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Efficacy of Octenidine Hydrochloride in Reducing *Clostridioides difficile* Spores on Stainless Steel Surfaces

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Abstract

Objective: The spores of *Clostridioides difficile* can survive on surfaces for several months, and act as a source of new and recurrent infections by fecal-oral route. The objective was to investigate the sporicidal efficacy of Octenidine Hydrochloride (OH) against *C. difficile* spores on stainless steel surfaces.

Methods: Suspensions containing ~1,000,000 *C. difficile* (ATCC 1870 and 1805) spores/ml were inoculated on steel discs and treated with 0%, 1%, 2%, 3%, 4% and 5% of OH in ethanol (70%) for 10 min. Viable attached spores were recovered from discs and enumerated by pour plating. In addition, discs were inoculated with levels of *C. difficile* spores/ml (100,000, 10,000, 1,000 and 100), and wiped with 1%, 3% and 5% of OH, followed by enumeration of residual spores on discs and wipes.

Results: OH decreased *C. difficile* spores on steel discs ($P < 0.05$). In both *C. difficile* strains, 5% OH reduced spores by a $\log_{10}$ reduction factor of 2.7 compared with controls. Similarly, wiping with OH reduced *C. difficile* spores on stainless steel surfaces by a $\log_{10}$ reduction factor of 4 per disc compared with controls. Additionally, residual spores on wipes reduced by more than a $\log_{10}$ reduction factor of 4 on wipes treated with 5% OH ($P < 0.05$).

Conclusions: The results suggest that OH could potentially be used as a disinfectant to reduce *C. difficile* spores on stainless steel surfaces.

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Introduction

Clostridioides difficile is a gram-positive, spore forming anaerobic pathogen, which causes a serious enteric disease in humans [1]. Annually, over 500,000 cases of *C. difficile* infections (CDI) are reported in the United States, which incur about $3 billion in healthcare and treatment costs [2]. *Clostridioides difficile* infections are transmitted through a fecal-oral route, and the majority of cases occur in healthcare facilities [3]. Although *C. difficile* is considered a nosocomial pathogen producing antibiotic-associated diarrhea, there has been a recent paradigm shift in the CDI epidemiology wherein there is an observed increase in the incidence of community-associated *C. difficile* infection (CA-CDI) [4]. Apart from the several environmental sources for pathogen prevalence, food animals and animal derived foods have been considered as a potential conduit to the increased reports of CA-CDI [5,6]. During the past decade, researchers have observed an increased prevalence of the porcine *C. difficile* ribotype 078 contributing to human CA-CDI [7]. Moreover, companion animals have also been considered a likely link for CA-CDI in humans [4]. A recent study by Rabold and coworkers (2018), revealed that the epidemiological analysis of factors among co-existing small companion animals and owner pairs supports the hypothesis for a potential zoonotic transmission due to the similarities in the molecular characteristics of *C. difficile* isolated in the human owner-pet pairs [8]. In addition, there is substantial evidence of fecal shedding of *C. difficile* in dogs and cats at veterinary facilities with similar prevalence rates among investigations [9-11]. A recent study identified a high contamination rate of *C. difficile* spores on shoes of veterinarians, which sheds light on the importance of disinfection of veterinary environments [12].

Ingested *C. difficile* spores germinate in the intestines of susceptible individuals and cause toxin-mediated colitis and diarrhea [1,13]. Infected patients shed highly resistant spores in their feces and contaminate the environment. These spores can survive on abiotic surfaces for up to five months [14,15]. Commonly contaminated hospital surfaces and equipment include floors, call buttons, windowsills, bedrails, toilets, bedside-tables, thermometers, commodes, blood-pressure cuffs, and intravenous catheters [14,16,17]. In addition, transmission through hands can occur when healthcare workers or patients come in contact with surfaces contaminated with *C. difficile* spores [17,18]. Table surfaces (i.e. examination rooms) floor surfaces (i.e. treatment and isolation rooms) and equipment (i.e. stethoscopes and thermometers) in veterinary hospital environments are also commonly contaminated with *C. difficile* [19]. An observational study including 30 patients with *C. difficile* found a 50% transfer rate on gloved hands following health-care worker examination of patient skin sites (chest, hand, abdomen and arm). This study also reported greater than a 50% transfer rate to health-care workers gloved hands after touching hospital surfaces such as call buttons, bed rails and tables [17]. Therefore, it is critical for hospitals and veterinary facilities to establish a routine and effective disinfection procedure against *C. difficile* spores to control transmission between humans, animals and across species. Commonly used hospital disinfection agents, such as quaternary ammonium-based and other surfactant-based detergents do not kill *C. difficile* spores, and alternatively may even increase sporulation capacity [16,20,21]. Recommendations from the UK Department of Health and US Center for Disease Control and Prevention recommend chlorine-based products generally with ten minutes of contact time or greater to control *C. difficile* spores [22-27]. This continued use of chlorine, especially high concentration, can cause skin irritation and respiratory distress in healthcare workers and patients, and lead to corrosion of hospital surfaces and equipment [28,29]. Considering the public health implications and the plausibility for the zoonotic potential of this pathogen, there is a need for a safe and effective alternative disinfectant that can be used routinely against *C. difficile* in hospitals and veterinary facilities.

