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Characterization of biofilm formation in uropathogenic *Staphylococcus aureus* and their association with antibiotic resistance

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ABSTRACT

Introduction: The increase in antibiotic resistance and biofilm formation in *Staphylococcus aureus* is a serious public health problem, complicating the treatment of infections caused by these bacteria, including urinary tract infections (UTIs). This research aimed to assess the correlation between antibiotic resistance and the capacity for biofilm formation in *S. aureus* strains isolated from patients with UTIs.

Methods: The antibiotic susceptibility testing was conducted using disc diffusion and broth micro-dilution method. The *bla*Z and *mec*A genes were investigated in all penicillin and cefoxitin-resistant *S. aureus* strains, using PCR. Biofilm formation was investigated using the Congo red agar and microtitre plate methods. All *S. aureus* strains were screened for genes encoding adhesion and biofilm formation (*spa*, *fnb* AB, *clf*A, *bap*) and intercellular adhesin (*ica*ADB and *ica*R).

Results: Among 20 strains of *S. aureus*, 55% were found to be multidrug-resistant (MDR) and methicillin-resistant (MRSA). All strains were biofilm producers, including 55% moderate biofilm producers and 35% strong biofilm producers. A significant correlation (p-*value* <0.01) was observed between MDR and non-MDR strains in terms of biofilm production. All strains were sensitive to linezolid, and 20% exhibited the constitutive clindamycin resistance phenotype. The *spa*, *fnb*B, and *clf*A genes were present in all strains followed by the *bap* (95%) and *fnb*A (90%). The *ica*A (90%) and *ica*B (60%) genes were most detected. The coexistence of *ica*A, *ica*ADB, and *ica*AD genes was significantly associated with MRSA strains. In contrast, the *ica*R gene was significantly detectable in the methicillin-susceptible group of strains.

Conclusion: This study uncovered a significant association between the formation of biofilms by uropathogenic *S. aureus* and antibiotic resistance, highlighting a noteworthy concern for public health.

Introduction

Staphylococcus aureus is a member of the coagulase-positive staphylococci, which are relatively uncommon causes of urinary tract infections (UTIs) in the general population (0.5–6%) (Yousefi et al., 2016). Nevertheless, they can be more prevalent in specific population groups such as elderly patients with urinary catheters or those with *S. aureus* bacteremia (Rafik et al., 2021; Hashemzadeh et al., 2021). If left untreated, the infection can escalate into a serious life-threatening situation (Karakonstantis and Kalemaki, 2018).

Abbreviations: bap, Biofilm-associated Protein; fnbA, Fibronectin-binding protein A; fnbB, Fibronectin-binding protein B; CA-SFM 2021, Antibiogram Committee of the French Microbiology Society; clfA, clumping factor A; CLSI, Clinical and Laboratory Standards Institute; CRA, Congo red agar microtitre plate methods; MAR, The multiple antibiotic resistance; MDR, multidrug-resistant; MICs, Minimum inhibitory concentrations; MRSA, Methicillin-resistant Staphylococcus aureus; spa, Staphylococcal protein A; TCP, Quantitative microtitre plate method.

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The increasing prevalence of multidrug-resistant (MDR) uropathogenic *S. aureus* poses a significant global health challenge. Multiple studies have shown that over 50% of *S. aureus* strains are completely resistant to the penicillin and cephalosporin antibiotic classes, leading to the term methicillin-resistant *S. aureus* (MRSA) (Yousefi et al., 2016). However, MRSA frequently demonstrates resistance to other commonly used antibiotics, such as aminoglycosides, fluoroquinolones, chloramphenicol, macrolides, and tetracycline (Piechota et al., 2018).

UTIs and other infections caused by MRSA are associated with a significantly high mortality and morbidity rate (Manandhar et al., 2018a). This is attributed to its capability to produce a wide range of virulence factors that aid in bacterial invasion, including factors involved in attachment, adhesion, and biofilm formation (Bissong and Ateba, 2022).

The formation of biofilms by MRSA and methicillin-sensitive *S. aureus* strains (MSSA) on the surfaces of medical equipment has a significant impact on increased resistance to antimicrobial treatment and host immune factors (Yousefi et al., 2016). The biofilm matrix formed by *S. aureus* strains can facilitate interactions among themselves and horizontally transfer genetic material through conjugation or transformation. This process allows the development of a resistome and virulome, potentially leading to the dissemination of genes associated with antibiotic resistance and virulence factors (Yousefi et al., 2016; Roy et al., 2022; Águila-Arcos et al., 2017). It is estimated that biofilms are responsible for over 80% of all infections in human communities (Piechota et al., 2018).

In recent years, research in several countries has begun to focus on the development of vaccines targeting MDR biofilm-forming *S. aureus* (Roche et al., 2003; Schroeder et al., 2009; Josefsson et al., 2001). The aim is to prevent infections caused by this bacterium, particularly in immunocompromised and chronic disease patients. Vaccine design is based on the detection and characterization of the polysaccharide intercellular adhesion and microbial surface components recognizing adhesive matrix molecules.

The production of *S. aureus* biofilms is most associated with the biosynthesis of intracellular polysaccharide adhesins (IcaADBC) (Neopane et al., 2018). This operon encodes for membrane proteins (IcaA, IcaD, and IcaC), an extra-cellular protein (IcaB), and a regulatory protein (IcaR) (Manandhar et al., 2018a). The *icaR* gene, positioned upstream of *ica*ADBC, belongs to the TetR family, which encodes proteins that repress the expression of *ica*ADBC genes (Peng et al., 2023). Additionally, several *S. aureus* adhesion genes are responsible for the adhesion of *S. aureus* to extracellular matrix proteins, facilitating cell aggregation and the accumulation of bacteria in the biofilm. These genes include *spa* (Staphylococcal protein A), *fnbB* (fibronectin-binding protein B), *clfA* (clumping factor A), *bap* (Biofilm-associated Protein), and *fnbA* (fibronectin-binding protein A) (Avila-Novoa et al., 2018).

