

Anti-Adhesion and Anti-Biofilm Effectiveness of Disinfectants Used In Hemodialysis against both *Staphylococcus Warneri* and *Staphylococcus Sciuri* Biofilms

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ABSTRACT

Biofilms are communities of microorganisms attached to a surface and included in an extracellular matrix making it resistant to exogenous deleterious agents. The aim of this study is to evaluate the anti-adhesive and anti-biofilm effect of five commercial disinfectants having different active principles (hydrogen peroxide, sodium hypochlorite, isopropyl alcohol and ethanol) on four *Staphylococcus* strains isolated from hemodialysis unit surfaces. The disinfectants anti-adhesive effect was estimated to an exceeding rate 70% for the various studied dilutions and 90% towards the pure products. Whereas the anti-biofilm effect showed an elimination rate varying between 10 % and 95 % according to the following parameters: active principle, time of contact, concentration and bacterial strain. Our study demonstrated that all tested products have an interesting anti-adhesive effect and that the peroxide of hydrogen is endowed with important anti-biofilm efficiency, followed by the alcoholic products and the sodium hypochlorite.

Keywords - anti-adhesive effect, anti-biofilm effect, disinfectants, *Staphylococcus warneri*, *Staphylococcus sciuri*

I. INTRODUCTION

Bacterial infections in hemodialysis have two origins related or unrelated to vascular access [1]. During their stay in hemodialysis services, the inert surfaces may represent the sites of microorganisms adhesion forming stronger biofilms resistant to antimicrobial agents [2].

Staphylococcus warneri and *Staphylococcus sciuri*, such as other Gram positive bacteria, produces structured aggregates called biofilms, protected by a matrix composed primarily of complex polysaccharides [3]. These biofilms form a physical barrier against the entry of antimicrobial agents, and are considered pathognomic of chronic infections among attained patients [4]. Indeed, the infection is a major cause of morbidity and mortality in patient with renal failure dialyzed, and is responsible for around 15% of deaths according to the National Institute of Diabetes and Digestive and Kidney Diseases.

All of these elements highlight the importance of studying the effectiveness of disinfectants used in the hemodialysis service against biofilms. Our work join within this framework, whose main objective is to evaluate the anti-biofilm and anti-adhesion potential of five disinfectants.

II. MATERIALS AND METHODS

2.1 Strains tests

The antibacterial activity was evaluated on four strains isolated from hemodialysis unit surfaces (three *Staphylococcus warneri* "3, 17, 20" and one *Staphylococcus sciuri* "9"). All strains were revived from glycerol stock cultures kept at -80°C and sub-cultured onto lysogeny broth (LB) agar plates and incubated at 37°C for 24 h. Prior to use in the adherence and biofilm experiments, the cells were harvested, washed twice in 0,1 M (KNO₃) and adjusted to 10⁷-10⁸ CFU/ml.

2.2 Products tests

In this study, the antimicrobial activity was investigated for five commercial disinfectants having different active principles summarized in Table 1. The anti-adhesive and anti-biofilm effect of different commercial disinfectants was tested on polystyrene flat-bottomed microtitre plates.

Table 1: The active principles of five tested disinfectants. P: Product.

Products	active principles	pH
P ₁	H ₂ O ₂ (2%)	2-3
P ₂	Ethanol (94 %), 1-propanol, wetting agents	6
P ₃	H ₂ O ₂ (50%) stabilized by argent	1,7
P ₄	isopropyl alcohol, cationic surfactants, alkaline complex	11,5-
P ₅	sodium hypochlorite (12°)	11,5

2.3 Determination of MIC

The Minimum Inhibitory Concentration of disinfectants studied on planktonic cells was determined using a broth dilution micro-method on polystyrene flatbottomed microtiter plates previously described by National committee for clinical Laboratory Standards [5]. The data from at least three replicates were evaluated and modal results were calculated.