Octenidine Hydrochloride (OH) is a bispyridinamine compound that has two active cation centers which bind to negatively charged components such as cardiolipin in bacterial cell membranes [30,31]. Since human cell walls do not contain cardiolipin, OH does not bind to eukaryotic cells, which makes the compound safe for use on skin and wounds [30,32,33]. The compound has a broad spectrum of activity against Gram-positive and Gram-negative bacteria [32,34-37]. It is used as an antiseptic on skin and wounds which identifies its safety as a routine disinfectant [32,38,39]. Further, OH has been proven effective in reducing the number of bacterial pathogens such as *Acinetobacter baumannii, Staphylococcus aureus*, *Staphylococcus epidermidis* and *Proteus mirabilis* on wounds [32,40,41]. Moreover, OH is used as a mouthwash, and was shown to eliminate plaque-forming microorganisms, including *Streptococcus mutans* [34,42-44]. In addition, OH showed antimicrobial effectiveness against planktonic cells and biofilms of *Listeria monocytogenes*, *S. aureus* and multi-drug resistant *A. baumannii* [45-48]. It has been observed that the development of bacterial resistance against OH is minimal, and based on animal studies, OH is neither carcinogenic nor mutagenic [39,49]. In this study, we investigated the efficacy of OH in reducing *C. difficile* spores on stainless steel surfaces for its potential use as a routine disinfectant in hospitals and veterinary facilities to reduce CDI transmission.

Materials and methods

Spore preparation

Clostridioides difficile spores were prepared using a previously published protocol with slight modifications [50]. Two hypervirulent *C. difficile* isolates (ATCC BAA 1870 and 1805) were grown in brain heart infusion broth supplemented with 5% yeast extract (Difco, Sparks, MD) in a Don Whitley A35 anaerobic work station (Microbiology Inc., Frederick, MD) in the presence of 80% nitrogen, 10% carbon dioxide and 10% hydrogen at 37°C for 24 h. The cultures were inoculated on to 6-well brain heart infusion agar plates by adding 150 µl of each culture separately followed by gentle rotation to evenly disperse the culture. After seven days of incubation, a loopful of colony was taken for Gram staining. When ~ 90% sporulation was visualized under a microscope, the spores were harvested from the wells as follows.

The wells of the six-well plate were flooded and gently washed with 1 ml of sterile ice-cold phosphate-buffered saline (pH 7.0). Following washing, the spore suspension was transferred to tubes for sedimentation by centrifugation (20,000 x g for 5 min, 4°C). The supernatant was removed with a pipette and the pellet was resuspended in 2 ml of sterile phosphate-buffered saline. Centrifugation and resuspension were repeated five times to remove cellular debris. The resuspended spores were heat-shocked at 60°C for 20 min to kill any remaining vegetative cells [51]. The spores were enumerated by serial dilution and plated on brain heart infusion agar supplemented with 0.1% (w/v) sodium taurocholate (BHIT) (Thermo Fisher Scientific, Pittsburg, PA). The plates were incubated anaerobically for 48 h and colonies enumerated. The spore solution was

divided into aliquots and diluted to 10,000,000 spores/ml. The spore stock was stored at -80°C.

