

## Isolation of Biofilm Producing Methicillin-Resistant *Staphylococcus aureus* from Hospitalized Orthopaedic Patients in Kano State, Nigeria

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### ABSTRACT

Biofilm formation and resistance to methicillin are among the factors that makes *Staphylococcus aureus* a very important human pathogen in both health-care and community settings. This study investigated methicillin-resistance among biofilm-producing *S. aureus* isolated from 49 orthopaedic in-patients within a 3 months period. Wound swabs, nasal swabs, bed swabs and urine samples were collected from each patient. The samples were cultured and screened for presence of *S. aureus* while the micro-titre plate method was used to detect biofilm producing isolates. PCR technique was finally used to detect the presence of *mecA* gene in methicillin resistant *S. aureus* (MRSA) isolates. Findings reveal 14.8% of bacterial isolates were *Staphylococcus aureus* of which 96.4% were biofilm-producers. However, strong biofilm producers constitute 11.1%. The *mecA* gene was detected in 15.8% of the MRSA isolates. Therefore, MRSA among biofilm-producing *S. aureus* is a potential threat primarily to the community of National Orthopaedic Hospital Dala and a major public health challenge.

**Keywords:** Biofilm, Methicillin-resistance *Staphylococcus aureus* (MRSA), *mecA* gene, Orthopaedic patients

### INTRODUCTION

*Staphylococcus aureus* isolates that are resistant to the isoxazolyl-penicillins such as methicillin, oxacillin and flucloxacillin are regarded as methicillin resistant *Staphylococcus aureus* (MRSA). These MRSA isolates have shown cross-resistance to all licensed  $\beta$ -lactam antibiotics (Loomba *et al.*, 2010). The expression of methicillin resistance in *S. aureus* strains is by virtue of acquired penicillin binding protein PBP2a, encoded by *mecA* gene (Chambers, 1999). Many MRSA isolates have the capacity to form biofilms through an *icaADBG*-independent mechanism, such as fibronectin-binding proteins A and B (FnbpA and FnbpB), as well as major autolysin (Tong *et al.*, 2015). Methicillin-resistant *Staphylococcus aureus* has been implicated in nosocomial infections, including orthopaedic infections (Hideki *et al.*, 2016).

Biofilm describes the association of micro-organisms in which the cells adhere to each

other on a living or non-living surface within a self-produced matrix of extracellular polymeric substances (Jamal *et al.*, 2015). Biofilms exhibit altered phenotype with regard to growth, gene expression and protein production (Donlan and Costerton, 2002). Biofilms confers protection to bacteria from stress or stimuli by providing a thick layer of extracellular proteins (Costerton, 1984; Chino *et al.*, 2017). A bacterial biofilm is formed through initial and irreversible attachment, micro-colony formation, biofilm maturation, and biofilm dispersion (Wei and Ma, 2013). Biofilm matrix is known to be made of polysaccharides, proteins, and nucleic acids that can be dissolved by enzymatic degradation (Fong and Yildiz, 2015). Proteinaceous components, such as the non-ribosomally generated peptide aureusimine (phevalin) in *Staphylococcus aureus* (Paharik and Horswill, 2016) and three exopolysaccharides (Psl, Pel, and alginate) in *Pseudomonas aeruginosa* (Wei and Ma, 2013; Fong and Yildiz, 2015) play important roles in

helping to maintain biofilm structure and confer resistant to antibiotics and disinfectants.

Many implant-based and chronic infections including those involving prosthetic heart valves, central venous catheters, urinary catheters, orthopaedic prostheses, penile prostheses, endocarditis, otitis media, osteomyelitis and sinusitis have been associated with *Staphylococcus aureus* biofilms (Costerton *et al.*, 1999; Bendouah *et al.*, 2006). Biofilm infections are difficult to treat, and *in vitro* susceptibility tests have shown that biofilms are considerably more resistant than planktonic cells to the action of antibiotics (Mohamed *et al.*, 2007). The mechanisms responsible for this resistance include the physical and chemical diffusion barrier formed by the exopolysaccharide matrix which hinders antimicrobial penetration, the existence of micro environments that antagonize the antibiotic action, the activation of stress responses that cause changes in bacterial physiology, and the stable and slower growth of these microorganisms due to nutrient limitation and the absence of antimicrobial targets (Coelho *et al.*, 2011). Initially, high resistance of biofilm bacteria to antibiotics was attributed to reduced drug penetration into the biofilm core caused by a physical barrier formed of extra-cellular polymeric substance (Suci *et al.*, 1994). However, reduced bacteria growth rate, active starvation response and changes in bacterial gene expression also contribute to biofilm resistance (Marque's *et al.*, 2015), making biofilm-associated infection very difficult to overcome.

