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Evaluation of Biofilm Formation by *Staphylococcus aureus* Recovered from Clinical Samples of Patients Attending a Tertiary-Care Hospital in North-Eastern Nigeria

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Abstract

Staphylococcus aureus is a classical pathogen that is implicated in a wide range of diseases. It is a resident flora of the human skin and can easily contaminate open wounds and gain access into circulation and inflict damage to the host. This study is designed to identify *Staphylococcus aureus* from various clinical samples and determine the isolates' ability to form biofilm. Eighty-six (86) clinical samples were collected aseptically from patients attending University of Maiduguri Teaching hospital. The samples were processed using standard microbiological methods for the identification of *S. aureus*. Samples were cultured on 5% blood agar and presumptive *S. aureus* isolates were further confirmed by biochemical identification at the Microbiology Laboratory of University of Maiduguri, Nigeria. Biofilm formation was analysed using three detection methods which comprise of Congo Red Agar (CRA) method, Tube Method (TM) and Microscopy Method (MM). Out of the eighty-six (86) samples processed, sixty-one (61) yielded positive growth of *Staphylococcus aureus*, which gives a bacterial recovery rate of 71.0%. The yield was found to be highest from blood samples (36.1%) and wound swab samples (18.0%). Majority of the isolates were alpha-hemolytic (50.2%) while the rest were beta-hemolytic (49.8%). Of the *S. aureus*, 29.5% of the isolates were good mucoid variants, 32.8% were strong mucoid variants while 13.1% were complete mucoid variants. 55.4% of isolates tested positive to biofilm formation according to the CRA method, 30.4% according to Tube method and 14.3% according to Microscopy method. Biofilm formation is a recipe for the chronicity of infection and if not detected, can delay therapy and increase the cost of management of an infectious diseases.

Keywords: *Staphylococcus aureus*; Nosocomial infection; Biofilm detection; Clinical samples; Congo red agar

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Introduction

Staphylococcus aureus is a gram-positive bacterium that colonized significant parts of healthy adult population and this aid in risk of diverse infections in both community and hospitalized individuals [1]. *S. aureus* is considered a key agent of hospital and community-acquired infections; hence complicated the load in management of diseases [2]. *S. aureus* have ability to adhere to surfaces of medical devices and host tissue, leading to the formation of biofilm [3]. The ability to form biofilm by this organism can serve as a recipe for the chronicity of infections, by aiding the organism in host-immune evasion and resistance to antimicrobial drugs [2]. Infections caused by biofilm-producing organisms are chronic in nature and mostly occur in hospitals. *S. aureus* has been described as a major cause biofilm-associated infection [4]. A study reported that the notoriety of infections caused by biofilm-producing *S. aureus* has led to increase in cost of managing illness globally in the form increase in hospital bill

and cost of hospital stay [5]. Biofim formation involves series of processes to include initial attachment to surfaces, accumulation of bacterial population and maturation of complex biofilm layer and subsequent dispersal in case of change in environmental conditions [6,7]. Furthermore, Studies indicate that *S. aureus* can form biofilm on host surfaces such as heart valves, bones, cartilage, and medical device such as catheters and orthopaedic devices [8].

S. aureus attaches firmly to those surfaces by either direct interactions with a device's polymer surface or by clinging to human matrix proteins after those proteins have covered the device [8]. And as such, *S. aureus* is considered as a key pathogen implicated in foreign-body infections [9,10]. Similarly, the organism also has ability to form biofilm on inanimate objects such as pipes or any foreign body [11]. In addition to biofilm formation, *S. aureus* use vast array of virulent determinants to overcome host defence mechanism such as extracellular toxins (hemolysin, leukotoxins), enzymes such as coagulases and proteases and, surface proteins (clumping factor, adhesins) as virulent factors [2]. The combinations of these virulence factors and ability of *S. aureus* to form biofilm increase challenge of treating bioflim-associated infections cause by *S. aureus* [12]. The clinical forms of *S. aureus* infections range from mild superficial skin infections to toxin mediated and severe life-threatening systemic presentations [2]. Infections such as *S. aureus* bacteraemia (SAB) reported widely, and incidences of pneumonia, infective endocarditis caused *S. aureus* infections and resistant form of Methicillin-Resistant *Staphylococcus aureus* (MRSA) were also reported in both community and hospitalized patients [2,5,13,14].