Sporicidal efficacy of OH against *C. difficile* on stainless steel surfaces

Octenidine hydrochloride (> 99% purity) was obtained from Dishman USA, Middlesex, NJ. This test procedure was based on the American Society of Testing Materials (ASTM) E2197–11 method for assessing the effect of a treatment on bacterial contamination [52]. Sterile stainless steel discs (16 mm diameter) were placed in a 12-well plate. The surface of each disc was inoculated with 100 µl of spore suspension containing ~1,000,000 spores. The inoculum was air dried at room temperature for 1 h. The disc treatments were as follows; an untreated control of *C. difficile* inoculation alone, an ethanol control (70%), and 5 treatments with 1%, 2%, 3%, 4%, or 5% OH dissolved in 70% ethanol. The treatments were added at a volume of 1 ml to fully submerge the disc and incubated at room temperature for 10 min. The discs were transferred aseptically with sterile forceps to 50 ml tubes containing 5 ml phosphate-buffered saline and glass beads. The tubes were vortexed for 2 min and sonicated for 2 min to recover spores from the disc surface. The solution was serially diluted three times and 1 ml of each dilution was added in duplicate to empty petri plates. The dilutions were pour-plated with BHIT agar (with *C. difficile* moxalactum norfloxacin (CDMN) supplement) and incubated anaerobically for 48 h after which colonies were enumerated.

Figure 1: The wiping apparatus to test the effect of Octenidine Hydrochloride (OH) wiping on *Clostridioides difficile* spores inoculated on stainless steel discs. Rotations per minute were controlled by the rotating bar settings and pounds of pressure were controlled by the weigh balance.

Efficacy of OH wipes in reducing *C. difficile* spores on stainless steel surfaces

The wiping experiments were conducted using a previously published protocol with minor modifications [53]. Wipes (Kimberly Clark™ WypAll™ X60 Wipers, Kimberly Clark, Irving, TX) were cut (4x4 cm) and sterilized by autoclaving. Stainless steel discs (16 mm diameter) were attached to petri plates and inoculated with 100 ul of spore suspension containing ~100,000 *C. difficile* spores (ATCC BAA 1870). The experiment was repeated with 100 µl of lower spore inoculations; i.e., 10,000, 1,000 and 100 spores. The inoculum was air-dried at room temperature for 1 h. The treatments of 1%, 3%, and 5% OH were applied on disk surfaces and allowed to incubate for 10 min, followed by immediate wiping. A wipe was pinned to a sterile rubber stopper attached to a stirring rod of a rotating overhead electric drill (Figure 1). Wipes were rotated mechanically with an electric drill for 10 sec at 60 rpm with a downward weight of 500 g. The wipe was stamped on the four quadrants of a BHIT agar plate (with CDMN supplement and 7% horse blood). The discs were pour-plated with BHIT agar (with CDMN supplement). The plates were incubated anaerobically for 48 h at 37°C after which bacterial colonies were enumerated.

Statistical analysis

All experiments were carried out in duplicate and the study was repeated three times. The data were analyzed using one-way ANOVA. Differences between the means were considered significantly different at $P < 0.05$.

Results

To investigate the effect of OH on *C. difficile* spore survival and recovery on stainless steel surfaces, the inoculated discs were treated with varying concentrations of OH. The spores were recovered from the discs after each treatment and enumerated by dilution and plating. In addition, the residual spores on the discs after spore recovery were also determined by pour-plating the discs in BHIT agar (with CDMN supplement). OH significantly reduced the number of spores of *C. difficile* strain ATCC BAA 1870 recovered from the stainless steel discs ($P < 0.05$). The treatment with 1% OH reduced spores by a $\log_{10}$ reduction factor of 1 (factor of 10); 2%, 3% and 4% OH reduced spores by a $\log_{10}$ reduction factor of 2 (factor of 100), and 5% OH reduced spores by a $\log_{10}$ reduction factor of 2.7 (factor of 317) compared with controls (Figure 2). Similarly, OH significantly reduced *C. difficile* strain ATCC BAA 1805 spores recovered from stainless steel discs ($P < 0.05$). The 1% and 2% OH treatments reduced spores by a $\log_{10}$ reduction factor of 0.5 (a factor of 5); 3% and 4% OH reduced spores by $\log_{10}$ reduction factor of 1 (a factor of 10) and 5% OH reduced spores by about a $\log_{10}$ reduction factor of 2 (factor of 100) compared with controls (Figure 3). In addition, there were no residual spores present on the surface of the discs treated with OH, as indicated by the absence of colonies after pour-plating the discs with BHIT agar (with CDMN supplement). However, untreated and ethanol-treated discs contained several residual spores, as indicated by the presence of colonies after pour-plating the discs with BHIT agar (with CDMN supplement).

