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MAJOR ARTICLE

A Cumulative Spore Killing Approach: Synergistic Sporicidal Activity of Dilute Peracetic Acid and Ethanol at Low pH Against *Clostridium difficile* and *Bacillus subtilis* Spores

Michelle M. Nerandzic,[1] Thriveen Sankar C,[2] Peter Setlow,[3] and Curtis J. Donskey[2,4]

[1]Research Service, Cleveland Veterans Affairs Medical Center, Ohio; [2]Case Western Reserve University School of Medicine, Cleveland, Ohio; [3]Department of Molecular, Microbial and Structural Biology, University of Connecticut Health Center, Farmington; and [4]Geriatric Research, Education and Clinical Center, Cleveland Veterans Affairs Medical Center, Ohio

Background. Alcohol-based hand sanitizers are the primary method of hand hygiene in healthcare settings, but they lack activity against bacterial spores produced by pathogens such as *Clostridium difficile* and *Bacillus anthracis*. We previously demonstrated that acidification of ethanol induced rapid sporicidal activity, resulting in ethanol formulations with pH 1.5–2 that were as effective as soap and water washing in reducing levels of *C difficile* spores on hands. We hypothesized that the addition of dilute peracetic acid (PAA) to acidified ethanol would enhance sporicidal activity while allowing elevation of the pH to a level likely to be well tolerated on skin (ie, >3).

Methods. We tested the efficacy of acidified ethanol solutions alone or in combination with PAA against *C difficile* and *Bacillus subtilis* spores in vitro and against nontoxigenic *C difficile* spores on hands of volunteers.

Results. Acidification of ethanol induced rapid sporicidal activity against *C difficile* and to a lesser extent *B subtilis*. The addition of dilute PAA to acidified ethanol resulted in synergistic enhancement of sporicidal activity in a dose-dependent fashion in vitro. On hands, the addition of 1200–2000 ppm PAA enhanced the effectiveness of acidified ethanol formulations, resulting in formulations with pH >3 that were as effective as soap and water washing.

Conclusions. Acidification and the addition of dilute PAA induced rapid sporicidal activity in ethanol. Our findings suggest that it may be feasible to develop effective sporicidal ethanol formulations that are safe and tolerable on skin.

Keywords. alcohol-based hand sanitizers; *Bacillus subtilis* spores; *Clostridium difficile* spores; hand hygiene; peracetic acid.

Effective hand hygiene is essential to prevent transmission of pathogens [1, 2]. Due to their efficacy and convenience, alcohol-based hand sanitizers have become the primary method of hand hygiene in healthcare settings, and they are commonly used in the community [3]. The antimicrobial activity of alcohols is due to their ability to denature proteins, resulting in germicidal activity against vegetative bacteria and many fungi and enveloped viruses [4]. However, alcohols lack activity against bacterial spores, and use of alcohol hand sanitizer does not reduce levels of spores on hands [5,6]. This gap in the spectrum of activity of alcohols is of critical importance because the spore-forming anaerobe *Clostridium difficile* is a major pathogen in healthcare facilities and in the community [7, 8]. In addition, the intentional use of *Bacillus anthracis* spores as an agent of bioterrorism remains a threat, and ethanol is ineffective in reducing levels of a surrogate of *B anthracis* on hands [9, 10]. Although soap and water hand washing and application of chlorine or hypochlorous acid solutions can reduce levels of *C difficile* and *Bacillus* spp spores on hands [5, 6, 10–12], these hand hygiene methods lack the convenience and efficiency of alcohol hand sanitizers.

As a novel approach for development of sporicidal hand hygiene solutions, we have investigated the potential to induce sporicidal activity in alcohol and other skin disinfectants through alteration of physical or chemical conditions that might degrade or allow penetration of spore coats [13, 14]. Chlorhexidine, a nonsporicidal cationic bisbiguanide, exhibited activity against *C difficile* spores in the presence of denaturing chemical and physical conditions such as elevated temperature, elevated pH, or alcohol [13]. Moreover, acidification, alkalinization, and heating of ethanol induced rapid sporicidal activity against *C difficile* and to a lesser extent *Bacillus thuringiensis* and *Bacillus subtilis* [14]. The sporicidal activity of acidified ethanol was enhanced by increasing ionic strength and mild elevations in temperature [14]. On skin, sporicidal acidified ethanol formulations with pH 1.5–2 were as effective as soap and water

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Correspondence: M. M. Nerandzic, BS, 1110 (W), Cleveland VA Medical Center, 10701 East Blvd., Cleveland, OH 44106 (michellenerandzi@aim.com).

