The sporicidal effect of Chlorine dioxide against Clostridium Difficile spores with and without presence of organic material

Version 1

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ABSTRACT

Introduction: Spore resistance make C. difficile and CDI difficult to control and prevent, especially in a hospital environment. The importance of finding an effective sporicidal agent is vital. Since the spore’s natural habitat is faecal material, evaluating sporicidal efficacy in its presence is important as organic material seem to hamper the efficacy. The objective of this study was to determine the sporicidal efficacy of the ClO₂-based agent LifeClean on C. difficile spores with and without the presence of organic material.

Material and Method: One strain of the PCR ribotype UK023 was studied in this experimental study. A 10⁸ spore inoculum was exposed during 10 minutes to LifeClean (1600 ppm), 70 % ethanol or water with and without organic material (5 % or 10 % FBS or 10 % faecal material).

Results: The log₁₀ reductions for LifeClean’s efficacy on spores were 2.48 with no organic material; 2.65 with 5 % FBS; 2.02 with 10 % FBS and 1.09 with 10 % faecal material. The effect of LifeClean in comparison to 70 % ethanol shows a p-value of 0.057.

Conclusion: Our results indicate that the efficacy of LifeClean was relatively low despite the high concentration of ClO₂ and contact time, but were not statistically significant due to few tests. However, these results may be due to the high inoculum used. We notice a decrease in efficacy of LifeClean with increasing amount of soiling. Further investigations are needed for evaluation in this area as well as for an acceptable spore environmental control.

Keywords: Clostridium difficile, spore, chlorine dioxide, foetal bovine serum, faecal material, sporicidal, disinfection.
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Appendix 1 PM; Klinisk handläggning av akut Clostridium difficile infection (CDI) (in Swedish)

Appendix 2 Vårdhygieniska riktlinjer; Städning; Vårdpersonal slutent vård (in Swedish)
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>C. difficile</td>
<td>Clostridium difficile</td>
</tr>
<tr>
<td>CDI</td>
<td>Clostridium difficile infection</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td>ClO₂</td>
<td>Chlorine dioxide</td>
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<tr>
<td>CFU</td>
<td>Colony forming unit</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>FBS</td>
<td>Foetal bovine serum</td>
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1. **INTRODUCTION**

1.1. **Clostridium difficile**

*Clostridium difficile* is a gram positive, spore forming, obligate anaerobe and a major nosocomial pathogen worldwide. Because of its strict anaerobic requirements, the infection is made possible by transmission through the metabolically dormant spore spreading via the oral-faecal route [1,2].

1.1.1. **Clostridium difficile infection**

Transmission of CDI may occur externally from direct or indirect contact via patient-to-patient transmission, healthcare workers or environment leading to ingestion of spores [3]. The spores seem to be volatile because of discovery of growing *C. difficile* bacterium in air samplings. This suggests that transmission via air may be a possible route of infection [4]. Infection may also occur internally through antibiotic usage leading to suppression of normal, healthy, intestinal micro-flora [5] and permitting overgrowth of existing *C. difficile* bacterium [2]. Host susceptibility and a change in colonic micro-flora seem to be required for infection to occur which is proposed to allow pathogenic species to overgrowth or make the mucosa more susceptible to toxin effects. Host defences are not completely understood, but the best defence seems to be maintenance of a normal intestinal micro-flora [1].

Risk factors such as hospitalization (providing a reservoir and a route for transmission of spores) [3], altered patterns of antibiotic usage, increasing age and susceptibility, use of nasogastric tubes, use of immunosuppression and acid reducing agents [6,7] may promote the development of infection. All antibiotics may predispose for CDI, however antibiotics most frequently associated with infection are cephalosporins, clindamycin and broad-spectrum penicillins [7].

Asymptomatic carriage occurs, mainly in children, and may be a vector for transmission [3] even though no diarrhoea is present. Symptoms may present if the carrier is treated with antibiotics and if the bacterium are pathogenic [1,8].
In susceptible patients, the spores germinate in the colon to form vegetative cells that adhere to colonic enterocytes [2] and produce toxins, Toxin A (“enterotoxin”) or Toxin B (“cytotoxin”), or both, thus initiating CDI [9]. The toxins enter cells via receptor-mediated-endocytosis and may alter cell function, distort the cytoskeleton and disrupt tight junction leading to an inflammatory response and clinical symptoms. These symptoms may present as mild self-limiting *C. difficile*-associated diarrhoea (CDAD), *C. difficile*-associated colitis (CDAC) or severe and fatal pseudomembranous colitis (PMC) [1,9]. *C. difficile* may also produce transferase (CDT), also known as “binary toxin”. However, its role in disease is unknown, yet may be cytotoxic and believed to facilitate adherence of the bacteria to enterocytes [10].

