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Research Article

Prevalence of Biofilm Forming Staphylococci from Clinical Isolates in University of Ilorin Teaching Hospital, North Central Nigeria.

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Abstract
Biofilm, a structural community of bacterial cells enclosed in a self-produced polymeric matrix which could adhere to inert or living surfaces was studied. Microorganisms that grow within the biofilm state possess several mechanisms that increase resistance to external antimicrobial treatments. The objective of the study was to determine the prevalence of biofilm forming staphylococci at the University of Ilorin Teaching Hospital. One hundred and sixty-eight staphylococcal isolates from different clinical specimens were collected in a non-repetitive manner and studied at the Microbiology Laboratory of University of Ilorin Teaching Hospital (UITH). These 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 450nm. The prevalence of biofilm formation among Staphylococcal isolates was 56.5%. S. aureus isolates had 52.8% while coagulase negative Staphylococci had 79.2% biofilm producers. Strong biofilm production was found to be highest in urine (35.7%), followed by wound swab (31%) and blood specimen (19.0%). Some of the wards of admission showed high prevalence of biofilm producers. Notable of the wards were General Outpatient Department (21.1%), Accident and emergency (16.8%) and Neonatal intensive care unit (13.7%). The prevalence of biofilm production at UITH is relatively high and of grave concern considering the devastating effect of antimicrobial resistance, therefore, there is a need to include biofilm detection protocol in the routine microbiological examination.

Key Words: Prevalence, Biofilm, Staphylococci, Clinical Isolates

INTRODUCTION

Biofilm is a structural community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to interior living surfaces (Ramakrishna *et al* 2014). 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 (Bjarnsholt *et al*., 2011). It connotes the lifestyles of aggregated, sessile, or attached microbes in any environment (Kreth and Herzberg, 2015). 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 extracellular polymeric substances (EPS) matrix. Between micro colonies, there are channels through which water flows (Archer *et al*., 2011).

Both Gram positive and Gram-negative bacteria form biofilm. Gram-positive microorganisms that form biofilm include: Bacillus sp., Listeria monocytogenes, Staphylococcus sp., Streptococcus sp. while the Gram-negative bacteria that form biofilm include Escherichia coli, Pseudomonas aeruginosa. The innate and adaptive arms of the immune system more effectively eliminate planktonic cells than microorganisms in biofilms. Microorganisms that grow within the biofilm state are thought to possess several mechanisms that increase resistance to external antimicrobial treatments when compared with bacteria in the planktonic state (Reiter *et al*., 2013). The EPS matrix of the biofilms reduces the penetration of antimicrobial agents into the biofilm community. The matrix barrier also acts as a defense mechanism against other external stimuli such as UV light and dehydration (Conlon *et al*., 2014). It has been reported that mature biofilms (≥7daysold)

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require500-5000 times the concentration of bactericidal agents necessary to kill plankton cells of the organism (Percival *et al*., 2015).

Bacteria involved in biofilm formation are highly refractory to antimicrobial treatment, resulting in serious consequences for the therapy of associated infections. The biofilm lifestyle affordsbacteriaa10-1000 fold increase in antimicrobial resistance compared to their planktonic counterparts, and many of these genetic changes render the constituent bacteria resistant to antimicrobials (Bjarnsholt *et al*., 2011; Hassan *et al*., 2011). Staphylococcus epidermidis in biofilm resists deposition of the antimicrobial complement component C3b and immunoglobulin G (IgG) on cell surfaces thereby reducing the opsonisation required for phagocyte mediated killing (Muhsin *et al*., 2015; Reiter *et al*., 2013; Belibasakis *et al*., 2012). Also, Staphylococcus aureus cells in biofilms, resist macrophage phagocytosis by circumventing bacterial recognition pathways mediated by toll-like receptors TLR2 and TLR9. The EPS mask the bacterial component that serves as the pathogen associated molecular patterns (PAMP) (Percival *et al*., 2015). Even in individuals with excellent cellular and humoral immune reactions, biofilm associated infections are rarely resolved by the host defense mechanisms. Chemotherapy only reverses the symptoms caused by planktonic cells released from the biofilm without destroying the biofilm (Onemu and Ophori, 2013). Staphylococcus aureus biofilm, once established, are recalcitrant to antimicrobial treatment and the host response, and therefore assist in the etiology of many recurrent infections (Bose and Ghosh, 2011).

