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Antibiotic susceptibility and biofilm pattern in *Staphylococcus aureus* from clinical sources in Diyala Governorate

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ABSTRACT

Staphylococcus aureus is a pathogenic bacteria that causes a variety of diseases, from skin infections to serious and fatal infections, due to producing a variety of virulence factors. Biofilm is the most important virulence factors that confers *S. aureus* the severity. This study aims to isolate *S. aureus* from different sites of the human body and investigate the distribution of antibiotic resistance and biofilm profiles and the relationship between them. In this study, 250 clinical specimens were collected from teaching laboratories in Baqubah Teaching Hospital, Diyala Province, Iraq, from which, fifty isolates were selected to conduct this study. Based on morphological and biochemical tests, the isolates were identified, and the VITEK2 system confirmed the identities of the isolates. The isolates were tested with 12 antibiotics belong to different groups. The isolates showed high resistance to Oxacillin (100%), azithromycin (68%). In contrast, a high susceptibility percentage was observed with nitrofurantoin (84%), chloramphenicol (90%), rifampin (76%), gentamicin (68%), clindamycin (70%), tetracycline (48%), norfloxacin (62%), trimethoprim-sulfamethoxazole (60%), levofloxacin (62%), and ciprofloxacin (60%). The biofilm formation test revealed that 22(44%) of *S. aureus* isolates were strong biofilm-forming, 26(52%) were moderate, and 2(4%) were weak biofilm-forming. This study revealed a correlation between the isolates with strong biofilm and their antibiotic resistance pattern. In conclusion, these recent results may provide a basis to treat *S. aureus* by developing new antibiotics.

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1. INTRODUCTION

Staphylococcus aureus is a Gram-positive coccus, pathogenic bacterium that is largely responsible for a variety of sever infectious diseases affect both humans and animals. The illnesses caused by *S. aureus* are vary widely in severity from simple infections of the skin and soft tissues to more serious and fatal disorders such as bacteremia or septicemia [1]- [2]. Antimicrobial resistance (AMR) is a globally concern and expected to cause 10 million deaths per year by 2050 [3]. *S. aureus* is aggressive microbe due to their frequently antibiotic resistance such as in methicillin-resistant *S. aureus* (MRSA). The infections by these bacteria are characterized with high mortality, morbidity, and longtime hospital stay [4]-[5]. The increase of antibiotic resistance by these bacteria is related to with biofilm formation leading to multidrug resistance in chronic diseases such as osteomyelitis, endocarditis, and wound infections [6]. *S. aureus* harbors many virulence factors that are responsible for several diseases such as Staphylococcal enterotoxins, staphyloxanthin, arginine catabolic mobile element, coagulase, hemolysins, α-toxin, and biofilm [7]. These virulence factors have several pathogenic functions, for instance, damage host tissue, adherence the bacteria to human tissue, and act as immunomodulators which disrupt host immunity [8]. Biofilms, in particular, have a vital role in healthcare research due to their association with chronic wounds, urinary catheter infections, pneumonia, and medical devices [9]- [10]. Biofilm formation is considered a major cause of hospital-acquired infections due to poor sanitizing of the medical equipment [11]. Biofilms are complex extracellular polymeric materials (EPS) secreted by microbial communities. Biofilms are adhered to inert or living surfaces by means of a self-produced matrix. In contrast to planktonic (free-floating bacteria), biofilms are more resistant to the host immune system and curing by antimicrobial agents [12]. The formation of biofilm by *S. aureus*, which is a major cause of hospital infections when the medical equipment is not properly sanitized, is the basis for infectious efficiency.

Furthermore, the majority of *S. aureus* bacteria are found in contaminated biofilms on surfaces, typically in contact with food such as stainless-steel tools [13].

The ability of *S. aureus* to synthesis biofilms plays a key role in the development of antibiotic resistance and chronic infections. Biofilm structures protect bacterial cells from antimicrobial agents and host immune responses. In line with this, [14] demonstrated a significant correlation between biofilm formation and antibiotic resistance among *S. aureus* isolates, indicating that strong biofilm producers show higher levels of multidrug resistance. The aim of this study is to isolate *S. aureus* from different sites of human body to investigate the distribution of antibiotic resistance in addition to biofilm profile.