1.1.2. Epidemiology

In Sweden, in 2012, the majority of CDI cases affected women (54 %). According to the annual report from Folkhälsomyndigheten in 2012, the median age for this infection was 74 years for women, respectively 73 for men [11].

Globally there has been an increase in CDI from the start of the 21st century and may be due to a more virulent and mortal strain of *C. difficile* (PCR ribotype UK027/SE10). In Sweden few of these cases of CDI have been detected and the total number of cases have been relatively stable for the last couple of years with an incidence of 85 per 100 000 [8,11].

In 2013, although for reason unknown, there was a distinct increase in the number of patients infected by the PCR ribotype UK023/SE25 [12]. There is a variety of many different ribotypes of *C. difficile* and every hospital seems to have its own set of frequently appearing ribotypes [13].

1.1.3. Diagnosis

Since the signs and symptoms of CDI are nonspecific, such as fever, abdominal pain and nausea, diagnosis may be challenging. Diagnosis include the presence of diarrhoea as well as stool samples detecting bacteria, toxins or toxin genes [1,8] via enzyme immunoassays, cell cytotoxicity assay, PCR or culturing [14,15]. Endoscopy or radiology may also reveal evidence for diagnosis [14].
1.1.4. Treatment and Prevention for Clostridium difficile infection

If a patient is treated with antibiotics and present with CDI, the first precaution is, if possible, to withdraw the antibiotics and maintain a positive fluid balance with fluid therapy, see Appendix 1. Spontaneous recovery occurs, primarily in mild cases. One study reported that 15% of a study population of patients with CDI recovered without antibiotic treatment [14]. The recommended treatment is metronidazole or vancomycin depending on severity [14], see Appendix 1. Faecal microbiota transplantation [16], or colectomy [8] (see Appendix 1), are potential treatments in severe cases. Relapse occurs in 15-20% since only the vegetative form of *C. difficile* is killed by the antibiotics [1], while the spores remain resistant [17].

Prevention control measures and hygiene routines, such as patient isolation, use of gloves, appropriate hand hygiene as well as improved disinfection procedures with sporicidal agents, should be applied [18].

1.2. Spores of Clostridium difficile

1.2.1. Sporulation

During CDI, *C. difficile* bacterium induces a sporulation pathway that produces spores by asymmetrical cell division, engulfment of the smaller cell (so called forespore) by the larger mother cell, followed by the formation of a layer around the forespore, resembling a cell wall. When the mother cell undergoes lysis the spore is released. The signals triggering sporulation have yet to be identified for *C. difficile*, although could be related to environmental stimuli such as nutrient depletion, chemicals or other unidentified stress factors [19]. The decision to enter sporulation seems to be complicated and is regulated by several factors. In many Clostridium species this may be regulated by several orphan histidine kinases that phosphorylate the transcriptional regulator Spo0A which seems to be involved in both the sporulation process and may also indirectly regulate toxin production [20].

1.2.2. Germination

Germination of *C. difficile* spores to form vegetative cells is triggered due to species-specific environmental signals, so called germinants [19]. Recent studies have made progress in this area leading to the discovery of certain germinants such as bile salts and its derivates (e.g. taurocholate) and co-germinants, such as L-glycine [21], potassium salts and phosphate [22] that may trigger germination, although the mechanism remain uncertain.
Once germination has begun, the spore cannot prevent the development, and is as weak and vulnerable as the vegetative cell. A potential strategy for eradicating transmission may be to trigger germination [23].

1.2.3. Spore structure

The spores consist of, inside and out, a core (with a complete copy of the chromosome and cytoplasmic components), followed by an inner membrane, a thick germ cell wall, a peptidoglycan-rich cortex, an outer membrane and a protein coat. The spores of *C. difficile* have an additional layer outside the coat, the exosporium, which has hair-like projections. However, there is a limited knowledge of the outermost proteins and their roll in disease [19].

Little is still known about *C. difficile* spores whereas more work has been done in *B. subtilis* spores. Although it seems as though they share similarities, it is important to take into account that they might be different in other aspects [19].

1.2.4. Spore resistance

Once the spores are shed into the environment they may survive for many months [24], while the vegetative cells, under aerobic conditions, may persist on dry surfaces only for 15 minutes and on moist surfaces for several hours [25].

