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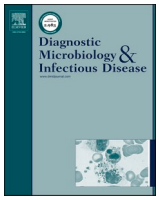

Molecular and phenotypic characterization of biofilm formation and antimicrobial resistance patterns of uropathogenic staphylococcus haemolyticus isolates in Casablanca, Morocco

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ABSTRACT

This study aimed to establish the correlation between antibiotic resistance and biofilm formation by *Staphylococcus haemolyticus* and to examine the impact of sub-inhibitory concentrations of antibiotics (sub-MICs) on biofilm formation.

Antibiotic susceptibility testing was conducted using the disk diffusion method, and biofilm formation was determined using Congo red agar and microtiter plate methods. Antibiotic resistance and biofilm-associated genes were detected using polymerase chain reaction.

The majority of the twenty-one *S. haemolyticus* isolates were multidrug-resistant, methicillin-resistant (MRSH) and biofilm producers, including 43 % of moderate biofilm producers. A significant correlation was observed between MRSH and MSSH isolates in terms of biofilm production. Vancomycin, gentamicin, and ciprofloxacin at their sub-MICs tended to promote biofilm formation. The *eno* gene was present in 76.2 % of strains, followed by *aap*, and *atlE*.

This study revealed a strong correlation between the biofilm-forming ability and antibiotic resistance in *S. haemolyticus*, which underlines a crucial public health issue.

1. Introduction

Coagulase-negative *Staphylococcus* (*CoNS*) is the most common etiological agent of several infections, including urinary tract infections (UTIs) [1]. *Staphylococcus haemolyticus* is a member of the skin microflora and is among the most commonly isolated *CoNS* species in UTIs (10–25 %) [2]. *S. haemolyticus* has the highest level of antibiotic resistance among *CoNS*, and plays a crucial role in the spread of resistance genes, contributing to the emergence of more virulent and epidemic clones [3]. This limited the number of therapeutic options available.

Biofilm formation is one of the main virulence factors of *S. haemolyticus* [3]. Furthermore, bacteria aggregated in biofilms can survive in harsh environments, including the presence of antimicrobials and the host immune system [3].

The process of biofilm production in *S. haemolyticus* is mediated by several adhesive proteins such as fibrinogen-binding proteins A and B (fnbA et B), biofilm-associated protein (bap), and agglutination factor A (clfA). Polysaccharide intercellular adhesins (PIA) are the main functional constituents of intercellular adhesion in *S. haemolyticus* biofilms. PIA are synthesized by enzymes encoded by the icaADBC operon [4].

During the antibiotic treatment of biofilm-related infections, bacteria may encounter sub-inhibitory concentrations of antibiotics (sub-MICs). However, the effects of antibiotics on biofilm formation remain unclear. Several studies have shown that biofilm formation by *S. haemolyticus* on abiotic surfaces is not inhibited by many antibiotics such as linezolid and vancomycin [3]. Other studies have shown that tigecycline/rifampicin combination is more effective against biofilm-forming *S. haemolyticus* [5].

This study aimed to phenotypically and genotypically characterize the antimicrobial susceptibility and biofilm formation of uropathogenic

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S. haemolyticus, and examine the effects of antibiotic sub-MICs on biofilm formation.

2. Materials and Methods

2.1. Bacterial strains

Twenty-one unduplicated *S. haemolyticus* isolates were collected from clinical samples of patients with UTIs in medical biology laboratories in Casablanca, Morocco, between January 2020 and December 2022. Of these patients, 71.43 % were male and 28.57 % were female, with ages ranging from 0 to 15 years (33.33 %), 15 to 25 years (19.05 %), 26 to 65 years (19.05 %), and over 65 years (28.57 %). According to medical records, 61.9 % of the patients had urinary catheters, 57.1 % had recurrent UTIs, and 14.3 % were immunocompromised.

Standard morphological and biochemical techniques, including mannitol Salt Agar (Biokar Diagnostics, Beauvais, France), catalase test, coagulase test, and hemolysin production, were used to identify the presence of *S. haemolyticus* isolates. Biochemical test results were used to confirm the *S. haemolyticus* strains using the Vitek 2® Compact 15 system identification card (BioMérieux, Marcy-l'Étoile, France) according to the manufacturer's guidelines.

