

Correlation between biofilm development and antibiotic resistance in *Staphylococcus haemolyticus*

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ABSTRACT

Aim To evaluate antibiotic resistance, biofilm formation, and the presence of virulence-associated genes in *Staphylococcus haemolyticus* isolates.

Methods Clinical specimens were obtained from patients in Mosul city. Antibiotic susceptibility was determined using the Kirby–Bauer disk diffusion method against fourteen antibiotics. Biofilm production was assessed by both the tube adherence method and the microtiter plate assay. Polymerase chain reaction (PCR) was employed to detect selected resistance and virulence genes.

Results The isolates exhibited high resistance rates to ampicillin (94.7%) and cloxacillin (94.7%). These antibiotics were tested separately to compare β -lactamase-labile (ampicillin) and β -lactamase-stable (cloxacillin) penicillin. All isolates were uniformly susceptible to vancomycin. Variable resistance patterns were observed with other antibiotics. All isolates demonstrated biofilm production. PCR analysis revealed the presence of the *SH* gene in 100% of isolates. *mecA* was detected in nine (out of 19; 47.3%) isolates, *tetK* in 10 (52.6%), and *ermC* in five (26.3%). Among the virulence genes, *hla* was detected in all 19 isolates, correlating with the observed complete haemolysis on blood agar, while *fnbB* was found in 14 isolates (73.7%). Regarding resistance genes, *mecA* was present in 9 isolates (47.3%), confirming methicillin resistance and indicating that these strains are methicillin-resistant *S. haemolyticus*.

Conclusion The study highlights the alarming antibiotic resistance, strong biofilm-forming ability, and high prevalence of virulence and resistance genes in *S. haemolyticus*, reinforcing concerns over the global spread of multidrug-resistant organisms.

Keywords: biofilm formation, PCR, *Staphylococcus haemolyticus*, virulence genes.

INTRODUCTION

Bacterial strains that are resistant to antibiotics pose a major risk to human health. Due to rising antibiotic resistance in common human infections, fewer antibiotics remain effective against infectious diseases (1). In routine clinical settings, coagulase-negative staphylococci (CoNS) are among the most frequently isolated microorganisms. These bacteria are part of the normal microbiota of the skin and mucous membranes, and their prevalence has increased steadily alongside advancements in medical technology, especially with the widespread use of implanted medical devices (2). Although they were traditionally considered low-virulence organisms, CoNS have emerged as significant pathogens in nosocomial environments. Various research studies have explored the processes involved in this shift and pinpointed several potential factors linked to disease causing abilities. This is especially evident, in *Staphylococcus*

aureus (3). *S. haemolyticus* is a species in the *Staphylococcus* genus that has a prominent presence in healthcare-related diseases because of its high level of resistance to antibiotics, like methicillin, and its strong ability to form biofilms (4). It usually acts as a pathogen that originates from the body's normal microbiota rather than being acquired externally (5).

Among CoNS types, *S. haemolyticus* linked with hospital originated infections display increased level of resistance against antibiotics (2). Additionally persuasive data indicate that this particular species can function as a source by transmitting genes for resistance to other *Staphylococcus* species (5). In immunocompromised host *S. haemolyticus* can cause serious opportunistic infections such as meningitis, inflammation of the heart inner lining, endocarditis, reaction problems in artificial joints, bacteraemia, sepsis, peritonitis and ear infections (6). Its association with infections related to devices like artificial joints, valves of the heart and vascular catheters is a major concern in clinical settings (4). Treating these infections can be quite challenging because the bacteria can stick to surfaces and create biofilms that make them hard to eradicate completely from medical equipment or devices and shield them against the body's immune defences and antibiotic treatments – leading to recurring and long lasting infections (5). This ability is further amplified by harmful factors, like haemolysins, which

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help bacteria damage host tissues and evade immune responses and adhesive proteins evade being cleared out effectively (3). In persons with impaired immunity opportunistic bacteria like *S. haemolyticus* can cause serious infections such as surgical site infections (6).

The emergence of drug-resistant *S. haemolyticus* strains is a serious concern in healthcare settings worldwide (7). This particular species is known for its role in spreading genes which could result in the rise of more aggressive and widespread variants (8,9). Its remarkable capacity to develop resistance, towards oxacillin, makes treating catheter related infections more challenging and raises the chances of sepsis occurrence leading to higher morbidity and mortality rates (10). The presence of insertion sequences (ISs) in the genome of *S. aureus* or *S. haemolyticus* facilitates gene gain or loss, leading to significant genomic alterations. This genetic reshuffling boosts the ability of bacteria, for acquiring resistance genes resulting in coping and persistence within hospital settings (11). Fibronectin binding proteins such as *fmbB* play a role in the creation of biofilms by facilitating cell aggregation or attachment to host receptors during the synthesis stage (13).