Studies on biofilm formation among clinical isolates of *Staphylococcus aureus* in North-eastern Nigeria is lacking. Hence, this study seeks to identify *S. aureus* isolates from clinical samples collected from patients in the study area and examine the rate of biofilm formation among those isolates.

Materials and Methods

Study area

This is a cross-sectional qualitative study that was conducted at the laboratory of the Department of Microbiology, University of Maiduguri, North-Eastern Nigeria.

Bacterial isolates and growth media

A total of 86 clinical samples (comprising of blood, sputum, wound swab, nasal wash, aspirates, body fluid, catheter tips, and urine) were collected asceptically from patients attending University of Maiduguri Teaching hospital, over three months period (November 2019 to January 2020). Samples were then processed using standard microbiological methods for the identification of *Staphylococcus aureus*. Samples were cultured on 5% blood agar, which was prepared according to the manufacturer's specification. Presumptive *S. aureus* isolates were further confirmed by biochemical identification at the Microbiology Laboratory of University of Maiduguri, Nigeria [15].

Biofilm formation evaluation

Biofilm formation was assessed using three (3) qualitative methods as described below.

Congo Red Agar (CRA) method

Confirmed *S. aureus* isolates were cultured on Congo Red Agar (CRA). CRA was prepared by adding 10 gram of nutrient agar powder, 0.4 gram of Congo Red agar (CRA) and 7.5 gram of NaCl into a conical flask. 500 ml of distilled water was added. Glucose (10 grams) and sucrose (10 grams) were added and the solution autoclaved at 121°C for 15 minutes at a pressure of 15 psi. The media is then poured into petri dishes and allowed to cool. Two sets of the media were made and supplemented with glucose and sucrose respectively. CRA was then inoculated with the test organism and incubated for 24 hours at 37°C. The formation of crystal black colonies is indicative of biofilm formation. The addition of the sugars is to enhance biofilm formation. *E. coli* was used as a control, and was incubated on the CRA plates to detect whether or not they produce black colonies. All the control species were incubated for 24 hours at 37°C on media with sugar supplementations [16].

Nutrient broth was prepared by adding 1.6 gram of nutrient broth powder to 200 ml of distilled water and heated for 10 minutes. 5 ml of the broth was then dispensed into test tubes and incubated for 1 hour at 37°C. To each test tube containing nutrient broth, 100 ml of sucrose was added, and *S. aureus* was inoculated and mixed, then incubated for one day at 37°C. This is applied to the *Staphylococcus* colonies and the control isolates. Similar procedure was done using glucose. The tubes were made dry, and then stained with 0.1% crystal violet. Biofilm formation was detected by the presence of a line on the wall of the tubes as described previously [17].

Identification of biofilm using microscopy methods

A loop full of test organism was inoculated into 2 sets of broth, 43 test tubes each for the test organisms in different sugar supplementation, while another tube for the control. The test tubes were divided to contained two different sugars, into each set; 100 µL of glucose and sucrose were added respectively. All sets were incubated for 24 hours at 37°C. After incubation, 50 µL of the test organisms from the test tubes were transferred into eppendorf tubes, then 20 µL of indian ink was added and 1 ml of distilled water were adequately mixed. Certain portion of the mixture was transferred on slides and viewed under microscope for possible formation of biofilm ring around the colonies [18].

Data were grouped as frequencies and percentages, and presented as tables and figures using Microsoft Excel program (2016).

Results

Sixty-one (61) out of the eighty-six (86) samples processed yielded a positive growth of *Staphylococcus aureus*, which gives

a bacterial recovery rate of 71.0%. The yield was found to be highest from blood samples and least among body fluid samples (36.1% and 1.6% respectively) **(Figure 1**).