2.4 Prevention Protocol

The prevention protocol was performed according to Leroy C. [6] with the following modifications; this protocol consist to add 200 µl of the product at each concentration tested (Table 3) with bacterial suspension per well and incubated for 8 hours to 37° C. After incubation, the biofilm is revealed with crystal violet as described in the paragraph 6.

2.5 Washing Protocol

The washing protocol was performed according to Leroy C. [6] with the following modifications; this protocol involves depositing 220 µl of the product at each concentration tested (Table 3) per well on biofilm preformed by 8 hours. After incubation to 10, 30 and 60 min, the biofilm is revealed with crystal violet as described in the paragraph 6.

Indeed, the biofilm is preformed by incubating a bacterial suspension distributed per well of a sterile 96-well microplate. After 8 hours of incubation, plates were washed three times with sterile distilled water to remove any loosely associated or planktonic bacteria.

2.6 Crystal Violet staining assay

Biofilm formation was indirectly assessed using the modified crystal violet assay as described previously [7]. In brief, after the incubation period, plates were washed three times with sterile distilled water to remove any loosely associated or planktonic bacteria. The plates were air-dried. The wells were then stained with 220 µl of 1% crystal violet and incubated at room temperature for 15 min following by three times wash with sterile distilled water. The semiquantitative assessment of biofilm formation was

performed by adding 220 µl of ethanol to destain the wells. 220 µl from each well was then transferred to a new plate and the absorbance determined at 550 nm. Sterility check and biofilm positive control were performed for each strain. The mean of the triplicate samples and the standard deviations were determined and plotted against EOC incubation time. The antimicrobial effect was measured by comparing the readings of the EOC treated biofilms to a positive and negative control.

2.7 Analysis Method

The micro titer screening method was used to quantitatively measure the removal efficacy of commercial disinfectants on biofilms of *Staphylococcus*. A measure of efficacy called Percentage Reduction (Percentage Removal) was used to evaluate the efficacy of five disinfectants.

$$\text{Percentage Reduction (Percentage Removal)} = [(C - B) - (T - B)] / (C - B) \times 100\%$$

Where:

B denotes, the average absorbance per well for blank (no biofilm, no treatment); C denotes the average absorbance per well for control wells (biofilm, no treatment) and T denotes the average absorbance per well for treated wells (biofilm and treatment).

III. RESULTS

3.1 MIC: Efficiency threshold of five commercial disinfectants

The MICs of disinfectants studied on planktonic cells were summarized in Table 2. These results showed that each test product has a specific action that varies depending on the microorganisms. Note that both products P3 and P4 have the same MIC, and it is the lowest MIC, hence their high efficiencies on bacteria in suspension.

Founding to the determined MICs, six dilutions were selected with increasing concentrations, to test the anti-adhesive and anti-biofilm disinfectants effect. Table 3 focuses on these dilutions.

3.2 Anti-adhesion effect

The prevention protocol was used to determine the activity of disinfectants on adherence ability of *Staphylococcus warneri* "3, 17, 20" and *Staphylococcus sciuri* "9". The disinfectants anti-adhesive effect was estimated to an exceeding rate 70% for the various studied dilutions and 90% towards the pure products. Product P1, P2 and P3 showed a highest percentage reduction (Fig.1). While both product P3 and P4 were analyzed by the same concentrations but the results showed that the product P3 present a higher anti-adhesive effect. Where P5 remains less powerful than P3 (Fig.1).

3.3 Anti-biofilm effect

Bacterial strains were exposed to the different tested disinfectants listed in Table 1 at different concentrations (Table 3) in triplicate, at three different time exposures (10, 30 and 60 minutes). The anti-biofilm effect showed an elimination rate varying between 10 % and 95 %, this removal percentage of biofilm increases by rising the time of treatment (Fig. 2). The hydrogen peroxide present a

highest anti-biofilm effect than other products. This was followed by alcoholic products and the sodium hypochlorite (Fig. 2). However, the active principle concentration in the product P3 is raised than product P1, this highest concentration increases its anti-biofilm effect. This ascertainment was noted even in raising the concentration for all tested products (Fig. 2).

