

**Original Article****Open Access****Characterization of biofilm formation in clinical urinary isolates of *Staphylococcus aureus* from five hospitals in Lagos State, Nigeria**¹Orjih, C. I., ²Ajayi, A., ^{1,3}Alao, F. O., ¹Adeleye, A. I., and ^{*2,4}Smith, S. I.¹Department of Microbiology, University of Lagos, Akoka, Nigeria²Molecular Biology and Biotechnology Department, Nigerian Institute of Medical Research, Lagos, Nigeria³Department of Biological Sciences, Bells University of Technology, Ota, Ogun State, Nigeria⁴Mountain Top University, Lagos-Ibadan Expressway, Ogun State, Nigeria*Correspondence to: stellaismith@yahoo.com**Abstract:****Background:** Biofilm formation by pathogens is of great clinical importance as it mediates persistence and resistance to antibiotics, hence posing difficulty in treatment and management of diseases. The aim of this study was to evaluate the biofilm forming potential of *Staphylococcus aureus* isolated from urine samples of females with urinary tract infection and to detect the presence of clumping factor (*clfA*) and intracellular adhesion (*icaA*) encoding genes.**Methodology:** A total of 50 *S. aureus* were obtained from urine samples of women in five hospitals in Lagos State, Nigeria. Isolates were confirmed by standard biochemical and novobiocin susceptibility tests. The isolates were screened for biofilm formation using three methods; Congo-red agar (CRA), tube, and tissue culture plate (TCP) methods. Detection of *clfA* and *icaA* genes was done by PCR.**Results:** The Congo red agar method showed that 39 (78%) of the isolates were biofilm producers while 11 (22%) were non-biofilm producers. However, the tube method indicated that 12 (24%) were strong biofilm producers, 26 (52%) were moderate biofilm producers, and 12 (24%) were non-biofilm producers. The standard TCP assay showed that strong biofilm producers (OD > 0.240) were 13 (26%), moderate biofilm producers were 22 (44%), and weak or non-biofilm producers (OD < 0.120) were 15 (30%). The tube method showed a good correlation with the TCP method for strong biofilm production. Ten (20%) isolates possessed *clfA* gene and 31 (62%) possessed *icaA* gene.**Conclusion:** The ability of *S. aureus* to form biofilm is a key risk factor that can increase morbidity and mortality from infections they cause. Hence, rapid and sensitive phenotypic methods can be used in screening for biofilm formation thereby providing data that can guide therapy and control of the pathogen**Keywords:** *Staphylococcus aureus*, Biofilm, Clumping factor, Intracellular adhesion

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Copyright 2021 AJCEM Open Access. This article is licensed and distributed under the terms of the Creative Commons Attribution 4.0 International License <http://creativecommons.org/licenses/by/4.0/>, which permits unrestricted use, distribution and reproduction in any medium, provided credit is given to the original author(s) and the source. Editor-in-Chief: Prof. S. S. Taiwo**Caractérisation de la formation de biofilm dans des isolats urinaires cliniques de *Staphylococcus aureus* provenant de cinq hôpitaux de l'État de Lagos, Nigéria**¹Orjih, C. I., ²Ajayi, A., ^{1,3}Alao, F. O., ¹Adeleye, A. I., et ^{*2,4}Smith, S. I.¹Département de microbiologie, Université de Lagos, Akoka, Nigéria²Département de biologie moléculaire et de biotechnologie, Institut nigérian de recherche médicale de Lagos, Nigéria³Département des sciences biologiques, Université de technologie Bells, Ota, Ogun State, Nigeria⁴Université Mountain Top, autoroute Lagos-Ibadan, État d'Ogun, Nigéria*Correspondance à: stellaismith@yahoo.com

Abstrait:

Contexte: La formation de biofilm par des agents pathogènes est d'une grande importance clinique car elle intervient dans la persistance et la résistance aux antibiotiques, ce qui pose des difficultés dans le traitement et la gestion des maladies. Le but de cette étude était d'évaluer le potentiel de formation de biofilm de *Staphylococcus aureus* isolé à partir d'échantillons d'urine de femmes atteintes d'une infection des voies urinaires et de détecter la présence de gènes codant pour le facteur d'agglutination (*clfA*) et l'adhésion intracellulaire (*icaA*).

