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Original article

Detection, Antibiotic Susceptibility, and Selected Virulence Factors of *Staphylococcus spp.* Isolated from Clinical Samples in Baquba City

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Abstract
Staphylococcus bacteria belong to the genus Staphylococcus of the family Micrococcaceae and are Gram-positive bacteria. This study aimed to investigate the prevalence of staphylococci in clinical samples, determine their virulence factors, biofilm formation ability, and their relationship to antibiotic resistance, with a focus on the role of the icaA and icaD genes in this phenomenon. One hundred clinical samples were collected from patients at Baqubah Teaching Hospital and cultured on nutrient media to identify staphylococci using catalase and coagulase tests. Virulence factors such as hemolysis and biofilm formation were assessed, and antibiotic susceptibility was tested using the disk diffusion method and the minimum inhibitory concentration (MIC). Polymerase chain reaction (PCR) was used to detect the presence of the icaA and icaD genes. Twenty-three staphylococcal species were isolated, of which 11 were coagulase-positive and 12 were coagulase-negative. The highest percentage of isolations was from skin swabs (52.2%), followed by nasal swabs (30.4%), and then urine (17.4%). S. aureus was the most common (47.8%), followed by S. epidermidis and S. saprophyticus. 47.8% of isolates demonstrated the ability to produce beta-hemolysin, and biofilm formation rates reached 86% for S. epidermidis and 82% for S. aureus and S. saprophyticus (60%). Antibiotics such as imipenem were most effective. According to the study's findings, most bacterial isolates could form biofilms quickly, and this capacity was frequently linked to the presence of the icaA/D genes. This link was not absolute, though, because isolates that tested positive for the genes did not form biofilms, and there were no group differences that were statistically significant ($P > 0.05$). The results of this study confirm that biofilm formation is a major factor in increasing antibiotic resistance in staphylococcal isolates, especially in isolates containing the icaA and icaD genes associated with biofilm formation, highlighting the importance of studying these genes within infection control programs
Keywords. Antibiotic Susceptibility Test, Virulence Factors, *Staphylococci*, *IcaA/D* Genes, MIC.

Introduction

Staphylococci are gram-positive bacteria that belong to the genus *Staphylococcus* in the family *Micrococcus*. They are divided into two groups based primarily on clinical and diagnostic criteria: coagulase-positive (CoPS) bacteria (e.g., *Staphylococcus aureus*) and coagulase-negative (CoNS) bacteria (e.g., *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*) [1]. *Staphylococci* are common bacteria that colonize mammalian tissues such as the skin, nose, and mucous membranes. *Staphylococcus aureus* is the primary pathogen, causing a wide range of clinical infections in humans, including bacteremia, endocarditis, and several infections associated with invasive medical equipment. In the meantime, coagulase-negative (CoNS), particularly *S. epidermidis,* as common carriers of hospital-acquired infection (nosocomial infections), particularly those linked with indwelling devices [2]. *Staphylococcus epidemiology* has become a big concern in humans and animals as antibiotic resistance has grown and infections have become more prevalent. In general, antibiotics can be used to treat most *Staphylococcal* infections; however, in recent years, various strains of *Staphylococcus* have demonstrated resistance to the most widely used antibiotics, including cephalosporins, gentamicin, tetracyclines, macrolides, lincosamides, and other β-lactams [3,4].

In Iraq, as in many neighboring countries such as Jordan, Lebanon, and Iran, epidemiological reports have shown an increase in the incidence of resistant *staphylococcal* isolates. Recent research conducted in northern Iraq (Dohuk, Tikrit, and Baghdad) has revealed extremely high levels of resistance to common antibiotics like erythromycin, oxacillin, and penicillin. In certain investigations, the percentage of isolates that are resistant to antibiotics has surpassed 70% in certain situations; the isolates are even resistant to vancomycin (VRSA), which makes treatment more difficult [5,6]. The danger of these strains is due not only to their resistance but also to their possession of multiple virulence factors, which are bacterial compounds that increase their ability to establish and thrive within the host, causing damage to the host and facilitating host colonization and immune resistance.

Staphylococci have a diverse set of virulence factors [7] that allow them to cross the body's natural barriers and immune defenses and spread to different tissues. *Staphylococcus* produces a variety of proteins, including cytotoxins such as hemolysins and leukocidins, which are among the most common toxins. The genes responsible for the production of these toxins, such as PVL, TSST-1, and ETA, are key factors in pathogenicity. Genes associated with biofilm formation also play a pivotal role, with *icaA* and *icaD* being the most prominent. These genes enhance the bacteria's ability to adhere to living and non-living surfaces and

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adapt to harsh environmental conditions, such as the presence of antibiotics or disinfectants. This ability contributes to biofilm formation, leading to chronic infections that are difficult to treat, especially in cases where implanted medical devices such as catheters or artificial joints are present [8]. In addition to genetic virulence factors, *S. aureus* secretes a group of enzymes, such as plasma coagulase, which is used as a diagnostic tool to identify this bacterial species, and catalase, which is a virulence factor in many bacterial pathogens [9].

As these bacteria become increasingly resistant to antibiotics and their resistance patterns change over time, it has become essential to accurately assess their physiological and genetic characteristics, including the sensitivity of clinical isolates to available antibiotics. This assessment contributes to understanding their resistance mechanisms and developing effective strategies to limit the spread of multidrug-resistant strains. This study aims to isolate and identify *Staphylococcus* spp species from different clinical samples collected from patients in Baqubah city hospitals, to evaluate their sensitivity pattern to antibiotics, and to detect virulence factors such as *icaA* and *icaD* genes responsible for biofilm formation, in addition to studying the effect of different concentrations of antibiotics on the biofilm using the minimum inhibitory concentration (MIC) test.

