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Research Article

Biofilm Formation in Methicillin Resistant *Staphylococci aureus* at the University of Ilorin Teaching Hospital, Ilorin

Oshin Oluwaseyi Babatunde*, Adejuyigbe Zacchaeus and Prof Charles Nwabuisi

Department of Medical Microbiology, University of Ilorin, Nigeria

***Corresponding Author:** Oshin Oluwaseyi Babatunde, Department of Medical Microbiology, University of Ilorin, Nigeria.

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Abstract

Biofilm is a structural community of bacterial cells enclosed in a self-produced polymeric matrix which could adhere to inert or living surfaces. It has also been discovered that 99.0% of bacteria exist in this community, with only 1.0% living in planktonic state and that 65.0% of microbial infections are associated with biofilms. Microorganisms that grow within the biofilm state possess several mechanisms that increase resistance to external antimicrobial treatments. The objective of the study is to determine the prevalence of biofilm forming ability amongst Methicillin resistance *Staphylococci aureus* at the University of Ilorin Teaching Hospital. One hundred and sixty eight (168) staphylococcal isolates from different clinical specimens were collected from the Microbiology Laboratory of University of Ilorin Teaching Hospital (UITH). The isolates were collected into 20% glycerol-brain heart infusion broth in vials and stored at -20oC for further processing. The isolates were re-characterized using standard microbiological techniques. Biofilm detection and quantification was carried out using modified Christensen's Microtitre plate method and the optical density determined at 450 nm.

The prevalence of biofilm formation among Staphylococcal isolates was 56.5%. Staphylococcal isolates showed moderate resistance to almost all the antibiotics (Gentamycin, Erythromycin, Tetracycline, Ciprofloxacin, Amoxicillin-Clavulanic acid, Cefuroxime and Linezolid) with Tetracycline (51.8%), Ciprofloxacin (42.3%), and Gentamycin (35.7%) with the highest resistance. Biofilm producers and non-biofilm producers exhibited 100% sensitivity to Linezolid. The prevalence of methicillin resistance among staphylococcal isolates was 44.6. There was a significant difference ($P < 0.05$) in the distribution of biofilm production among Staphylococcal isolates.

The prevalence of biofilm production at UITH is relatively high (56.5%) and of grave concern considering the devastating effect of antimicrobial resistance. Linezolid still remain a drug of choice in managing Staphylococcal infections and also infections caused by methicillin resistant staphylococci. And there is a need to include biofilm detection protocol in the routine microbiological examination with an objective to curbing antimicrobial resistance.

Keywords: Biofilm Formation; Methicillin Resistance; *S. aureus;* Northern Nigeria; Health Facilities

Introduction

Biofilm is a structural community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to inert or living surfaces [1]. Majority of microbial cells live in distinct communities. It has been known that 99.0% of bacteria exist in this community, with only 1.0% living in planktonic state and that 65.0% of microbial infections are associated with biofilms. It connotes the lifestyles of aggregated, sessile, or attached microbes in any environment [2-4].

Biofilm formation is regulated by different genetic and environmental factors. Genetic studies have shown that bacterial mobility, cell membrane proteins, extracellular polysaccharides and signalling molecules play significant roles in biofilm formation [5]. Basic structural units of a biofilm are micro colonies, separate communities of bacterial cells embedded into extracellular polymeric substances (EPS) matrix. These micro colonies are in most cases mushroom- shaped or rod-like and they can consist of one or more types of bacteria. Depending on bacteria type, micro colonies consist of 10.0-25.0% of cells and 79.0-90.0% of EPS matrix. Between micro colonies, there are channels through which water flows [5].

