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Molecular Detection of Biofilm-Producing *Staphylococcus aureus* Isolates from National Orthopaedic Hospital Dala, Kano State, Nigeria

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Abstract

This study evaluated biofilm formation and antibiotic susceptibility in 36 clinical *S. aureus* isolates recovered from orthopaedic patients and detected the presence of intercellular adhesion and adhesin genes. *Staphylococcus aureus* was isolated from nasal swab, wound and urine specimens collected from orthopaedic patients in National Orthopaedic Hospital Dala, Kano over a period of three months. The isolates were identified using rapid identification kit for *Staphylococcus* species. The antibiotics susceptibility of the isolates was determined using modified disc diffusion method. Phenotypically, the biofilm formation was assessed using the Congo red agar method and microtitre plate assay. Polymerase chain reaction (PCR) analysis was used to detect biofilm-associated genes and characterize the isolates. The isolation rate of *S. aureus* from the samples (n = 134) was 26.8%, mainly from nasal swab (36%) and wound swab (36%). A total of 19 (52.7%) of the isolates showed positive for slime production. Majority of the isolates 29/36 (81.6%) were biofilm positive with only 2 (5.5%) and 5 (13.8%) as strong biofilm-formers and moderate biofilm-formers respectively. Molecular evaluation of the biofilm-associated genes in 12 *S. aureus* isolates revealed the prevalence of *bbp* genes (25%), *clfA* genes (16.6%) and the *icaA* (8.3%). None of the isolates harboured the *fnbA* and *cna* genes. There is no significant difference ($P > 0.05$) in the antibiotic resistance pattern between biofilm-positive and biofilm-negative *S. aureus* isolates. This result revealed that phenotypically most of the *S. aureus* isolates were biofilm formers but few of them chromosomally harbour the biofilm-associated genes.

Keywords

Staphylococcus aureus, Biofilm, Intercellular Adhesion and Adhesin Genes, Orthopaedic Patients, Microtitre Plate, Congo Red Agar

1. Introduction

Staphylococcus aureus has been an important human pathogen throughout history and causes a range of clinical infection worldwide [1]. *Staphylococcus aureus* has the unique ability of being persistent in causing diseases, ranging from minor skin infections to fatal necrotizing pneumonia [2]. Orthopaedic patients with implanted medical device such as central venous catheters, cardiac valves, and pace makers and artificial joints are most vulnerable to *S. aureus* infection [3]. When biofilm-associated *S. aureus* infections occur, they are difficult to treat by conventional procedure and may only be resolved by surgical removal of the focus of infection or removal of the device [4].

The adhesion stage of *S. aureus* is mediated by a protein family of staphylococcal microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) such as extracellular matrix protein: fibronectin binding proteins (*Fnb*A and *Fnb*B), collagen binding protein (*cna*), elastin binding protein (*Ebp*S), fibrinogen binding protein, (*Fnb*), Bone sialoprotein-binding protein (*bbp*), clumping factor (*clfA* and *clfB*) [5] [6], and the aggregation stage is conducted by the synthesis of polysaccharide intercellular adhesin (PIA) molecule [7]. Research has shown that the intracellular adhesion (*ica*) operon is essential for the control of biofilm production [8]. The *ica* locus, consisting of the gene *ica*ADBC, encodes the proteins mediating the synthesis of polysaccharide intercellular adhesion (PIA) molecule [9].

Adherence to surfaces/tissues, avoiding or invasion of the immune system and causing harmful toxic effect to the host are products of an array of factors expressed by *S. aureus* biofilm [10]. Recently, scientific observations have shown that, cells in biofilms differ from those of their free-floating counterparts due to possession of different genotypic and phenotypic characteristics which make them persistent and more resistant to antibiotics [11] [12].

Staphylococcus aureus biofilm structures were observed in a porcine wound colonization model using multiple microscopic techniques [13]. Similarly, a murine cutaneous wound model also demonstrated that *S. aureus* biofilm delays re-epithelialization and healing which was specifically dependent on *S. aureus* biofilm development [14].

Biofilms exert increased protection from the host immune system and an increased resistance to antibiotic therapy in comparison to their planktonic counterparts [15]. Organisms that produce biofilm show much greater resistance to antibiotics than their free living counterparts. This increase in drug re-

sistance is partly due to the penetration barrier that biofilm present to antimicrobials [16].