Antimicrobials such as beta-lactams are preferred for the treatment of staphylococcal infections. However, production of beta-lactamase enzymes, coded by *blaZ* that hydrolyzes the beta-lactamic ring, and production of low-affinity penicillin binding protein (PBP2a), coded by *mecA*, may lead to antimicrobial resistance (Pehlivanoglu and Yardimci, 2012). These resistance genes can be acquired by bacteria via horizontal gene transfer (HGT). A very important process resulting to the

emergence of new resistant pathogen is conjugative plasmid-mediated HGT (Schiwon *et al.*, 2013). Biofilm provides an ideal environmental situation for conjugation to occur between bacteria within the biofilms, thereby allowing the exchange of genetic materials between them (Águila-Arcos *et al.*, 2018). Also, bacterial conjugation can induce biofilm formation since the cell-to-cell contact required for gene exchange is favored by the close proximity of bacteria required for biofilm formation (D'Alvise *et al.*, 2010). This link between biofilms and bacterial conjugation increases both the risk of biofilm-related infections and the spread of virulence factors. In this study, we investigated the capability of *Staphylococcus aureus* isolated from orthopaedic patients on admission to produce biofilm as well as determine methicillin-resistance among these biofilm-producing *Staphylococcus aureus*

## MATERIALS AND METHODS

### Sample Collection

Ethical approval was obtained from the hospital's Ethical Committee for the purpose of this research and informed consent was obtained from each patient. The sample size was determined according to methods described by Kadam and Bhalerao (2010) and a total of 49 patients on admission in the National Orthopaedic Hospital Dala (NOHD), Kano were recruited for this study. The gender and age of each patient was noted and recorded. From each patient, 4 categories of samples were collected: mid-stream Urine (U), wound swab (W), nasal swab (N) and patient's bed swab (B). A total of 189 samples were collected and were numbered serially. Samples collected were transported in sterile ziploc (plastic) bags contained in ice packs to the Microbiology Laboratory, Department of Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria.

### **Staphylococcus aureus Isolation and Identification**

The clinical samples were inoculated into freshly prepared nutrient broth (Oxoid Ltd., Basingstoke, Hampshire, England) and incubated at 37°C for 24 h. A loop-full of growth from the nutrient broth medium was streaked onto Mannitol Salt Agar (Oxoid Ltd., Basingstoke, Hampshire, England) and incubated for 24 h at 37°C (Public Health England, 2017). Discrete, round, single, golden-yellow colonies were presumptively identified as *Staphylococci*. The isolates were characterized by conducting the following tests: gram stain, catalase, coagulase (Ochei and Kolhatkar, 2008; Chandra and Mani, 2011). Microgen™ Staph-ID System was used to identify the isolates.

### **Detection of Biofilm Producing Staphylococcus aureus Isolates**

The micro-titre plate method as modified by Merrit *et al.* (2005) was used to screen for biofilm producing *Staphylococcus aureus* isolates. The cells were grown for 24 h at 37°C in 2% glucose and 2% sucrose supplemented Brain Heart Infusion (BHI) broth (Oxoid Ltd., Basingstoke, Hampshire, England). After preparing 1:100 µl dilution of the cells in supplemented BHI broth, 150 µl of the cell suspension was used to inoculate sterile flat-bottomed, 96-well polystyrene micro-titre plate in triplicate and 150 µl of un-inoculated supplemented BHI broth was dispensed into three wells of the micro-titre plate to serve as control. The micro-titre plate was then incubated for 48 h at 37°C. After incubation, the suspension was poured off and the wells washed three times in normal saline to remove unfixed microbial cell then allowed to dry in an inverted position. The dried wells were subsequently stained with 250 µl of 0.1% crystal violet solution and incubated 25°C for 20 minutes. The excess stain was poured off and wells washed three (3) times with normal saline and dried for 30 minutes at 25°C. Positive results were seen as the presence of a layer of stained materials adhered to the inner wall of the wells.