2.2. Antibiotic susceptibility testing

Antimicrobial susceptibility testing of the *S. haemolyticus* strains was performed using the automated VITEK 2® COMPACT 15 system. Following the Antibiogram Committee of the French Microbiology Society (CA-SFM 2021) guidelines, each strain was categorized as susceptible (S), susceptible dose-dependent (I), or resistant (R) [6].

Fosfomycin, gentamicin, vancomycin, ciprofloxacin and teicoplanin susceptibility was investigated using the broth micro-dilution method with Muller Hinton broth (Oxoid, Thermo Scientific™) medium, and the results were interpreted according to the CA-SFM (2021) guidelines [6].

S. haemolyticus strains resistant to at least one antibiotic from three or more antibiotic classes are defined as multidrug-resistant (MDR).

2.3. Detection of β-lactamase production and methicillin resistance

Phenotypic β-lactamase activity was assessed based on the appearance of the inhibition zone edge around the penicillin G 1-unit disk (Thermo Fisher Scientific™ Oxoid™) obtained using the disk diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI) [7]. Methicillin resistance was tested using a cefoxitin disk (30μg) under standard susceptibility testing conditions by plating on Mueller-Hinton (MH, Bio-Rad, Marnes-la-Coquette, France) agar, followed by incubation at 35 ± 2 °C for 20±4h. The inhibition zone diameters were measured in millimeters and the results were interpreted in accordance with CA-SFM (2021) guidelines [6]. The *mec*A gene was tested in all phenotypically cefoxitin-resistant *S. haemolyticus* isolates and *bla*Z was tested in the genomes of all penicillin-resistant isolates by PCR amplification.

2.4. Screening of inducible clindamycin resistance

In accordance with CLSI M100-S28 recommendations [8], the D-zone test on MH agar was used to screen all erythromycin-resistant *S. haemolyticus* strains for inducible resistance to clindamycin (iMLSB).

2.5. Biofilm formation assay

Two phenotypic techniques, the quantitative microtiter plate method (TCP) and Congo red agar (CRA), were used to assess biofilm formation by *S. haemolyticus*. The CRA culture method was carried out according to the procedure documented by Freeman et al. (1989) [9], and TCP, a quantitative method, was used as described by Christensen et al. (1985) [10]. Experiments were carried out in triplicate for each strain and biofilm production was classified as negative, low, moderate or strong.

2.6. Effect of vancomycin, gentamicin and ciprofloxacin sub-MICs on biofilm formation

The experiments were conducted in sterile 96-well polystyrene plates. The strains were incubated with MIC/2, MIC /4, MIC /8 MIC, and MIC/16 MIC of antibiotics supplemented in brain heart infusion broth (BHI, Biokar Diagnostics, Beauvais, France) at 35±2 °C for 48h. A negative control well without antibiotics or bacteria was used. After incubation, biofilm formation was quantified using crystal violet staining. The tests were performed in triplicate. The following formula was used to calculate the rate of inhibition of biofilm formation [11]:

$$\text{Inibition biofilm formation } (\%) = \frac{ODc - ODt}{ODc} \times 100$$

- **ODc:** Optical density of the biofilm formed without antibiotics.
- **ODt:** optical density of biofilm formation in the presence of an antibiotic.

2.7. DNA extraction

The genomic DNA was extracted using the InstaGene (Bio-Rad Laboratories, USA) extraction kit in accordance with the manufacturer's instructions. The DNA concentration and purity of the extracts were determined based on the measurement of the OD 260/280 ratio using a Nano Drop-ND-1000 spectrophotometer instrument (Thermo Fisher Scientific, Wilmington, U.S.A).

