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Comprehensive Evaluation of Biofilm-Producing Staphylococci: Correlation with Multidrug Resistance and Comparison of Phenotypic Detection Methods

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KEYWORDS

Biofilm formation, MRSA, Multidrug resistance, Staphylococcus aureus, Nosocomial infections, Antibiotic susceptibility

ABSTRACT:

Background: Staphylococcus species are known to form biofilms, which are major virulence factors that cause persistent infections, antimicrobial resistance, and device-associated complications in health care facilities. The clinical correlation of accurate detection and its clinical correlation is a crucial element in effective management.

Aim: To isolate and determine Staphylococcus species of clinical samples and assess their biofilm-forming capacity against antimicrobial resistance.

Methods: A case control study was carried out as a prospective study on 250 clinical samples (200 cases and 50 controls). Standard microbiological methods were used to identify isolates, and the KirbyBauer method was used to detect antimicrobial susceptibility. The formation of biofilms was evaluated by using the Congo Red Agar (CRA), Tube Method (TM), and Tissue Culture Plate (TCP) assay (gold standard). Chi-square test was used to perform statistical analysis.

Results: Biofilm was produced much more in cases (78.5%) than in controls (16.0%) ($p<0.001$). The most common isolation was Staphylococcus aureus (71.6%). The resistance of methicillin was detected in 67.0 percent of the isolates, and 80.6 percent of the MRSA strains were biofilm producers. TCP method was most sensitive (78.0) to biofilm detection. Maximum sensitivity of linezolid was observed and high resistance to penicillin (61.2%), and erythromycin (45.2%) was observed. Production of biofilms was closely linked with chronic illnesses (94.87%).

Conclusion: Multidrug resistance, chronic comorbidity, and extended stay in the hospital are closely related to biofilm formation. TCP is the most stable detection process. To manage biofilm related staphylococcal infections, early diagnosis and specific antimicrobial interventions are needed.

Introduction:

Staphylococci exist mainly as commensals but can easily transform into opportunistic infections when host defenses are weak [1]. They invade human skin and mucosal surfaces everywhere. Particularly noticeable is this shift in healthcare settings, when patients are more likely to contract infections due to invasive operations, implanted medical equipment, and impaired immune systems [2]. Ray et al. (2021) biofilm is a group of sessile communities that attaches itself to biotic or abiotic surfaces through extracellular polymeric compounds and pili. There is a great variety of microbial species in the human body, which are spatially and temporally distributed differently. Examples of natural microbes that form biofilms include those that can be found in the human body in diverse locations such as the mouth, stomach, skin, vagina, and lung and heart tissue. Dysbiosis may lead to the development of dental and stomach biofilms that may cause numerous disorders [3]. Staphylococci are especially harmful due to their ability to form biofilms, or communities of microbes that are incorporated into a polymeric matrix external to the cell [4]. Such biofilms play a crucial role in the development of medical device-related infections as well as long-term infections [5].

The process of biofilm growth in bacteria brings a lot of change in the physiological condition of the bacteria, and they become resistant to antimicrobial agents and immune system of the host [6]. The metabolic rate,

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gene expression, and synthesis of extracellular molecules, including proteins, extracellular DNA and so-called "polysaccharide intercellular adhesion (PIA)" are also altered in biofilm cells [7]. Bacteria in biofilms can be as much harder to kill as bacteria in planktonic environments (up to a thousand times) because of these changes, making them more challenging to treat and making infections that persist more common [8].

The most clinically significant biofilm makers among staphylococci are Staphylococcus aureus and “coagulase-negative staphylococci (CoNS)”, specifically Staphylococcus epidermidis. [9] While S. aureus is associated with aggressive infections due to its broad virulence factors, CoNS are very effective at establishing biofilms on abiotic surfaces such as catheters, prosthetic joints, and implants [10]. This capability makes them the major cause of nosocomial infections, especially in patients with indwelling medical devices [11]. Wojtyczka et al. (2014), like this study, found that biofilm-embedded bacteria are highly susceptible to drugs and immune clearance, thus infections are persistent, recurrent, and hard to eradicate. The presence of bacterial biofilms on orthopedic implants contributes to frequent infections due to the ability to protect the pathogens against the defense mechanisms of the host, as well as reduce the effect of antibiotics. Phenotypic methods that are simple (e.g., tube method, Congo Red Agar (CRA)) are also economical in terms of screening biofilm generation, especially in resource-constrained contexts. Have good sensitivity and specificity, though not as standardized as quantitative assays, and can help identify strong biofilm-forming isolates, which may need more intensive treatment [12].

Accurate biofilm identification is consequently required for appropriate clinical management and infection prevention [13]. Several phenotypic approaches, including “Congo red agar (CRA), tube adherence test, and microtiter plate (MTP)” assay, are commonly employed for biofilm detection [14]. The MTP method is regarded as the gold standard due to its quantitative character and increased sensitivity [15]. Jaiswal et al. (2025) similar of this study combination to effectively identify and measure biofilm-forming capacity in clinical isolates.) Like this work, Biofilm-embedded bacteria are highly susceptible to drugs and immune clearance and therefore are persistent, recurrent, and difficult to clear infections. The presence of bacterial biofilms on orthopedic implants contributes to frequent infections due to the ability to protect the pathogens against the defense mechanisms of the host, as well as reduce the effect of antibiotics. Simple phenotypic methods (e.g., tube method, “Congo Red Agar (CRA)” are also cheap in terms of screening biofilm generation in resource-limited settings. Good sensitivity and specificity, but not as standardized as the quantitative assays, can be used to recognize strong biofilm-forming isolates, which might require more aggressive treatment [16]. However, differences in laboratory techniques and interpretation criteria frequently result in variations in results [17]. Genotypic approaches targeting biofilm-associated genes such as the icaADBC operon give further insights; however, may not necessarily correlate with phenotypic expression [18].

1.1. Biofilm formulation as a key virulence factor

One of the most significant virulence mechanisms of staphylococci, which contributes greatly to their pathogenicity and to their maintenance in the clinical setting, is the generation of biofilms [19]. A biofilm is a group of bacterial cells that have developed their extracellular polymeric substance (EPS) and that adheres to biotic or abiotic surfaces [20]. This matrix comprises primarily polysaccharides such as “polysaccharide intercellular adhesion (PIA)”, proteins, teichoic acids, and extracellular DNA, which, as a combination, provide structural stability and protection to the bacterial community [21].

Staphylococci have the capacity to form biofilms, which enables them to withstand unfavorable environments like exposure to the host immune system and antimicrobial agents [22]. The physiological and genetic alterations in bacterial cells of biofilms include reduced metabolism, altered gene expression, and stress response pathways [23]. The changes lead to higher antibiotic tolerance, which usually requires doses that are several-fold higher than those that are effective against planktonic cells [24]. In addition, the thick matrix serves as a diffusion barrier, which reduces infiltration of antibiotics and enables survival of persistent cells [25]. To identify whether there was an overlap in the associated genes which is statistically significant, Stewart et al. (2015) compared these genes to lists of genes that were generated independently of

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each other to confirm the stress responses and the other potential antibiotic-defensive mechanisms in place. The gene's interests were biofilm response to tobramycin or ciprofloxacin drug efflux pumps, acyl homoserine lactone quorum sensing, osmotic shock, thermal shock, hypoxic stress, and stationary phase growth. The findings correspond to an idea that several genes controlled by overlapping starvation or stress signals are involved in increasing the resilience of a P. aeruginosa biofilm to ciprofloxacin [26].

An increase in the resistance to antibiotics among staphylococci has an impact on the therapeutic treatment of such diseases [27]. Diseases such as methicillin-resistant Staphylococcus aureus (MRSA) have found their way to the hospitals and communities [28]. The manufacture of biofilms adds to the problem by reducing how antibiotics penetrate and passing the genes of resistance to one another within the bacterial population [29]. Then the use of biofilm-forming staphylococci sometimes might involve a prolonged course of treatment, increased dosages of anti-bacterial drugs, or even surgery [30]. The new concerns on emergence of antibiotic resistance on staphylococci influence the treatment therapy of such diseases [31]. Conditions and even strain such as the methicillin-resistant Staphylococcus aureus commonly referred to as (MRSA) have been spread in both hospitals and communities [32]. The situation is worsened by biofilm production that restricts the penetration of antibiotics and promotes the transfer of resistance genes between the bacterial population [33]. This makes biofilm-producing staphylococci infections sometimes require prolonged treatment, higher doses of antibiotics, or even [34]. Biofilm generation has a major impact on the physiological features of bacterial cells, including changes in metabolic activity, gene expression, and production of extracellular components such as polysaccharide intercellular adhesin (PIA), proteins, and DNA [35]. These alterations let bacteria survive by lowering the potency of antimicrobial drugs and insulating them from host immunological responses [36].

