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Effect of Sodium Hypochlorite Exposure on Infectivity of *Cryptosporidium parvum* Oocysts for Neonatal BALB/c Mice

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Oocysts of *Cryptosporidium parvum* suspended in 5.25, 2.63, or 1.31% aqueous sodium hypochlorite (Clorox laundry bleach) for 10, 30, 60, or 120 min at 21°C were administered by gastric intubation to neonatal BALB/c mice. Microscopic examination of intestinal tissue sections revealed developmental stages of *C. parvum* in all of the mice.

The oocyst stage of the protozoan parasite *Cryptosporidium parvum* is a widespread environmental contaminant. Oocysts ingested in food or water or through direct contact with feces can initiate infection in the intestinal epithelia of animals and humans, resulting in morbidity or mortality. To reduce the risk of infection through environmental contamination, a disinfectant active against the oocyst stage is needed. Laundry bleach is widely used and recommended as a disinfectant for *C. parvum* oocysts in veterinary laboratories (4). A recent letter in a medical journal recommended cleaning surfaces with full-strength bleach for over 15 min to reduce transmission of *C. parvum* in group homes for human immunodeficiency virus-infected patients (9). Despite such recommendations, neither the effective concentration nor the exposure time required for bleach to render *C. parvum* oocysts noninfectious has been documented.

Effects of diluted sodium hypochlorite (NaOCl) laundry bleach have been reported as preliminary (limited) findings or in vitro tests. Tests such as excystation of sporozoites from oocysts (1, 11, 13, 17) or exclusion of dye from oocysts (2, 3, 16) have been used to indicate viability after exposure to laundry bleach without testing for infectivity. Because techniques and experimental designs in these studies have not clearly and unequivocally demonstrated the effects of highly concentrated laundry bleach versus exposure time on infectivity of *C. parvum* oocysts, the present study was undertaken.

Source of oocysts. Oocysts of the AUCP-1 isolate were obtained from feces of an experimentally infected calf. They were cleaned of fecal debris and host tissue as previously described (7, 10) to eliminate competitive effects on the disinfectant and maximize any anticryptosporidial activity. They were less than 1 month old when used.

Bioassay for infectivity. Thirteen BALB/c mouse dams, each with five pups 1 to 3 days old, were purchased from the National Cancer Institute, Frederick, Md. Each litter, housed in a separate cage, had fresh water and dry, pelleted mouse chow (Agway Prolab Animal Diet 3000; Agway, Inc., Syracuse, N.Y.) available at all times. One litter was used to bioassay oocyst infectivity for each of 11 NaOCl exposure procedures. All mice were killed by CO_2 overexposure 96 h later. One uninfected litter served as a negative control.

Histology and quantification. Five-millimeter segments of the ileum, cecum, and colon from each mouse were fixed in 10% neutral buffered formalin for histology. The sections were stained with hematoxylin and eosin. Five or more fields of 100 epithelial cells in each segment were examined microscopically. A score of 0, 1, 2, 3, or 4 (0%, 1% or less, 2 to 33%, 34 to 66%, or 67% or more, respectively) designated the percentage of epithelial cells parasitized. Cumulative scores were recorded for each mouse. Mean cumulative scores and standard errors for groups are shown in Fig. 1. Data were subjected to analysis of variance with significance at $P \leq 0.01$. Means with the same letter were not significantly different on the basis of the Duncan multiple-range test.

Experimental design. Twelve tubes, each containing 900,000 purified oocysts, were centrifuged at 1,000 × *g* for 10 min. Pelleted oocysts were resuspended in 1 ml of distilled water or an NaOCl solution at 21°C. Oocysts were exposed for 10, 30, 60, or 120 min to undiluted bleach (Clorox) and to bleach diluted 1:1 with distilled water, achieving NaOCl concentrations of 5.25 and 2.63%, respectively. Other oocysts were exposed for 10, 30, and 60 min to bleach diluted 1:3 with distilled water, achieving an NaOCl concentration of 1.31%. At the desired exposure time, 14 ml of Hanks' balanced salt solution (HBSS) at 5°C was added to each oocyst suspension and the tube was centrifuged at 1,000 × *g* for 10 min. HBSS wash steps were repeated three times. Oocysts in each tube were resuspended in 300 μl of HBSS. Each pup received 150,000 oocysts in 50 μl of HBSS by gastric intubation with a 25-gauge gavage needle.