Methods

Collection of the specimens

This study collected 100 specimens from various sources between December 2023 to September 2024, including normal skin swabs 40, nasal swabs 32, and urine swabs 28 from patients attending Baqubah Teaching Hospital in the Governorate of Diyala

Specimens' cultivation and colony characteristics

Staphylococcus was identified using the conventional culture detection method by culturing the samples on blood agar, nutrient agar, and mannitol salt agar and incubating at 37°C for 24h for growth. To guarantee the effectiveness of the culture media and the precision of the diagnostic procedures, positive controls were incorporated during the cultivation and identification of bacterial isolates using verified reference bacterial strains of the same genus or species. Furthermore, sterile, uninoculated slides and medium were employed as negative controls to make sure that the instruments and supplies were free of any possible contamination. The samples were initially characterized by observing the cultural characteristics of the growing colonies in terms of colony shape, size, height, edge, colour, and their effect on the medium, such as hemolysis on blood agar, and mannitol fermentation on mannitol salt agar [10]. The culture media came from two brands that are often used in microbiological labs: Oxoid (UK) and HiMedia Laboratories Pvt. Ltd. (India).

Microscopic examination

To view the growing bacterial cells under a microscope, a tiny colony was put on a sterile glass slide using the sterile inoculating loop and mixed with a drop of distilled water, spread out, and dried before being heat-fixed on a Bunsen burner. It was stained with Gram stain to observe the bacterial cells' shape and how they interact with the Gram stain [11]. A compound light microscope, from Olympus CX23 – Japan, with a 100× objective lens and immersion oil, was used to examine the slides.

Biochemical tests and virulence factors of Staphylococcus isolates [12].

Catalase test

A bacterial colony is transferred by a loop to a clean glass slide by mixing the colony with sterile wooden sticks and adding drops of 3% H2O2 reagent. We immediately noticed the formation of bubbles, indicating a positive result.

Slide coagulase test

In order to examine the clumping factor, a drop of human blood plasma was placed on a glass slide. Colonies growing on blood agar medium between the ages of 18-24h were then added, and the mixture was thoroughly mixed. The test was considered positive if clumping appeared within [5-10] seconds. As a negative control, a drop of the bacterial suspension with the physiological solution was placed on a different glass slide

Oxidase test

One colony of the bacterial culture was placed onto filter paper that had been saturated with one or two drops of oxidase reagent using sterilized wooden sticks. The appearance of a purple color indicates that the test is positive.

Growth on Mannitol salt agar

A selective medium for *Staphylococcus* bacterial isolates is the Mannitol Salt Agar. This medium was used to culture these isolates and incubated for 24h at 37°C, to distinguish *Staphylococcus aureus* from non-

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mannitol-fermenting species, as their colonies are golden yellow on this medium as a result of their fermentation of the mannitol sugar and their color changes. As for the species that do not ferment mannitol sugar, their colonies appear pink or white as a positive result.

Novobiocin sensitivity test

Using a sterile swab, the bacterial culture broth was spread overnight on a Mueller-Hinton agar plate. Before incubating the plates, a 5 µg disk of novobiocin was placed on the agar medium. After incubating the plates at 37°C for 24h, the zone of inhibition around the disk was measured. Novobiocin is an antibiotic that inhibits DNA unpacking and repackaging during bacterial replication and the cell cycle [13].

Haemolysin production

Blood agar base medium containing 5%human blood was used to detect hemolysin enzyme production. Bacteria were inoculated onto this medium and incubated for 24h at 37°C. After incubation, a clear zone appeared surrounding cultivated colonies with hemolysin positive (β-hemolysis) [14].

Detection of motility

A motility medium was used to detect bacterial motility after bacteria were incubated on this medium and incubated for 24h at 37°C. A positive result indicates the spread of motile organisms in the medium and from the site of inoculation [10].

Detection of biofilm formation

A microtiter plate assay was used to assess the ability of bacterial isolates to form biofilms, according to the methodology described in reference [15]. Isolates were cultured in nutrient broth medium and incubated at 37°C for 24 hours. The density of the bacterial suspension was then adjusted using the same medium as a diluent until a McFarland standard of 0.5 was reached. Two hundred microliters of the bacterial suspension were distributed into three wells of a 96-well, flat-bottom polystyrene plate, and the plate was again incubated at 37°C for 24 hours. Following incubation, the wells were washed three times with distilled water to remove non-adherent cells and then allowed to dry completely at room temperature. To fix adherent cells, 200 µL of pure methanol was added to each well, then the wells were stained with 200 µL of 0.5% crystal violet solution for 15 minutes. Excess dye was removed by repeated washing with distilled water, and 200 µL of ethanol was then added to each well to dissolve the crystal violet attached to the adherent cells. Wells containing only bacteria-free nutrient broth medium were used as a negative control. According to reference [16], absorbance (OD) was measured at a wavelength of 630 nm using an ELISA reader. The measured values reflect the extent of biofilm formation by the studied isolates. Bacterial isolates were classified based on their absorbance compared to the absorbance of control wells (ODc), according to the following criteria: Non-biofilm producer: If OD ≤ ODc, Moderate biofilm producer: If OD < OD ≤ 2×ODc, Strong biofilm producer: If OD > 2×ODc.

