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Biofilm Formation and Antimicrobial Susceptibility Pattern of *Staphylococcus aureus* Clinical Isolates from Two Healthcare Facilities in Zaria

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: Antibiotic resistance is a public health challenge worldwide. There is a huge global concern about the increased drug-resistant *S. aureus* and the development of multiple resistance to several drugs. A well-designed surveillance study has been found to be a fundamental approach in the control of antimicrobial resistance.
Objective: This study investigated the biofilm formation and antimicrobial susceptibility pattern of *Staphylococcus aureus* from two healthcare facilities in Zaria.
Methods: A total of 200 presumptive Staphylococcal isolates from clinical specimens were collected and identified by conventional methods. *Staphylococcus aureus* isolates were tested against a panel of antibiotics using the modified Kirby-Bauer disk diffusion method, Methicillin-resistant *Staphylococcus aureus* (MRSA) were tested using cefoxitin disk, and Micro broth dilution method for Vancomycin Minimum Inhibitory Concentration (MIC). The biofilm-forming ability of the isolates were analyzed quantitatively using the microtitre plate method.
Results: Of the 200 presumptive staphylococcal isolates, 22(11%) were *Staphylococcus aureus*. The antibiotic resistance pattern of the isolates shows high resistance to tigecycline (100%), vancomycin (100%), clindamycin (40.9%), and tetracycline (40.9). The occurrence of MRSA in this study was 18.8% and MDR (was 68.2%). The biofilm-forming ability of the *Staphylococcus aureus* isolates is; weak biofilm formers 16 (72.7%), moderate biofilm formers 5 (22.7%), and strong biofilm former 1 (4.5%).
Conclusion: There is need for more research to ascertain the relationship between biofilm formation and antimicrobial resistance in *Staphylococcus aureus*. Close monitoring of antimicrobial resistance is necessary as it helps to design tangible actions that will yield the greatest impact to control the spread of resistant organisms.

Keywords*: Staphylococcus aureus*; Multidrug resistance; Susceptibility; Biofilm

INTRODUCTION

The spread of multidrug resistance (MDR) *S. aureus* is a public health concern. In the last twenty years, efforts have been made worldwide to address the rapid increase in antibiotic resistance, including monitoring the use of antibiotics in hospitals (Allerberger*et al*., 2008; Charani and Holmes, 2019; Tarrant *et al*., 2019). A well-designed quantitative system for the surveillance of spread in antimicrobial resistance is a fundamental approach in the control of antimicrobial resistance (Altorf-Vander Kuli *et al*., 2017). Surveillance of antimicrobial resistance (AMR) monitors changes in microbial populations, permits the early detection of resistant strains of public health importance, and supports the prompt notification and investigation of outbreaks (WHO, 2021).

In 2017, WHO published its list of pathogens for which new antimicrobial development is urgently needed to focus and guide research related to new antibiotics (WHO, 2017). Within the comprehensive list, ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens were designated "priority status" (Oliveira *et al*., 2020). *Staphylococcus aureus* is a major opportunistic human pathogen that causes a wide range of diseases, from mild (furuncles, carbuncles, impetigo, cellulitis etc) to life threatening (endocarditis, sepsis, toxic shock syndrome *etc*) (Tong *et al*., 2015; Gnanamani*et al*., 2017). Acquisition of multi-drug resistance in *S. aureus* represents a major problem in hospital settings (Stefani et al., 2012) and in the community (Matouke and Nour, 2019). The emergence of antibiotic resistance in MRSA strains and unavailability of therapeutic options for managing the MRSA infections remains a challenge to healthcare (Kanbi and Mbe, 2012; Yousefi*et al*., 2017). There is a huge global concern about the increased drug resistant *S. aureus* and development of multiple resistance to several drugs such as penicillins, tetracyclines, macrolides and aminoglycosides (Thati*et al*., 2011; Mohammad *et al*., 2014).

The high prevalence of antibiotic resistance in *S. aureus* clinical isolates is found to be caused by intensive use of topical and systemic antimicrobial agents in health care settings (Zhou *et al*., 2012). *Staphylococcus aureus* strains have two states; planktonic and biofilm states (Parastan*et al*., 2020). Biofilm formation in *Staphylococcus aureus* is a basic human and animal health concern (Achek*et al*., 2020). Compared to bacteria in the planktonic state, bacteria in the biofilm state are significantly more resistant to antibiotics and appear to be multidrug resistant (Wu *et al*., 2019). The matrix is a barrier for the entrance of antibiotics to a deeper layer of bacteria in biofilm (Fisher *et al*., 2017).