Histologic findings. Results of the present study show for the first time that *C. parvum* oocysts exposed to undiluted laundry bleach for as long as 120 min are infectious for animals. Although bleach is widely used as a bacterial and viral disinfectant, the present findings indicate that under practical conditions it is not an effective disinfectant for *C. parvum* oocysts.

Scores indicated that exposure of oocysts to 1.31, 2.63, and 5.25% NaOCl for as long as 120 min at 21°C significantly reduced the number of infected epithelial cells but did not eliminate infectivity for mice (Fig. 1). Stages of *C. parvum* were found in 179 of 180 segment sections. Groups that received oocysts exposed for 120 min to 5.25 or 2.63% NaOCl had the lowest scores. It is unclear why groups that received oocysts exposed for 60 min to 1.32% NaOCl or for 120 min to 2.63% NaOCl had lower scores than groups that received oocysts exposed for the same times to 5.25% NaOCl (Fig. 1). Tissues

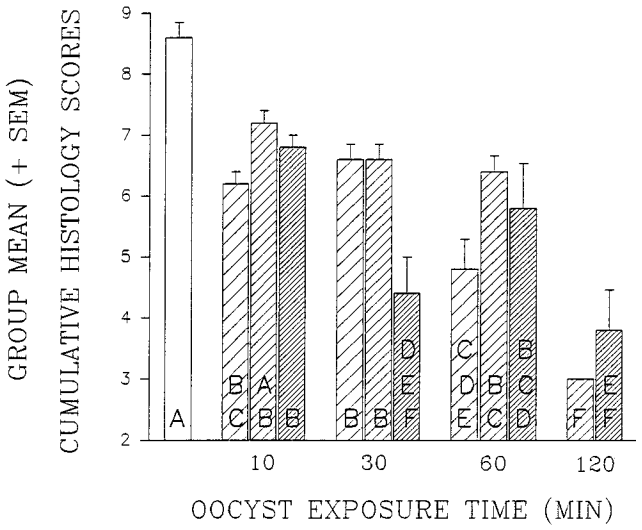

FIG. 1. Plot of duration of oocyst exposure to 5.25, 2.63, and 1.31% NaOCl versus mean group histology scores ± the standard error of the mean. The relationship of histologic scores to the percentage of intestinal epithelial cells infected with *C. parvum* is described in the paragraph on histology and quantification. □, control; ▨, 1.32% NaOCl; ▨, 2.63% NaOCl; ▨, 5.25% NaOCl.

from uninfected controls contained no *C. parvum*, suggesting that no accidental infections occurred.

The present findings extend those of previous studies. Tests with disinfectants used routinely in veterinary laboratories indicated that *C. parvum* oocysts suspended in homogenized intestinal tissue retained infectivity for mice after 18 h in 3% NaOCl (4). However, the number of oocysts, quantity of tissue debris, and final dilution of disinfectant were not reported. Another report considered purified oocysts suspended in 1% NaOCl for 30 min at 22 and 37°C viable on the basis of excystation data (1). When walls of *C. parvum* oocysts exposed to 5.25% NaOCl appeared collapsed, other oocysts exposed to 1.75 or 1.05% solutions for 12 min on ice were excysted (14). In still another study, excystation of *C. baileyi* exposed for 30 min at 25°C to 50% laundry bleach was reduced but not eliminated (17).

