

Review

# Effect of Certain Disinfectants and Antibiotics on the Biofilm Formed by *Staphylococcus Aureus* Isolated from Medical Devices at the University Hospital Center of Sidi Bel Abbes (Algeria)

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## Article history

Received: 03-04-2019

Revised: 10-05-2019

Accepted: 12-09-2019

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**Abstract:** *Staphylococcus aureus* is one of the species that are most frequently isolated from medical devices. The ability to produce a biofilm is an important step in the pathogenesis of these staphylococci; biofilm formation is strongly dependent on the environmental conditions and also on the antibiotics and disinfectants used in the treatment of infections. In this study, 28 *staphylococcus aureus* isolated from medical devices at the University Hospital Center of Sidi Bel Abbes (in Northwestern Algeria) were analyzed to detect the formation of biofilm by culture on Red Congo Agar (RCA). The Tube Method (TM) and tissue Culture Plate (TCP) techniques were also used to investigate the effect of penicillin, ethanol and Betadine on the preformed biofilm. It has been found that 19 strains produced a bacterial slime on the Congo red medium, 7 strains produced a biofilm by the tube method, 2 of which are highly productive. In addition, 9 strains produced a biofilm on polystyrene micro-plates; this number was higher in the presence of penicillin and ethanol with 19 and 11 biofilm producing strains, respectively. On the other hand, no biofilm was formed in the presence of Betadine. It is important to examine the response of biofilms following an imposed external constraint such as disinfectants and antibiotics in order to develop new strategies to combat bacterial biofilms but also to better control their formation.

**Keywords:** *Staphylococcus aureus*, Biofilm, Medical Devices, Disinfectants, Antibiotics

## Introduction

*Staphylococcus aureus* is one of the major causes of nosocomial and community-acquired infections. This germ is responsible for acute and chronic infections; most of them are due to the ability of these germs to adhere to medical implants and form a biofilm (Verma and Singh, 2015).

The biofilm is recognized as the most predominant form of development of bacteria in nature. These bacteria are complex communities of microorganisms, embedded in a

self-secreted matrix of Extracellular Polymeric Substances (EPS) (Kara Terki *et al.*, 2013).

Biofilms form on the surface of most materials, whether biological or not. According to the Center for Disease Control and Prevention (CDC), 65% of bacterial infections are due to the presence of biofilms. In addition, infections associated with biofilms constitute a major clinical problem and are the cause of increased mortality; they generally require higher costs of medical treatment (Bellifa *et al.*, 2016; Chessa *et al.*, 2016; Badran *et al.*, 2015).

The development of biofilms depends strongly on the environmental conditions in which they are formed; it also depends on the different parameters these biofilms are subjected to. Any change in any of these parameters is usually perceived as stress and can trigger a particular response within the biofilm at any time (Ouchar Mahamat *et al.*, 2013).

It is therefore essential to study the response of biofilms to an imposed external constraint, such as the action of disinfectants and antibiotics, in order to develop adequate strategies to fight biofilms but also to control and eventually limit their formation. The purpose of the present work fits within this particular context, which is to isolate *Staphylococci* from medical devices at some departments of the University Hospital Center of Sidi Bel Abbes for the purpose of evaluating the capacity of isolated strains to adhere to a surface and form a biofilm. This work aims also to investigate the influence of some disinfectants and antibiotics on the formation of staphylococcal biofilms.

## Materials and Methods

### *Bacterial strains*

The strains selected in this study were isolated from medical devices obtained from the Departments of Reanimation, Urology and Internal Medicine at the University Hospital Center (CHU) of Sidi Bel Abbes, a city located in northwestern Algeria.

### Identification

After ablation of the devices, the microbiological analysis was carried out using the "Brun-Buisson" technique" (Brun-Buisson, 1994) which consists in rinsing the catheter lumen with saline solution and vortexing its intravascular end before culturing on a Chapman agar medium; this operation allows for the selection of the *staphylococci*.

Bacterial identification was performed by conventional methods, such as colony morphology, Gram stain, catalase production, coagulase assay and also by the API STAPH system (Biomérieux®).