Collecting antimicrobial resistance data is an essential approach to defining the scope of the resistance problem, developing interventions that improve the appropriate application of the antimicrobial agents, and decreasing resistance selection pressure (Núñez-Núñez*et al*., 2018). This study investigated the antibiotic susceptibility pattern and biofilm production capability of *S. aureus* clinical isolates from two healthcare facilities in Zaria.

METHODOLOGY

Scope of the study

Presumptive Staphylococcal isolates obtained from specimens submitted to the Medical Microbiology unit of Ahmadu Bello University Medical Centre, Samaru, and Ahmadu Bello University Teaching Hospital, Shika, over a three-month period were collected and analyzed in the Microbiology Laboratory, Department of Pharmaceutical Microbiology, Ahmadu Bello University, Zaria, Nigeria.

Sample Collection

A total of 200 consecutive, non-duplicated presumptive staphylococcal isolates from urine 38(19%), blood 49 (24.5%), sputum 15 (7.5%), urethra 8 (4%), wound 38 (19%), skin 1 (0.5%), ear swab 6 (3%), high vaginal swab (HVS) 28 (14%), Endocervical swab (ECS) 6 (3%), Throat swab 7 (3.5%), eye swab 1 (0.5%), stool 3 (1.5%) submitted to the Microbiology Department of the two selected hospitals were collected over a three-month period from September, 2019 to December, 2019, and transported to Microbiology Laboratory, Department of Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria for further analysis.

Identification and Confirmation of *Staphylococcus aureus* Isolate

Isolates were collected on Nutrient Agar slants, all the isolates were inoculated on Mannitol Salt Agar (MSA) and incubated at 37°C for 24 hours (Alsaimary, 2011). Colonies with distinct features of small yellow were sub-cultured on Nutrient Agar to obtain pure colonies. Preliminary identification of the isolates were based on morphological characteristics such as Gram reaction, shape and arrangement of cells, and biochemical ability to ferment Mannitol, Catalase and Coagulase production using standard microbiological methods. The isolates were confirmed as *S. aureus* on Microgen Staph Identification kit according to the manufacturer's instruction.

Determination of Antibiotic susceptibility of *Staphylococcus aureus* isolates

The modified Kirby-Bauer disc-diffusion method was used to determine the antibiotic susceptibility pattern of the confirmed *S. aureus* isolates to a panel of twelve (12) antibiotics. A sterile swab stick was used to inoculate the standard inoculum (1.5 x 10^8cfu/mL) of the test organisms evenly on the surface of Muller

Hinton agar (MHA) and was allowed to stand for 5 minutes to dry. Commercially prepared disc of Cefoxitin (FOX, 30 μg), Ciprofloxacin (CIP, 5μg), Amoxicillin-Clavulanate (AMC, 30μg), Gentamicin (GEN, 30μg), Clindamycin (DA, 2μg), Erythromycin (ERY, 15μg), Quinupristin-dalfopristin(QD, 15μg), Tigecycline (TGC, 15μg), Tetracycline (TET, 30 μg), Linezolid (LZD, 10μg), Rifampicin (5μg) from Oxoid limited were aseptically placed on the inoculated MHA using a sterile antibiotic disc dispenser (Oxoid Ltd, Basinstoke, Hampshire, England). The plates were then incubated at 37◦C for 18 hours, after which the inhibition zone diameter for each of the antibiotics were measured and interpreted using the interpretative chart provided by EUCAST 2019.

Detection of Vancomycin Resistant *Staphylococcus aureus*Isolates

Standard Microbroth dilution method was used to determine Vancomycin resistance in the *S. aureus* isolates (Balouiri*et al*., 2016). Two-fold dilution of the Vancomycin powder (0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256 µg/mL) was prepared in a Mueller Hinton Broth medium using a 96-well microtitration plate. Each well was inoculated with a standardised microbial suspension adjusted to 0.5 McFarland scale. After mixing, the wells were incubated at 37◦C for 24 hours. To determine the MIC endpoint, the results were interpreted according to EUCAST, 2019.

Determination of Multiple Antibiotic Resistance Index

The Multiple Antibiotic Resistance Index was determined as the ratio of the number of antibiotics to which the *S. aureus* isolates were resistant, to the total number of antibiotics against which the organisms were tested.