Genomic features of *S. haemolyticus* are defined by its abundance of insertion sequences and its extensive multidrug resistance, strongly associated with its involvement in hospital infections. This species has become more associated with infections that are resistant to multiple drugs and is a rising concern for public health because it lingers in hospital environments and can survive on medical devices (14-16).

Although *S. haemolyticus* is the most frequently isolated species among coagulase-negative staphylococci (CoNS), there is still limited understanding of its biological characteristics, resistance mechanisms, and pathogenic potential. This knowledge gap, particularly regarding its high antibiotic resistance and virulence profiles, underscores the need for further investigation.

This study was conducted to better understand the antibiotic resistance patterns and to provide molecular characterization of resistance and virulence-associated genes in *S. haemolyticus* isolates recovered from various clinical specimens.

The aim of this study was to clarify the antibiotic resistance patterns and provide molecular characterization of resistance and virulence factor genes in *S. haemolyticus* isolates obtained from a variety of clinical samples.

MATERIALS AND METHODS

Materials and study design

A total of 450 clinical samples were collected from various sources, including urine, blood, sputum, and eye swabs, from both male and female patients of different age groups attending hospitals in Mosul City, during the period from July 2024 to November 2024.

The study was approved by the Ethical Committee of the Department of Biology, College of Science, University of Tikrit, Iraq.

Methods

Isolation and identification. Conventional methods were initially used for the isolation and identification of *S. haemolyticus*. Samples were streaked on blood agar and mannitol salt agar (containing 7.5% NaCl) to selectively isolate salt-tolerant staphylococci (17). After aerobic incubation at 37 °C for 24

hours, colonies were Gram-stained and subjected to standard biochemical tests, including catalase, oxidase, DNase, coagulase, Voges-Proskauer (VP), nitrate reduction, pyrrolidonyl arylamidase (PYR), urease, novobiocin sensitivity, and polymyxin B sensitivity. Polymyxin B disks can be used to help differentiate *Staphylococcus epidermidis* from most other coagulase-negative staphylococci, and bacitracin disks were used to differentiate *Staphylococcus haemolyticus* from other novobiocin-susceptible staphylococci (18).

Although conventional biochemical tests were performed, the VITEK system was subsequently used to confirm identification and antibiotic sensitivity for standardization and accuracy purposes in species-level confirmation.

Antibiotic susceptibility test. The Kirby-Bauer disk diffusion method was performed using 14 antibiotics (Bioanalyse, Turkey): tetracycline (30 µg), clindamycin (2 µg), vancomycin (30 µg), meropenem (10 µg), cefoxitin (30 µg), trimethoprim (10 µg), azithromycin (15 µg), levofloxacin (5 µg), oxacillin (1 µg), amikacin (10 µg), ampicillin (25 µg), cloxacillin (5 µg), amoxicillin (25 µg), and gentamicin (10 µg).

Methicillin resistance was identified via screening for reduced susceptibility to cefoxitin (30 µg) and oxacillin (1 µg), following CLSI guidelines (19).

The ability to form biofilm was tested using the tube adherence test. The bacterial isolates were inoculated in heart and brain infusion broth (BHI) and incubated at 35 °C for 48 hours. The tubes were washed, and then stained with 0.1% crystal violet. Biofilm formation was evaluated visually and categorized as: no biofilm, mild, moderate, or strong producers (20). The second method used was testing the microtiter plate for bacterial suspensions in BHI, incubated in 96-well plates at 37 °C for 24 hours. Wells were then washed with saline, stained with 0.1% crystal violet, and excess dye removed using 99% ethanol. Biofilm biomass was quantified by measuring optical density (OD) at 630 nm using an ELISA reader (21).

Genomic DNA was extracted using the Geneaid kit (Taiwan) according to the manufacturer's protocol. DNA concentration and purity were measured spectrophotometrically (22).

Six genes were amplified via PCR: the SH gene (species confirmation), three antibiotic resistance genes (*mecA*, *tetK*, *ermC*), and two virulence genes (*hla*, *fmbB*). PCR was carried out using GoTaq G2 Green Master Mix (Promega, USA) in 20 µL reactions, including 200 ng DNA template and 2 µM of each primer. PCR products were visualized on 2% agarose gels stained with Redsafe. A 100 bp DNA ladder (New England Biolabs, UK) was used.