In Morocco as in other African countries, no studies have been conducted to characterize biofilm-forming uropathogenic *S. aureus*. Therefore, the objective of the current study was to characterize the MRSA and MSSA uropathogenic's isolated strains. To achieve this, we assessed their biofilm formation capacity and examined the presence of genes involved in cell adhesion and biofilm formation. Additionally, we investigated the relationship between the biofilm-forming capacity of *S. aureus* strains and their antibiotic resistance.

Materials and methods

Bacterial strains

Twenty unduplicated strains of *S. aureus* responsible for UTIs were collected from the bacteriology laboratory of the Pasteur Institute of Morocco, as well as from other private medical laboratories in the greater Casablanca region, Morocco, between January 2020 and December 2022.

Standard morphological and biochemical techniques, including mannitol fermentation on Mannitol salt agar (Biokar Diagnostics, Beauvais, France), Gram stain, catalase, and coagulase tests, were utilized to confirm *S. aureus* strains. The results of the biochemical tests were used for species identification using the Vitek 2® Compact 15 automated system identification (bioMérieux, Marcy-l'Étoile, France) following the manufacturer's guidelines.

Antimicrobial drug susceptibility testing

Antimicrobial susceptibility testing of *S. aureus* strains was performed against cefoxitin (30 μg), levofloxacin (5 μg), gentamicin (10 μg), tobramycin (10 μg), erythromycin (15 μg), clindamycin (2 μg), tetracycline (30 μg), tigecycline (15 μg), and fusidic acid (10 μg). using the disk diffusion method on Mueller Hinton agar medium (Bio-Rad, Marnes-la-Coquette, France). The VITEK 2® COMPACT 15 system (bioMérieux, Marcy-l'Étoile, France) was used for testing penicillin-G, linezolid, and trimethoprim/sulfamethoxazole.

The findings were interpreted according to the guidelines set by the Antibiogram Committee of the French Microbiology Society (CA-SFM 2021) (CASFM2021,). Each isolate was categorized as sensitive (S), susceptible dose dependent (I), or resistant (R).

The fosfomycin, teicoplanin, and vancomycin susceptibility were investigated using the broth micro-dilution method. The results were interpreted by the CA-SFM (2021) guidelines (CASFM2021,).

The multiple antibiotic resistance (MAR) indexes was calculated using the following formula (Ayandele et al., 2020):

$$MAR\ \ Index = \frac{The\ \ antibiotics\ \ number\ \ to\ \ which\ \ the\ \ strains\ \ are\ \ resistant}{Total\ \ number\ \ of\ \ antibiotics\ \ tested}$$

Detection of β-lactamase production and methicillin resistance

Phenotypic β-lactamase activity was assessed by observing the appearance of the inhibition zone edge around the penicillin G disk (1 unit) (Thermo Fisher Scientific™ Oxoid™) using the disk diffusion method recommended by Clinical and Laboratory Standards Institute (CLSI) (VET01, 2023). A negative test result was indicated by a fuzzy "beach-like" edge appearance, while a positive test result was indicated by a sharp "cliff-like" edge appearance (Ferreira et al., 2016). *S. aureus* ATCC 25923 and ATCC 29213 were utilized as negative and positive controls, respectively.

The resistance to methicillin was assessed using a cefoxitin disk (30 μg) under standard susceptibility testing conditions. The method involved plating on MH agar medium and subsequent incubation at 35 °C ± 2 °C for 20 ± 4 h. The diameter of the inhibition zone was measured in millimeters. Strains were categorized as MRSA if the zone of inhibition was greater than 22 millimeters in diameter, indicating methicillin resistance. Conversely, strains with a zone of inhibition less than 22 millimeters in diameter were classified as MSSA. *S. aureus* ATCC 25923 and ATCC 43300 were used as negative and positive controls, respectively.

Screening of inducible clindamycin resistance

In accordance with CLSI M100-S28 recommendations (Thapa et al., 2021), the D-zone test on MH agar was used to screen all erythromycin-resistant *S. aureus* strains for inducible resistance to clindamycin. The procedure involved placing an erythromycin (15 μg) disc at a distance of 15 mm from a clindamycin (2 μg) disc on a MH agar plate that was previously inoculated with a 0.5 McFarland standard bacterial suspension. Following overnight incubation at 35 °C ± 2 °C, the results of this induction test led to the identification of three distinct phenotypes of *S. aureus* strains (Ambachew et al., 2022):

- Moderate sensitive phenotypes (MS): *S. aureus* strains that are resistant to erythromycin and sensitive to clindamycin, but without a D-shaped zone.
- Inducible clindamycin resistance phenotype (iMLSB): *S. aureus* strains that are resistant to erythromycin, sensitive to clindamycin, and exhibit a D-shaped zone.
- Constitutive clindamycin resistance phenotype (cMLSB): *S. aureus* strains that are resistant to both clindamycin and erythromycin.

Biofilm formation assay

Two phenotypic techniques, the quantitative microtitre plate method (TCP), and the Congo red agar (CRA), were used to assess the formation of biofilms by *S. aureus* strains.

Qualitative evaluation of biofilm formation

The qualitative phenotypic detection of *S. aureus* slime production using the CRA culture method was conducted following the documented procedure by Freeman et al (Freeman et al., 1989).

S. aureus strains were inoculated onto CRA medium and incubated aerobically at 35 °C ± 2 °C for 20 ± 4 h. The cultures were conducted in triplicate. Based on colony morphology, colony color, and intensity of slime production, the results were recorded as strong (very black), moderate (almost black), or weak (black). Conversely, *S. aureus* strains with red colonies were classified as strains incapable of biofilm formation (Manandhar et al., 2018b).

Quantitative evaluation of biofilm formation

TCP, a quantitative method was used as described by Christensen et al (Christensen et al., 1985). Initially, a *S. aureus* colony was isolated from a fresh agar plate and inoculated into 3 mL of brain heart infusion broth (BHI, Oxoid, Thermo Scientific™). The culture was incubated at 35 °C ± 2 °C under agitation (150–250 rpm) for 20 ± 4 h.

After incubation, 20 μL of the bacterial inoculum was mixed with 180 μL of BHI and inoculated into individual 96-well, disposable, sterile, clear, polystyrene, non-treated surface, U-bottom tissue culture microplates (*Citotest, Jiangsu, China*). BHI broth without cells served as a negative control to verify media sterility. The TCP was incubated at 35 ℃ ± 2 °C for 48 h.

