofilm-producing *S. aureus* strains pose a heightened threat in clinical settings. Additionally, the disparity in biofilm detection rates using CRA across different studies suggests methodological limitations and the need for standardised diagnostic approaches.

In the present study, 15 strains of *S aureus* produced biofilm in Congo-red agar (CRA), which is consistent with the study by Mariana et al. (2009), who found that 78% out of 100 isolates were positive for producing biofilm formation in this medium. The present study is also consistent with the study by Arciola et al. (2001), who found that 14 out of 23 isolates were positive for producing biofilm. At the same time, Taj et al. (2012) found that 4 (3.47%) out of 63 isolates were positive for biofilm production and concluded that the CRA method is not recommended for the investigation of the biofilm formation by *S aureus*.

The present findings demonstrated *icaA* and *icaD* genes in 15 (28.8%) of the *S. aureus* biofilm-producing isolates. In the study by Arciola et al. (2001), the slime-producing strain of *S. epidermidis* ATCC 35984 was positive for both *icaA* and *icaD* genes, which gave a 188-bp band and a 198-bp band, respectively. Another study by Mahmood & Hussein (2022) found that isolates of *S. aureus* were identified in 29 (19.3%) of 150 samples, of which 9 (24.1%) isolates were biofilm-generating isolates, which means that they form black colonies on CRA. At the same time, in the study by Mariana et al. (2009), the PCR method detected *icaA* and *icaD* genes in all the 100 tested isolates of *S. aureus*, which formed intense black pigmented colonies on the Modified Congo-Red Agar. Some studies explained

the contradictory findings by stating that a variety of factors, such as the presence of glucose, lactose, or proteases in the culture media, the surface area and type of surface (rough or smooth), porosity, and charge on the surface, all influence the development of biofilms. Variations can also be caused by the presence of foreign bodies, the genome of the clinical isolate of *S. aureus*, and different types of samples. Furthermore, human cell receptors, tissues, and matrix proteins can all be bound by *Staphylococcus* species (Lotfi et al., 2014; Tong et al., 2015).

A total of 52 *S. aureus* isolates were tested for their antibiotic susceptibility to nine selected antibiotics (listed in Table 3) using the Kirby-Bauer disk diffusion method. The diameter of the inhibition zones around the antibiotic discs was measured and compared with the NCCLS guidelines (2018). The resistance rates among *S. aureus* isolates ranged 3.8% to 86.5%, highlighting a significant variability in the antibiotic effectiveness.

Table 3
Antibiotic susceptibility patterns of the *S. aureus* isolates (n = 52) based on the Kirby-Bauer disk diffusion method

Antibiotics	S (%)	R (%)
Augmentin	47 (90.4)	5 (9.6)
Ciprofloxacin	18 (34.6)	34 (65.4)
Oxacillin	7 (13.7)	45 (86.5)
Cotrimoxazole	49 (94.2)	3 (5.8)
Erythromycin	19 (36.5)	33 (63.5)
Tetracycline	17 (32.7)	35 (67.3)
Rifampicin	50 (96.2)	2 (3.8)
Vancomycin	52 (100.0)	–
Gentamicin	52 (100.0)	–

The observed resistance rates were as follows: 9.6% to Augmentin, 65.4% to Ciprofloxacin, 86.5% to Oxacillin, 5.8% to Cotrimoxazole, 63.5% to Erythromycin, 67.3% to Tetracycline, and 3.8% to Rifampicin. The most effective antibiotics were Vancomycin and Gentamicin, both demonstrating 100% efficacy, followed by Rifampicin (96.2%), Cotrimoxazole (94.2%), and Augmentin (90.4%). By contrast, the least effective antibiotics were Erythromycin (36.5%), Ciprofloxacin (34.6%), Tetracycline (32.7%), and Oxacillin (13.7%).