Thermocycling conditions were standardized, and only annealing temperatures (Table 1).

Statistical analysis

Categorical variables, such as the presence or absence of resistance and virulence genes, were compared using the χ^2 test. A $p < 0.05$ was considered statistically significant.

RESULTS

Nineteen *S. haemolyticus* isolates were recovered from 450 clinical samples. On blood agar, the isolates formed opaque white colonies with clear zones of complete haemolysis. Identification was confirmed based on morphological and biochemical characteristics: coagulase-negative, catalase-positive, DNase-positive, oxidase-negative, variable reactions in

Table 1. Primer sequences and PCR conditions used in this study

Gene	Sequence (5'→3')	Product size (bp)	Annealing temp (°C)	Reference No
<i>SH</i>	F: GGTCGCTTAGTCGGAAACAAT R: CACGAGCAATCTCATCACCT	271	52	(34)
<i>hla</i>	F: TGGGCCATAAACTTCAATCGC R: ACGCCACCTACATGCAGATTT	72	53	(33)
<i>fnbB</i>	F: TAAATCAGAGCCGCCAGTGGAG R: GTCCTTGCGCTTGACCATGTTC	416	57	(12)
<i>mecA</i>	F: TAGAAATGACTGAACGTCCG R: TTGCGATCAATGTTACCGTAG	154	50	(34)
<i>tetK</i>	F: GTAGCGACAATAGGTAATAGT R: GTAGTGACAATAAACCTCCTA	360	46	(35)
<i>ermC</i>	F: GCTAATATTGTTTAAATCGTCAATTCC R: GGATCAGGAAAAGGACATTTTAC	572	47	(35)

mannitol fermentation, Voges-Proskauer, nitrate reduction, and PYR tests, and susceptibility to novobiocin and polymyxin B. Molecular confirmation was performed by detecting the *SH* gene specific to *S. haemolyticus* (Figure 1).

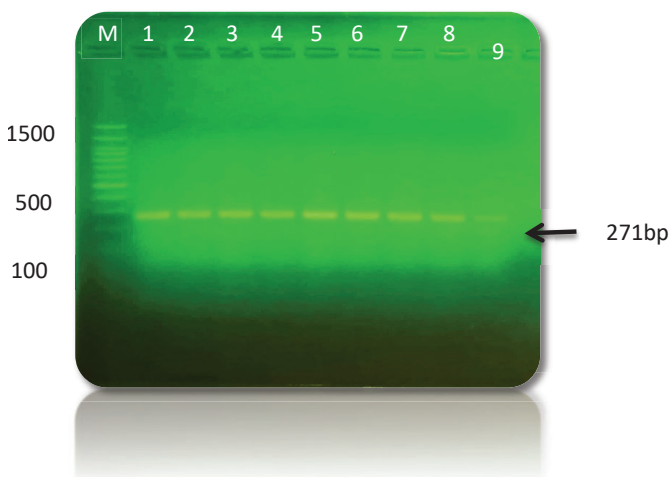


Figure 1. Gel electrophoresis image showing PCR-amplified SH gene bands from confirmed *S. haemolyticus* isolates

Antimicrobial susceptibility testing revealed diverse resistance patterns (Figure 2). The highest resistance rates were observed for ampicillin and cloxacillin (94.7%), fusidic acid (89.4%), azithromycin (84.2%), and gentamicin (78.9%). Moderate resistance was noted for tetracycline (63.1%), levofloxacin, amikacin, and trimethoprim (57.8% each), amoxicillin (52.6%), and oxacillin (47.3%). Lower resistance rates were found for cefoxitin (31.5%), clindamycin (15.7%), and meropenem (10.5%). All isolates were fully susceptible to vancomycin. Notably, a high number of isolates demonstrated multidrug resistance (MDR), particularly those resistant to methicillin. Out of 450 clinical samples, nineteen *S. haemolyticus* isolates were obtained and identified through conventional biochemical

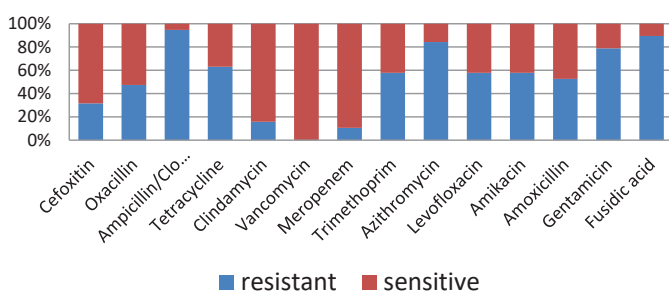


Figure 2. Antibiotic resistance rates

tests and confirmed by the VITEK system and PCR targeting the *SH* gene. These 19 isolates were selected for further analysis based on confirmed identification and distinct colony morphology.