2. MATERIALS AND METHODS

2.1 Collection the specimens and identification of bacterial isolates

Two hundred and fifty clinical isolates were collected from patients in Baqubah Teaching Hospital, Diyala Province, Iraq. The clinical specimens were obtained from nose, sputum, burns, wounds, blood, throat, urine, and ears. Sterile culture media of Mannitol Salt Agar and Blood Agar were utilized to cultivate the isolates. The growing colonies were recognized based on morphological characteristics including size, shape, margin, colour, consistency and their transparency. Microscopic examination by Gram stain technique and biochemical tests including catalase, coagulase, oxidase, DNase, and gelatinase tests were applied. Finally, the isolates identification was confirmed by automated VITEK-2 system. The pure isolates were kept at 4ºC on nutrient agar for a short-term storage.

2.2 Antibiotic susceptibility of identified *S. aureus*

Twelve standard antibiotics disc (purchased from Condalab, Madrid, Spain) were used to examine the antibiotic susceptibility of 50 isolates by Kirby-Bauer testing. These antibiotics included: Oxacillin (1 µg), azithromycin (15 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), trimethoprim-sulfamethoxazole (25 µg), norfloxacin (10 µg), tetracycline (30 µg), clindamycin (2 µg), gentamicin (10 µg), rifampin (5 µg), chloramphenicol (30 µg), and nitrofurantoin (300 µg). Bacterial suspension was adjusted to 0.5 McFarland turbidity (1.5 x 10^8 CFU/ml) and used to inoculate sterile Petri dishes of Mueller Hinton agar. Six discs per plate were applied after streaking the isolates by sterile cotton swab. Plates were incubated for a 24 hr at 37ºC. The results were recorded in accordance with the Clinical and Laboratory Standards Institute (CLSI) [15].

2.3 Biofilm formation assay

The ability of 50 isolate of *S. aureus* to form biofilm was conducted by crystal violate assay in 96-well flat-bottom polystyrene microplate. In brief, one loopful of each tested isolate was mixed with 2 ml of Tryptic Soya Broth (TSB) (with 2% glucose) and incubated at 37 °C for 24 hr. Each well in microplate was filled with 200 µL of the diluted culture broth. The plate was incubated at 37°C for 48 h. The plate contents were discarded by gentle tapping and the wells were washed three times with 200 µL of normal saline. A 300 µL of 99% methanol for 15min and aliquot of 100 µL of 0.1% crystal violet was then added for 15 min. The excess dye was carefully removed from the stained wells and gently washed with distilled water. The plate was allowed to dry at room temperature and then 200 µL of 95% ethanol was added to dissolve the unbound dye. The absorbance at 630 nm was measured used to quantify the biofilm formation. The experiment was performed in triplicate. The criteria that used to interpret biofilm formation was according to [16].

3. Statistical analysis

The collected data were statistically analyzed by application SPSS software (version 23, Statistical Package for Social Science, Chicago, IL, USA). Chi-square was applied for the comparison of the data. Results were expressed as means ± standard deviation (SD) and considered significantly different when p value ≤ 0.05.

3. RESULTS AND DISCUSSION

3.1 Phenotypic profile of *S. aureus*

Fifty *S. aureus* isolates were obtained from total of two hundred and fifty clinical specimens collected from laboratory of Baqubah Teaching Hospital, Diyala Province, Iraq. The source of isolation was nose, sputum, burns, wounds, blood, throat, urine, and ears. The isolates were identified based on their phenotypic characteristic, microscopic examination, biochemical tests, and the VITEK®-2 system. When the bacteria were grown on blood agar, they exhibit small, yellowish, spherical, smooth, and opaque colonies. It is often a β-hemolysis pattern after 24 hours incubation due to lysis of blood cells (Figure 1A). The isolates were cultured on Mannitol Salt Agar (MSA), which is a selective differential medium for *S. aureus*, resulting in tiny to medium in size, round, slightly mucoid or shiny colonies and yellow in color due to the fermentation of mannitol sugar in the medium (Figure 1B). Both blood hemolysis and mannitol salt agar are considered critical and distinct tests in *S. aureus* distinguishing from other staphylococci species [17]. *S. aureus* is typically forming circular, convex, and smooth colonies on nutrient agar. The colonies are usually about 1-3 mm in diameter, with a golden-yellow or white in color, which is characteristic of *S. aureus*. The edges of the colonies are well-defined, and they often have a shiny appearance (Figure 1C).

