Efficacy of Glutaraldehyde Disinfectant Against *Cryptosporidium parvum* in the Presence of Various Organic Soils

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The opportunistic protozoan *Cryptosporidium parvum* is highly resistant to disinfectants, including those specifically used for processing reused medical equipment in hospitals. *C. parvum* oocysts were dried onto glass and steel grooved penicylinders and challenged with 2.5% glutaraldehyde solution in the presence of 3 types of soil with exposures at 10 min, 90 min, and 10 h. The influence of organic soils on disinfection was measured with 5% fetal bovine serum (FBS), 10% FBS, and 5 mg mucin/mL. An in vitro excystation procedure and cell culture infection assay were used to determine survivability of oocysts after the germicide challenge. In the presence of organic soil, all oocysts removed from carriers excysted and infected cell monolayers after all germicide contact times. However, excystation was observed only from oocysts that received no protection from organic soil after 10 h exposure. In these samples, no infection was observed in the cell monolayers. The results of this research demonstrate the importance of thorough cleaning of medical equipment before disinfection.

*Cryptosporidium parvum* is an intracellular enteric protozoan that causes self-limiting diarrhea in immunocompetent individuals. In immunocompromised individuals, such as those with HIV or transplant recipients, it causes chronic diarrhea and malabsorption, which in some cases can lead to death (1, 2).

At present, there is no effective treatment for cryptosporidiosis (1–3). Reports on encountering this organism during routine endoscopic exams are becoming more prevalent (4). Asymptomatic carriers of *C. parvum* have been diagnosed during esophagogastroduodenoscopy and the possibility of transmission via endoscopes has been raised (4, 5). The ability of *C. parvum* to withstand chemical disinfection is well known (6–8). The AOAC sporicidal test (9) is used to distinguish between chemistries that kill dry spores and those that cannot. Those that can kill dry spores are considered the highest level of disinfectants and are tested to validate that they kill mycobacteria, vegetative bacteria, and hydrophilic and lipophilic viruses. There is no evidence that chemistries that can kill bacterial spores are also able to inactivate *C. parvum* oocysts.

Publications concerning the operational use and care of endoscopes recommend that it is safe to reuse an endoscope after only a 20 min soak in a 2.5% glutaraldehyde-based disinfectant (10, 11). Manual cleaning of an endoscope can remove approximately 99% of viable pathogens on its external surface and internal chambers, if done properly (11). Depending on the manufacturer, the labeled high-level disinfection times for 2.5% glutaraldehyde are 45–90 min at room temperature. It has been documented that a 45 min soak time is necessary to kill high numbers of viable pathogens (11). Previous work has shown that clean suspensions of high-level disinfectants do not inactivate *C. parvum* oocysts (12). Furthermore, even cleaning the interior chambers of these devices probably does not remove all viable pathogens (11). For these reasons, it is important to determine if oocysts, which may be in patient material associated with medical devices, could be inactivated during a typical germicide exposure. *C. parvum* is extremely resistant to commonly used high-level disinfectants and, therefore, may pose a health risk when associated with reusable medical devices.

The objective of this research was to determine the protection given to *C. parvum* oocyst in the presence of different organic soils when challenged with 2.5% glutaraldehyde. The germicide was tested in the presence of no organic soil and with organic soils in the form of 5% fetal bovine serum (FBS), 10% FBS, and 5 mg mucin/mL. The organic soils mimic the biological fluids that may be found on medical devices. A cell culture assay determined the loss of oocyst infectivity. An excystation assay compared the viability of treated and untreated oocysts.

**Experimental**

**Reagents and Materials**

(a) *C. parvum* oocysts.—Purchased commercially from Immucell Corp. (Portland, ME). Oocysts were received in sterile phosphate-buffered saline (PBS) under refrigeration.
Once received, oocysts were diluted to $1 \times 10^6$/mL and stored in sterile PBS at 4°C until needed.

(b) **Glazed glass penicylinder.**—8 × 8 mm cloning cylinder.

(c) **Stainless steel penicylinder.**—8 × 10 mm (Fisher Scientific, Fairlawn, NJ). Steel penicylinders were grooved internally with 1/4-28 spiral-pointed tap (FDA-WEAC machine shop, Winchester, MA).

(d) **Ultrasonic bath**

(e) **Minicentrifuge**

(f) **Cells.**—HCT-8 [ileocecal colorectal adenocarcinoma, colon; American Type Culture Collection (ATCC), Rockville, MD; No. CCL-244].

(g) **Cell medium.**—RPMI 1640 with NaHCO3. Medium supplemented with FBS, L-glutamine, penicillin, streptomycin solution, and HEPES buffer.

(h) **Cell culture flasks.**—75 cm$^2$.

(i) **Slides.**—8-well.

(j) **CO$_2$ incubator**

(k) **Tergitol—1%.**

(l) **Microscope.**—BMAX 50 with Nomarski interference contrast.