Due to the limited knowledge in *C. difficile* spores, similarities to the spores of *B. subtilis* will be considered [19]. Due to different mechanisms, the spores are insensible to heat, radiation, and several enzymes and chemicals, such as disinfectants frequently used in hospital setting. These mechanisms include the many layers of the spores, low water content, low permeability, protection of spore DNA from small α/β-type acid-soluble proteins (SASP), and repair of DNA during germination [26]. Some of the disinfectants used in hospitals appear to even enhance sporulation rate [27]. Recent studies have discovered that *C. difficile* bacterium produces biofilm in vitro and it is possible that it may serve to enhance the protection of bacterium and spores against antibiotics and host defences [28].

All the abilities that amplify *C. difficile* spore resistance make *C. difficile* and CDI difficult to control and prevent, especially in a hospital environment. The importance of finding an effective sporicidal agent is vital.
1.3. Sporicidal agents

The majority of all cleaning agents used in hospital environment are not sporicidal [29], although evidence suggests that spores are physically removed from hands if rubbed off with soap and water [30].

The selection of sporicidal agents is limited and divided into alkylating or oxidizing agents which are furthermore all highly reactive. The agent efficacy may be influenced by several factors, such as pH, concentration of agent, contact time, level of soiling, temperature and type of surface. Commonly used oxidizing disinfectants such as chlorine-based agents and hydrogen peroxide [31] seem to be sporicidal. Several disinfectants have been proven to suppress vegetative cell growth only [27,32]. In this study, we will consider a chlorine dioxide-based agent.

1.3.1. Chlorine dioxide

ClO₂ is a powerful oxidizing agent and seems to have a broad biocidal activity against bacteria, viruses, fungi and protozoa [33-36]. However, the mechanism for its inhibition of microorganisms is unknown, although there are many speculations from early studies ranging from protein or protein-synthesis inactivation, reaction with amino acids or free fatty acids leading to an increase in permeability and cell destruction [37]. Because of its disinfecting ability ClO₂ has been used in sanitizing of food and water [33].

ClO₂ has a confirmed sporicidal effect although not fully understood. The mechanism may be due to inner membrane damage or damage leading to defective development in the germination process. The spore’s coat may be an important structure for ClO₂ resistance [38].

Usage produces potentially harmful disinfection by-products, although to a lesser extent than of chlorine due to different reactions with organic material which makes ClO₂ less odorous. ClO₂ is therefore considered an alternative disinfectant to chlorine [39]. The efficacy of ClO₂ seems to be mainly dependent on its concentration and duration of contact [40].
1.4. Terminal cleaning after CDI patient

Virkon™ 3 %, a potassium monopersulfate-based agent with oxidizing capacity, is used for terminal cleaning after patients with CDI in Örebro (see Appendix 2), although different methods are used in different hospitals in Sweden since no specific agent has been proven to be superior to others [41]. However, Virkon™ seem to only have a minor effect on *C. difficile* spores [29,32].

1.5. Organic material

It is known that organic material hampers the effect of many disinfectants which makes it difficult to eliminate all microorganisms without thorough cleaning before biocidal treatment. The soiling is suggested to provide a physical barrier, thus protecting the microorganisms from cleaning detergents [42,43].

2. Objective

The objective of this experimental study was to determine the sporicidal effect of the ClO₂-derivated agent LifeClean on the spores of *C. difficile* with and without the presence of organic material. LifeClean was expected to show sporicidal effect, as well as a lesser effect with organic soiling. This is the hypothesis for this trial.

3. Material and Method

In the absence of European *Clostridium difficile* sporicidal standards we designed a reproducible carrier test modified according to AOAC (Association of Official Agricultural chemists) [44].

3.1. Isolates

The selected *C. difficile* strain for this study was PCR ribotype UK023/SE25 due to its relatively high incidence in Sweden [12] and because of its spores’ poor ability of clumping together, seen continuously in previous experiments at the Örebro University hospital (Karin Johansson, personal communication). Therefore, this ribotype is relevant and a more simple strain to work with when counting colonies. The strain was received from European Centre for Disease Prevention and Control (ECDC) and had been isolated from a patient suffering from diarrhoea, stored at -70°C until studied.
3.2. Chlorine dioxide

The ClO₂-based agent used for this study is a gaseous ClO₂ dissolved in water, LifeClean Slutstäd/Ytdesinfektion (1600 ppm available ClO₂; LifeClean International AB; Uddevalla, Sweden), with surfactants. The manufacture states that LifeClean kills the C. difficile spores within 1 minute via depletion of the spore wall followed by chemical interaction inside the spore core. The agent is also effective against several other microorganisms [45].

