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Ethanol and Isopropyl Alcohol Exposure Increases Biofilm Formation in *Staphylococcus aureus* and *Staphylococcus epidermidis*

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ABSTRACT

Introduction: Alcohols, including ethanol and isopropyl alcohol, are used in clinical practice for disinfection and infection prevention. Recent studies, however, demonstrate that alcohols may enhance biofilm production in *Staphylococci*.

Methods: We quantified biofilm formation in the presence of ethanol and isopropyl alcohol in six different, well-characterized strains of *Staphylococcus epidermidis* and *Staphylococcus aureus*. After 24 h of biofilm development, each strain was exposed to normal saline (NS), ethanol, or isopropyl alcohol (40%, 60%, 80% and 95%) for additional 24 h incubation. Adherent biofilms were stained and optical density was determined. Viability of strains was also determined after alcohol exposure.

Results: Ethanol increased biofilm formation in all six strains compared to normal saline ($p < 0.05$). There was increased biofilm formation with increasing ethanol concentration. Isopropyl alcohol also increased biofilm formation with increasing alcohol concentration in all six strains ($p < 0.01$ vs NS). The slime-negative, chemical mutant strain of *S. epidermidis* increased biofilm formation after exposure to both alcohols, likely reverting back its primary phenotype through modulation of the intercellular adhesin repressor. All strains demonstrated viability after exposure to each alcohol concentration, though viability was decreased.

Conclusion: Ethanol and isopropyl alcohol exposure increases biofilm formation of *S. aureus* and *S. epidermidis* at concentrations

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used in clinical settings. Ethanol and isopropyl alcohol did not eradicate viable *Staphylococci* from formed biofilm.

INTRODUCTION

Staphylococci, including *Staphylococcus epidermidis* and *Staphylococcus aureus,* are common biofilm-forming pathogens [1]. They frequently cause implant and catheter-associated infections, and are a significant cause of morbidity and mortality [1]. Previous studies have demonstrated increased biofilm production of *S. epidermidis* and *S. aureus* after exposure to different alcohols, including ethanol at concentrations above 40% [2, 3]. This is important since isopropyl alcohol is commonly used as a cutaneous disinfectant and ethanol is used in catheter lock solutions for the treatment and prevention of catheter-related bloodstream infections (CRBSI) [1, 4]. Although ethanol-based catheter lock solutions, including combinations with isopropyl alcohol, have been advocated for the prevention and management of CRBSI at concentrations between 25% and 100%, ethanol-based lock solutions may have unintended consequences since CRBSI are frequently caused by biofilm-forming bacteria [5, 6]. Additionally, ethanol use in lock solutions has been demonstrated to have other deleterious effects [5, 6].

We compared the effects of ethanol and isopropyl alcohol on *Staphylococcal* biofilms using a semi-quantitative microtiter plate assay to better understand the effect of these alcohols on biofilm formation. We also measured the viability of biofilm-embedded bacteria after exposure to ethanol or isopropyl alcohol.

MATERIALS AND METHODS

Bacterial Strains

Five ATCC *Staphylococcal* strains were evaluated: a biofilm-producing *S. epidermidis* strain (ATCC 35984; RP62A [ATCC®, Manassas, Virginia]) and its isogenic, slime-negative, biofilm-deficient mutant derived from chemical mutagenesis (M7), two biofilm-forming methicillin-susceptible *S. aureus* strains (ATCC 35556 and ATCC 29213) and a biofilm-forming methicillin-resistant *S. aureus* strain (MRSA; ATCC 43300) [7–10]. ATCC 35984, ATCC 43300, and ATCC 29213 were originally isolated from clinical sources, including a catheter sepsis (ATCC 35984). Additionally, one known biofilm-forming clinical MRSA strain (L32; from blood at the Providence Veterans Affairs Medical Center) was tested [11].