Méthodologie: Un total de 50 *S. aureus* a été obtenu à partir d'échantillons d'urine de femmes dans cinq hôpitaux de l'État de Lagos, au Nigéria. Les isolats ont été confirmés par des tests standard de sensibilité biochimique et à la novobiocine. Les isolats ont été criblés pour la formation de biofilm en utilisant trois méthodes; Méthodes de gélose au rouge Congo (CRA), de tubes et de plaques de culture tissulaire (TCP). La détection des gènes *clfA* et *icaA* a été réalisée par PCR.

Résultats: La méthode de l'agar rouge du Congo a montré que 39 (78%) des isolats étaient des producteurs de biofilm tandis que 11 (22%) n'étaient pas des producteurs de biofilm. Cependant, la méthode du tube a indiqué que 12 (24%) étaient de puissants producteurs de biofilm, 26 (52%) étaient des producteurs de biofilm modérés et 12 (24%) n'étaient pas des producteurs de biofilm. Le test TCP standard a montré que les producteurs de biofilm forts (DO > 0,240) étaient 13 (26%), les producteurs de biofilm modérés étaient de 22 (44%) et les producteurs de biofilm faibles ou non (DO < 0,120) étaient de 15 (30%). La méthode du tube a montré une bonne corrélation avec la méthode TCP pour une forte production de biofilm. Dix (20%) isolats possédaient le gène *clfA* et 31 (62%) possédaient le gène *icaA*.

Conclusion: La capacité de *S. aureus* à former un biofilm est un facteur de risque clé qui peut augmenter la morbidité et la mortalité dues aux infections qu'elles provoquent. Par conséquent, des méthodes phénotypiques rapides et sensibles peuvent être utilisées dans le dépistage de la formation de biofilm, fournissant ainsi des données qui peuvent guider la thérapie et le contrôle du pathogène.

Mots clés: *Staphylococcus aureus*, Biofilm, Facteur d'agrégation, Adhésion intracellulaire

Introduction:

Biofilms are communities of bacteria embedded in a self-produced extracellular matrix made of exopolysaccharides (EPSs) containing proteins and some macromolecules such as DNA. They can form on both biotic and abiotic surfaces (1). Studies have shown using scanning electron microscopy (SEM) and other molecular techniques that human tissues and medical implants are colonized by biofilms. Biofilm protects the microorganism from host defences and impedes delivery of antibiotics thereby resulting in the persistence of infections (2,3).

Staphylococcus aureus is an opportunistic pathogen implicated in skin and soft tissue infections. It exists in the nasopharynx, skin, eye, intestine and urogenital tract as normal flora. However, it can breach the skin barriers through the wound or surgical incision and cause infection. Furthermore, it has the ability to adhere to and form a biofilm on tissues or medical indwelling devices (4). *S. aureus* initially adheres to a solid substrate, after which cell-cell adhesion occurs; and the bacteria then multiply to form a multilayered biofilm encased in EPS.

Biofilm formation involves the production of polysaccharide intercellular adhesin, which depends on the expression of the intercellular adhesion molecule operon (*icaADBC*) which encodes three membrane proteins (*IcaA*, *IcaD*, and *IcaC*) and one extracellular protein (*IcaB*). In addition, several surface

proteins have been involved in the biofilm formation process in staphylococci including biofilm-associated protein; surface protein G, fibronectin-binding proteins and staphylococcal protein A (5). Biofilm formation by *S. aureus* can lead to delay in re-epithelialization of infected tissues, ultimately increasing healing time. *S. aureus* biofilms have been associated with chronic wounds such as diabetic foot ulcer, pressure sores and venous ulcers (6). Detachment of matured biofilm of *S. aureus* is a prerequisite for the dissemination of wound infection (7).

In Nigeria, there is paucity of data on studies that characterize biofilm formation in pathogens of clinical origin. Hence this study is aimed at characterizing biofilm formation in clinical *S. aureus* isolates.

Materials and method:

Study setting and subjects

The subjects were non-pregnant women presenting with urinary tract infection in five public hospitals; Lagos University Teaching hospital, General Hospital Marina, General Hospital Gbagada, General Hospital Shomolu, and General Hospital Orile-Agege in Lagos State, Nigeria. Samples were collected between February 2015 and April 2017.