Biofilm formation in *Staphylococcus aureus* isolates

The confirmed *S. aureus* isolates were also screened quantitatively for their ability to form biofilm by microtitre plate (MTP) method according to the work of Christensen *et al*., 1985 and modified by Merrit*et al*., 2005. The isolates were grown overnight for 24 hours at 37˚C in brain heart infusion (BHI) broth supplemented with 2% glucose. The cultures were diluted 1 µL in 10 mL medium and 150 µL of the cell suspension was used to inoculate sterile flat-bottomed 96-well polystyrene microtitre plate and incubated for 48 hours at 37℃. After 48 hours, the suspension was poured off and the wells washed three (3) times in three (3) different trays of normal saline to remove any unfixed microbial cell and leave only those fixed to the surface of the wells within a biofilm matrix and dried in an inverted position. The dried wells were stained with 250 µl of 0.1% crystal violet solution in water and incubated at room temperature for 20 minutes. The excess stains were poured off and wells washed three (3) times in three (3) different trays of normal saline and dried for 30 minutes at room temperature. A positive result was seen as the presence of a layer of stained materials adhered to the inner wall of the wells. Interpretation of biofilm production was performed as per the criteria described by Stepanovic*et al*, 2007, and the bacteria were categorized into biofilm nonproducers, or weak, moderate or strong biofilm producers.

Biofilm produced was quantified by adding 250 µL of ethanol-acetic acid (95:5 vol/vol) to destain the wells obtained from the preceding test, then 100 µl from each well was transferred to a new microtitre plate and the optical density (OD) of the solution were measured at a wavelength of 492 nm using a microtiter plate reader.

The uninoculated medium was used, as control, to determine the negative control (OD) and the cut-off value (ODc) = (average OD value of negative control + 3 × standard deviation of negative control). The experiment was repeated three times separately for each strain and the average values were calculated with standard deviation.

RESULTS

Sample collection

The 200 non–duplicate presumptive Staphylococcal clinical isolates obtained were made up of 41 (20.5%) from ABUMC and 159 (79.5%) from ABUTH. The distribution of isolates by source include urine 38(19%), blood 49 (24.5%), sputum 15 (7.5%), urethra 8 (4%), wound 38 (19%), skin 1 (0.5%), ear swab 6 (3%), HVS 28 (14%), ECS 6 (3%), Throat 7 (3.5%), eye 1 (0.5%), stool 3 (1.5%). A total of 115 (57%) were from female patients while 85 (42.5%) were from male patients.

Isolation and Identification of *S. aureus* Isolates.

Preliminary identification by growth on Mannitol Salt agar recorded 162 (81%) growth, while Gram's reaction showed 160 (80%) to be Gram positive Staphylococcal isolates. Catalase test was positive in

151 (75.5%), while coagulase test shows 29 (14.5%) isolates were presumptively *S aureus*. Confirmation test using Staph ID kit showed 22(11%) of the isolates to be *S. aureus*. Of the 22 *S. aureus* clinical isolates, 8 (36.4%) were from male and 14 (63.6%) were from female patients. The age group range of the patients harbouring the isolates are 0 – 17 years 8(36.4%), 18 – 40 years 12 (54.5%), 41 - above years 2 (9.1%) as shown in table 1. Majority of the isolates were from wound 11 (50%), blood 5 (22.7%), and urine 3 (13.6%), as shown in Table 2.

Table 1: Percentage Distribution of *S. aureus* Isolates among Age-Group and Gender

Age range (years)	Subjects harbouring *S. aureus* isolates		Total
	Male	Female	
0 – 17	4(18.2%)	4(18.2%)	8(36.4%)
18 – 40	4(18.2%)	8(36.4%)	12(54.5%)
41 and above	0(0%)	2(9.1%)	2(9.1%)
Total	8(36.4%)	14(63.6%)	22(100%)

Table 2: Distribution of *S.aureus* Isolates by specimen.

Isolate Source	*S.aureus* (n=22) No (%)
Urine	3(13.6)
ECS	1(4.5)
Wound	12(54.5)
Blood	5(22.7)
Ear swab	1(4.5)

Key: ECS – Endo-cervical swab

Antibiotic Susceptibility of *S. aureus* Isolates.

