Molecular Detection of the Relationship between Biofilm Production and Antibiotic Resistance in Staphylococcus Haemolyticus

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Abstract: To evaluate antibiotic resistance, biofilm formation, and the detection of virulence-associated genes this study was conduct. Clinical specimens samples were obtained from patients in Mosul city. susceptibility of Antibiotic was assessed using the Kirby-Bauer method with nineteen antibiotics. Biofilm production was tested using the tube adherence and microtiter plate methods. PCR was used to identifying virulence and resistance-related genes. Results showed a 94.7% resistance to ampicillin/cloxacillin and ceftriaxone, and 100% sensitivity to vancomycin and nitrofurantoin, with variability in resistance to other antibiotics. Biofilm formation was observed in all isolates. The PCR results showed that the SH gene was present in all isolates. The mecA gene, linked to methicillin resistance, was found in 88.88% of isolates, the tetK gene (tetracycline resistance) in 83.33%, and the ermC gene (erythromycin resistance) in 26.31%. Virulence factor genes, hla and fnbB, were detected in 100% and 73.68% of isolates, respectively. The findings highlight the significant antibiotic resistance, biofilm-forming ability, and horizontal gene transfer in Staphylococcus haemolyticus, emphasizing the global concern of multidrug-resistant bacteria.

1 INTRODUCTION

Bacterial strains that are resistant to antibiotics pose a major risk to human health. Fewer antibiotics are still effective against infectious diseases due to rising antibiotic resistance in common human infections [1]. In ordinary clinical care, coagulase-negative staphylococci (*CoNS*) are among the most commonly recovered microorganisms. Over the past few decades, their prevalence has risen gradually in tandem with medical advancements, particularly with regard to the use of foreign body devices [2]. *CoNS* have become important pathogens in nosocomial settings due to their function in preserving homeostasis. Numerous research have looked into the molecular causes of this development and have found several potential virulence factors related to the pathogenicity of *S. aureus* [3].

A vital component of the Staphylococcus genus, *S. haemolyticus* is important in Healthcare-associated illnesses due of its great resistance to drugs like methicillin and its ability to produce biofilms [4]. Is a nosocomial infection-causing acquired opportunistic pathogen (5). Compared to other coagulase-negative staphylococci, *S. haemolyticus* strains, especially those causing hospital-acquired infections, are more resistant to antibiotics. There is compelling evidence that it can transfer resistance genes to other types of staphylococci. Meningitis, endocarditis, prosthetic joint infections, bacteremia, sepsis, peritonitis, and otitis media are among the severe infections associated with this bacterium, especially in individuals with weakened immune systems [6]. Because a strain that is resistant to various drugs is becoming a serious hazard in healthcare facilities around the world [7]. It also plays a significant function in the propagation of resistance genes, which can lead to the production of more virulent and an epidemic clones [8], [9].

The exceptional ability to develop antibiotic resistance, especially against oxacillin, limits the available treatment options for catheter-associated infections that do not respond to methicillin. Additionally, this may increase the risk of sepsis and raise the morbidity and mortality rates of patients [10]. The existence of insertion sequences *(Iss)* in this organism facilitates gene addition and deletion, promoting frequent genomic rearrangements that enable the acquisition of drug resistance genes, hence enhancing adaption and survival in hospital environments [11]. Is well known for its capacity to form biofilms, which are crucial for the transmission of disease [12]. The formation of biofilms, which contribute to hospital-acquired infections such as

catheter-associated diseases, is the main characteristic of pathogenic isolates. Additionally, they secrete substances called hemolysins, enterotoxins, and fibronectin-binding proteins that enhance bacterial adhesion and invasion [6]. In the synthesis stage of biofilm formation, the fibronectin binding proteins (*fnbB*) are crucial. By homophilic contacts or protein binding to receptors on the surface of nearby cells, *fnbB* facilitates the formation of biofilms [13].