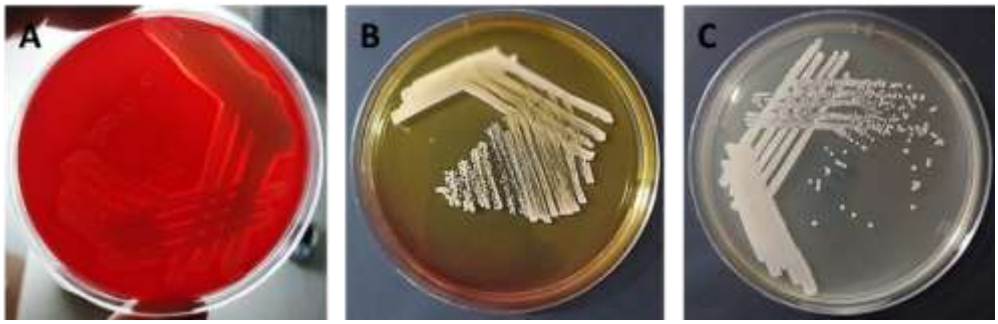

Figure 1. Growth characteristics of *S. aureus* on various media after 24 hours of incubation at 37 °C. (A) Blood agar shows smooth, spherical colonies with obvious areas of β-hemolysis surrounding the colonies. (B) Mannitol salt agar shows strong growth and yellow color of the medium due to mannitol fermentation. (C) Nutrient agar shows the characteristic creamy white round colonies with smooth edges characteristic of *S. aureus*.

When examined under a microscope using Gram stain, *S. aureus* appears as Gram-positive cocci arranged in grape-like clusters. Their purple color is due to the bacteria retaining the crystal violet stain after staining because of their high density of peptidoglycan in the cell wall. To confirm the diagnosis of bacteria, the 50 isolates of *S. aureus* bacteria had been subjected to some biochemical tests that are considered primary identification involved coagulase test, catalase test, gelatinase test, DNase test and oxidase test. *S. aureus* was identified as positive in coagulase test indicated by the formation of a clot or gel-like substance in the test tube due to the presence of free coagulase produced by *S. aureus* (Figure 2A). Coagulase play a role in the development of abscesses in host tissues and enhance the bacterial pathogenicity to cause fatal sepsis [18]. In catalase test, a gas bubbles formed by the enzyme which catalyzes conversion the hydrogen peroxide into water and oxygen indicated a positive test (Figure 2B). The key function of catalase enzyme is to prevent the accumulation of toxic amounts of hydrogen peroxide, which is used to differentiate between the *Streptococcus* and *Staphylococcus* genera [19]. *S. aureus* is generally gelatinase-positive, producing gelatinase enzyme that hydrolyzes and liquefies the gelatin after cooling (Figure 2C).