### Detection of Biofilm Formation

#### *Method of Red Congo Agar*

The Congo red test was performed as previously described by Freeman *et al.* (1989). The medium consisted of brain heart infusion broth (BHIB, 37 g/L), sucrose (50 g/L), agar no.1 (10 g/L) and Congo red stain (0.8 g/L). Congo red was prepared as a concentrated aqueous solution and autoclaved at 121°C for 15 min, separately from the other medium constituents and was then added to the mixture when the agar had cooled to 55°C. The plates were inoculated and incubated aerobically for 24 to 48 h at

37°C. Biofilm producers form black colonies on CRA, whereas non-producers form red colonies. The Congo red dye directly interacts with certain polysaccharides, forming colored complexes (Jain and Agarwal, 2009).

#### *Tissue Culture Plate (TCP) Method*

Quantitative determination of biofilm formation in 96-well microplates was performed according to Christensen *et al.* (1985) recommendations, but with changes the in incubation time which was extended to 48 hours. After culturing the bacterial strains in the BHIB medium and incubating for 18h at 37°C, the mixture is diluted 1/100 in fresh (BHIB) medium. Then, the wells of a 96-well plate are filled with 0.2 ml of this dilution and incubated at 37°C. The microplate wells are then washed 3 times with distilled water, dried in the inverted position and stained with 0.5% (P.V) crystal violet solution.

The adherent cells are resuspended in 95% ethanol solution and the absorbance is measured at 540 nm using an Auto Reader ELISA reader (Model 680, Biorad, UK). The isolates are then classified into three categories as: (a) not-adhering, with an optical density less than 0.120; (b) weakly adhering, with an optical density greater than 0.120 or less than or equal to 0.240 and (c) strongly adhering, with an optical density greater than 0.240.

#### *Tube Method*

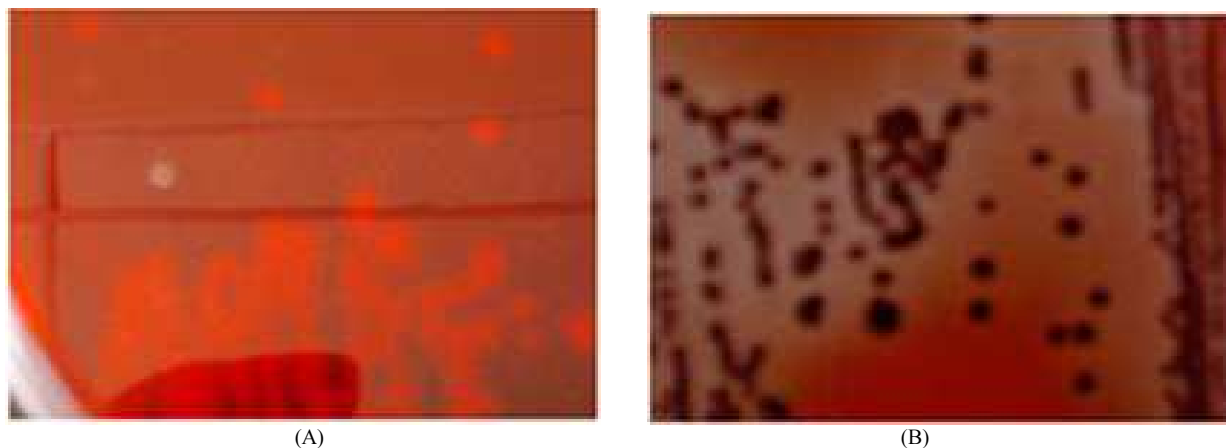
This technique, developed in 1982 by Christensen *et al.* provides a qualitative assessment of the biofilm formation. From a young culture of 24h, a colony is sown in 10 mL of Brain Heart Infusion Broth (BHIB) supplemented with 2% sucrose. After incubation at 37°C for 24h, the tubes are washed with Phosphate Buffered Saline (PBS) at pH = 7.3 and then dried. Each tube is then stained with crystal violet (0.1%) for 5 min. Once the dye is removed, the tubes are washed with distilled water and allowed to dry. The formation of the biofilm is considered positive when a visible film doubles the wall of the tube as well as its bottom. The formation of a ring at the liquid interface is not indicative of biofilm formation (Mathur *et al.*, 2006).