The abundance of insertion sequences and resistance to many drugs are two characteristics of its genome that cause hospital infections, this strain has been linked to an increase in infections and cases of multidrug resistance, which makes it a serious concern to public health [14], [15]. Given its remarkable adaptability and capacity for survival in hospital settings, particularly on medical equipment, *S. haemolyticus* emerges as a key contributor to nosocomial infections brought on by multiresistan [16]. Although being, the second most normally discovered *CoNS*, little is known about its virulence factors and antibiotic resistance.

Clarifying antibiotic patterns and molecular classification of resistance and virulence factor genes in *S. haemolyticus* isolates from different clinical samples is the goal of this investigation.

2 MATERIAL AND METHODS

2.1 Study Design

Isolation and Identification: 450 samples were collected from various medical sources, including urine, blood, sputum, and eye swabs, and different ages of both females and males from Ibn Al-Athir Hospital for Children and Ibn Sina General Hospital for the period from July 2024 to November 2024.

2.2 Methods

Traditional method: Using the streaking technique from the clinical sample, the organism is isolated or cultured on a solid medium containing agar and blood. Staphylococci can be isolated from samples that may contain other microbes using mannitol salt agar with 7.5% sodium chloride, which facilitates the growth of salt-resistant staphylococci [17]. After incubating them aerobically for 24 hours at 37 degrees Celsius, the isolates are stained with Gram stain and subjected to standard biochemical tests, including catalase, oxidase, DNase, coagulase, VP, nitrate, PYR, urease, novobiocin, and polymyxin B sensitivity [18].

Confirmed method: Commercial biochemical test systems like VITEK2 species-level identification. Established biochemical techniques and recently created substrates serve as the foundation for the GP identity card. Forty-three biochemical assays are used to measure resistance, enzymatic activity, and carbon source utilization.

Antibiotic susceptibility test: Nineteen tablets of antibiotics were used (Bioanalyses) : Penicillin G (10 µg), Tetracycline (30 µg), Clindamycin (2 µg), Vancomycin (30 µg), Meropenem (10 µg), Ceftriaxone (30 µg), Cefotaxime (10 µg), Ceftazidime/clavulanic acid (30/10 µg), Cefoxitin (30 µg), Trimethoprim (10 µg), Azithromycin (15 µg), Levofloxacin (5 µg), Oxacillin (1 µg), Amikacin (10 µg), Ampicillin/Cloxacillin (25/5 µg), Amoxicillin (25 µg)., Gentamicin (10 µg), Nitrofurantoin (100 µg), and Fusidic acid (10 µg).

Methicillin resistance was identified through preliminary phenotypic viewing for diminished sensitivity of the isolates to cefoxitin (30 µg) and oxacillin (1 µg) disks (Mast, UK) utilizing the disk diffusion method, in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (2023) [19] standards.

Biofilm formation assay was performed in accordance with the: a) Tube Adherence Test (TM). Brain Heart Infusion (BHI) broth was inoculated with tested strains and incubate at 35°C for 2 days. Test tubes were dyed with crystal violet, and experiments were conducted three times, categorized as nonexistent, mild, moderate, and powerful [20].

Biofilm formation test using Microtiter Plate: Bacterial isolates are added to brain heart infusion medium, incubated for 24 hours at 37°C. fill the first well of the first row with 200 microliters of the negative control, or non-bacterial brain-heart infusion medium. Then, 200 microliters of the bacterial suspension are added to the first three wells of the third row. The wells are cleaned with saline solution, stained with 0.1% crystal violet, and then diluted with 99% ethanol. The optic-density (OD) is v using ELISA equipment at a wavelength of 630 nano-meters [21].

Molecular identification of genes linked to antibiotic resistance and pathogenicity: The genetic DNA of all *S. haemolyticus* strains under investigation was extracted using the Gene aid kit (Taiwan) the recommended steps from the manufacturer were followed. After that, Measurements were made of the genetic DNA's content and purity [22]. To identify the relevant genes in the genetic DNA of *S. haemolyticus*, six sets of primers were used to confirm the bacteria at the

genetic level using the *SH* gene and to identify the virulence factors *fnbB*, *hla* and the antibiotic resistance genes *mecA*, *tetK*, *ermC*, as shown in Table 2.

