Detection of biofilm formation in multi-drug resistant *Staphylococcus aureus* among clinical isolates in Zaria metropolis, Kaduna, Nigeria

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Abstract

Antimicrobial resistance are associated with several virulence factors such as biofilm. The biofilm formation ability of *S. aureus* complicates effective therapeutic management of infections.

In this research, we intend to observe biofilm formation in *S. aureus* using two *in vitro* phenotypic methods: tissue culture plate and Congo red agar as well as biofilm associated genes *Ica A B & D* and *16SrRNA*. A total of 150 presumptive coagulase positive *staphylococcal* isolates from all specimens (blood, urine, high vaginal swab, wound swab, ear swab, endocervical swab) submitted to the microbiology laboratory of the selected hospitals were collected. Seventy-three (73) or 48.7% of the isolates were identified as coagulase positive *Staphylococcus* using microbiology standard methods (tube coagulase test, growth on mannitol salt agar & Dnase agar). Using microgen *staph* identification kit, the isolates were further characterized into various *Staph spp.* Disc agar diffusion method was used for eleven (11) antibiotics susceptibility test while vancomycin screening agar and BMD (Broth Micro dilution) MIC test was used for vancomycin susceptibility evaluation. Indirect observation of biofilm was performed using congo red agar (CRA) and Microtitre plate assay method. PCR and sequencing were conducted on the isolates to molecularly detect the presence of *16SrRNA, IcaA, IcaB, IcaD genes.*

Susceptibility of *S aureus (21%)* isolates tested against 12 different categories of antibiotics shows 4(27%) not multidrug resistant (MDR), 7(47%) were MDR and 4 (27%) were extensively drug resistant (XDR). High percentage (74%) of Multiple antibiotic resistant index at ≥0.3 suggested that the isolates originated from an environment where antibiotics are often used. Biofilm assessment of MDR *S aureus* isolates revealed that 9(82%) and 11 (100%) of the isolates were biofilm formers using Congo Red Agar method and Microtiter plate assay respectively. All isolates amplified with *16SrRNA* primer signifying all are *S. aureus*, 100% of the isolates amplified with *IcaA*, 90% isolates amplified with *IcaD*, and 50% isolates amplified with *IcaB*. We can conclude that there is high prevalence of Biofilm forming *S. aureus* strains in the hospitals studied. This result should be born in mind by clinicians while prescribing medication for *S. aureus* positive conditions. Hospital managements should be proactive in preventing the preservation of the biofilm formers in their hospital environment through adequate sanitary and disinfection protocols. It is also necessary for clinician to note that nitrofurantoin, linezolid and vancomycin were most effective against the biofilm producing *S. aureus* and microtitre plate assay method was

observed to be most reliable assay in the study of biofilm.

Keywords: MDR, *S. aureus*, MAR, isolates, nosocomial, biofilm

Introduction

Bacteria have developed many ways by which they become resistant to antimicrobials. Phenotypic resistance, such as biofilm formation appears as a result of exposure of bacteria to antibiotics (Insha *et al*.,2022).
Most nosocomial infections (80%) are biofilm-associated infections, and the responsible pathogens today have high levels of resistance to most existing antibiotics commonly used in the hospitals such as beta lactams (amoxicillin, ampicillin) and the third-generation antibiotic cephalosporin. (Ibukun *et al*., 2019; Eduardo *et al*., 2016).
Biofilm formation is one of the resistance strategies of many pathogenic bacteria which makes them more difficult to treat than their planktonic counterparts (De *et al*., 2016).
Bacteria protected within biofilm exopolysaccharides are up to 1,000 times more resistant to antibiotics than planktonic cells (free-floating) (Lowrence *et al*., 2018) This poses serious challenges to regimen selection and severely complicates treatment options **(**Yelin *et al*., 2018).
S. aureus is the leading organism in that aspect that is responsible for a number of community acquired and nosocomial infections. It is the most frequent germ found in biofilms related infections worldwide (Maneesha *et al*., 2019). Biofilms are organized assemblage of sessile bacterial cells protected by an extracellular matrix (ECM). In clinical scenario *S. aureus* forms biofilms on the surfaces of the catheters (intravenous catheters, urinary catheters, dialysis catheters etc.) and implanted medical devices (fluid shunts, joint prostheses and pacemakers etc). *S. aureus* in the biofilms are resistant to antibiotics and innate host defence (Maneesha *et al*., 2019). The biofilm formation ability of *S. aureus* complicates effective therapeutic management of infections. This study showed, for the first time, the prevalence of biofilm forming strains of *S. aureus* amongst *S. aureus* positive specimens collected from two hospitals located in Zaria, Kaduna state.