Antibiotic susceptibility test

According to the Clinical and Laboratory Standards Institute (CLSI-2024), the Kirby-Bauer Method on Mueller-Hinton agar was used to assess each isolate's susceptibility to the six antibiotics listed in (Table 1). A standard reference strain of the genus *Staphylococcus* spp. was employed as a positive control to compare the diameter of growth suppression with the clinical isolates under investigation, ensuring the validity of the performance of antibiotics and culture medium, as well as the accuracy of the results. To prepare the bacterial suspensions, a single colony grown on nutrient agar was transferred to 5 ml of distilled water (D.W.), with the goal of forming a bacterial suspension for each isolate at a concentration equivalent to 1.5 x 10^8 CFU/ml, compared to the 0.5 McFarland standard. The Mueller-Hinton agar medium is covered with bacterial suspensions using a sterile swab stick. The plate is then streaked in three different directions and allowed to dry for 15 minutes at room temperature. The plates were incubated at 37°C for 18-24h after the antibiotic discs were moved to the plate using an antibiotic disk dispenser at a rate of six discs per plate, fixed on the plate's surface, and left for 30 minutes. According to [17], the bacteria were classified as S-Sensitive, I-Intermediate, or R-Resistant based on zones of inhibition surrounding the antibiotic disc.

Table 1. The Classes of Antibiotics and Concentration

Classes of antibiotics	Antibiotics	Concentration (µg / disk)
Aminoglycosides	Gentamicim	10
Carbapenems	Imipenem	10
Fluoroquinolones	Ciprofloxacin	5
	Levofloxacin	5
Sulfonamide	Trimethoprim/sulfamethoxazole	1.25/23.75
Glycopeptide	Vancomycin	30

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Determination of Minimum Inhibitory Concentration (MIC)

Using the serial dilution method in Mueller-Hinton broth, the minimum inhibitory concentration (MIC) of erythromycin, tetracycline, trimethoprim/sulfamethoxazole, and methicillin against isolates of *Staphylococcus* spp. was ascertained. The antibiotics were produced in serial dilutions with concentrations ranging from 2 to 1024 µg/ml. To tubes with varying antibiotic concentrations, a bacterial suspension with turbidity equal to 0.5 McFarland standard was introduced. After 18 to 24 hours of incubation at 37°C, the minimum inhibitory concentration (MIC) was identified as the antibiotic concentration that inhibited bacterial growth.

Polymerase Chain Reaction assay

Using specially created primers, the target genes *icaA* and *icaD* in *Staphylococcus* isolates were found using the polymerase chain reaction (PCR). Each experiment had a positive control made of known DNA containing the target genes and a negative control made up of all reaction components other than the DNA template to guarantee the precision and integrity of the reaction. DNA concentration was determined using a Quantus™ Fluorometer, after mixing 1 µL of sample with 199 µL of diluted QuantiFluor® dye, and leaving the mixture at room temperature for 5 minutes. To create a stock solution (100 pmol/µl), triple primers were purchased from Macrogen and dissolved in nuclease-free water. A working solution of 10 pmol/µl was then created by diluting 10 µl of the stock in 90 µl of water, and it was kept at -20°C for use in the reaction. Conventional PCR was used to screen for the *icaA*/*icaD* genes in all *Staphylococcus* spp. isolates. Initial denaturation at 94°C for 5 minutes, 38 cycles of denaturation(94°C,30s), annealing(57°C,45s), extension (72°C, 45 s), and a final extension at 72°C for 7 minutes comprised the reaction's protocol. After electrophoresizing the reaction products in a 2% agarose gel with 1x TAE buffer for 80 minutes at 80 V, the gel was stained with RedSafe to reveal the bands that formed.

Statistical analysis of data

Based on the findings, the data was analyzed and organized in a database using Microsoft Excel 2010. Tables of frequency distribution were prepared to classify the data into common categories, and data were represented as percentages, facilitating descriptive analysis and graphical representation to clearly illustrate and interpret the results. The chi-square test was used to identify significant differences between the number of isolates and the source of isolation. The distribution of different bacterial species (*S. aureus*, *S. epidermidis*, and *S. saprophyticus*) was also analyzed according to biofilm production categories (strong, medium, weak, and non-productive). Additionally, the proportions of different bacterial species were compared in terms of their ability to produce biofilms and their association with ICAA/D gene-positive isolates. The following levels were used to determine if a result was statistically significant: $P < 0.05$ (high statistical significance), $P < 0.01$ (high statistical significance), and $P < 0.001$ (strong statistical significance) [18].

Results

Isolation of Staphylococcus spp

Of the 100 clinical samples collected, 23 (23%) tested positive for *Staphylococcus* spp. These isolates were distributed across different sample types, including skin swabs, urine swabs, and nasal swabs **(**Table 2).

Table 2. Distribution of CoPS and CoNS isolates among different clinical sample types

Sample type	Number of clinical samples (%)	CoNS Isolates (n/%)	CoPS Isolates (n/%)	Total *Staphylococcus* spp (n/%)
Skin swabs	40 (40%)	7(17.5%)	5(12.5%)	12(30%)
Nasal swabs	32 (32%)	3(9.4%)	4(12.5%)	7(21.8%)
Urine swabs	28 (28%)	2(7.1%)	2(7.1%)	4(14.3%)
Total samples	100 (100%)	12 (12%)	11 (11%)	23(23%)

p > 0.05

It was observed through laboratory culture of clinical samples that 23 samples produced a positive laboratory culture result, representing a percentage of 23%, and those that gave a negative result were 67 samples. The number of isolates of coagulase-positive *Staphylococcus* (CoPS) obtained from all *staphylococcal* isolates is 11 isolates, constituting a percentage of 47%, while the number of isolates of coagulase-negative *staphylococci* (CoNS) obtained from all *Staphylococcus* isolates is 12 isolates, constituting a percentage of 52%. The results of the Chi-square test showed that there was no statistically significant difference in the distribution of coagulase-positive and coagulase-negative *Staphylococcus* (CoPS and CoNS) between the different sample types, with a p-value of 0.654 (>0.05).