3.3. Culture

The strain was inoculated on anaerobe agar plate (5 % horse blood, defibrinated [SVA, Uppsala, Sweden], 4.57 % Fastidious Anaerobe Agar [50 % Peptone, 26 % Agar, 11 % Sodium Chloride, 2 % Soluble Starch, 2 % L-Arginine, 2 % Glucose, 2 % Sodium Pyrovate, 1 % L-Cysteine, 1 % Sodium Succinate, 0.8 % Sodium Bicarbonate, 0.5 % Sodium Pyrophosphate, 0.02 % Hemin, 0.002 % Vitamin K] (Acumedica, Neogen Corp. Lansing, MI, USA), 0.1 % taurocholate [Sigma-Aldrich; Saint Louis, MO, USA]) and incubated anaerobically at 36°C for 48 hours.

3.4. Spore suspensions

The agar plates were left to sporulate in room temperature for 7 days. The colonies were then removed from the plates and dissolved with RO-water (Cold sterile reverse osmosis-water). The suspensions were exposed to an alcohol shock with Absolut finsprit 99.5 % (99.5 % Ethanol, 0.5 % water; Kemetyl AB; Haninge, Sweden), which was diluted from 99.5 % to 70 % ethanol with 0.85 % NaCl-solution (Riedel de Haen; Seelze, Germany), and stored at 4°C for 1-2 hours to eliminate vegetative cells and possible contaminants. To obtain the required concentration of spores, the suspensions was centrifuged 4 times at 3000 x g in 4°C for 20 minutes, washed with RO-water between centrifugation, re-suspended in smaller volumes of RO-water and stored at 4°C for no more than 2 weeks.

3.5. Spore counting

The spore suspensions were determined by visual enumeration with a Bürkers chamber (0.05 mm depth; Assistent; Glaswarenfabrik Karl Hecht GmbH & Co KG; Sondheim v. d. Rhön, Germany) and a light microscope (Eclipse 50i, Nikon Instruments Europe BV, Amsterdam, Netherlands). Spores were counted in 10 D-squares, resembling 1/8000 μL, and the CFU per
mL was calculated to receive the inoculum of spores. The spore counts in the spore suspensions were 1.51 x 10^9 and 1.76 x 10^9 CFU/mL.

3.6. Organic material

FBS (EU Standard, South America, Brazil; BioWhittaker, Lonza Walkersville Inc.; Walkersville, MD, USA) 5 % and 10 %, and 10 % faecal material (derived from faeces samples negative for C. difficile) were diluted from 100 % concentrations with 0.85 % NaCl-solution. This was used to stimulate a more credible environment and to evaluate the sporicidal efficacy in the spores’ natural habitat. The volume of spore suspension required for the tests was centrifuged at 3000 x g in 4°C for 20 minutes, and re-suspended with the same amount of volume of 5 %, 10 % FBS, or 10 % faecal material solutions.

3.7. Positive Controls

0.85 % NaCl-solution and 70 % ethanol diluted from 99.5 % with 0.85 % NaCl-solution.

3.8. Spore viability

The spore suspensions were inoculated on sterile glass carriers and air dried. All the tests with LifeClean were made in duplicate.

0.2 mL of agent or positive control (LifeClean, 70 % Ethanol or 0.85 % NaCl-solution), were added to the inoculum and allowed to act for 10 minutes.

The carriers were put into sterile, 500 mL glass bottles, containing 250 mL 0.85 % NaCl-solution on a Gyrotory Shaker Model G2 (New Brunswick Scientific Co Inc., Edison, NJ, USA) at 200 rpm for 20 minutes to prevent the agent from further action.

A first sample from the glass bottles was put on an agar plate.

A second sample was diluted in two steps, in 1 to 10, with 0.85 % NaCl-solution and from these dilutions two separate samples were put on agar plates.

A third sample was filtrated by DOA Vacuum Pump (Gast Manufacturing, Inc.; Benton Harbor, MI, USA) through membrane filters (S-Pak Filters, 47 mm diameter, 0.45 μL pore size; Merc Millipore; Billerica, MA, USA), and washed with 100 mL Buffered Peptone water (PENAL) (98.4 % RO-water, 0.71 % di-Sodium Hydrogen Phosphate Dihydrate [Merck, Whitehouse Station, NJ, USA], 0.42 % Sodium chloride [Riedel de Haen], 0.35 % Potassium phosphate [Merck], 0.1 % Peptone Bacto [Becton, Dickinson and Company (BD), Franklin Lakes, NJ, USA]) before the filter was put on an agar plate. The rest of the spore solution in
the glass bottle was poured through another filter, washed with PENAL, and also put on an agar plate.