The hemolytic pattern of *S. aureus* identified revealed that 50.8% were alpha hemolytic, 49.2% were beta hemolytic while none was found to exhibit the gamma hemolytic pattern. The mucoid aspect of isolates was also determined. 24.6% of isolates identified were weak mucoid variants, 29.5% were good mucoid variants, 32.8% were strong mucoid variants while 13.1% were complete mucoid variants (**Figures 2 and 3**).

The rate of biofilm formation among *S. aureus* isolates identified was found to be 51.0% (31/61). Analysis of the sensitivity of the three biofilm detection methods revealed that 55.4% of isolates tested positive to biofilm formation according to the CRA method, 30.4% tested positive according to the Tube method while 14.3% tested positive according to Microscopy method **(Table 1 and Figure 4).**

The results of staining techniques using Indian ink on the species and viewed under microscope. All the isolates formed a ring-like appearance indicating presence of biofilms **(Figure 5).**

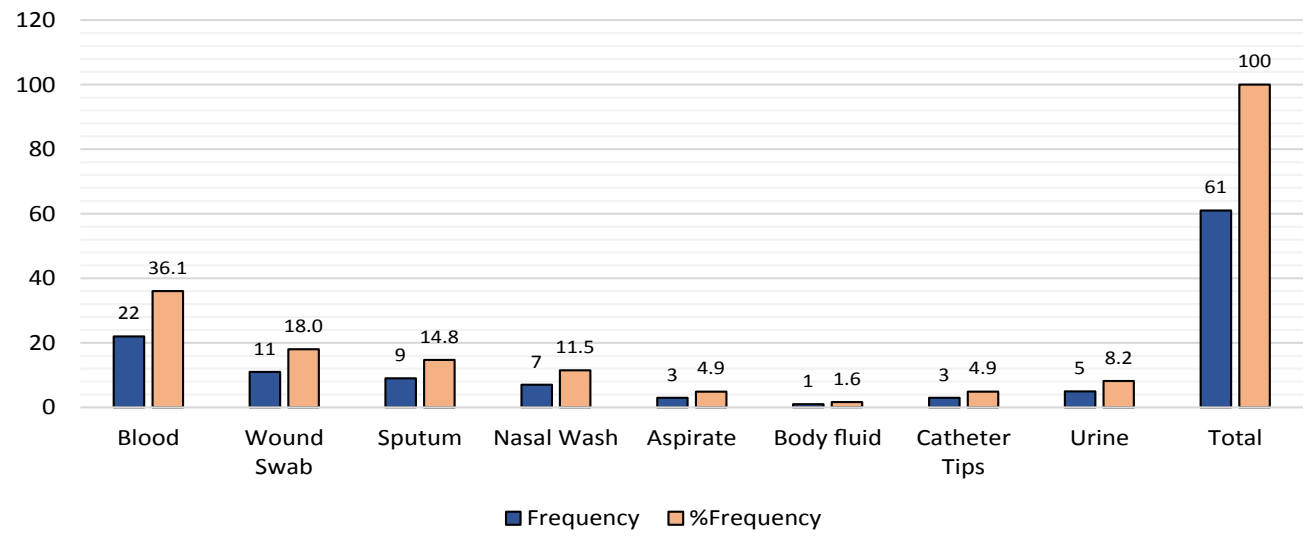

Figure 1 Identification of *Staphylococcus aureus* from clinical samples collected from patients attending University of Maiduguri Teaching hospital, Maiduguri.