Methods

Study Area

This study was carried out using two selected hospitals within Zaria metropolis. Two hospitals (ABUTH Shika and ABU Medical Centre Main campus Zaria, Kaduna State) were selected for this study based on their patients' population, location and representatives' area of Zaria metropolis.

Collection of clinical Isolates

A total of 150 suspected coagulase positive *staphylococcal* isolates from all specimens (blood, urine, high vaginal swab, wound swab, ear swab, endocervical swab) were sampled through the microbiology unit of the selected hospitals over a period of 6 months and transported in a sterile ice park to Pharmaceutical Microbiology Lab Ahmadu Bello University (ABU), Zaria. Ethical clearance was collected from the Health Research Ethics Committee, ABUTH Shika and Commitee On Use of Human Subject For Research, ABU Medical Centre, Samaru Zaria.

Identification of *S. aureus* isolates

Microbiology standard procedures ((tube coagulase test, growth on mannitol salt agar & Dnase agar) Microgen *Staph* identifcation kit (bioMerieux, Inc, Durham, USA) was used to identify the *Staph spp* isolates. The procedure was carried out according to the manufacturer's instructions

Antibiotic susceptibility test

Kirby–Bauer Disk diffusion tests was performed for each of the isolates previously identified following the method recommended by the Clinical Laboratory Standard Institute. A disk containing the following antibiotics, gentamicin (10µg), ceftaroline (30µg), trimethoprim/sulphamethoxazole (1:19) (25µg), ciprofloxacin (5µg), chloramphenicol (30µg), cefoxitin (30µg), erythromycin (30µg), nitrofurantoin (30µg), clindamycin (30µg), vancomycin (30µg), doxycycline (30µg), linezolide (30µg) (Oxoid Ltd. Basingstoke, London) was used.

Determination of multiple antibiotics resistance (MAR) index

The Multiple Antibiotic Resistance (MAR) index was determined for each isolate by dividing the number of antibiotics to which the organisms is resistant to by the total number of antibiotics tested (Paul *et al*., 1997; Christopher *et al*., 2013).

$$\text{MAR Index} = \frac{\text{Number of antibiotics to which isolate is resistant}}{\text{Total number of antibiotics tested}}$$

Determination of vancomycin susceptibility

The vancomycin minimum inhibitory concentration (MIC) of *S. aureus* was determined based on 0.5 McFarland standards by using Micro dilution Broth and Vancomycin screening agar 6µg/ml BHI (Brain Heart Infusion agar) CLSI 2020.

Visible growth was recorded after 24 hours incubation at 37°C in each concentration.

Assessment of biofilm formation using Congo red agar (CRA) Based Method

The agar medium to be used was prepared by adding 37 g of the Brain Heart Infusion (BHI) powder, 50 g of sucrose and 10 g of agar in 1 L of distilled water. The mixture was then autoclaved for 15 min at 121° C. The agar solution was cooled down to about 50° C and a solution of Congo red (8 g/L) was added and mixed. The media was poured into the Petri dishes and allowed to solidify. The solid plates were inoculated with the microorganisms and incubated at 37° C for 24 h. The plates were read the next day and the organisms considered positive (biofilm-producers) if black colonies were observed on the Congo red agar and negative (non-biofilm producers) if pink, or red-orange colonies were observed on the Congo red agar (Idrees *et al*., 2021).