Biofilm formation was evaluated using two methods. In the tube adherence test, 18 out of 19 isolates (94.7%) produced biofilms, with 12 classified as moderate producers and 6 as either weak or strong producers. In comparison, the microtiter plate assay showed that 17 isolates (89.5%) were biofilm positive, with varying levels of biofilm biomass compared to the negative control.

Molecular detection confirmed the presence of the *SH* gene in all 19 isolates. Among resistance genes, *mecA* was detected in nine (out of 19; 47.3%) isolates, *tetK* in 10 (52.6%), and *ermC* in five (26.3%) (Figure 3).

Among the virulence genes, *hla* was detected in all 19 isolates, correlating with the observed complete haemolysis on blood agar (Figure 3B), while *fnbB* was found in 14 isolates (73.7%) (Figure 3A). Regarding resistance genes, *mecA* was present in 9 isolates (47.3%), confirming methicillin resistance and indicating that these strains are methicillin-resistant *S. haemolyticus* (MRS_H), which is in agreement with previous studies (Figure 3C). Additionally, *tetK* was detected in 10 isolates (52.6%) (Figure 3D), and *ermC* in 5 isolates (26.3%) (Figure 3E).

DISCUSSION

This study demonstrated a clear link between biofilm production and antibiotic resistance in *S. haemolyticus*, reinforcing its role as a challenging pathogen to treat. The high level of resistance, particularly against β -lactam antibiotics such as ampicillin and cloxacillin, indicate therapeutic limitations (23). However, the effectiveness of vancomycin offers a reliable treatment option (24).

The previous term “environmental/comensals” was vague; it refers to the natural habitat of *Staphylococcus* spp., which is part of the normal microbiota of human skin and mucous membranes. However, under clinical conditions, *Staphylococcus* spp., including *S. haemolyticus*, may act as opportunistic pathogens (25,26).

The significant biofilm-forming ability of *S. haemolyticus* isolates was confirmed using both the tube adhesion and microtiter plate methods. Differences in biofilm-forming capacity between isolates may reflect differences in virulence potential (27,28). These biofilms protect the bacteria from immune responses and antibiotic treatments, enhancing their survival and pathogenicity (29,30).