2.8. PCR amplification

All *S. haemolyticus* isolates were screened for the presence of genes coding for adhesion and biofilm formation, using specific primers and PCR amplification conditions (Supplementary Table 1 and 2). The PCR reaction was performed using 2 μL (200 ng) of the DNA template, 10 μL of 5 x MyTaq Reaction Buffer (Bioline Reagents, London, UK), 1 μL of MyTaq DNA Polymerase, and 1 μL of each primer at a concentration of 20 pM, and double-distilled water was added up to 50 μL. PCR products were separated by electrophoresis on a 1.5 % agarose gel (Sigma, Darmstadt, Germany) and visualized using ultraviolet light.

2.9. Statistical analysis

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. Statistical significance was set at $P \leq 0.05$. However, for independent groups, the nonparametric Wilcoxon-Mann-Whitney rank sum test was used.

To determine the correlation between the different genes involved in biofilm-forming *S. haemolyticus* strains, bivariate analysis was used to calculate Pearson's correlation coefficient (ρ). The "Hierarchical Cluster" function of IBM SPSS Statistics 26 was used to conduct the cluster analysis and determine all pairwise dissimilarity distances between isolates.

3. Results

According to our results, all *S. haemolyticus* isolates were catalase-positive and coagulase-negative, and 95.23 % (20/21) of the strains produced hemolysin.

3.1. Antibiotic resistance and biofilm formation

S. haemolyticus has exhibited high resistance against penicillin G

(100 %), followed by fusidic acid (90.48 %), tetracycline (90.48 %), and fosfomycin (85.71 %). In contrast, S. haemolyticus was highly susceptible to linezolid (100 %) and vancomycin (95.24 %). Furthermore, 95.2 % (20/21) of the strains tested were categorized as S. haemolyticus MDR (Table 1).

All penicillin-resistant S. haemolyticus strains harbored blaZ gene, and 85.71 % of isolates were positive for mecA gene. In addition, 19.05 % of S. haemolyticus strains exhibited the iMLSB phenotype (Table 2).

Based on the TCP findings, 95 % of S. haemolyticus strains demonstrated the ability to generate biofilms at various levels. Strong, moderate, and weak biofilm formations were produced by 5, 43, and 48 % of the isolates, respectively. In contrast, biofilm production was detected in 90 % of S. haemolyticus isolates using CRA approaches, with only one isolate not producing biofilms. Additionally, 5 %, 38 %, and 52 % of the strains characterized as very black, black, and almost black, respectively. MRSH strains demonstrated a higher biofilm production ability than MSSH strains. However, according to the Mann-Whitney U test, there was a significant association between biofilm biomass and methicillin resistance among S. haemolyticus isolates ($p = 0.037$).

Among the nine *S. haemolyticus* isolates evaluated as moderate biofilm producers by TCP, 22.22 % had the iMLSB phenotype, while 30 % of the weak biofilm-producing strains exhibited the iMLSB phenotype (Table 2).

3.2. Sub-MICs effects of antibiotics on biofilm formation

The MICs of vancomycin and ciprofloxacin were found to be very narrow, ranging from 1 to 2 mg/L and 0.25 to 0.5 mg/L, respectively, while the MICs of ciprofloxacin for 71.42 % of *S. haemolyticus* plankton cells were 0.5 mg/L (Supplementary Table 3).

Analysis of the influence of sub-MICs on *S. haemolyticus* biofilm formation showed that the presence of vancomycin, gentamicin, and ciprofloxacin sub-MICs promoted biofilm formation on polystyrene surfaces in most of the isolates (Table 3).

The sub-MICs of vancomycin led to a dose-dependent increase in *S. haemolyticus* biofilm formation, with 81.8 %, 83.3 %, 91.66 %, and 100 % of biofilm formation enhanced at MIC/2, MIC/4, MIC/8, and MIC/16 concentrations, respectively, compared to the control. Moreover, the inducing effect of gentamicin and ciprofloxacin on biofilm formation was observed in 60 % and 75 % of the strains at a concentration of MIC/2, respectively, and this effect remained consistent even as the concentration of the antibiotic decreased (Supplementary Figure 1).

Table 1
The resistance profile of S. haemolyticus isolates to antibiotics.