Microtitre plate assay method

Three wells of sterile 96-microtiter polyester U-bottomed plate were filled with 200 µl of bacterial suspension (dilution 1:100 with fresh medium). Negative control contained broth only. After incubation for 24 h at 37°C, wells were washed three times with 250 µl of distilled Water. After 15 min, plates were stained for 5 min with 0.2 ml of 2% crystal violet per well. Excess stain was removed and rinsed off by placing the plates under running tap water. The plate was air-dried.

The adherent cells were resolubilized with 160 µl of 95% (V/V) methanol per well (Christensen *et al*., 1985). The optical density (OD) of each was measured at 570 nm.

The adherence ability of tested isolates was classified into four categories based on the obtained OD as strongly adherent (OD570 ≥ 3.0) +++, moderately adherent (OD570 ≥ 1.5– 2.0) ++, weakly adherent (OD570 ≤ 0.5– 1.0) +, and nonadherent (OD570 < 0.5) - , (OD570 of negative control). (Alcaráz, Satorres, Lucero, & Centorbi, 2003).

Molecular analysis of MDR *S. aureus*

The preparation of the bacteria cell was carried out using the method described by (Dubey, 2009). An overnight culture of the bacteria isolates was subcultured in LB broth for 48 h. This is to ensure increased cell mass and yield. DNA extraction was carried out

according to Zymogen[R] manufacturer protocols. Detection of biofilm associated genes using primers**:** *IcaA, IcaB, IcaD,* including *16SrRNA* primers were carried out using PCR as shown in table 1.
PCR was performed with the following thermal settings conditions: 5 min at 94°C for initial enzyme activation, followed by 40 cycles of amplification consisting of denaturation at 94°C for 1 min for *Ica genes*, annealing at 57°C and extension at 72°C for 1 min

Primers	Sequence ($5^0 \rightarrow 3^0$)	Amplicon Size (bp)	References
16S rRNA Gene	F (5′-AGA GTT TGA TCC TGG CTC AG-3′) R (5′ TAC GGT TAC CTT GTT ACG ACTT-3′)	700	Gumaa *et al* 2021
icaA (F)	ACACTTGCTGGCGCAGTCAA	188	Barbara *et al*.,2018
icaA (R)	TCTGGAACCAACATCCAACA		
icaD (F)	ATGGTCAAGCCCAGACAGAG	198	Barbara *et al*.,2018
icaD (R)	AGTATTTTCAATGTTTAAAGCAA		
icaB () (R)	ATCATTAGGTAAAATGTCTGGACATGATCCA	433	Barbara *et al*.,2018
(F)	GCATCAASTGTATTGGATAGCAAAAGC		

Statistical analysis:

Data obtained in this study were expressed as Mean ± Standard Deviation (SD) using SPSS version 20. Results were recorded as averages and percentages.

Results

A total of 73 isolates were identified as coagulase positive *Staphylococcus species* in which *S aureus* 15 *(21%),* were isolated from all specimens (blood, urine, high vaginal swab, wound swab, ear swab, endocervical swab) submitted to the laboratory microbiology unit of the selected hospitals.
Nine (60%) of the 15 positive samples were isolated from wound. The remaining 6 were isolated from blood 2(13%), ear 2(13%) and High vaginal swab 2(13%). As shown in table 1.
Overall, multi drug resistant (MDR) isolates were 11(73%) with majority from wound 9 (81.8%) and blood samples 2(18.2%). The overall antimicrobial susceptibility revealed linzolide, nitrofurantoin (73%) and Vancomycin (67%) to be the efficacious and promising drug in MDR against *S aureus* infections whereas high resistance was observed among the MDR isolates, Cefoxitin 73% (MRSA) and doxycyline 73%, Sulphamethoxazole trimethoprim 67% and ciprofloxacin, 60% as shown in table 2.
MAR (Multiple antibiotic resistance) index revealed 11 isolates (74%) with MAR index greater than 0.2 suggested that the isolates originated from an environment where

antibiotics are often used and 4 (26%) isolates with MAR index less than 0.2.