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Identification of Staphylococcus spp

The colony characteristics, microscopic examination, and biochemical tests listed in (Table 3) were used to diagnose the isolated bacteria.

Colony characteristics

All 23 isolates were cultivated for 24 hours at 37°C on nutrient agar, mannitol salt agar, and blood agar. Initial identification of bacterial isolates from the genus *Staphylococcus* was based on their phenotypic traits when cultured on nutrient agar. Growing on this medium, *Staphylococcus* bacteria appeared to be large, circular, slightly raised, and opaque white colonies. Then its color became pale yellow when it was grown for a longer period, and this agrees with what Isa *et al*. [19]. *Staphylococcus aureus* bacteria appeared by growing on Blood agar medium, producing relatively large circular colonies, slightly raised, yellow to golden, surrounded by a transparent area, as a result of its beta-hemolytic activity on blood agar. As for isolation, the *S. epidermidis* and *S. saprophyticus* appear in the form of small white, opaque, smooth colonies and are not surrounded by a transparent area, indicating their inability to produce blood hemolytic [20]. Mannitol salt agar is a differential and optional medium because it has a high proportion of salts (7.5–10%) that the *Staphylococcus* genus can tolerate. The medium also contains mannitol sugar and methyl red reagent, where the bacteria *Staphylococcus aureus* appeared its colonies are yellow, with a diameter of 2-3 mm, capable of fermenting mannitol sugar and producing acidic products, which is attributed to the reason for changing the color of the medium from red to yellow, while *S. epidermidis* and *S. saprophyticus* colonies appeared white, with a diameter of 1-2 mm, unable to ferment mannitol sugar, nor is it the medium turns yellow [21].

Microscopic examination

Through microscopic examination, the shape of the cells, their organization, the way they are assembled, and their interaction with Gram stain were observed. When examining *Staphylococcus* under the optical microscope and after staining them with Gram stain using a regular optical microscope and an oil lens, it was found that they are gram positive cells with a spherical shape with a diameter of approximately 1 mm, gathered in the form of grapes clusters and single, spherical (single cocci), pairs, or tetrad shapes, and this is what he mentioned Matar, [22].

Biochemical tests

The bacterial isolates under study were subjected to biochemical tests Table 3, and all isolates appeared positive for the catalase test, distinguishing the genus *Staphylococcus* from *Streptococci*. An oxidase test was also performed, in which all isolates appeared to be negative, and this test was used to initially distinguish between the genus *Staphylococci* and the genus *Micrococci*. After identifying the isolates at the genus level, they were distinguished at the species level, based on a slide coagulase test, where all the isolates that gave yellow colonies on the Mannitol salt agar medium showed a positive test for test. In contrast, the white colonies growing on the same medium gave a negative result for this test. The coagulase test may sometimes show false results due to the type and nature of the plasma used, the duration of incubation, and the degree of coagulation, in addition to the possibility of producing this enzyme from another bacterial species [23]. All isolates *of S. saprophyticus* were novobiocin-resistant.

Table 3. The results of diagnostic tests for Staphylococcus spp

Test	*S. aureus*	*S. epidermidis*	*S. saprophyticus*
Mannitol fermentation	Mannitol fermented	Mannitol non-fermented	Mannitol non-fermented
Hemolysis when growing on blood agar	β-hemolysis	Non-hemolysis	Non-hemolysis
Pigment	+ (Golden yellow pigmentation)	White-gray	White-gray
Gram stain	+	+	+
Oxidase	-	-	-
Catalase	+	+	+
Coagulase	+	-	-
Novobiocin resistance	+	-	+
Number of positive strains (n)	11	7	5

\+ *Positive;* - *Negative*

Distribution of Staphylococcus spp according to the source

Twenty-three isolates out of 100 clinical specimens were positive for *Staphylococcus* species (Table 4). These isolates came from the following sources: There were 4 isolates from urine swabs, 7 from nasal swabs, and 12 from skin swabs. Skin contained the largest percentage of *Staphylococcus* (52.2%), followed by nasal specimens (30.4%), and urine specimens (17.4%).

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Table 4. The numbers and percentages of Staphylococcus among different clinical specimens.

Type of specimens	No. of specimens & (%)	No. of *Staphylococcus* (%)	Percentage of isolates to specimens
Skin swabs	40 (40%)	12 (52.2%)	30%
Nasal swabs	32(32%)	7 (30.4%)	21.8%
Urine swab	28 (28%)	4 (17.4%)	14.3%
Total	100 (100%)	23 (100%)	23%

p-value = 0.312

p-value (0.312) is greater than 0.05, which means that there is no statistically significant difference between different types of samples in the percentage of Staphylococcus presence.

Distribution of Staphylococcus spp according to the species

Three species of *Staphylococcus* were isolated during our research. Of which, the number of *S.aureus* 11(47.8%) isolates, *S.epidermidis* 7(30.4%) and *S.saprophyticus* 5(21.7%) (Figure 1).