Antibiotics	*S. haemolyticus* isolates (%)		
	MRHA (85.71 %, n = 18)	MSHA (14.29 %, n = 3)	Total (%)
Penicillin G	18(100)	3 (100)	21 (100)
Fusidic Acid	17(94.4)	2 (66.7)	19 (90.48)
Tetracycline	16 (88.9)	3 (100)	19 (90.48)
Erythromycin	11 (61.9)	2 (66.7)	13 (61.9)
Fosfomycin	16 (88.9)	2 (66.7)	18 (85.71)
Tobramycin	7 (38.9)	0 (0)	7 (33.33)
Teicoplanin	2 (11.1)	1 (33.3)	3 (14.29)
Gentamicin	4 (22.2)	0 (0)	4 (19.05)
Clindamycin	4 (22.2)	1 (33.3)	5 (23.81)
Tigecycline	5 (27.8)	1 (33.3)	6 (28.57)
Levofloxacin	7 (38.9)	0 (0)	7 (33.3)
Vancomycin	0 (0)	1 (33.33)	1 (4.76)
Trimethoprim-sulfamethoxazole	5 (27.8)	0 (0)	5 (23.81)
Linezolid	0	0	0
Total	21		

The difference in biofilm forming capacity between resistant and susceptible strains, which were prompted the formation of strong biofilms in vitro when incubated with sub-MICs of vancomycin, gentamicin, and ciprofloxacin (Supplementary Figure 2).

Biofilm formation was stimulated by vancomycin treatment in a dose-dependent manner and was significantly increased in the vancomycin-resistant *S. haemolyticus* strain compared with the susceptible strain by MIC/2 ($P < 0.001$), MIC/4 ($P < 0.001$), MIC/8 ($P < 0.001$), and MIC/16 ($P < 0.001$) of vancomycin, compared with incubation in the absence of vancomycin.

S. haemolyticus treated with sub-MICs of gentamicin exhibited significantly stronger biofilm formation than untreated *S. haemolyticus*, for both gentamicin-sensitive and gentamicin-resistant strains. In addition, ciprofloxacin-resistant strains were significantly more likely to form biofilms than ciprofloxacin-sensitive strains when incubated with sub-MICs of ciprofloxacin (Supplementary Figure 2).

3.3. Molecular detection of biofilm-related genes

In this study, the *eno* gene was present in 76.2 % of the *S. haemolyticus* strains, followed by *aap* (42.8 %), *ica*D (42.8 %), and *atlE* (23.8 %). Meanwhile, 9.5 % of the isolates carried *embP* and *icaA* genes. The presence of *fnb*A and *fnb*B genes were observed in 28.57 % and 28.6 % of *S. haemolyticus* strains, respectively. The *bap, clf*A, *ica*B and *ica*R genes were not detected in any of the isolates. There was a significant association between the occurrence of *atlE* ($p = 0.033$), *embp* ($p = 0.014$), and *aap* ($p < 0.001$) genes and the different categories of biofilm-producing isolates (Table 4).

Bivariate analysis of *S. haemolyticus* biofilm formation-related genes revealed significant positive correlations between *embP* and *atlE* ($\rho = 0.58$, $p < 0.01$), *aap* and *atlE* ($\rho = 1$, $p < 0.001$), *bla*Z and *fnb*A ($\rho = 0.645$, $p < 0.01$), and *aap* and *eno* genes ($\rho = 0.484$, $p < 0.05$) (Fig. 1).

3.4. Hierarchical clustering of S. haemolyticus isolates

Hierarchical analysis of *S. haemolyticus* was performed to establish five groups based on the results of the genotypic and phenotypic characterization of antibiotic resistance and biofilm formation. Group 1 comprised six strains (USH 4, 21, 13, 2,7 and USH 19), cluster 2 comprised six strains (USH 6, 17, 5, 10, 8, and USH 9), cluster 3 included one isolate (USH 15), group 4 comprised two strains (USH11 and USH 14), and group 5 comprised six strains (USH1,20, 3, 12, 18, and USH 16) (Fig. 2).

