

# Prevention and Treatment of *Pseudomonas Aeruginosa*-Based Biofilm with Ethanol

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**ABSTRACT** **Background:** Although indwelling catheters are increasingly used in modern medicine, they can be a source of microbial contamination and hard-to-treat biofilms, which jeopardize patient lives. At times 70% ethanol is used as a catheter-lock solution due to its bactericidal properties. However, high concentrations of ethanol can result in adverse effects and in malfunction of the catheters.

**Objective:** To determine whether low concentrations of ethanol can prevent and treat biofilms of *Pseudomonas aeruginosa*.

**Methods:** Ethanol was tested at a concentration range of 0.625–80% against laboratory and clinical isolates of *P. aeruginosa* for various time periods (2–48 hours). The following parameters were evaluated following ethanol exposure: prevention of biofilm formation, reduction of biofilm metabolic activity, and inhibition of biofilm regrowth.

**Results:** Exposing *P. aeruginosa* to twofold ethanol gradients demonstrated a significant biofilm inhibition at concentrations as low as 2.5%. Treating pre-formed biofilms of *P. aeruginosa* with 20% ethanol for 4 hours caused a sharp decay in the metabolic activity of both the laboratory and clinical *P. aeruginosa* isolates. In addition, treating mature biofilms with 20% ethanol prevented the regrowth of bacteria encased within it.

**Conclusions:** Low ethanol concentrations (2.5%) can prevent in vitro biofilm formation of *P. aeruginosa*. Treatment of previously formed biofilms can be achieved using 20% ethanol, thereby keeping the catheters intact and avoiding complications that can result from high ethanol concentrations.

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**KEY WORDS:** catheters, central lines, healthcare-associated infections, ethanol, *Pseudomonas aeruginosa*

Biofilms are communities of bacteria, usually attached to surfaces and embedded in a self-secreted matrix that present major challenges in modern medicine. In fact, it was estimated that close to 80% of all infections are caused by biofilm-forming organisms [1]. Biofilms increase the risk of failed antibiotic therapy. For example, biofilm-forming *Pseudomonas aeruginosa* was found to be 1000 times more resistant to antibiotics and more resistant to the host immune response, than cells in suspension [2,3].

Biofilms are especially problematic in the context of medical and indwelling devices such as orthopedic implants and catheters. Central venous catheters are increasingly used for administration of medications, fluids, blood products and nutrition, blood collection, and hemodialysis. Infections, including central line-associated bloodstream infections (CLABSIs), are the most common complication of such devices, with mortality rates ranging from 12 to 25% [4]. A survey of 183 hospitals showed that infections related to medical devices accounted for 26% of healthcare-associated infections [5]. These biofilm-based CLABSIs are often difficult to eradicate, despite prolonged antibiotic treatment [3,6,7].

Ethanol penetrates biofilms and has bactericidal activity against most CLABSI pathogens through several mechanisms of action, including membrane disruption and interference with cell division and protein synthesis. Thus, local application of ethanol has been suggested as an intervention strategy to prevent and treat these infections [1,8,9]. The "lock" technique is usually used; namely, a small volume of 70% ethanol is instilled to fill the catheter lumen for 6–24 hours, and is then withdrawn [8]. Ethanol lock reduced the rates of CLABSI under diverse settings by 44–96% [10–14], and the rates of catheter replacement by 65–77% [11,13]. Likewise, when used in addition to systemic antibiotics in patients with CLABSI, ethanol lock increased the cure rates from the infection [15], salvaged the central catheter [15–17], and eradicated the causative organisms [16,17]. A meta-analysis concluded that ethanol lock is effective in preventing [8,18] and treating [8] CLABSI.

However, using 70% ethanol in central catheters is associated with some adverse effects. Mild self-limited systemic effects include nausea, headache, and a taste of alcohol [4,19]. More concerning are the observed effects on catheter integrity with plasma protein precipitation [4,19], elution of polymers from the catheter's surface [19], and catheter thrombosis and occlusion [18,20,21].

It was suggested that using lower concentrations of ethanol and/or shorter exposure times may mitigate these effects [4]. These avenues have not been comprehensively studied. The aim of the present study was to systematically evaluate in vitro the

concentrations and exposure time of ethanol needed for preventing and treating *P. aeruginosa*-based biofilm, using a static microplate model. *P. aeruginosa* was used as it is a major clinically-relevant biofilm-forming microorganism that can cause life-threatening nosocomial infections, and is often resistant to antibiotics [1,3].