Ethical approval

Ethical approval for this study was obtained from the Human Research and Ethical Committee (HREC) of the Lagos University Teaching Hospital with code number ADM/

DCST/HREC/879 and Health Research Ethics Committee of the Gbagada General Hospital with code number GBGH/705/100

Bacterial isolates

One millilitre of the urine sample was inoculated into 9mL of brain heart infusion broth (Oxoid, Basingstoke, UK) and incubated for 24 hours at 37°C. A loopful of broth culture was then streaked onto mannitol salt agar (Oxoid, Basingstoke, UK) and incubated for 24 hours at 37°C. Yellow colonies were picked and sub-cultured on nutrient agar to obtain pure colonies. Standard biochemical tests and novobiocin susceptibility test to phenotypically confirm *S. aureus* were carried out according to Cheesbrough (8), with *S. aureus* ATCC 29213 as quality control strain.

Biofilm formation assay

Biofilm formation assays were performed according to Mathur et al., (9) and Cavant et al., (10) with slight modifications. All 50 *S. aureus* isolates were tested by three *in vitro* screening procedures for their ability to form biofilm

Tube method

Staphylococcus aureus isolates were inoculated into brain heart infusion broth (Oxoid, Basingstoke, UK) and incubated for 24 hours at 37°C. Thereafter, spent broth were decanted and tubes were washed with phosphate buffered saline (pH 7.3) and allowed to air dry, and then stained with 0.1% crystal violet solution. Excess stain was discarded and tubes were washed three times with sterile distilled water and dried. A positive result was indicated by the presence of visible film lining the wall and bottom of the tube. The absence of a film or presence of only a stained ring at the liquid air interface was considered as a negative result. Biofilm forming potential were categorized base on the intensity of film as strong (+++), moderate (++) , weak (+) (9). *S. aureus* ATCC 29213 was used as positive control

Congo-Red Agar (CRA) method

Suspension of *S. aureus* isolates were inoculated onto brain heart infusion agar (Oxoid, Basingstoke, UK) supplemented with 5% sucrose and 0.8 g/L Congo red. This was

done in triplicate and incubated at 37°C for 24 to 48 hours. Culture plates that displayed black colonies with a dry crystalline consistency were noted as positive for biofilm formation while plates that had pink colonies with occasional darkening at the center were considered as weak or non-biofilm formers.

Tissue Culture Plate (TCP) method

Staphylococcus aureus isolates were inoculated into brain heart infusion broth (Oxoid, Basingstoke, UK) supplemented with 2% sucrose and incubated for 18 hours at 37°C. A dilution of 1 in 100 with fresh medium was done and 200µL was dispensed into individual wells of sterile 96 well tissue culture plates. Positive control *S. aureus* ATCC 29213 strain was included in separate wells, while negative control was sterile broth medium. The tissue culture plates were incubated for 18 hours at 37°C, after which the content of each well was gently removed by tapping the plates. The wells were washed four times with 200µL of phosphate buffer saline (pH 7.2) to remove free-floating bacteria. Biofilm formed by adherent organisms in plate were fixed with 2% sodium acetate and stained with 0.1% crystal violet. Excess stain was washed off gently with deionized water and dye incorporated by the adherent cells was solubilized by adding 200µL of 33% glacial acetic acid (Merck, Darmstadt, Germany).

The optical density (OD) of each well was determined with an Emax® Plus Microplate Reader (Molecular Devices San Jose, CA) at wave length 570nm. The experiment was performed in triplicate and repeated three times. Absorbance was calculated by subtracting the OD₅₇₀ of control from that of the test assays OD₅₇₀ with mean value determined for each isolate. Data obtained were used to classify OD₅₇₀ values < 0.120 as non-biofilm producers, OD₅₇₀ values between 0.120-0.240 as moderate biofilm producers, and OD₅₇₀ values > 0.240 as strong biofilm producers.

Molecular detection of *icaA* and *clfA* genes

DNA extraction was done using the method of Suvajdžić et al., (11). PCR was performed by targeting specific primers listed in Table 1. A 20µL PCR reaction containing 4µl (5x) FIREPol master mix [7.5mM MgCl₂, 1Mm