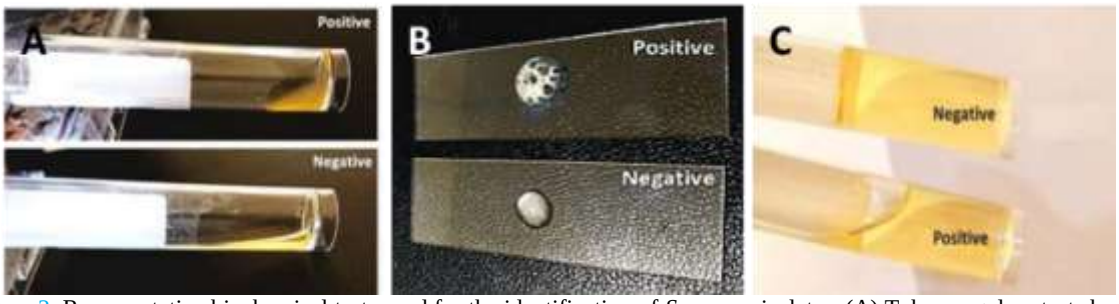

Figure 2. Representative biochemical tests used for the identification of *S. aureus* isolates. (A) Tube coagulase test shows clot formation in plasma indicating a positive result. (B) Catalase test performed on a glass slide shows immediate bubble formation after addition of hydrogen peroxide. (C) Gelatin hydrolysis test shows liquefaction of gelatin medium in positive isolates.

S. aureus grown on DNase test agar with toluidine blue shows pinky color around the colonies, indicating a positive DNase test. In bacteria, deoxyribonucleases (DNases) act as virulence factor, hydrolyzes nucleic acids of the host producing oligonucleotides. It plays a key role in bacterial growth, maturation of biofilm and enables bacteria to evade the immune system [7]. The oxidase test shows negative reaction, which mean that it does not produce cytochrome c oxidase and therefore the colorless reagent is not turn to dark purple or bluish color (Table 1) [20]. Oxidase acts as a virulence factor, enabling pathogens to manipulate the host environment, evade immune responses, and damage tissues. This facilitates bacterial survival, colonization, and increased pathogenicity within the body [21]. VITEK-2 system, an accurate identification test, revealed that all the isolates were *S. aureus* according to the gram-positive card.

Table 1. Bio-chemical tests that applied to identify *S. aureus* isolates.

Biochemical tests	Test result
Coagulase	+
Catalase	+
Gelatinase	+
DNase	+
Oxidase	-
VITEK-2 system	+

3.2 Distribution of *S. aureus* isolates based on clinical specimens sources

The percentage of *S. aureus* isolates from various clinical sources varied depending on the source. The highest percentage of *S. aureus* isolates was in nasal swabs 22(44%) followed by burn swabs 8(16%). Sputum swabs revealed the lowest percentages of *S. aureus* isolates with 1(2%) as indicated in Figure (3). The percentage of nasal isolates in this study is higher than that in Kurdistan region (37.6%) [22]. Also, in another study conducted in the city of Kirkuk, the prevalence of *S. aureus* in the nose among the population was about (16.6%) [23], which is lower than the rate in the current study. *S. aureus* is colonize different sites in human body. The most important site to colonize these bacteria is nose. Approximately 20% to 30% of people have *S. aureus* in their bodies at various locations such as the neck, axilla, nose, and perineum [24]. The risk of surgical site infections by *S. aureus* is linked to nasal *S. aureus* carriage alone, increasing the risk by two to ten times [24]. The reason for the change in the isolation percentage is that a number of environmental risk variables, including age, seasonal variation, and certain diseases, influence the colonization of these bacteria [25]. Also, many studies revealed that the socio-demographic factors play an additional important role in the nasal transmission of *S. aureus* infection. Nasal colonization with *S. aureus* is associated with a history of cigarette and Khat plant consumption, as well as with the sharing of personal items. This association may result from frequent hospital visits, poor personal hygiene. The transmission mechanism is from contaminated hands to the nose through temporary carriers of the bacteria on the hands of healthy individuals and skin-to-skin contact [26].

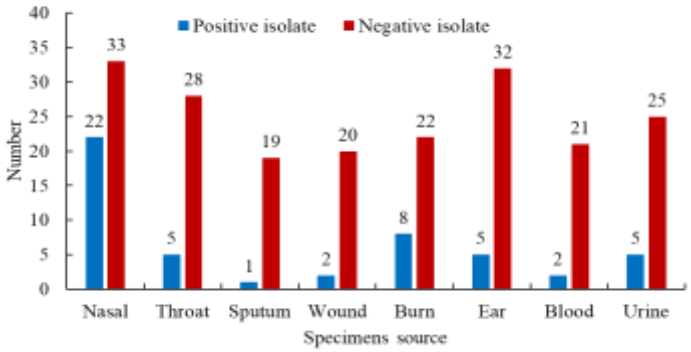

Figure 3. Distribution of *S. aureus* isolates obtained from different clinical samples. Bar graph shows the number of positive isolates (blue bars) and negative isolates (red bars) recovered from nasal swabs, throat swabs, sputum, wound swabs, burn swabs, ear swabs, blood and urine samples. Nasal samples showed the highest number of positive isolates (n = 22), followed by burn samples (n = 8), while sputum samples showed the lowest number of positive isolates (n = 1).