**Procedures**

(a) **Oocyst viability.**—Oocysts were excysted for viability as described by Black et al. (6) before each experiment and were used in experiments only if excystation was 70% or higher. Oocyst numbers were determined by hemacytometer counts and the concentration was adjusted as needed for each experiment.

(b) **Preparation of cylinders.**—Before inoculation, all cylinders were washed in 1% Tergitol followed by a rinse in double-distilled water; soaked in 1N NaOH for 30 min; rinsed with double-distilled water 3 times; soaked in 70% ETOH for 30 min; rinsed again with double-distilled water 3 times; and autoclaved at 121°C for 30 min.

(c) **Inoculation of cylinders.**—Cylinders were inoculated to determine the infectivity and percent excystation of oocysts after germicide treatment. Sterilized cylinders were placed in 18 × 150 mm culture tube containing $1 \times 10^6$ oocysts/mL in 2 mL PBS. The cylinders were soaked in the suspension for 15 min, and then removed and air-dried for 15 min on sterile filter paper. After drying, the carriers were placed into a second culture tube containing 2 mL 2.5% glutaraldehyde. After the exposure had expired, 2 mL sterile PBS was added to dilute the germicide, and the culture tube containing the cylinder was placed in a sonic bath for 5 min to remove adhering oocysts. The carrier was then removed from the test tube, and the supernate was centrifuged at 5000 × g for 3 min. The supernate was discarded and the sample was washed twice with sterile PBS. After washing, samples received either 100 μL warmed cell culture medium or 100 μL excystation fluid.

(d) **Excystation of treated oocysts.**—Recovered oocysts were excysted by being placed in a suspension of 0.5% trypsin and 2.5% sodium taurocholate and then warmed to 37°C for 90 min (3). A positive control consisting of untreated oocysts was also excysted for viability comparisons. Percent viability was determined with the following formula:

$$\text{Viability, } % = \frac{\text{EO} + \text{PEO}}{\text{EO} + \text{PEO} + \text{IO}} \times 100$$

where $\text{EO} =$ excysted oocyst, $\text{IO} =$ intact oocyst, and $\text{PEO} =$ partially excysted oocyst.

(e) **Cell culture.**—HCT-8 cells were grown in 75 cm$^2$ cell culture flasks in a 37°C, 5% CO$_2$-95% air, humidified incubator. Cells were removed from the surface of flasks by trypsinization in a solution of 0.25% (w/v) trypsin and 0.53mM EDTA in cell culture PBS. The cell culture medium, as described by Upton et al. (13), was RPMI 1640 supplemented with 15mM HEPES, 100 U penicillin/mL, 100 μg streptomycin/mL, FBS, 200mM L-glutamine, and NaHCO3. For cell maintenance, 5% FBS was used; 10% FBS was used when oocysts were inoculated onto cell monolayer.

(f) **Inoculation of oocysts onto cell monolayer.**—The method used to inoculate recovered oocysts onto cell monolayers was described by Upton et al. (13). HCT-8 cells were removed from cell culture flasks by trypsinization 16 h before inoculation of oocysts. Recovered cells were mixed on a Vortex mixer and counted with a hemacytometer. Cells were distributed into each well of the 8-well glass slide. Each well received $1 \times 10^5$ HCT-8 cells in 100 μL cell culture medium. Before inoculation, cell culture medium was removed from wells.

The inoculating suspension, consisting of 100 μL growth medium and 100 μL oocyst suspension, was added to wells 1–5. Well 6, which represented the positive control, received fresh oocysts to ensure infectivity. Well 7, used as a negative control, received only cell culture medium. The slides were then placed into a 37°C incubator. Sporozoites emerged from excysting viable oocysts and infected the host cells. Removal of oocyst toxins occurred 2 h post-inoculation (PI) as described by Eggleston et al. (14), and 200 μL fresh growth medium was added to monolayers. Nonviable oocysts were removed during cell culture fluid changes. Slides were then returned to the incubator.

After 72 h, slides were removed from the incubator and observed under 100×-oil immersion with Nomarski interference contrast.
used as contrast for the presence of later life-cycle forms (type I and II meronts, macrogametes, and microgametocytes) in the parasitic vacuole. This vacuole is formed around the infecting sporozoite in the region between the host cell membrane and outer cytoplasm. Host cell microvilli surround the intracellular attached end of the sporozoite from which the later life-cycle forms develop.

The criteria for scoring a positive cell culture infection were the observation of these extracytoplasmic parasitic vacuoles containing sporozoites and later life-cycle forms. When no vacuoles with life-cycle forms were observed in 10 000 host cells, the tissue culture was scored as not infected. Quantitation of oocysts and life-cycle forms was therefore not performed, because the assay was based on presence/absence of life-cycle forms.