## PATIENTS AND METHODS

### BACTERIAL STRAINS AND GROWTH CONDITIONS

Two strains of *P. aeruginosa* were used: the laboratory strain *P. aeruginosa* PAO1 [22] and the clinical strain *P. aeruginosa* 10955, isolated from a patient with CLABSI hospitalized at Schneider Children's Medical Center. For the experiments, bacteria were grown overnight at 37°C under agitation (250 rounds per minute [rpm]) in Tryptic Soy Broth (TSB, DIFCO, Bethesda, MD, USA).

### PREVENTION OF *P. AERUGINOSA*-BASED BIOFILM FORMATION WITH ETHANOL

A static crystal violet assay for biofilm formation was used, essentially as previously described [22]. Briefly, after overnight growth in TSB, bacteria were diluted in a fresh TSB medium to obtain a working stock solution of an OD<sub>595</sub> of 0.1 (corresponding approximately to 108 colony-forming units [CFU]/ml). For experiments, the stock solution was transferred into a 96-well plate (De-Groot, Israel) and diluted to a final concentration of  $5 \times 10^7$  CFU/ml. Ethanol was added at twofold dilutions in distilled water to a final concentration ranging from 0.625–80% and control wells contained no ethanol. The plates were then incubated at 37°C for 20 hours. On the following day, the supernatant in each well was removed, and the wells were rinsed three times with distilled water to remove the unattached bacteria (i.e., planktonic cells). The wells were then stained with 1% crystal violet (CV, Sigma, USA) for 15 min, and thoroughly washed with distilled water. The stain was eluted from the biofilms by the addition of absolute ethanol for another 15 minutes and quantified by optical density measurement at OD<sub>595</sub> [22]. All measurements were followed by blank subtraction (i.e., subtraction of ethanol incubated with medium alone).

### TREATMENT OF PRE-FORMED BIOFILM WITH ETHANOL

A static XTT assay examining the metabolic activity of a pre-formed biofilm after ethanol treatment was conducted essentially as previously described [23]. *P. aeruginosa* biofilm was grown as previously described. After overnight growth, ethanol at concentrations of 70%, 40%, 30%, 20%, 10% or 0 (control) were added for durations of 2, 4, 8, or 24 hours. Following the ethanol treatment, the supernatant in each well was removed and the wells were rinsed three times with distilled water to remove planktonic bacteria. The wells were then supplemented with 250 µl XTT working solution (0.5 mg/ml XTT and 1 µM

menadione, both purchased from Sigma, Germany) and incubated for 4 hours. XTT is converted to the soluble colored formazan in a manner correlated with microbial viability [21]. The absorbance was read at 490 nm, and the anti-biofilm activity of ethanol was calculated as a percentage of viability compared with ethanol-free controls that were regarded as 100%.

### RE-GROWTH TESTING AFTER EXPOSURE TO ETHANOL

*P. aeruginosa* biofilm was grown as described above. On the following day, the wells were rinsed three times with sterile water and subsequently supplemented with ethanol at a concentration ranging from 10% to 70% for 48 hours. The wells were then washed three times with sterile water, and 200 µl of basal medium 2 (BM2) were added to each well. The growth of the bacteria was monitored at OD<sub>595</sub> for 15 hours (overnight).

### STATISTICAL ANALYSES

Assays were performed in duplicate, with at least three independent experiments. The nonparametric Mann-Whitney U test was used to compare biomass and metabolic activity results at various ethanol concentrations vs. ethanol-free controls and among various treatment durations. Statistical analyses were performed using the IBM SPSS Statistics 23 software package (IBM Corp., Armonk, NY, USA). A *P* value < 0.05 was considered statistically significant.