To make sure no spores had passed through the filters a control sample from the filtrate was obtained during the test. The controls were centrifuged at 3000 x g in 4°C for 20 minutes, and re-suspended with 0.85 % NaCl-solution and put on selective *C. difficile* agar plates (5 % Horse blood, defibrinated [SVA, Sweden], 4.57 % Fastidious Anaerob Agar [Acumedia Neogen Corp., USA], Clostridium difficile Supplement [500 mg/L D-Cycloserine, 16 mg/L Cefoxitina; Bergman Labora AB, Danderyd, Sweden]).

The remaining spores on the glass carrier were removed by double-S cotton swab and put to a 0.85 % NaCl-solution and diluted in three steps in 1 to 100 with NaCl-solutions. From these dilutions three separate samples were put on agar plates.

The agar plates were then incubated anaerobically at 36°C for 48 hours.

The colonies on the agar plates were visually counted.

**3.9. Log$_{10}$ reduction calculation**

Every colony on the agar plates were estimated to be derived from one viable spore (CFU). From every agar plate with a countable (0-300) amount of CFU, a calculation was made to quantify the remaining viable spores in the 250 mL spore-solution in the glass bottle with the glass carrier. From these values, a median value, converted to log$_{10}$, was calculated for all separate tests for evaluation of the sporicidal effect.

**3.10. Statistical analyse**

Wilcoxon rank-sum test was used to evaluate the statistical significance of the efficacy for LifeClean in comparison to 70 % ethanol. Excel 2010 was used to calculate the exact p-value.

**3.11. Ethical consideration**

No ethical application was submitted to the Regional Ethical Review Board (EPN). The *C. difficile* strain used in this trial was collected from an infected patient, although there was no association between the strain and the patient, why the identity remain unknown. However, faecal material was collected from two patients who were randomly selected from a reservoir of faecal samples negative for *C. difficile* by laboratory personnel. After sampling was made, the jars, with identification tags, were disposed, and there were no linkage of the patients to the study. The risk of infection for investigators or other personnel was slim due to careful infection control routines.
4. RESULTS

4.1. Spore viability counts

The results are seen in Figure 1, showing the LifeClean and 70 % ethanol efficacy in \(\log_{10}\) viable CFU counts with increasing amount of soiling compared to the water control. The median CFU value was evaluated for all separate tests in Figure 1. Due to the low amount of tests made in this study the CFU values varied in the different samples in the tests. Therefore, the median, mean and range values for each test are presented in Table 1. The two spore inoculums used, counted in the Bürker chamber, were both \(>10^8\) CFU.

![Graph showing median log10 reduction factor of viable counts](image)

**Figure 1:** The median \(\log_{10}\) factor of viable counts, after 10 minutes of exposure, with and without organic material. The values shown for LifeClean are the mean values of duplicates.
4.2. Log\textsubscript{10} reduction

The CFU reductions were calculated with the log\textsubscript{10} median value of viable CFU counts after exposure to LifeClean subtracted with the respectively water control, because of the probability that not all spores were viable in the counted inoculum. These calculations were 2.48 log\textsubscript{10} with no organic material; 2.65 log\textsubscript{10} with 5 % FBS; 2.02 log\textsubscript{10} with 10 % FBS and 1.09 log\textsubscript{10} with 10 % faecal material. These results are suggesting that with increasing amount of soiling, except for the sample with 5 % FBS, the efficacy of LifeClean decreases. The results also indicate that the difference in the efficacy between LifeClean with no organic soiling up to 10 % faecal material was 1.39 log\textsubscript{10}.

4.3. LifeClean efficacy in comparison to 70 % ethanol

The results indicate, but were not significant using Wilcoxon rank-sum test (p= 0.057), that LifeClean was more effective than 70 % ethanol in inactivating spores in all the tests made in this study. 70 % ethanol seems to be less effective than LifeClean with increasing amount of soiling, seen in Figure 1.