The development of a mature biofilm may take several hours to several weeks depending on the system [6]. Both gram positive and gram negative bacteria form biofilm. Gram positive microorganisms that form biofilm include *Bacillus* spp, *Listeria monocytogenes*, *Staphylococcus* spp, *Streptococcus* spp. Gram negative bacteria that form biofilm include *Escherichia coli*, *Pseudomonas aeruginosa*. The main pathogen of implant infections is staphylococci that cause 80.0% of all prosthetic infections [7]. Adherence of Staphylococci to medical devices or indwelling devices depends on a number of factors. The physiochemical properties of the device and surface appendages [Wall Techoic Acid (WTA), Lipoteichoic Acid (LTA), Accumulation Associated Protein (Aap), Autolysin A and E (AltA &E)] of the microorganism must be compatible for a long lasting association. In the adherence to living cells (cases of Otitis media, endocarditis), cell wall-anchored (CWA) proteins such as the fibrinogen-binding protein SdrG/Fbe of *S. epidermidis* and fibrinogen/fibronectin-binding proteins FnBPA and FnBPB and clumping factors A and B of *S. aureus* play integral roles [8]. Several staphylococcal surface proteins that mediate primary adherence of bacteria such as clumping factors A and B, fibrinogen-/fibronectin-binding proteins FnbA and FnbB of *S. aureus* or the fibrinogen-binding protein SdrG/Fbe of *S. epidermidis* that are cell wall-anchored proteins (CWA) also promote intercellular adhesion and the aggregation of bacteria in *ica*-independent biofilm formation [9-11].

The ability of microorganisms to be organised in a biofilm community confers increased antimicrobial resistance thereby depleting the pool of available antimicrobials. Antimicrobial resistance is a global threat to the health sector worldwide and so it is therefore important to carry out this study, which will give information on the relationship between biofilm formation and antimicrobial resistance and also the prevalence of the trait among staphylococcal clinical isolates at UITH.

Material and Methods

Isolate collection, storage and reactivation

Staphylococcal isolates were collected into sterile glycerol broth in vials. Five (5 ml) of glycerol broth was dispensed into each vial. The vials were autoclaved and allowed to cool at room temperature. Aliquots of each isolate were inoculated into each vial. The isolates were then maintained as a stock kept at -20^{o}C before the commencement of the research work.

Identification of staphylococcal isolates

Cultural re-characterization

The vials with the isolates were removed from the cold storage and allowed to thaw at room temperature. Aliquot from each vial was then inoculated on 5% sheep blood agar (Oxoid, UK) and a selective medium, Mannitol salt agar (HiMedia-MSA). They were incubated aerobically at 37oC for 24hour. The plates were examined after 24hours of growth.

Interpretation of result

Staphylococcus aureus ferment mannitol to produce a yellow colony while *S. epidermidis* and *S. saprophyticus* retain the red colouration of the MSA.

Staphylococcus aureus colonies on solid media were smooth, raised, and glistening and some strains form gray to deep golden yellow colonies. A convex colony with yellowish pigment and porcelain-like pigment on blood agar was indicative of *S. aureus* [12,13].

Gram staining

This was carried out to differentiate between the morphology of gram positive and gram negative according to Chessbough [14].

Quality control

- Gram positive: *Staphylococcus aureus* ATCC25923
- Gram negative: *Escherichia coli* ATCC25922.

Catalase test

This test was used to differentiate catalase producing staphylococci from non-catalase producing streptococci.

Coagulase test

This test was used to differentiate between *S. aureus* and the CoNS (*S. epidermidis*, *S. saprophyticus*).

Biofilm detection and quantification assay

The study employed a microtiter plate biofilm assay. The assay in its typically used form is derived from a protocol published by Christensen., *et al*. [15].

Principle

The protocol entails growing staphylococci in microtiter dishes for a desired period of time and then the well will be washed to remove planktonic bacteria. Cells adhering to the wells are then subsequently stained with a dye that allows visualization of the attachment pattern.

Materials

Staphylococci isolates, 0.1% (w/v) crystal violet (May and Baker) in water, 30% v/v acetic acid in water, Brain Heart Infusion broth (Oxoid, UK - 2% sucrose), 96 well-flat bottom polystyrene (Grenier Bio-one), small tray, and ELISA reader (Multiskan Ex V2.3).

Methods

- Staphylococcal isolates from overnight culture plates were emulsified with normal saline in universal bottles. The suspensions were standardized to 0.5 McFarland standards.
- Individual wells of sterile 96 well flat bottom polystyrene plate was inoculated with 20µl of the prepared suspension. 180µl BHIB was then added to each well. The first 8 wells served as the control and 200µl of sterile BHIB was added to each of the eight wells.
- The plates were then incubated at 37oC for 24 h.
- After incubation, contents of each well were removed by gentle tapping to remove planktonic bacteria from each microtiter dish.
- One hundred and fifty (150) µl of 0.1% crystal violet solution was added for staining to each well including the negative control for 10minute at room temperature.
- The Microtiter dish was shaken-out over the waste tray to remove the crystal violet solution and inverted on paper towels to remove excess liquid.
- The plates were then dried in the hot air oven for one hour.
- Before reading, 200 µl of 33% acetic acid [16] was added to each stained well and incubated for 10- 15 minutes at room temperature.
- The plates were read using the ELISA plate reader.
- The optical density (OD) of each well was then measured at 450 nm with an ELISA reader at the University of Ilorin Teaching Hospital Microbiology Laboratory [17,18].