The *S. aureus* isolates were generally resistant to Tigecycline (100 %), Vancomycin (100%), Tetracycline (40.9%), Clindamycin (40.9%) but sensitive to Linezolid (90.9%), Ciprofloxacin (81.8%), Gentamicin (81.8%), Cefoxitin (81.8%), Amoxicillin-Clavulanic acid (77.3%), and Rifampicin (59.1%) while intermediately resistant to Quinupristin-dalfopristin (72.7%), Clindamycin (45.5%) and Erythromycin (38.4%) as shown in figure 2.

AntibioticResistance Pattern of *S. aureus* Isolates

The isolates were classified based on their pattern of resistance. A total of 15 (68.2%) of the *S. aureus* isolates were MDR, 2 (9.1%) were XDR and no PDR *S. aureus* were isolated as shown in figure 3.

Multiple Antibiotic Resistance Index (MARI) of the *S. aureus* isolates

All the *S. aureus* isolates have MAR index ≥ 0.2 indicating they originated from environments where antibiotics are often used (Table 6).

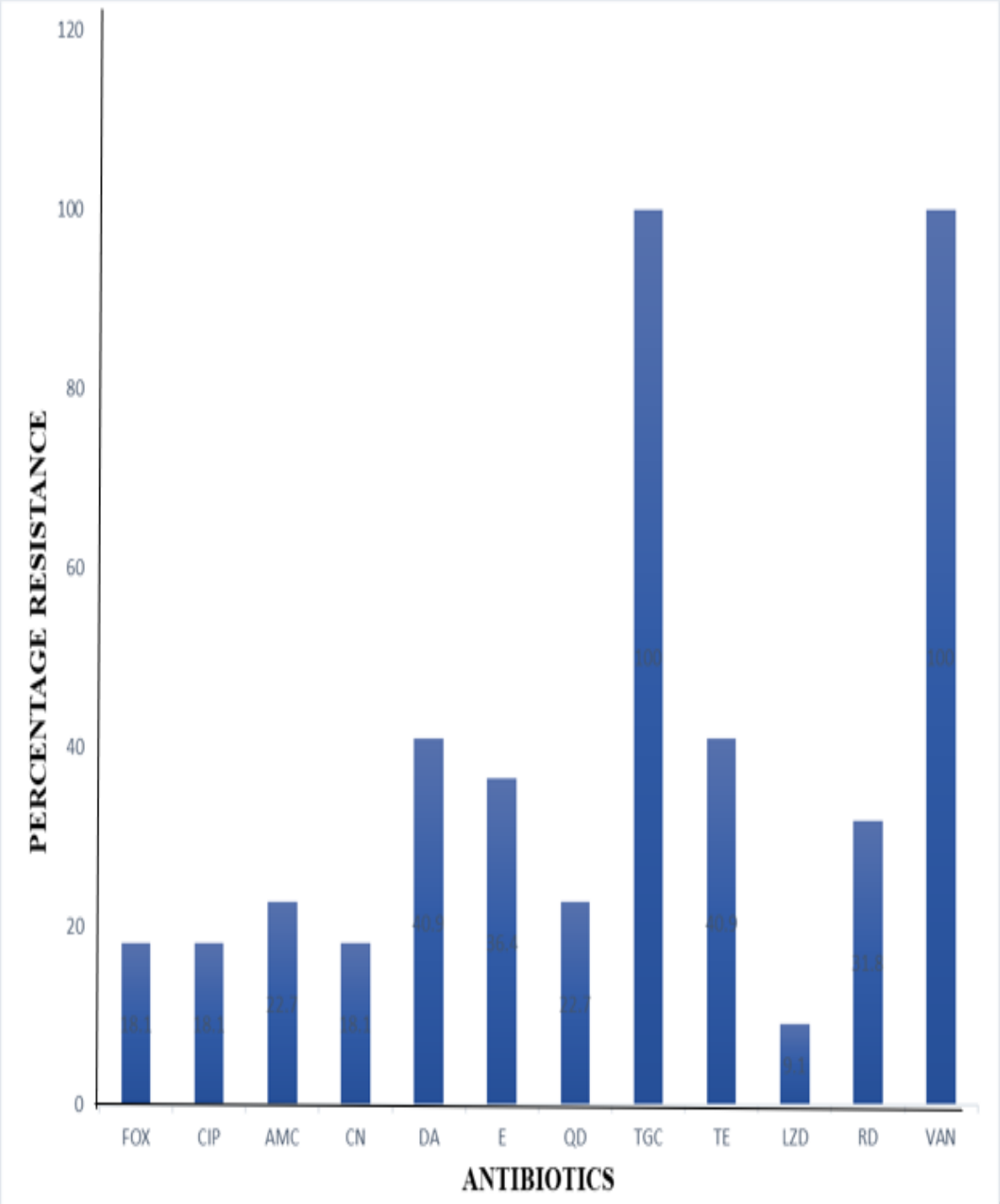

Figure 1: Antibiotic-Resistant Pattern of *S. aureus* Isolates