Agents tested

Ethanol (Pharmco-aaper, Brookfield, CT, USA) and isopropyl alcohol (Acros, New Jersey, USA) were evaluated at concentrations of 40%, 60%, 80%, and 95% in sterile water for 24 h exposure. Normal saline (NS) was used for comparison.

Medium

Strains were grown overnight on Tryptic Soy Agar (TSA, Becton–Dickinson, Sparks, MD, USA). Supplemented Tryptic Soy Broth (STSB; Becton–Dickinson, Sparks, MD, USA) with 1% glucose, 2% sodium chloride, 25 mg/L calcium, and 12.5 mg/L magnesium was used to optimize biofilm production in the biofilm assay [12, 13].

Biofilm Formation Assay

Quantification of biofilm formation was conducted using the microtiter plate assay first described by Christensen et al. [14] and modified as described [8, 11–13]. Briefly, a 0.5 McFarland standard of overnight growth of test strains was diluted into STSB. Inocula ($\sim$6.5 $\log_{10}$ CFU/mL) were verified by plating. The inoculated medium was dispensed into wells of sterile flat-bottom 96-well polystyrene tissue culture plates (Costar no. 3596; Corning Inc., Corning, NY, USA). Plates were incubated statically at 37 °C. After 24 h of biofilm development, broth was removed and replaced with test solution and incubated at 37 °C for an additional 24 h. The solution was then removed and the plates were carefully rinsed three times with NS to remove planktonic bacteria. Adherent bacteria were dried overnight and stained with 2% crystal violet solution (Becton–Dickinson, Sparks, MD, USA). The crystal violet was then resolubilized in 95% ethanol and the optical density (OD) of stained adherent bacterial films was read at 570 nm using a SpectraMax M2 Spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Viability

Viability of biofilm-embedded *Staphylococci* was evaluated using a similar 96 well plate assay [15]. After inoculation, incubation and alcohol or NS exposure as above, media was removed and wells were carefully rinsed three times with NS to remove planktonic bacteria. Wells were then filled with 200 μL of NS and plates were sonicated for 20 min in a water bath sonicator (Fisher Scientific FS20, Pittsburg, PA, USA) to disperse adherent biofilms. Viability was determined in quadruplicate on two occasions

by plating aliquots from each strain and alcohol concentration. Plate counts were determined after 24 h incubation. The lower limit of detection for this method is 2.0 $\log_{10}$ CFU/mL.

Statistical Analysis

OD and log CFU/mL were compared between groups using analysis of variance (ANOVA) with Tukey's post hoc test [16]. Data is presented as the mean OD with standard error of the mean using at least eight replicates for each strain and test solution combination. Statistical analysis was conducted using SPSS (release 20; SPSS, Inc. Chicago, IL). A p value of $<$0.05 was considered significant. Each alcohol concentration was compared to NS, and mean difference (change) in OD between alcohol and NS was determined, with a corresponding p value. Mean differences in OD are presented as a range for all the strains in the results.

Compliance with Ethics

This article does not contain any new studies with human or animal subjects performed by any of the authors.