The (PCR) was conducted utilization the *GoTaq* G2 Green Master Mix from Promega (USA) in a 20-microlitre reaction volume for molecular identification. We added 200 nanograms of DNA template, with a primer concentration of 2 micromoles each, next the producer guidance. The PCR produce were divided on a 2% agarose gel and staining with red safe dye. A 100 base pair DNA ladder from New England Bio Labs, UK.

2.3 PCR Conditions

The PCR program including an primary denaturation at 95°C for 3 min., following by denaturing at 94°C for 45 seconds, annealing at the temperatures specified in Table 1, and expansion at 72°C for 45 seconds. A final extension step was performed at 72°C for 3 minutes.

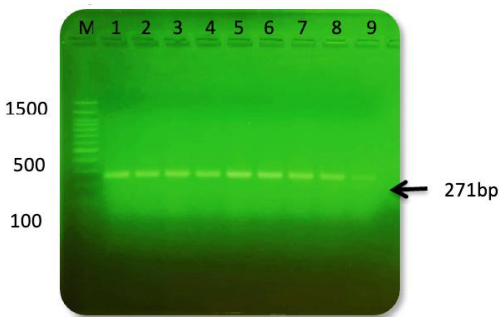

Figure 1: Agarose Gel electrophoresis for SH gene in S.haemolyticus .Lane (M): p DNA Lanes (1-9)PCR product of SHgene (271 bp).

Table 1: Biofilm production method.

Step	Description
1. Bacterial Culture Preparation	The isolates were grown in TSB medium at 37°C for 24 hours.
2. Inoculation	A standardized bacterial suspension (0.5 McFarland) was prepared and inoculated into 96-well polystyrene plates.
3. Incubation	Plates were incubated at 37°C for 24-48 hours.
4. Washing	Wells washed for 3 times by PBS
5. Staining	Biofilms were stained with 0.1% crystal violet for 15 minutes.
6. Washing & Drying	Excess stain was removed, and plates were air-dried.
7. Quantification	Absorbance was measured at 570 nm to determine biofilm formation levels.

Table 2: Primer DNA sequences used in this study.

Gene	Sequences (5'-3')	Product Size (bp)	Annealing Temperature (°C)	Source of Primer
SH	F: GGTCGCTTAGTCGGAACAAT R: CACGAGCAATCTCATCACCT	271	52	(Pereira et al., 2010)
mecA	F: TAGAAATGACTGAACGTCCG R: TTGCGATCAATGTTACCGTAG	154	50	(Pereira et al., 2010)
tetK	F: GTAGCGACAATAGGTAATAGT R: GTAGTGACAATAAACCTCCTA	360	46	(Manoharan et al., 2021)
ermC	F: GCTAATATTGTTTAAATCGTCAATTCC R: GGATCAGGAAAAGGACATTTTAC	572	47	(Manoharan et al., 2021)
hla	F: TGGGCCATAAACTTCAATCGC R: ACGCCACCTACATGCAGATTT	72	53	(Pinheiro et al., 2015)
fnbB	F: TAAATCAGAGCCGCCAGTGGAG R: GTCCTTGCGCTTGACCATGTTC	416	57	(Eltwisy et al., 2021)

3 RESULTS

Number of 450 clinical samples yielded nineteen isolates (4.4%) were positive for S. haemolyticus. The growth of the bacteria on blood agar is characterized by opaque white colonies surrounded by a zone of complete hemolysis.

All isolates were negative for oxidase, DNase, and coagulase tests, variable in mannitol fermentation, VP, nitrate, and PYR tests, positive for the catalase test, sensitive to polymyxin B, and susceptible to novobiocin. The results were confirmed using the VITEK2 technique.

All isolates identified by morphological and biochemical methods were confirmed by molecular methods using the SH diagnostic gene for S. haemolyticus bacteria, as shown in Figure 1.