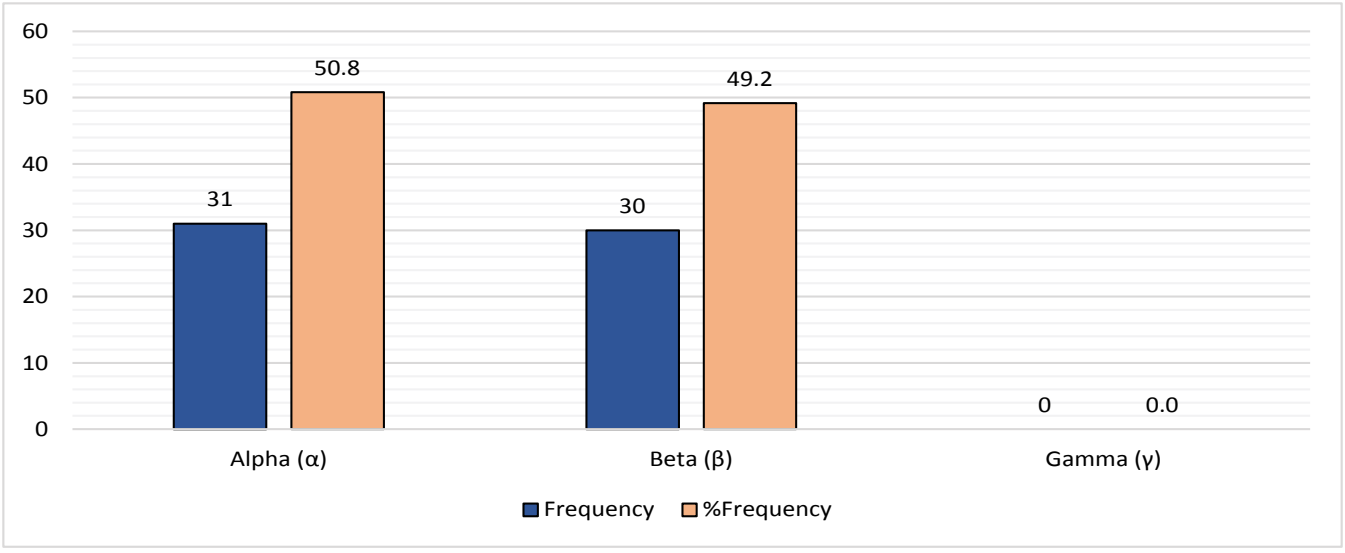

Figure 2 Characterization of *S. aureus* isolates identified based on their hemolytic pattern.

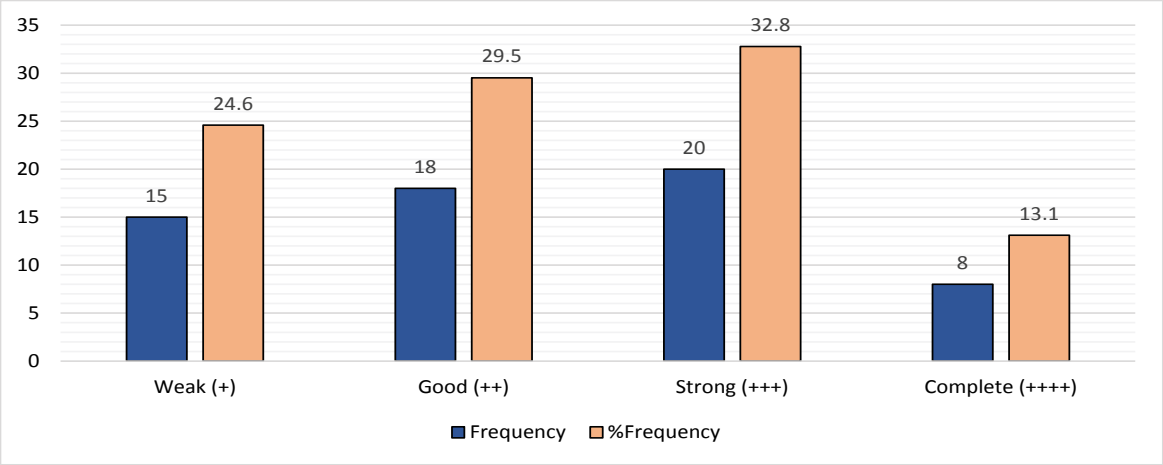

Figure 3 Characterization of *S. aureus* isolates identified based on mucoidity.