1.1. AIM

To isolate and identify Staphylococci from clinical samples, and assess their biofilm-forming potential in connection with antibiotic resistance

1.2. Objective

2. Isolation of Staphylococci species from various clinical samples
3. Characterization of isolated Staphylococci species by conventional and molecular methods
4. Antibiotic sensitivity pattern of isolated strains by the Kirby-Bauer disc diffusion technique and automated ID/AST
5. Screening of strain for MRSA
6. Detection of biofilm formation by various standard methods

2. Material and Method

2.1. Study design and setting

A prospective case-control study was carried out over one year following clearance from the Institutional Ethical Committee of Government Medical College, Mandi (tertiary care center) "(clearance No. HFW(H)/SLBSGMC/IEC/2018-127)". All individuals provided informed consent before their enrollment in the study.

2.2. Study Population

➢ **Inclusion criteria**

- Patient admitted for > 48 hours
- Patients possessing indwelling medical devices (urinary catheter, intravenous catheter, central line, Ryle's tube, endotracheal tube, chest tube, surgical drain, prosthetic implants)
- Patients receiving total parenteral nutrition or medications
- Patients with chronic infections

➢ **Exclusion criteria**

- Patients without indwelling medical devices
- Patient discharged within 48 hours

2.3. Case and control

Cases comprised inpatients aged ≥1 month with culture-confirmed Staphylococcus infection occurring after 48 hours of hospitalization and the presence of at least one indwelling device. Controls included outpatients or freshly hospitalized patients (within 48 hours) with uncomplicated infections caused by Staphylococcus. Patients who have a history of decontamination therapy, polymicrobial illnesses, or those who declined consent were excluded.

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2.4. Sample collection and processing

Clinical specimens, including pus, blood, urine, sputum, catheter tips, pleural fluid, and ascetic fluid, were collected aseptically and delivered to the laboratory within two hours. Samples were accurately labeled with patient information, date, time, and collection place.

❖ **Sample procedures were followed for device-associated samples**

- Urinary catheter samples: collected using a sterile syringe after disinfecting the catheter port
- Ryle's tube samples: gastric content aspirated aseptically
- Blood samples: collected from the intravenous catheter site
- Drain sample collected under strict aseptic conditions

2.5. Isolation and identification of staphylococci

Specimens were inoculated onto Blood agar and MacConkey agar and incubated aerobically at 37°C for 18 to 24 hours. Colonies were assessed for morphology, hemolysis, pigmentation, and consistency.

- Identification of isolates was performed using:
- Gram staining (gram – positive cocci in clusters)
- Catalase test
- Slide and tube coagulase tests
- Biochemical tests (mannitol fermentation, urease, and ornithine decarboxylase novobiocin susceptibility)

2.6. Antimicrobial susceptibility testing

The antimicrobial susceptibility of Staphylococcus isolates was tested using the Kirby-Bauer disc diffusion method on Mueller-Hinton agar. A 0.5 McFarland standardized inoculum was utilized, and the plates were incubated at 37°C for 18-24 hours. Antibiotics from several classes were examined, and zones of inhibition were interpreted using established guidelines. Cefoxitin (30µg) was utilized to detect MRSA. Staphylococcus aureus ATCC 25923 was used as quality control. Multidrug-resistant isolates were those that could withstand three or more antibiotic classes.

- Zone diameters were interpreted as per standard guidelines
- The methicillin resistance was detected with Cefoxitin (30µg).
- Quality control was done using Staphylococcus aureus "ATCC 25923."

❖ Multi-drug resistance (MDR) was defined as resistance to three or more classes of antibiotics

2.7. Detection of biofilm formulation

Three traditional phenotypic media were used to evaluate biofilm development in Staphylococcus isolates, namely: Congo Red Agar (CRA), tube adherence, and the microtiter plate (MTP) assay. In the CRA technique, organisms were inoculated on Congo red agar and incubated at 37 °C for 24-48 hours. Black crystalline colonies were used to indicate the presence of biofilm formation, and red or pink colonies were used to indicate the absence of production. The tube adherence method was conducted using the bacterial cultures in the broth, incubating the cultures, followed by washing the cultures and staining the cultures with crystal violet; the occurrence of a visible film on the inside wall of the tube confirmed the formation of biofilms. The gold standard assay, the microtiter plate assay, was used to measure the extent of biofilm development by labeling adherent cells and using a spectrophotometer to measure optical density to enable the classification of the isolates as weak, moderate, or robust biofilm producers.

2.8. Data analysis

All data obtained were compiled, coded, and inserted into a suitable statistical software program to be analyzed. Descriptive statistics used included the frequency, percent, mean and SD to generalize on the frequency of Staphylococcus isolates, formation of biofilms and resistance patterns against antibiotics. The presence of multidrug resistance (MDR), and biofilm production was correlated using the assistance of the relevant statistical tools, such as Chi-square and Fisher exact test. The sensitivity, specificity, and the accuracy of various phenotypic techniques (CRA, tube method, and MTP test) were compared against the MTP method (reference standard). The p-value of 0.05 was thought to be statistically significant. Results were tabulated, graphed and charted in easily comprehensible forms.

3. Results

The present study was done on 250 clinical samples (200 cases and 50 controls) to assess the distribution of Staphylococcus species, their biofilm-forming capacity,

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and related antimicrobial resistance patterns. These findings were systematically assessed according to demographics, clinical features, microbiological, and biofilm detection through different methods of phenotypic. Case and control groups were compared to find out the relevance of biofilm production, methicillin resistance, and clinical risk factors including prolonged hospital stay and related chronic diseases. The results are given below in a well-organized form with relevant statistical analysis.

Table 1: Distribution of age between groups.

		Group		Total
		Case	Control	
AGE	<1 year	8	0	8
		4.0%	0.0%	3.2%
	1-10 years	7	0	7
		3.5%	0.0%	2.8%
	11-20 years	7	5	12
		3.5%	10.0%	4.8%
	21-30 years	27	11	38
		13.5%	22.0%	15.2%
	31-40 years	27	8	35
		13.5%	16.0%	14.0%
	41-50 years	28	6	34
		14.0%	12.0%	13.6%
	51-60 years	47	8	55
		23.5%	16.0%	22.0%
	61-70 years	30	5	35
		15.0%	10.0%	14.0%
	71-80 years	16	5	21
		8.0%	10.0%	8.4%
	81-90 years	3	2	5
		1.5%	4.0%	2.0%
Total		200	50	250
		100.0%	100.0%	100.0%

"Chi-Square" = 12.646; p value = 0.179

Interpretation:

The age distribution of the study participants revealed that most of the cases were found in the 51 years and above (23.5%), 61 years and above (15.0%), and 41 years and above (14.0%) age group which means that middle-aged and older people were more affected.

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Control group on the other hand had a relatively larger number of younger people, especially those within the age group of 21-30 years (22.0%). Minimal representation was done in pediatric age groups (<10 years) and only seen in cases. In general, the pattern of distribution between the two groups was not significantly different in many age categories and the difference was found to be non-significant (p = 0.179) indicating that age was not significantly related to occurrence of infection among this study population.