After incubation, the contents of each well were carefully removed, and the free-floating planktonic bacterial cells were eliminated by washing them four times with 200 μL of sterile phosphate-buffered saline (PBS) with a pH of 7.2, followed by air-drying. Next, the wells were stained with 1% crystal violet for 15 min, rinsed thoroughly three times with deionized water, and the dye was dissolved in 96% ethanol for 15 min.

The optical density (OD) was measured at 590 nm using an automatic micro-ELISA reader. Triplicate experiments were performed for each strain and repeated three times. According to the recommendations described by Stepanovic et al (Stepanović et al., 2007)., the OD values of adherent bacterial cells were used to categorize biofilm production as negative, weak, and high. *S. aureus* reference strains ATCC 25923 and ATCC 6538 were used as positive controls.

Bacterial DNA extraction

All *S. aureus* strains were cultured on Mannitol Salt Agar at 35 °C ± 2 °C for 20 ± 4 h. The genomic DNA of the bacterial strains was extracted from pure cultures using the InstaGeneTM template (Bio-Rad Laboratories, USA) following the manufacturer's instructions. The purified DNA was subsequently stored at − 20 °C until it was used as a template for PCR.

Molecular detection of genes

To confirm the identification of the *S. aureus* species, we utilized a PCR method to screen for the presence of the *nuc* gene, which encodes

Table 1
Target genes and their primer pairs used in this research.

Target gene	Protein function	The nucleotide sequences of the primers (5'→3')	PCR product (bp)	References
nuc	Nuclease	F: GCGATTGATGGTGATACGGTT R: AGCCAAGCCTTGACGAACTAAAGC	270	(Al-Amery et al., 2019)
mecA	Methicillin resistance	F: GATATCGAGGCCCGTGGATT R: ACGTCGAACTTGAGCTGTTA	642	(Pajohesh et al., 2022)
blaZ	Penicillin resistance	F: TACAACTGTAATATCGGAGGG R: CATTACTCTTGGCGGTTTC	861	(Ghaioumy et al., 2021)
icaA	Intercellular adhesion A	F: TCTCTTGCAGGAGCAATCAA R: TCAGGCACTAACATCCAGCA	188	(Zalegh et al., 2022)
icaD	Intercellular adhesion A	F: ATGGTCAAGCCCAGACAGAG R: CGTGTTTTCAACATTTAATGCAA	198	(Polysaccharide, 2023)
icaB	Intercellular adhesion A	F: TTGCCTGTAAGCACACTGGA R: GGAGTTCGGAGTGACTGCTT	735	(Zalegh et al., 2022)
icaR	Intercellular adhesion A	F: TTCCAGAAAATTCCTCAGGCG R: TCAGAGAAGGGGTATGACGG	266	(Zalegh et al., 2022)
fnbB	Fibronectin binding protein B	F: ACGCTCAAGGCGACGGCAAAG R: ACGCTCAAGGCGACGGCAAAG	197	(Ghaioumy et al., 2021)
clfA	Clumping factor A	F: CCGGATCCGTAGCTGCAGATGCACC R: GCTCTAGATCACTCATCAGGTTGTTCAGG	1000	(Ghaioumy et al., 2021)
Bap	Biofilm-associated Protein	F: CCCTATATCGAAGGTGTAGAATTG R: GCTGTTGAAGTTAATACTGTACCTGC	971	(Solati et al., 2015)
fnbA	Fibronectin binding protein A	F: ACGCTCAAGGCGACGGCAAAG R: ACCTTCTGCATGACCTTCTGCACC	191	(Ghaioumy et al., 2021)

F: forward; R: reverse

Table 2
PCR cycling conditions.

Target gene	Temperature (°C) / Time					Number of cycles
	Initial denaturation	Cycling conditions			Final extension	
		Denaturation	Annealing	Extension		
nuc	94/5 min	94/30 s	55/30 s	72/1 min	72/10 min	35
icaA			55.5/1 min			
icaD			55.5/1 min			
mecA	95/1 min	94/1 min	52/ 1 min	72/30 s		
icaB			54/1 min			
icaR			54/1 min			
blaZ	94/ 5 min	94/ 60 s	55/ 60 s	72/2 min		32
fnbB	94/ 4 min	94/45 s	58/ 60 s	72/ 40 s	72/5 min	30
clfA						
Bap	94/4 min	94/ 45 s	58/ 40 s	72/40 s		30
fnbA						

Table 3
Antibiotic resistance profile and biofilm-forming capacity of S. aureus isolates.

Antibiotics	*S. aureus* isolates N (%)				Ability to produce biofilm (%)		
	MRSA (n = 11)	MSSA (n = 9)	Total (n = 20)	*P value*	Strong (n = 7)	Moderate (n = 11)	Weak (n = 2)
Penicillin G	11(100)	8 (88.89)	19 (95)	0.45	6/7 (85.71)	11/11 (100)	2/2 (100)
Fusidic Acid	11(100)	4 (44.44)	15 (75)	0.008*	4/7 (57.14)	9/11 (81.81)	2/2 (100)
Tetracycline	8 (72.73)	5 (55.56)	13 (65)	0.64	4/7 (57.14)	9/11 (82)	1/2 (50)
Erythromycin	8 (72.73)	1 (11.11)	9 (45)	0.009*	2/7 (28.57)	6/11 (54.54)	1/2 (50)
Fosfomycin	8 (72.73)	1 (11.11)	9 (45)	0.009*	2/7 (28.57)	7/11 (63.63)	1/2 (50)
Tobramycin	6 (54.55)	0	6 (30)	0.014*	1/7 (14.28)	4/11 (36.36)	1/2 (50)
Teicoplanin	5 (45.45)	2 (22.22)	7 (35)	0.374	2/7 (28.57)	4/11 (36.36)	1/2 (50)
Gentamycin	4 (36.36)	0	4 (20)	0.094	1/7 (14.28)	3/11 (27.27)	0
Clindamycin	3 (27.27)	1 (11.11)	4 (20)	0.59	0	4/11 (36.36)	0
Tigecycline	4 (27.27)	0	4 (20)	0.218	2/7 (28.57)	0	1/2 (50)
Levofloxacin	4 (27.27)	0	4 (15)	0.218	1/7 (14.28)	3/11 (27.27)	1/2 (50)
Vancomycin	1 (9.09)	2 (22.22)	3 (15)	0.56	0	2/11 (18.18)	0
Trimethoprim sulfamethoxazole	1 (9.09)	0	1 (15)	1	0	1/11 (9.09)	0
Linezolid	0	0	0	1	0	0	0

