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Comparison of Three Methods for the Detection of Biofilm Formation by Clinical Isolates of *Staphylococcus aureus* Isolated in Casablanca

Achmit Mohamed[1], Ait Mhand Rajaa[2], Zerouali Khalid[3], Mellouki Fouad[4], Rhallabi Naima[5]

[1, 2, 4, 5] Laboratory of Virology, Microbiology and Quality / Eco-toxicology and Biodiversity, Faculty of Sciences and Techniques, Mohammedia, Morocco
[3]Laboratory of Microbiology, Ibn Rochd University Hospital, Faculty of Medicine and Pharmacy, Casablanca, Morocco

Abstract: ***Biofilms are group of microorganisms encased in a matrix of extracellular polysaccharide (slime), called polysaccharide intercellular adhesin (PIA). They have been associated with a variety of chronic and persistent infections. Staphylococcus aureus is from bacteria which have high ability to form a biofilm. In this study, detection of biofilm production by Staphylococcus aureus was done by using three different methods: tube method (TM), congo red agar method (CRA) and tissue culture plate method (TCP), three repetitions were made for every method. Among of 117 strains of Staphylococcus spp isolated between October 2015 and January 2016 at the University Hospital Ibn Rochd in Casablanca, 74 were identified as Staphylococcus aureus. 58 isolates were detected as biofilm producer by TCP method, 49 by TM and 42 by CRA method. We can conclude from our study that the TCP method is a more quantitative and reliable method for the detection of biofilm formation by Staphylococcus aureus as compared to TM and CRA methods.***

Keywords: Biofilm, *Staphylococcus aureus*, Tube method (TM), Congo red agar method **(**CRA), Tissue culture plate method (TCP).

1. Introduction

Staphylococccus aureus is the most frequent cause of nosocomial and community-acquired infections and is recognized as the most frequent causes of biofilm-associated infections.

The ability of *Staphylococcus aureus* to form biofilms helps the bacteria to survive in hostile environments within the host and is considered to be responsible for chronic or persistent infections [1, 2].

Biofilm consists of multilayered cell clusters embedded in a matrix of extracellular polysaccharide (slime), called polysaccharide intercellular adhesin (PIA), which facilitates the adherence of these microorganisms to biomedical surfaces and protect them from host immune system and antimicrobial therapy [3].

The synthesis of PIA is mediated by the products of the chromosomal ica gene (intercellular adhesion), which are organized in an operon structure. This operon contains the icaADBC genes, in addition to the icaR gene which exerts a regulatory function and is transcribed in the opposite direction. Once this operon is activated, four proteins are transcribed, IcaA, IcaD, IcaB and IcaC, which are necessary for the synthesis of PIA [4-6].

The three methods broadly used for the phenotypic identification of biofilm-producing strains are Tube method (TM), Congo red agar method **(**CRA) and Tissue culture plate method (TCP) [1, 7- 8].

The Aim of this study is to compare three different methods for detection of biofilm formation in *Staphylococcus aureus* in order to determine the most reliable method.

2. Materiel and Methods

A total of 117 strains of *Staphylococcus spp* were isolated between October 2015 and January 2016 from different samples from patients hospitalized in various services of the University Hospital Ibn Rochd in Casablanca. Isolates were initially identified by standard microbiological techniques including Gram stain, catalase test, coagulase test and mannitol fermentation [9]. API Staph gallery (Biomerieux, France) was performed for identification of different *Staphylococcus* strains then biofilm ability formation was detected for each isolate by Tube method (TM), Congo red agar method **(**CRA) and Tissue culture plate method (TCP).

Tube method (TM)

Biofilm production was investigated by the tube adherence test proposed by Christensen et al.. 10 ml of Trypticase soy broth with 1% glucose was inoculated with a loopfull of test organism from overnight culture on nutrient agar individually. Broths were incubated at 37 °C for 24 hours. The cultures were decanted and tubes were washed with phosphate buffer saline pH 7.3. The tubes were dried and stained with 0.1% crystal violet. Excess stain was washed with deionized water. Tubes were dried in inverted position and observed for biofilm formation. Biofilm Production was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined and amount of biofilm formation was scored as 0-absent, 1-weak, 2-moderate, 3-strong [10, 11].

