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Genotype-phenotype characteristics of biofilm formation in clinical isolates of *Staphylococcus aureus* in acute tonsillitis

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Staphylococcus aureus is not a primary etiological agent of acute tonsillitis but may contribute to disease progression and persistence, particularly in polymicrobial infections. Its ability to form biofilms can complicate treatment and contribute to persistent infection. However, the relationship between the presence of adhesion genes and their phenotypic expression remains unclear. The objective of the study was to comprehensively evaluate biofilm formation in clinical *S. aureus* isolated from patients with acute tonsillitis using combined genotypic and phenotypic approaches. A total of 14 *S. aureus* isolates were analysed. Biofilm formation was assessed using phenotypic methods: microtiter plate assay, tube method, and microscopy. Exopolysaccharide production was evaluated using Congo red agar and a modified liquid medium. PCR was performed to detect the *icaABCD* operon, *clfA*, *clfB* genes as well as the species-specific markers (nuc, au-nuc). All isolates carried the full set of adhesion-related genes. However, phenotypic analysis revealed a variability in the biofilm formation: According to the microtiter plate assay, 7.1% of the isolates were non-producers, 42.8% weak, 21.4% moderate, and 28.6% strong biofilm producers. Using three phenotypic methods, 71.4% of the isolates showed complete agreement in their scores, and a strong correlation between the methods within the study sample. All isolates tested positive on Congo red agar and broth. The presence of adhesion genes alongside variable phenotypic expression may indicate the probable involvement of regulatory mechanisms in biofilm formation. Combining genotypic and phenotypic methods provides a comprehensive assessment of the biofilm-forming potential of clinical isolates of *S. aureus*.

Keywords: biofilm formation; adhesion genes; *ica* operon; *clfA*; *clfB*; tonsillitis; phenotypic methods; PCR; Congo red agar.

Introduction

Biofilm formation is a key virulence factor of bacterial pathogens, including *Staphylococcus aureus*, contributing to persistence and treatment resistance in infections such as acute tonsillitis. Tonsillitis, defined as inflammation of the palatine tonsils, is a common condition accounting for approximately 1.3% of outpatient visits (Naumenko et al., 2025; Nimmana & Paterek, 2025). The bacterial aetiology of the disease is primarily caused by group A β-haemolytic streptococcus, may be caused by *Staphylococcus aureus*, *Streptococcus pneumoniae*, or *Haemophilus influenzae*. *Staphylococcus aureus* plays a particular role in recurrent tonsillitis because it persists in tonsil tissue long-term due to its ability to form biofilm (Kravets et al., 2020; Di Pietro et al., 2024, Ng, 2025). Biofilm formation enhances the virulence factors and colonisation potential of staphylococci (Hammoudi & Mohammed, 2025), allowing them to adapt to the competitive environment of the biotope (Sannasiddappa et al., 2011; Shevchenko, 2023). The ability to form biofilms complicates the course of infections and limits the effectiveness of antibacterial therapy, leading to adverse medical and economic outcomes (Kravets et al., 2022; Empitu et al., 2025).

Although numerous methods exist for studying biofilm formation, their standardisation remains problematic. Classical approaches (such as the methods by Christensen et al. (1982) and modern microtiter plate assays) often yield variable results due to differences in medium composition, aeration conditions, and adhesion properties of bacterial strains (Voronkina et al., 2015). It has been experimentally proven that, according to a standardised protocol, only 10.0–12.5% of strains showed high biofilm-forming ability *in vitro*, which highlights the risk of false-negative conclusions when using only one test (Voronkina et al., 2015). An alternative phenotypic method, Congo red agar (CRA), is used to detect exopolysaccharide production (Freeman et al., 1989). However, this method is inherently subjective due to visual interpretation of colony pigmentation and demonstrates low concordance with quantitative crystal violet assays (Moreno et al., 2023). The variability of phenotypic results necessitates the investigation of genetic determinants of adhesion. Biofilm formation is closely associated with the expression of the *icaABCD* operon, which encodes proteins responsible for the synthesis of polysaccharide intercellular adhesin (PIA) (Toledo-Arana et al., 2005; Atshan et al., 2011). However, as gene expression is regulated by complex multilevel mechanisms (Peng et al., 2023), phenotypic outcomes may vary depending on experimental conditions and methodological approaches (Basnet et al., 2023).