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hand washing in reducing levels of *C difficile* spores [14]. Although these findings are promising, it is anticipated that sporicidal ethanol formulations with less acidic pH will be required to assure safety and tolerability with repeated use.

Peracetic acid (PAA) is a sporicidal oxidizing agent used for disinfection of hard surfaces, waste water, and heat-labile instruments [15–17]. Although irritating to the skin and respiratory tract at high concentrations, PAA is well tolerated on skin at concentrations of ≤2000 ppm (0.2% w/v) [6, 18]. Peracetic acid at 2000 ppm combined with surfactant (90-second application time) was more effective than a tap water wash (30-second application) for removal of *C difficile* spores from skin [6]. Alcohol enhances the efficacy of PAA [19, 20]. Dilute PAA combined with ethanol is used for disinfection of tissue and bone grafts [21, 22], and 2000 ppm PAA combined with 80% ethanol has been proposed as a hand sanitizer due to its efficacy against enveloped viruses [23]. It has recently been demonstrated that *B subtilis* spores with defective coats are greatly sensitized to PAA, purportedly because PPA is allowed greater access to its site of sporicidal action on the inner membrane [24]. Based on these data, we hypothesized that the combination of dilute PAA and acidified ethanol would result in synergistic killing of *C difficile* and *Bacillus* spp spores, and the addition of dilute PAA would result in an effective sporicidal acidified ethanol hand hygiene solution with pH > 3.

METHODS

Ethics Statement

The Institutional Review Board of the Cleveland Veterans Affairs Medical Center approved the study protocol (reference number: 11009-H10 approved May 18, 2011) for hand hygiene studies. For the hand hygiene studies, a non-toxigenic *C difficile* strain purchased from the American Type Culture Collection (ATCC) was used, and participants provided verbal informed consent to participate. Verbal informed consent was obtained because the study was considered minimal risk. Participant consent was recorded in study documents. The Cleveland Veterans Affairs Institutional Review Board approved the consent procedure.

Spore Strains and Growth Conditions

Two *C difficile* strains cultured from patients with *C difficile* infection in Cleveland and 1 strain purchased from the ATCC were used. VA 17 is an epidemic (cdtB$^+$) restriction endonuclease analysis (REA) BI strain, and VA 11 is a nonepidemic (cdtB$^-$) REA J strain; both isolates are toxigenic (tcdA$^+$, tcdB$^+$) strains. American Type Culture Collection 43593 is a non-toxigenic (*tcdA*, *tcdB*$^-$) strain from serogroup B. *Clostridium difficile* cultures were incubated at 37°C for 48 hours in a Whitley MG1000 anaerobic workstation (Microbiology International, Frederick, MD) on prereduced cycloserine-cefoxitin-brucella agar containing 0.1% taurocholic acid and lysozyme 5 mg/L (CDBA) [25].

Bacillus subtilis 168 was donated by Peter Setlow (UConn Health Center, Farmington, Connecticut). Strain 168 spores were cultured on trypticase soy agar containing 5% sheep blood (Becton Dickinson, Franklin Lakes, NJ) under aerobic conditions at 37°C for 24 hours.

Preparation of Spores

Clostridium difficile spores were prepared as previously described [26]. In brief, prereduced brain-heart infusion plates were spread with 100 µL of a 24-hour *C difficile* suspension and incubated for 1 week in an anaerobic incubator. Spores were harvested from the plates using sterile swabs and 8 mL ice-cold, sterile, distilled water. Spores were washed 5 times by centrifuging and resuspending in distilled water. Vegetative material was removed by density gradient centrifugation in Histodenz (Sigma Aldrich, St. Louis, MO). Before testing, spore preps were confirmed by phase contrast microscopy and malachite green staining to be >99% dormant, bright-phase spores.

Bacillus subtilis spores were prepared at 37°C on 2× SG medium agar plates and harvested, cleaned, and stored as previously described [27]. Spores were separated from vegetative material by density gradient centrifugation in Nycodenz (Axis-Shield, Oslo, Norway). Spores were confirmed by phase contrast microscopy and malachite green staining to be >99% dormant, bright-phase spores.