3.1 Antibiotic Susceptibility

The findings demonstrated a distinct variation in the isolates' resistance to the employed antibiotics (see Fig. 2). The isolates showed resistance to ampicillin/cloxacillin and ceftriaxone at a rate of (94.7%), fusidic acid (89.4%), azithromycin (84.2%), gentamicin (78.9%), cefotaxime (73.6%), penicillin G and tetracycline (63.1%), levofloxacin, ceftazidime/clavulanic acid, and amikacin (57.8%), amoxicillin (52.6%), clindamycin (15.7%), and meropenem (10.5%). While the isolates were all susceptible to the medicines nitrofurantoin and vancomycin, some isolates exhibited multidrug resistance (MDR), especially in those that showed resistance to methicillin.

Eighteen strains (94.73%) produced biofilms in the tube out of 19 isolates, according to the results of the biofilm formation test conducted using the tube adhesion method. The isolates' capacities to form biofilms differed; twelve isolates formed the biofilm in a moderate manner, while six isolates were classified as weak or strong. Additionally, the microtiter Plate method was used to examine S. haemolyticus capacity to produce biofilms, and 17 out of 19 isolates (89.47%) had favourable results. When compared to the control sample, the results showed varying degrees of biofilm formation.

3.2 Molecular Studies

As for the virulence genes used in the study, found that 19 out of 19 isolates tested positive for the hla gene, which is 100% (see Fig. 3). This is consistent with the phenotypic characterization of S. haemolyticus that produces complete hemolysis on blood agar, The results of their study were (94.7%) and (100%) respectively for the appearance of the hla gene.

As for fnbB gene it was found 14 out of 19 isolates test positive with a gene appearance rate of 73,68% out of a total of 19 isolates. Detailed results for fnbB gene detection are shown in Fig. 4. Eight positive isolates of the mecA gene were detected out of nine resistance isolates, with the gene appearing at a rate of 88.88%. (see Fig. 5)

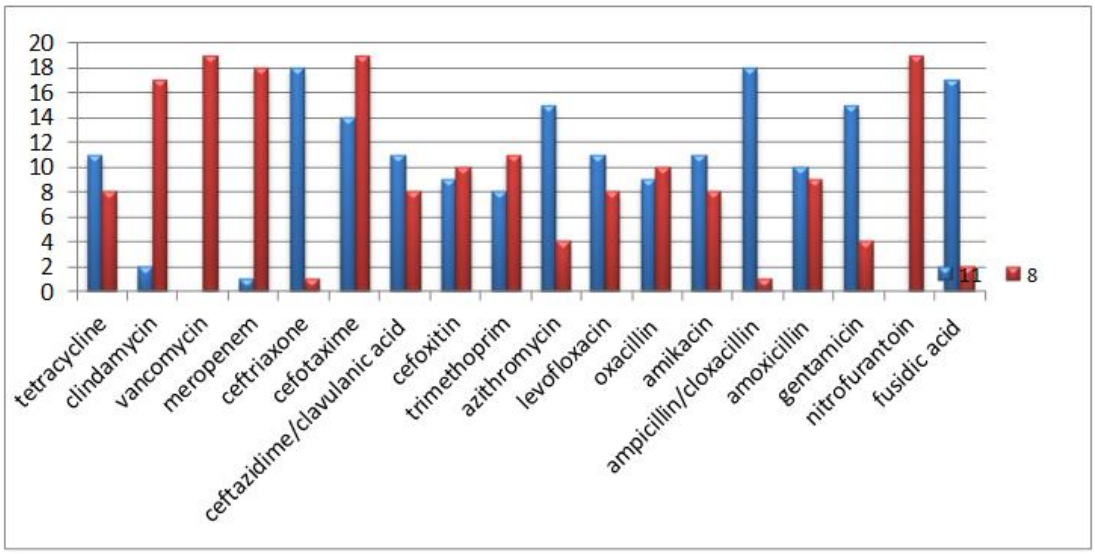

Figure 2: Antibiotic resistance profiles of Staphylococcus haemolyticus Isolate.