Biofilm assessment of MDR *S aureus* isolates revealed that 9(82%) and all MDR 100% were biofilm formers using Congo Red and Microtiter plate assay respectively as shown in table 4.

All isolates at polymerase chain reaction PCR amplified with *16SrRNA* fig1 signifying all are *S. aureus*, 100% amplified with *IcaA* fig2, 90% amplified with *IcaD* fig3 and 50% amplified with *IcaB*. Fig4. Signifying they are carrying the intercellar adhesin genes responsible for biofilm formation.

Source distribution of *S. aureus*

Table 1; *S. aureus* was mostly isolated from wound, HVS, ear and blood

S/N	Sample source	ABUTH		ABUMEC		Total	
		No	%	No	%	No	%
1	Blood	2	13	0	0	2	13
2	Ear	2	13	0	0	2	13
3	HVS	2	13	0	0	2	13
4	Wound	9	60	0	0	9	60
	TOTAL	15	100	0	0	15	100

Antibiotics resistant profile of *S. aureus* N=15

Table 2; Antibiogram analysis of isolated *S. aureus* strains with respect to different antimicrobial categories revealed resistance pattern ranging from 27 to 73%.

S/No	Antibiotics	I (%)	R (%)	Total (%) n=15
		N (%)	N (%)	N (%)
1	Cefoxitin	0(0)	11(73)	11 (73)
2	Chloramphenicol	2(13)	4 (27)	6 (40)
3	Ceftaroline	4(27)	4(27)	8 (54)
4	Ciprofloxacine	1(7)	8(53)	9 (60)
5	Clindamycin	1(7)	7(47)	8 (54)
6	Erythromycin	3(20)	6(40)	9 (60)
7	Doxycycline	2(13)	9(60)	11(73)
8	Gentamicin	0(0)	6(40)	6 (40)
9	Linezolide	0(0)	4(18)	4 (27)
10	Nitrofurantoin	2(13)	2(13)	4 (27)
11	Vancomycin	3(20)	2(13)	5 (33)
12	Sulphamethoxazole trimethoprim	0(0)	10 (67)	10 (67)

Degree of resistance *S. aureus*

Table 3; Susceptibility of *S aureus (21%)* isolates tested against 12 different categories of antibiotics shows 4(27%) Non MDR, 7(47%) MDR, 4 (27%) XDR. as shown in table 3

S/No	Degree of Resistance	No	%
1	Non- MDR	4	27
2	MDR	7	47
3	XDR	4	27
4	PDR	0	0
	TOTAL	15	100

Biofilm forming *S. aureus* isolate

Table 4; Biofilm phenotype on CRA, and adherence capacity on the Microtitre Plate as shown in table below

Method	**Weak**	**Moderate**	**Strong**	**Total(n)**	**%**
Congo Red	1	0	8	9	82
Microtitre plate assay	1	2	8	11	100

Summary on relationships among biofilm detection methods

Table 5; Relationships among Biofilm phenotype on CRA, slime production, adherence capacity on the Microtitre Plate and presence of adhesion genes (*icaA*, *icaD*, *icaB)* of MDR *S. aureus* is shown below

S/No	**Isolate code**	Biofilm phenotype on CRA	Slim synthesis	Adherence OD570 nm ± SD ∗	Adherence ability	Presence of adhesion genes *icaA*, *icaD*, *icaB*		
1	TH01	Strong black	Producer	3.23 ± 0.986	+++	+	+	+
2	TH02	Strong black	Producer	3.09 ± 0.236	+++	+	+	+
3	TH03	Strong black	Producer	3.06 ± 0.896	+++	+	+	+
4	TH04	Strong black	Producer	3.51 ± 0.889	+++	+	+	-
5	TH05	Bordeaux red	Non-Producer	0.91 ± 0.632	+	+	-	-
6	TH06	Reddish black	Producer	2.01 ± 1.639	++	+	+	-
7	TH07	Strong black	Producer	3.86 ± 0.127	+++	+	+	+
8	TH08	Strong black	Producer	3.09 ± 0.698	+++	+	+	-
9	TH09	Strong black	Producer	3.32 ± 0.325	+++	+	+	+
10	TH10	Bordeaux red	Non-Producer	0.98 ± 0.639	+	+	+	+
11	TH11	Strong black	Producer	3.32 ± 0.645	+++	+	+	-

Susceptible antibiotics in MDR biofilm formers *S. aureus*

Table 6: antibiotics observed to be susceptible to biofilm formers.