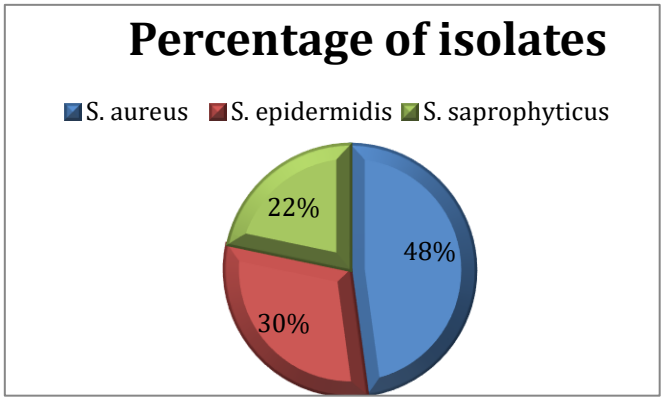

Figure 1. Frequency and percentage of each Staphylococcus spp

Virulence Factors of Staphylococcus

Although they do not contribute to bacterial growth, virulence factors are essential to the ability of bacterial isolates to cause illnesses. Hemolysin, motility, and biofilm formation are selected as representative Staphylococcus virulence factors in this investigation.

Hemolysin production

In this study, we observed that 11(47.8%) *staphylococcal* isolates were beta-hemolysin producers and 12(52%) did not produce beta-hemolysin on a blood agar

Motility production

Twenty-three (100%) of the isolates were nonmotile. To colonize the host and form biofilms, motility is required. It also mediates the first surface interactions [24].

Biofilm formation

The results showed in (Table 5) that 82% of *S. aureus* isolates were capable of forming biofilms, similarly, 86% of *S. epidermidis* isolates. As for *S. saprophyticus* isolates, 60% showed varying degrees of biofilm formation. Biofilm production was classified based on absorbance at OD = 630 nm, with isolates divided into strong, intermediate, and weak producers. Statistical analyses revealed no significant difference ($p > 0.05$) between the different bacterial species regarding the distribution of biofilm formation degrees (strong, intermediate, weak, and non-producers).

Table 5. Classification of Staphylococcus bacterial isolates according to their ability to produce biofilm

Bacterial Species	Strong Producer (%)	Moderate Producer (%)	Weak Producer (%)	Non-Producer (%)	Total Isolates	Biofilm Producers
S. aureus	5(45.5%)	2(18.2%)	2(18.2%)	2(18.2%)	11	82%
S. epidermidis	4(57.1%)	1(14.3%)	1(14.3%)	1(14.3%)	7	86%
S. saprophyticus	1(20%)	1(20%)	1(20%)	2(40%)	5	60%

**p-value > 0.05*

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S. epidermidis showed a higher biofilm-forming capacity than *S. aureus* and *S. saprophyticus*, consistent with its role in medical device and catheter infections.

Antibacterial susceptibility test of S. aureus

In the current study, while performing an antibiotic susceptibility test (Figure 2), *S. aureus* isolates revealed that a lower percentage of antibiotic resistance was seen against imipenem at 8% and sensitivity to it by 92%. Followed by trimethoprim/sulfamethoxazole, the percentage resistance was 9%, and the sensitivity to it by 91%. The percentage of resistance to ciprofloxacin by 27% and the sensitivity to it by 73%. While the percentage of resistance to gentamycin by 36.3% and sensitivity to it by %63.7 %. The *S. aureus* isolates were resistant to levofloxacin by 44% and sensitive to it by 56%. Resistant to vancomycin by 45.5% and sensitive to it by 54.5%.

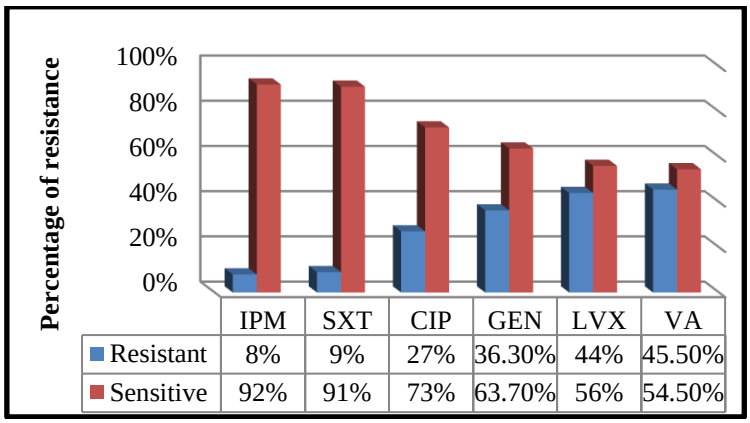
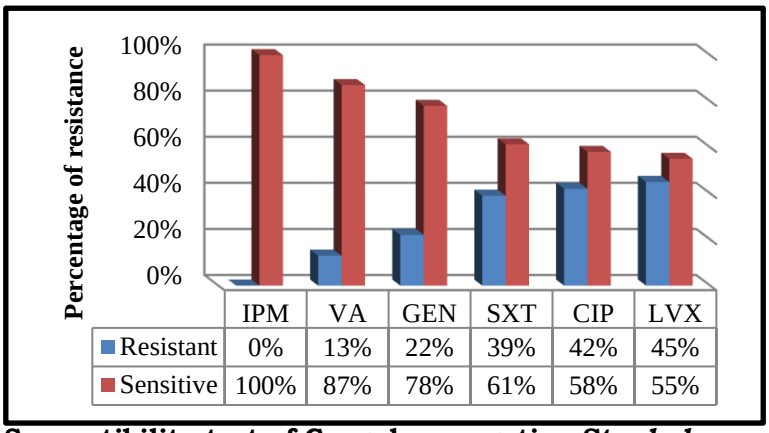


Figure 2. **Susceptibility test of *S. aureus* isolates**

IPM = Imipenem, SXT= Trimethoprim/ sulfamethoxazole, CIP = ciprofloxacin, GEM = Gentamycin, LVX = Levofloxacin, VA = Vancomycin

Antibacterial susceptibility test of Coagulase-negative Staphylococci CONS

In the current study (Figure 3), the results showed that CONS isolates revealed a lower percentage of antibiotic resistance against imipenem by 0% and sensitivity to it by 100%. Followed by vancomycin, the percentage of resistance was 13%, and sensitivity was 87%. The percentage of resistance to gentamycin was 23% and sensitivity was 78%. The percentage of resistance for trimethoprim/sulfamethoxazole was 38.7%, and the sensitivity to it of 61.3%.42% resistance to ciprofloxacin and sensitivity to it by 58%. Finally, the percentage of resistance to levofloxacin was 45.1% and sensitivity to it was 54.9%.