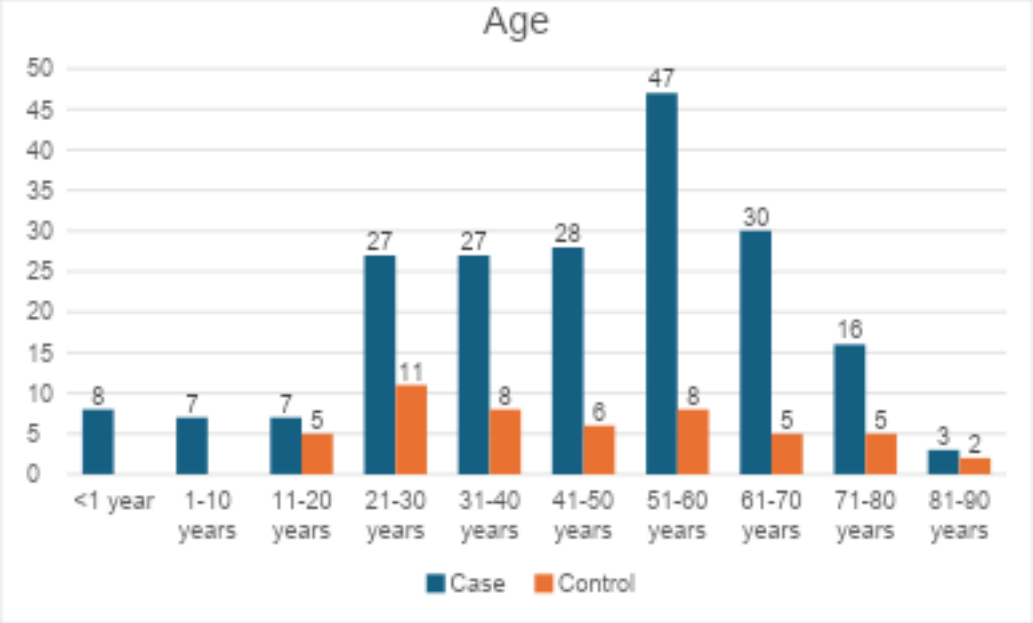

Figure 1: Age Distribution (Cases vs Controls)

Table 2: Distribution of sex between groups

		Group		Total
		Case	Control	
SEX	F	109	29	138
		54.5%	58.0%	55.2%
	M	91	21	112
		45.5%	42.0%	44.8%
Total		200	50	250
		100.0%	100.0%	100.0%

"Chi-Square" = 0.082; p value = 0.775

Interpretation:

The distribution of participants in the study by sex showed a small female preponderance both in cases and controls with females representing 54.5% of cases and 58.0% of controls and males representing 45.5% of both cases and controls. Generally, the female population constituted 55.2 of the total population of the study as compared to 44.8 males. There was no significant difference in sex-wise occurrence as both case and control groups had a similar distribution of gender. This is an indication that there was no substantial effect of gender on the spread of infections in the current study.

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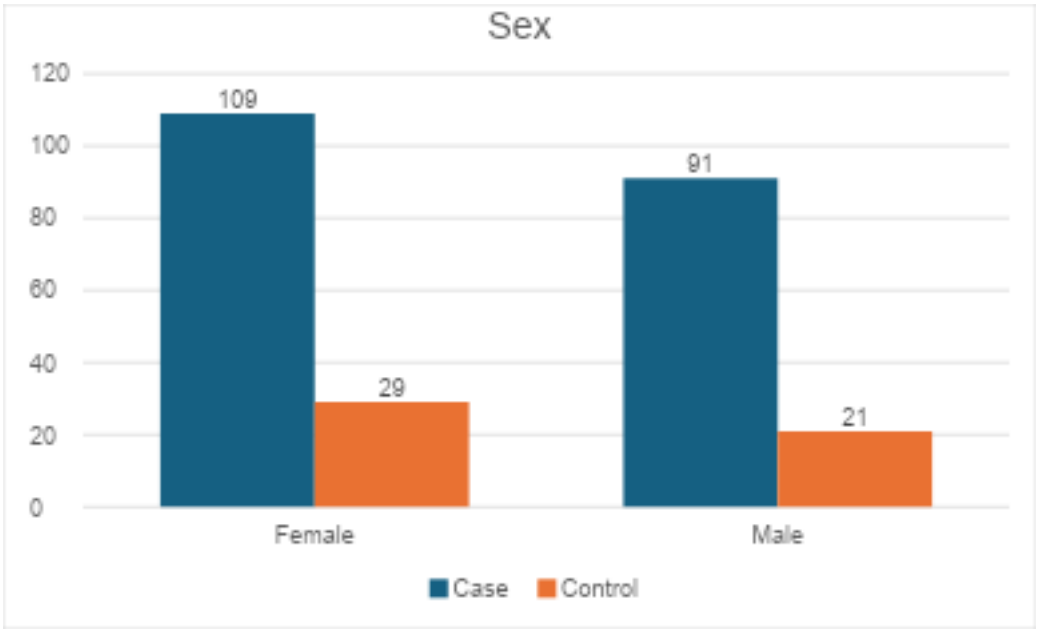

Figure 2: Sex Distribution (Cases vs Controls)

Table 3: Distribution of hospital stay (days) between groups

		Group		Total
		Case	Control	
Hospital days	<5 days	14	38	52
		7.0%	76.0%	20.8%
	11-15 days	61	0	61
		30.5%	0.0%	24.4%
	16-20 days	63	0	63
		31.5%	0.0%	25.2%
	21-25 days	12	0	12
		6.0%	0.0%	4.8%
	5-10 days	50	12	62
		25.0%	24.0%	24.8%
Total		200	50	250
		100.0%	100.0%	100.0%

"Chi-Square" = 125.574; p value<0.001

Interpretation:

The allocation of hospital stay showed a significant variance between the case and control groups. The patients in the case group were also of long hospital stay with 31.5% requiring 16-20 days and 30.5% requiring 11-15 days of stay with only 7.0% requiring a stay of less than 5 days. Conversely, the control group

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had a higher proportion of shorter hospital stays with 76.0% of patients being under a stay of less than 5 days and none of the patients being under a stay of more than 10 days. A similar percentage in both groups (25.0% cases and 24.0% controls) remained 5-10 days. Overall, these results suggest that cases had a strong relationship with length of stay in hospital, which implies a close relationship between length of stay and cases of infections.

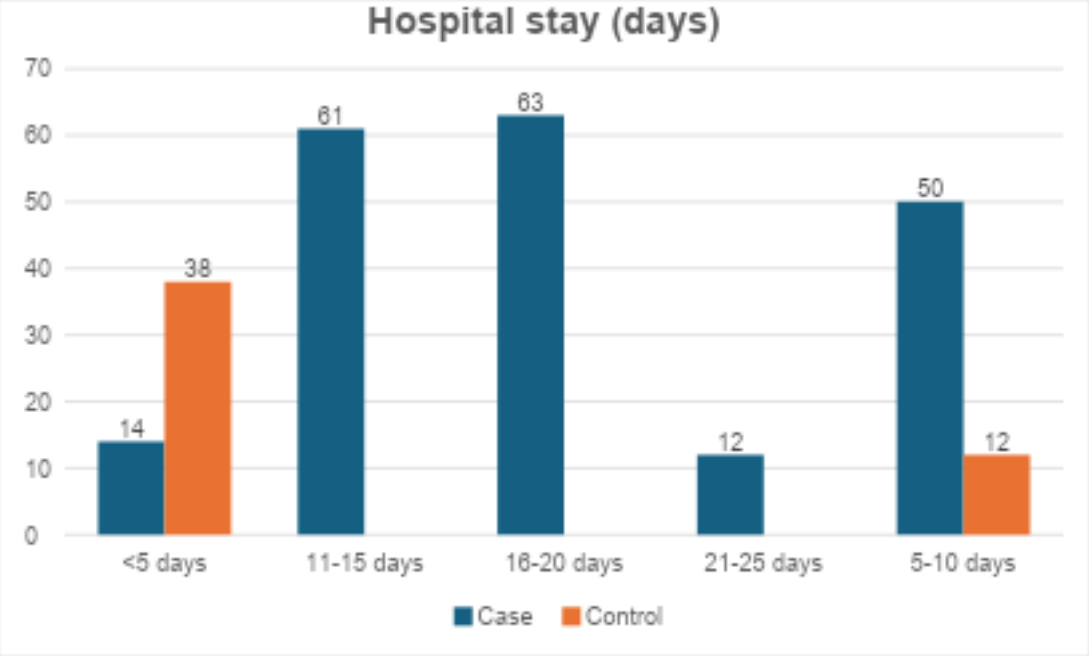

Figure 3: Hospital Stay Distribution (Cases vs Controls)

Table 4: Distribution of biofilm production in Staphylococcus isolates between groups

		Group		Total
		Case	Control	
BIOFILM PRODUCTION	NEGATIVE	43	42	85
		21.5%	84.0%	34.0%
	POSITIVE	157	8	165
		78.5%	16.0%	66.0%
Total		200	50	250
		100.0%	100.0%	100.0%

"Chi-Square" = 69.630; p value<0.001

Interpretation:

Production of biofilms between the case and control groups was strikingly different between the Staphylococcus isolates. A large proportion of bi-film producers (78.5) and non-producers (21.5) were observed in the case group. Conversely, the control group exhibited mostly biofilm-negative isolates (84.0%), with just 16.0% producing biofilms. Overall, 66.0% of the total isolates were biofilm positive. These results suggest that biofilm-forming Staphylococcus

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isolates are closely related to cases of infection, implying that biofilm-forming Staphylococcus isolates contribute to the pathogenesis and persistence of diseases.