S/no	Isolate code	Susceptible antibiotics
1	TH01	S9= DA, CPT, FOX, C, CIP, LZD, E, NI, GM
2	TH02	S9= DA, CPT, C, CIP, LZD, E, NI, VAN, GM
3	TH03	S6= STX, DA, C, LZD, E, NI,
4	TH04	S6= STX, DA, LZD, E, NI, VAN
5	TH05	S5= CPT, C, LZD, NI, VAN
6	TH06	S4= LZD, DA, NI, VAN
7	TH07	S4= LZD, CPT, NI, VAN
8	TH08	S1= GM
9	TH09	S1= CPT
10	TH10	S1= VAN
11	TH11	S1= C

STX Trimethoprim/Sulphamethoxazole (1:19), DA Clindamycin, CPT Ceftaroline, FOX Cefoxitin, CIP Ciprofloxacin, LZD linezolide, E Erythromycin, NI Nitrofurantoin, GM Gentamicin, DO doxycycline, VAN Vancomycin C Chloramphenicol

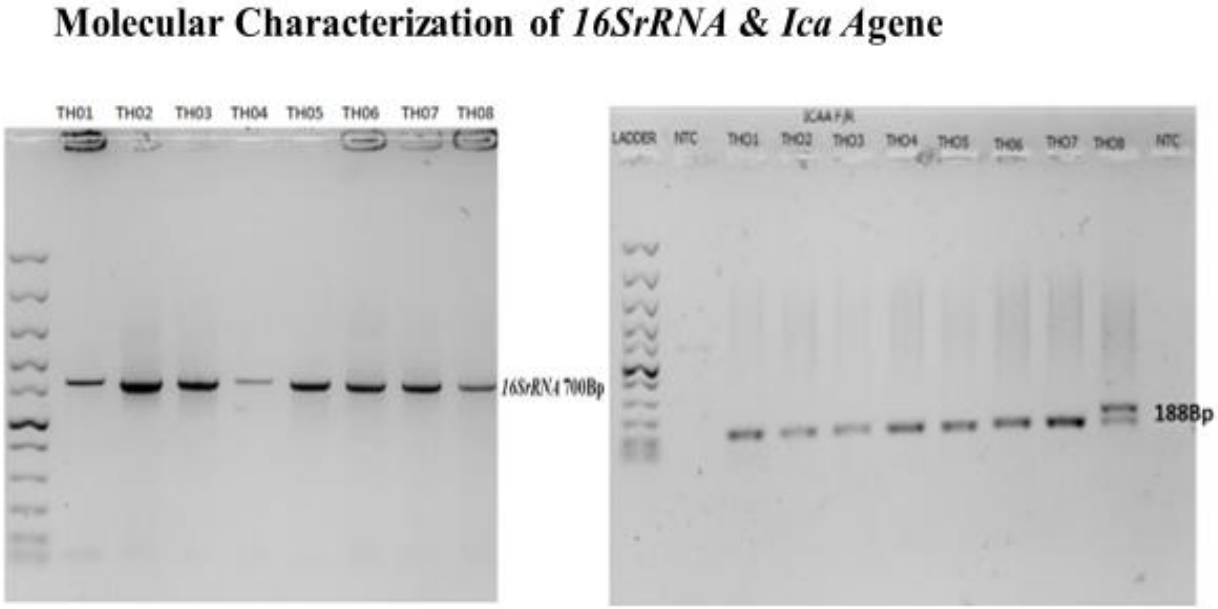

Figure 1: Electrophoretic gel of *16SrRNA* 700Bp amplified from *S. aureus* isolates.