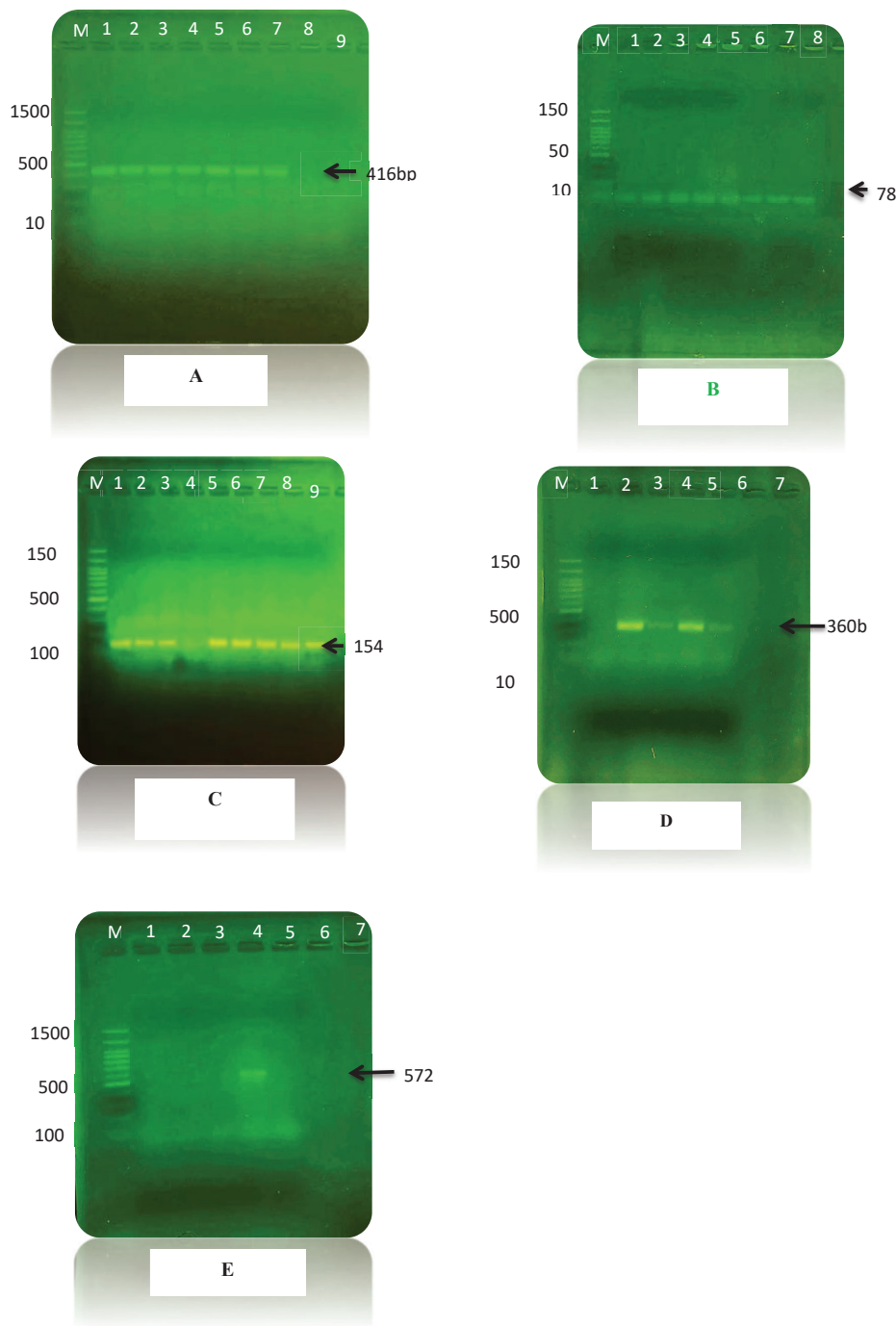


Figure 3. Agarose gel electrophoresis of *S. haemolyticus* (Lane(M): 100 bp DNA ladder (BioLabs) for: A) *fnbB*, Lanes (1-9) PCR product of *fnbB* gene (416 bp); B) *hla* gene, Lanes (1-8) PCR product of *hla* gene (78 bp); C) *mecA* gene, Lanes (1-9) PCR product of *mecA* gene (154 bp); D) *tet k* gene, Lanes (1-7) PCR product of *tet k* gene (360 bp); E) *ermC*, Lanes (1-7) PCR product of *ermC* gene (572bp)

The presence of virulence-related genes was also notable. The *hla* gene, which encodes alpha-haemolysin, was present in all isolates and corresponds with the complete haemolysis observed on blood agar, indicating its strong role in pathogenicity (31-33). Additionally, 73.68% of the isolates carried the *fnbB* gene, which facilitates adhesion and biofilm formation by binding fibronectin on host cells.

Regarding resistance genes, 47.3% of the isolates harbored the *mecA* gene, definitively classifying them as MRSH. The report of 88% methicillin resistance is confusing and should be clarified: all *mecA*-positive strains are methicillin-resistant by definition. Moreover, 52.6% of the isolates carried the *tetK* gene (tetracycline resistance), and 26.3% harboured the *ermC* gene (macro-

lide, lincosamide, and streptogramin B resistance) (34,35). These results emphasize the importance of monitoring resistance mechanisms and applying infection control measures effectively in order to manage infections associated with *S. haemolyticus*.

The research highlighted the resistance characteristics of *S. haemolyticus* strains found in samples in Mosul and their capacity for forming biofilms effectively. These strains showed resistance against β lactam, aminoglycosides and macrolides while they remained vulnerable, to vancomycin. The existence of *mecA*, *tetK* and *ermC* genes grants the bacteria resistance against methicillin, tetracycline and macrolide antibiotics respectively. Simultaneously, virulence elements like *hla* and *fnbB* increase their capacity for causing infections.

In conclusion, these findings highlight the significance of enhancing infection prevention guidelines and exploring treatment options for addressing *S. haemolyticus* infections.

AUTHOR CONTRIBUTIONS

The research idea and methodology were developed by the author, with input from the first researcher, who focused on the implementation and analysis the plan; both researchers collaborated on writing the manuscript.

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Conflicts of interest: None.

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