Interpretation of result

The optical density cut-off value (ODc) is defined as three standard deviations above the mean optical density (OD) of the negative control. These four categories are: 79

- Non-biofilm producer when OD≤ ODc,
- Weak biofilm producer when ODc < OD ≤ 2 × ODc,
- Moderate biofilm producer when 2 × ODc < OD ≤ 4 × ODc
- Strong biofilm producer when 4 × ODc < OD [19].

Quality control

Negative control: uninoculated broth.

All the materials and methods that are used to complete the study should be mentioned.

Antimicrobial susceptibility testing

The antimicrobial susceptibility pattern of each isolate was carried out using modified Kirby Bauer disc diffusion method based on the clinical laboratory standard institute guidelines.

Principle

Antibiotics impregnated discs diffuse in the agar causing an inhibition of growth around the disc and visually seen as a zone of clearance around the discs.

Materials

Staphylococcal isolates, antibiotics discs, forceps, 0.5 McFarland solution, Mueller Hinton agar, and normal saline [20].

Methods

- Sterilized inoculating loop was used to take fresh colonies of staphylococcal isolates to prepare suspension.
- Inoculum suspension was made with saline and the turbidity of the suspension was standardized using 0.5 McFarland standards (1.5 X 10^8 CFU/ml).
- The microorganism suspension was thoroughly shaken to make sure it was well-mixed.
- Sterile swab stick was then used to evenly inoculate the surface.
- Sterilized forceps was used to apply the antibiotic discs within 15 minutes of inoculating the MHA plate.
- After antibiotics application, the plates were then incubated at 35oC for 24hrs with the agar side up.
- After incubation, the plate was read, measured against a light source using a ruler. And interpretation was done using the antibiotics breakpoints according to the CLSI guidelines [20].

The following antibiotics discs were used to detect the antimicrobial susceptible pattern of the isolates: Gentamycin, Erythromycin, Tetracycline, Ciprofloxacin, Amoxicillin-Clavulanic acid, Cefuroxime, Ceftriaxone and Linezolid (Oxoid, UK).

Quality control

S. aureus ATCC 25923.

Interpretation of result

The zone of inhibition of the test plates was read according to the clinical break point using Clinical Laboratory Standard institute guideline [20]. The isolates were reported as sensitive, intermediate and resistant to the various antibiotics depending on the sizes of the zones of inhibition

Methicillin resistance test

The test was carried out using the modified Kirby Bauer disc diffusion method. Cefoxitin (Oxoid, UK-30µg) disc was used to test for meticillin resistance in coagulase positive Staphylococci.

Principle

Cefoxitin, which is a potent inducer of the mecA regulatory system, is being widely used as a surrogate marker for detection of mecA gene-mediated methicillin resistance.

Materials

Cefoxitin disc (30 µg), Mueller Hinton agar, Staphylococcal isolates.

Methods

- Sterilized inoculating loop was used to take fresh colonies of staphylococcal isolates to make a suspension using normal saline and 0.5 McFarland standards.
- The microorganism suspension was thoroughly agitated to make a homogenous mixture. The MHA plate was then swabbed entirely with the swab stick dipped in the suspension.
- Sterilized forceps was used to apply the antibiotic discs within 15 minutes of inoculating the MHA plate.
- The plates were incubated at 35oC for 24hrs with the agar side up.
- After incubation, the plates were read, zone of inhibition measured against a light source and interpreted according to the CLSI guidelines [20].

Quality control

S. aureus ATCC 43300.

Interpretation of result

Using a measuring ruler, isolates were categorized as susceptible (S ≥22 mm) or resistant (≤21) for *Staphylococcus aureus* depending on the diameter of the inhibition zone.

Data analysis

sData was analyzed using SPSS 20.0 and Microsoft Excel 2010.

Results

Distribution of isolates

A total of one hundred and sixty eight (168) Staphylococcal isolates from different clinical specimens submitted to the Microbiology Laboratory of the University of Ilorin Teaching Hospital was obtained for this study.