Table 2: Minimum Inhibitory Concentrations of the products tested on four strains (*Staphylococcus warneri* “3, 17, 20” and one *Staphylococcus sciuri* “9”). P: Product

Products	Strain 3	Strain 9	Strain17	Strain 20
P1	1/40	1/40	1/40	1/80
P2	1/10	1/20	1/40	1/10
P3	1/640	1/640	1/640	1/640
P4	1/640	1/640	1/640	1/640
P5	1/16	1/16	1/32	1/80

Table 3: Dilutions used for testing the anti-adhesion and anti-biofilm effects of the five disinfections tested. P: product.

Products	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5	Dilution 6
P1	1/40	1/16	1/8	1/4	1/2	Pure
P2	1/10	1/8	1/6	1/4	1/2	Pure
P3	1/640	1/64	1/16	1/4	1/2	Pure
P4	1/640	1/64	1/16	1/4	1/2	Pure
P5	1/32	1/16	1/8	1/4	1/2	Pure

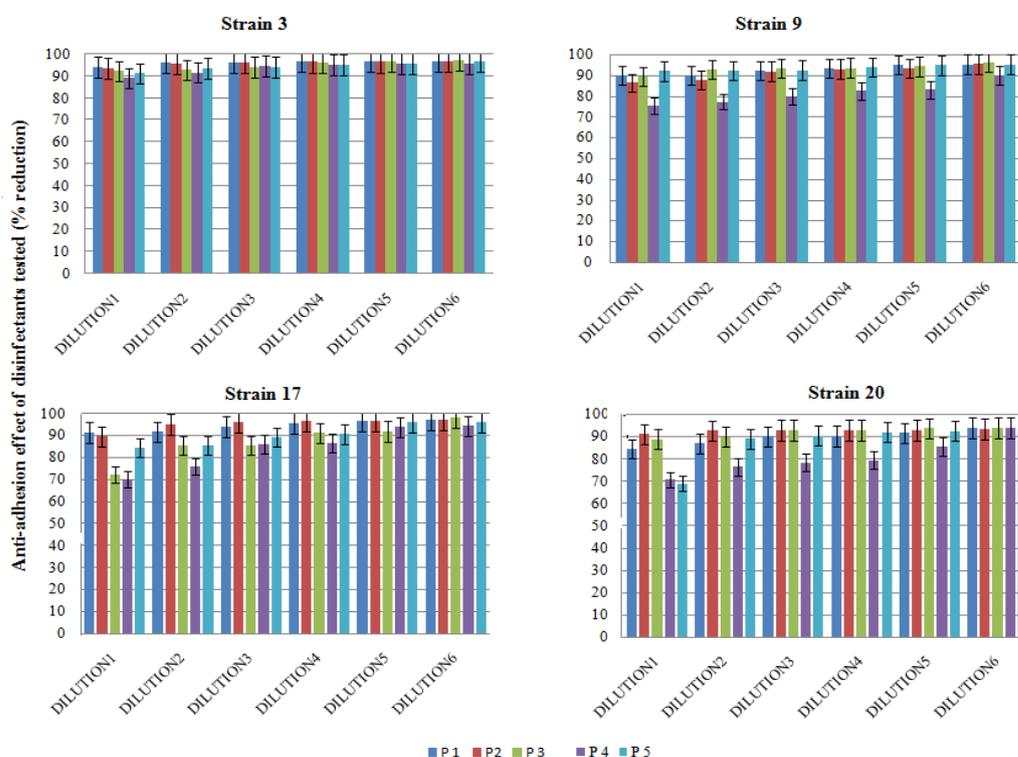
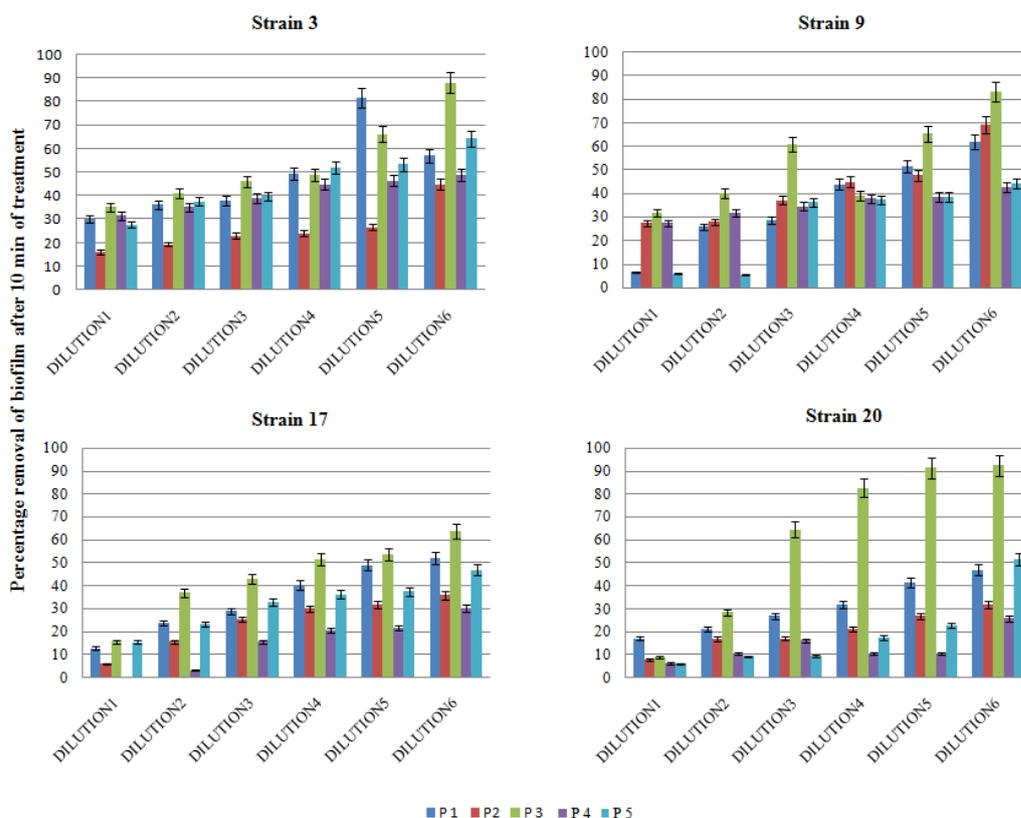
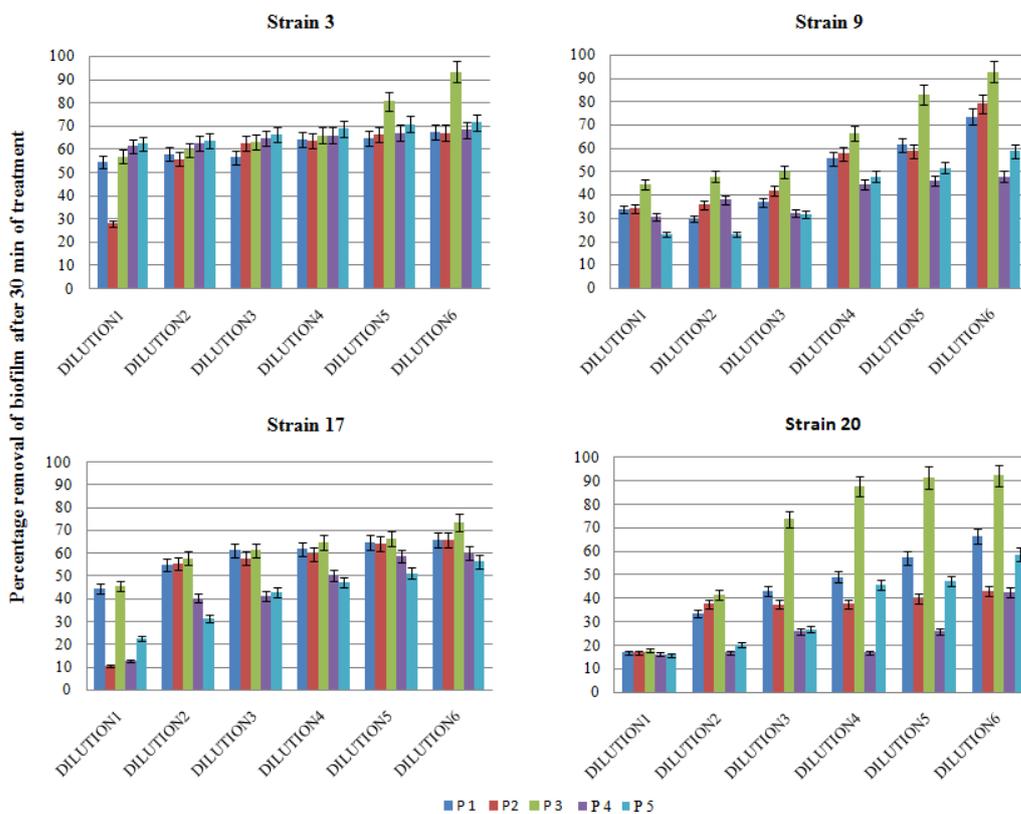