Cystic fibrosis, native valve endocarditis, otitis media, periodontitis and chronic prostatitis all appear caused by biofilm associated microorganisms (Chen and Wen, 2011). A spectrum of in dwelling medical devices or other devices used in the healthcare environment have been shown to harbor biofilms, resulting in measurable rates of device-associated infections (Conlon *et al*.,2014; Peterson *et al*., 2011).The of microorganisms to be organized in a biofilm community confers increased antimicrobial resistance thereby depleting the pool of available antimicrobials. Antimicrobial resistance is an increasing public health problem and a challenge worldwide. The prevalence of resistance in S. aureus also is on the increase globally. Biofilm formation has been described as a possible attribute to the resistance of pathogenic microorganism and a major contributor to the establishment of nosocomial infections (Hassan *et al*., 2011). Plasmid exchange occurs at higher rate in biofilms, increasing the chances of developing naturally occurring and antimicrobial-induced resistance (Percival *et al*., 2015). This, coupled with the fact that exchange between species possibly increases antimicrobial resistance.

The prevalence of resistant Staphylococcus spp. {Methicillin resistant S. aureus and coagulase- negative staphylococci (CoNS)} is on the increase in Nigeria especially in teaching hospitals (Fadeyi *et al*., 2010; Onemu and Ophori, 2013). The increase in antimicrobial resistance exchange mechanism, such as biofilms formation underscores the importance of this study. Therefore, the contributions of Staphylococci biofilm formation in antimicrobial resistance exchange mechanism and its sources at the UITH, north central Nigeria was studied.

MATERIALS AND METHODS

This study was carried out at the Medical Microbiology Laboratory of the University of Ilorin Teaching Hospital (UITH), a tertiary health care centre and one of the referral centres in Kwara State, north central Nigeria. Purposive, non-probability sampling technique was adopted for the study, using a descriptive cross-sectional design that allows inclusion of all patients from whom staphylococcal isolates were recovered. Staphylococcal isolates were recovered from different clinical specimens (pus, sputum, urine, blood, High Vaginal Swab (HVS), rectal swab, eye discharge, wound swab, aspirate, endocervical swab) at the Microbiology Laboratory of UITH.

Ethical approval: Ethical approval/clearance was sought and obtained from the Ethical Review Committee (ERC) of the University of Ilorin Teaching Hospital (UITH/ERC/2019/021).

Sample size: The sample size for the study was determined using Fisher's formula as modified by Araoye, 2003 where 168 samples were obtained and studied.

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-20oC before the commencement of the research work.

IDENTIFICATION OF STAPHYLOCOCCAL ISOLATES

Culture and 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 37oCfor24hours. The plates were examined after24 hours 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 (Tille, 2014; Brooks *et al*., 2013).

Colonial morphological characteristics: Staphylococcus aureus isolates appeared as translucent, entire colonies with golden colouration of 2-3mm in diameter. The colonies are raise, smooth and glistening. S. epidermidis isolates appeared as greyish-white, raised, circular, smooth, glistening, and translucent to slightly opaque, cohesive colonies S. saprophyticus isolates appeared as raised to slightly convex, circular, entire, smooth, and glistening. The staphylococcal

isolates were identified using an identification workflow (Tille, 2014).

BIOFILM DETECTION AND QUANTIFICATION ASSAY

The study employed a microtiter plate biofilm assay according to the method of Christensen and co-workers (Christensen *et al*., (1985).

Principle: The protocol entails growing staphylococci in microtiter dishes for a desired period of time and washing the well 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 (BHIB, Oxoid, UK – 2% sucrose), 96 well-flat bottom polystyrene (Grenier Bio-one), small tray and ELISA reader (MultiskanExV2.3).