The Effect of Acidic pH on Sporicidal Activity of Ethanol

We previously demonstrated that reducing the pH of 70% ethanol to pH 1.3–2.0 induced rapid sporicidal activity against *C difficile* at room temperature [14]. However, *B subtilis* spores remained resistant to killing at this pH range. To determine whether sporicidal activity could be induced in ethanol against the more resistant *B subtilis* spores, the pH of 70% ethanol and deionized water (acid control) was reduced further with hydrochloric acid (HCl) to a pH range of 0.8–4. Ten microliters of *B subtilis* and *C difficile* spores (approximately 10^6 colony-forming units [CFU]) were incubated for 5 minutes in 1 mL of the pH-adjusted ethanol or water at 22°C. The reaction was quenched by neutralizing 1:1 in Dey-Engley neutralization broth (BD Biosciences, San Jose, CA). Neutralized samples were serially diluted in deionized water, drop-plated, and cultured as described previously. To increase the sensitivity of enumeration for samples with high levels of spore killing, 1 mL of the neutralized spore suspensions was spread-plated. After incubation, $\log_{10}$ CFU reduction of spores was determined by calculating the difference in $\log_{10}$ CFU recovered from baseline (pH altered water) and experimental groups (pH altered ethanol).

Efficacy of Aqueous Versus Alcoholic Peracetic Acid for Killing of *Clostridium difficile* Spores

The effect of ethanol on the sporicidal activity of dilute PAA was assessed for *C difficile* strains VA 17 and ATCC 43593. Peracetic acid solutions were prepared to a final concentration of

450 ppm in sterile deionized water or 70% ethanol. Final PAA concentrations were measured using a PAA titration kit (LaMotte Company, Chestertown, MD). The pH of the solutions was left unaltered (approximately pH 3.5) or adjusted with HCl (pH 3.0, 2.5, 2.0, 1.5, and 1.0). Ten microliters of spores (approximately 10^6 CFU) was inoculated into 1 mL water (baseline), pH adjusted ethanol (pH 2.5 and 1.5), and PAA solutions and incubated at room temperature for 3 minutes. The reaction was quenched by neutralizing 1:1 in Dey-Engley neutralization broth. Neutralized samples were serial diluted in deionized water, drop-plated, and cultured as described previously. After incubation, $\log_{10}$ CFU reduction of spores was determined by calculating the difference in $\log_{10}$ CFU recovered from baseline (water) and experimental groups. Experiments were performed in triplicate.

Effect of Dilute Peracetic Acid on Sporicidal Activity of Acidified Ethanol

The effect of the addition of dilute PAA on the activity of acidified ethanol against *C difficile* strain VA 17 and *B subtilis* was tested at 22°C. The pH of specified test solutions was adjusted to 2.5 or 1.5 with HCl. Ten microliters of spores (approximately 10^6 CFU) was inoculated into 1 mL water (baseline), pH-adjusted ethanol, PAA at 450, 650, or 1500 ppm, pH-adjusted PAA at 450 and 650 ppm, ethanol plus PAA at 450 and 650 ppm, and pH-adjusted ethanol plus PAA at 450 and 650 ppm and incubated for 3 minutes. The reaction was quenched by neutralizing 1:1 in Dey-Engley neutralization broth. Neutralized samples were serial diluted in deionized water, drop-plated, and cultured as described previously. After incubation, $\log_{10}$ CFU reduction of spores was determined by calculating the difference in $\log_{10}$ CFU recovered from baseline (water) and experimental groups. Experiments were performed in triplicate.

Efficacy of Dilute Peracetic Acid and Acidified Ethanol Solutions for Reducing *Clostridium difficile* Spores on Hands

A modification of the "Standard Test Method for Determining the Bacteria-Eliminating Effectiveness of Hygienic Handwash and Handrub Agents Using the Fingerpads of Adults" (American Society for Testing and Materials E 2276–10) was used to determine the efficacy of test solutions against non-toxigenic *C difficile* spores [28]. Each fingerpad of both hands were contaminated with 10 μL of a liquid inoculum containing 6 $\log_{10}$ CFU of ATCC 43593 spores. The fingerpads were rubbed together until the inoculum was dry. Hand contamination levels were measured using a modified fingerpad sampling method. The fingerpads of each hand were rubbed with slight friction against the bottom of a 150 mm × 15 mm Petri dish filled with 25 mL Dey-Engley neutralizer for 30 seconds. The neutralizer was collected from the Petri dish, serially diluted, and plated on CDBA media to determine *C difficile* counts. Log_{10} reductions were calculated by subtracting $\log_{10}$ CFU recovered after hand hygiene treatment from $\log_{10}$ CFU recovered from hands without treatment.

