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ACTIVITY OF THREE DISINFECTANTS AND ACIDIFIED NITRITE AGAINST *CLOSTRIDIUM DIFFICILE* SPORES

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ABSTRACT

OBJECTIVE: To identify environmentally safe, rapidly acting agents for killing spores of *Clostridium difficile* in the hospital environment.

DESIGN: Three classic disinfectants (2% glutaraldehyde, 1.6% peracetyl ions, and 70% isopropanol) and acidified nitrite were compared for activity against *C. difficile* spores. Four strains of *C. difficile* belonging to different serogroups were tested using a dilution–neutralization method according to preliminary European Standard prEN 14347. For peracetyl ions and acidified nitrite, the subjective cleaning effect and the sporicidal activity was also tested in the presence of organic load.

RESULTS: Peracetyl ions were highly sporicidal and yielded a minimum 4 $\log_{10}$ reduction of germinating spores already at short exposure times, independent of organic load conditions. Isopropanol 70% showed low or no inactivation at all exposure times, whereas glutaraldehyde and acidified nitrite each resulted in an increasing inactivation factor (IF) over time, from an IF greater than 1.4 at 5 minutes of exposure time to greater than 4.1 at 30 minutes. Soiling conditions did not influence the effect of acidified nitrite. There was no difference in the IF among the 4 strains tested for any of the investigated agents. Acidified nitrite demonstrated a good subjective cleaning effect and peracetyl ions demonstrated a satisfactory effect.

CONCLUSIONS: Cidal activity was shown against *C. difficile* spores by glutaraldehyde, peracetyl ions, and acidified nitrite. As acidified nitrite and peracetyl ions are considered to be environmentally safe chemicals, these agents seem well suited for the disinfection of *C. difficile* spores in the hospital environment (*Infect Control Hosp Epidemiol* 2003;24:765-768).

Hospital environments are considered to be one of the most important reservoirs for and areas of transmission of *Clostridium difficile* spores.[1-4] Symptomatic patients are known to excrete a large number of organisms in feces, and bacterial spores are found in abundance in the environment of patients with *C. difficile*–associated diarrhea (CDAD).[3] *C. difficile*, either as vegetative organisms or as spores, can also be isolated from the stools of asymptomatic patients. Thus, under the ecologic pressure of antibiotics, the hospital ward can be a potential focus where introduced and disseminated *C. difficile* spores may be ingested, germinate, and induce symptoms in susceptible hosts.[5] In one study of patients colonized with *C. difficile*, it was demonstrated that most patients acquired the organism during hospitalization.[6] Patient-to-patient transmission via contaminated environmental surfaces and the hands of healthcare workers has been suggested as an important mechanism of *C. difficile* acquisition during outbreaks of CDAD.[3-6] However, in another study, patient-to-patient transmission was not convincingly shown.[7]

Vegetative forms of *C. difficile* are killed when exposed to air, but their spores are resistant to oxygen, desiccation, and most commonly used disinfectants[2,8,9] and may persist in the hospital environment for long periods of time (months and probably years). Thorough cleaning of surfaces using detergents in combination with separate, clean equipment for each patient are accepted measures for reducing the number, density, and transmission of spores in the hospital environment. However, the efficacy of different cleaning and disinfection strategies for the environmental control of *C. difficile* contamination is not well documented.[10] Thus, evidence is lacking to support routine environmental decontamination by the use of either disinfectants or detergent-based formulas.[11,12] Furthermore, there is a need for studies of the efficacy of disinfectants against *C. difficile* spores, particularly on surfaces.

The aim of our study was to identify environmentally safe, rapidly acting chemicals against *C. difficile* spores and to examine their effectiveness in the presence of organic load. To our knowledge, this is the first evaluation of the activity of acidified nitrite as a disinfecting agent against *C. difficile* spores.