Key: Cefoxitin (FOX), Ciprofloxacin (CIP), Amoxicillin-Clavulanate (AMC), Gentamicin (CN), Vancomycin (VAN), Clindamycin (DA), Erythromycin (ERY), Quinupristin-dalfopristin(QD), Tigecycline (TGC), Tetracycline (TE), Linezolid (LZD), Rifampicin (RD).

Table 3: Antibiotic Resistance Pattern of *S. aureus* Isolates

Isolate	Resistance Pattern	Number of Antibiotics
Th118	AMC, DA, E, QD, DA, TGC, TE, RD, FOX, VAN	9
Mc022	FOX, CIP, AMC, CN, E, QD, TGC, TE, VAN	9
Th117	CN, DA, E, QD, TGC, RD, FOX, VAN	8
Th136	DA, E, QD, TGC, TE, LZD, RD, VAN	8
Th102	CIP, AMC, CN, TGC, TE, FOX, VAN	7
Mc001	CN, DA, TGC, TE, LZD, RD, VAN	7
Th084	DA, E, TGC, TE, RD, VAN	6
Th130	DA, E, TGC, RD, VAN	5
Th159	CIP, AMC, TGC, TE, VAN	5
Th109	DA, E, TGC, VAN	4
Th122	DA, QD, TGC, VAN	4
Th129	DA, TGC, RD, VAN	4
Th137	CIP, TGC, TE, VAN	4
Th009	AMC, TGC, VAN	3
Mc003	TGC, TE, VAN	3
Th025	TGC, VAN	2
Th043	TGC, VAN	2
Th077	TGC, VAN	2
Th135	TGC, VAN	2
Th157	TGC, VAN	2
Mc009	TGC, VAN	2
Th085	TGC, VAN	2