A crossover design was used such that each of the 10 volunteers was exposed to 1 of the 10 disinfection procedures no more than once every 24 hours. The order of the hand disinfection procedures for each volunteer was assigned using a computer-generated random numbers list designed to allow all agents or procedures to be tested 6 times. The person reading the plates was blinded to the test product. The hand disinfection interventions included 1 mL ethanol-based hand sanitizer gel (Purell, GOJO Industries, Akron, OH), 1 mL 0.05% triclosan liquid soap (STERIS Corporation, Mentor, OH), 1 mL 450 ppm PAA (unaltered pH approximately 3.0), and 1 mL of the following ethanol-based solutions: 70% ethanol pH 2.5; 70% ethanol pH 1.5; 70% ethanol plus 450, 1200, or 2000 ppm PAA (unaltered pH > 3.0); 70% ethanol pH 1.5 plus 450 ppm PAA; and 70% ethanol pH 2.5 plus 450 ppm PAA. For the soap and water hand wash, fingerpads were rubbed vigorously with liquid soap for 20 seconds, rinsed with water until soap was completely removed, and patted dry with paper towels. For the PAA and ethanol-based handrub agents, fingerpads were rubbed together until dry.

Data Analysis

Data were analyzed with R statistical software (version 3.1.1). Continuous data were analyzed using unpaired *t* tests. For skin model experiments, one-way analysis of variance was performed to compare the mean log reductions. A post hoc Tukey honestly significant difference test was conducted to test all pairwise differences between group means.

RESULTS

Acidification Induces Sporicidal Activity in Alcohol

Clostridium difficile and *B subtilis* spores were not killed in water adjusted to pH 0.8–4.0. There were no significant differences between the $\log_{10}$ CFU reductions of the 3 strains of *C difficile* tested (VA 11, VA 17, and ATCC 43593); therefore, data for the strains were pooled. With a 5-minute dwell time, *C difficile* spores were reduced in a dose-dependent fashion as the concentration of acid was increased (Figure 1). A $\geq$2 $\log_{10}$ CFU reduction of *C difficile* spores was observed when ethanol solutions were adjusted to pH < 2.0 at room temperature. *Clostridium difficile* spores were significantly more susceptible to killing by acidified ethanol solutions than *B subtilis* spores ($P < .001$). *Bacillus subtilis* spores were reduced by approximately 1 $\log_{10}$ CFU when the pH of ethanol was adjusted to 0.8, but no significant reduction of *B subtilis* spores was observed for any of the other pH-adjusted solutions.

Ethanol Enhances the Sporicidal Efficacy of Dilute Peracetic Acid

There were no significant differences between the $\log_{10}$ CFU reductions of the 2 strains of *C difficile* tested (VA 17 and ATCC 43593); therefore, data for the strains were pooled. After 3 minutes of incubation, the presence of ethanol significantly enhanced the activity of dilute PAA (450 ppm) at pH 1.5 and