Figure(3). **Susceptibility test of Coagulase-negative *Staphylococci* CONS**

IPM = Imipenem, VA = Vancomycin, GEM = Gentamycin, SXT= Trimethoprim/ sulfamethoxazole, CIP = ciprofloxacin, LVX = Levofloxacin

Biofilm-associated genes (icaA, icaD)

The study's findings demonstrated that the majority of isolates had a high capacity to form biofilms, with 82% of Staphylococcus aureus isolates, 86% of Staphylococcus epidermidis isolates, and 60% of Staphylococcus saprophyticus isolates exhibiting this capacity. 64% of S. aureus isolates, 71% of S. epidermidis isolates, and 40% of S. saprophyticus isolates tested positive for the icaA gene when genes linked to biofilm formation were examined. The icaA gene positivity rate among the isolates that produced biofilms was 33% for S. saprophyticus, 67% for S. aureus, and 67% for S. epidermidis. 72% of S. aureus isolates, 71% of S. epidermidis isolates, and 40% of S. saprophyticus isolates had the icaD gene. The icaD gene positivity percentage for isolates that produced biofilms was 33% for S. saprophyticus, 67% for S.

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epidermidis, and 78% for S. aureus (Table 6). Statistical analysis showed no significant differences between groups (P> 0.05), suggesting that the differences may be due to random variation.

Table 6. Percentage of biofilm production and presence of icaA and icaD genes in the studied bacterial species

Bacterial Species	Total number of isolates (n)	Percentage of Biofilm Producers	Number of isolates positive for the icaA gene (n/%)	Number of biofilm-producing isolates positive for the icaA gene (n, %)	Number of isolates positive for the icaD gene (n, %)	Number of icaD gene positive isolates among biofilm producers (n/%)
S.aureus	11	9(82%)	7(64%)	6 (67%)	8 (72%)	7 (78%)
S.epidermidis	7	6(86%)	5(71%)	4 (67%)	5 (71%)	4 (67%)
S.saprophyticus	5	3(60%)	2(40%)	1 (33%)	2 (40%)	1 (33%)

**p-value > 0.05*

Effect of different concentrations of antibiotics on biofilms (Minimum inhibitory concentration)

Staphylococcus aureus isolates demonstrated significant resistance to methicillin, with MICs exceeding 4 µg/ml in all isolates, indicating the presence of MRSA strains. For trimethoprim/sulfamethoxazole, the MIC varied from 0.5 to 2 µg/ml, and most isolates were susceptible, with the exception of a few isolates with MICs as high as 4 µg/ml, which may indicate the beginning of the development of partial resistance. In *S. epidermidis*, biofilm-producing isolates were more resistant than non-producing isolates. High MICs were recorded for erythromycin, ranging from 8 to 32 µg/ml, and for tetracycline, ranging from 16 to 64 µg/ml. For trimethoprim/sulfamethoxazole, the MIC ranged between 1 and 4 µg/ml in non-biofilm-producing isolates, while it rose to 8 µg/ml in biofilm-producing isolates, reinforcing the role of biofilm in reducing treatment efficacy. As for *S. saprophyticus*, isolates showed varying resistance, with the MIC for erythromycin ranging from 8 to 32 µg/ml, while some isolates recorded high MICs for tetracycline, ranging from 16 to 64 µg/ml. For trimethoprim/sulfamethoxazole, the MIC ranged between 1 and 4 µg/ml in non-biofilm-producing isolates, while it reached 8 µg/ml in biofilm-producing isolates, indicating the role of biofilm in increasing resistance of these isolates to the antibiotic.

Discussion

The purpose of this discussion is to evaluate, interpret, and contrast the findings of the current study with those of earlier research. The isolation rate recorded in this study (23%) is an indicator of the prevalence of *Staphylococcus* among the clinical samples studied. Among the recovered bacterial isolates, the proportion of coagulase-negative *Staphylococcus* (CoNS) was slightly higher (52%) than that of coagulase-positive *Staphylococcus aureus* (CoPS) (47%). This is approximately similar to Hirose *et al.* [25], which was CoPS (33.1%) and CoNS (51.8%). Our study disagrees with Sigudu *et al.* [26], who found that of the 32 different species identified, CoPS were the most frequently isolated (74.7%), followed by CoNS (18.9%). During the distribution of *Staphylococcus* spp according to the species, three species of *Staphylococcus* were isolated during our research. Of which, the number of *S.aureus* 11(47.8%) isolates, *S.epidermidis* 7(30.4%) and *S.saprophyticus* 5(21.7%). Our study's findings agree with those of Azih and Enabulele [27], conducted in Nigeria, which were *S.saprophyticus* (28.3%) and *S.epidermidis* (26.7%).

Our study disagrees with the results of Fazal *et al.* [28], which were *S.epidermidis* (20.2%), *S.saprophyticus* (19.1%), and *S.aureus* (15.7%). The difference in percentages obtained agrees or disagrees with other previous studies. The agreement and difference are due to several reasons, the most important of which is the difference in the methods and means by which the samples were taken, the nature of the samples, the type of study, the sample size, the geographical region from where the samples were collected, and the season of sample collection in addition to the health culture that differs from one person to another. Their number, or the level of cleanliness of the hospital environment and the tools used, all led to the noticeable discrepancy in the percentages [29]. Each of these factors has a part in the variation in *staphylococci* presence. The skin was the most frequent source of positive isolates (52.2%), followed by the nose (30.4%) and urine (17.4%), according to an analysis of positive isolates by sample location.