## Effect of Certain Antiseptics and Antibiotics on Biofilm Formation using the TCP Technique

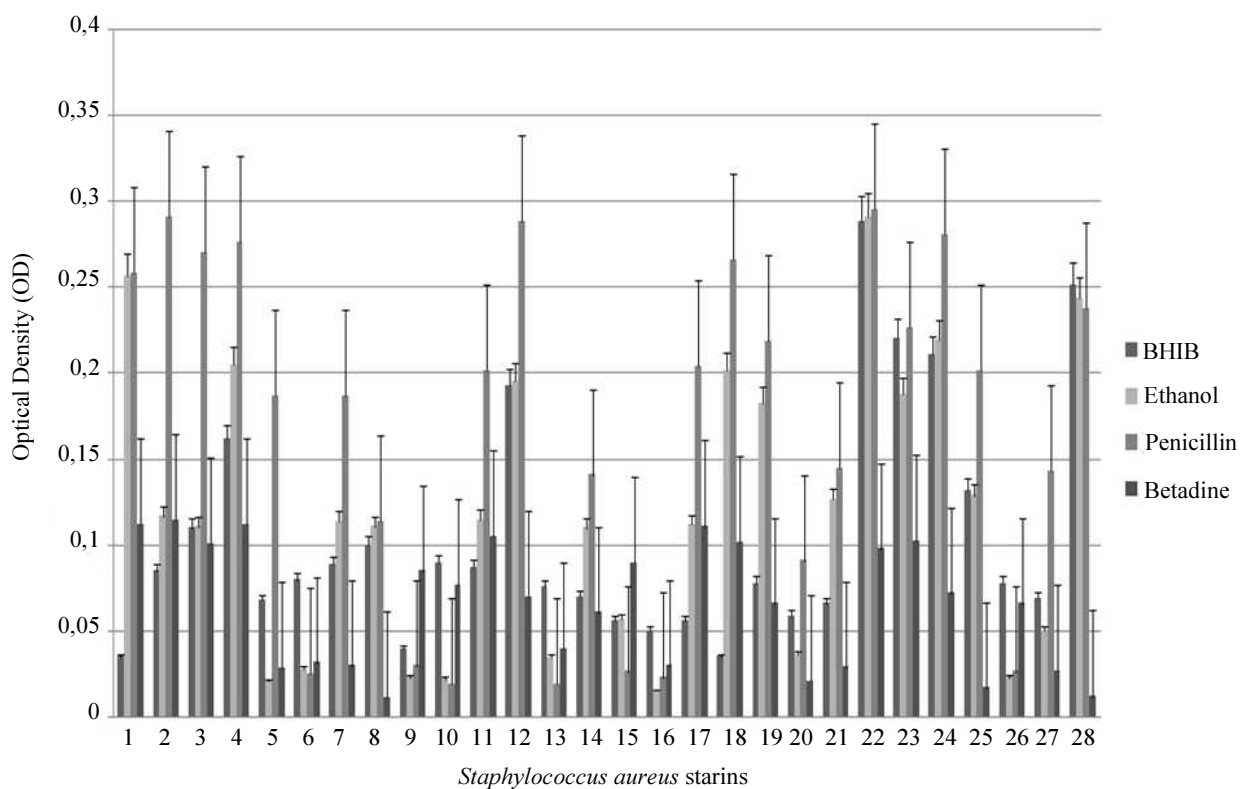
### *Antiseptics and Antibiotics under Study*

The main antiseptics used at the Hospital University Center of Sidi Bel abbes are polyvidone iodine (PVPI) and ethyl alcohol at 70°. As for antibiotics, penicillin G is by far the most widely used antibiotic in the hospital.

Polyvidone iodine is marketed under the name of Betadine® 10% (Laprophan laboratory). Also, the ethyl alcohol at 70° is prepared at the laboratories of the hospital pharmacy of the University Hospital by diluting the alcohol at 90° and penicillin G (1 million); it is marketed by SAIDAL laboratories.