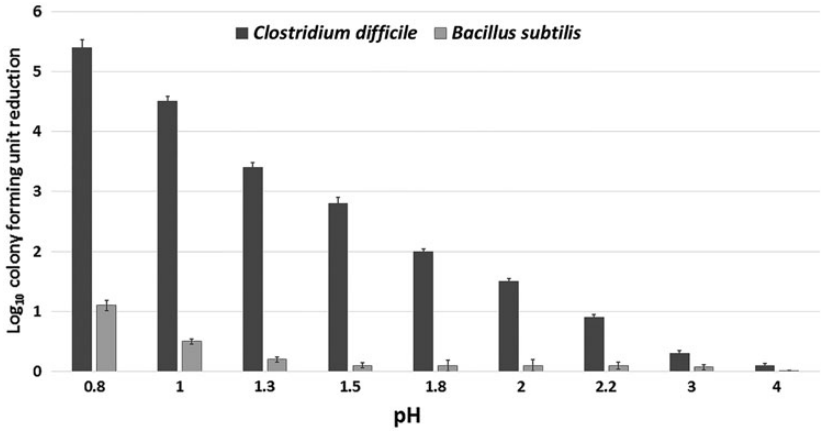

Figure 1. Acidification induces sporicidal activity in ethanol. Six $\log_{10}$ colony-forming units (CFU) of *Clostridium difficile* (VA17, VA11, and American Type Culture Collection [ATCC] 43593) and *Bacillus subtilis* spores were exposed to 70% ethanol solutions adjusted to pH 0.8–4 for 5 minutes at room temperature. Log_{10} CFU reduction of spores was determined by calculating the difference in $\log_{10}$ CFU recovered from baseline (pH altered water) and experimental groups (pH altered ethanol). The means of data from triplicate experiments are presented. Error bars indicate standard error.

1.0 ($P < .01$ for aqueous vs alcoholic PAA at pH 1.5 and 1.0) (Figure 2). However, PAA solutions with a pH > 1.5 were unaffected by the presence of ethanol.

After 10 minutes of incubation, ethanol significantly enhanced the sporicidal activity of PAA solutions for all pHs assessed, including PAA with no added HCl at pH 3.5 ($P < .01$ for each comparison). The degree to which ethanol enhanced the sporicidal activity of PAA solutions increased as the pH was lowered (ie, approximately 0.5 $\log_{10}$ CFU reduction for pH 3.5 alcoholic PAA solutions and approximately 2 $\log_{10}$ CFU reduction for pH 1.5 alcoholic PAA solutions).

Acidified Ethanol and Dilute Peracetic Acid Exert Synergistic Sporicidal Activity Against *Clostridium difficile* and *Bacillus subtilis* Spores

With a 3-minute dwell time, PAA at concentrations of 450, 650, and 1500 ppm (pH approximately 3.5) reduced *C difficile* and *B subtilis* spores in a dose-dependent fashion (Figure 3). The addition of acidified ethanol to 450 and 650 ppm PAA significantly