Diabetic foot wound patients with *S. aureus* colonization have a 2-fold increase in healing time [17]. Biofilm protection of microorganisms from opsonophagocytosis and antibiotics make *S. aureus* capable of causing chronic infection and sepsis an economically important organism in nosocomial infections [18].

In this study, we screened 36 *S. aureus* isolates from nasal swabs, wound swab and urine specimens of orthopaedic patients by microtitre plate method for determining their ability to form biofilm. This study evaluated the biofilm formation and antibiotics resistance pattern in 36 clinical *S. aureus* isolates from orthopaedic patients.

2. Materials and Methods

2.1. Study Design and Study Area

This descriptive study was conducted at Ahmadu Bello University, Zaria, Kaduna, Nigeria. Samples were collected from National Orthopaedic Hospital Dala (NOHD) after approval by the ethics committee of the hospital. NOHD is a tertiary health care institution located in northwest Nigeria, it is also a referral hospital for other hospitals and states such as Kaduna, Zamfara, Sokoto, and Kebbi. The hospital has well equipped nine wards and an emergency unit.

2.2. Collection and Identification of Bacterial Isolates

A total of 134 clinical samples were collected from nasal swabs, wound and urine specimens of orthopaedic patients. The samples were cultured based on standard microbiological techniques and a total of 36 *S. aureus* isolates were identified using Microgen™ Staph-ID System (Microgen, Surrey, UK).

2.3. Quantitative Detection of Biofilm Formation

The 36 *S. aureus* isolates were also screened quantitatively for their ability to form biofilm by microtitre plate (MTP) method according to the work of Christensen [19] and modified by Merrit [20].

The isolates were grown overnight for 24 hours at 37˚C in brain heart infusion broth (BHI) supplemented with 2% glucose and 2% sucrose. 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˚C. 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 in the well 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 stain were poured off and wells washed three (3) times in three (3) different trays of normal saline and dried for 30 minutes at room tem-

perature. A positive result was seen as the presence of a layer of stained materials adhered to the inner wall of the wells.

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 630 nm using a microtitre plate reader.

The uninoculated medium was used, as control, to determine the negative control (OD). 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.

2.4. Classification of Adherence

The biofilm ability of the tested strains was classified into four (4) categories based on the OD, Stepanovic [21]: non-adherent (OD < ODc), weakly adherent (ODc < OD < 2XODc), moderately adherent (2XODc < OD < 4XODc), and strongly adherent (4XODc < OD).

2.5. DNA Extraction and PCR Amplification

A typical isolate was cultivated in 1 ml TSB for 24 h at 37˚C. The bacterial genomic DNA of 12 selected strains was extracted with a ZR Fungal/Bacterial DNA MiniPrep™ (USA) as recommended by the manufacturer.

Amplification of biofilm-forming genes was carried out using PCR (Bio-Rad DNA Machine) thermal cycler after an external optimization of the reaction to ensure a better amplification with specific primers. The PCR master mix contain 1.0 µl each of forward and reverse primers, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.15 mmol/L dNTP, 1.25 IU *Taq* DNA polymerase and 1 µL of prepared DNA (0.5 µg) template was added to the final volume. Running conditions (denaturation, annealing and extension) are described by Pournajaf [22].

2.6. Statistical Analysis

Data was analysed using Statistical Package for Social Sciences (SPSS) Version 21. Descriptive analysis such as percentages was used in the analysis.

3. Results

We found that, out of 36 strains of *S. aureus* identified 13 (36%) were from wound swabs, 13 (36%) were from nasal swabs and 10 (28%) were from urine samples.

Using antibiotics susceptibility testing, we found out the percentage biofilm production in CRA and MTP from resistant *S. aureus* strains in each antibiotic agent tested as shown in **Figure 1**. Isolates resistant to clindamycin were all biofilm producers (100%) in MTP, then (96.5%) of isolates resistant amoxicillin,

oxacillin, norfloxacin and gentamicin were also biofilm producers in CRA, while only 10.5% and 6.89% in MTP and CRA respectively were found to be the least biofilm producers among the resistant isolates.

Phenotypically, a total of 19 (52.7%) and 17 (47.2%) qualitatively showed positive and negative slime production respectively when observed on CRA as shown in **Figure 2**. The MTP used for the assessment of biofilm-forming ability of the 36 clinical isolates is presented in **Figure 3** based on adherence ability classification as described by Stepanovic [21]. Only 2 (5.5%) were strongly-adherent while 22 (61.1%) of the isolates were weakly-adherent recording the highest percentage as presented in **Figure 4**. Using a paired T-test for CRA and MTP in each case, P-value of 0.0931 and 0.0948 was observed between the biofilm formers and non-biofilm formers respectively.