**Table 1:** The median, mean and range values of viable spore counts, made from calculating the colonies on the many agar plates made from the different samples of in the separate tests. CFU (colony forming unit)

<table>
<thead>
<tr>
<th>Test 1: No organic matter</th>
<th>Median viable CFU</th>
<th>Mean viable CFU</th>
<th>Range of viable CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>LifeClean 1/2</td>
<td>3.68 x 10^5</td>
<td>6.22 x 10^5</td>
<td>1.40 x 10^5 - 2.03 x 10^6</td>
</tr>
<tr>
<td>LifeClean 2/2</td>
<td>3.45 x 10^4</td>
<td>5.56 x 10^4</td>
<td>0 - 2.03 x 10^5</td>
</tr>
<tr>
<td>Ethanol control</td>
<td>7.09 x 10^6</td>
<td>2.58 x 10^7</td>
<td>5.57 x 10^6 - 6.48 x 10^7</td>
</tr>
<tr>
<td>Water control</td>
<td>6.04 x 10^7</td>
<td>6.04 x 10^7</td>
<td>4.28 x 10^7 - 7.80 x 10^7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test 2: 5 % fetal bovine serum</th>
<th>Median viable CFU</th>
<th>Mean viable CFU</th>
<th>Range of viable CFU</th>
</tr>
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<tbody>
<tr>
<td>LifeClean 1/2</td>
<td>2.74 x 10^5</td>
<td>2.81 x 10^5</td>
<td>0 - 5.00 x 10^5</td>
</tr>
<tr>
<td>LifeClean 2/2</td>
<td>5.50 x 10^3</td>
<td>7.62 x 10^3</td>
<td>0 - 2.40 x 10^4</td>
</tr>
<tr>
<td>Ethanol control</td>
<td>7.94 x 10^5</td>
<td>1.24 x 10^7</td>
<td>5.00 x 10^5 - 4.76 x 10^7</td>
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<tr>
<td>Water control</td>
<td>6.20 x 10^7</td>
<td>6.20 x 10^7</td>
<td>5.80 x 10^7 - 6.61 x 10^7</td>
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<tr>
<th>Test 3: 10 % fetal bovine serum</th>
<th>Median viable CFU</th>
<th>Mean viable CFU</th>
<th>Range of viable CFU</th>
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<tbody>
<tr>
<td>LifeClean 1/2</td>
<td>3.75 x 10^5</td>
<td>4.32 x 10^5</td>
<td>2.00 x 10^5 - 8.76 x 10^5</td>
</tr>
<tr>
<td>LifeClean 2/2</td>
<td>7.00 x 10^5</td>
<td>7.68 x 10^5</td>
<td>2.96 x 10^5 - 1.38 x 10^6</td>
</tr>
<tr>
<td>Ethanol control</td>
<td>3.30 x 10^6</td>
<td>1.90 x 10^7</td>
<td>3.25 x 10^6 - 5.04 x 10^7</td>
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<tr>
<td>Water control</td>
<td>4.59 x 10^7</td>
<td>4.59 x 10^7</td>
<td>1.82 x 10^7 - 7.36 x 10^7</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Test 4: 10 % fecal material</th>
<th>Median viable CFU</th>
<th>Mean viable CFU</th>
<th>Range of viable CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>LifeClean 1/2</td>
<td>2.45 x 10^6</td>
<td>3.02 x 10^6</td>
<td>1.00 x 10^6 - 5.60 x 10^6</td>
</tr>
<tr>
<td>LifeClean 2/2</td>
<td>4.50 x 10^6</td>
<td>4.30 x 10^6</td>
<td>3.20 x 10^6 - 5.20 x 10^6</td>
</tr>
<tr>
<td>Ethanol control</td>
<td>3.65 x 10^7</td>
<td>6.65 x 10^7</td>
<td>9.01 x 10^6 - 6.40 x 10^7</td>
</tr>
<tr>
<td>Water control</td>
<td>4.30 x 10^7</td>
<td>4.30 x 10^7</td>
<td>3.20 x 10^6 - 8.28 x 10^7</td>
</tr>
</tbody>
</table>
5. DISCUSSION

5.1. Introduction to discussion

Patients with CDI or developing a nosocomial CDI tend to prolong hospital duration stay and affect patient care leading to increasing costs [46]. These patients shed large amounts of both vegetative cells and spores into the environment and every surface contaminated with spores may become a vector for transmission, even the hands of healthcare workers [47]. Effective environmental spore control [27,48] may contribute to reduced infection rate of CDI in hospitals.

5.2. Efficacy of LifeClean on C. difficile spores

In this small trial, LifeClean, had relatively low efficacy on C. difficile spores despite 10 minutes of exposure and high concentration of ClO₂. Nevertheless, the results indicate that LifeClean was more effective than 70 % ethanol, but did not reach a significant difference. A larger study may reveal a more significant result.