4. Discussion

S. haemolyticus remains one of the most frequently isolated *CoNS* in UTIs, particularly those associated with implanted medical devices, notably in immunocompromised patients and neonates [12]. In addition, the increasing number of *S. haemolyticus* in these infections is closely linked to their antimicrobial resistance to the majority of commonly prescribed antibiotics, their ability to survive in the hospital environment, and their ability to adhere to the surfaces of biomaterials and form biofilms [13]. The aim of this study was to investigate the relationship between antibiotic susceptibility and biofilm-forming capacity in *S. haemolyticus* isolates.

To the best of our knowledge, this is the first study to report the prevalence and phenotypic and genotypic characterization of antibiotic resistance and biofilm formation in *S. haemolyticus* strains isolated from UTIs in Africa.

The results of our study revealed that the rate of resistance to numerous antibiotics, including penicillin G, fusidic acid, erythromycin, and fosfomycin, was higher in *S. haemolyticus* isolates. This could be due to the overuse of antibiotics, induction of selective pressure, presence of resistance genes in *S. haemolyticus*, and its dissemination in the hospital

Table 2
Results of the methicillin resistance test and inducible clindamycin resistance test on S. haemolyticus isolates.

Phenotypes	*Number of resistant isolates*			Ability to produce biofilm			
	MRHA (n = 18)	MSHA (n = 3)	All (%)	Moderate (n = 9)	Weak (n = 10)	Strong (n = 1)	No-biofilm formation (n = 1)
cMLSB	4 (22.22)	1 (33.33)	5 (23.81)	0	3 (30)	1 (100)	0
iMLSB	4 (22.22)	0	4 (19.05)	2 (22.22)	3 (30)	0	0
MS	6 (33.33)	1 (33.33)	7 (33.33)	5 (55.55)	2 (20)	0	0
E-S, CL-S	4 (22.22)	1 (33.33)	5 (23.81)	2 (22.22)	2 (20)	0	1 (100)

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

Table 3
The effect of Sub-MIC of vancomycin, gentamicin and ciprofloxacin on biofilm formation in S. haemolyticus isolates.

Resistant to none antibiotics	Sub- MIC	Effect on biofilm formation	Strains in phenotype
	MIC /2	V↑ None G↑ C↑	USH 4, 2
		V↑ G↑ C↑	USH 9, 19, 21,10
		V↑ G↓ C↓	USH 8, 5
		V↑ G↓ C↑	USH 17, 6
		V↓ G↓ C↓	USH 13, 7
	MIC /4	V↑ G↑ C↑	USH 4, 2, 9, 17, 19, 21, 10
		V↑ G↓ C↓	USH 8, 5, 6
		V↓ G↓ C↓	USH 13, 7
	MIC /8	V↑ G↑ C↑	USH 4, 2, 9, 17, 19, 21, 10
		V↑ G↓ C↓	USH 8, 5, 6
		V↓ G↓ C↓	USH 13, 7
	MIC /16	V↑ G↑ C↑	USH 4, 2, 9, 17, 19, 21, 10
		V↑ G↓ C↓	USH 8, 5, 6
		V↓ G↓ C↓	USH 13, 7
Vancomycin	MIC /2	V↑ G↑ C↑	USH 15
	MIC /4	V↑ G↑ C↑	USH 15
	MIC /8	V↑ G↑ C↑	USH 15
	MIC /16	V↑ G↑ C↑	USH 15
Gentamicin	MIC /2, MIC /4, MIC /8, MIC /16	V↑ G↑ C↑	USH 1, 12, 20
		V↑ G↓ C↓	USH 18
Ciprofloxacin	MIC /2, MIC /4, MIC /8, MIC /16	V↑ G↑ C↑	USH 3, 12, 11, 14, 16, 20
		V↑ G↓ C↓	USH 18
Gentamicin and Ciprofloxacin	MIC /2, MIC /4, MIC /8, MIC /16	V↑ G↑ C↑	USH 12, 20
		V↑ G↓ C↓	USH 18
		V↑ G↓ C↓	USH 8

↑: Increased biofilm formation; ↓ Decreased biofilm formation; V: vancomycin; G: gentamicin; C: ciprofloxacin; MIC: Minimal inhibitory concentration; USH: uropathogenic Staphylococcus haemolyticus.