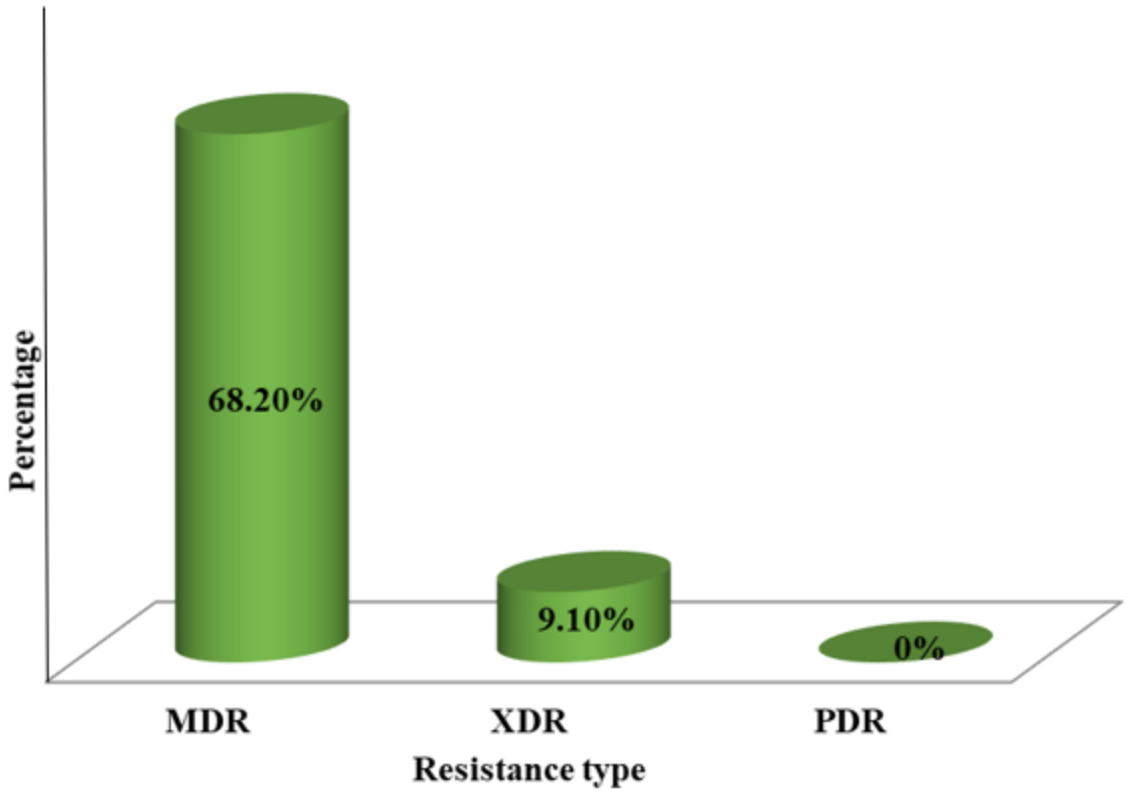

Figure 2: Percentage Distribution of Resistance Types in the *S. aureus* Isolates
Key: Multidrug Resistance (MDR), Extended Drug Resistance (XDR), Pan Drug Resistance (PDR)

Table 4: Percentage Distribution of Methicillin Resistance *S. aureus* Isolates

Sample type	MRSA (%)	MSSA (%)	Total (%)
Urine	0 (0.0)	3 (13.6)	3 (13.0)
ECS	0 (0.0)	1 (4.5)	1 (4.5)
Wound	2 (9.1)	12 (54.5)	12 (83.6)
Blood	1 (4.5)	5 (22.7)	6 (27.2)
Ear swab	1 (4.5)	1 (4.5)	2 (9.0)
	4 (18.2)	18 (81.8)	22 (100.0)

Key: Methicillin Resistant *Staphylococcus aureus* (MRSA), Methicillin Susceptible *Staphylococcus aureus* (MSSA)

Table 5: Multiple Antibiotic Resistance Index (MARI) of *Staphylococcus aureus* Isolates.

MARI	No of Isolates	Percentage %
0	0	0
0.1	0	0
0.2	7	31.8
0.3	6	27.3
0.4	2	9.1
0.5	1	4.5
0.6	2	9.1
0.7	2	9.1
0.8	2	9.1
0.9	0	0
1.0	0	0

Qualitative Analysis of Biofilm Formation in *S. aureus* Isolates.

All of the 22 *Staphylococcus aureus* isolates tested were biofilm formers. The distribution of the biofilm-forming *S. aureus* isolates showed that 11 (54.5%), 5 (22.7), 3 (13.6%), 1 (4.5%), and 1 (4.5%) were from wound, blood, urine, ECS, and ear swab respectively as shown in table 6.

Quantitative Analysis of Biofilm Formation in *S. aureus* Isolates.

The *S. aureus* isolates were classified according to their biofilm-forming ability; non-biofilm formers 0 (0%), weak biofilm formers 16 (72.7%), moderate biofilm formers 5 (22.7%), and strong biofilm former 1 (4.5%) as shown in table 6.

Table 6: Percentage Distribution of the biofilm producing *S. aureus* isolates by source.

Biofilm production	Wound (%)	ECS (%)	Ear swab (%)	Urine (%)	Blood (%)	Total (%)
Non-biofilm formers	0 (0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	**0(0.0)**
Weak biofilm formers	10(44.5)	1(4.5)	1(4.5)	2(9.0)	2(9.0)	**16(72.7)**
Moderate biofilm formers	1(4.5)	0(0.0)	0(0.0)	1(4.5)	3(13.6)	**5(22.7)**
Strong biofilm formers	1(4.5)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	**1(4.5)**
Total	12(54.5)	1(4.5)	1(4.5)	3(13.6)	5(22.7)	22(100)