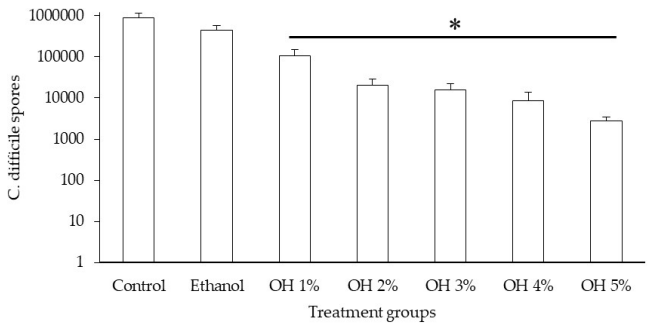

Figure 2: The effect of Octenidine Hydrochloride (OH) on *Clostridioides difficile* spores (ATCC BAA 1870) inoculated on stainless steel discs. Treatments 1%, 2%, 3%, 4% and 5% OH dissolved in ethanol (70%) compared with control (no treatment) and ethanol (70%) treatments. * indicates treatments with significant difference at $P < 0.05$.

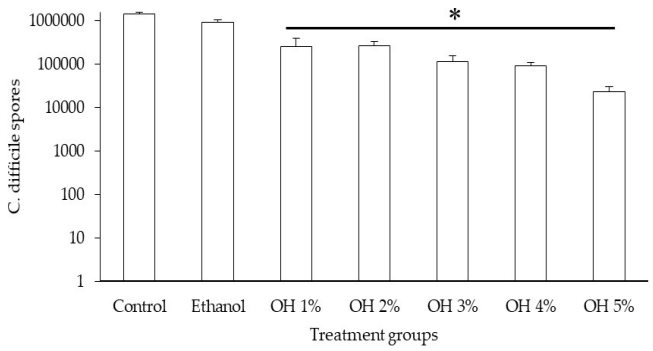

Figure 3: The effect of octenidine hydrochloride on *Clostridioides difficile* spores (ATCC BAA 1805) inoculated on stainless steel discs. Treatments 1%, 2%, 3%, 4% and 5% OH dissolved in ethanol (70%) compared with control (no treatment) and ethanol (70%) treatments. * indicates treatments with significant difference at $P < 0.05$.

In the wiping experiment, stainless steel discs were inoculated with varying concentrations of *C. difficile* spores in log increments. The discs were then treated with different concentrations of OH and wiped with a dry wipe after 10 min of contact time. The sporicidal efficacy of each treatment was expressed in log reduction by observing complete absence of spores at each inoculation level. In addition, after each wiping, the wipe was stamped on to the four quadrants of a BHIT agar plate (with CDMN supplement) in order to estimate the spore survival on the wipe. *C. difficile* spores were inoculated at the level of 100,000, 10,000, 1000 and 100 on to the disc surface and treated with OH at 1%, 3% and 5% levels for 10 min followed by wiping. The results indicated that 5% OH completely inactivated spores on the discs inoculated with 10,000 spores, which suggests a $\log_{10}$ reduction factor of 4 (factor of 10,000) in *C. difficile* (ATCC BAA 1870) spores (Table 1). However, several colonies appeared on discs with control treatments (untreated control and ethanol control). Treatments with 1% OH and 3% OH resulted in a $\log_{10}$ reduction factor of 3 (factor of 1,000) in spores (Table 1). In addition, the agar plates stamped with wipes containing 5% OH reduced spore counts by a $\log_{10}$ reduction factor of 4 (reduction factor of 10,000) (Table 2), whereas in 3% and 1% OH treatments provided spore reduction by a factor of 2 (factor of 100). However, colonies were observed in all plates in the untreated control and ethanol control groups (Table 2).

Table 1: Recovery of *Clostridioides difficile* spores from stainless steel discs.

C.difficile inoculation level	Control	Ethanol	1% OH	3% OH	5% OH
100,000spores/disc	+	+	+	+	+
10,000spores/disc	+	+	+	No colonies	No colonies
1,000 spores/disc	+	+	No colonies	No colonies	No colonies
100 spores/disc	+	+	No colonies	No colonies	No colonies

Note: The (+) represents the presence of *C. difficile* colonies on the BHIT plate recovered from stainless steel discs.

Table 2: Recovery of residual Clostridioides difficile spores from wipes.

C.difficile inoculation level	Control	Ethanol	1% OH	3% OH	5% OH
100,000spores/disc	+	+	+	+	+
10,000 spores/disc	+	+	+	+	No colonies
1,000 spores/disc	+	+	+	+	No colonies
100 spores/disc	+	+	No colonies	No colonies	No colonies

Note: The (+) represents the presence of *C. difficile* colonies on the BHIT plate recovered from wipes.