Standards for evaluation of sporicidal efficacy have been developed and according to some of these achieving ≥ 3 log₁₀ reduction in spore viability (where inoculums were 10^5 or 10^6 CFU/mL) have been considered a sporicidal efficacy of relevance [43,49]. Others have demanded ≥ 6 log₁₀ reduction when using inoculums of 10^6. However, in this trial we used an inoculum of >10^8 CFU/mL which correlated to the standard method used for this trial experiments [44]. Some studies have evaluated certain ClO₂-based agents with significant sporicidal effect [43]. Usage of a lower spore concentrate inoculum may have resulted in achievement of the criterions mentioned above. In this trial, LifeClean do not accomplish a very high log₁₀ reduction, although since lacking specific criteria for the acceptable log₁₀ reduction with this inoculum, evaluation of sporicidal efficacy is complicated.

Unexpectedly, the test with 5 % FBS soiling resulted in the best effect of LifeClean. Although there might have been a faulty in one of these tests whereas the two median duplicate values varied from 5.50x10^3 to 2.74x10^5, seen in Table 1. There is a possibility that the latter were more accurate. A possible laboratory faulty may have resulted in the wide range of the test with 70 % ethanol with 5 % FBS, from 5.00x10^5 to 4.76x10^7, seen in Table 1.

When spore counts were made in the Bürker chamber there was no possibility to distinguish non-viable spores from viable, which resulted in a higher estimated CFU/mL value of the inoculum. Therefore, the received CFU reduction values were calculated from the
water control, and not the first counted inoculum, resulting in a concentration range between $10^7$ and $10^8$, seen in Figure 1. Since water has no sporicidal effect, the viable CFU-count received from the water controls were considered the absolute viable CFU concentration inoculum used in the tests.

5.3. Efficacy of LifeClean in presence with organic material

It is known that the efficacy of most disinfectants and other sanitizing agents quickly drops in presence with organic material, including chlorine-based agents and Virkon™ [27,32]. However there is evidence that some ClO$_2$-based agents may have some effect despite soiling [43]. In this study we did receive similar findings, although not significant.

From the results in Figure 1 we notice a decrease in efficacy of LifeClean with an increasing amount of soiling. This decrease was to be expected due to the many studies indicating a reducing effect of oxidizing agents with the presence of organic material [27,32,42,43]. Our study confirms these results. Because the natural habitat of the spores is faecal material, it is important that sporicidal agents are effective in presence of faecal soiling. Therefore, challenging these agents in the presence of organic material is essential for evaluation of sporicidal efficacy and clinical relevance. With 10 % faecal solution used in this trial real-life conditions were approached.

5.4. Evaluation of chlorine dioxide as a sporicidal agent

The results from different studies using ClO$_2$ have varied [43,50].

Speight et al [43] evaluated several oxidizers, including several ClO$_2$-based agents. During 1 minute of exposure, 12 of the 19 ClO$_2$-based agents tested managed a 3 to $>4 \log_{10}$ reduction of a $10^6$ C. difficile spore inoculum under clean conditions. 8 of these agents managed the same criteria under dirty (3 % albumin) conditions. However, during 60 minutes of exposure, 18 of the ClO$_2$-based agents achieved these criteria under clean conditions, yet only 9 agents under dirty conditions. The ClO$_2$-concentrations and other contents of these agents were uncertain due to anonymous content profiles, although the most effective of the agents seem to have a higher concentrate of ClO$_2$, or in other ways have a more rapid effect.

Perez et al [40] evaluated a liquid ClO$_2$-based agent (600 ppm) among other oxidizing disinfectants on C. difficile spores with exposure of organic soiling and found a $\log_{10}$ reduction of $>6$ from an inoculum of $10^7$ under 10 minutes.
Compared to our experiments, the results in these two studies were more statistically significant. Despite the lower ClO$_2$-concentration used in Perez et al [40], and the shorter contact time of 1 minute in Speight et al [43], the results showed a greater sporicidal efficacy of the agents. However, the difference in results between these studies compared to our trial may be due to several factors, such as usage of a lower spore concentration inoculum, a lower load of organic soiling and a greater amount of detergent used in proportion to the inoculum.

The ideal sporicidal agent should have a rapid acting, strong sporicidal capacity with and without presence of organic soiling in addition to be easy to use and harmless to the environment as well as to personnel and patients, with no need for cleaning before or after the treatment.

Terminal cleaning after a CDI patient in Örebro with Virkon$^\text{TM}$ 3 % include pre-clean and mechanical rubbing, and thereafter a final rub off after treatment, see Appendix 2. However, the manufacture for LifeClean only recommends two of these procedures, which are pre-clean in combination with mechanical rubbing [45].