Congo red agar method (CRA)

The medium composed of Brain heart infusion broth (37 mg/l), sucrose (5 mg/l), agar number 1 (10 mg/l) and Congo red dye (0.8 mg/l). Congo red stain was prepared as concentrated aqueous solution and autoclaved at 121°C for

15 minutes. Then it was added to autoclaved Brain heart infusion agar with sucrose at 55°C. Plates were inoculated with test organism and incubated at 37°C for 24 to 48 hours aerobically. Slime-producing strains form black colonies, whereas non producing strains develop pink colonies [8].

Tissue Culture Plate Method (TCP)

The tissue culture plate method was conducted as previously described [11]. Briefly, the bacterial suspension grown in trypticase soy broth (TSB) was supplemented with 1% glucose and diluted for 1:100. 200 µl of this dilution was poured into the wells of sterile flat-bottomed 96-well polystyrene tissue culture plates (200 µl of TSB supplemented with 1% glucose was used as the negative control) and incubated 24 hours at 37°C. All tests were performed in triplicate. Washing was then performed three times for each well with sterile phosphate-buffered saline (PBS; pH 7.2). After that, the fixation step was done by air drying. Subsequently, the adherent biofilm layer was stained by crystal violet for 15 minutes at room temperature. This was followed by the washing steps. Then the plates were air dried and resolubilized with ethanol (95%) for 30 minutes. Optical density (OD) of stained adherent bacteria was determined with an Absorbance Microplate Reader (model EL×800) at wave length of 630 nm. The experiment was repeated three times separately for each strain and the average values were calculated with standard deviation (SD). Optical density cut-off (ODc) was determined. Formation of biofilm by isolates was analyzed and categorized relying on the absorbance of the crystal violet-stained attached cells.

ODc = (average OD negative control + 3 standard deviation of negative control)

Weak biofilm = ODc up to ODc

Moderate = 2 ODc up to 4 ODc

Strong biofilm = more than 4 ODc

3. Results and Discussion

Staphylococci are most often associated with serious infections. The predominant species isolated in these infections are *Staphylococcus aureus*. Their attachment to host tissue and medical implants by forming biofilm, play an important role in the persistence of chronic infections.

In a total of 117 clinical isolates of *Staphylococcus spp.* isolated from patients hospitalized in various departments of the University Hospital Ibn Rochd of Casablanca, 74 strains were identified as *Staphylococcus aureus.*

There are various methods for biofilm detection. In this study we tested 74 clinical isolates of *Staphylococcus aureus* for their ability to form biofilm by three phenotypic methods Tube method (TM), Congo red agar method **(**CRA) and Tissue culture plate method (TCP).

Tube method could detect 49 (66.2%) of 74 *Staphylococcus aureus* as biofilm producers, which are correlating well with the results of khan et *al*. and Gunti et *al*. who reported 63.7 % and 60 % respectively of positivity among *Staphylococcus aureus* [13, 14]. Among this 49 biofilm producers 27 (55%) as week, 13 (26.5%) as moderate and 9 (18.5%) as strong biofilm producers (fig. 1).

It's noted for this technique the presence of high variability among the three repetitions. Similarly, it was difficult to differentiate between strains weak biofilm producers and non producers. We confirm the hypothesis already made by several authors that tube technique cannot be recommended as general screening test to identify biofilm-producing isolate [10, 11].