The clinical specimens that yielded the staphylococcal isolates are blood (50, 29.8%), urine (27, 16.1%), sputum (9, 5.4%), eye swab (9, 5.4%), Wound swab (51, 30.4%), throat swab (2, 1.2%), STI swab (10, 6.0%), aspirate (9, 5.4%), and ear swab (1, 0.6%).

Relationship between biofilm production and methicillin resistance in *S. aureus* Biofilm Production

Biofilm Production	*S. aureus*		Total N (%)	X^2	P- value
	MRSA N (%)	MSSA N (%)			
Biofilm Producer	45 (77.6)	31 (36)	76 (52.8)	6.141	P < 0.001
Non Biofilm Producer	13 (22.4)	55(64)	68 (47.2)		
Total	58 (40.3)	86 (59.7)	144 (100)		

Table 1

Forty five of 58 (77.6%) MRSA isolates were biofilm producer compared with to 31 of 84 (36.9%) of MSSA isolates. There was a significant difference in the distribution of biofilm producers among *S. aureus* isolates (P < 0.001).

Discussion

In this study, 168 staphylococcal isolates were obtained and re characterized for analyses and 144 (85.7%) isolates were coagulase positive Staphylococci.

The staphylococcal isolates were obtained from different clinical specimens. Statistical Analyses showed that there was no significant difference in the distribution of the organisms among the clinical specimens (P > 0.05). The highest incidence of *S. aureus* was in wound (29.9%) which was consistent with a study by Nwoire., *et al*. [21] who documented that 37% of *S. aureus* isolates were obtained from wound specimens. The high incidence of Staphylococcal isolates in wound specimen could be attributed to the superficial location of some wounds with the presence of Staphylococci species on the skin as microflora and the ability of Staphylococci to survive in deep wound being facultative anaerobes.

The prevalence of biofilm forming Staphylococci in this study was found to be 56.5%. A prevalence of 52.8% was found among the S. aureus isolates and this result was similar to a study by Abirami., *et al*. [22] in India where biofilm prevalence among S. aureus isolates was 53.4%. A slightly lower prevalence of 35.6% was obtained among *S. aureus* isolates in Yaoundé [23] and in Zaria, 48.2% prevalence was obtained [24]. There was a significant difference (P < 0.05) in the distribution of biofilm production among Staphylococcal isolates. Based on the strength/ability of production, there was higher percentage of strong biofilm producers (42, 25%) than the other biofilm formation categories [weak producer (24, 14.3%), moderate producer (29, 17.3%)]. In the study conducted by Abirami., *et al*. [22], moderate biofilm producers had the highest prevalence (74%) as compared to this study where there was a relatively low prevalence of moderate biofilm producers (17.3%). This difference may not be unconnected from study design and quantification method. The several mechanisms of biofilm formation in Staphylococci have been a major challenge in the management of staphylococcal infections because of antimicrobial resistance.

The highest percentage of strong biofilm producers in urine could be as a result of catheters facilitated urine. In a study conducted by Walker., *et al*. [25] it was discovered that catherization potentiates Staphylococci infection in the urinary tract. Catherization in humans damaged the bladder resulting in the release of host protein fibrinogen (Fg). In the study, SEM analysis indicated that MRSA forms biofilm like communities on the implants that incorporated host components, including Fg. Also in a study conducted by Kawamura., *et al*. [26] in Japan, MRSA strains from patients with device related orthopaedic infection were more likely to be strong biofilm formers than those from patients with device non-related infection. It was also established in that study that biofilm forming capacity is associated with the pathogenesis of catheter-related urinary tract infections. In a study by Gad., *et al*. [27] strong biofilm formation in urine specimen was also found to be a factor of catherization.

In this study, wound specimen had the highest number of biofilm producers (37.9%), followed by blood (24.2%) and urine (17.9%). The result did not agree with that of Abdel Hasim, Kassem and Mahmoud [28] where blood specimen had the highest percentage of biofilm producers (82.6%).

Forty three (25.6%) staphylococcal isolates were resistant to Cefuroxime, 48 (28.8%) isolates were resistant to Amoxicillin-clavulanic. Forty nine (29.2%) isolates were resistant to Ceftriaxone. Seventy-one (42.3%) isolates were resistant to Ciprofloxacin. While 87 (51.8%) isolates were resistant to Tetracycline. All the staphylococcal isolates used in the study were susceptible to Linezolid. In the study conducted by Abirami., *et al*. [22], there was similarity in the antimicrobial susceptibility pattern (Erythromycin-63.7%). Also, all staphylococcal isolates were susceptible to Linezolid in that study. Also, in a study conducted by Filho., *et al*. [29], all isolates used in the study were susceptible to Linezolid.