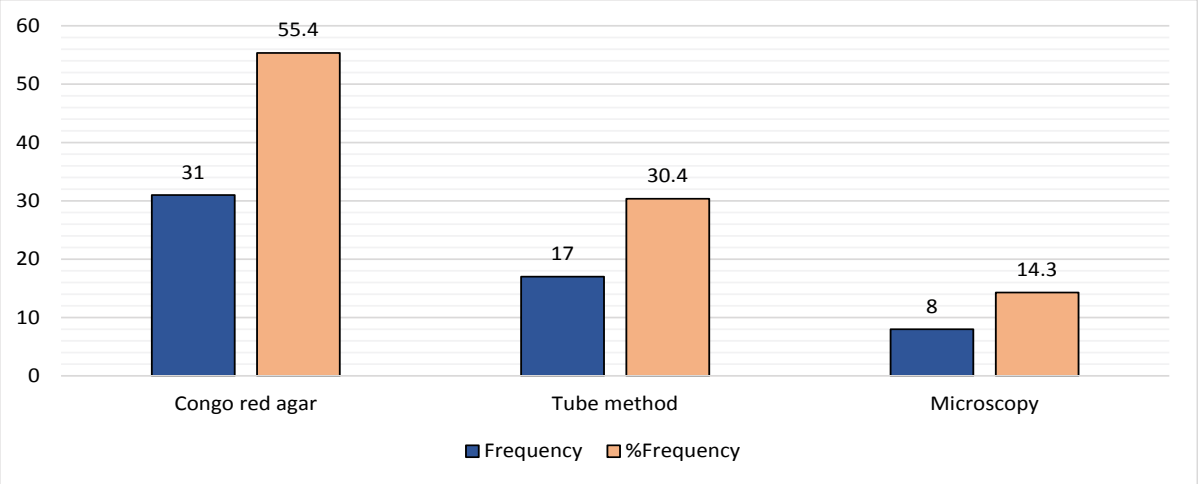

Figure 4 Comparative analysis of biofilm detection among the three (3) biofilm detection methods used in this study.

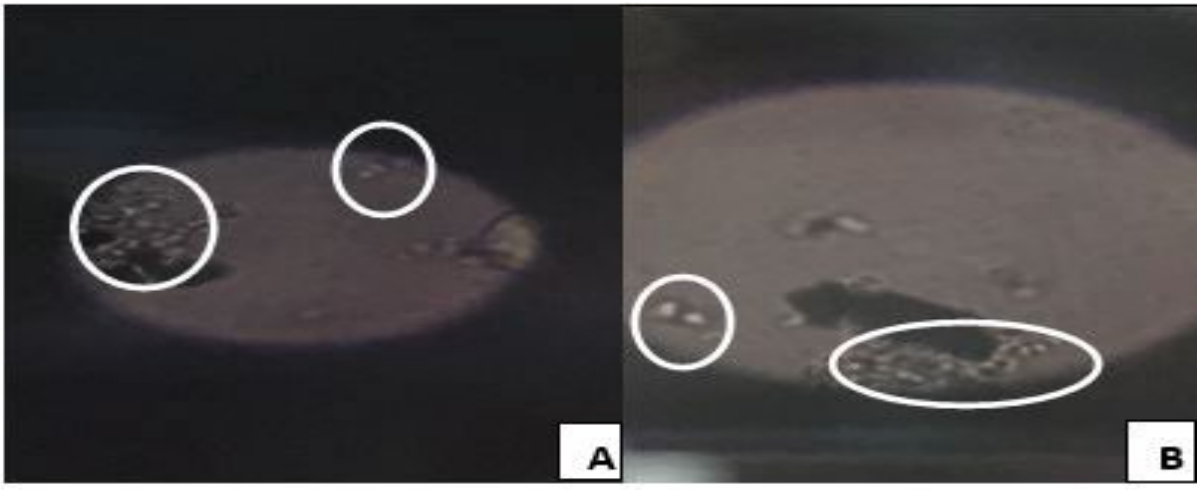

Figure 5 Biofilm production by *S. aureus* colonies as observed under the microscope (A and B). The black ring surrounding the cream white colonies indicate biofilm formation.