Table 4
The frequency of S. haemolyticus isolates carrying biofilm-forming genes.

Biofilm formation	Weak (n = 10)	Moderate (n = 9)	Strong (n = 1)	All (n = 21)	*P value*
*ica*A	1 (10 %)	1 (11.11 %)	0 (0 %)	2 (9.5 %)	0.971
*ica*D	4 (40 %)	3 (33.33 %)	1 (100 %)	9 (42.8 %)	0.387
bap	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	-
*clf*A	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	-
*ica*B	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	-
*ica*R	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	-
*fnb*A	2 (20 %)	4 (44.44 %)	0 (0 %)	6 (28.57 %)	0.518
*fnb*B	1 (1 %)	2 (22.22 %)	0 (0 %)	3 (14.28 %)	0.814
*atl*E	0 (0 %)	4 (44.44 %)	1 (100 %)	5 (23.8 %)	0.033
embp	0 (0 %)	1 (11.11 %)	1 (100 %)	2 (9.5 %)	0.014
eno	6 (60 %)	9 (100 %)	1 (100 %)	16 (76.2 %)	0.051
aap	0 (0 %)	8 (88.88 %)	1 (100 %)	9 (42.8 %)	<0.001

setting, which poses a dangerous risk, as this bacterium can store resistance genes and transfer them to other species [13]. The *mecA* gene is present in over 85 % of *S. haemolyticus* strains, resulting in a high number of MRSH, which has led to major restrictions in the use of β-lactam antibiotics. *S. haemolyticus* shares a similar mechanism of methicillin resistance with *S. aureus*, which is mediated by modified transpeptidase enzymes (PBP2a) that cross-link peptidoglycan layers by forming pentaglycine bridges in peptidoglycan. This modified PBP2 binds methicillin with much lower affinity, resulting in therapeutic failure [13].

For severe MRSH infections, vancomycin and linezolid are considered the primary choices for first-line therapy [14]. In our study, linezolid showed higher effectiveness against all *S. haemolyticus* isolates, while a decreased susceptibility was observed for vancomycin and other antibiotics such as gentamicin, clindamycin, and levofloxacin. These findings align with previous investigations [15,16]. 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 [17].

Biofilm formation is one of the most crucial virulence factors that enhance the invasive characteristics of *S. haemolyticus* and its capacity to cause infections [18]. Also, it is an ideal structure for protecting bacteria from the host immune response and actions of antimicrobial and environmental agents [19]. In the present study, the majority (95.23 %) of *S. haemolyticus* isolates were capable of forming biofilms, and 42.8 % were moderate biofilm producers. However, in a study by Pinheiro et al. (2016), the frequency of *S. haemolyticus* biofilm producers was 21.5 % in blood cultures [12]. Furthermore, Kot et al. (2018) reported that the strongest biofilm was formed by strains from infected skin wounds, compared to other infected organs (e.g., the respiratory tract) [20].

The formation of biofilm is enabled by the intercellular adhesion of surface protein polysaccharides, which are regulated by the *ica*ADBC genes. Among the *ica* genes, *ica*D is the most common and is present in 42.8 % of *S. haemolyticus* strains. Furthermore, a few strains harbored *ica*A and *ica*D genes and no strains harbored *ica*B and *ica*R genes. Our research findings corroborate the hypothesis that unlike *S. aureus* and *S. epidermidis, S. haemolyticus* biofilms capacity do not possess genes for accumulation-related proteins and biofilm-related proteins, and it has been observed that bacterial strains are capable of forming biofilms even in the absence of *ica* genes, which could imply the existence of distinct mechanisms for biofilm formation that are not reliant on intercellular polysaccharide adhesion [13]. Other genes associated with biofilm formation were also analyzed. The *eno* gene was prevalent in the majority of *S. haemolyticus* strains, and 42.8 %, 23.8 %, and 9.5 % of these isolates carried *aap, atl*E, and *emb*P, respectively. In addition, there was a significantly higher occurrence of these genes in strongly or moderately forming biofilms than in weakly or non-forming biofilms, indicating that these genes contribute significantly to primary attachment, intracellular adhesion, and biofilm formation, independently of or in association with *ica* genes [21].