Therefore, a combined approach integrating genotypic and phenotypic methods is required for accurate assessment of the biofilm-forming potential of *S. aureus* strains. Such an approach enables not only the detection of adhesion-related genes but also the evaluation of their functional expression (Melo et al., 2013; Ferreira et al., 2014; Kryvtsova, et al., 2020). Furthermore, the persistence of opportunistic and antibiotic-resistant oropharyngeal microbiota, particularly following viral respiratory infections, increases the clinical relevance of such investigations (Romanyuk et al., 2022).

The objective of this study was to comprehensively evaluate the biofilm-forming potential of clinical *S. aureus* isolates obtained from patients with acute tonsillitis by combining genotypic analysis of adhesion genes with phenotypic methods for detecting exopolysaccharides production.

Materials and methods

This study used *Staphylococcus* spp. strains isolated from the oropharynx of patients presenting with symptoms of acute tonsillitis who attended primary healthcare centres in Ternopil, Ukraine, between 2023 and 2025. Bacteriological examination and preliminary identification were performed in accordance with standard microbiological methods (Minukhin et al., 2014). Egg-yolk salt agar (Farmactiv, Kyiv) was used for the selective isolation of staphylococci.

Staphylococcus aureus isolates were identified following standard laboratory protocols, including morphological assessment (Gram staining) and biochemical characterisation (catalase, coagulase, lecithinase tests) (Ukrmediasnab LLC, Dnipro). Coagulase-negative staphylococci were differentiated based on susceptibility to novobiocin (5 μg) using commercial discs (Ukrmediasnab LLC, Dnipro; Farmactiv, Kyiv). Identification of *S. aureus* was confirmed by PCR detection of the species-specific nuc gene. As this study utilized previously collected bacterial strains from a laboratory collection, ethical approval was not required. All data were anonymised and no patient-identifiable information was used.

Adhesion was assessed using glass coverslips (24 × 24 mm) placed in Petri dishes (Chimlaborreaktyv LLC, Kyiv) according to the method described by Christensen et al. (1982). The isolates were cultured in meat peptone broth (MPB; Chimlaborreaktyv LLC, Kyiv) supplemented with 1% glucose (Darnytsia, FF, PrJSC, Kyiv) and incubated for 24–48 hours. Following incubation, the coverslips were fixed and stained with 0.1% crystal violet solution for 10 minutes (gentian violet; Chimlaborreaktyv LLC, Kyiv). Microscopic examination was performed using a light microscope (TM MICROmed, Poltava, Ukraine) under oil immersion (Chimlaborreaktyv LLC, Kyiv) at 1,000× magnification. The degree of adhesion was assessed using a semi-quantitative scale based on visual evaluation of adherent cell density in the microscopic field: 0 – no adherent cells observed; 1 – weak adhesion (single cells or small clusters covering <25% of the field of view); 2 – moderate adhesion (formation of microcolonies covering 25–75% of the field of view); 3 – strong adhesion (dense aggregates or continuous biofilm covering >75% of the field of view). At least 10 randomly selected fields of view were analyzed for each sample.

Visual assessment of biofilm formation was performed using the method described by Christensen et al. (1982). An overnight culture was inoculated into test tubes containing 5 mL of MPB (Chimlaborreaktyv LLC, Kyiv) supplemented with 1% glucose (Darnytsia, FF, PrJSC, Kyiv) and incubated at 37 °C for 24 hours. After incubation, the medium was removed, and the tubes were fixed, stained with crystal violet, and air-dried. A positive result was defined as the presence of a visible coloured film along the walls and at the bottom of the tube. Ring formation at the air-medium interface was not considered indicative of biofilm formation.