* Statistically significant

the *S. aureus* nuclease enzyme (Al-Amery et al., 2019). Additionally, PCR amplification was employed to examine all penicillin-resistant and cefoxitin-resistant *S. aureus* strains for the presence of the genes encoding β-lactamase (*blaZ*) and penicillin-binding protein 2a (*mec*A), respectively (Pajohesh et al., 2022; Zalegh et al., 2022). Furthermore, we investigated the presence of genes encoding polysaccharide intercellular adhesion (*ica*A, *ica*D, *ica*B, *ica*R) and microbial surface components recognizing adhesive matrix molecules (*fnb*B, *clf*A, *bap*, *fnb*A) in all isolated strains using specific primers and PCR amplification conditions (Tables 1 and 2) (Polysaccharide, 2023; Ghaioumy et al., 2021; Solati et al., 2015).

The PCR reaction was performed using 2 μL of the DNA template, 10 μL of 5x MyTaq Reaction Buffer (Bioline Reagents, USA) containing 5 mM of dNTPs, 15 mM of $MgCl_2$, stabilizers, and enhancers at optimal concentrations, which eliminates the need for optimization, additionally, 1 μL of MyTaq DNA Polymerase and 1 μL of each primer at a concentration of 20 pmol were added. The total volume was adjusted to 50 μL with double-distilled water. The primer pairs and cycling conditions used in the PCR are listed in Tables 1 and 2.

After PCR amplification, 10 μL of the reaction product was separated by electrophoresis in a 1.5% agarose gel (Sigma, Darmstadt, Germany) stained with 0.5 μg/mL ethidium bromide (Sigma) in 1 × Tris-acetate EDTA buffer for 30 min and then visualized under ultraviolet light.

Statistical analysis

The data were analyzed using IBM SPSS Statistics 26 (IBM, Armonk, NY, USA). Categorical data were analyzed using the chi-square test and Fisher's exact test. A p-value ≤ 0.05 was considered statistically significant. However, for independent groups, the nonparametric Wilcoxon-Mann-Whitney rank sum test was used.

Cluster analysis and pairwise dissimilarity distances between strains were performed. This method simultaneously analyzes several variables, such as antibiotic resistance, the intensity of biofilm formation, and the occurrence of genes involved in biofilm formation in each strain.

Pearson correlation coefficients (ρ) were calculated to determine the correlation between different genes involved in biofilm-forming *S. aureus* strains.

Results

Antibiotics resistance patterns

All 20 *S. aureus* strains were resistant to at least one of the 13 antibiotics, high resistance was observed against penicillin G (95%), followed by fusidic acid (75%) and tetracycline (65%). On the other hand, low resistance was observed against gentamycin (20%), clindamycin (20%), levofloxacin (15%), and vancomycin (15%) (Table 3). Meanwhile, 55% of *S. aureus* strains were multidrug-resistant, showing resistance to at least three different antibiotic classes. *S. aureus* strains showed the highest drug resistance, with a MAR index ranging from 0.14 to 0.71(Table 4).

Antibiotic resistance and the biofilm formation ability of S. aureus strains

Based on the TCP approach findings, all *S. aureus* strains demonstrated the ability to generate biofilm at various levels. Strong,

Table 4
Resistance phenotypes of S. aureus strains and MAR index.

Resistance phenotype	Number of isolates (%)	MAR index
P, Tm, G, E, Te, Tg, FA, Tei, F, Le	2(10)	0.71
P, Tm, E, Te, Tg, FA, Vn, Tei, F, CL	1(5)	0.71
P, Tm, E, Te, Tg, FA, F, CL	1(5)	0.57
P, Tm, Gm, E, Tei, FA, F, Le	1(5)	0.57
P, E, Te, FA, Vn, Tei, F	1(5)	0.50
P, Tm, Gm, E, FA, SXT, F	1(5)	0.50
P, E, Te, FA, F, CL	1(5)	0.43
P, E, Te, FA, Tei, F	1(5)	0.43
P, FA, Vn, Tei	1(5)	0.29
P, E, CL	1(5)	0.21
P, Te, F	1(5)	0.21
P, Te	5(20)	0.14
P, FA	2(10)	0.14
P, Lev	1(5)	0.14

P: Penicillin G; FA: Fusidic Acid; Te: Tetracycline; E: Erythromycin; F:Fosfomycin; Tm: Tobramycin; Tei: Teicoplanin; G: Gentamycin; CL: Clindamycin; Tg: Tigecycline; Le: Levofloxacin; Vn:Vancomycin; SXT:Trimethoprim sulfamethoxazole; Ln: Linezolid

Table 5
The ability to produce biofilm in MRSA and MSSA isolates.

	Biofilm formation	MRSA (n = 11)	MSSA (n = 9)	Total (n = 20)
Tissue culture plate method (**TCP**)	**Strong**	4 (36%)	3 (33%)	7 (35%)
	Moderate	6 (55%)	5 (56%)	11 (55%)
	Weak	1 (9%)	1 (11%)	2 (10%)
Congo red agar method (**CRA**).	**Very black**	5 (46%)	1 (11%)	6 (30%)
	Black	4 (36%)	3 (33%)	7 (35%)
	Almost black	1 (9%)	4 (45)	5 (25%)
	Red	1 (9%)	1 (11%)	2 (10%)

moderate, and weak biofilms were produced by 35%, 55%, and 9% of these strains, respectively. On the other hand, biofilm production was detected in 90% of *S. aureus* strains using CRA approaches, with only two strains not producing biofilm. Additionally, 30%, 35%, and 25% of the strains produced colonies that were characterized as very black, black, and almost black, respectively (Table 5).