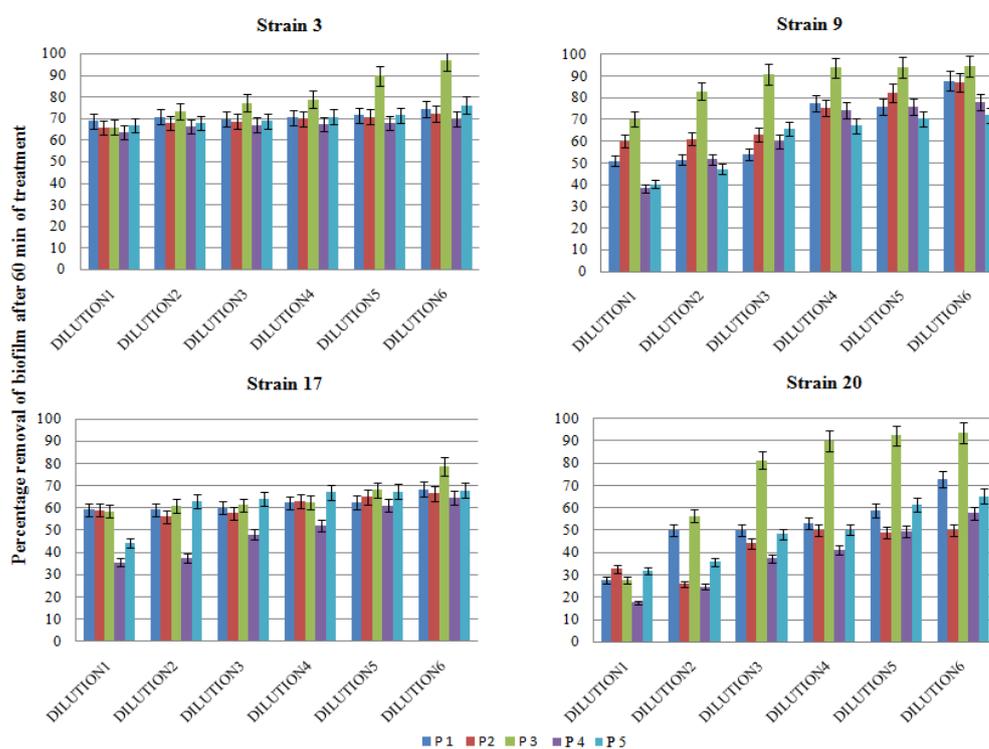
Figure 1: the anti-adhesion efficacy of five disinfectants, in four strains (*Staphylococcus warneri* “3, 17, 20” and *Staphylococcus sciuri* “9”), expressed as reduction percentage. P: product.



(a)



(b)



(c)

Figure 2: anti-biofilm effect of the five disinfectants tested, in four strains (*Staphylococcus warneri* “3, 17, 20” and *Staphylococcus sciuri* “9”), expressed as removal percentage after three times of treatment, (a): 10 min, (b): 30 min, (c): 60 min. P: product.