The biofilm-forming ability of the isolates was quantified using 96-well microtiter plates (Chimlaborreaktyv LLC, Kyiv) according to Stepanović et al. (2007). An overnight culture was prepared in sterile saline (0.9% NaCl; Novopharm-Biosynthesis, Zvyagel) and standardised to 0.5 McFarland (1.5×10^8 CFU/mL) using a DEN-1 densitometer (BioSan SIA, Riga, Latvia, 2018). Each well contained 150–200 μL of MPB (Chimlaborreaktyv LLC, Kyiv) supplemented with 1% glucose (Darnytsia, FF, PrJSC, Kyiv) and 50 μL of the bacterial suspension. Plates were incubated at 37 °C for 24 hours. After incubation, the wells were washed three times with sterile saline (0.9% NaCl; Novopharm-Biosynthesis, Zvyagel), air-dried, and fixed with 96% ethanol (PJSC Fitopharm, Kyiv) for 10 minutes. Biofilms were stained with 0.1% crystal violet solution (gentian violet; Chimlaborreaktyv LLC, Kyiv) for 10 minutes. After washing, the bound dye was solubilised with 200 μl of 95% ethanol (PJSC Fitopharm, Kyiv) and the absorbance was measured at a wavelength of 570 nm using a Multiskan FC spectrophotometer (Thermo Fisher Scientific, USA, 2015). The cut-off OD (OD_c) was defined as the mean OD of the negative control plus three standard deviations:

ODc = mean OD negative control + (3 × SD negative control).

The results were interpreted as follows: OD ≤ OD_c – non-producer; OD_c < OD ≤ 2×OD_c-weak producer; 2×OD_c < OD ≤ 4×ODc – moderate producer; OD > 4×OD_c – strong producer. All OD values were blank-corrected using 95% ethanol (PJSC Fitopharm, Kyiv) as the control solvent prior to analysis.

A modified tube test was used to evaluate slime production (Atshan et al., 2011). An overnight culture of microorganisms was inoculated into tubes containing 5 mL of tryptic soy broth (Chimlaborreaktyv LLC, Kyiv), which was supplemented with 1% glucose (Darnytsia, FF, PrJSC, Kyiv) and 0.04% Congo red dye (Chimlaborreaktyv LLC, Kyiv). The cultures were then incubated at 37 °C for 24 hours. Results were evaluated based on colour changes in the broth and sediment, as well as the formation of black precipitates (a positive result indicates active slime production). This modified assay was used as an additional qualitative approach to assess slime production in a liquid medium, complementing the Congo red agar method, which evaluates colony morphology on solid media.

Slime production was determined qualitatively using the method of Freeman et al. (1989) with modifications (Darwish & Asfour, 2013; Triveni et al., 2018). The medium consisted of tryptic soy agar (Chimlaborreaktyv LLC, Kyiv), supplemented with sucrose (36 g/L; Chimlaborreaktyv LLC, Kyiv), and Congo red dye (0.8 g/L; Chimlaborreaktyv LLC, Kyiv). Plates were incubated at 37 °C for 24–48 hours and then kept at room temperature for 2–4 days. Slime producer strains formed black colonies, whereas non-producers formed red colonies.

To confirm the species identification and evaluate biofilm-related genetic determinants, PCR analysis was performed targeting the *nuc* gene (for *S. aureus* identification), as well as genes of the *ica* operon and clumping factor (*clf*) genes.

A single isolated colony was selected from an overnight culture and suspended in phosphate-buffer saline (Chimlaborreaktyv LLC, Kyiv) until the turbidity reached 0.5 McFarland (equivalent to 1.5 × 108 CFU/mL). Aliquots of 200 μL were used for DNA extraction. Genomic DNA was extracted using the IndiSpin Pathogen Kit (Indical Bioscience, Germany) following the manufacturer's protocol. The purified DNA was stored at –20 °C until further PCR analysis.