Figure 2: Electrophoretic gel of ***IcaA*** 188Bp amplified from *S. aureus* isolates.

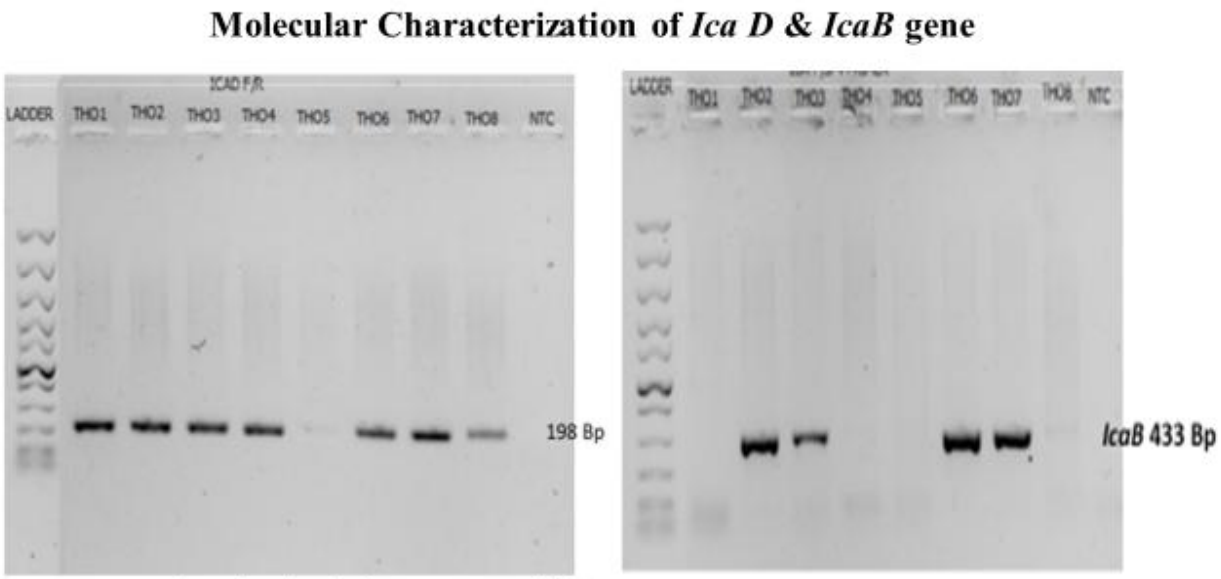

Figure 3: Electrophoretic gel of ***IcaD*** 198Bp amplified from *S. aureus* isolates

Figure 4: Electrophoretic gel of ***IcaB*** 433Bp amplified from *S. aureus* isolates.

Discussion

Biofilm producing *S. aureus* isolated from clinical samples are of clinical significance as biofilm are associated with resistance to antimicrobial agents and chronic infections. So, a reliable and easy method for their diagnosis is necessary (Abdul-helim *et al.,* 2018).

Techniques adopted by Microbiologists to study and evaluate *S. aureus* biofilm are either direct evaluation based on the measurement of thickness of a biofilm, or indirect techniques that quantify through the study its different constituents or the activity of the bacterial cells within the biofilm (Idrees *et al.,* 2021)

Our study tested clinical isolates of multidrug resistant *S. aureus* for their ability to form a biofilm using three screening procedures. The procedures are, microtiter plate assay method is a standard quantitative technique used to detect biofilm production, Congo red Agar method which gives a qualitative, sensitive and positive predictive value and Biofilm-associated genes method through conventional PCR, which presents sharper specificity, sensitivity and time efficient.

Overall, the percentage of multi drug resistant (MDR) isolates observed in these studies was 73% This was within the same range as the 78.3% in Nepal reported by Maharjan *et al. (*2022), 68.0 % in central Nigeria reported by Abdullahi and Iregbu, (2018) and 71.4 % in Iran reported by Eftikhar *et al*., (2017). MDR *S. aureus* isolates are often linked to inappropriate use of antibiotics, whether in human care as in self-medication or in veterinary, and poor or inadequate infection control and prevention practices (Abdulaziz *et al*., 2022). The three reports cited above were all from developing countries where the use of antibiotics may not be under strict control.