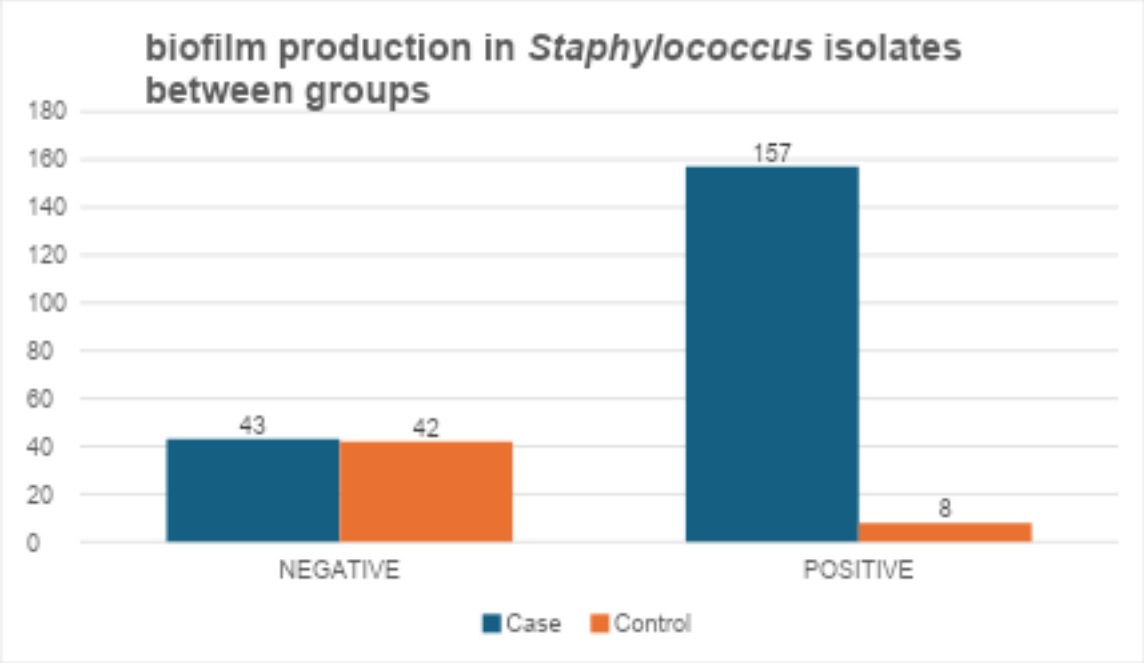

Figure 4: Biofilm Production (Cases vs Controls)

Table 5: Distribution of cases according to Chronic Disease

CHRONIC DISEASE ASSOCIATED	Number of Chronic Disease Associated	Percent
CVA	1	0.50
HYPOTHYROIDISM	1	0.50
TUBERCULOSIS	1	0.50
ASTHMA	1	0.50
CA BREAST	1	0.50
CAD	1	0.50
"CHRONIC OBSTRUCTIVE PULMONARY DISEASE"	1	0.50
CHRONIC OSTEOMYELITIS	1	0.50
CKD	8	4.00
CLD	1	0.50
COPD	8	4.00
CORONARY ARTERY DISEASE	1	0.50

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CRF	2	1.00
CVA	2	1.00
DIABETES MELLITUS	12	6.00
ENDOMETRIAL CARCINOMA	1	0.50
FEBRILE SEIZURES	1	0.50
HEPATITIS	2	1.00
HIV INFECTION	3	1.50
HYPERTENSION	5	2.50
HYPOTHYROIDISM	1	0.50
IHD	3	1.50
LIVER CIRRHOSIS	8	4.00
MULTIPLE ORGAN DYSFUNCTION SYNDROME	1	0.50
PSORIASIS VULGARIS	1	0.50
TUBERCULAR MENIGITIS	2	1.00
TUBERCULOSIS	7	3.50
URETERO-PELVIC JUNCTION OBSTRUCTION	1	0.50

Interpretation:

The patterns of cases distribution based on the related chronic diseases demonstrated that there was a wide variety of comorbidities amongst patients. Diabetes mellitus (6.0%), chronic kidney disease (4.0%), chronic obstructive pulmonary disease (COPD) (4.0%), and liver cirrhosis (4.0%) were the most prevalent comorbidities, and tuberculosis (3.5%), was also significant. Other diseases like hypertension (2.5%), HIV infection (1.5%), and ischemic heart disease (1.5%), among others, existed in smaller proportions, whereas other diseases like CVA, hypothyroidism, asthma, carcinoma, and others were only present in sporadic cases (≤1%). Overall, the results show that a substantial percentage of patients had chronic conditions, especially metabolic and systemic ones, and which can lead to higher vulnerability to infections and biofilm-related complications.

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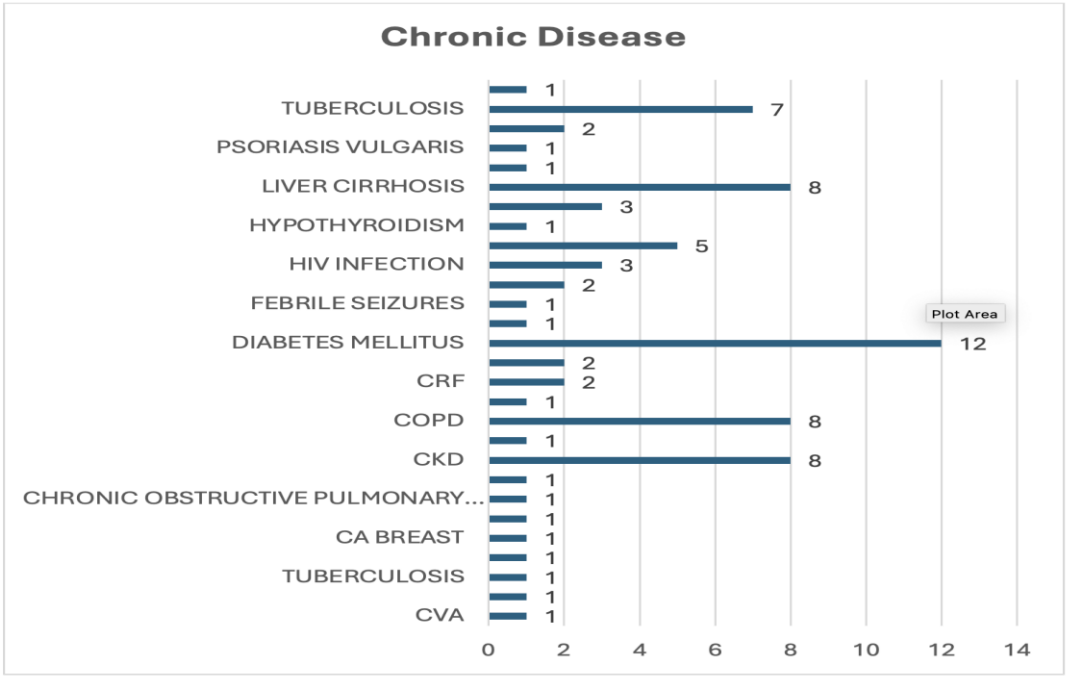

Figure 5: Distribution of Chronic Diseases in Study Population

Table 6: Distribution of organisms between groups

		Group		Total
		Case	Control	
ORGANISM	*S.aureus*	143	36	179
		71.5%	72.0%	71.6%
	S.epidermidis	36	10	46
		18.0%	20.0%	18.4%
	S.haemolyticus	11	0	11
		5.5%	0.0%	4.4%
	S.hominis	6	2	8
		3.0%	4.0%	3.2%
	S.lugdunensis	1	0	1
		0.5%	0.0%	0.4%
	S.saprophyticus	3	2	5
		1.5%	4.0%	2.0%
Total		200	50	250
		100.0%	100.0%	100.0%

Chi-Square = 4.463 ; p value = 0.485

Interpretation:

The distribution of the Staphylococcus species indicated that Staphylococcus aureus was the most common organism with 71.6% of the total number of isolates with equal number in both instances (71.5) and controls (72.0) cases. This was followed by Staphylococcus epidermidis (18.4%), once again with similar

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distribution in the two groups. Other coagulase-negative staphylococci like S. haemolyticus, S. hominis, S. lugdunensis and S. saprophyticus were found in significantly lower percentages. Interestingly, S. haemolyticus was found in the case group only. But total comparison revealed no statistically significant differences in organism distribution between cases and controls (2 = 4.463, p = 0.485), which means that the kind of Staphylococcus species was both similar in case and controls.