Biofilm was quantified by first reconstituting the microtitre plate with 250 µl of ethanol:acetic acid (95:5 v/v) after which 100 µl was transferred to a new microtitre plate. Absorbance (OD) was read at 630 nm using a microplate reader. Absorbance of the uninoculated wells (negative control) was used to calculate the cut off (ODc) as follows:

$ODc = \text{Average OD value of negative control} + 3 \times \text{standard deviations of negative control}$ .

The ODc value was used to classify biofilm producing capability of isolates into four categories: non-adherent ( $OD < ODc$ ), weakly adherent ( $ODc < OD < 2 \times ODc$ ), moderately adherent ( $2 \times ODc < OD < 4 \times ODc$ ), and strongly adherent ( $4 \times ODc < OD$ ) (Stepanovic *et al.*, 2007).

### **Detection of mecA Gene in Methicillin-Resistant Staphylococcus aureus Isolates**

Biofilm producing isolates were subjected to agar-disc diffusion method (EUCAST, 2018) for antibiotic susceptibility test using 30 µg cefoxitin disc (Oxoid Ltd., Basingstoke, Hampshire, England) to determine methicillin resistance. Nine phenotypically methicillin-resistant *S. aureus* (MRSA) strains were randomly selected as representatives of the nineteen (19) phenotypical MRSA isolates for polymerase chain reaction (PCR). Briefly, genomic DNA was extracted using Qiagen genomic DNA extraction kit, from an overnight MRSA culture. A 25 µl reaction mix was prepared in microfuge tubes containing 12.5 µl master mix (Biolabs), 1 µl each of forward and reverse *mecA* primer (5'-AAA ATC GAT GGT AAA GGT TGG C -3' and 3'- AGT TCT GCA GTA CCG GAT TTG C -5') respectively with a base pair of 533bp (Inqaba Biotec), 5.5 µl nuclease free water and 5 µl of the extracted DNA. The tubes were vortexed briefly to mix then placed in a thermal cycler (Applied Biosystem 9700) for DNA amplification using the following PCR cycling protocol: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, an extension at 72°C for 1 minute and final extension at 72°C for 7 minutes. PCR

products (amplicons) were electrophoresed on 1.5% agarose gel and visualized after ethidium bromide staining on a UV trans-illumination gel documentation system (Bio-Rad).

### Data Analysis

Data was presented as mean  $\pm$  standard deviation and percentages were calculated where necessary. Results of *Staphylococcus aureus* and MRSA distribution are presented as bar-charts and in tabular form while PCR electrophoretogram was presented as plates.

### RESULTS

Table 1 presents summary of gender and ages of selected subjects for this study. Male participants were 42 (85.7%), while 7 (14.3%) were female. Among these participants, 3 (6.1%) were within 1 – 17 years, 38 (77.6%) were within 18 – 40 years and 8 (16.3%) were above 40 years. Table 1 also shows the age distribution of participants in this study.

### Identification of *Staphylococcus aureus* and biofilm producers

Twenty eight (28) samples were positive for *Staphylococcus aureus* out of which 2 (7.1%) were from bed swabs, 12 (42.9%) were from wound swabs, 8 (28.6%) were from nasal swabs, while 6 (21.4%) were isolated from urine. Classification of biofilm producing strains is presented in Table 2. Twenty seven (27) isolates (96.4%) had biofilm production capabilities and the distribution showed that most of the biofilm producing strains (12 or 42.9%) were wound sample isolates. However 64.3% of all the biofilm producing isolates were weak biofilm Producers.