RESULTS

Ethanol exposure increased biofilm in all strains (Fig. 1a). In five strains, the amount of biofilm increased with increasing ethanol concentration. At 60%, 80% or 95% ethanol, more biofilm was produced than after exposure to NS (mean difference in OD vs NS 0.25–1.23, $p < 0.02$). One strain, the prolific biofilm-forming *S. epidermidis* ATCC 35984, demonstrated the inverse trend of decreased biofilm production with higher ethanol concentration, which was significantly
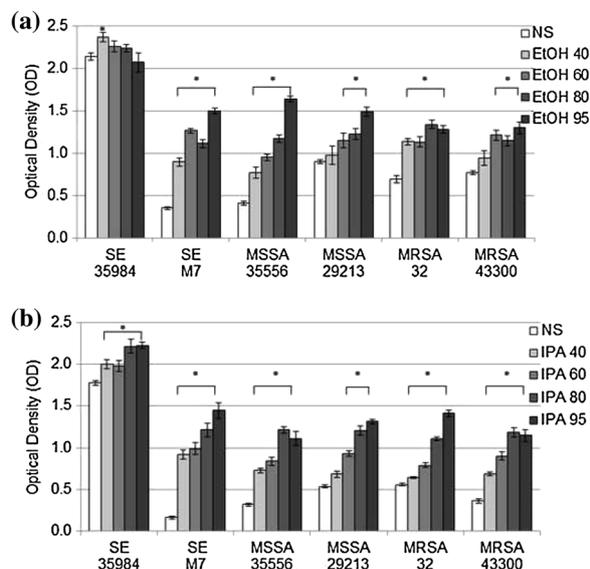
Fig. 1 Biofilm production after ethanol (**a**) or Isopropyl Alcohol (**b**) exposure for 24 h. Mean ± SEM optical density (OD) at 570 nm of stained biofilms in 96 well plates after 24 h exposure to 40%, 60%, 80%, and 95% alcohols compared to normal saline 0.9% (NS) ($n = 8$ each). *SE 35984 S. epidermidis* ATCC 35984, *SE M7 S. epidermidis* M7, *MSSA 35556* methicillin-susceptible *S. aureus* ATCC 35556, *MSSA 29213* methicillin-susceptible *S. aureus* ATCC 29213, *MRSA 32* methicillin-resistant *S. aureus* clinical strain L32, *MRSA 43300* methicillin-resistant *S. aureus* ATCC 43300, *EtOH* ethanol, *SEM* standard error of the mean. (*Asterisk*) Statistically significant compared to NS ($p < 0.05$). SE 35984 $p = 0.04$; SE M7 $p < 0.01$; MSSA 35556 $p < 0.01$; MSSA 29213 $p < 0.02$; MRSA 32 $p < 0.01$; MRSA 43300 $p < 0.01$. *IPA* isopropyl alcohol. (*Asterisk*) Statistically significant compared to NS ($p < 0.01$ for all)

different between 40% and 95% ethanol (−0.29, 95% CI 0.03–0.55, $p < 0.02$). However, differences between other concentrations were not statistically significant. Isopropyl alcohol exposure (Fig. 1b) led to increased biofilm in all strains tested, with higher biofilm production for 60%, 80%, and 95% alcohol compared to NS (mean difference in OD vs NS 0.15–1.28, $p < 0.01$).

Viable bacteria remained at all concentrations of both ethanol and isopropyl alcohol with a range up to 2.93 $\log_{10}$ CFU/mL

after ethanol exposure and 3.01 $\log_{10}$ CFU/mL after isopropyl alcohol exposure. NS exposure yielded 2.35–4.4 $\log_{10}$ CFU/mL, depending on strain. For *S. epidermidis* ATCC 35984 and M7, the quantity of viable bacteria was reduced by all of the alcohol conditions tested ($p < 0.03$). Cell counts were not significantly reduced by alcohol exposure for any of the *S. aureus* strains tested. For all strains, viable cell count tended to decrease with increasing alcohol concentration, but these differences were not statistically significant. Some bacterial counts (CFU/mL) reached the 2.0 $\log_{10}$ CFU/mL lower limit of detection, but viable bacteria were present for each strain-alcohol concentration combination tested.

DISCUSSION

Our results are similar to a previous study demonstrating increased *S. aureus* biofilm formation after ethanol exposure [2], however, there are conflicting reports on the viability of those biofilm bacteria [17, 18]. We found these bacteria within biofilm were viable, although viability was decreased compared to NS-exposed biofilm. In contrast to previous reports [4, 19], bacteria in biofilm were not eradicated after alcohol exposure. This may be due to different methods used to remove the biofilm from 96 well plates, as prior studies removed biofilm using cotton swabs [4, 19], whereas we sonicated the well plates.