Variable	*Staphylococcus aureus* (%)	Biofilm formation (%)*
Positive	61 (71.0)	31 (51.0)
Negative	25 (29.0)	30 (49.0)
Total (%)	86 (100)	61 (100)
*based on CRA method		

Table 1: Determination of Biofilm formation among *S. aureus* identified from patients attending University of Maiduguri Teaching hospital, Maiduguri.

Discussion

Biofilm formations of clinical isolates have long been considered threat for the treatment of infections complicated by biofilm-producing strains [19]. *Staphylococcus aureus* is a classical pathogen that has been identified in various anatomical sites of the human body. It is a resident flora of the human skin and can contaminate open wounds on the skin surface and sometimes gain entry into normally sterile sites such as the blood from which it can be distributed to other parts of the body. In this study, we have observed a very high contamination rate of clinical samples by *Staphylococcus aureus*. Blood and wound swab samples were the most contaminated. All *S. aureus* isolates identified were either alpha-hemolytic or beta-hemolytic. Nwankwo and Nasiru [20] reported similar findings while working on *S. aureus* identified from clinical samples collected from patients attending a tertiary hospital in Kano, Northwestern Nigeria. Several studies elsewhere in Nigeria reported similar findings [21,22].

The presence of *S. aureus* in wounds and blood is an indication of a potential wound infection and bacteremia respectively. Contamination of wounds could be a consequence of poor personal hygiene and inappropriate exposure of wounds. *S. aureus* would naturally be suspected to contaminate such a wound because of its status as a resident flora of the skin. *S. aureus* bacteremia has been documented across all age groups and carries 20%-35% mortality rate [23]. In a study conducted by Ladhani et al. [24] where they analyze staphylococcal bacteremia among children admitted in a rural Kenyan hospital, they reported that almost half of the patients that were later diagnosed with bacteremia due to *S. aureus*, presented without a staphylococcal focus of infection and as a result, they had a 14-fold increase in mortality. This can be attributed to the fact that they have not received a specific antistaphylococcal antibiotic on admission.

In the current study, 61/86 (71%) *Staphylococcus aureus* strains were recovered from clinical samples according to standard protocols and testing using *in vitro* testing methods for the detection of biofilms. *E. coli* strains were used as negative control. The study employed Congo red agar, Test tubes method and Microscopy for the detection of biofilm production by *S. aureus*. In this study, the rate of biofilm-producing isolates was found to be 55.4%, 30.4% and 14.3% for CRA, TM and MM methods respectively.

Several studies have investigated biofilm production by *Staphyococci* species from clinical origin using different methods [6,7, 25-27]. In this study, it was found that all the methods used in the work, were able to detect biofilms formation among the isolated strains. The results of biofilms production by the 56 (92%) *S. aureus* strains were assessed by the production of either biofilm black colonies on (CRA) the formation of a turbid ring in a tube or Indian ink surrounded the cells, indicating presence of EPS and can be viewed under microscope. We observed that the isolates in this study were slime producers, and this finding is in agreement by the previous reports [28,29]. Interestingly, the control used, *E. coli* strains were not biofilms producers, this observation in the current study is in agreement with previous reports [30,31]. In this study, a significant majority of the *S. aureus* isolates identified were strong mucoid variants. In addition, more than half of the isolates identified in this study were biofilm producers. Biofilm formation has also been associated with increased resistance and/or tolerance to antimicrobial drugs. Ibrahim et al. [32] (2020) reported that biofilm forming isolates of *S. aureus* exhibit a highly reduced susceptibility to antimicrobial drugs. They further affirmed that extreme forms of antimicrobial resistance such as multidrug resistance and pan drug resistance are more pronounced among biofilm forming strains of *S. aureus*.

In the present study, three methods for the detection of biofilms were employed in this study and Congo red agar (CRA) method was observed to be the most sensitive. This is contrary to the report of Arslan and Özkardes, [33] who observed that Tube method was the most sensitive. Hassan et al. [34] observed that Tissue Culture Plate (TCP) is the most sensitive method and recommend the use of it as a general screening method for detection of biofilm producing bacteria in laboratories.