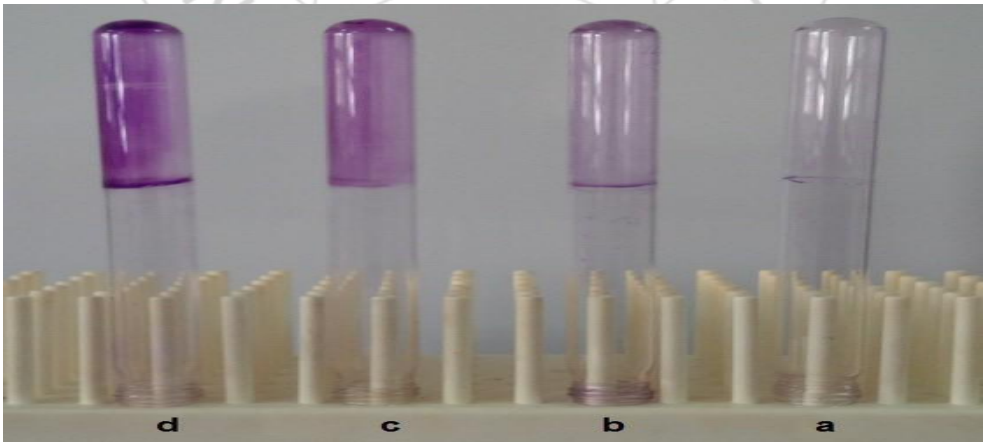

Figure 1: Detection of biofilm producers by Tube method. a: Non biofilm producer; b: Weak biofilm producer; c: Moderate biofilm producer and d: strong biofilm producer

The search for biofilm production by CRA method showed that among the 74 clinical isolates of *Staphylococcus aureus* 42 (56.8 %) are biofilm producers against 32 (43.2%) non-biofilm producers (fig. 2). Similar results were reported by Namvar (2013) who reported 65% positive results with congo red agar and Khan et al. (2011) who reported 47.79% positive results [15]. However Mathur et al. (2006), Taj et al. (2012) and Bose et al. (2009) reported a less number of biofilm production by *Staphylococci* species by this method. As researchers have only recently found that PIA/PNAG (polysaccharide intracellular adhesions / poly N-actyl glucosamine) have little input in the biofilm matrix of *Staphylococcus aureus* so cannot be detected by the CRA method [16-18].

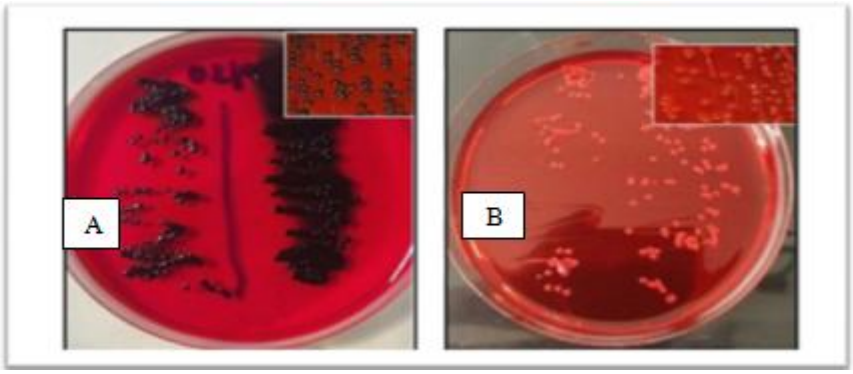

Figure2. Detection of biofilm production by Congo Red Agar method. A: black colonies of biofilm producer *Staphylococcus aureus*. B: pink colonies of non biofilm producer *Staphylococcus aureus*

By TCP, the standard method, 58 (78%) *Staphylococcus aureus* were positive for biofilm production. these were agree with Gunti et al. (2013) and Deka et al. (2014) who found that 76% and 83% respectively were positive slime producing staphylococci by TCP, but that was more than found by Mathur et al (2006) and Khan et al (2011) who reported 57.8% and 58% respectively positive results [19]. Among this 58 biofilm producers, TCP method detected 6 (8, 1 %) as strong, 12 (16, 2 %) as moderate and 40 (54 %) as weak biofilm producers (fig. 3).

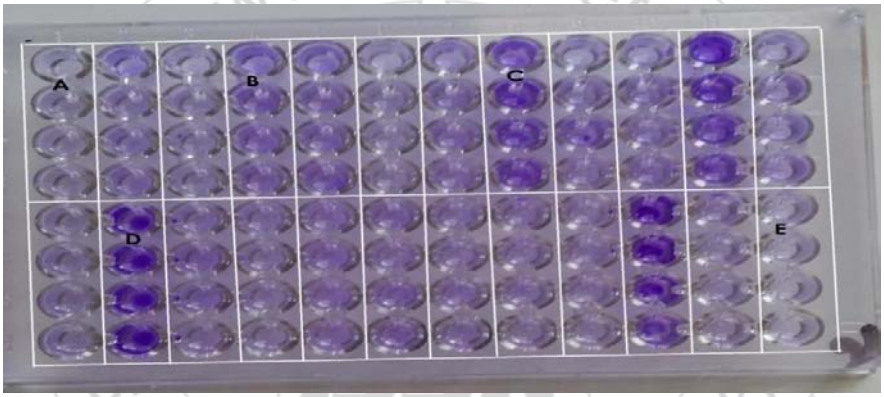

Figure 3: Detection of biofilm production by tissue culture plate method (TCP). A: non biofilm producer, B: week producer biofilm, C: moderate producer biofilm, D: strong producer biofilm and E: negative control