About 10 of the 12 resistance isolates tested positive for the tet-K gene, representing 83.33% (see Fig. 6).

Nineteen isolates the *ermC* gene was detected in around 5 of them, with a frequency of 26.31% (see Fig. 7).

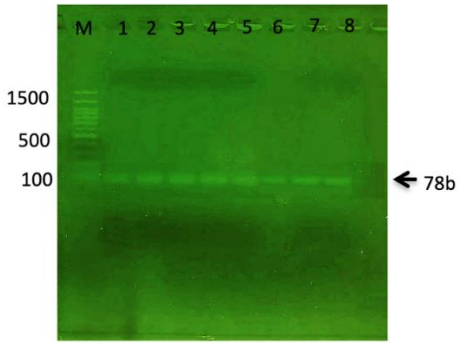

Figure 3: Agarose Gel electrophoresis for hla gene in S. haemolyticus. Lane(M):100 bp DNA ladder Lanes (1-8) PCR product of hla gene (78 bp).

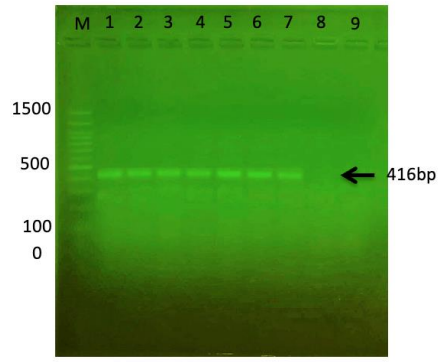

Figure 4: Agarose Gel electrophoresis for fnbB gene in S.haemolyticus. Lane(M):100 bp DNA ladder Lanes (1-9) PCR product of fnbB gene (416 bp).

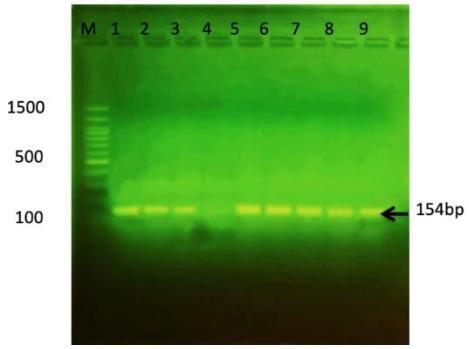

Figure 5: Agarose Gel electrophoresis for mecA gene in S.haemolyticus. Lane(M):100 bp DNA ladder ,Lanes (1-9) PCR product of mecA gene (154 bp).

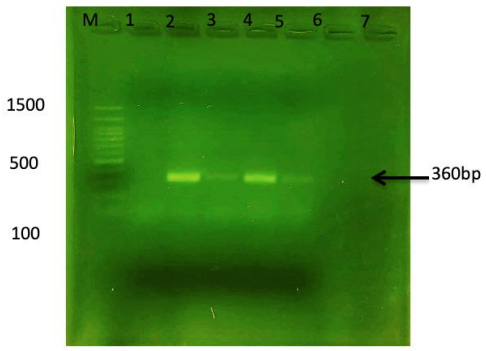

Figure 6: Agarose Gel electrophoresis for tet k gene in S.haemolyticus. .Lane(M):100 bp DNA ladder (BioLabs), Lanes (1-7)PCR product of tet k gene(360 bp).

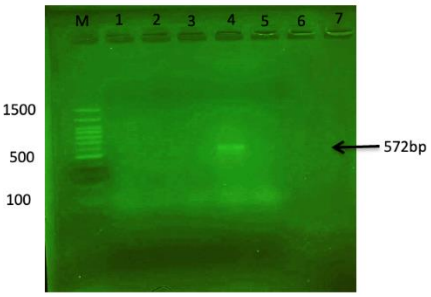

Figure 7: Agarose Gel electrophoresis for ermC gene in S.haemolyticus.Lane(M): 100 bp DNA ladder (BioLabs),Lanes (1-7)PCR product of ermC gene (572bp).