IV. DISCUSSION

Recently, scientific interest in the anti-biofilm properties of disinfectants has increased remarkably [4, 8, 9]. In this study, we initially assessed the efficacy of five disinfectants on inhibiting the growth of planktonic strains. The MICs results show obviously that the four strains are sensitive to the tested products. For comparison, we note that both products P1 and P3 had the same active ingredient (hydrogen peroxide), while their action is different, for the simple reason of their different concentration of H₂O₂. In addition, the P3 is stabilized by argent, which explains its bactericidal effect [10]. Thus efficacy of the product P4 is mainly due to one of its active ingredient which is isopropyl alcohol; the most widely used and known to be effective against bacteria [11, 12]. According to the literature, alcoholic compounds act by denaturing proteins, such as solvents or dehydrating agents [13]. In conclusion, the sensitivity of tested strains towards the five products that explains their effectiveness on bacteria in suspension.

Concerning the anti-adhesive effect, excellent activity was marked against the four strains studied. This efficiency could not reach 100%, this can be explained by the presence of proteins, whether in the

LB culture medium or in the matrix protecting biofilm, decreasing their anti-adhesive effect [3, 14]. While the results of the anti-biofilm effect show that the removal rate is somewhat important. This effect depends on four parameters: (i) the active principle, (ii) the concentration of the product, (iii) the contact time (iiii) and the tested strains.

We found that the product P3 containing hydrogen peroxide (H₂O₂) has a substantial elimination percentage versus to alcohol and chlorine (sodium hypochlorite) products. The anti-biofilm effect of H₂O₂ reside in its ability to pass through the biofilm and generate free radicals degrading the polysaccharides that constitute a barrier to protect the bacteria against biocides degrading. While the effectiveness of the product P3 is accentuated by the addition of argent, in addition to its antimicrobial and anti-adhesive properties. Moreover, the chlorine-based product present a lower removal biofilm percentage than H₂O₂. Indeed, several studies have shown that the anti-biofilm effect of chlorine is important on young biofilms aged some hours, which explains our results since the study was conducted on a mature biofilm. In addition, we can add that the concentration 38 mg/l of chlorine corresponding to 12 ° of bleach was not sufficient to remove all of the biofilm [8, 15].

Alcohol families studied showed that the isopropyl alcohol has a low MIC relative to the ethanol product that means its important effect on planktonic bacteria. However, the anti-biofilm effect noted a greater important with isopropyl alcohol, however it is lower than the hydrogen peroxide products. A study was performed on *Staphylococcus epidermidis* biofilm, showed that the ethanol wash is responsible for the induction of biofilm formation [16].

The biofilm elimination rate which reaches not 100% can be explained by the resistance to disinfectants. Several mechanisms have been given. Some studies have pointed to mechanisms involving in particular that the barrier formed by the biofilm, share of its organic consistency, it prevents antimicrobials or antibiotics access by limiting their diffusion or their repulsion [17]. This may be due to electrostatic repulsion or sequestration by the surface polymers [18]. Other studies have suggested that this resistance causes a slow or incomplete penetration of disinfectants to biofilm. Furthermore, the presence of a neutralizing disinfectant microenvironment [12, 19, 20] or by inhibition of certain active principles such as the inhibition of oxidants by the presence of proteins, inducing poor diffusion of the product within the biofilm [21].

V. CONCLUSION

Bacterial biofilm communities present a tank of virulence and interbacterienne transmission genes resistance. Their presence in hospitals especially in hemodialysis services represents a major problem of public health. Despite the antibiotic sensitivity possibility of strains studied, their ability to form biofilms makes them susceptible to acquire resistance genes. This is evident with intercellular communication called quorum sensing. Indeed, an adequate and consistent control of products used for cleaning and disinfecting is required. In addition, periodic assessment of effectiveness or resistance carries a major interest. Ensuring thus, the reduction of patient morbidity and number of hospitalizations and improved quality of life.

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