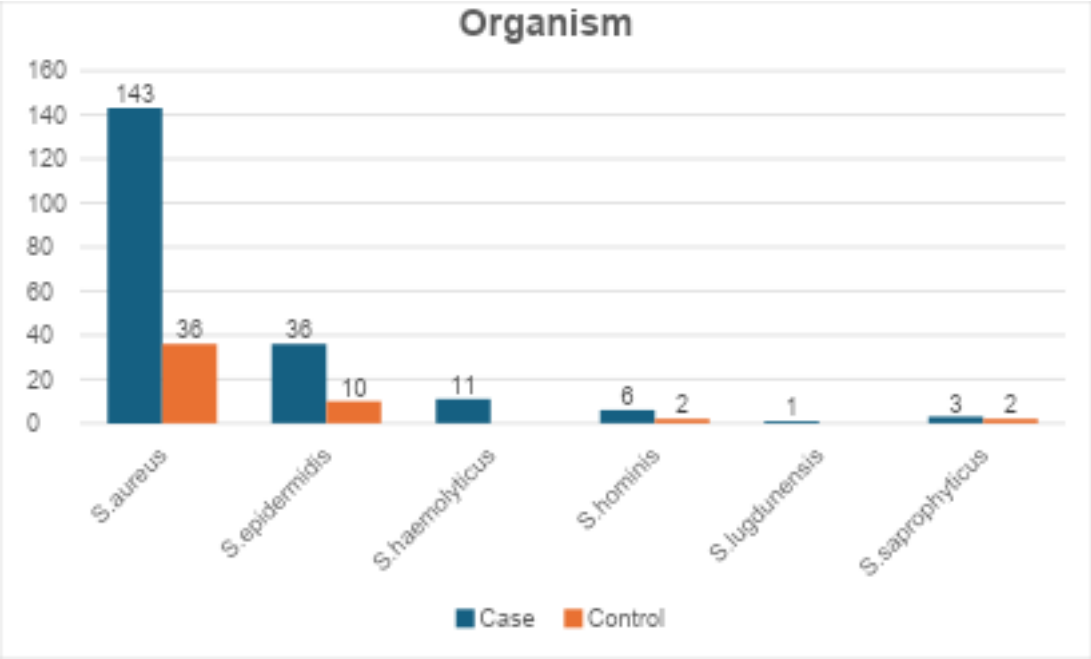

Figure 6: Distribution of Staphylococcus Species (Cases vs Controls)

Table 7: Comparison of Biofilm Production by "Congo Red Agar (CRA), Tube Method, and Tissue Culture Plate" Among Case Staphylococcal Isolates

	CRA		TM		TCP	
BIOFILM PRODUCTION	No.	%	No.	%	No.	%
HIGH	29	14.5	45	22.5	79	39.5
MODERATE	49	24.5	96	48.0	77	38.5
NON/WEAK	122	61.0	59	29.5	44	22.0
TOTAL	200	100.0	200	100.0	200	100.0

Interpretation:

The comparison of biofilm formation of the case isolates using various phenotypic techniques showed significant difference in the detection capacity. The Tissue Culture Plate (TCP) technique was found to identify the greatest percentage of strong biofilm producers (39.5%), then Tube Method (22.5%), and finally, Congo Red Agar (CRA) method revealed only 14.5%. On the same note moderate biofilm production was the most observed by the Tube Method (48.0%), in comparison to TCP (38.5%) and CRA (24.5%). Conversely, CRA method had the most non/weak producers (61.0%), which means it was less sensitive. Comprehensively, these results indicate that TCP

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method is most sensitive and reliable technique of detecting biofilm formation followed by Tube Method, but CRA is less effective especially in detecting strong biofilm producers.

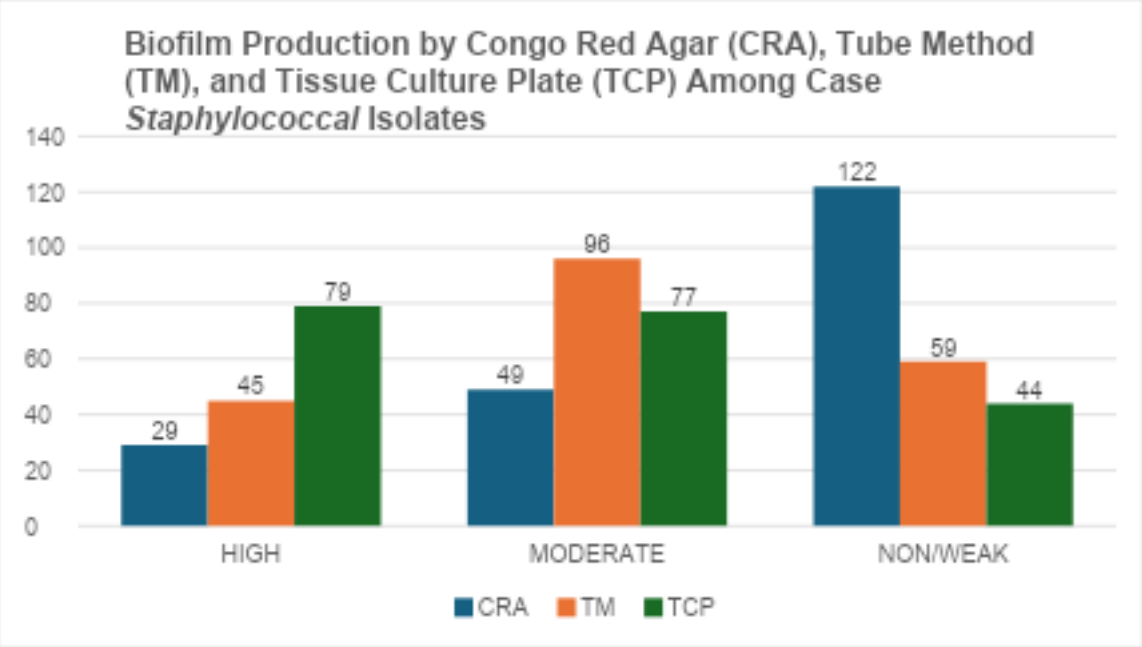

Figure 7: Comparison of Biofilm Detection Methods (CRA vs TM vs TCP)

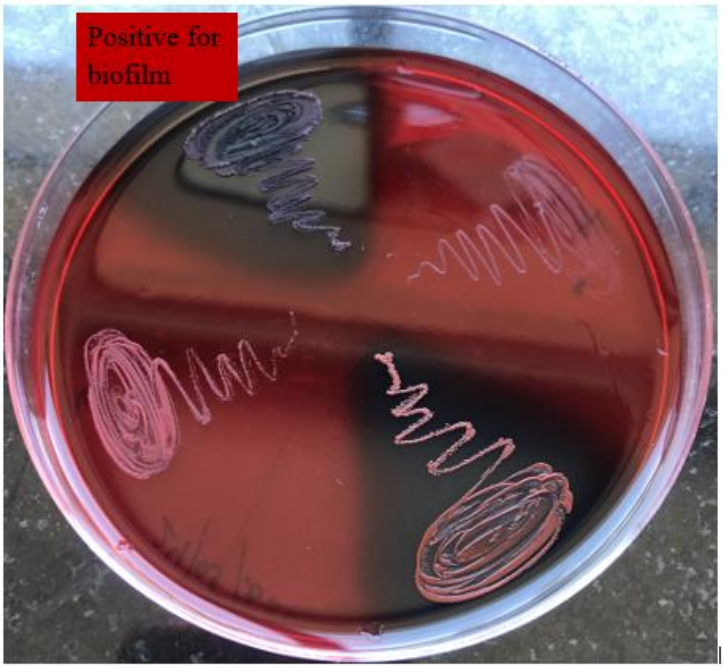

Figure 8: Congo Red Agar (CRA) Method

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Figure 9: Biofilm production on congo red agar

Table 8: Comparison of TM and TCP in detection of biofilm production in cases

	TM	Percent	TCP	Percent
POSITIVE	141	70.5	156	78.0
NEGATIVE	59	29.5	44	22.0
Total	200	100.0	200	100.0

"Chi Square" =113.957; p value<0.001

Interpretation:

The evaluation of the Tube Method (TM) versus Tissue Culture Plate (TCP) method of detecting biofilm production in case isolates revealed that the TCP method detected a larger percentage of biofilm-positive isolates (78.0) than did the Tube Method (70.5%). In line with this, the proportion of biofilm negative isolates was less with TCP (22.0%) compared to TM (29.5%). These results suggest that although both techniques are useful in the detection of biofilm, TCP method is more sensitive and has more detection limits indicating that it is the technique of choice as the gold standard in measuring biofilm formations in Staphylococcus isolates.