Previous studies support these findings. El-Kady (2015) reported a 93.75% sensitivity of the isolates to Vancomycin, while Danelli et al. (2020) in Southern Brazil found all the isolates susceptible to this drug. Alternative studies (Rashwan et al., 2006; Munck-hof et al., 2009; Prates et al., 2010; Khorvash et al., 2012; and Jasim& Al-Moosawi, 2014) have confirmed that the sensitivity to Vancomycin was 100%. This high sensitivity may be linked to the limited clinical application of Vancomycin, perhaps due to its cost, which minimizes the likelihood of resistance development.

Danelli et al. (2020) reported some similarities and differences compared to the present study, since they have found high sensitivity rates to Ciprofloxacin (86.3%), Gentamicin (89.2%), Sulfamethoxazole / Trimethoprim (88.5%), Tetracycline (92.8%), and Rifampicin (99.3%). By contrast, the present study demonstrated lower sensitivity rates for Ciprofloxacin (34.6%) and Tetracycline (32.7%), however, similar findings for Gentamicin (100.0%), Co-trimoxazole (94.2%), and Rifampicin (96.2%) were also reported.

The resistance to Augmentin in this study was 9.6% (5 out of 52 isolates), contradictory to the results of Nour El-Din et al. (2021), who demonstrated 18% resistance rate (29 out of 85 isolates). Moreover, El-Kady (2015) revealed a 100% resistance to Oxacillin, which agrees with 86.5% resistance rate (45 out of 52 isolates) of this study. The high resistance to Oxacillin and other beta-lactam antibiotics is expected due to the beta-lactamase formation by *S. aureus*, alongside the frequent and inappropriate use of these antibiotics, which initiates selective pressure favoring resistant strains (Khalili & Sharifi-Yazdi, 2009).

The range of antibiotic sensitivity in the antibiotic sensitivity detected in this study could be induced by other factors, such as, geographic differences, alterations in antibiotic usage policies, and the rate of resistance genes in different populations. Prolonged surveillance and antibiotic monitoring are fundamental to direct the intensifying resistance of *S. aureus* and confirm the effectiveness of the present treatment options.

The investigation of *icaA* and *icaD* genes in 15 (28.8%) of the biofilm-generating isolates further intensifies the interpretation of biofilm formation mechanisms in *S. aureus*. The expression of these genes align with the biofilm production, as demonstrated by previous studies. The genetic element of the biofilm formation determines the requirement of molecular diagnostic tools in assessing the pathogenic capacity of collected sample isolates. However, alteration in detection rates over studies emphasize the complexity of biofilm regulation, which is encouraged by milieu factors, strain-specific genomic differences, and exogenous stimuli such as nutrient availability and surface properties.

The outcomes of this study are notable in both clinical and public health settings. The rate of *S. aureus* nasal colonization demonstrated the need for serious infection control policies, particularly in healthcare facilities where carriers can function as pools for hospital-acquired infections. The validated biofilm-production capacity of these sample isolates reflects that management protocol should merge approaches aimed at biofilm breakage to facilitate the antibiotic efficacy. Moreover, the genetic understandings gained from this study postulated a foundation for improving novel treatment modalities that direct against biofilm inhibition.

Conclusion

This study revealed that the *S. aureus* strains collected from participants with nasal carriage infections were positive for both *icaA* and *icaD* genes and generated biofilm. Moreover, the study underscores the role of *S. aureus* in human infections, especially because bacteria can resist the exogenous severe milieu by producing a biofilm that protects them from antibiotics and the host immune system. Moreover, the isolated *S. aureus* demonstrated a recurrent resistance to certain antibiotics, ranging 3.8% to 76.9%. Vancomycin and Gentamicin, with 100% inhibition of the isolates of *S. aureus*, were the most efficient antibiotics. The agreement of these results with earlier findings determined the global issue regarding *S. aureus* infections and the necessity for improved detection, prevention, and treatment strategies. Future studies should be directed toward finding alternative therapeutic options, such as antibiofilm agents, to combat *S. aureus*-associated infections effectively.

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