DISCUSSION

Staphylococcus aureus is an opportunistic pathogen causing many life-threatening infections, which may lead to prolonged hospital stay, additional antibiotic use, increased healthcare-associated costs, high morbidity and mortality (Akhtar Danesh *et al.,* 2019). The incidence of *S. aureus* in this study was 11%, it's almost similar to the work of Garba *et al.* (2018) which reported 14.6% *Staphylococcus aureus* from clinical isolates but lower than the 21.3% incidence reported by Sunday *et al*. (2020) in Keffi, Nigeria. Although this study showed a lower prevalence of *S. aureus* when compared to other studies, it should be considered a pathogen of great concern. The highest incidence (54.5%) of *S. aureus* was found in wound swabs, dissimilar to the findings of Gaire *et al*. 2021 who recorded the highest occurrence in urine (41.8%) but agrees with the findings of Nwoire *et al.* (2013) who recorded a high occurrence of *S. aureus* from the wound (29.4%). This could be because the skin's normal flora which *S aureus* is part of, can easily get access to the wound. In this study, female subjects were found to be more vulnerable (63.6%) to *S. aureus* infections, this agrees with the work of Chibueze *et al*. (2017) in Zaria, and of Ifediora *et al*. (2019) in Abia State but didn't agree with the findings of Kumurya *et al*. (2017) who reported the highest occurrence in males. This could be due to the difference in length of the study period, the number of study sites, and sample size. In this study, the highest frequency of *S. aureus* (54.6%) was observed in the 18 – 40 years age group. This could be because they are the active group and are always exposed to contaminated environments.

Resistance to commonly used antibiotics is often encountered in *S. aureus* (Guo *et al*., 2020). In this study, the highest resistance (100%) was found with tigecycline and vancomycin. This did not agree with the work of Gitua *et al*. (2018) who reported 98.2% and 95.1% susceptibility to tigecycline and vancomycin respectively. However, this finding of high resistance to vancomycin and tigecycline is a concern considering the fact that they are not drugs that are routinely prescribed in the two healthcare facilities. The isolates recorded 40.9% resistance to tetracycline which is similar to the report of Gitua *et al*., (2018). MRSA was tested using a cefoxitin disc. The occurrence of MRSA in this study was 18.8%, which is similar to the findings of Dilnessa and Bitew, (2016) with 17.5% MRSA. Multidrug resistance (MDR) *S. aureus* from this study was 68.2%, which agrees with the findings of Upreti *et al*., (2018). Extended drug resistance (XDR) was 9.1% and no Pan drug resistance (PDR) *S. aureus* were isolated from this study. The high percentage of resistance recorded in this study may be due to inappropriate prescription and unnecessary use of antimicrobials in the study area. The *S. aureus* isolates were highly susceptible to linezolid (90.9%), cefoxitin, ciprofloxacin, and gentamicin at 81.8%. This is in agreement with the findings of Onaolapo *et al*. (2016), Nsofor *et al*. (2016), and Olowo-Okere *et al*. (2017), this implies that these drugs are still effective and could be used in

the treatment of *Staphylococcus aureus* infection in the locality.

All 22 (100%) *Staphylococcus aureus* isolates were biofilm formers. Weak biofilm formers 16 (72.7%), moderate biofilm formers 5 (22.7%), and strong biofilm former 1 (4.5%). This is contrary to the work of Abdulrahim *et al*. (2019), who recorded 5.5% and 13.8% as strong and moderate biofilm formers respectively in Kano. Isolates from wounds produced the highest occurrence of biofilm (54.5%). Biofilm formation depends on some factors such as environment, availability of nutrients, geographical origin, types of specimen, surface adhesion characteristics, and genetic makeup of the organism (Neopane *et al*., 2018). These factors must have contributed to the high prevalence of biofilm formation recorded in this study. Of the 22 (100%) biofilm formers, 14 (54.5%) were MDR. Compared to bacteria in the planktonic state, bacteria in the biofilm state are significantly more resistant to antibiotics and they appear to be multidrug-resistant (Wu *et al*., 2019). This is because the matrix of the biofilm reduces the penetration of some antibiotics and also protects the persisters from the immune system (Fisher *et al*., 2017).

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CONCLUSION

Staphylococcus aureus isolates from this study were most susceptible to linezolid, gentamicin, ciprofloxacin, and rifampicin, thus, they are still useful for the treatment of infections caused by *S. aureus* in this region. All *Staphylococcus aureus* isolates produced biofilm which might be the reason for high resistance. High resistance to tigecycline and vancomycin observed in this study is of concern and there is need to conduct further research involving larger samples of Staphylococcus aureus so as to have a broader and holistic picture of the situation. Continuous surveillance of antimicrobial susceptibility of *S. aureus* is essential for the detection of emerging antibiotic resistance trends and the development of suitable curbing strategies

ETHICAL CONSIDERATIONS

Ethical approval (ABUTHZ/HREC/W35/2021) was obtained from the Ethical Committee of Ahmadu Bello University Teaching Hospital for use of human subjects for research.

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