We also found an increase in biofilm formation with increasing alcohol concentration. Only one strain, the prolific biofilm-forming *S. epidermidis*, decreased biofilm formation with increasing concentrations of ethanol. This strain was likely near maximal biofilm production possible in this assay. Small variations in

biofilm formation are possible, as demonstrated by the differences in NS-exposed biofilm between the ethanol and isopropyl alcohol experiments. The differences in biofilm comparing other ethanol concentrations, such as 40% and 80% or 60% and 80% are not statistically significant for this strain.

The bactericidal effect of alcohol depends upon dehydration and denaturation of proteins [20]. Mixtures of alcohols and water (60–90% v/v) are more effective because proteins are denatured more quickly in the presence of water [20, 21]. Ethanol also causes leakage of the plasma membrane, disrupting bacterial growth and metabolism [22]. The impact of dehydration on cell death in the presence of alcohols may not be observed in catheter lock solutions since these do not dry, however denatured proteins and leaking membranes may still lead to decreased viability. The high concentrations of ethanol in catheter lock solutions increase biofilm formation in *Staphylococci* and also predisposes to catheter dysfunction and plasma protein precipitation [6].

Staphylococcus epidermidis M7, the isogenic slime-negative, biofilm-deficient mutant of *S. epidermidis* ATCC 35984 demonstrated increased OD with exposure to both alcohols; however, they were not as dense as the prolific biofilms of ATCC 35984. M7 was derived from ATCC 35984 through mitomycin C-induced mutations. M7, sometimes referred to as an accumulation-negative mutant, is distinguished from ATCC 35984 because it lacks a 140 kDa antigen called accumulation-associated protein, but it has been found to have a 200 kDa protein with similar homology [23, 24]. This strain does not accumulate on glass and polystyrene surfaces [23], but it accumulates on polyvinyl chloride disks and has been shown to produce biofilm [25–28]. The exact mechanism for the mutation is unknown but is believed to be due to alteration of the intercellular adhesin (*ica*) gene [10]. The *ica* gene regulates production of polysaccharide intercellular adhesin, the major exopolysaccharide produced in *S. epidermidis* and *S. aureus* biofilm [29]. Ethanol increases *Staphylococcal* biofilm formation by increasing *ica* expression through modulation of the repressor, *icaR* [2, 3, 29, 30]. It is possible that alcohol exposure and subsequent increase in *ica* expression allowed accumulation and biofilm formation of this strain in polystyrene plates. To our knowledge, this is the first report of any alcohol exposure to cause the M7 strain to increase biofilm formation.

Regarding limitations, we tested a small number of strains, including one clinical isolate which may have different biofilm-forming behavior. The crystal violet used in this study stains cells and does not differentiate between viable and nonviable cells or quantify extracellular matrix production. Also, we did not characterize the composition or matrix production of the biofilms. We considered that alcohol may denature bacteria in biofilm, allowing for greater penetration of the crystal violet. However, differences in biofilm formation could be observed between wells even before the crystal violet stain was added. This also would not account for the increase in *ica* expression noted previously [30]. Viability may be underestimated using this method, since some adherent cells were visible in the bottom of wells after 20 min of sonication, particularly the prolific biofilm-forming ATCC 35984. Sonication of well plates can fail to release cells completely [31]. There was also a tendency for the number of bacteria to be higher in the center of the well plate than along the edges where evaporation was higher, further suggesting that dehydration played a role in cell viability.

CONCLUSION

Staphylococci exposed to clinically relevant concentrations of ethanol and isopropyl alcohol increase biofilm formation; however, the viability of these biofilm-embedded bacteria was diminished. Future research should determine the impact of these findings on the use of various alcohol preparations in the management and prevention of infections due to biofilm-forming *Staphylococci*.

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Compliance with ethics guidelines. This article does not contain any new studies with human or animal subjects performed by any of the authors.

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