In the current study, we examined the modulatory effects of sub-MICs of vancomycin, gentamicin, and ciprofloxacin on *S. haemolyticus* adhesion and biofilm formation. These antibiotics were selected because

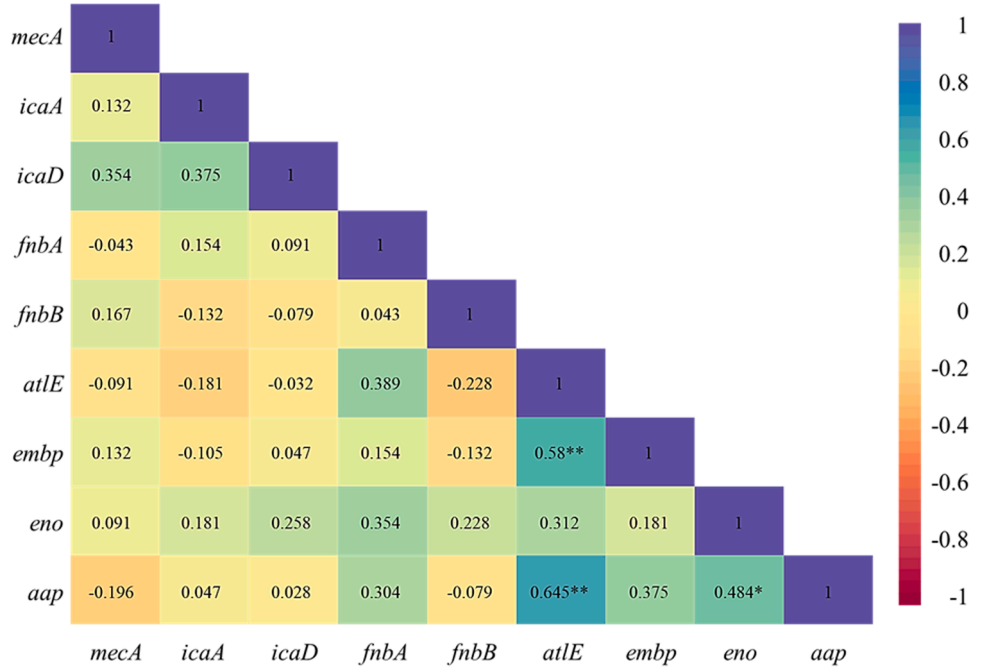

Fig. 1. Correlation matrix heatmap indicating the correlations between the biofilm formation-related genes of S. haemolyticus strains. The color represents Pearson's correlation coefficient and its intensity represents the coefficient's value (Shades of blue are positive correlations and shades of orange-brown are negative correlations). *P $\leq$ 0.05, **P $<$ 0.01.