### Results and Discussion

**Number of adhered oocysts recovered from carriers.**—The number of recovered oocysts varied between those that received organic soils and those that were inoculated without soil. In the absence of soil, the number of recovered oocysts was greater from steel grooved penicylinders than from glass penicylinders (Table 1). This difference increased with the introduction of soils. The glass penicylinder surface is smooth and allows oocyst suspensions to dry more evenly. The steel penicylinder is grooved on its internal surface, allowing pockets of oocysts to build up and providing extra protection against germicide treatment.

The original inoculum for the carriers was $10^6$ oocysts/mL contained in 2 mL. The lowest number of oocysts recovered from a carrier was 4.73 logs from the glass penicylinder in the absence of soil. The infectious dose of *C. parvum* is thought to be as

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<th>SDb</th>
<th>Control excystation, %</th>
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*Table 2. Percent excystation post-germicide exposure in absence and presence of soils*

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a Average of triple replicates.

b SD = Standard deviation.
low as 30 oocysts. In fact, the oocysts from this sample treated with glutaraldehyde infected the cell monolayer at both 10 and 90 min exposure; however, infection was not observed after a 10 h treatment. Endoscopes might be exposed to glutaraldehyde for approximately 45–90 min. Therefore, oocysts remaining on the carrier are capable of causing infection if they remain on a medical device throughout its reprocessing.

(b) Determination of viability by excystation.—The addition of soil to the oocyst suspensions appears to have helped to increase the number of oocysts excysting. Excystation percentages of soil-protected oocysts were higher than those in the absence of soils. Table 2 shows that the addition of mucin to the sample provided the most protection at all exposure times. Percent excystation was the highest with the introduction of mucin. After 10 min exposure with no soil, the mean excystation was 22%, whereas with 5% FBS, 10% FBS, and mucin, excystation was 30, 52, and 53%, respectively. At 90 min exposure, the percentage of excysted oocysts increased compared with that of oocysts receiving no soil. Although low numbers of sporozoites excysted from oocysts recovered from both penicylinders, infection of the cell monolayer still occurred after all exposure times from oocysts that received soils. It is evident from these studies that organic soils decreased the effectiveness of the germicide.

(c) Determination of viability by cell culture assay.—In a previous study (12), higher numbers ($10^5$ and $10^6$) of oocysts survived a 10 h glutaraldehyde treatment without soil and were infectious for cell monolayers. In the present study, oocysts inoculated onto carriers with and without soils infected cell monolayers after 10 and 90 min exposure. C. parvum oocysts were capable of surviving the germicide treatment without the added protection of soils. This finding agrees with previous data generated at WEAC (12). However, no infection was observed after a 10 h soak in germicide for oocysts with no soil (Tables 3 and 4). Protection from different soils was seen at 10 h exposure. All oocysts that were recovered from both cylinder types containing soil remained infectious for the cell monolayer after a 10 h exposure (Tables 3 and 4).

C. parvum oocysts have been observed in endoscopic biopsies of asymptomatic patients (4). Furthermore, other publications indicate that endoscopes may not always be properly cleaned (11). For this reason, the data generated from this research should be a cause for concern. Oocysts in the presence of soils readily infected cell monolayers after 10 h immersion. Presently, endoscopic disinfection is recommended using 20–90 min exposure to a 2.5% glutaraldehyde-based disinfectant (10). As evident from these data, organic material preserved oocysts in the presence of the germicide. Oocysts may be desiccated if endoscopes are allowed to dry over an extended time. However, because endoscopes are used more than once a day, desiccation of the organism in the presence of organic soil would be unlikely.

The antimicrobial activity of high-level disinfectants is negatively affected by the presence of organic soil; therefore, it is extremely important that all devices presented for disinfection be thoroughly cleaned. Cleaning of critical and semicritical medical devices is defined as the removal of all foreign material from the external and internal surfaces of the device. This cleaning must precede disinfection (10). It has been shown that cleaning alone is very effective in removing pathogenic organisms (10), and lower numbers of oocysts may be removed if proper cleaning and disinfection protocols are followed. However, as evident from this research, 90 min

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</tbody>
</table>

Average of triple replicates.

Table 4. Infectivity results in absence and presence of organic soils with glass penicylinders
exposure did not inactivate low numbers of oocysts, which re-

mained infectious for cell culture.

In summary, this research has shown that even in the ab-

sence of organic soil, a typical high-level disinfection proce-

dure cannot kill high numbers of \textit{C. parvum}. Thorough clean-

ing and proper disinfection protocols must be followed. A 10 h

disinfectant soak in clean suspensions inactivated the lower

numbers of oocysts. However, although a 20–90 min soak is

thought to be sufficient to kill most microorganisms, this

work showed that the survivability of oocysts increased in

the presence of organic soils. Therefore, without efficient

cleaning and proper disinfection of devices, \textit{C. parvum} is

likely to remain viable even after the 20–90 min soak that is

presently recommended for high-level disinfection. It is evi-
dent that this organism is resistant to glutaraldehyde-based
disinfectants and may pose a possible health risk in the hos-
pital environment.

References