**Fig. 1:** CRA plate test (A): Non slime producing strains / (B): slime-producing strains



**Fig. 2:** Biofilm formation of *Staphylococcus aureus* strains on BHIB, ethanol 70%, Penicillin and betadine 10%. Adherent bacterial biofilms were stained with Crystal violet as described in Materials and methods. A strain was considered biofilm-positive, if its OD was higher or equal to 0.120,  $P^* < 0.05$  (t-test). Data are representative of 3 replicate experiments

### Biofilm Assay

After forming a 48-hr young biofilm by the TCP technique (previously described), the 96-well microplate are rinsed 3 times with distilled water and dried. Then, Penicillin G (1million U.I), Betadine 10% (an iodinated derivative) and ethyl alcohol 70°

are added to the biofilm. The microplate is then formed and incubated for 24 h.

After incubation, the wells of the microplate are carefully rinsed, dried and stained with crystal violet according to the standard technique. The Optical Density (OD) is measured at 490 nm by the ELISA reader.

**Table 1:** Results of biofilm formation by *Staphylococcus aureus* isolated from medical devices

Strain	Unit	Medical device	TCP					TM	Production of slime
			BHIB	Ethanol	penicilin	betadine			
S1	Urology	U.C	-	++	++	-	-	+	
S2	Urology	U.C	-	-	++	-	-	+	
S3	Urology	U.C	-	-	++	-	-	+	
S4	Urology	U.C	+	+	++	-	+	+	
S5	Urology	U.C	-	-	+	-	+	+	
S6	Urology	U.C	-	-	-	-	-	+	
S7	Urology	U.C	-	-	+	-	-	+	
S8	Urology	U.C	-	-	-	-	-	-	
S9	Urology	U.C	-	-	-	-	-	-	
S10	Urology	U.C	-	-	-	-	-	-	
S11	Urology	U.C	-	-	+	-	-	-	
S12	Urology	U.C	+	+	++	-	+	+	
S13	Urology	U.C	-	-	-	-	-	-	
S14	intensive care	C.V.C	-	-	+	-	-	-	
S15	intensive care	C.V.C	-	-	-	-	-	-	
S16	intensive care	C.V.C	-	-	-	-	-	-	
S17	intensive care	C.V.C	-	-	+	-	-	+	
S18	intensive care	C.V.C	-	+	++	-	+	+	
S19	intensive care	C.V.C	-	+	++	-	+	+	
S20	intensive care	C.V.C	-	-	-	-	-	+	
S21	intensive care	C.V.C	-	+	+	-	-	+	
S22	intensive care	C.V.C	++	++	+	-	++	+	
S23	Internal Medicine	U.C	+	+	+	-	-	+	
S24	Internal Medicine	U.C	+	+	++	-	-	+	
S25	Internal Medicine	U.C	+	+	+	-	-	+	
S26	Internal Medicine	C.V.C	-	-	-	-	-	+	
S27	Internal Medicine	C.V.C	-	-	+	-	-	+	
S28	Internal Medicine	C.V.C	++	++	+	-	++	+	

C.V.C: central venous catheters, U.C: urinary catheters, (++) : biofilm forsmation good, (+): biofilm formation average, (-) :non biofilm formation

## Results

### Samples

A total of 42 samples were taken at the Sidi Bel Abbes Teaching Hospital; a number of 28 strains were retained, after biochemical identification using the API 20 staph gallery and the coagulase test. All the strains were identified as *staphylococcus aureus*.

### Results of the Red Congo Agar Technique

The search for slime production on Congo red medium revealed that 19 out of 28 strains, isolated from medical devices, are slime-producing and show black colonies with a dry-consistency crystalline on the Congo Red Agar. This aspect is due to the production of exo-polysaccharides that react with Congo red, while 09 strains were non-slime producing and showed red colonies on the same medium (Fig. 1).

### Results of the Tissue Culture Plate Technique

Quantitative determination of biofilm formation within all our strains was performed by microplate technique (TCP), as shown in Fig. 2.

Using the BHIB growth medium, only 9 strains produced biofilm; 7 of them are low producing and 2 high producing of biofilms (Table 1).

### Results of the Tube Method

The results obtained by the Tube Method (Table 1) are different from those obtained by the TCP technique; indeed, only 7 strains produce biofilms by this technique, 2 of which are highly productive.