3.3 Antibiotics susceptibility pattern of *S. aureus*

In the present study, the results of sensitivity of the isolates to the antibiotics reported as resistant, intermediate, and sensitive according to the Clinical and Laboratory Standard Institute published [15]. The fifty isolates of *S. aureus* were subjected to the antibiotic susceptibility test using 12 different types of antibiotics belong to different classes. The test showed a significant rate in resistance to certain antibiotics. The complete resistance (100%) was found against Oxacillin indicated that all the isolates under the study are MRSA isolates. The higher number of the isolates in this study displayed resistance to azithromycin (68%), ciprofloxacin (38%), levofloxacin (38%), trimethoprim sulfamethoxazole (38%), norfloxacin (32%), tetracycline (30%), clindamycin (30%), gentamicin (28%), and rifampin (24%). In contrast, fewer *S. aureus* isolates revealed a low percentage of resistance against chloramphenicol (8%), and nitrofurantoin (4%) (Figure 4).

The differences in bacterial resistance could be due to various factors. Some of these factors are related to the mode of action of the antibiotic and others can be related to the genetic variation of each isolate. *S. aureus* is resistant to multiple antibiotics for several reasons. One reason is that the bacteria can acquire new genes through horizontal gene transfer, allowing them to quickly incorporate resistance genes from other bacteria. Another reason is that *S. aureus* undergoes mutations in their chromosomal genes, leading to changes that confer resistance [8]. Other resistance mechanisms including secretion enzymes such as beta-lactamases, which can break down antibiotics such as penicillins. Moreover, bacteria modify antibiotic target sites, preventing the drugs from binding to them and exerting their effects [27]. Biofilm formation also contributes to antibiotic resistance by impeding the penetration of antibiotics to reach the site target in bacteria [28]. Oxacillin (belonging to the penicillin group) acts as an inhibitor of cell wall synthesis. This resistance is mediated by blaZ gene located on Tn552-like transposons or transposons remnants which encodes β-lactamase. These enzymes hydrolyze the lactam ring in the penicillin structure [8].

The resistance to Azithromycin by *S. aureus* can be due to different mechanisms. It can inhibit protein synthesis by binding to the bacterial ribosome. Alternatively, *S. aureus* bacteria can develop resistance by actively pumping azithromycin out of the cell via an efflux pumps system. The resistance may also be due to mutations in the ribosomal RNA (rRNA), which can lead to reduced antibiotic binding; overuse of azithromycin, which leads to the spread of resistant strains; or gene transfer, where resistance genes can be transferred between bacteria through horizontal gene transfer, allowing resistance to spread [29].

A study conducted in Diyala Province, Iraq, by [30] found that all S. aureus isolates isolated from different clinical sources were 100% resistant to oxacillin. Our results agreed with a study carried out on *S. aureus* isolated from nasal carriage in Diyala Province by [31]. Their results showed that all isolates were 100% resistant to oxacillin. Resistance rates to other antibiotics were also high to azithromycin, tetracycline, gentamicin, ciprofloxacin, and trimethoprim-sulfonamide by 94%, 72%, 32%, 14%, and 20%, respectively. In a study carried out by [32] in Diyala Province, reported that the percentage of resistance to Azithromycin was high by 84% and 36% for chloramphenicol. [33] obtained 50 *S. aureus* isolates from 220 clinical samples were resist to Oxacillin (76%), Ciprofloxacin (42%), Gentamycin (20%), Clindamycin (74%), Tetracycline (40%), Rifampicin (6%) and Trimethoprim/Sulfamethoxazole (14%).