Biofilm producing MDR *S. aureus* isolates revealed that 82% of the isolates were biofilm producers when CRA method was performed with 72.7% of the isolates to be strong biofilm producers and 9.3% to be weak biofilm producers. Also, when Microtitre plate Assay (MtP) method was performed, 100% of the isolates had biofilm adherence capacity revealing 72.7% isolates with strong adherence capacity, 18.2% isolates with moderate adherence and 9.1% isolates with weak adherence capacity. Similar finding

(83%) has been reported for CRA method (Cafiso *et al*., 2004) following the addition of glucose 1% w/v in the congo red medium which is consistent to what was carried out in this study. Lower positive percentage (0.9%) has been associated with the exclusion of glucose 1%w/v as was reported by Abdul-Helim *et al.* (2018). Also, sensitivity has been attributed to the technique of autoclaving congo red stain before adding it to the agar.

The result observed using microtitre plate assay in our study (100%) were higher than the 69% reported by Abdul-helim *et al.,* 2018 using the TLC method. this could be attributed to Non specificity with crystal violet dyes, variation in biofilm biomass and washing step (Idrees *et al.,* 2021).

However, Oliveira and Cunha, 2010 reported higher sensitivity of 89% and specificity of 100% for biofilm detection. Furthermore, Andrade *et al*. (2022) reported 94.6% biofilm in MDR *Staph aureus* using microtitre plate assay while Neopane *et al*. (2018) reported 86.7% biofilm in MDR *S. aureus*. Microtiter plate assay method is a gold standard for quantitative detection biofilm production (Andrade *et al*. 2022).

Majority of the isolates in this study were subjected to genotypic detection of *16SrRNA*, *icaA*, *icaD*, and *icaB,* genes through PCR. The data of PCR analysis revealed that, 100% of the isolates amplified with *16SrRNA* signifying that all isolates are *S. aureus*, 100% of the isolates also amplified with *icaA*, 90% of the isolates amplified with *icaD* while 50% amplified with *icaB*.

In line with our results, Andrade *et al*. (2022) reported the carriage of *IcaADB* genes in all MDR *S. aureus* while Gowrishankar *et al*. (2016) reported *icaADBC* genes in 84.13% of isolates.

Although several studies have reported no correlation between biofilm formation and disease outcome in *S. aureus* infections (Kwiecinski *et al*., 2019), many reports demonstrated the significance of surface components in the biofilm of *S. aureus* such as the product of *icaADBC* operon. The polysaccharide, intercellular adhesin (PIA), is a major component of extra polysaccharide EPS in *S. aureus* biofilm. It is cationic in nature and is considered essential for the pathogenicity of *S. aureus*. PIA plays a significant role in colonization, biofilm formation, biofilm-related infections, immune evasion, resistance to antimicrobials and phagocytosis (Abdul-helim *et al.,* 2018).

Being a nosocomial pathogen and due to its ability to form biofilms on both biotic and abiotic surfaces, *S. aureus* infections are complicated and difficult to eradicate. Such surface-related and medical device-associated *S. aureus* biofilms, if not properly sanitised and left unsterilized, can cause infections in patients. It has been revealed from the results that there were different degrees of susceptibility of the biofilm forming *S. aureus* to various antibiotics, Therefore, continuous monitoring of the antimicrobial susceptibility pattern for the selection of appropriate therapy is very crucial.

Conclusion

Biofilm formation in *S. aureus* were detected in this study which poses a therapeutic challenge in treatment of infections. However, nitrofurantoin, linezolid and vancomycin were observed to be effective against the MDR *S. aureus* biofilm producers in this locality. Continuous determination of the antibiotic susceptibility pattern is very necessary for effective regimen selection. Microtitre plate assay method has been shown to be a reliable assay in the study of biofilm forming MDR *S. aureus.*

Conflict of Interest: The authors declare that they have no conflict of interest.

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