The biological traits of *S. aureus*, a bacterium that colonizes the skin and nasal mucosa, are in line with this. According to a recent study by Medeiros *et al.* [30], adults have a nasal colonization rate of 20–33% with *S. aureus*, whereas children have a nasal colonization rate of above 50%. The skin serves as a significant reservoir for this bacterium, particularly in patients with wounds or in crowded medical environments [31]. The low percentage of urinary isolates is explained by the fact that, according to a recent systematic study by Flores-Mireles AL, *et al.* [32], *Staphylococci* are responsible for less than 10% of UTIs, and *S. aureus* is rarely regarded as a primary cause of UTIs in comparison to *E. coli*.

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The *staphylococcal* isolates were cultivated on blood agar containing 5% human blood to verify their capacity to produce hemolysin. According to the findings, 12 isolates (52.2%) lacked any hemolysis pattern, whereas 11 isolates (47.8%) produced β-hemolysin. Our study's findings agree with those of Azih and Enabulele [27], in which 29 *staphylococcal* isolates 29(38.16%) were β-hemolysin producers, and 33(43.42%) had no hemolysis pattern (γ-hemolysis). The most important virulence factor is hemolysin production, which is connected with neurotoxicity and cytotoxicity in the cell and can damage red blood cells to remove iron. Recent research has demonstrated that hemolysins, particularly α and β, function as pore-forming toxins that can directly cytolyze cells, making them essential for infection and tissue penetration [33].

Biofilms are essential for increasing bacterial persistence in the host and for boosting resistance to immunomodulators and antibiotics. As a result, evaluating biofilm formation is a crucial step in figuring out how harmful the bacterial isolates are. The results indicate a clear variation in the ability of different *Staphylococcus* species to produce biofilms. *S. epidermidis* showed the highest production rate, followed by *S. aureus*, while *S. saprophyticus* had the least biofilm-forming capacity. For *S. aureus*, the results showed that 82% of isolates had varying degrees of biofilm production, with 45.5% being strong producers, 18.2% being intermediate producers, and 18.2% being weak producers .The results of this study are consistent with the findings of Zaki *et al.* [34], where the percentage of *S.aureus* isolates producing biofilms was 80%. This percentage is comparable to our findings. This indicates that the majority of isolates are capable of forming biofilms, reinforcing the pathogenic role of *S. aureus*, capable of colonizing and cause chronic infections, particularly in implanted medical devices and chronic wounds [35]. *S. epidermidis* showed the highest ability to produce biofilms, with 86% of isolates being producers to varying degrees, with 57.1% being strong producers, 14.3% being intermediate producers, and 20% being weak producers .The results for *S. epidermidis* are also consistent with those of Mirzaei *et al.* [36], where all 54 *S. epidermidis* isolates were biofilm-producing. This ratio reflects the known role of *S. epidermidis* in biofilm formation, making it a major factor in infections associated with medical devices, such as intravenous catheters and artificial joints [37].

In contrast, *S. saprophyticus* was the least capable of biofilm production, with only 60% of isolates producing biofilms, of which 20% were strong producers, 20% were intermediate producers, 20% were weak producers, and 40% were unable to produce biofilm. This is inconsistent with the results of Rafiee and Ghaemi [38], who evaluated 35 *S. saprophyticus* isolates using the microplate method and found that 91% were biofilm producers. These results are also consistent with those of Akbar *et al.* [39] from 50 isolates, 3 isolates, 6% were found to be highly capable of forming biofilms, 6 isolates, 12% were intermediate producers, 36 isolates, 72% were weak producers, and 5 isolates, 10% were not capable of forming biofilms. This may explain why *S. saprophyticus* is primarily associated with acute urinary tract infections rather than chronic infections associated with medical devices, where biofilms play a lesser role in their survival and spread than other species [40]. Overall, these findings confirm the pivotal role of biofilms in promoting bacterial resistance to environmental factors and antibiotics, contributing to the difficulty of treating infections associated with these strains, particularly in hospital and clinical settings. The variation in the ability of different species to produce biofilms may be related to genetic and environmental differences that influence the gene expression of factors responsible for biofilm formation [41] [42].

The study showed a lower rate of resistance, but it remained resistant to some, indicating the need for greater monitoring of its resistance. Overall, our results revealed high antibiotic susceptibility rates. As opposed to previous research, which has demonstrated a significant rate of antibiotic resistance. Differences in antibiotic prescribing practices, antimicrobial use policies, and accessibility to healthcare resources may contribute to the differences in resistance rates and could be due to differences in the strains of *staphylococcal* isolates obtained and may be related to differences in antibiotic use in different settings and selective pressure, sample size of these bacterial isolates in the current study, type and structure of antibiotics, the doses used, the origin of the manufacturing company, the use of antibiotics without a proper prescription by a specialist, the use of antibiotics without laboratory guidance, and misuse of the drug through inappropriate concentrations and/or incorrect dosing schedule [43].