## RESULTS

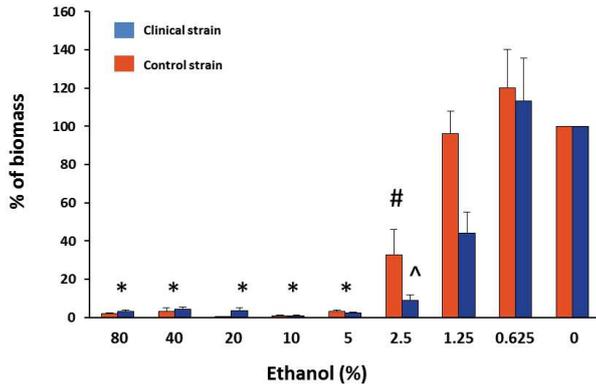
### PREVENTION OF *P. AERUGINOSA*-BASED BIOFILM FORMATION WITH ETHANOL

The effect of various concentrations of ethanol on biofilm formation by *P. aeruginosa* in a static assay is shown in Figure 1. Ethanol concentrations of 5% to 80% reduced the biofilm biomass of both the control and clinical isolates of *P. aeruginosa* to near zero compared to no treatment, with high statistical significance (*P* < 0.001). Notably, an ethanol concentration of 2.5% reduced the biofilm biomass of the clinical and laboratory strains by 91% (*P* = 0.002) and 67% (*P* = 0.003), respectively [Figure 1]. Lower ethanol concentrations did not demonstrate a significant effect on biofilm formation.

### TREATMENT OF PRE-FORMED BIOFILM WITH ETHANOL

The effect of ethanol on the metabolic activity of pre-formed *P. aeruginosa* biofilm, as determined by a static XTT assay, is shown in Figure 2. Ethanol treatment reduced the metabolic activity with a clear dependence on concentration and treatment duration. Treatment with 70% ethanol reduced the activity to near zero after 2, 4, 8, and 24 hour treatments (*P* < 0.001) in both the control laboratory [Figure 2A] and clinical [Figure 2B] strains of *P. aeruginosa*. Treatment with 40% ethanol reduced the activity to zero after 8 hours, but not after 2 or 4 hours (control strain) or 2 hours (clinical strain). Similar trends were ob-

**Figure 1.** The effect of various concentrations of ethanol on preventing biofilm formation by *P. aeruginosa* in a static 20-hour assay. Biofilm biomass was determined by the crystal violet assay. Data are presented as percentages of the results of ethanol-untreated controls, which were designated as 100%. Results are shown as mean and standard error  
\* $P < 0.001$ , # $P = 0.003$ , ^ $P = 0.002$



served with lower concentrations of ethanol [Figure 2]. For all ethanol concentrations, the differences between 2 hour and 24 hour treatments were statistically significant ( $P = 0.004$ ).

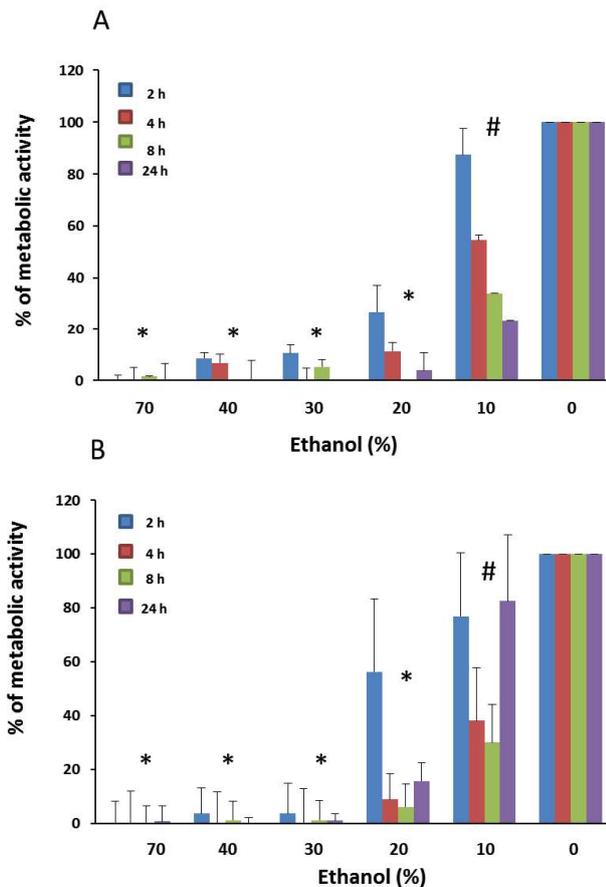
**RE-GROWTH TESTING AFTER EXPOSURE TO ETHANOL**

The ability of *P. aeruginosa* biofilm to recover from the ethanol treatment for 48 hours was tested by washing out the ethanol and replacing it with BM2 growth medium. As shown in Figure 3, ethanol at concentrations of 20% to 70% most likely killed all the bacteria in the developed biofilm as no re-growth was detected, while the growth curve displayed following 10% ethanol treatment showed considerable re-growth, suggesting that the biofilm bacteria survived treatment at this ethanol concentration.