In a study conducted by [34], all the isolates exhibited resistance to oxacillin (100%), the resistance to ciprofloxacin and tetracycline was 31.7% and 31.7%, respectively, while resistance to Trimethoprim sulfamethoxazole by 17.5% which is similar to the results of our study. *S. aureus* can develop antibiotic resistance due to increase the mutation rate when exposes to environmental stress by using sub-MIC of antibiotic and bacteriophages [8]. According to the current results, *S. aureus* isolates were classified as multidrug-resistant (MDR). Specifically, 30 out of 50 isolates (60%) were classified as MDR. The widespread occurrence of multidrug-resistant organisms (MDROs) is a major concern worldwide. These organisms are known as hospital-acquired pathogens, leading to increased morbidity and mortality rates and a significant economic loss to healthcare systems. A significant factor contributing to the rise in antibiotic resistance is the inappropriate and repeated use of antimicrobial agents [35]. The current study was close related to the results of the study of [30] in Diyala, Iraq, as they showed that 48(96%) of the *S. aureus* isolates were MDR.

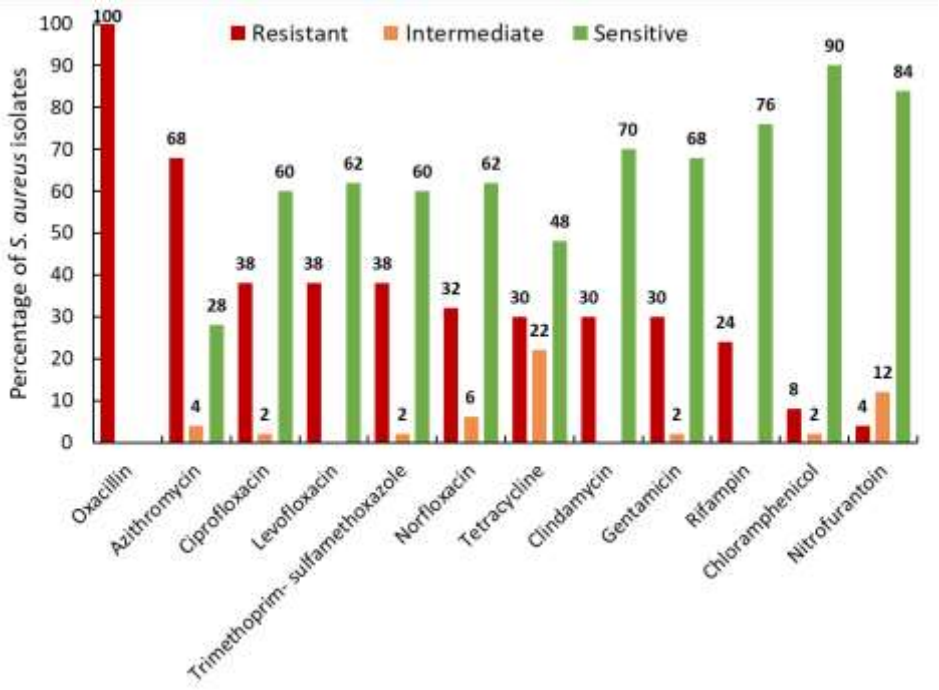

Figure 4. Antibiotic sensitivity profiles for *S. aureus* isolates determined by the Kirby-Bauer disk diffusion method. The percentage of isolates resistant (red bars), intermediate (orange bars) and sensitive (green bars) is shown for each antibiotic tested. High resistance was observed for oxacillin and azithromycin, while high sensitivity was found for rifampin, chloramphenicol and nitrofurantoin.