Discussion

Approximately 10%–25% of hospitalized patients and 4%–20% of residents in long-term care facilities are colonized with *C. difficile* [2,54]. The shedding of spores in the hospital environment by infected individuals is the major cause of *C. difficile* transmission in healthcare facilities [1]. *C. difficile* spores are extremely resistant to physical and chemical disinfectants, and can reside on surfaces for several months [15,22,31]. Clostridioides difficile has been isolated from the rooms of infected patients in healthcare settings in a range of 2.9% to 75% [18]. Further, in healthcare environments, *C. difficile* contamination has been identified in 49% of rooms occupied by CDI patients compared with 29% of rooms occupied by asymptomatic CDI carriers suggesting shedding of *C. difficile* [3,55]. In addition, equipment shared between patients such as bed-side tables, blood pressure cuffs and other surfaces such as floors and toilets can be contaminated with *C. difficile* spores, thereby serving as a potential source for acquiring the infection [14]. A transmission rate above 50% was observed after healthcare workers touched hospital surfaces such as call buttons and bed rails, exemplifying the requirement for disinfection to prevent CDI transmission [17]. Therefore, it is necessary to disinfect hospital rooms daily with an effective and safe antimicrobial against *C. difficile* spores. More importantly, the higher positivity rate for toxigenic *C. difficile* among livestock and companion animals stresses the importance of adopting disinfection protocols in the premises of veterinary hospitals to reduce the plausible transmission of *C. difficile* by veterinarians to the community [4,12].

Generally, chlorine-based disinfectants with manufacturer's claims for sporicidal activity have not resulted in adequate disinfection as determined by the labelled contact time against *C. difficile* spores on simulated clean or dirty environments [25]. Moreover, the effective reduction of *C. difficile* spores on simulated surface carriers with organic load requires a 1:10 dilution of 6.15% sodium hypochlorite for a minimum contact time of ten minutes [56]. With such long holding times for disinfection, certain drawbacks for the use of hypochlorites include unpleasant odor, damage to hospital surfaces and also potential respiratory exposure issues in patients and healthcare associated employees [25,29].

Our results indicate that OH significantly reduced *C. difficile* (strains ATCC BAA 1870 and ATCC BAA 1805) spores on stainless steel discs compared with controls ($P < 0.05$) (Figure 2 & 3). In addition, wiping with 5% OH treatment reduced *C. difficile* spores by a $\log_{10}$ reduction factor of 4 (factor of 10,000) on stainless steel disc surfaces (Table 1), thereby suggesting the potential for OH to be utilized as a surface wipe. Previously, it was reported that wiping with non-sporicidal agents alone removed 2.9 log of *C. difficile* spores, while wiping with a sporicidal agent

yielded a greater reduction of 3.9 log [57]. However, these researchers did not determine the population of residual spores on the wipes. The efficacy of OH for reducing spores was generally found to increase with OH concentration, since the contact time was constant. For example, in the first experiment, where inoculated discs were immersed with various treatments, reductions in spore counts on discs increased with increased OH concentration, with 5% treatment leading to the maximum $\log_{10}$ reduction by a factor of 2.7 (factor of 317) (Figures 2 & 3). Similarly, in the wiping study, 5% OH was most effective, reducing the spore population by a $\log_{10}$ reduction factor of 4 (factor of 10,000) followed by 3% OH which reduced by a $\log_{10}$ reduction factor of 2 (factor of 100) (Table 1). Likewise, the residual spore population on the wipes were also lowest in the 5% treatment, followed by 3% and 1% treatment groups (Table 2).

In conclusion, the results of this study suggest the potential use of OH as a disinfectant on hospital stainless steel surfaces to reduce *C. difficile* spores. Although the mechanisms behind the sporicidal effect of OH are not known, OH exerts its antimicrobial effect against bacterial cells by binding to the negatively charged bacterial cell envelope, and disrupting the functions of the cell [31]. A neutralizer was not included in the experimental methodology because there is a lack of evidence for a universal neutralizer agent for octenidine hydrochloride [33,48,58,59]. Octenidine hydrochloride is stable within a wide pH range of 1.6 to 12.2, and is not sensitive to hydrolysis from light, harsh chemical or physical conditions. Thus, the safety and stability of OH make it an ideal disinfectant for routine use in hospitals. However, large-scale and long-term efficacy studies in clinical and veterinary settings are warranted before recommending routine OH use in hospitals and veterinary facilities.

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