The results of the current study showed a variation in the rates of resistance of *Staphylococcus aureus* to antibiotics compared to previous studies: Low resistance to imipenem (8%) and high sensitivity to it (92%) this finding agreed with Akanbi *et al.*,[44] which the sensitivity was 96% and disagreed with Suaréz-Del-Aguila *et al.*, [45] which the sensitivity was 100%. Followed by trimethoprim/sulfamethoxazole, the percentage resistance was 9%, and sensitivity to it by 91%; this result agreed with Derakhshan *et al.* [46], the sensitivity was 97.6 %. Ciprofloxacin resistance (27%), which agreed with the results of Adhikari *et al.* [47], whose resistance was 80%, and disagreed with Bitew *et al.* [48], whose resistance was 22.2%. Gentamicin resistance (36.3%) is consistent with Liu *et al.* [49], who reported a similar resistance rate (36.1%), but differs from Maharjan *et al.* [50], who reported a higher resistance rate of 77.58%. Levofloxacin resistance (44%) agreed with Nda Mefo'o *et al.* [51], and sensitivity to it by 56%. Vancomycin resistance (45.5%) and sensitivity (54.5%) are consistent with the study by Akanbi *et al.* [44], which showed sensitivity of 50%. CONS isolates showed complete sensitivity to imipenem (100%), a result inconsistent with that of Al-Suadi [52], who reported a sensitivity of 83.3%. For vancomycin, the resistance rate (13%) agrees with the result of Moawad *et al.* [53], who found that 13% of CONS were resistant to vancomycin. Gentamicin

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resistance (23%). This outcome was in agreement with Deyno *et al.* [54], who found that 27% of CONS were resistant to gentamycin. Levofloxacin resistance (45.1%) disagrees with Nicolosi *et al.* [55], whose resistance was 55%. Ciprofloxacin resistance (42%). This result disagreed with Debnath and Sande [56], whose resistance was 67.33%.

For trimethoprim/sulfamethoxazole, the resistance rate (38.7%) is partially consistent with Nicolosi *et al.* [55], who reported a resistance rate of 30%. From the above results, it can be said that Imipenem was the most effective drug compared to the others, and this may be because these antibiotics are less commonly used in our area. Trimethoprim/sulfamethoxazole showed high susceptibility to *S.aureus* isolates with a rate of 91%. Ciprofloxacin, levofloxacin, and vancomycin were the least resistant antibiotics. Vancomycin showed higher efficacy against CONS and for *S. aureus*, which is a concern, as this is an antibiotic of last resort and the highest quality antimicrobial for the treatment of true MRSA infections. The preferred medication for treating MDR-MRSA infections is vancomycin. However, frequent monitoring of vancomycin susceptibility and routine testing should be performed. The use of vancomycin should be limited to maintain its value. *S. aureus* and *Staphylococci* CONS were highly susceptible to gentamycin. *S. aureus* has dynamic qualities that lead it to lose susceptibility to first- and second-generation aminoglycosides, which can be explained by the microorganism's quick adaptive properties [57].

The study's findings corroborate the prevalence of MRSA strains by showing significant rates of *S. aureus* resistance to methicillin (MIC = 4 µg/mL). Recent research showing the worldwide expansion of MRSA strains, especially in hospital settings, is in line with this pattern [58]. These strains are particularly challenging to treat because of their high propensity to build biofilms [59]. The majority of isolates remained susceptible to trimethoprim/sulfamethoxazole (MIC ≤ 2 µg/mL); however, the presence of some isolates with MIC = 4 µg/mL indicates the emergence of partially acquired resistance, which is supported by recent studies that demonstrate a gradual increase in resistance to this compound [60]. The study discovered that isolates that produced biofilms had comparatively high percentages of *icaA* and *icaD* gene-positive. These results support the hypothesis that the icaA/D genes play a key role in biofilm formation. However, they are not the sole factor, as other genetic and environmental factors also influence this process [61]. These findings point to a broad correlation between bacteria's capacity to produce biofilms and the presence of *icaA/D* genes. This correlation was not entirely true, though, as some isolates tested positive for the genes but failed to form a biofilm, indicating the possible inclusion of additional genes or regulatory factors. These genes are linked to the synthesis of poly-N-acetylglucosamine (PNAG), a polymeric compound that contributes to bacterial cell adhesion and the formation of the biofilm's basic structure, impeding antibiotic penetration and increasing the bacteria's ability to survive in harsh environments [62]. The fact that some isolates tested positive for the genes but did not produce biofilms suggests that the gene's presence is insufficient on its own. According to recent research, these genes' real expression is influenced by a number of environmental factors as well as other regulatory factors like agr, sarA [37] [59]. The results of this study indicate that biofilm production plays a key role in enhancing bacterial resistance to antibiotics.

This is consistent with numerous studies that have confirmed that biofilm-producing isolates are more resistant than non-producing ones. Based on these findings, it becomes imperative to search for new therapeutic strategies that target biofilm disassembly or inhibit the genes responsible for its production, in addition to developing treatments based on combinations of antibiotics with different mechanisms to limit the spread of resistance. Regular screening for resistance genes in clinical isolates may also help develop more precise strategies to combat bacterial infections and reduce the chances of treatment failure. Therefore, further studies are needed to understand the mechanisms regulating icaA/D gene expression and the impact of various factors on biofilm production.

Conclusion

The study confirms that the icaA/D genes play a key role in biofilm formation, but their expression is influenced by environmental and genetic factors. Bacterial species also vary in their ability to form membranes, with *S. epidermidis* being the most prolific. Film-producing isolates exhibited greater resistance to antibiotics, particularly methicillin and erythromycin, reinforcing the role of membranes in bacterial protection. More research is required to fully understand these genes' regulatory processes and how they affect antibiotic resistance. In addition to the fact that the genetic analysis was restricted to the icaA and icaD genes alone, without looking at other genes or regulatory factors that might be crucial in biofilm formation, the study's most important limitations are the small number of bacterial isolates examined, which may limit the results' generalizability on a larger scale.

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Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript. Conflicts of Interest

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