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METHODS

Bacterial Strains

We tested one reference strain of *C. difficile* (CCUG 37779) from serogroup A, obtained from the Culture Collection at the University of Gothenburg, and three clinical strains originally isolated from patients at our hospital. The strain Cl-382 had a polymerase chain reaction (PCR) ribotype pattern identical to that of the reference strain CCUG 9018, and strain Cl-385 had the same PCR ribotype pattern as the serotype C and reference strain CCUG 37766. The PCR ribotype of strain Cl-388 did not correspond to that of any reference strain in the library of the Swedish Institute for Infectious Disease Control, but is currently one of the most common types of *C. difficile* in Swedish hospitals.

Growth of Bacteria and Preparation of Spores

The strains were streaked on blood agar and grown anaerobically for 48 hours. Several colonies of each strain of *C. difficile* were then added to 5 mL of brain–heart infusion broth and incubated anaerobically for 48 hours. Thereafter, the culture was vortexed, mixed with 5 mL of 95% ethanol, vortexed again, and left at room temperature for 30 minutes. After centrifugation at 3,000 $\times$ g for 20 minutes, the supernatant was discarded and the pellet containing spores plus killed bacteria was suspended in 0.3 mL of water. Viable spore counts were performed by serial dilutions on blood agar. The spore counts in the resulting suspensions were 3×10^5 to 2×10^6 per milliliter and Gram staining verified that 85% to 90% of the bacterial structures were spores.

Disinfectants and Acidified Nitrite

The disinfectants tested were 70% isopropanol (vol/vol), Wavicide-200 (2% glutaraldehyde, Promagent AB, Malmö, Sweden), and Perasafe (1.6% peracetyl ions equivalent to 0.26% peracetic acid; Antec Int. Ltd, Sudbury, United Kingdom). Acidified nitrite was prepared by mixing 0.1 M of citric acid with an equal volume of 0.1 M of sodium nitrite at room temperature. All acids used were always freshly prepared as described or recommended by the manufacturer at the start of each experiment.

Efficacy Test

A dilution–neutralization method was used according to preliminary European Standard prEN 14347.[13] Test methods were modified according to our hospital protocol to provide anaerobic conditions during the experiments.

Disinfection testing was performed by thoroughly mixing 0.2 mL of the spore suspension with 0.8 mL of disinfecting agent. Samples (0.1 mL) were taken from the reaction mixture after 5, 15, and 30 minutes in 0.8 mL of neutralizer (for isopropanol and glutaraldehyde, 1.0% histidine in tryptone soya broth; for peracetyl ions and acidified nitrite, 0.5% sodium thiosulfate in tryptone soya broth) and 0.1 mL of water to prevent further inactivation, and left for 5 minutes. Samples of the final mixture and 10-fold dilutions thereof were then seeded on blood agar and incubated anaerobically at 35°C for 48 hours. Colonies from germinated spores were counted and expressed as colony-forming units (CFU)/mL. For peracetyl ions and acidified nitrite, the test was performed at low-level and high-level soiling conditions (presence of bovine serum albumin at a final concentration of 0.3% and 3%, respectively). To describe the sporicidal effect of the disinfecting agents, the results are given as the inactivation factor (IF). The IF was calculated as the $\log_{10}$ CFU reduction of the viable count from the initial inoculum. The maximum IF was dependent on the starting inoculum, but was approximately 4 $\log_{10}$ CFU at the time of sampling. According to the requirements of prEN 14347, the test conditions were validated regarding the spore count of the stock suspension, toxicity of the water, and sporistatic activity of the neutralizers.

Surface Cleaning Test

The cleaning effect of peracetyl ions and acidified nitrite on surfaces was determined using a carrier test.[14] Five milliliters of fresh human blood without any additives was placed on the 4 central steel plates of 16 in a square and dried. Twenty milliliters of the liquid disinfectant was poured onto the plates. After an initial contact time of 5 minutes, the surface of the test plates was subjected to cleaning for 2 minutes using a cloth attached to a mop. The mop was worked by hand 10 times to and fro with constant pressure. The cleaning effect is described as the subjective cleaning factor, which was the number of cleaning movements (to and fro) with the mop necessary before the spot was no longer seen by the naked eye.