3.4 Biofilm formation profile in *S. aureus* isolates

The biofilm was quantified to determine percentage formation of biofilm by *S. aureus* isolates. The ELISA reader was used to determine the amount of biofilm created by adhesion to the surface wells, which was expressed as a numerical value of absorbance at 630 nm. The distribution of biofilm-forming ability among *S. aureus* isolates was significantly different between the three categories ($\chi^2 = 18.64$, df = 2, $P < 0.001$). Among the vast array of virulence factors in *S. aureus*, biofilm formation represents the key factor that affects the emergent of antibiotic resistance in these bacteria [36]. All *S. aureus* isolates studied were 100% biofilm-forming, regardless of whether they were strong, moderate, or weak biofilm-forming. The results showed that 22(44%) of *S. aureus* isolates were strong biofilm-forming, 26(52%) were moderate, and 2(4%) were weak biofilm-forming, as shown in Figure (5). In a study conducted on *S. aureus* isolated from a different clinical specimen in Baghdad, 38% were strong, 32% moderate, and 9% weak biofilm-forming [37]- [38] reported 47.7% of *S. aureus* produce strong biofilm, 38.6% were moderate and the rest of the isolates (13.6%) were weak biofilm forming. In pathogenic *S. aureus*, the biofilm formation occupies a crucial role in acquire and development antibiotic resistance. Moreover, the biofilm gives the bacteria a survival advantage, increasing strain resistance by up to 1500 times [36]. The variation in biofilm formation degree in *S. aureus* isolates is

due mainly to genetic factors (*ica* operon, *agr* and *sarA* genes). Moreover, within the biofilm, the percentage of dead cells, matrix, and live adhering bacteria may vary greatly depending on the infection site [39].

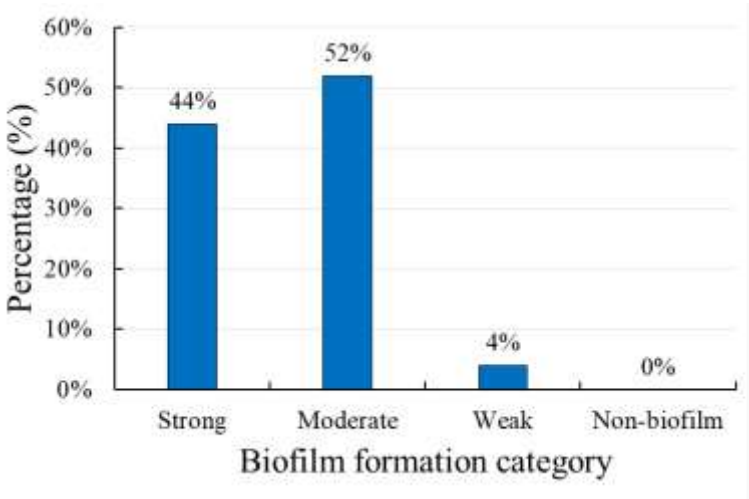

Figure 5. Percentage of biofilm formation produced by *S. aureus* isolates (A). Microtitre plate screening for biofilm formation by crystal violet test of *S. aureus* isolates.

3.5 Relationship between biofilm pattern and antibiotic resistance

The formation of biofilms is essential for increasing the resistance of *S. aureus* to antibiotics. Therefore, developing successful ways to address biofilm-associated infections caused by *S. aureus* requires an understanding of these intricate mechanisms. As shown in (Figure 6), there is clear evidence that there is a common antibiotic resistance between the isolates with strong and medium biofilm production. Most of antibiotic resistance were accompanied by a high percentage of strong biofilm production. The strong biofilm isolates were resistant to oxacillin by 100%. The resistance to Rifampin and clindamycin was also high in strong biofilm isolates by 75% and 73.3%, respectively. However, not all the antibiotics revealed the same behavior, for example, the resistance to Chloramphenicol appear in weak (25%), and moderate (25%) and 50% with strong biofilm producers. Moreover, Ciprofloxacin and Levofloxacin showed the same pattern of resistance for weak biofilm (10.5%) and moderate (26.3%) and (63.2%) with strong biofilm isolates. Biofilms play a crucial role in the spread of antibiotic resistance through several factors such as the interaction of the biofilm matrix with antibiotics that can delay or reduce their bioactivity [40, 41]. Antibiotics (e.g., beta-lactams) that target actively growing cells are less effective against the slow-growing or non-growing cells that create biofilms. In the biofilm matrix, bacteria are able to exchange the genetic material and produce enzymes, such as proteases and β-lactamases that can breakdown β-lactams and some antibiotics. Also, the matrix acts a physical barrier preventing the antibiotic penetration to the next layers in biofilm. In matrix, some antibiotics (e.g., aminoglycosides) are bind to negatively charged of eDNA [41].