### Effect of Ethanol, Penicillin and Betadine on the Biofilm

For the purpose of assessing the impact of environmental conditions on the *staphylococcus* biofilm, the three substances were added after a period of 48 h. This has led to an increase in the number of strains capable of forming a biofilm by the TCP technique; indeed, 11 strains produced a biofilm in the presence of ethanol and 19 strains gave a biofilm in the presence of penicillin which appears to stimulate greater biofilm formation. Indeed, 8 strains are found to be good biofilm producers and 11 are moderate biofilm producers. Moreover, only 3 strains are highly biofilm producers

and 11 are moderate producers in the presence of ethanol (Table 1 and Fig. 2).

On the other hand, no strain could form a biofilm in the presence of Betadine.

## Discussion

*Staphylococcus aureus* is one of the most common germs that are responsible for foreign body infections of central venous catheters, mechanical heart valves and urinary catheters. Their major virulence factors are the ability to produce an extracellular matrix and form a biofilm; this makes the clinical treatment extremely difficult (Derek *et al.*, 2017).

Early detection of staphylococcal biofilms may be one of the essential steps for the prevention and treatment of infection on medical devices (Martín-López *et al.*, 2002).

The present article aims mainly to evaluate the formation of biofilms by *staphylococcus aureus* isolated from medical devices at the Hospital University Center of Sidi Bel Abbes using RCA, TM and TCP techniques.

The results obtained reveal that 19 out of 28 strains can produce slime by culture on Congo Red medium. These results turn out to be in good agreement with those found by Arciola *et al.* (2001) who reported significant slime production in *staphylococcus aureus* (60.8%).

The production of slime is revealed by the appearance of black colonies on Congo Red medium (Fig.1). This aspect is mainly due to the production of Polysaccharide Intercellular Adhesin (PIA) that reacts with the culture medium. Described for the first time in *Staphylococcus epidermidis*, by Mack *et al.* (1992), the PIA is generally located on the surface of the cell. It is coded by the locus *ica* and plays an important role in intercellular adhesion; as a result, PIA has been recognized as a crucial factor in the colonization of medical equipment by staphylococci (Cramton *et al.*, 2001; Martín-López *et al.*, 2002; Derek *et al.*, 2017).

The results obtained by the TCP technique reveal that only 9 out of 28 strains can produce a biofilm in the BHIB medium. These results are in agreement with the observations of other authors who have shown that few strains are biofilm-forming in a growth medium without supplement (sugar, antibiotics or NaCl) (Cho *et al.*, 2002; Johannes *et al.*, 2002; Mathur *et al.*, 2006).

In our study, it was found that 7 of the 28 strains could produce a biofilm by the Tube technique. Also, a good correlation was noted between the Tissue Culture Plate (TCP) method and the Tube Method (TM) for the high biofilm producing strains (Table 1). It is worth noting that a large variability in the positive and negative biofilm classification was observed by the Tube Method (TM). Similarly, it was difficult to differentiate between biofilm low-producing and non-producing strains. Therefore, the present work allows us to confirm the hypothesis already formulated by several authors,

including Mathur *et al.* (2006), according to which the Tube Method (TM) cannot routinely be recommended.

Consequently, the TCP technique should be more widely utilized as it is considered as a standard test for the detection of biofilm formation. This method has been recognized as being the most sensitive, accurate and reproducible for the determination of staphylococcal biofilm formation. It allows for a quantitative assessment in order to compare the adhesion of different strains and also to examine a large number of isolates simultaneously (Racha *et al.*, 2012).

Furthermore, it is a quantitative, practical and economical technique that allows for the identification of optimal culture factors and conditions for biofilm formation (Castro Melo *et al.*, 2013).

Indeed, biofilm formation is a complex phenomenon, which can be affected by many factors, particularly the surrounding environment (Branger *et al.*, 2007). In order to study the influence of certain factors of the surrounding environment at the Hospital University Center of Sidi Bel Abbes on the biofilm of *Staphylococcus aureus*, it was decided to use the TCP technique described above. It was noted that after the addition of penicillin and ethanol, the number of biofilm-forming strains increased to 11 in the presence of ethanol and to 19 in the presence of penicillin. These observations are consistent with those made in other studies that suggest a strong dependence between growth conditions and biofilm formation in staphylococci (Mathur *et al.*, 2006; Kevin *et al.*, 2016).