The PCR primers were synthesised commercially by Macrogen (Seoul, South Korea). PCR reactions were performed in 25 μL volumes containing 12.5 μL of OneTaq Quick-Load 2 × Master Mix with standard buffer (New England Biolabs, USA), 0.5 μM of each primer (final concentration), 1 μL of template DNA, and nuclease-free water to make up the required volume. Amplification was performed using a Stratagene Mx3005P real-time PCR system (Agilent Technologies, USA, 2015), under the following conditions: initial denaturation at 94 °C for 1 minute; followed by 35 cycles of denaturation at 94 °C for 30 seconds, primer-specific annealing temperatures (Table 1) for 30 seconds, and elongation at 68 °C for 30 seconds, and a final elongation step at 68 °C for 5 minutes. Nuclease-free water served as a negative control in each PCR run.

Data are presented as the mean ± standard deviation (m ± SD). Correlation analysis between the phenotypic methods was performed using Kendall's (τ) and Spearman's (ρ) rank correlation coefficients. The chi-squared (χ^2) goodness-of-fit test was employed to assess the uniformity of isolate distribution across biofilm formation categories. Statistical significance for all tests was defined as $P < 0.05$. Methodological algorithm of the study is presented in Figure 1.

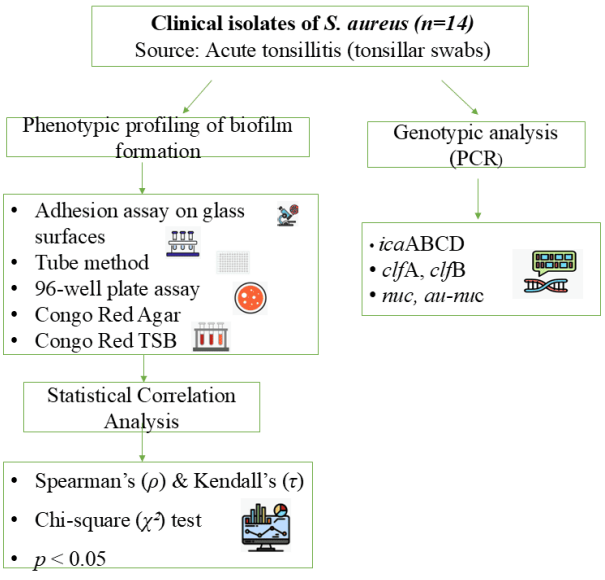

Fig. 1. Methodological algorithm of the study

Table 1
Primer sequences and thermal cycling conditions used in this study

Target gene (locus)	Forward primersequence (5'→3')	Reverse primer sequence (5'→3')	Product size (bp)	T_α (°C)	Reference
Nuc (*Staphylo-coccus* spp.)	GGCCGTGTTGAACGTGGTCAAATCA	TIACCATTTCAGTACCTTCTGGTAA	370	55	https://journals.asm.org/doi/full/10.1128/jcm.39.7.2541-2547.2001
au-nuc (*S. aureus*)	TCGCTTGCTATGATTGTGG	GCCAATGTTCTACCATAG C	359	55	https://journals.asm.org/doi/full/10.1128/jcm.01232-09
icaA	TCTGGAACCAACATCCAACA	ACACTTGCTGGCGCAGTCAA	188	50	https://www.mdpi.com/2076-0817/10/4/427
icaB	TAATAAGCATTAATGTTCAATT	ATGGGACGGATTCCATGAAAAAGA	900	50	https://www.mdpi.com/2076-0817/10/4/427
icaC	TCTAATCTTTTTCATGGAATCCGT	AGAATCGTGAAGTATAGAAAATT	1100	48	https://www.mdpi.com/2076-0817/10/4/427
icaD	AGTATTTTCAATGTTTAAAGCAA	ATGGTCAAGCCCAGACAGAG	198	48	https://www.mdpi.com/2076-0817/10/4/427
clfA	CGTTTCTTCCGTAGTTGCATTTG	ATTGGCGTGGCTTCAGTGCT	292	52	https://pmc.ncbi.nlm.nih.gov/articles/PMC193818/
clfB	TCGCACTGTTTGTGTTTGCAC	ACATCAGTAATAGTAGGGGCAAC	205	52	https://pmc.ncbi.nlm.nih.gov/articles/PMC193818/