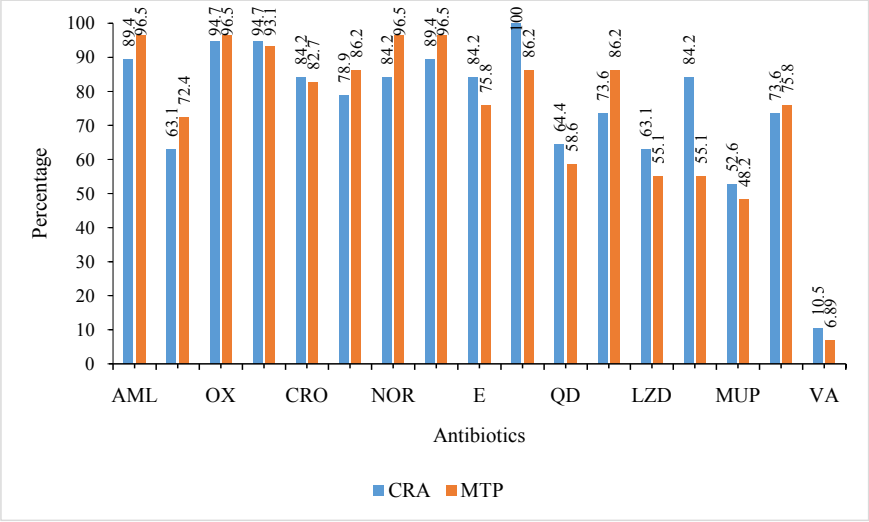

Figure 1. Percentage biofilm formation in Congo red agar and Microtitre plate methods among *S. aureus* resistant isolates. Keys: CRA = Congo red agar; MTP = Microtitre plate; NOR = Norfloxacin; CIP = Ciprofloxacin; QD = Quinupristin-dalfopristin; LZD = Linezolid; CN = Gentamicin; SXT = Trimethoprim-sulfamethoxazole; TE = Tetracycline; MUP = Mupirocin; VA MIC = Vancomycin minimum inhibitory concentration; FOX = Cefoxitin; AMC = Amoxicillin-clavulanic acid; E = Erythromycin; DA = Clindamycin; AML = Amoxicillin; OX = Oxacillin; CRO = Ceftriaxone; C = Chloramphenicol.

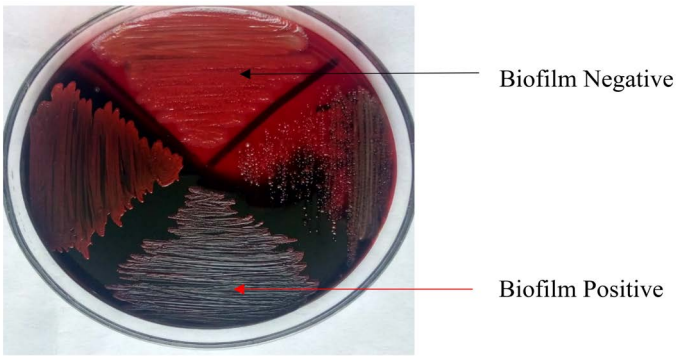

Figure 2. *Staphylococcus aureus* biofilm formation on congo red agar.

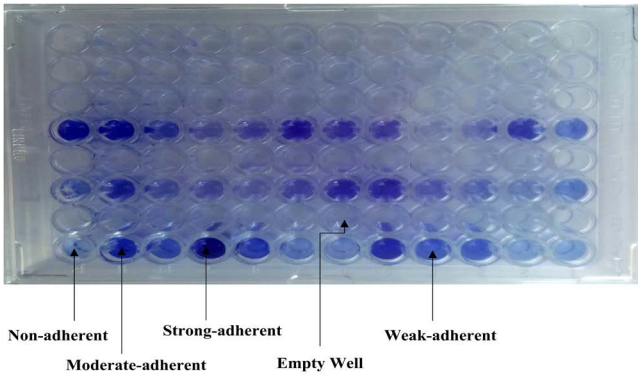

Figure 3. Microtitre plate indicating the adherence ability of the isolates.