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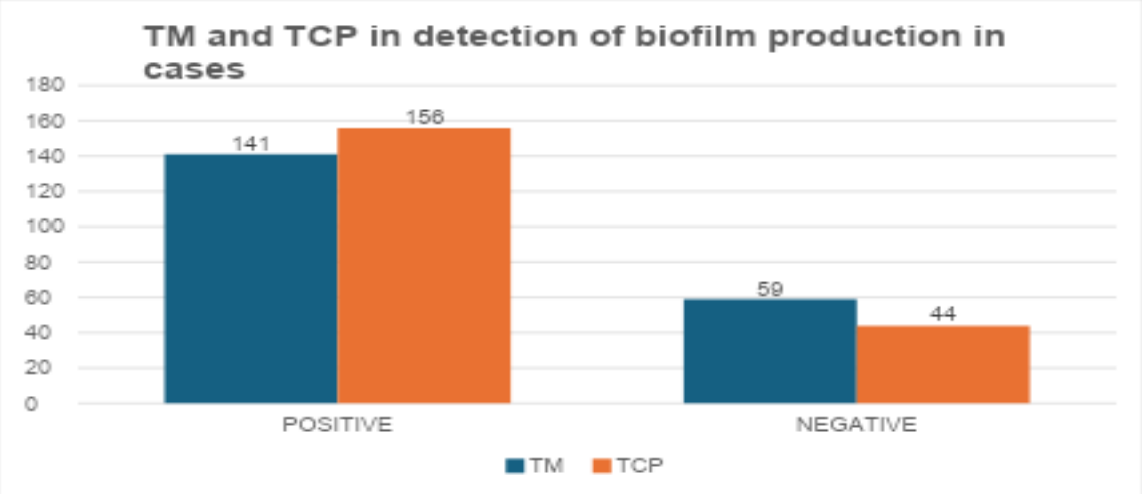

Figure 10: Comparison of TM and TCP for Biofilm Detection

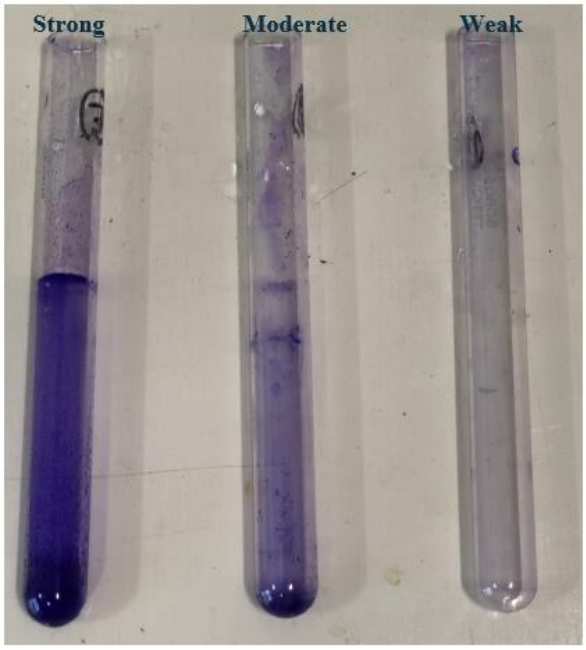

Figure 11: Tube Method (TM)

Table 9: Species distribution of methicillin-resistant and methicillin-sensitive amongst various isolates of Staphylococcus in cases

		MRS	MSS	Total
ORGANISM	S.aureus	97	46	143
		67.8%	32.2%	100.0%
	S.epidermidis	21	15	36
		58.3%	41.7%	100.0%

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	S.haemolyticus	11	0	11
		100.0%	0.0%	100.0%
	"S.hominis"	4	2	6
		66.7%	33.3%	100.0%
	S.lugdunensis	1	0	1
		100.0%	0.0%	100.0%
	"S.saprophyticus"	0	3	3
		0.0%	100.0%	100.0%
Total		134	66	200
		67.0%	33.0%	100.0%

Chi square = 13.269; p value = 0.021

Interpretation:

The prevalence of methicillin resistance among the Staphylococcus isolates in cases revealed that most of the isolates (67.0 percent) were methicillin-resistant with only a minority (33.0 percent) being methicillin-sensitive. S. aureus contained the greatest number of isolates with 67.8 percent being methicillin-resistant, which demonstrates a great load of MRSA. In the same vein, S. epidermidis had a 58.3% methicillin resistance, with S. haemolyticus and S. lugdunensis displaying a hundred percent resistance (100%), indicating close correlation to resistant infections. Conversely, S. saprophyticus isolates were all methicillin sensitive (100%). Overall, the results reveal that methicillin resistance is highly common in most Staphylococcal species especially in clinically significant isolates, highlighting the increasing problem of antimicrobial resistance in these infections.

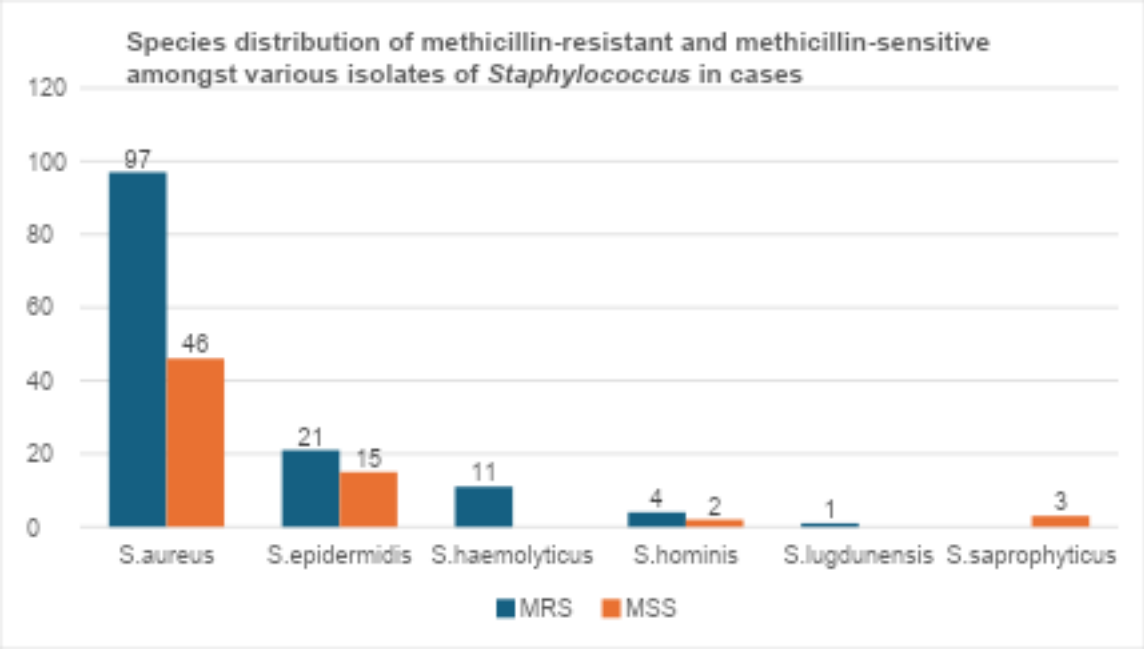

Figure 12: MRSA vs MSSA Distribution

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Table 10: Biofilm Production Among Methicillin-Resistant Staphylococcus Isolates in cases

		BIOFILM PRODUCTION		Total
		NEGATIVE	POSITIVE	
ORGANISM	S.aureus	19	78	97
		19.6%	80.4%	100.0%
	S.epidermidis	5	16	21
		23.8%	76.2%	100.0%
	S.haemolyticus	2	9	11
		18.2%	81.8%	100.0%
	S.hominis	0	4	4
		0.0%	100.0%	100.0%
	S.lugdunensis	0	1	1
		0.0%	100.0%	100.0%
Total		26	108	134
		19.4%	80.6%	100.0%

Chi square = 1.477; p value = 0.831

Interpretation:

The biofilm production among methicillin-resistant Staphylococcus isolates showed that methicillin resistance and biofilm production were highly correlated. In general, 80.6% of the isolates resistant to methicillin were biofilm producers, and only 19.4% were non-producers. Of the species, S. aureus was 80.4% biofilm positive, followed by S. haemolyticus (81.8) and S. epidermidis (76.2). It is interesting to note that S. hominis and S. lugdunensis showed 100 percent biofilm production albeit in lesser amounts. These results reveal that most methicillin-resistant strains are mainly robust biofilm formers, which points to their increased virulence and persistence and the clinical difficulty of treating such infections because of combined resistance and biofilm-mediated protection.