### Methicillin Resistance in Biofilm Producing *Staphylococcus aureus* Isolates

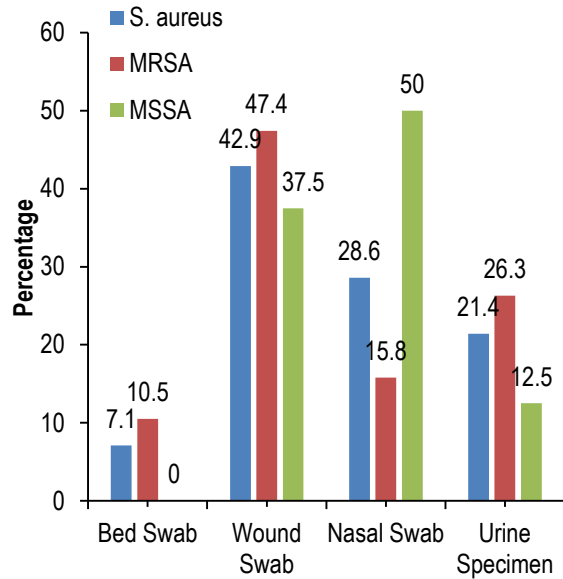
Out of the 27 biofilm producing *S. aureus* isolates tested for phenotypic expression of methicillin resistance, 19 (67.9%) of the isolates were MRSA while 9 (32.1%) were methicillin susceptible *S. aureus* (MSSA). Distribution of the MRSA and MSSA isolates across the sample collected is shown in Figure 1.

Table 1: Demography of Patients that Participated in this Study.

Age Range in Years	Percentage of Patients Recruited		All Patients
	Male	Female	
1 – 17	2 (4.1%)	1 (2%)	3 (6.1%)
18 – 40	32 (65.3%)	6 (12.3%)	38 (77.6%)
41 – above	8 (16.3%)	0 (0)	8 (16.3%)
<b>Total</b>	<b>42 (85.7%)</b>	<b>7 (14.3%)</b>	<b>49 (100%)</b>

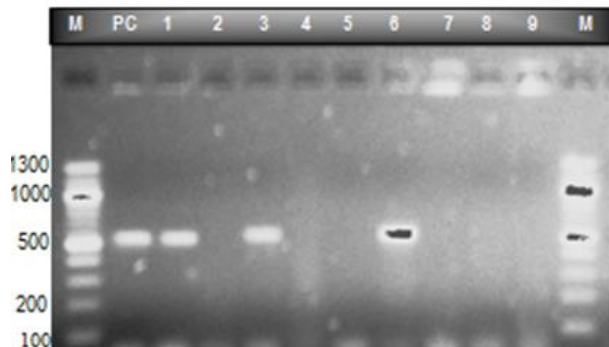
Table 2: Classification and Distribution of Biofilm Produced by the *S. aureus* Isolates.

Biofilm Production	Bed	Wound	Nasal	Urine	All Samples
Non Biofilm Producer	0 (0%)	0 (0%)	1 (12.5%)	0 (0%)	<b>1 (3.6%)</b>
Weak Biofilm Producer	1 (50%)	8 (66.7%)	6 (75%)	3 (50%)	<b>18 (64.3%)</b>
Moderate Biofilm Producer	0 (0%)	3 (25%)	0 (0%)	3 (50%)	<b>6 (21.4%)</b>
Strong Biofilm Producer	1 (50%)	1 (8.3%)	1 (12.5%)	0 (0%)	<b>3 (10.7%)</b>
<b>Total</b>	<b>2 (7.1%)</b>	<b>12 (42.9%)</b>	<b>8 (28.6%)</b>	<b>6 (21.4%)</b>	<b>28 (100%)</b>



**Figure 1:** Distribution of *Staphylococcus aureus*, MRSA and MSSA Isolates by Specimen.

The *mecA* gene was detected in 3 of the 9 randomly selected biofilm producing MRSA isolates. The *mecA* genes detected were all from wound swap samples (Plate 2).



**Plate 2:** Electrophoretogram of Amplified *mecA* gene (533bp) *Staphylococcus aureus* Isolates.

**Key:** Lane PC: Positive Control; Lane 1: 49W; Lane 2:33U; Lane 3: 23W; Lane 4: 46B; Lane 5: 7N; Lane 6: 26W; Lane 7: 50W; Lane 8:24U; Lane 9: 3W; Lane M: 100bp Molecular DNA ladder; and bp= Base pair.

## DISCUSSION

*Staphylococcus aureus* are successful as commensal or pathogenic organisms because they adapt rapidly to selective pressures imparted by the human host (Malachowa and DeLeo, 2010). The highest occurrence of

*Staphylococcus aureus* observed in this study was within the group of 18 to 49 years, Ribeiro *et al.* (2013) also reported similar occurrence of Staphylococcal infection for surgical site infection in orthopaedic surgery within the age group of 18 to 48 years. Although, Crémet *et al* (2012) noted a higher risk in orthopaedic surgical infections among patients with ages of 50 years and above.