Methods: The method of Stepanovic and co-workers was followed (Stepanovic *et al*., 2007). Briefly, Staphylococcal isolates from overnight culture plates were emulsified with normal saline in universal bottles. The suspensions were standardized to 0.5 McFarland standard. Then individual well sofa sterile 96-well microtiter flat bottom polystyrene plate was inoculated with20 µL of the prepared suspension. Thereafter, 180µL BHIB was added to each well. The first 8 wells served as the control and received 200 µL of sterile BHIB. The plate was incubated at 37oC for 24 h. After incubation, content of each well were removed by gentle tapping to remove planktonic bacteria from each microtiter plate. One hundred and fifty (150 µL of 0.1% crystal violet solution was added for staining in each well including the negative control for 10 min at room temperature. The microtiter plate was shaken-out over the waste tray to remove the crystal violet solution and inverted on paper towels to remove excess liquid. The plate was dried in the hot air oven for one hour. Just before reading, 200 µL of 33% acetic acid was added to each stained well and incubated for 10-15 min at room temperature. The plate was read using the ELISA plate reader. The optical density (OD) of each well was measured at 450nm with an ELISA reader at the University of Ilorin Teaching Hospital Microbiology Laboratory.

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:

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 (Chai *et al*., 2017).

Data Analysis: Data generated were analyzed using statistical package for social sciences (SPSS) version 21.0 and results presented in tables and a figure. Biofilm producers and non-biofilm producers were compared using X2 analysis at a significant level of P<0.05.

RESULTS

Distribution of isolates

The 168 Staphylococcal isolates were from; 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 swab1 (0.6%). Of the 168 isolates, 24 isolates were identified as CoNS. A total of 95 staphylococcal isolates were biofilm producers given a prevalence rate of 56.5%. Seventy six of 144(52.8%) S. aureus produced biofilm while19 of 24 CoNS (79.2%) produced biofilm P= 0.16, (Table 1)

Seventy three (43.5%) Staphylococci were non-biofilm producer, 24(16.7%) were weak biofilm producers, Twenty-nine (17.3%) were moderate biofilm producers while forty-two (25%) Staphylococcal isolates were strong biofilm producers (Figure 1).

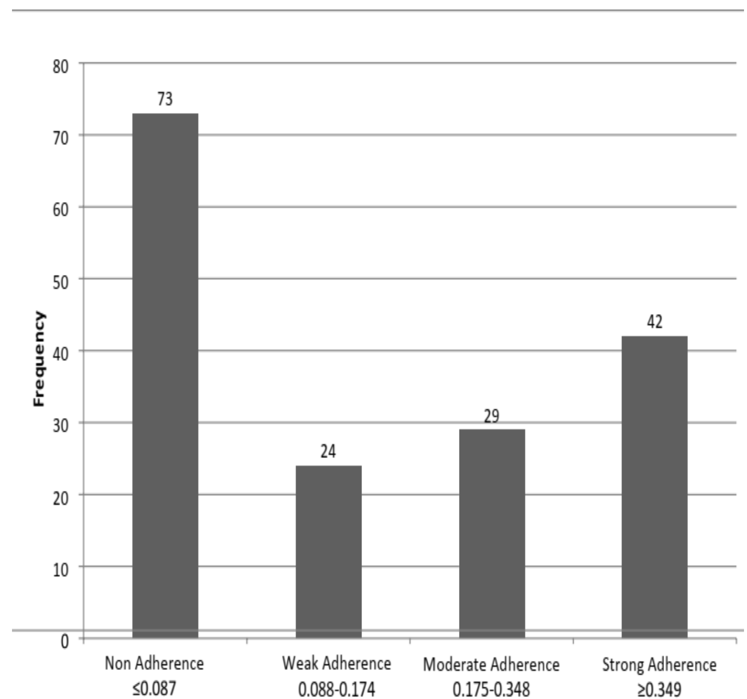

Figure 1: Biofilm forming strength/adherence of staphylococcal isolates

Urine specimen (15, 88.2%) had the highest number of strong biofilm producers and closely followed by wound swab and blood having 13(36.1%) and 8 (34.8%) respectively. Blood specimen had the highest percentage of moderate biofilm producer (11, 47.8%) followed by wound swab (11, 30.6%). Wounds had the highest number of weak biofilm producers (12, 33.3%) (Table 2)