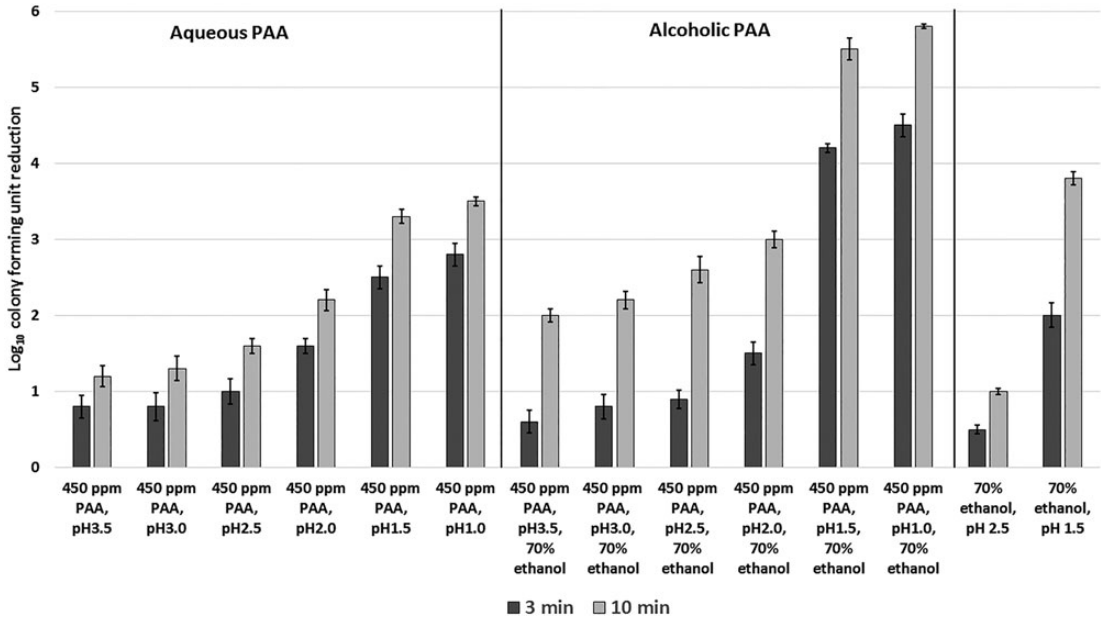

Figure 2. Ethanol enhances the sporicidal efficacy of dilute peracetic acid (PAA). Six $\log_{10}$ colony-forming units (CFU) of *Clostridium difficile* (VA17 and American Type Culture Collection [ATCC] 43593) spores were exposed to aqueous and alcoholic PAA solutions (450 ppm) and incubated at room temperature for 3 or 10 minutes. The pH of the solutions was either left unadjusted (pH 3.5) or lowered to 3.0, 2.5, 2.0, 1.5, or 1.0. Log_{10} CFU reduction of spores was determined by calculating the difference in $\log_{10}$ CFU recovered from baseline (water) and experimental groups. The means of data from triplicate experiments are presented. Error bars indicate standard error.

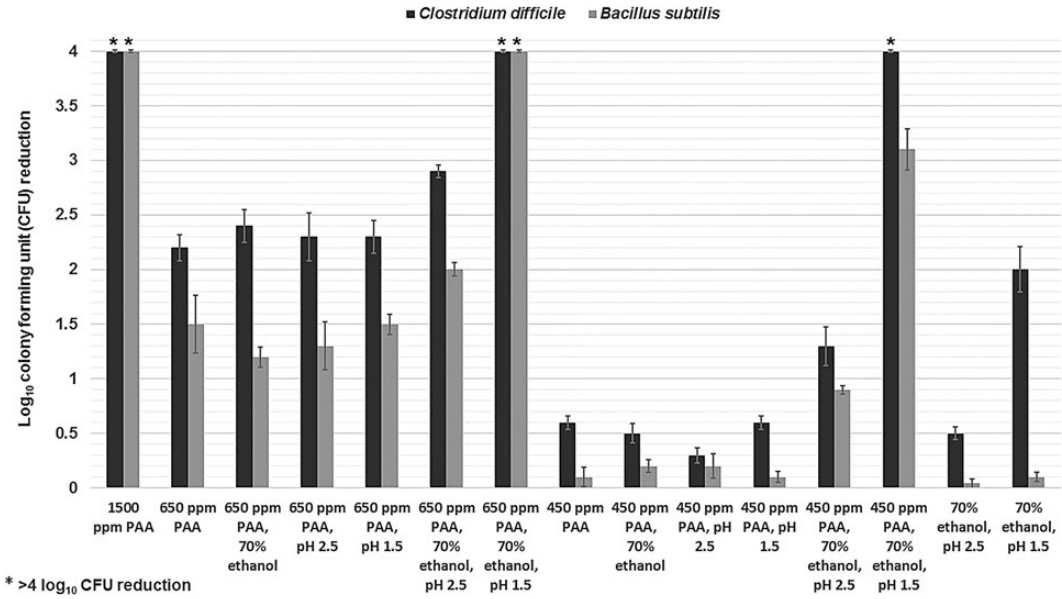

Figure 3. Acidified ethanol and peracetic acid (PAA) exert synergistic sporicidal activity against *Clostridium difficile* and *Bacillus subtilis* spores in vitro. Six $\log_{10}$ colony-forming units (CFU) of *C difficile* (VA17) and *B subtilis* spores were exposed to PAA alone (450, 650, and 1500 ppm) or in combination with 70% ethanol and reduced pH (unadjusted, 2.5, and 1.5). Spores suspensions were incubated at room temperature for 3 minutes. $\log_{10}$ CFU reduction of spores was determined by calculating the difference in $\log_{10}$ CFU recovered from baseline (water) and experimental groups. The means of data from triplicate experiments are presented. Error bars indicate standard error.

enhanced killing of *C difficile* and *B subtilis* spores by >2 $\log_{10}$ CFU and >1 1$\log_{10}$ CFU, respectively ($P < .001$ for each comparison), whereas PAA with the addition of acid (pH 1.5 or 2.5) alone or ethanol alone did not similarly enhance killing.