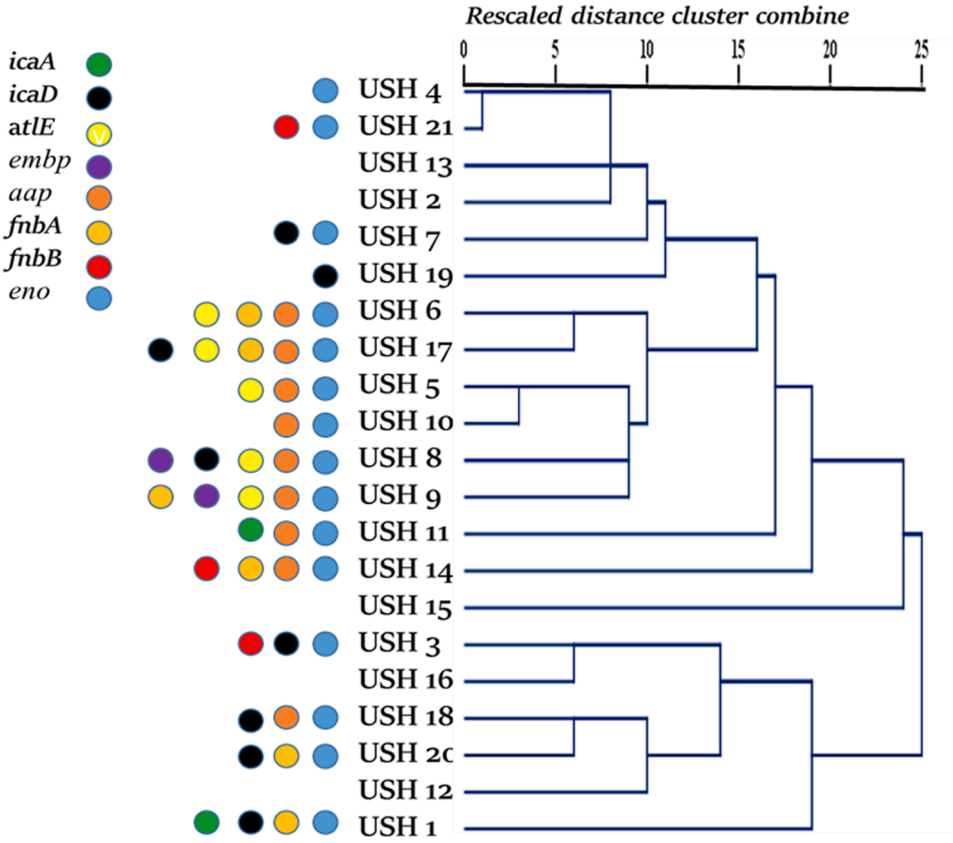

Fig. 2. Hierarchical clustering of uropathogenic S. haemolyticus isolates (USH) was based on their phenotypic (antimicrobial resistance and biofilm formation capacity) and genotypic (antimicrobial resistance and biofilm formation genes) characteristics. Each vertical line represents the strain. The x-axis describes the similarity distances between the strains and between the groups. The presence of genes is indicated by colored circles next to the isolate name.

they are the most appropriate and widely used antibiotics for the treatment of *S. haemolyticus* infections. Treatment with vancomycin, gentamicin, and ciprofloxacin sub-MICs contributed to the development of a hyper-adhesive character in *S. haemolyticus* and increased its biofilm-forming capacity. Recently, few studies have focused on the influence of sub-MICs of antibiotics on the biofilm formation ability, and virulence of staphylococci. Pereira-Ribeiro et al. reported that the majority of *S. haemolyticus* strains continue to generate biofilms in vitro in the presence of sub-MICs of the antibiotics tested [3]. Amit Kumar et al. reported that sub-MICs of antibiotic stress increased biofilm formation ability by altering bacterial surface properties and promoting bacterial attachment to these surfaces [22].

Preventing bacterial exposure to low concentrations of antimicrobial agents is a crucial strategy for reducing infections caused by biofilm-forming *S. haemolyticus*. In addition, assessing the MICs of antibiotics against biofilm-forming bacteria in routine medical laboratories is a critical step that should complement the traditional MICs tests performed on planktonic bacteria.

5. Conclusion

S. haemolyticus has shown resistance to almost all currently effective antibiotics. Linezolid and vancomycin remain the most practical choices for the treatment of MRSA, with necessary caution to avoid the emergence of resistance. This study demonstrated a significant relationship between biofilm-forming genes and the emergence of antibiotic resistance. These results provide information that could facilitate attempts to control drug-resistant bacteria, including the development of vaccines targeting biofilm-producing MDR strains.

CRediT authorship contribution statement

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

Declaration of competing interest

No conflict of interest.

Ethical approval

This study was approved by the internal ethics committee of the Biological Geology Department of the polydisciplinary faculty of Sultan Moulay Slimane University, Beni Mellal, and by the Ethics Committee for Biomedical Research of the Faculty of Medicine and Pharmacy of Mohammed I University, Oujda, Morocco, approval number: 15/2023.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.diagmicrobio.2024.116483.

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