Results

The analysis of the biofilm-forming capacity among 14 clinical isolates of *S. aureus* revealed a pronounced phenotypic variability with the studied population. The integrated approach, including microscopy on glass coverslips, allowed differentiating the strains based on their adhesive activity. The null hypothesis (H_0) assumed no statistically significant differences in the frequency distribution of isolates across categories of adhesion intensity categories. Adhesion intensity varied among isolates: 14.3% (n = 2) were classified as strong (3 points), 35.7% (n = 5) as moderate (2 points), and 35.7% (n = 5) as weak (1 point). Only 14.3% (n = 2) showed no adhesion (0 points). The chi-squared (χ^2) goodness-of-fit test yielded $\chi^2 = 2.57$ (df = 3, P = 0.444), which is lower than the critical value (7.82; P = 0.05), indicating no statistically significant deviation from a uniform distribution. The microscopic examination (oil immersion, 1,000×) revealed irregularly shaped bacterial aggregates. Most aggregates were discrete, although in some fields, partial merging of clusters was observed.

The analysis of the intensity of biofilm formation by 14 *S. aureus* isolates using the microtiter plates enabled their classification based on the calculated optical density cut-off (OD_c) (Fig. 2).

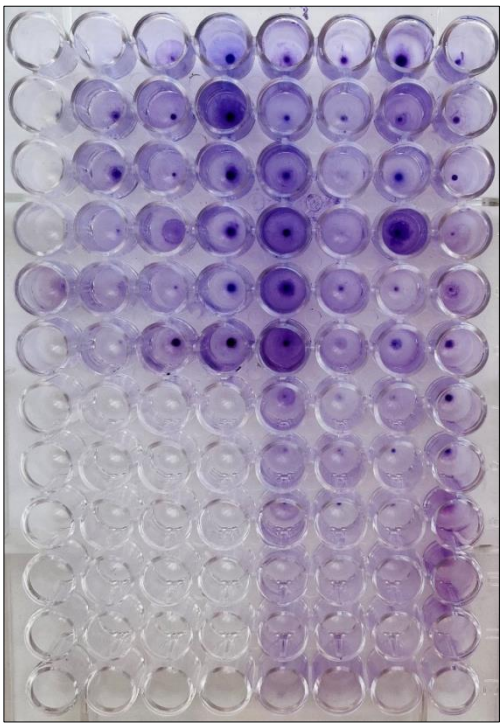

Fig. 2. Macroscopic evaluation of *S. aureus* biofilm formation in a 96-well microtiter plate

Based on negative control values (OD = 0.0003; SD = 0.004), the cut-off was defined as OD_c = 0.0137. Accordingly, one isolate (7.1%) was classified as a non-producer (OD ≤ 0.0137), six isolates (42.8%) as weak producers (0.0137 < OD ≤ 0.0274), three isolates (21.4%) as moderate producers (0.0274 < OD ≤ 0.0548), and four isolates (28.6%) as strong producers (OD > 0.0548). The OD_{570} values ranged from 0.0137 to 0.095. The distribution of OD values and the corresponding median values for each group are presented in a box plot (Fig. 3).

The distribution of *S. aureus* isolates according to the tube method showed that only one isolate (7.1%) did not form a biofilm, five isolates (35.7%) demonstrated weak biofilm formation, five (35.7%) moderate, and three (21.4%) strong biofilm formation.

All isolates demonstrated positive results in both assays for exopolysaccharide (slime) production, including the liquid medium assay and the Congo red agar (CRA) method. Positive reactions were indicated by black pigmentation in broth and black colony formation on solid medium.