Table 1: List of primers targeted to detect *icaA* and *clfA* genes

Primer	Sequence (5'-3')	Product Size	Reference
<i>icaA</i> -FW	GAGGTTAAAGCCAACGCACTC	151	(12)
<i>icaA</i> -RV	CCTGTAACCGCACCAAGTTT		
<i>clfA</i> -FW	ACCCAGGTTTCAGATTCTGGCAGCG	165	(12)
<i>clfA</i> -RV	TCCGCTGAGTCGGAATCGCTTGCT		

dNTPs, 0.4M Tris-HCl, 0.1M (NH₄)₂SO₄, 0.1% Tween-20, FIREPol DNA Polymerase) (Solis BioDyne, Estonia)], 0.6µL forward primer, 0.6µL reverse primer, 4µL DNA template and 10.8µL nuclease free water, was carried out in a Master cycler (Eppendorf AG, Hamburg, Germany). Amplification conditions consist of initial DNA denaturation at 95°C for 5min, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 45°C for *clfA* and 54°C for *IcaA* for 30s and extension at 72°C for 2min, followed by a final extension at 72°C for 10min. Amplification products were analysed by electrophoresis at 100V for 60min in 2% agarose gel stained with ethidium bromide and visualized under ultraviolet trans-illuminator (Cleaver Scientific Ltd). A 100bp DNA ladder (Solis BioDyne, Estonia) was used as a molecular weight marker.

Statistical analysis

Microsoft Excel (Microsoft Cooperation, 2013 USA) was used to generate graphics and determine percentages

Results:

The three phenotypic biofilm characterization methods used yielded varying results. The CRA method indicated that 39 (78%) of the isolates were biofilm producers while 11 (22%) of the isolates were non-biofilm producers as shown in Fig 1. However, the tube method indicated that 12 (24%) were strong biofilm producers, 26 (52%) were moderate biofilm producers and 12 (24%) were non-biofilm producers. The standard TCP assay showed that strong biofilm producers (OD > 0.240) were 13 (26%), moderate biofilm producers were 22 (44%) and weak or non-biofilm producers (OD < 0.120) were 15 (30%). The tube method showed a good correlation with the TCP for strong biofilm producers as shown in Fig 2.

Despite the phenotypic display of biofilm formation by the *S. aureus* isolates, only 10 (20%) harboured clumping factor encoding gene (*clfA*) while the intracellular adhesion encoding gene (*icaA*) was detected in 31 (62%) isolates as shown in Fig 3.

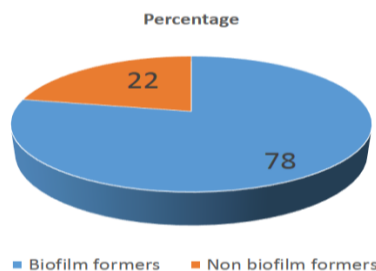


Fig 1: Frequency (%) of biofilm formation among *Staphylococcus aureus* isolates by Congo red agar method

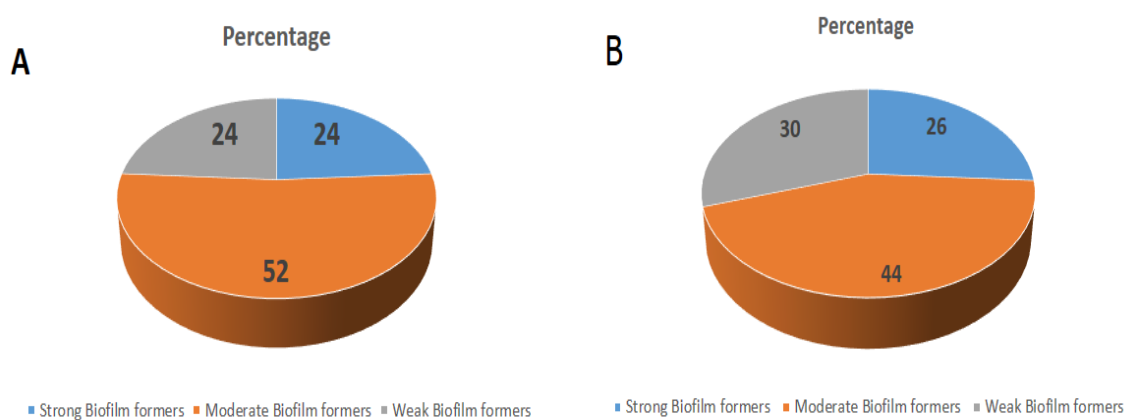
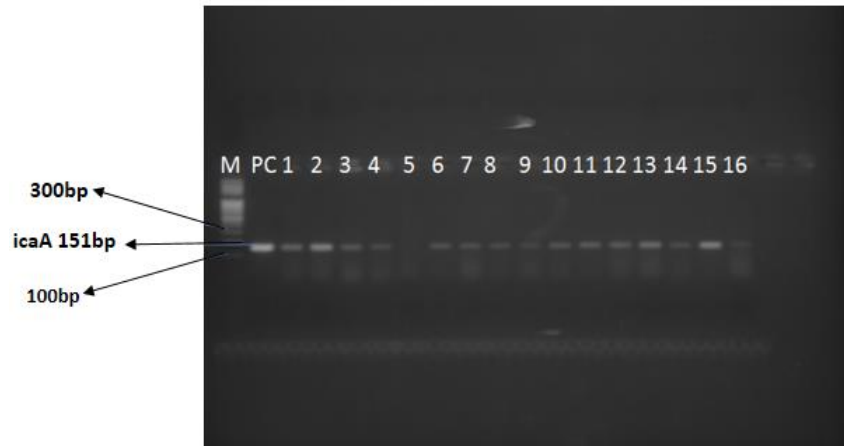