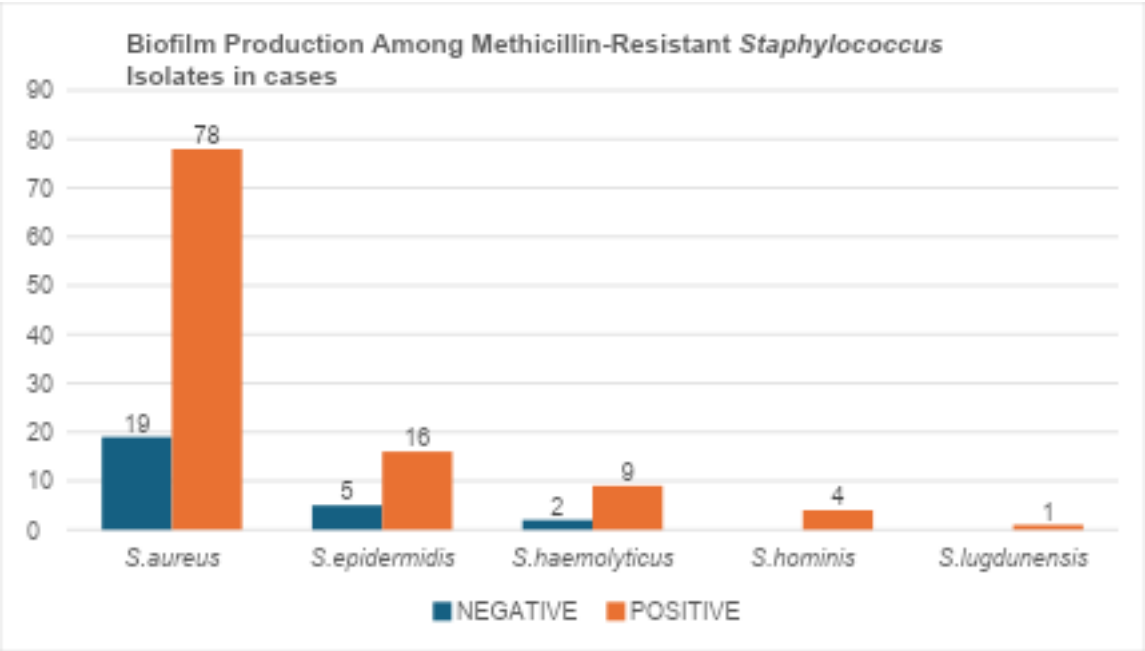

Figure 13: Biofilm Production in MRSA Isolates

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Table 11: Antibiotic resistance pattern in biofilm producers

Antibiotics	No. of resistant isolates	Percentage
Cefoxitin	116	46.4
AZM	30	12
Amikacin	2	0.8
Cefazolin	26	10.4
Clindamycin	78	31.2
Gentamicin	20	8
Levofloxacin	22	8.8
Ampicillin	76	30.4
Cotrimoxazole	35	14
Ciprofloxacin	3	1.2
Erythromycin	113	45.2
Penicillin G	153	61.2
Rifampin	9	3.6
Minocycline	3	1.2
Linezolid	4	1.6
Moxifloxacin	19	7.6
Tetracyclin	15	6.0

Interpretation:

The pattern of antibiotic resistance in the biofilm-producing Staphylococcus isolates showed that the isolates were very resistant to several widely used antibiotics. Penicillin G showed the greatest resistance (61.2%), followed by Cefoxitin (46.4%), and Erythromycin (45.2%), which is significant to note that the burden of 8-lactam and macrolide resistance was high. Clindamycin (31.2%), and Ampicillin (30.4%), had moderate resistance, and lower resistance was observed with other antibiotics like Cotrimoxazole (14.0%), Azithromycin (12.0%), and Cefazolin (10.4%). Interestingly, low resistance was reported against Amikacin (0.8%), Ciprofloxacin (1.2%), Minocycline (1.2%), and Linezolid (1.6%) indicating that these would remain effective. All in all, the results suggest that isolates that form biofilms have an impressive amount of multidrug resistance, especially against the most common antibiotics, making it difficult to treat and making it necessary to consider specific antimicrobial treatment.

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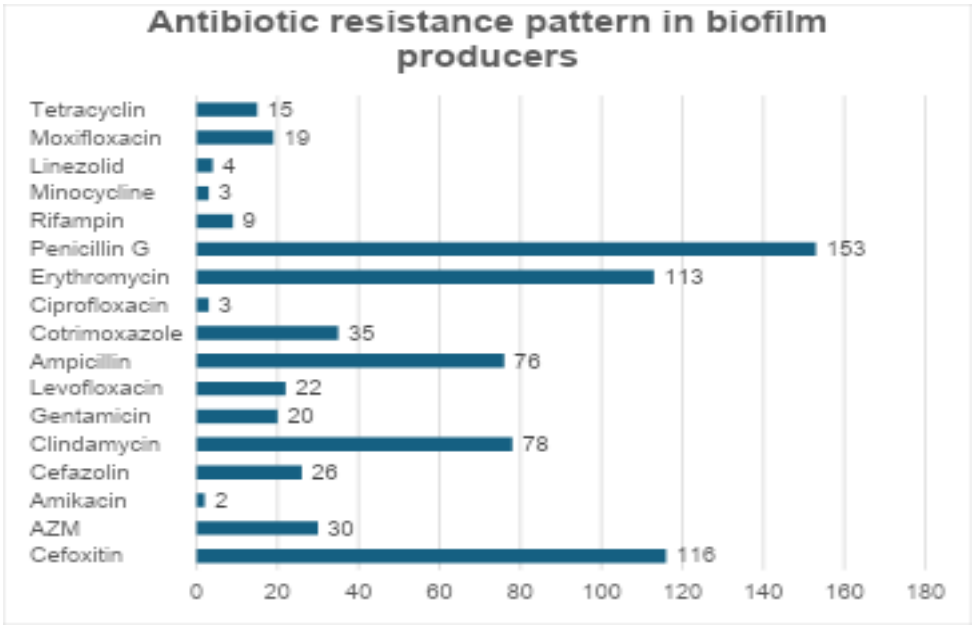

Figure 14: Antibiotic Resistance in Biofilm Producers

Table 12: Antibiotic sensitivity pattern of various Staphylococcus isolates from patients

		Organism							
		S.epidermidis	**S.aureus**	**S.epidermidis**	**S.haemolyticus**	**S.hominis**	**S.lugdunensis**	**S.saprophyticus**	**Total**
Antibiotics	CEFOXITIN	0	63	15	0	3	0	3	84
	AZM	0	17	2	0	0	0	0	19
	AMIKACIN	0	1	2	0	0	0	0	3
	CEFAZOLIN	S	0	3	6	0	3	0	1
	CLINDAMYCIN	0	97	23	2	7	0	3	132
	DAPTOMYCIN	0	0	2	2	1	0	0	5
	GENTAMICIN	0	30	20	4	8	1	2	65
	LEVOFLOXACIN	0	8	7	1	2	0	0	18
	AMPICILLIN	0	12	2	0	1	0	0	15
	COTRIMOXAZOLE	1	88	19	7	2	1	3	121
	CEPHALEXIN	0	1	0	0	0	0	0	1
	ERYTHROMYCIN	0	59	8	0	2	0	1	70
	NITROFURANTOIN	0	37	5	0	0	0	5	47