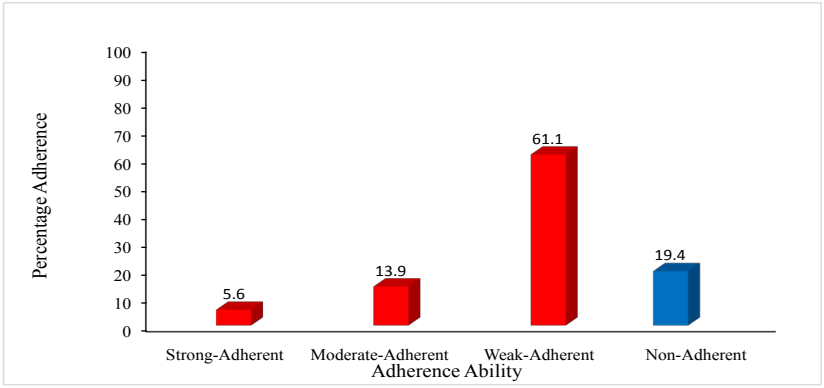

Figure 4. Percentage classification of isolates adherence ability.

Table 1 presents the forward and reverse primers used for the polymerase chain reaction with their nucleotide sequence and amplicon sizes for each gene, using a multiplex polymerase chain reaction. Genetically, 16.6% of *clfA* and 25% of *bbp* amplifications were observed while no *cna* amplification was observed as shown in **Figure 5**. Only 8.3% of *icaA* showed amplification while *fnbA* did not show any amplification among the 12 isolates tested as shown in **Figure 6**.

Table 1. Intercellular adhesion and adhesins genes primers.

Genes	Primers	Nucleotide Sequence	Amplicon Sizes (bp)
fnbA	Forward	5'-GCGGAGATCAAAGACAA-3'	1279
	Reverse	3'-CCATCTATAGCTGTGTGG-5'	
cna	Forward	5'TTCACAAGCTTGGTATCAAGAGCATGG-3'	452
	Reverse	3'-GAGTGCCTTCCCAAACCTTTTGAGC-5'	
bbp	Forward	5'-TCAAAAGAAAAGCCAATGGCAAACG-3'	956
	Reverse	3'-ACCGTTGGCGTGTAACCTGCTG-5'	
icaA	Forward	5'-ACACTTGCTGGCGCAGTCAA-3'	1000
	Reverse	3'-TGTTGGATGTTGGTTCCAGA-5'	
clfA	Forward	5'-GGCTTCAGTGCTTGTAGG-3'	2000
	Reverse	3'-TTTTCAGGGTCA ATATAAGC-5'	

Keys: *fnbA* (Fibronectin binding proteins); *cna* (Collagen binding protein); *bbp* (Bone sialoprotein-binding protein); *icaA* (Intercellular adhesion); *clfA* (Clumping factor).

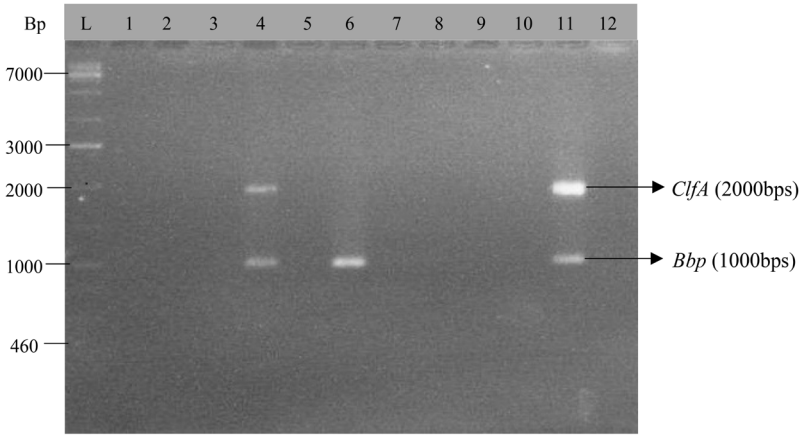

Figure 5. Amplicons of the *clfA* and *bbp* genes of *Staphylococcus aureus* producing biofilm with a size of 2000 bp and 1000 bp respectively.