RESULTS

Glutaraldehyde, peracetyl ions, and acidified nitrite each demonstrated satisfactory activity against *C. difficile* spores already after 15 minutes (99% to 99.9% reduction of viable counts) (Table 1). In contrast, 70% isopropanol included as a negative control showed little or no measurable sporicidal activity after 30 minutes. Peracetyl ions showed a high IF after brief exposure times (1 and 5 minutes), independent of the amount of organic load (Table 2). The effect of acidified nitrite was not influenced by a low level or high level of dirt. However, the efficacy of acidified nitrite was decreased twofold (data not shown) when not prepared 24 hours before testing. No difference in the IF among the strains of *C. difficile* was observed. Validation of the test conditions showed a stable spore count suspension, no toxicity of the water, and no sporistatic activity of the neutralizers (data not shown).

Cleaning of the blood from the metal carriers by the standardized mop and the disinfectant was accomplished by three strokes to and fro for acidified nitrite and six strokes for peracetyl ions, as judged by the naked eyes. Thus, a subjective cleaning effect of 3 was demonstrated for acidified nitrite and 6 for peracetyl ions.

DISCUSSION

Nosocomial outbreaks of CDAD have been linked to the spread of *C. difficile* spores via floors and other sur-

TABLE 1
MEAN INACTIVATION FACTOR AND STANDARD DEVIATION OF PERACETYL IONS, GLUTARALDEHYDE, ACIDIFIED NITRITE, AND ISOPROPANOL AGAINST *CLOSTRIDIUM DIFFICILE* SPORES

	Inactivation Factor* (± SD)			
Exposure Time (min)	Peracetyl Ions	Glutaral-dehyde	Acidified Nitrite	Isopro-panol
5	> 4.1 = NG (0)	1.5 (0.5)	1.4 (0.3)	0.2 (0.1)
15	> 4.1 = NG (0)	2.5 (0.5)	3.1 (0.4)	0.3 (0.1)
30	> 4.1 = NG (0)	> 4.1 = NG (0)	> 4.3 = NG (0)	0.4 (0)

SD = standard deviation; NG = no growth.
*Mean values of 4 strains tested in triplicate.

TABLE 2
MEAN INACTIVATION FACTOR AND STANDARD DEVIATION OF PERACETYL IONS AND ACIDIFIED NITRITE AGAINST *CLOSTRIDIUM DIFFICILE* SPORES IN THE PRESENCE OF ORGANIC LOAD

	Inactivation Factor* (± SD)			
	Peracetyl Ions		Acidified Nitrite	
Exposure Time (min)	BSA 0.3%	BSA 3%	BSA 0.3%	BSA 3%
1	3.9 (0.6)	3.8 (0.6)	-	-
5	> 4.1 = NG (0)	> 4.3 = NG (0)	1.3 (0.3)	1 (0.1)
15	> 4.1 = NG (0)	> 4.3 = NG (0)	3.8 (0.2)	3 (0.6)
30	> 4.1 = NG (0)	> 4.3 = NG (0)	> 4.3 = NG (0)	> 4.3 = NG (0)

SD = standard deviation; NG = no growth; BSA = bovine serum albumin.
*Mean values of 4 strains tested in triplicate.

faces in the rooms of symptomatic and asymptomatic patients.[2,3,6] Thus, the choice of disinfectant may be important for the persistence of *C. difficile* spores in the hospital environment.

The impact of surface disinfection for reducing nosocomial CDAD is debatable.[15] Most studies dealing with disinfectants have examined their impact on *C. difficile* spores for instrument disinfection,[8,16,17] whereas few studies have examined their effect for surface disinfection.[3,15] Mayfield et al. reported that the incidence of CDAD was reduced after the environmental disinfectant was changed from a quaternary ammonium agent to a 10% hypochlorite solution in the rooms of patients with CDAD in a bone marrow transplantation unit.[15] In another study, during an outbreak of CDAD, surface contamination of *C. difficile* was decreased to 21% of the initial levels with the use of unbuffered hypochlorite, and the outbreak subsequently ended. The use of phosphate-buffered hypochlorite in that study was shown to be even more effective, as its use resulted in a 98% reduction in surface contamination.[3]