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	NORFLOXACIN	0	3	0	0	0	0	0	3
	PENICILLIN G	0	9	5	0	0	0	0	14
	RIFAMPIN	0	9	22	5	8	0	0	44
	MINOCYCLINE	0	38	7	0	0	0	1	46
	LINEZOLID	1	178	45	9	8	0	5	246
	MOXIFLOXACIN	0	26	13	2	4	1	0	46
	TETRACYCLINE	1	110	26	6	5	1	1	150
	CIPROFLOXACIN	0	5	3	1	1	0	0	10

Interpretation:

The pattern of antibiotic sensitivity of Staphylococcus isolates proved to have a significant variation in the various species. The highest sensitivity (246 isolates) was exhibited by linezolid on nearly all species, which suggested that it was very effective against S. aureus and coagulase-negative staphylococci. Likewise, tetracycline (150 isolates), clindamycin (132) and cotrimoxazole (121) were found to be sensitive. Gentamicin (65), erythromycin (70) and nitrofurantoin (47) were moderate. Conversely, antibiotics like ciprofloxacin (10), norfloxacin (3) and cephalexin (1) were found to be less sensitive. S. aureus provided the greatest number of sensitive isolates to most antibiotics, followed by S. epidermidis and other CoNS, indicating that although various antibiotics are still effective, resistance to commonly used antibiotics continues to be an issue requiring careful antibiotic choice based on the pattern of susceptibility.

Table 13: Association of biofilm producing Staphylococcus species with Chronic Disease

Chronic Disease	Biofilm producers	Percentage
Present (n=78)	74	94.87
Absent (n=172)	91	52.90
Total	165	

Interpretation:

There was a strong relationship between the production of biofilm and association with chronic disease in Staphylococcus isolates. Of the patients with chronic illness (n=78) 94.87% of the isolates (74 isolates) were biofilm producers and in the patients that did not have chronic illness (n=172) 52.90% (91 isolates) of the patients showed biofilm generation. This difference suggests that the production of biofilm is much more abundant in patients with underlying chronic illnesses, implying that comorbidities can predispose the individuals to infection by biofilm-forming organisms. These results indicate the presence of chronic diseases as a significant risk factor of staphylococcal infections that are persistent and difficult to treat.

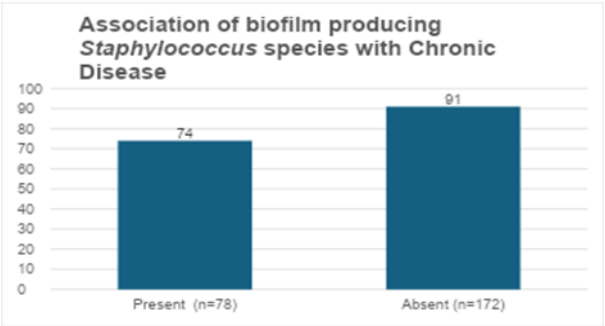

Figure 15: Biofilm vs Chronic Disease Association

4. Discussion

This current research identifies bi-film formation as an important phenomenon in Staphylococcal isolates and its close correlations with antimicrobial resistance, especially in hospital patients. The percentage of

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isolates of the cases producing biofilms (78.5) was significantly higher than that of controls (16.0), showing that the formation of biofilms is mainly linked to nosocomial infections and device-related issues. Similar results were published by Howden et al. (2023) who highlighted that Staphylococci have increased pathogenicity in hospitals since they are able to survive on medical equipment and circumvent the host defense. The longer hospital stays seen in the cases also supports this association since, long hospitalization offers good environment in the establishment and survival of biofilms.

The preponderance of Staphylococcus aureus (71.6) among isolates in the current study is in line with previous studies by Cheung et al. (2021) who reported that S. aureus is the most clinically relevant species because of the wide range of virulence factors and adaptability. Also, the coagulase-negative Staphylococci, including S. epidermidis also exhibited significant biofilm formation, which is consistent with the results of Franca (2023), who claimed that they play a significant role in device-related infections. Despite the biofilm production being seen in a variety of species, statistical analysis showed no significant differences, implying that biofilm production is a ubiquitous virulence factor in Staphylococci and not species specific.

The assessment of phenotypic approaches showed that Tissue Culture Plate (TCP) method identified the greatest number of biofilm producers (78.0%), and the Tube Method (70.5) and Congo Red Agar (39.0) identified the lowest number of biofilm producers. This fact supports the results of Sultan and Nabiel (2019), who have stated that TCP is the most sensitive and reliable technique to detect biofilms since it is a quantitative method. The decreased sensitivity of CRA in the current study is also in line with past studies, which report that CRA can underestimate biofilm production because of variation in colony morphology and interpretation criteria. The high correlation between TM and TCP also indicates that TM can be an effective screening tool in resource-restricted environments, albeit it might not identify all biofilm-producing isolates.

A remarkable observation of this research is that the most common cases (67.0 percent) are methicillin-resistant Staphylococcus isolates, which is like other studies by Espadinha et al. (2013), indicating that the MRSA has been widely distributed in hospitals and the community. The close interrelation between resistance to methicillin and biofilm formation (80.6% of MRSA isolates) confirms the idea that biofilm formation may also play a role in increasing antimicrobial resistance. Almatroudi (2025) made the same observations and emphasized that biofilms not only promote the transfer of horizontal genes but also shield bacteria against the penetration of antibiotics, thus, fostering multidrug resistance. It is however unfortunate that the statistical significance of the variation in biofilm production between the various MRSA species was not statistically significant implying that resistance and biofilm formation are closely related but not species-specific.

The pattern of antibiotic resistance in the current research showed that Penicillin G (61.2%), Cefoxitin (46.4%), and Erythromycin (45.2%) had high resistance and thus, the moderate effect of the widely used antibiotics. These results are congruent with the previous research by Gnanamani et al. (2017), who stated a high level of resistance in Staphylococcal isolates because of the extensive use of antibiotics. On the contrary, Linezolid showed the greatest sensitivity to all the isolates, which was also observed by Tuon et al. (2023), who found Linezolid to be a promising therapeutic agent against biofilm-associated infections. The comparatively low resistance to various drugs including Minocycline and Ciprofloxacin also indicates that they may be useful as alternative treatment.

Of particular interest is the relationship between biofilm production and chronic disease that was found in the present study, where 94.87% of patients with comorbid conditions harbored biofilm-producing isolates. The finding is consistent with Percival et al. (2015), who indicated that the presence of chronic conditions like diabetes and renal disorders results in an immunocompromised environment to support biofilm persistence and chronic infection. Moreover, the prevalence of biofilm formation in device-related samples (catheter tips and drain samples) supports the importance of medical devices as a key reservoir of biofilm-related infections as outlined by Di Domenico et al. (2022).

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In general, the results of this research are very clear to back the hypothesis that biofilm formation is an important virulence factor that leads to persistent infection, long hospitalization, and augmented antimicrobial resistance among the Staphylococcal isolates. The better results of the TCP technique emphasize the significance of the correct biofilm testing in clinical laboratories and the high rates of MRSA and antibiotic resistance warn about the necessity of prudent use of antibiotics and effective infection control methods. These findings agree with the available literature and further support the clinical importance of biofilm-associated infections in health care facilities.

5. Conclusion

The current study shows that biofilm formation is a significant virulence factor among the isolates of Staphylococcal, especially in hospital-acquired infections, and its prevalence is significantly high among the cases compared to the controls, which implies that biofilm formation is strongly associated with nosocomial conditions, prolonged hospital stay, and device-related infections. The most common organism was Staphylococcus aureus and coagulase-negative staphylococci that lead to biofilm-related infections. The Tissue Culture Plate (TCP) method was the most sensitive and reliable method of detection followed by the Tube Method and the least sensitive method was Congo Red Agar. Methicillin resistance was found in a high percentage of isolates and biofilm-producing strains were more resistant to frequently used antibiotics, particularly β-lactams and macrolides, which illustrate the role of biofilm in antimicrobial resistance, although Linezolid was still highly effective. Also, the association between biofilm production and chronic diseases was found to be strong, and it is supposed that immunocompromised patients are more susceptible. In general, Staphylococci form biofilms can be a serious clinical problem, and their detection, rational use of antibiotics, and strict measures of infection control are the key to successful control.

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