Table 1: Distribution of biofilm producer and non-biofilm producers in the wards

WARD	*S. aureus*			CoNS		
	Biofilm Producer n (%)	Non-Biofilm Producer n (%)	Total N	Biofilm Producer n (%)	Non- biofilm Producer n(%)	Total N
A/E	14(18.4)	7(1.1)	21	2(10.5)	1(20)	3
ENT	2(2.6)	2(2.9)	4	0(0.0)	0(0.0)	0
E/ CLINIC	0(0.0)	1(1.4)	1	1(5.3)	0(0.0)	1
MMW	5(38.5)	8 (11.8)	13	2(10.5)	0(0.0)	2
FMW	4(6.6)	5(7.4)	9	3(15.8)	0(0.0)	3
MSW	1(1.3)	1(1.4)	2	0(0.0)	0(0.0)	0
FSW	3(3.9)	0(0.0)	3	0(0.0)	1(20)	1
GOPD	17(22.4)	11 (16.2)	28	3(15.8)	0(0.0)	3
SOPD	0(0.0)	0(0.0)	0	1(5.3)	0(0.0)	1
EPU	4(5.3)	10 (14.7)	14	3(15.8)	1(20)	4
O/G	4(5.3)	2(2.9)	6	2(10.5)	0(0.0)	2
UROLOGY	1(1.3)	2(2.9)	3	0(0.0)	0(0.0)	0
IVF	1(1.3)	0(0.0)	1	0(0.0)	0(0.0)	0
NICU	11(14.5)	8 (11.8)	19	2(10.5)	1(20)	3
PAEDWARD	0(0.0)	1(1.4)	1	0(0.0)	1(20)	1
CHESTCLINIC	2(2.6)	1(1.4)	3	0(0.0)	0(0.0)	0
BURN/ SUR UNIT	1(1.3)	2(2.9)	3	0(0.0)	0(0.0)	0
OTHERS	6(7.9)	7 (10.3)	13	0(0.0)	0(0.0)	0
Total	**76(52.8)**	**68(47.2)**	**144**	**19(79.2)**	**5(20.8)**	**24**

X^2 = 5.83, P =0.02, N = Total ***S. aureus*** and total CoNS, n = sub-total in biofilm producers and non-Biofilm Producers, A/E = Accident and Emergency, ENT = Ear, nose and Throat, MMW = Male Medical Ward FMW, Female Medical Ward, FSW = Female Surgical Ward, MSW = male Surgical Ward, SOPD = Surgical Out-Patient Ward, GOPD = General Out- Patient Wards, NICU = Neonatal Intensive care Unit, IVF = *In vitro* fertility Ward, EPU = Emergency Pediatric Ward, O/G = Obstetrics and Gynecology, Others include NHIS and undocumented records

Table 2: Percentage distribution of biofilm producers among clinical specimens

Clinical specimen	Weak Biofilm Producer N (%)	Moderate Biofilm Producer N (%)	Strong Biofilm Producer N (%)	Total Biofilm producers per each specimen
Blood	4(17.4)	11(47.8)	8(34.8)	23
Urine	0(0.0)	2(11.8)	15(88.2)	17
Sputum	1(50.0)	1(50.0)	(0.0)	2
Eye swab/discharge	1(25.0)	1(25.0)	2(50.0)	4
Wounds swab/biopsy/pus	12(33.3)	11(30.6)	13(36.1)	36
Throat swab	1(50.0)	0(0.0)	1(50.0)	2
STI swab	3(50.0)	1(16.7)	2(33.3)	6
Aspirate/Effusion	2(40.0)	2(40,0)	1(20.0)	5
Total	**24(25.3)**	**29(30.5)**	**42(44.2)**	**95**

DISCUSSION

The prevalence of staphylococcal isolates obtained from this study shows that both coagulase negative Staphylococci and coagulase positive Staphylococci are present in the different clinical specimens, at the University of Ilorin Teaching Hospital. However there was no significant difference in the distribution of these organisms from the clinical specimens (P>0.05). The highest incidence of S. aureus was in the wound (29.9%) which was consistent with a study by Nwoire *et al.* (2013) who documented that 37% of S. aureus isolates 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. Also, the location of the wounds exposed them to microorganisms originating from the environment, health professionals and medical devices as asserted in a study by Fadeyi *et al* (2010). In the study, it was established that MRSA carrier of health care workers was high especially in critical care units.