Genera closely related to *Cryptosporidium* also appear to be resistant to NaOCl disinfection. In the first report on in vitro cultivation of *Eimeria tenella*, bacteria were removed from oocysts by exposure to undiluted Clorox for 15 to 30 min (12). The same exposure process has been used to prepare many other avian and mammalian *Eimeria* species for in vitro cultivation. *Toxoplasma gondii* oocysts exposed to 6% NaOCl (Purex bleach) for 24 h were infectious for mice (5). Recovery of large quantities of clean, viable *Sarcocystis* sporocysts has been optimized by incubating intestinal mucosa scrapings in cold 5.25% NaOCl for 30 min (6). Sporocysts of *Sarcocystis cruzi*, *S. capracanis*, and *S. tenella* have been incubated in 2.6% NaOCl at 4°C for 30 min to enhance excystation (6).

Other chlorination procedures for destroying oocysts of *C. parvum* have yielded mixed results. Exposure to 80 ppm of free chlorine (prepared from NaOCl) at 25°C for 2 h was reported to render oocysts noninfectious for 22 mice (11). Others reported that exposure to 8 to 16 g of free chlorine per liter, independently of temperature and pH, was required to kill oocysts by 24 h or that oocyst viability was reduced 70.5% after exposure to 1 g of free chlorine per liter at 10°C for 24 h (16). Data reflecting oocyst inactivation by exposure to chlorine dioxide, which is many times more effective than free chlorine, do not agree. Mice infected with oocysts exposed to 0.43 ppm for 30 min had oocyst output reduced 94.3% (13), whereas mice infected with oocysts exposed to 1.3 ppm for 1 h (three times the concentration for twice the exposure time) had a similar level of reduction (11). Compared with those of ozone (11), the concentration levels and exposure times required for free chlorine or chlorine compounds to reduce or eliminate oocyst infectivity are suboptimal.

Dye exclusion test. The vital dye trypan blue was used in this study as a possible indicator of oocyst viability. Twelve tubes each containing 10^7 *C. parvum* oocysts were exposed to 5.25, 2.63, or 1.31% NaOCl for 10, 30, 60, or 120 min. At the desired exposure time, NaOCl was rinsed from all tubes by centrifugation with HBSS as described above. Pelleted oocysts were mixed 1:1 (vol/vol) with 1 ml of 0.4% trypan blue (catalog no. 8154; Sigma Chemical Co., St. Louis, Mo.) and incubated at room temperature for 30 to 60 min. Every oocyst in each suspension examined by bright-field microscopy excluded dye, suggesting that all were viable. These observations do not correlate with histologic findings of fewer stages of *C. parvum* in mice that received oocysts exposed to bleach. Previously, investigators have used vital dyes as indicators of *C. parvum* oocyst inactivation or viability with various degrees of success. Although oocysts were rendered noninfectious for mice after exposure in water to 72.4°C or higher temperature for 1 min (7), 75.4% of the oocysts heated to 75°C excluded 0.2% trypan blue dye, suggesting they were still viable (7a). These studies indicated that trypan blue dye exclusion does not correlate with infectivity. The reason for this finding is unknown. The oocyst wall appears intact and impermeable although sporozoites within the oocyst are damaged or killed. In another study, the vital dyes fluorescein diacetate and propidium iodide were deemed not useful (no explanation provided) whereas in vitro excystation and infectivity for mice were used to evaluate effects of free-chlorine exposure on oocysts (11). In contrast, others indicated that a combination of two vital fluorogenic dyes was a useful indicator of oocyst viability, defined in terms of excystation (2, 3, 15, 16). Oocysts were considered viable when 4′6-diamidino-2-phenylindole (DAPI) entered sporozoite nuclei but propidium iodide was excluded from oocysts. With DAPI and propidium iodide, oocyst viability was assessed after exposure to environmental and experimental conditions. In one such study, oocysts were considered viable after slow freezing and thawing in water (15). In contrast, when oocysts and sporozoites subjected to 26 different freezing-thawing protocols were tested for infectivity, none were infectious for mice (8). Such differences between in vitro tests for viability and in vivo tests for infectivity indicate the importance of demonstrating a strong correlation between methods to ensure valid interpretation and clear understanding of test results.

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