Fig 2: Frequency (%) of strong, moderate and weak biofilm formation among *Staphylococcus aureus* isolates by the tube (A) and TCP (B) methods



Lane M: 100bp molecular marker, Lane PC: Positive control, Lane 1-16 with the exception of Lane 5 are positive for *icaA* gene

Fig 3: Agarose gel image showing the 151 bp *icaA* gene detected in *Staphylococcus aureus* isolates

Discussion:

Biofilm forming *S. aureus* has been linked to life threatening infections that defies antimicrobial therapy due to resistance, which encourage persistence and easy dissemination (12). In this study, the TCP assay method revealed that 13 (26%) of the *S. aureus* isolates screened were strong biofilm formers, 44% were moderate biofilm formers, and 30% were weak or non-biofilm formers. This is similar to the findings of Mathur *et al.* (9) who reported 14.47%, 39.4%, and 46.0% as strong, moderate and weak *S. aureus* biofilm formers respectively. This method gives the best discrimination between strong, moderate and non-biofilm formers as it used cut-off values.

Although the tube method correlated well with the TCP strong biofilm formers in this study, weak formers were difficult to discriminate from non-biofilm formers. The tube method detected slightly lower number of strong biofilm formers with 24%, which was lower in comparison to the TCP method with 26%. However, the observation of Flemming *et al.*, (13) showed that TCP method detected 69% of biofilm formers, whereas, TM detected only 36%. The authors further opined that this method could discriminate between strong and moderate biofilm formers since it is a quantitative method.

In this study, CRA detected 78% *S. aureus* isolates that were biofilm formers and 22% non-biofilm formers. However, this is at variance with previous observations made by Mathur *et al.*, (9) and Taj *et al.*, (14) who reported very low percentage 5.3%, and 3.4% of biofilm formers respectively by the CRA

method. In a similar study by Sharvari and Chitra (15), a higher percentage (25.3%) was reported. The higher percentage reported in our study using the CRA method could be attributed to a modification in the preparation of the CRA in our laboratory by filter sterilization of the Congo red before addition to the molten autoclaved brain heart infusion agar.

Clumping factor A (*clfA*) has been reported to mediate adhesion to fibrinogen thereby enhancing the formation of biofilm in *S. aureus*. In the report of Wolz *et al.*, (16), staphylococcal *clfA* cloned into *Streptococcus gordonii* and *Lactococcus lactis* which lack surface adhesion potentially gained the ability and were able to cause endocarditis in an animal model. PCR assay of *clfA* in this study revealed that 24% of the *S. aureus* isolates possessed the gene. In a similar study, Nourbakhsh and Namvar (17) reported a slightly higher carriage rate (41.4%) of *clfA* gene in *S. aureus* isolated from nosocomial infection in Isfahan Iran. As opposed to the low occurrence of *clfA* genes in our isolates, majority (62%, n=31) of them carried *icaA* gene. Kifaya *et al.*, (18) reported that all *S. aureus* isolates assayed for *icaA* gene were positive for the gene and they all phenotypically formed biofilm, which agrees with our study where all 31 isolates carrying *icaA* gene phenotypically formed biofilm. The correlation of biofilm formation and the possession of *icaA* gene was also reported by Namvar *et al.*, (12).

Conclusion:

Biofilm formation by microbial pathogens remains a great threat in the healthcare sector because infections caused by them are

associated with increased morbidity and mortality, as well as contributing to significant financial burden on the economy. Hence, the use of nanoparticles that have been shown to compromise integrity or destroy biofilms, may be explored in tackling the challenges of biofilm forming pathogens.

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