The isolates were distributed based on wards of admission of the patients from whom the specimens were recovered. GOPD had the highest number of isolates followed by A/E and NICU, while the least number of isolates came from IVF ward and SOPD. The distribution of these isolates based on the reasons why the patients visited the hospital showed that sepsis had the highest number of isolates. This was followed by UTI while the least isolate came from patients with burn infection, ulcer and Otitis media.

The prevalence of biofilm forming Staphylococci in this study was found to be 56.5%.Aprevalence of 52.8% was found among the S. aureus isolates and 79.2% among the CoNS isolates. This result was similar to a study by Abirami *et al.*(2016) 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 (Eyoh *et al.*,2014) while in Zaria,48.2% prevalence was obtained (Chibueze *et al.*, 2017). There was a significant difference in the distribution of biofilm production among Staphylococcal isolates. The result of this study was also in agreement with a study by Ramakrishna *et al* (2014) where higher biofilm prevalence was found among the CoNS isolates (38% S. aureus and 84% of CoNS). However, some researchers reported higher biofilm prevalence in S. aureusas compared to CoNS (Houston *et al.*, 2011; Conlon *et al.*, 2014; Chika *et al.*, 2018; AbdelHalim *et al.*, 2018). This might not be unconnected with differences in study design and sources of stains isolated. Based on the strength/ability of production, there was a 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.* (2016), moderate biofilm producer 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 be as a result of the difference in study design and quantification method. There was a higher prevalence (92%) of biofilm formation among CoNS isolates in a study conducted by Veena *et al.* (2015) also in India. In that study, 45% and 30% CoNS isolates were strong and moderate biofilm producers respectively. The finding is relatively similar to this study where 50% and 16.6% CoNS isolates were strong and moderate biofilm producers respectively. The mechanisms of biofilm formation in Staphylococci have been a major challenge in the management of staphylococcal infections because of antimicrobial resistance (Meritt *et al.*, 2011; Hassan *et al.*, 2011).

Sixteen (16.8%) Staphylococcal isolates were biofilm producers in the A/E ward while 20(21.1%) Staphylococcal isolates in the GOPD ward were biofilm producers. The presence of biofilm in these wards is of grave concern in the management of infection and development of antimicrobial resistance. All CoNS isolates from eye clinic, MMW, FMW, GOPD, SOPD and O/G were positive for biofilm production.

Eleven (37.93%) isolates from blood and wound swab/discharge specimens were moderate biofilm producers followed by isolates from urine and aspirate/ effusion specimen. Out of the seventeen isolates from urine sample, fifteen (35.7%) constituted the strong biofilm production and from these fifteen seven were from, seven were from catherized patients. 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.*(2017) 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. Also, in a study conducted by Kawamura *et al.* (2011) 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.* (2009) strong biofilm formation in urine specimen was also found to be a factor of catherization. This study showed that wound specimen had the highest number of biofilm producers followed by blood and urine.

CONCLUSION

The prevalence of biofilm forming Staphylococci in this study is relatively high considering the consequential effect of antimicrobial resistance in the development to biofilm. The high prevalence of biofilm forming staphylococci in the wards of admissions are also of grave concern. Wards of critical needs (NICU and A/E) showing relatively high biofilm prevalence emphasizes the importance of prompt intervention. Wound specimens exhibiting high prevalence of biofilm categories established the fact that the wound domain is a favorable environment for the initiation and development of biofilm, therefore prompt and adequate attention must be given to wound infections. We recommend that biofilm detection and quantification should be included in the routine microbiological investigations.

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