Luther *et al.* (2015; Redelman *et al.*, 2012) showed that ethanol encourages biofilm formation in all strains studied. Similarly, El-Banna *et al.* (2010) showed that antibiotics also promote biofilm formation in staphylococci isolated from medical devices at the University Hospital Center of the City of Alexandria (Egypt).

Based on our 2013 results (Kara Terki *et al.*, 2013) and those found in the literature, it has been found that the *ica* operon expression, which is responsible for the production of extracellular polysaccharides in *staphylococcus aureus*, depends on the environmental conditions, such as growth media composition, temperature, osmolarity, the presence of oxygen and sub-inhibitory concentrations of antibiotics; it was also revealed that large concentrations of NaCl increase biofilm formation and strongly induce *ica* expression in staphylococci. The presence of divalent cations, such as calcium and magnesium, increases the production of polysaccharides, which leads to an amplification of biofilm formation. In staphylococci, the expression of the *ica* ADBC genes can also be influenced by other environmental conditions, such as oleic acid and iron limitation (Gotz, 2002; Chaieb *et al.*, 2005; Derek *et al.*, 2017).

On the other hand, the resistance of biofilm-included bacteria to antibiotics and disinfectants is well acknowledged. According to some authors, this resistance is attributed to certain factors, such as the

bacteria physiology, the power of matrices and many others (Olsen, 2015). Scientific work on the subject shows that there is a strong link, direct or indirect, between the architecture of the biological structure and its resistance to antimicrobial action (Abdel Halim *et al.*, 2018). Indeed, the multiplication of adherent cells and the production of an extracellular matrix lead to the development of a complex structure in which the biocides and antibiotics can meet diffusion problems, which limits their effectiveness. This structure controls the establishment of nutrient, oxygen and metabolite product concentration gradients, resulting in chemical and nutritional heterogeneity within the biofilm (Stewart, 2015). In response to their local microenvironment, the cells can then evolve into tolerance phenotypes by physiological changes and/or by the expression of specific genes. Therefore, the overall resistance of the bacterial community appears to be a multi-factorial structural-dependent process that involves local phenomena. Several factors may explain this strong resistance. The polymeric matrix, which acts as a barrier to reduce or prevent the diffusion of antimicrobial agents and the low concentration gradients of oxygen and certain nutrients, cause some cells in the biofilm to be metabolically inactive and may even remain in dormant form. These dormant bacterial cells are probably responsible for much of the tolerance associated with biofilms (Wojtyczka *et al.*, 2014; Thien-Fah *et al.*, 2001). Repeated exposure to disinfectant and antibiotics concentrations can generate some physiological adaptations that further delay the subsequent tolerance of the biofilm. When a community of adherent bacterial cells is subject to antibiotics and disinfectants, only a few are able to resist (Amiyare *et al.*, 2015). On the other hand, in the presence of Betadine (polyvidone iodine), the optical density decreases in all the strains and none of them is able to form a biofilm.

The results of Essayagh *et al.* (2010) confirm ours which reveal that PVPI is the best of antiseptics studied. In fact, only six strains (4.6%) out of the 130 tested, could resist to the PVPI that was available at the pharmacy, while 40 (30.7%) were resistant to iodinated alcohol and 20 (15.4%) to alcohol at 70°C. Chemical analysis also confirms this finding. Indeed, PVPI is a stable molecule consisting of an iodine complex and a water soluble organic agent that slowly transports and releases iodine. This structure makes the PVPI less irritating, less allergenic and more stable over time; while iodized alcohol and ethyl alcohol at 70°C become stable after fifteen days and one month, respectively, after the date of their preparation (Clevenot *et al.*, 2003; Vaillant, 2005).

## Conclusion

Although a lot of research has been carried out in the field of biofilms, current treatments for their

development are still limited. However, recent findings from basic research have identified mechanisms or regulatory pathways that represent potential therapeutic approaches. However, it is likely that biofilms develop some strategies to resist to anti-infective agents and disinfectants, which requires new adaptive, elaborate and systematic plans of action to combat them.

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