Acidified Ethanol and Dilute Peracetic Acid Reduce Levels of *Clostridium difficile* Spores on Skin

A soap and water hand wash reduced *C difficile* spores by approximately 1.7 $\log_{10}$ CFU, whereas commercial ethanol-based hand sanitizer did not (Figure 4). At pH 1.5, 2.5, and 3.2–3.8, the addition of PAA 450 ppm to acidified ethanol resulted in a modest but consistent reduction in spore recovery; however, the differences were not statistically significant. At pH 3.2–3.8, the addition of PAA at 1200 or 2000 ppm significantly enhanced reductions in *C difficile* spores versus ethanol at pH 3.2–3.8. The reduction by 2000 ppm PAA plus acidified ethanol pH 3.2–3.8 was not significantly greater than the reduction by 2000 ppm PAA alone ($P > .05$). The reductions in *C difficile* spores achieved by ethanol pH 1.5, PAA 450 ppm plus ethanol pH 1.5, PAA 1200 ppm plus ethanol pH 3.2–3.8, PAA 2000 ppm plus ethanol pH 3.2–3.8, and PAA 2000 ppm were not significantly different from the reduction by soap and water hand wash ($P > .05$).

DISCUSSION

Alcohol-based hand sanitizers play a crucial role in preventing acquisition of pathogens, but they have no activity against bacterial spores [1–3, 5–7]. We previously reported that acidification induces sporicidal activity in ethanol [14]. On skin, acidified ethanol formulations with pH 1.5–2 were as effective as soap and water hand washing in reducing levels of *C difficile* spores [14]. In this study, we expand on those findings by reporting that the addition of dilute PAA results in synergistic enhancement of activity of acidified ethanol against *C difficile* and *B subtilis* spores in vitro. On hands, the addition of PAA enhanced spore reductions by acidified ethanol, although to a relatively modest degree in comparison to in vitro results. More importantly, the addition of PAA resulted in identification of ethanol formulations with pH >3 that were as effective as soap and water wash for reduction of spores on hands. Our findings suggest that it may be feasible to develop effective sporicidal ethanol formulations that are safe and tolerable on skin.

Although no adverse effects or discomfort were noted among the volunteers participating in hand hygiene experiments, safety and tolerability on skin will be important concerns for future development of sporicidal ethanol formulations. In healthy individuals, the skin surface is mildly acidic with pH 4–6 and has been termed an "acid mantle" [29, 30]. Alkaline soaps have been shown to cause dispersal of the resident skin microbiota and loss of epidermal barrier function [29, 30]. In contrast, mildly acidic skin products with pH 3.5–4 are considered optimal to preserve the resident skin microbiota and function [29, 30]. Our goal has therefore been to identify enhancements that raise the pH of acidified ethanol solutions to ≥3 while maintaining efficacy. Although dilute PAA has been well tolerated on skin [6, 18], it is an oxidizing agent that could have adverse