The bactericidal activity of acidified nitrite has been described.[18,19] In our study, acidified nitrite, generated by the acidification of sodium nitrite ($NaNO_2$) with citric acid, was tested for the first time as a disinfecting agent and demonstrated good sporicidal activity, which was not influenced by low-level or high-level soiling conditions. However, a freshly prepared solution was important for the efficacy of acidified nitrite. Nitric oxide–related agents have previously been shown to have antimicrobial activity against a variety of pathogenic microorganisms including *C. perfringens*.[20,21] It is believed that reactive nitrogen oxides (nitric oxide donors), rather than the nitrite itself, are responsible for the sporicidal activity of acidified nitrite.[22] Acidified nitrite is considered harmless to staff and to almost all surfaces because it is known to be synthesized by mammalian cells (N. Benjamin, PhD, written communication, February 10, 2003).[20]

Peracetyl ions have been described to have excellent sporicidal activity (Antec Int. Ltd, unpublished data, 1998). In this study, sporicidal activity within 5 minutes with a maximum IF of more than 4 $\log_{10}$ was seen in both low-level and high-level soiling conditions. These results are in accordance with other studies of peracetyl ions in suspension tests. Thus, Holton et al. (written communication, January 21, 1999) showed no growth of *C. difficile* after 5 minutes of exposure to peracetyl ions from an initial inoculum of 1.5×10^7 spores, even in the presence of organic load. Perasafe is harmless, according to its manufacturer (V. Croud, Technical Director, written communication, February 12, 2003) and peracetic acid has been reported as an environmentally safe agent by Rutala and Weber.[23]

In concordance with other studies, 2% glutaraldehyde is a sporicidal disinfectant used in most hospitals for the disinfection of endoscopes.[17,24-27] In our study, a greater than 4.1 $\log_{10}$ reduction was noted within 30 minutes. However, exposure to glutaraldehyde is considered to be hazardous and has been known to cause asthma and dermatitis in healthcare workers.[28]

The activity of alcohols is probably due to their ability to denature proteins. Although they are rapidly bactericidal, they have low activity against bacterial spores.[27] As expected, 70% isopropanol showed no measurable sporicidal activity after 30 minutes in our study.

In this study, a dilution–neutralization method was used according to prEN 14347, which demands at least a reduction of 4 $\log_{10}$ in viable counts within 120 minutes or less to be classified as a sporicidal disinfectant. In our test, an initial inoculum of 10^6 CFU/mL was used, and we could demonstrate a maximal reduction of 4 $\log_{10}$ CFU, which was the limit of detection in our assay. Our test showed good precision, and because glutaraldehyde, acidified nitrite, and peracetyl ions comply with prEN 14347, they can be considered as sporicidal agents.

Wilcox and Fawley[12] showed that an epidemic strain of *C. difficile* produced significantly more spores than did the nonprevalent strains, and that sporulation was further enhanced when the strain was cultured in feces exposed to subinhibitory concentrations of cleaning agents with-

out chlorine as a base. In our study, we examined clinical toxigenic strains of *C. difficile* from different serotypes, of which one has been described to cause nosocomial outbreaks.[29-31] No difference was found regarding susceptibility to the different chemicals among the spores of the tested strains.

To evaluate the subjective cleaning effect of acidified nitrite and peracetyl ions on organic matter, a modified surface cleaning test was used.[14] In this test, a low subjective cleaning effect generally means that the substance has a satisfactory or good cleaning effect for blood on a metal surface. Water has a good subjective cleaning factor of 2, 45% isopropanol a satisfactory subjective cleaning factor of 6, and 70% ethanol a bad subjective cleaning factor of 10 or greater. In our study, a good subjective cleaning effect for acidified nitrite equal to water was found and the subjective cleaning factor for peracetyl ions was satisfactory.

Glutaraldehyde, peracetyl ions, and acidified nitrite showed cidal activity against *C. difficile* spores. Because acidified nitrite and peracetyl ions are considered to be environmentally safe chemicals and show a good or satisfactory cleaning effect, they seem well suited for the disinfection of *C. difficile* spores in the hospital environment.

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