The striking observation made in the current study is the role of polysaccharides in the formation of biofilm. In the current study, it was found that the sugar used enhance the formation of biofilm, this was evident that the first set of CRA with no sugar supplementation did not yield biofilm colonies. This observation agreed with previous studies [35,36]. In these study sugar, glucose where added to detect biofilm formation by *S. epidermidis* and *S. aureus* in Lennox broth, the concentrations were given in an increasing manner from 0 to 320 mg/dL in 20 mg/dL intervals [28,29]. Biofilm was grown for 24 hours for *S. epidermidis* and 48 hours for *S. aureus.* Furthermore, Khangholi and Jamalli [36] demonstrated that biofilm mass was increased at higher glucose concentration for both species with a threshold response at 0 to 20 and 160 to 200 mg/dL for *S. epidermidis* and 200 to 240 mg/dL for *S. aureus*, similar to observed in this study when high concentration of glucose and sucrose were used, the black colonies appeared more readily. Thus, the presence of glucose and sucrose lead to the development of a stronger biofilm colonies. Pereira et al., [37] extensively studied the role of glucose in the proliferation of biofilm matrix in the presence of high sugar concentrations, which could be a reason as sugars play important role in bacterial growth and metabolisms. In another study, different sugar, galactose was showed to facilitate formation of biofilm by *B. subtilis*, a gram positive bacteria like *S. aureus* [38]. Consistently with this study, the authors revealed that sugar required for the biosynthesis of EPS as a nucleotide sugar substrate and thus for matrix production. Hence, it is obvious that sugar metabolism plays a central role in biofilm formation by bacteria.

In the tube methods, 17(30.4%) isolates turned out to be biofilm producing organism. Similarly, this method correlated well with the culture method on CRA for detection of biofilms. Though, it is difficult to differentiate the strength of the biofilm production. This work agreed with another report [39]. Furthermore, Hassan et al., [39] suggested that tube method cannot be employed as a general screening test to detect biofilm producing bacteria. Interestingly, another study noted that tube method detected more biofilm in *S. epidermidis* than culture method [40]. In the work, the authors found out of 147 isolates of *S. epidermidis*, TM detected biofilm formation in 79 (53.7%) and CRA detected in 64 (43.5%) isolates. They showed that TM is better for biofilm detection than CRA. In the same trend, in another study by Baqai et al., [41], reported high biofilm formation by tube method than CRA, and concluded that CRA method showed very little correlation with the other methods and parameters of sensitivity, specificity and accuracy.

This study also employed use of microscope to detect biofilm formation by *S. aureus.* Several imaging methods have been reported to have detected biofilm formation and cell viability as demonstrated in previously [42]. However, light microscopy remains a useful base-line technique to provide a visual identification of biofilm formation. In the current study, Indian ink was used but other authors also suggested various dyes such as Periodic Acid-Schiff (PAS), Haematoxylin and eosin (H & E), and brown and brenn gram staining have been recently reported to be more practical, cheaper and reliable methods for detection of bacterial biofilms in different infection foci [43-49]. The detection of biofilm by this practical and cost-effective staining method has been described as quantitative detection of biofilm biomass.

Conclusion

In conclusion, we report the isolation of mucoid strains of *S. aureus* from clinical samples. A significant portion of those isolates were found to produce biofilms. We found Congo red agar as the most sensitive biofilm detection method compared to Microscopy and Tube method. Biofilm production is an impediment to antimicrobial therapy and if not detected, can make an infection chronic and its control delayed.

Since this study only demonstrated the presence of biofilm in the isolates phenotypically, it is recommended that further study should explore by using the molecular techniques. There is need for more information on the mechanism of biofilm formation at a molecular level and observe its association with other microbial processes such as virulence and antibiotic resistance. Furthermore, use of molecular methods such as the Polymerase Chain Reaction (PCR), could amplifies the genes involved in biofilm production, and will significantly help to supports the methods used in this study, and also complements other methods used elsewhere.

Conflict of Interest

The authors have no conflict of interest to declare.

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