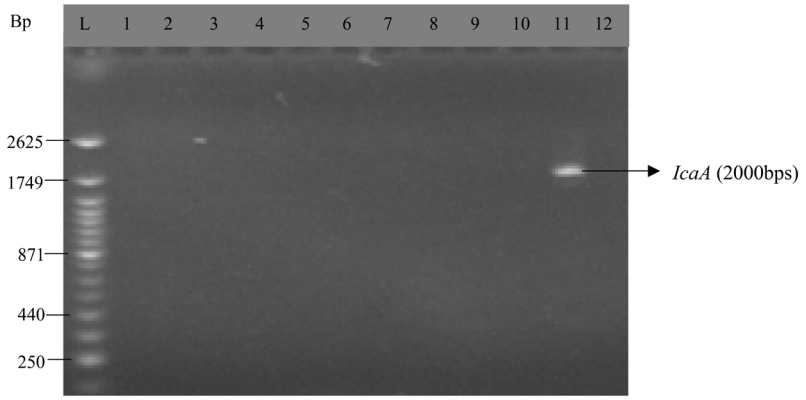

Figure 6. Amplicon of the *icaA* gene of *Staphylococcus aureus* producing biofilm with a size of 2000 bp.

4. Discussion

Biofilm formation in or on medical equipment and devices such as implants, may increase the number and severity of nosocomial infections; thus, it is important that attempts be undertaken to remove these antibiotic resistance factors [23]. The qualitative method (CRA) and quantitative method (MTP) showed biofilm formation in the *S. aureus* isolates for 52.7% and 80.6% respectively. Using a paired T-test for CRA and MTP in each case, P-value of 0.0931 and 0.0948 was observed between the biofilm formers and non-biofilm formers respectively. This indicates that, there is no significant difference ($P > 0.05$) in the antibiotics resistance pattern of biofilm-positive and biofilm-negative *S. aureus* isolates, which signifies that isolates biofilm forming ability may be a contributing factor in the resistance pattern observed in NOHD. A similar study conducted in Yaoundé, Cameroun also stated that there was no significant difference in the percentage of MDR among biofilm producers and non-biofilm producers for both medical and non-medical personnel [24]. Although, Fitzpatrick [25] has found more MDR strains among biofilm producers than non-biofilm producers.

Quantitatively, only 5.6% of the *S. aureus* are strong biofilm formers while 61.1% of them are weak biofilm as presented in **Figure 4**. This may be a sign that their biofilm forming ability is simultaneously influencing the resistance pattern of the isolates or have been masked, since most of the isolates already harbour resistant genes to the antibiotics tested. This result is in line with the study of Eyoh [24] where they recorded highest prevalence of 35.6% from weak biofilm-formers in isolates of both medical and non-medical personnel.

Over the years, scientists have being making attempt to understand the mechanism involved in biofilm formation, although studies have shown the expression of some genes involved in biofilm production [26] [27].

Among twelve (12) *S. aureus* isolates tested, the lowest prevalence was detected in *icaA* gene (8.3%), although, it is critical to biofilm elaboration, allowing bacteria to adhere to one another and also promote adherence to other molecules. Investigations comparing biofilm cells with planktonic cells, showed that the *ica* gene can be considered necessary for the initiation of biofilm development [23]. In this study, 2 (16.6%) *S. aureus* strains harbour the *clfA* gene which brings about fibrinogen binding as a result mediate induced platelets aggregation. This will initiate clumping to surfaces (animate or inanimate).

The *bbp* gene has high affinity for various extracellular adherence and capable of modulating inflammatory response, it has also been associated with osteomyelitis and arthritis in humans [28], the bone sialoprotein binding protein is one among the adhesin genes that is significantly associated with hematogenous tissue infections in human [29]. The 25% prevalence of *bbp* detected in this study may be because clinical samples were from orthopaedic patients. This result obtained in the study is lower than that reported by Montanaro [30] where 74% co-occurrence of bone sialoprotein-binding (*bbp*) and collagen-binding (*cna*) genes from orthopaedic implant infections. Several other studies, have not detected *bbp* gene in all their isolates [31] [32] though, their *S. aureus* isolates were not of orthopaedic origin.

5. Conclusion

The phenotypic and genotypic expression of biofilm formation among antibiotics resistant *S. aureus* makes them a potential threat and challenging pathogens with ability to causing infections in humans, especially among orthopaedic patients. This may result in treatment failure and persistency of infections among community and hospital inhabitants.

Limitations

All the female orthopaedic patients declined giving consent to participate in this research, reasons are mostly based on religious belief, no consent from their spouses and lack of personal interest. Financial constrain was also a challenge, which lead to molecularly detecting only 12 biofilm forming isolates out of all the phenotypically expressed biofilm producers.

Ethical Approval

Permission to conduct this study was granted by the hospital research ethics committee (NOHD/RET/ETHIC/60) and informed consent of patients to participate in the study was sought before commencement of sample collection.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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