S. aureus exhibited moderate sensitivity to Cefuroxime (92, 63.9%), Erythromycin (88, 61.1%), Gentamycin (81, 56.3%), Ciprofloxacin (80, 55.6%), Tetracycline (73, 50.7%). MRSA exhibited relatively low sensitivity to Ceftriaxone (14, 23.7%), Erythromycin (29, 49.2%), Gentamycin (19, 32.2%), Ciprofloxacin (21, 35.6%), and Tetracycline (17, 28.8%). Analysis showed that there was an increased resistance among the methicillin resistant staphylococci.

This study showed that there was high number of biofilm producers among isolates resistant to the antimicrobials used in the study. According to Sauer., *et al*. [30] and Anderson., *et al*. [31], biofilm formation is accompanied by significant changes in gene and protein expression, which confers resistance to antimicrobial agents. The relationship between antimicrobial resistance and biofilm formation was illustrated. The results showed that there was higher percentage of biofilm producers among methicillin resistant staphylococci than methicillin susceptible Staphylococci. Forty five (77.65%) MRSA were biofilm producers compared to the 31 (36.9%) MSSA. There was also a statistical difference in the distribution of biofilm production among *S. aureus* isolates. The result of this study was similar to one conducted by Samie and Shivambu [32] where 56% biofilm producers were MRSA and 48% among the MSSA isolates. There was a statistical significance in the distribution in that study. The result of this study was closely related to the outcome of the one conducted by Abdel Halim, Kassem and Mahmoud [28] where 75% of MRSA were biofilm producers. Twenty six (44.1%) methicillin resistant staphylococci which were Cefuroxime resistant were biofilm producers as compared to 9 (25%) meticillin susceptible staphylococci that were biofilm producers. Fifty nine (78.7%) methicillin resistant Staphylococci were biofilm producers as compared to 36 (38.7%) methicillin susceptible Staphylococci that were biofilm producers. This pattern of relationship was obtained in all the antimicrobials used except for Linezolid. Analysis showed that there was an overall significant difference in the susceptibility pattern among biofilm and non-biofilm producers in this study ($P < 0.05$). This result was in concordance with a study by Abirami., *et al*. [22] where there was a significant association between antimicrobial susceptibility pattern and biofilm production.

The prevalence of methicillin resistance in this study was 44.6%. Fifty-eight (40.3%) *S. aureus* isolates were methicillin resistant. This was similar to a study conducted by Abirami., *et al.* [22] where 44.8% MRSA was obtained. A higher prevalence (56.4%) was obtained among *S. aureus* isolates in Abakaliki in a study carried out by Chika., *et al*. [33]. The result of this study showed that there was a significant difference ($P < 0.05$) in the distribution of methicillin resistance among staphylococcal isolates. Antimicrobial resistance is a global menace that has become an issue of concern in the world. Staphylococci in biofilm are known to exhibit resistance to chemotherapy in the process of treatment. Resistance to methicillin has been a top concern to health practitioners because resistance to methicillin implies resistance to other β-lactam antimicrobial agents currently available [20]. The multidrug resistant ability of MRSA has become an increasing problem in hospital and a grave concern in the management of staphylococcal infections.

Conclusion

The prevalence of biofilm forming Staphylococci in this study is relatively high considering the consequential effect of antimicrobial resistance in the development of biofilm. The findings from the study also emphasized the increasing trends in the development of antimicrobial resistance using the biofilm concept. The work showed a correlation between biofilm production and resistance to antimicrobial. There was a significant difference in the biofilm

production to antimicrobial resistance. And it can actually be deduced that biofilm formation is a survival mechanism for pathogenic microorganisms against chemotherapeutic management. Oxazolidinones (Linezolid) is still a drug of choice in the treatment of staphylococcal infections and also infections by methicillin resistant staphylococci.

The growing prevalence of methicillin resistance among staphylococci isolates was also obtained in this study and there is a need to regulate the usage and prescription on antimicrobial in the management of infections.

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Conflict of Interest

No conflict of interest.

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