For the comparison between the TCP and CRA approaches, an agreement measure test was conducted to assess the biofilm production ability between the two methods. The total agreement was found to be 42.85% and Krippendorff's alpha (ordinal) reliability was calculated as 0.42, indicating a moderately significant association between the detection approaches.

The majority of strains that formed strong biofilms were penicillin-resistant *S. aureus* strains (85.71%) and tigecycline-resistant strains (57.14%). On the other hand, all vancomycin and clindamycin-resistant strains formed moderate biofilms (Table 8). Furthermore, moderate biofilm production was observed in 100% of penicillin-resistant strains and 81.81% of strains with fusidic acid resistance (Table 3).

The Mann-Whitney U test revealed a significant association between MDR and non-MDR strains in biofilm production (p-value <0.01). When comparing MDR and non-MDR strains, it was demonstrated that MDR strains exhibited stronger biofilm formation.

The ability to produce biofilm in penicillin-resistant and methicillin-resistant strains

Among the *S. aureus* strains, except for one strain, 19 (95%) penicillin-resistant were β-lactamase producers and harbored the *blaZ* gene. Fifty-five percent (n = 11) of the total *S. aureus* strains were cefoxitin-resistant and harbored the *mec*A gene. These MRSA strains were significantly (p-value<0.05) more resistant to fusidic acid, erythromycin, fosfomycin, and tobramycin. On the other hand, the most effective antibiotics against MRSA strains were linezolid (100%) and vancomycin (90.91%) (Tables 3 and 8).

In the TCP approach, the majority of the analyzed MRSA strains were moderate biofilm producers (55%), followed by strong biofilm producers (36%). Only 9% of the MRSA strains exhibited weak biofilm formation. On the other hand, in the CRA method, among the MRSA strains, 46% produced very black colonies, 36% produced black colonies, and 9% produced almost black colonies (Table 5). Overall, in both approaches, there was no significant association between resistance to methicillin and the level of biofilm formation.

Regarding the association between methicillin resistance and biofilm formation capacity, MRSA strains demonstrated a higher biofilm production ability compared to MSSA strains. However, according to the Mann-Whitney U test, there was no significant association between biofilm biomass measured at 590 nm absorbance and methicillin resistance among the *S. aureus* strains (p-value = 0.469).

The ability to produce biofilm in clindamycin-resistant strains

Both antibiotics, erythromycin, and clindamycin, were effective against fifty-five percent of *S. aureus*. On the other hand, 20% of *S. aureus* strains were cMLSB phenotypes, 15% were MS and 10% were iMLSB. Additionally, no significant correlation was found between the iMLSB phenotype and MRSA (Table 6).

Among the 7 (35%) *S. aureus* strains evaluated as strongly biofilm-producing by TCP, 29% exhibited the cMLSB phenotype, and 14% exhibited the MS phenotype. Similarly, for the *S. aureus* strains determined to be moderately biofilm-producing, 18% exhibited the cMLSB and MS phenotypes, while 9% exhibited the iMLSB phenotype (Table 6).

According to the TCP approach, there was no significant correlation between the development of biofilms and inducible clindamycin resistance among *S. aureus* strains.

Detection of genes encoding polysaccharide intercellular adhesion and microbial surface components recognizing adhesive matrix molecules

All uropathogenic *S. aureus* strains included in this study harbored *fnb*B and *clf*A genes. The presence of *ica*A, *ica*B, *bap*, and f*nb*A genes was observed in 18 (90%), 12 (60%), 19 (95%), and 18 (90%) strains, respectively. Additionally, 40% of strains carried the *ica*D and *ica*R genes.

There was no significant difference (p-value > 0.05) in the occurrence of *fnb*B, *clf*A, *bap*, *fnb*A, *icaR* and *ica*ADB genes among the different categories of biofilm-producing *S. aureus* strains.

The *ica*ADB and *ica*AD genes were detected in 8 (38.10%) strains. Additionally, there was a significant correlation between MRSA strains and the occurrence of the *ica*A ($p < 0.05$), *ica*ADB ($p < 0.01$), and *ica*AD genes ($p < 0.01$). Conversely, the *ica*R gene was significantly detected in MSSA strains ($p < 0.01$). There was no significant relationship (p-*value*> 0.05) between the acquisition of the *fnb*B, *clf*A, *bap*, and *fnb*A genes and methicillin resistance among uropathogenic *S. aureus* strains..

Bivariate analysis revealed significant positive correlations between *blaZ* and *bap*, *ica*B and *mec*A genes, *mec*A and *ica*D genes, *ica*D and *mec*A, *ica*D and *ica*B, and *fnb*A and *bap*. Furthermore, there are significant negative correlations between *ica*R and *ica*B, *ica*R and *mec*A, and *ica*R and *ica*D genes (Table 9).

Hierarchical clustering of S. aureus strains

Four groups were established through hierarchical clustering analysis of twenty uropathogenic *S. aureus* strains based on antibiotic resistance, genes involved in biofilm formation, and biofilm formation intensity. Group 1 is composed of 9 strains (S5, S10, S3, S14, S13, S20, S2, fS9, and S4). Cluster 2 includes 3 strains (S7, S12, and S18). Group 3 comprises 4 strains (S11, S1, S15, and S16), and group 4 includes 4 strains (S19, S8, S17, and S6) (Fig. 1).

Table 6
The results of the inducible clindamycin resistance test in *S. aureus* isolates and their ability to form biofilm.

Phenotypes	*Number of resistant isolates*				Ability to produce biofilm			
	MRSA (n = 11)	MSSA (n = 9)	All (n = 20)	*p* value	Strong (n = 7)	Moderate (n = 11)	Weak (n = 2)	All (n = 20)
cMLSB (E-R, CL-R)	3 (27.27)	1 (11.11)	4 (20)	0.59	2 (29%)	2 (18%)	0	4 (20%)
iMLSB (E-R, CL-S)	1 (9.09)	1 (11.11)	2 (10%)	1	0	1 (9%)	1 (50%)	2 (10%)
MS (E-R, CL-S)	3 (27.27)	0	3 (15)	0.089	1 (14%)	2 (18%)	0	3 (15%)
E-S, CL-S	4 (36.36)	7 (77.78)	11 (55)	0.092	4 (57)	6 (55%)	1 (50%)	11 (55%)

E: Erythromycin; CL: Clindamycin; R: resistant; S: sensitive

Table 7
The frequency of *S. aureus* isolates carrying biofilm-forming genes.