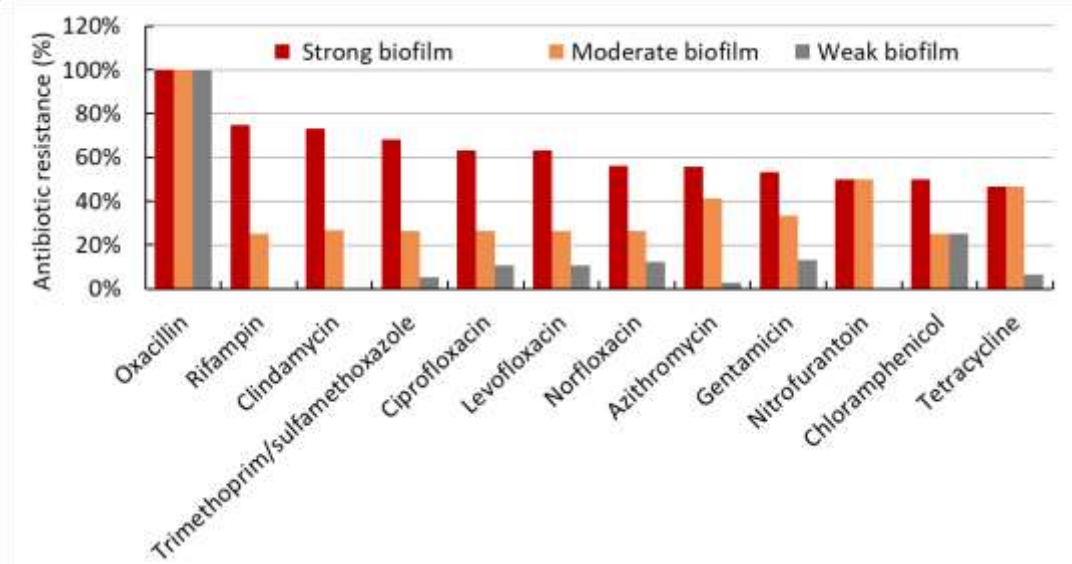

Figure 6. Relationship between biofilm-forming ability and antibiotic resistance among *S. aureus* isolates. Bar graph shows the percentage of antibiotic-resistant isolates classified according to their biofilm-forming ability (strong, moderate and weak). Strong biofilm-producing isolates showed high rates of resistance to most antibiotics tested, including oxacillin, rifampin, clindamycin, trimethoprim-sulfamethoxazole, and fluoroquinolones, while moderate and weak biofilm producers generally showed low levels of resistance.

4. CONCLUSION

S. aureus is an opportunistic pathogen that lives in the skin, nasal cavity and various places in the human body, and is responsible for a wide range of infections ranging from mild skin diseases to serious life-threatening diseases. In this study, fifty *S. aureus* isolates were recovered and identified using standard microbiological methods. Isolates were further evaluated for antibiotic sensitivity and biofilm-forming ability. All isolates showed resistance to oxacillin, a β-lactam antibiotic effective against methicillin-resistant *S. aureus* (MRSA). High resistance was also observed against azithromycin (68%). In contrast, lower resistance rates were found for rifampin (24%), chloramphenicol (8%) and nitrofurantoin (4%). Biofilm formation testing showed that 44% of the isolates were strong biofilm producers, 52% were moderate producers, and only 4% showed weak biofilm-forming ability. A clear correlation between the degree of biofilm formation and antibiotic resistance patterns was observed, suggesting that stronger biofilm-producing isolates show higher antimicrobial resistance. These conclusions highlight the clinical importance of biofilm-associated resistance in *S. aureus* infection. Therefore, the results of this study may contribute to the development of new antimicrobial strategies aimed at controlling biofilm formation and reducing the pathogenic potential of *S. aureus*.

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