**DISCUSSION**

This study systematically examined in vitro the concentration and duration of ethanol that are needed to prevent or treat foreign body-based *P. aeruginosa* biofilm. The new findings included the demonstration that low concentrations of ethanol successfully prevented biofilm formation and treated mature biofilms of *P. aeruginosa*, and that the treatment results were significantly dependent on the duration of ethanol exposure. In this study, 5% ethanol was the minimal concentration required for inhibiting biofilm formation of laboratory and clinical *P. aeruginosa* strains, while 2.5% ethanol caused only partial inhibition of biofilm formation. Treatment of established mature biofilms required higher concentrations of ethanol, although relatively low levels, such as 20%, were still effective. Treatment

**Figure 2.** The effect of various concentrations of ethanol and durations of exposure on treating pre-formed biofilm, as determined by the metabolic activity in a static XTT assay. Data are presented as percentages of the results of ethanol-untreated controls, which were designated as 100%. Results are shown as mean and standard error. **[A]** *P. aeruginosa* laboratory control strain. Statistical significance (all treatment periods) \* $P < 0.001$ , # $P = 0.006$ . **[B]** *P. aeruginosa* clinical strain 10955. Statistical significance for the differences among the treatment periods \* $P < 0.001$ , # $P = 0.019$



results were also time-dependent: 20% ethanol exposure for 8 hours was fully effective, while shorter durations of exposure of 2 and 4 hours resulted in only partial biofilm elimination [Figure 2]. Moreover, relapse experiments showed that pre-exposure to a range of ethanol concentrations (20–70%), followed by alcohol removal and fresh media supplementation, did not enable the regrowth of dormant bacteria [Figure 3]. This suggests that a low ethanol concentration, can eradicate the bacteria encased within the biofilm.

These in vitro findings are clinically relevant. *P. aeruginosa* is a major cause of persistent biofilm infections, for example in individuals with indwelling medical devices or in the airways of

patients with cystic fibrosis [1,3,6]. Because these biofilm-based infections are very difficult to eradicate [7], and as ethanol can penetrate biofilms and does not seem to provoke bacterial resistance [24], it has been studied as a therapeutic agent in these settings [8,15,17].

Currently, a solution of 70% ethanol is used clinically, but it can interfere with catheter integrity [4,19,20] and is sometimes associated with systemic and local reactions [4,18,19,21]. Regarding the treatment duration, 12 to 24 hours is usually recommended [8,15,17], although shorter duration might be required clinically, so that the central catheters can remain available for administration of fluids, medications and/or nutrition [8].

Our findings are in agreement with several reports in other microorganisms and other settings. Low concentrations of ethanol were effective in vitro against biofilms related to *Staphylococcus aureus* and *Candida albicans* [25]. Schilcher et al. [20] examined the effects of various ethanol concentrations (7–70%) as a lock treatment on catheters tips in the presence of plasma. They found that ethanol concentrations greater than 28% resulted in protein precipitation that could lead to catheter occlusion and recommended to limit ethanol concentrations used for catheter lock treatments to 28%, as indeed suggested by several reports [4,21]. Other reports suggested protocols based on a short exposure time to ethanol [13,15]. However, a systematic checklist evaluating the concentration- and duration-dependent effectiveness of ethanol in preventing and treating *P. aeruginosa*-based biofilm was lacking and led to our study.

Based on our findings, future studies will examine the optimal ethanol concentrations and treatment durations on other pathogens and on polymicrobial biofilms. In addition, to achieve higher effectiveness, the combination of low ethanol concentrations with antimicrobial agents or with novel anti-biofilm agents seems promising [15,17,24] and should be studied.

## CONCLUSIONS

We have systematically elucidated in vitro the concentrations and durations of ethanol that are needed to prevent and eliminate *P. aeruginosa*-based biofilms. Our findings provide evidence that low concentrations of ethanol have the potential to prevent biofilm formation and to eradicate existing ones; hence, preventing the regrowth of bacteria. Clinical studies are warranted to follow our study and confirm the efficacy of low-ethanol concentrations as catheter lock therapy and the optimal conditions of its use. These additional studies are needed for translating our in vitro findings to the clinical arena, which will hopefully reduce the systemic and local adverse effects that are associated with the currently used 70% ethanol [18,20,21].

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