The highest occurrence of *S. aureus* isolates in this study was however recorded from wound swabs (41.9%) followed by nose swab samples with 28.6%. A study conducted in Adis-Ababa recorded a 14.3% occurrence of *S. aureus* in tested samples and 33.3% of the samples were nasal swabs (Dilnessa and Bitew, 2016). Colonization of patient's nasal cavity bears risk of transferring this organism to the wound, either by way of aerosols or by the patient's hands, especially when the patients fail to wash hands after coming into contact with nose cavity or while sneezing. Similarly, prolong use of bed spread could contribute to the cross contamination of wounds as this organism was demonstrated to be present on bed spreads (Figure 1). High occurrence of biofilm formers (96.4%) reported in this study could be linked to the slow progression in wound healing process among orthopaedic patients. The biofilm matrix is known to be a vital factor in preventing antibiotics from reaching the infecting organisms within the matrix (Marque's *et al.*, 2015), thereby conferring resistance (bacteriostatic or bactericidal) on the organisms within the biofilm matrix. Biofilm production by *Staphylococcus aureus* has been identified as an important factor contributing to the pathogenesis, antibiotic resistance and virulence (Stewart and Costerton, 2001). Biofilm producing *Staphylococcus aureus* have been implicated as causative agents in diseases including septicaemia, endocarditis, osteomyelitis and nosocomial infections caused by *Staphylococcus aureus* (Kim *et al.*, 2008). Methicillin resistant *Staphylococcus aureus* (MRSA) infections continues to be a serious and dreadful challenge as their prevalence is reported to be increasing exponentially (Sanders *et al.*,

2011) especially among biofilm formers. Like other  $\beta$ -lactam antibiotics, methicillin interferes with the final step of cell wall biosynthesis by inhibiting the cross-linkage between peptidoglycan units that make up a major component of the cell wall of Gram positive bacteria (Kohanski *et al.*, 2010). This study reported a high phenotypic prevalence of MRSA (67.9%) in orthopaedic patients, and only 33.3% genetically harbour the *mecA* gene from the MRSA isolates. Similar findings by Adhikari *et al.* (2017) reported a 29.1% presence of *mecA* gene in *S. aureus* isolates. Absence of *mecA* gene in isolates classified as MRSA phenotypically could be attributed to the *mecC* gene that also confers resistance to methicillin. Other factors that may be responsible for the methicillin resistance is hyper-production of  $\beta$ -lactamase by these isolates (Adhikari *et al.*, 2017). Olayinka *et al.* (2009) reported the absence of *mecA* gene in 36 *Staphylococcus aureus* isolates that were phenotypically MRSA positive but were hyper-producers of the  $\beta$ -lactamase enzyme. Elhassan *et al.* (2015) also reported 61.5% *Staphylococcus aureus* isolates classified as MRSA phenotypically, but did not detect presence of *mecA* gene in their genomic DNA.

Evidences however suggest that the *mecA* gene is contained in a mobile gene element called the Staphylococcal cassette chromosome *mec*, from which the gene can undergo horizontal gene transfer (HGT) and insert itself into the host species, (Hanssen and Ericson, 2006). Due to the proximity or adherence of bacteria (*Staphylococcus aureus*) cells to each other within biofilms, resistant genes that confer resistance such as the *mecA* gene are easily transferred from one cell to other cells through HGT, thereby making the whole biofilm community resistant to methicillin and other antibiotics.

## CONCLUSION

This study detected *mecA* gene in MRSA among the biofilm producing *Staphylococcus aureus* isolated from in-patients of the National

Orthopaedic Hospital Dala (NOHD), Kano. The presence of resistant strains of *Staphylococcus aureus* in a clinical setting makes them a potential threat of medical importance and a major public health challenge. Therefore, regular monitoring of isolates circulating in a particular hospital or community is essential to tackle the spread of multidrug resistant pathogens like MRSA.

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## Oche *et al*: Isolation of Biofilm Producing Methicillin-Resistant *Staphylococcus Aureus* from.....

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