Biofilm formation	Strong (n = 7)	Moderate (n = 11)	Weak (n = 2)	All (n = 20)	*p value*
icaA	6 (86%)	10 (91%)	2 (100%)	18 (90%)	1
icaB	3 (43%)	8 (73%)	1 (50%)	12 (60%)	0.53
icaD	1 (14%)	6 (55%)	1 (50%)	8 (40%)	0.28
icaR	2 (29%)	6 (55%)	0	8 (40%)	0.35
fnbB	7 (100%)	11 (100%)	2 (100%)	20 (100%)	1
clfA	7 (100%)	11 (100%)	2 (100%)	20 (100%)	1
bap	6 (86%)	11 (100%)	2 (100%)	19 (95%)	0,45
fnbA	6 (86%)	10 (91%)	2 (100%)	18 (90%)	1

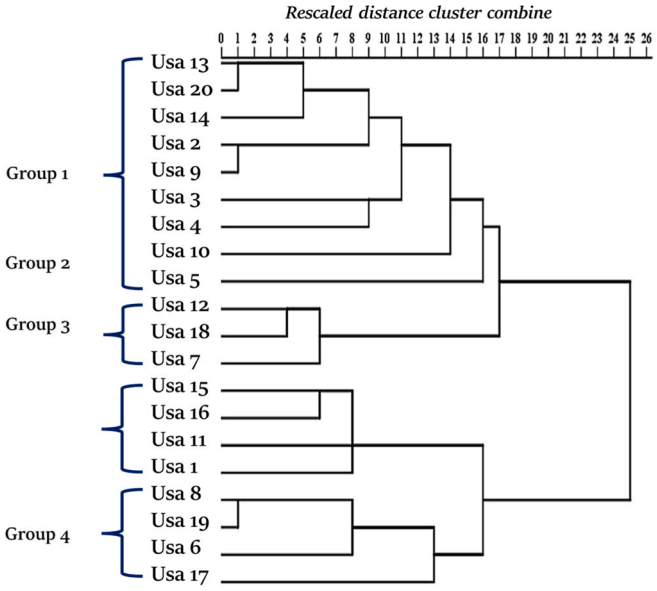

Fig. 1. Hierarchical clustering of uropathogeneic *S. aureus* isolated strains.

Discussion

Biofilm-forming *S. aureus* plays a significant role in the pathogenesis of UTIs as it confers resistance to antimicrobials and other environmental factors, while also providing protection against immune reactions. Moreover, it is associated with persistent or recurrent infection (Piechota et al., 2018). Studying biofilm-forming *S. aureus* contributes to the prevention, management, and treatment of UTIs caused by this bacteria (Manandhar et al., 2018b; Leshem et al., 2022). In the current study, we investigated the relationship between biofilm formation and multidrug resistance in *S. aureus* UTIs by assessing the antibiotic susceptibility profile and the occurrence of biofilm-forming MSSA and MRSA in vitro using phenotypic and genotypic approaches.

In this study, the prevalence of MRSA (55%) in uropathogenic *S. aureus* strains was higher than that reported in other studies, which ranged from 27 to 43% in UTIs (Looney et al., 2017; Mitiku et al., 2021). Furthermore, MRSA strains exhibited a higher antibiotic resistance profile compared to MSSA strains, with significant differences observed for fosfomycin, tobramycin, and erythromycin. The finding of this research are consistent with previous studies conducted in Ethiopia (Mitiku et al., 2021) and Iran (Yousefi et al., 2016). For severe MRSA infections, vancomycin and linezolid are considered the primary choices for first-line therapy (Maharjan et al., 2022). In our study, linezolid showed higher effectiveness against all *S. aureus* strains, while a decreased susceptibility was observed for vancomycin (75%) with MIC values between 8 and 32 μg/mL (Table 8), and other antibiotics such as gentamycin, clindamycin, and levofloxacin. These findings align with previous investigations (Yousefi et al., 2016; Manandhar et al., 2018b; Selim et al., 2022). Furthermore, the emergence of vancomycin-resistant *S. aureus* is primarily attributed to the irrational use of antibiotics, the availability of over-the-counter antibiotics without prescriptions, and the selective environment of hospitals (Loomba et al., 2010).

In our research, the CRA and TCP methods were employed to investigate the phenotypic production of biofilm in *S. aureus*. CRA was used as a screening test, while TCP served as a reference technique (Bissong and Ateba, 2022). Although CRA is one of the simplest techniques used, it is necessary to consider the potential for evident errors when employing these methods (Neopane et al., 2018). As a result, a moderately significant association in *S. aureus* biofilm formation was observed between the CRA and TCP approaches. Moreover, several recent studies have validated the accuracy of the TCP method by assessing sensitivity, specificity, as well as positive and negative predictive values (Neopane et al., 2018; Leshem et al., 2022; Harika et al., 2020). Furthermore, TCP is affordable and widely available, factors that are particularly crucial to consider in developing countries such as Morocco. Therefore, TCP is recommended for biofilm detection for routine biology medical laboratory work in both public and private hospitals.

In our current study, the majority of *S. aureus* strains (55%) were classified as moderate biofilm producers. Similar findings have been reported in several studies (Yousefi et al., 2016; Piechota et al., 2018; Manandhar et al., 2018b). Previous studies have indicated that biofilm-producing strains of *S. aureus* tend to exhibit higher levels of resistance to various antibiotics compared to planktonic cells, primarily due to the presence of a resistant polymeric matrix that hampers antibiotic penetration (Harika et al., 2020; Katongole et al., 2020). Consistent with the findings of Manandhar et al (Manandhar et al., 2021). and Saber et al (Saber)., our study demonstrates that the majority of MRSA and MSSA strains were moderate biofilm producers, and there was no significant correlation observed between methicillin resistance and biofilm formation.