These three methods are specific for the biofilm detection of *Staphylococcus aureus* but with a different sensitivity. Analysis of the results shows that the TCP method is most sensitive to the detection of a bacterial biofilm, while the RCA method seems to be the least effective (fig. 4).

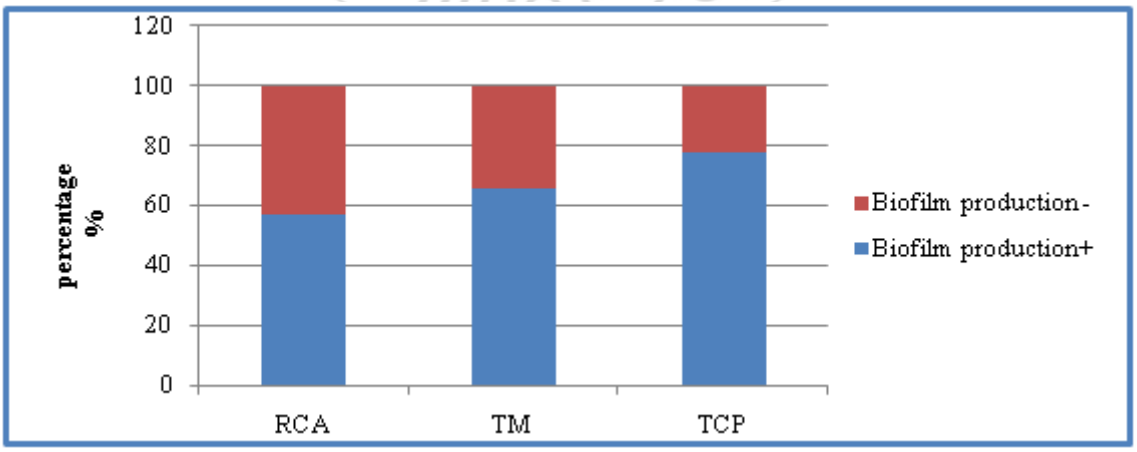

Figure4: Biofilm detection by three methods

The tube method correlated well with the TCP method for producing strains of biofilm. Our results agree with those of Knobloch et al., (2002), Mathur et al., (2006) and Oli et al., (2012) [20]. This data indicates that the TCP method is an accurate and reproducible method for screening and this technique can serve as a reliable quantitative tool for

determining biofilm formation by clinical isolates of staphylococci.

4. Conclusion

In conclusion, we observed the adhesion capacity of 74 clinical isolates of *Staphylococcus aureus* we have demonstrated that commensal bacteria have high capacity to form biofilms as well on hydrophilic surfaces than on hydrophobic surfaces. Each method of the three methods used for detection of biofilm formation has its advantages and disadvantages, as well as their specific indication. But TCP method is most widely used and was considered as standard test for detection of biofilm formation because it allows an easy and quantitative classification of the staphylococcal isolates.

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Author profile

Mohamed Achmit is a Ph.D. student in laboratory of virology, microbiology and quality / Eco-toxicology and biodiversity, Faculty of Sciences and Techniques, Mohammedia, Morocco. This article represents related work he accomplished during his Ph.D.

Rajaa Ait Mhand is University Professor, specialty: Microbiology and molecular biology, Faculty of Sciences and Techniques Mohammedia, Morocco.

Naima Rhallabi is University Professor, specialty: Microbiology, Faculty of Sciences and Techniques Mohammedia, Morocco.

Khalid Zerouali is University Professor, specialty: Microbiology, Faculty of medicine and pharmacy Casablanca, Morocco.

Fouad Mellouki is University Professor, specialty: Pharmacological sciences, Faculty of Sciences and Techniques Mohammedia, Morocco.