Lawley et al [29], show a greater sporicidal effect of other oxidizing agents than of Virkon$^\text{TM}$ 1 %, with a difference of 2 log$_{10}$ CFU reductions, with a $10^6$ C. difficile spore inoculum, as well as an undetectable effect of 70 % ethanol on the spores.

Vohra et al [32] evaluate Virkon$^\text{TM}$ 1 % in comparison to chlorine-based agents, with and without the presence of organic material of a $10^3$ C. difficile spore inoculum. The chlorine-based agent had a stable sporicidal efficacy both with and without organic soiling. However, Virkon$^\text{TM}$ 1 % had a lesser sporicidal effect, as well as a 10-fold decrease in log$_{10}$ reduction with the presence of organic soiling. The study also show different efficacy of the agents on various surface types, as well as on a diversity of ribotypes.

These studies evaluate a 1 % concentration of Virkon$^\text{TM}$ which may result in a lower effect than of a 3 % concentrate. The results from the studies may however be associated to the low amount of soiling, prolonged contact time, low spore inoculum, low load of organic soiling, and higher amount of detergent used in proportion to the inoculum.

In summary, the results from previous studies [29,32,40,43] indicate that with increasing amount of soiling the importance of prolonged contact time and usage of a greater amount of detergent is needed. Furthermore ClO$_2$ seems to have a greater effect on a lower spore concentration inoculum which indicates that pre-clean is crucial as an attempt to remove as much spores as possible. However, pre-clean may disperse the spores without actual
removing. Adequate cleaning is therefore required for spore control [43,50]. In comparison to the study showing that Virkon™ 1% had a 10-fold decrease of $\log_{10}$ reduction with organic soiling [32] it seems as though presence of organic material hampers Virkon™ to a greater extent than oxidizing agents, and that Virkon™, compared to hypochlorite and ClO$_2$, has a lesser sporicidal effect [29,32]. However, a 3% concentrate of Virkon™ may be more effective. In our study we show a 2-fold decrease of $\log_{10}$ reduction when using ClO$_2$ with no organic material in comparison to an increasing amount of soiling.

5.5. Further improvements and investigations

Before a change in terminal cleaning method should be considered, there are several features left to investigate. First, evaluating the toxicity of LifeClean and the potential hazards for personnel, patients and environment. Second, studying the effect of ClO$_2$ in comparison to other agents, as well as examine the efficacy on surfaces frequently appearing in hospital environment in addition to different contact times more likely to occur in hospital setting. Third, evaluating the efficacy of ClO$_2$ on various *C. difficile* ribotypes as well as the efficacy of different ClO$_2$-concentrations.

5.6. Limitations

Few tests were made which provided less accurate results. Colonies on agar plates were not counted if exceeded 300, which resulted in a lower amount of CFU-counts to receive median values.

Only one ribotype and one strain of this ribotype was investigated for this study. There is a possibility that other *C. difficile* ribotypes and strains possess different resistance mechanisms to ClO$_2$.

The spore inoculums used for this trial contained a high amount of spores which may have contributed to a less reliable result than expected.

There is a possibility that there were remaining spores left in the glass bottle or on the glass carrier since we did not make absolutely sure there were not. This would have provided a false positive result instead of the proper value.

Improper laboratory methods, due to inexperience, may have resulted in false positive or false negative result resulting in incorrect conclusions.
6. **CONCLUSION**

The results received from this study indicates that the ClO₂-based agent LifeClean do have a sporicidal effect on *C. difficile* spores, although relatively low despite the high concentration of ClO₂ and contact time. A decrease of the efficacy was noticed with an increasing amount of organic soiling. However our findings were not significant due to the low amount of experiments in this trial.

Before a change of sporicidal agents in terminal cleaning is to be considered, further investigation of ClO₂ and LifeClean will hopefully be encouraged by our results. Evaluation in this area is needed for a tolerable environmental spore control.

7. **ACKNOWLEDGEMENT**

I would like to thank my supervisor, MD, PhD and associated professor, Hans Fredlund, for all the support and guidance throughout my writing process. I would also like to thank my laboratory supervisor, PhD Karin Johansson for preparing my cell cultures, as well as BMA Eva Forsberg, for all the help and patience when performing the laboratory method. Finally I would like to thank all the personnel at the microbiology laboratory at the Örebro University hospital for answering all of my questions.

8. **REFERENCES**