Clindamycin is among the most recommended antibiotics for the treatment of MRSA infections (Maharjan et al., 2022). However, there is an increased incidence of inducible clindamycin resistance among biofilm-forming strains of *S. aureus*, particularly MRSA. Consequently, the risk of antibiotic failure is high when clindamycin is used to treat *S. aureus* infections with inducible clindamycin resistance (Maharjan et al., 2022). Furthermore, 29% of strongly biofilm-producing *S. aureus*

Table 8
The resistance pattern, MAR index, MIC values for vancomycin, fosfomycin, and teicoplanin, biofilm formation ability, and biofilm genes of 20 *S. aureus* strains.

Strain	MIC values (μg/mL)			Resistance pattern	MAR index	Biofilm formation ability	Biofilm genes
	Vn	**F**	**Tei**				
Usa 1	2	≥ 128	4	P, Tm, G, E, Te, Tg, FA, Tei, F, Le	0.71	Strong	*spa, clfA, fnbB, bap, fnbA, icaA, icaB*
Usa 2	2	≤ 8	2	-	0.14	Strong	*spa, clfA, fnbB, icaR*
Usa 3	32	≤ 8	≤ 0.5	P, FA, Vn, Tei	0.29	Moderate	*spa, clfA, fnbB, bap, icaA, icaR*
Usa 4	2	≤ 8	2	-	0.14	Moderate	*spa, clfA, fnbB, bap, fnbA, icaA, icaR*
Usa 5	8	64	4	P, E, Te, FA, Vn, Tei, F	0.50	Moderate	*spa, clfA, fnbB, bap, fnbA, icaA, icaR*
Usa 6	2	≥ 128	2	-	0.57	Moderate	*spa, clfA, fnbB, bap, fnbA, icaA, icaB*
Usa 7	2	≤ 8	2	-	0.14	Moderate	*spa, clfA, fnbB, bap, fnbA, icaA, icaB, icaR*
Usa 8	2	≥ 128	4	P, Tm, G, E, Te, Tg, FA, Tei, F, Le	0.71	Moderate	*spa, clfA, fnbB, bap, fnbA, icaA, icaB, icaD, icaR*
Usa 9	2	≤ 8	2	-	0.14	Moderate	*spa, clfA, fnbB, bap, fnbA, icaA, icaR*
Usa 10	≤ 0.5	≤ 8	≤ 0.5	-	0.21	Strong	*spa, clfA, fnbB, bap, fnbA, icaA, icaR*
Usa 11	8	≥ 128	4	P, Tm, E, Te, Tg, FA, Vn, Tei, F, CL	0.71	Moderate	*spa, clfA, fnbB, bap, fnbA, icaA, icaB, icaD*
Usa 12	≤ 0.5	≤ 8	≤ 0.5	-	0.14	Moderate	*spa, clfA, fnbB, bap, fnbA, icaA, icaB, icaD*
Usa 13	2	≤ 8	2	-	0.14	Strong	*spa, clfA, fnbB, bap, fnbA, icaA, icaB*
Usa 14	1	≤ 8	≤ 0.5	P, Te, F	0.21	Moderate	*spa, clfA, fnbB, bap, fnbA*
Usa 15	2	64	2	P, E, Te, FA, F, CL	0.43	Moderate	*spa, clfA, fnbB, bap, fnbA, icaA, icaB, icaD*
Usa 16	2	64	4	P, E, Te, FA, Tei, F	0.43	Strong	*spa, clfA, fnbB, bap, fnbA, icaA, icaB, icaD*
Usa 17	2	≥ 128	2	P, Tm, Gm, E, FA, SXT, F	0.50	Weak	*spa, clfA, fnbB, bap, fnbA, icaA, icaB, icaD*
Usa 18	1	≤ 8	≤ 0.5	-	0.14	Strong	*spa, clfA, fnbB, bap, fnbA, icaA, icaB, icaD*
Usa 19	2	64	4	P, Tm, Gm, E, Tei, FA, F, Le	0.57	Strong	*spa, clfA, fnbB, bap, fnbA, icaA, icaB, icaD*
Usa 20	1	≤ 8	≤ 0.5	-	0.14	Weak	*spa, clfA, fnbB, bap, fnbA, icaA*

P: Penicillin G; FA: Fusidic Acid; Te: Tetracycline; E: Erythromycin; F:Fosfomycin; Tm: Tobramycin; Tei: Teicoplanin; G: Gentamycin; CL: Clindamycin; Tg:Tigecycline; Le: Levofloxacin; Vn:Vancomycin; SXT:Trimethoprim sulfamethoxazole; Ln: Linezolid

Table 9
Pearson's correlation coefficients (ρ) for the mecA, blaZ, bap, fnbA, icaR and icaADB genes.

		blaZ	*mecA*	*icaA*	*icaB*	*icaD*	*icaR*	*bap*	*fnbA*
blaZ	**Pearson correlation**	1							
	Sig. (2-tailed)	-							
mecA	**Pearson correlation**	0254	1						
	Sig. (2-tailed)	0281	-						
icaA	**Pearson correlation**	,688**	0369	1					
	Sig. (2-tailed)	0001	0110	-					
icaB	**Pearson correlation**	0254	1000**	0369	1				
	Sig. (2-tailed)	0281	0000	0110	-				
icaD	**Pearson correlation**	0187	,739**	0272	,739**	1			
	Sig. (2-tailed)	0429	0000	0246	0000	-			
icaR	**Pearson correlation**	-0281	-,492*	-0068	-,492*	-,458*	1		
	Sig. (2-tailed)	0230	0027	0776	0027	0042	-		
bap	**Pearson correlation**	1000**	0254	,688**	0254	0187	-0281	1	
	Sig. (2-tailed)	0000	0281	0001	0281	0429	0230	-	
fnbA	**Pearson correlation**	,688**	0369	,444*	0369	0272	-0408	,688**	1
	Sig. (2-tailed)	0001	0110	0050	0110	0246	0074	0001	-

* The correlation is significant at the 0.05 level (two-tailed).
** The correlation is significant at the 0.01 level (two-tailed).

strains exhibited the cMLSB phenotype. Similarly, *S. aureus* strains categorized as moderately biofilm-producing displayed the cMLSB (18%) and iMLSB (9%) phenotypes. These findings are consistent with previous investigations conducted by Manandhar et al (Manandhar et al., 2021). and Maharjan et al (Maharjan et al., 2022). Therefore, it is important to incorporate a simple test such as the D-test for clindamycin resistance induction into routine medical biology laboratory testing. This test is crucial for guiding the treatment of *S. aureus* infections and is therefore recommended (Belbase et al., 2017).