3 DISCUSSION

The research shows a link between the development of biofilms and resistance to antibiotics in *S. haemolyticus*, reinforcing its identity as a bacteria that's hard to treat with multiple drugs. The elevated levels of resistance seen against ampicillin/cloxacillin and ceftriaxone point to a hurdle in therapy treatmen = [23] .Potential therapeutic options are available, due, to the effectiveness of vancomycin and nitrofurantoin [24]. Most of the isolates having mecA suggest a methicillin resistance mechanism widely, which complicates treatment approaches even more [25]. The discovery of tetK and ermC genes displays how S.haemolyticus can resist tetracyclines and macrolides effectively [26]. The impressive capability of forming biofilms, in each isolate boosts the survival and resilience of bacteria by facilitating the transfer of resistance and virulence genes, like *hla* and *fnbB* [27], [28].

These results highlight the importance of enhancing infection prevention practices and exploring treatment approaches, for addressing

multidrug *S.haemolyticus* infections. The significant increase, in resistance levels seen in *S. haemolyticus* strains to drugs like β lactams and aminoglycosides underscores the escalating problem of drug resistance, in environments [29,30]. The fact that many people respond well to vancomycin and nitrofurantoin indicates that these antibiotics continue to be treatment choices [31], [32]. The significant link, between methicillin resistance and multi drug resistance (MDR) underscores the importance of *mecA* mediated resistance. The findings, on biofilm formation show that the majority of strains are capable of sticking and creating biofilms. This process boosts the lasting power of bacteria and their resistance, to antibiotics [33], [34].

The differences, in how biofilms are formed were observed in both the tube adhesion and microplate methods. This indicates that certain strains could be more concerning in infections because they show stronger adherence capabilities [35], [36]. These discoveries highlight the importance of exploring treatment approaches and implementing infection control measures, against infections caused by *S. haemolyticus* due the protective function of biofilms for bacteria, against the bodys immune system and antibiotics. The molecular study shows that *S. haemolyticus* isolates contain a number of genes related to virulence and resistance [37], [38]. The widespread identification of the *hla* gene corresponds with the behavior noted on blood agar plates. This highlights its significance, in causing disease [39,40]. The occupancy of the *fnbB* gene, in 73.68 % of the isolation indicates an ability, for adhesion and biofilm creation boosting bacterial survival and potential for causing infections. The significant occurrence of the *mecA* gene, at 88% in methicillin strains provides more evidence for the connection, between creating biofilms and resistance to multiple drugs [41], [42]. The identification of *tetA* (83%) and *ermB* (26%) genes illustrates how *S. haemolyticus* can withstand tetracyclines and macrolides respectively, in settings. These discoveries highlight how crucial it is, for surveillance practices observing resistance mechanisms and applying infection control strategies, against *S. haemolyticus* related infections [43], [44].

5 CONCLUSIONS

This research emphasizes the levels of resistance pattern, towards antibiotics and the ability of S. haemolyticus strains from samples in Mosul to form biofilms efficiently. These strains exhibited resistance against β lactams and aminoglycosides well as macrolides but displayed consistent susceptibility towards vancomycin and nitrofurantoin. The presence of mecA,tetK and ermC genes authenticates their resistance against drugs; meanwhile virulence factors such as genes likehlaand fnbbcontribute towards their nature. These discoveries highlight the importance of implementing infection control procedures and exploring alternative therapeutic approaches, for tackling infections associated with S.haemolyticus.

Results showed a 94.7% resistance to ampicillin/cloxacillin and ceftriaxone, and 100% sensitivity to vancomycin and nitrofurantoin, with variability in resistance to other antibiotics. Biofilm formation was observed in all isolates. The PCR results showed that the SH gene was present in all isolates. The mecA gene, linked to methicillin resistance, was found in 88.88% of isolates, the tetK gene (tetracycline resistance) in 83.33%, and the ermC gene (erythromycin resistance) in 26.31%.

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