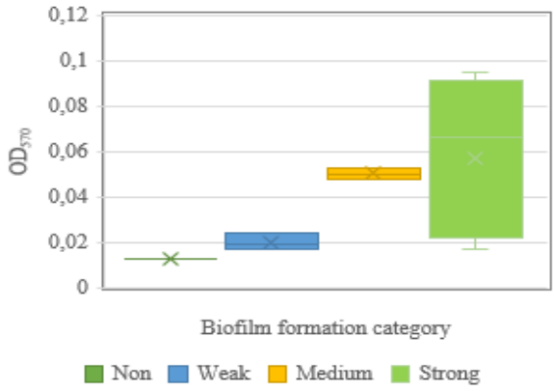

Fig. 3. Biofilm-forming capacity of *S. aureus* isolates quantified by the 96-well microtiter plate assay: the vertical axis scale was set from 0 to 0.12 with major increments of 0.02 to ensure uniform visualization

A comparison of three phenotypic methods (microscopy, tube method, and microtiter plate assay) demonstrated a high level of agreement. Complete concordance of scores (0–3) across all three methods was observed for 10 of 14 isolates (71.4%, Fig. 4). Discrepancies were observed in four isolates, with differences not exceeding one point.

The correlation analysis using Spearman's (ρ) and Kendall's (τ) coefficients confirmed strong positive relationships among all the methods (Table 2), with the highest correlation observed between the microtiter plate assay and microscopy.

PCR analysis confirmed that all studied *S. aureus* isolates (14/14, 100%) carried the *icaABCD* operon, as well as *clfA* and *clfB* genes. All isolates were also positive for the *nuc* gene, confirming species

identification. These findings indicate the presence of genes associated with adhesion and biofilm formation in all isolates.

Table 2
Heatmap of the semi-quantitative assessment of biofilm-forming capacity in *S. aureus* isolates using different phenotypic methods

Isolate	96-well assay	Tube method	Microscopy
1	0	0	0
2	1	1	0
3	1	1	1
4	1	1	1
5	1	2	1
6	2	2	2
7	2	2	2
8	2	2	2
9	3	3	3
10	3	3	2
11	3	2	2
12	3	3	3
13	1	1	1
14	1	1	1

Notes: biofilm production was scored as: 0 – non, 1 – weak, 2 – moderate, 3 – strong; the color intensity represents the score assigned by each method.

Table 3
Correlation analysis of phenotypic methods for the assessment of biofilm formation in *S. aureus* isolates (n = 14)

Methods	Kendall's Tau, τ	Spearman, ρ	P-value
96-well plate / tube method	0.89	0.915	0.0000043
96-well plate / microscopy	0.88	0.932	0.0000012
Tube method / microscopy	0.86	0.903	0.0000097

Discussion

Biofilm formation is considered a key virulence factor of *S. aureus*, contributing to its persistence in the tonsillar epithelium and potentially promoting recurrent tonsillitis (Hammoudi & Mohammed, 2025; Judi & Naser, 2025). Tonsillitis accounts for approximately 1.3% of outpatient visits and is defined as inflammation of the palatine tonsils caused by viral or bacterial pathogens (Nimmana & Paterek, 2025). Although most cases are caused by viruses, bacteria (particularly *S. aureus*) play a significant role in polymicrobial association and lead to complications (Pallon et al., 2021; Nimmana & Paterek, 2025). Despite numerous studies investigating the genetic determinants of adhesion, the relationship between gene carriage and phenotypic expression of biofilm formation remains inconsistent (Atshan et al., 2011; Goyal et al., 2014). In the present study, a combined phenotypic and genotypic analysis of 14 clinical *S. aureus* isolates from patients with acute tonsillitis was performed.

The molecular analysis confimed the presence of the *icaABCD* operon and *clfA* and *clfB* genes in all isolates. Similar data were found in clinical MRSA isolates in a Malaysian hospital, where the frequency of detection of the *icaA* and *icaD* genes was very high in all isolates (Mariana et al., 2009).