In our research, significantly higher rates of biofilm production were observed among MDR strains compared to non-MDR strains (p-value = 0.00398). These findings are consistent with the results reported by Neopane et al (Neopane et al., 2018). Bacteria that develop in biofilms inherently exhibit resistance to commonly used antibiotics due to the protective nature of biofilm communities. Moreover, biofilm formation facilitates horizontal gene transfer between bacteria, leading to the dissemination of drug-resistance markers, including MDR and other virulence genes. As a result, available treatment options become limited (Manandhar et al., 2021).

Numerous research studies have demonstrated the relationship between *S. aureus* biofilm formation and the presence of the *ica* operon. In our study, the majority of *S. aureus* strains were found to contain the *ica*A gene; however, the strains exhibited variations in terms of biofilm biomass, categorized as high, moderate, or low. This finding is consistent with previous investigations conducted by Chen et al (Chen et al., 2020). and Merino et al (Merino et al., 2009). It is possible that these biofilm-producing strains utilize additional mechanisms, such as protein A, *S. aureus* surface proteins, or fibronectin-binding proteins, which play critical roles in biofilm formation (Merino et al., 2009).

Furthermore, it revealed a significantly higher occurrence of the *ica*A gene in biofilm-producing MRSA strains compared to MSSA strains. Recent studies have confirmed the significant relationship between the *ica*A gene and methicillin resistance (Piechota et al., 2018; Usun Jones et al., 2022).

The presence of the *ica*A, *ica*ADB, and *ica*AD genes was significantly associated with MRSA strains rather than MSSA strains. This finding aligns with the findings presented by Piechota et al (Piechota et al., 2018). and Sherry et al (Usun Jones et al., 2022). Moreover, the

expression of the *ica*R gene showed an inverse relationship with the *ica*A, *ica*B, and *ica*D genes, highlighting the significance of the *ica*R gene in biofilm control (Mohammadi Mollaahmadi et al., 2021). In our research, the *ica*R gene was detected in 38.57% of *S. aureus* strains and was significantly associated with MSSA strains (p-value=0.006). Similar findings were reported by Mohammadi Mollaahmadi et al (Mohammadi Mollaahmadi et al., 2021). These results suggest that biofilm formation in MSSA is dependent on the *ica*R gene, and environmental regulation plays an important role in the expression of *ica*R.

The attachment factor genes *clf*A and *fnb*B were present in all uropathogenic *S. aureus* strains capable of producing biofilms, and a high percentage of these strains (95% and 90%, respectively) carried the *bap* and *fnb*A genes. This finding is consistent with the study conducted by Pajohesh et al (Pajohesh et al., 2022)., where it was concluded, based on phenotypic and genotypic assays, that strains harboring the *ica*ABD genes, *clf*B, *fnb*B, *clf*A, and *fnb*A are potential biofilm producers. Additionally, the biofilm-associated protein (*bap*) plays a significant role in *S. aureus* adhesion and biofilm formation.

A limited number of studies have been conducted to investigate the association between biofilm formation and antibiotic resistance in *S. aureus* strains (Saber et al.). Further studies are necessary to evaluate this correlation, as biofilm formation is a complex process involving numerous variables and components (Manandhar et al., 2021). However, previous studies have suggested that early antibiotic abuse may contribute to biofilm development (Huang et al., 2022). Nevertheless, more studies on the effects of exposure to sub-inhibitory concentrations of antibiotics are needed to test this hypothesis.

Conclusion

In conclusion, significantly higher rates of biofilm production were observed among MDR strains compared to non-MDR strains, which serve as crucial virulence factors and act as barriers against effective infection treatment. The prevalence of MRSA (55%) was higher, no significant correlation was observed between methicillin resistance and biofilm formation. All strains were sensitive to linezolid antibiotic, and a third of strongly biofilm-producing *S. aureus* strains exhibited the cMLSB phenotype. Genotypic characterization revealed a significant correlation between MRSA strains and the occurrence of the *ica*A, *ica*ADB, and *ica*AD genes. Conversely, the *ica*R gene was significantly detected in MSSA strains. These results can contribute to the enrichment of molecular and phenotypic data for the development of vaccines targeting biofilm-producing MDR *S. aureus*.

Ethics approval and consent to participate

This study was approved by the internal ethics committee of the Biology-Geology department of the Poly-disciplinary Faculty of Sultan Moulay Slimane University, Beni Mellal, and by the ethics committee for biomedical research of the Faculty of Medicine and Pharmacy of Oujda of Mohammed I University, Oujda, Morocco.

Authorship statement

All the authors meet the ICMJE authorship criteria. Literature search: AR, DA, RH, RA, TM, BA. Figures and tables: AR, DA, RH, RA, TM, BA. Study design: AR, DA, RH, RA, BA. Data collection: AR, DA, RH, RA, BA. Data analysis and interpretation: AR, DA, RH, RA, BA. Materials: AR, BA, HB, AK, NK. Writing: AR, BA All authors read and approved the final manuscript.

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CRediT authorship contribution statement

Kaotar Nayme: Supervision, Methodology. **Ressmi Amina:** Resources, Investigation, Data curation. **Raqraq Habiba:** Software, Resources, Data curation. **Asmaa Dihmane:** Resources, Investigation, Data curation. **Aniba Rafik:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Abouddihaj Barguigua:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Conceptualization. **Khalil Abdelouahed:** Software, Resources. **Berrougui Hicham:** Resources, Methodology, Conceptualization. **Mohammed Timinouni:** Supervision, Methodology.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

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