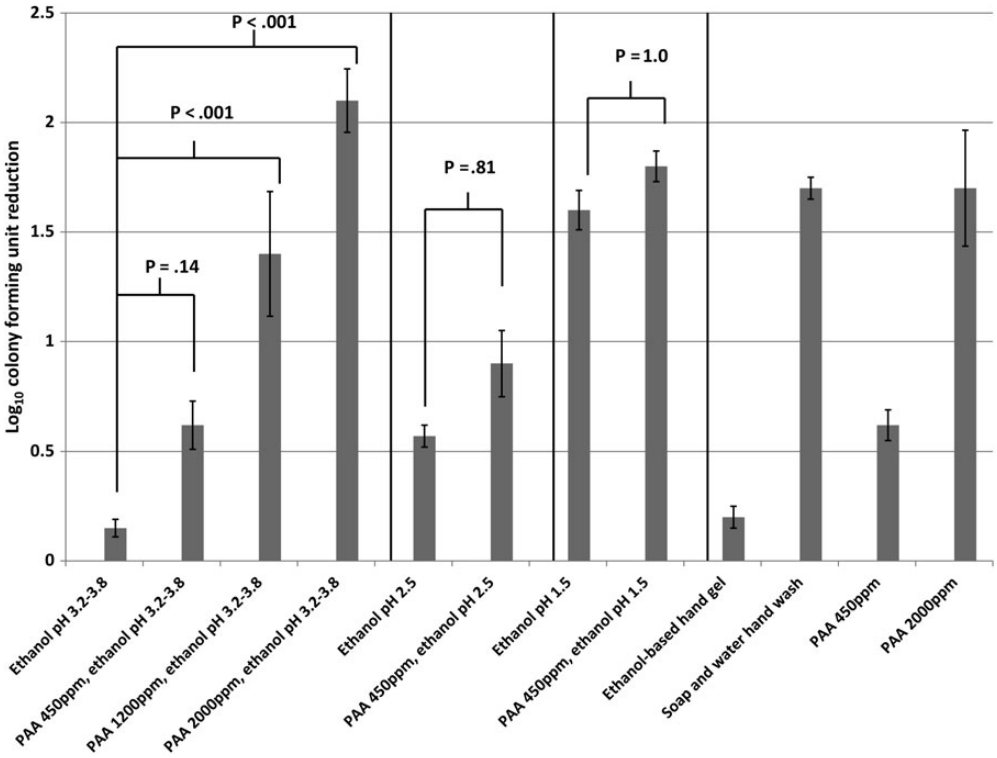

Figure 4. Acidified ethanol and peracetic acid (PAA) reduce levels of non-toxigenic *Clostridium difficile* (ATCC 43593) spores on skin. One milliliter test solution was applied with rubbing to contaminated finger pads. For soap and water hand wash, 1 mL soap was applied to finger pads, rubbed for 20 seconds, rinsed, and then patted dry with paper towels. Log_{10} colony-forming units (CFU) reduction of spores was determined by calculating the difference in log_{10} CFU recovered from treated versus untreated finger pads. The means of data from triplicate experiments are presented. Error bars indicate standard error.

effects with repeated use. Thus, additional studies are needed to identify solutions with the lowest concentration of PAA required to maintain efficacy.

Additional studies are needed to identify the mechanism of sporicidal activity of acidified ethanol plus PAA solutions. Protein denaturation occurs upon exposure to acid and alcohol as a result of disruption of basic protein residues and alteration of electrostatic interactions in proteins, respectively [31, 32]. We propose that protein denaturation by the acidified ethanol solutions tested here facilitate rapid penetration of the spore coat, enabling ethanol and PAA to reach targets within the spore core. This proposal is consistent with previous demonstrations that conditions that denature proteins may induce sporicidal activity in chlorhexidine and lysozyme [33–36], and that mutation of the spore coat protein *cotA* of *C difficile* results in a defect in the outer spore coat that induces ethanol susceptibility [37]. It is also consistent with the demonstration that *B subtilis* spores with defective coats are sensitized to PAA treatment, purportedly because PAA is allowed greater access to its site of sporicidal action on the inner membrane [24]. This evidence is corroborated by findings that alcohol enhances the bactericidal, virucidal, and sporicidal effects of dilute PAA, both in previous studies, and in the current study where it is demonstrated for the first time for *C difficile* spores [19–23].

Bacillus spores, particularly those of *B subtilis*, were more resistant to killing by PAA and acidified ethanol solutions than *C difficile* spores. The greater susceptibility of *C difficile* could potentially be due to differences in protein structure of *C difficile* vs *Bacillus* spp spore coats; recent proteomic studies have revealed major differences in the spore coat and exosporium of *C difficile* and *Bacillus* spp spores [38]. Although *Bacillus* spp spores were relatively resistant to acidified ethanol, they were very susceptible to acidified ethanol in combination with PAA. Differences in spore preparation technique, sporulation medium, and age of spores have previously been shown to affect the thermal resistance of *C difficile* and *Bacillus* spp spores [39, 40]. In the current study, differences due to spore preparation technique cannot be ruled out because *C difficile* and *B subtilis* spores require preparation under dissimilar conditions.

CONCLUSIONS

In summary, we report that the addition of dilute PAA results in synergistic enhancement of activity of acidified ethanol against *C difficile* and *B subtilis* spores in vitro. On hands, the addition of PAA enhanced spore reductions by acidified ethanol, resulting in identification of acidified ethanol formulations with pH >3 that were as effective as soap and water wash for reduction of *C difficile* spores on hands. These findings suggest that it may be

feasible to develop effective sporicidal ethanol formulations that are safe and tolerable on skin. Future studies need to be conducted to determine the impact of sporicidal ethanol formulations on reduction of *C difficile* in a healthcare setting.

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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