The high prevalence of adhesion genes among clinical isolates—particularly *clfB* (85.4%), *eno* (81.1%) and *icaD* (77.0%)—was confirmed in a meta-analysis of 53 studies (Sharifi et al., 2025). These findings are consistent with the high frequency of genetic determinants of adhesion observed in our sample of *S. aureus* isolates. Similarly, studies of isolates from chronic wound infections revealed a high prevalence of *icaA* (76%), *icaD* (74%), *clfA* (72%), and *clfB* (70%), and the presence of *icaAD* genes correlated with the phenotypic ability to form biofilms (Demir et al., 2020). However, despite the universal presence of these genes, the intensity of biofilm formation varied significantly, suggesting the involvement of complex regulatory mechanisms. Although all isolates demonstrated exopolysaccharide production, this did not directly correspond to biofilm formation intensity, indicating that matrix production alone is insufficient for the development of mature biofilms.

The phylogenetic analysis of the *icaA* gene in a large population of *S. aureus* (Kravets et al., 2025) demonstrated high nucleotide sequence conservation among isolates from diverse geographical locations. Such a conservation likely explains its ubiquitous presence in our studied isolates and emphasizes its fundamental role in the biosynthesis of the polysaccharide intercellular matrix. The differences in the intensity of biofilm formation observed in our study are probably due to the specificities of *ica* operon regulation. This process is modulated by various factors, including variations in *icaR* expression, the quorum-sensing system (*agr*), and global regulators (*sarA*, *sigB*), which can influence the phenotypic manifestation of biofilm formation regardless of the presence of structural genes.

The comparative analysis of the three phenotypic methods showed a high level of agreement in the results for 71.4% of the isolates, while the correlation analysis revealed a strong positive relationship among the methods ($\rho > 0.9$; $P < 0.001$). It should be noted that microscopy-based assessment primarily reflects initial adhesion rather than mature biofilm formation. This confirms their reliability for semi-quantitative assessment of biofilm formation. Despite previously reported variability of phenotypic methods, the high level of agreement observed in this study may be attributed to the use of standardized experimental conditions. However, the results should be interpreted with caution due to the limited sample size. Similar recommendations regarding the advisability of combining phenotypic methods to increase the reliability of the assessment are provided in the study by Atshan et al. (2011).

The studied sample of *S. aureus* isolates demonstrated positive results in tests for exopolysaccharide production, confirming their capacity for exopolysaccharide production. The informative value of the modified Congo red agar method for detecting biofilm formation was confirmed in the work of Mariana et al. (2009).

The presence of adhesion genes is not always accompanied by an equivalent level of phenotypic expression in biofilm formation. Biofilm development is controlled by a complex network of regulatory mechanisms. These findings confirm the reliability of the methods, including quorum-sensing systems, global regulators (*sarA*, *sigB*), the *icaR* repressor, and the influence of environmental factors (Peng et al., 2023). Therefore, the presence of structural genes is a necessary but insufficient condition for *in vitro* biofilm formation. The results obtained indicate that the manifestation of this virulence factor in clinical *S. aureus* isolates is determined by the efficiency of transcriptional control and the coordinated functioning of these regulatory systems. A limitation of the study is the small sample size (n = 14), which restricts the extrapolation of the results to larger populations. Furthermore, the use of qualitative PCR allowed only for the detection of the presence of genes, rather than their expression levels. Incorporating RT-qPCR in further studies would enable an assessment of the direct relationship between the expression of adhesion genes and the intensity of biofilm formation.

The results support the use of combined genotypic and phenotypic approaches for comprehensive evaluation of the biofilm-forming potential of clinical *S. aureus* isolates. This approach may have significant practical importance for identifying strains with enhanced persistence potential in clinical settings.

Conclusion

A comprehensive genotypic and phenotypic analysis of clinical *S. aureus* isolates from patients with acute tonsillitis demonstrated the universal presence of the *icaABCD* operon, *clfA*, and *clfB* genes, alongside a marked variability in the biofilm formation. The high concordance among the phenotypic methods ($\rho > 0.9$; $P < 0.001$) supports their combined use for semi-quantitative assessment of this trait. Importantly, the results indicate that the presence of adhesion-related genes does not necessarily correspond to the level of phenotypic biofilm expression, suggesting a key role of regulatory mechanisms. These findings highlight the importance of integrating genotypic and phenotypic approaches for the accurate evaluation of the biofilm-forming potential of clinical isolates.

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