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Resistance of *Cryptosporidium parvum* oocysts following commercial bleach treatment

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Abstract : We investigate the resistance of *Cryptosporidium* (*C.*) *parvum* oocysts to commercial bleach treatment. The viability and infectivity of *C. parvum* oocysts suspended in 100, 50, 25, 12.5, 6.3 or 3.2% aqueous commercial bleach for 10, 30, 60, 120 or 180 min at room temperature were assessed by nucleic acid Syto-9 staining, histologic examination of ileum and infectivity to immunosuppressed neonatal C57BL/6N mice. Although the viability was decreased compared with normal oocysts, all oocysts in contact with serially diluted commercial bleach for 180 min were alive by nucleic acid dye Syto-9 staining. And, microscopic examination of ileum sections revealed developmental stages of *C. parvum* in all mice. The oocyst shedding patterns between mice infected with oocysts contacted with commercial bleach and normal control mice were not significantly different each other. Although commercial bleach is widely used as a bacterial and viral disinfectant, the present findings indicate that it is not an effective disinfectant for *C. parvum* oocysts under practical conditions. Authors conclude that, therefore, it is undesirable to recommend commercial bleach as a disinfectant for *C. parvum* oocysts.

Keywords : *Cryptosporidium parvum*, sodium hypochlorite

Introduction

The oocyst stage of the protozoan parasite, *Cryptosporidium* (*C.*) *parvum* is a wide spread environmental contaminant. Ingestion of oocysts in food, 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, an active disinfectant against the oocyst stage is unconditionally needed. Laundry bleach is widely used and recommended as a disinfectant for *C. parvum* oocysts in veterinary laboratories [4]. Heald and Bartlett [7] recommended cleaning surfaces with full strength bleach for over 15 min to reduce transmission of *C. parvum* in group home for human immunodeficiency virus-infected patients. 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 commercial bleach (diluted sodium hypochlorite, NaOCl) on *C. parvum* have been reported as preliminary findings or *in vitro* tests by investigators. Tests such as excystation of sporozoites from *C. parvum* and avian *Cryptosporidium* oocysts [13, 17] or exclusion of dye from *C. parvum* oocysts [2, 3, 16] have been used to indicate viability after exposure to laundry bleach without testing for infectivity utilizing bioassay. Inasmuch as techniques and experimental designs in these studies have not clearly and unequivocally demonstrated the effects of highly concentrated commercial bleach versus exposure time on infectivity of *C. parvum* oocysts, the present study comprising especially bioassay was undertaken to clarify those subjects to evaluate suitability of the bleach as a disinfectant for the parasite.

Materials and Methods

Source of oocysts

The Iowa isolate of *C. parvum* oocyst and animals

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were kindly provided by the Department of Animal, Dairy and Veterinary Science, Utah State University, USA. Oocysts were maintained by passage in experimentally infected mice in the Department of Veterinary Parasitology, Chonbuk National University (Korea) and purified from feces using discontinuous sucrose gradients [1]. Purified oocysts were stored in 2.5% potassium dichromate solution at 4℃ for less than 4 months prior to use. The purified oocysts were further purified using a cesium chloride gradient [8].

Animals

Female C57BL/6N mice (Simonson Laboratories, USA) maintained in the Department of Veterinary Parasitology, Chonbuk National University aged 6 to 8 weeks and weighting 15 to 20 g each were used in this experiment. The mice were immunosuppressed with dexamathasone phosphate (DEXp) (Sigma Chemical, USA) administered *ad libitum* in drinking water (10 μg/mL) [22]. They were maintained in isolation during the course of the experiment and were housed in wire-floored cages. The cages were placed on trays containing 1.8% potassium dichromate solution to prevent the feces from drying out.

Experimental design

Thirty tubes, each containing 2×10^6 purified oocysts, were centrifuged at 1,000 rpm for 10 min. Pelleted oocysts were resuspended in 1 mL distilled water and were then exposed for 10, 30, 60, 120 or 180 min to undiluted commercial bleach (5.25% sodium hypochlorite, Clorox) and to bleach serially diluted 1 : 1 with distilled water at room temperature, achieving bleach concentrations of 100, 50, 25, 12.5, 6.3 or 3.2%, respectively. At the desired exposure time, oocyst inocula were prepared by washing oocysts with distilled water 3 times to remove the commercial bleach. Oocysts in each tube were resuspended in 100 μL distilled water. At the desired time, five immunosuppressed mice were given 2×10^5 washed oocysts in each tube by gastric intubation with a 25-gauge gavage needle, respectively. Meanwhile, another five immunosuppressed mice inoculated with 2×10^5 fresh oocysts served as a control. Infection experiment for oocyst shedding together with histologic examination of ileum was accomplished each tube, respectively.

Nucleic acid staining

Using the procedure described by Kim and Healey [9], 1×10^5 preserved oocysts were incubated with 250 μ mol nucleic acid dye Syto-9 (Molecular Probes, USA) in microcentrifuge tubes at 37℃ for 60 min in the dark. Oocysts were examined then under fluorescent microscopy and a total of 200 intact oocysts and/or empty shells were counted: positive staining represents dead cells. The percentages of viable oocysts were calculated according to the equation: {(number unstained oocysts) ÷ (number oocysts – number empty shells)} × 100. Each five determinations of three times repetitions were performed per sample.

Enumeration for oocyst shedding

In early times, Yang and Healey [22] reported that a large number of *C. parvum* oocysts was found in fecal samples obtained in immunosuppressed C57BL/6N mice inoculated with the protozoa on days 4~16 PI. To monitor oocyst shedding, fecal collection from mice infected with 2×10^5 oocysts in contact with commercial bleach started on day 4 and continued daily until on 16 day PI. The feces from each mouse were sieved through a succession of steel mesh screens, the smallest of which had a pore size of 250 μm. Subsequently, oocysts were harvested using discontinuous sucrose gradients and washed in 0.025 M phosphate buffered saline. The purified oocysts were then resuspended in 2.5% potassium dichromate solution and stored at 4℃ and later counted using a Fuchus-Rosenthal hemocytometer under a microscope.

Histologic examination

All mice were killed by CO_2 overexposure on 16 day PI. According to the histology service for routine histological processing, five centimeter segments of the ileum from each mouse were fixed in 10% buffered formalin, embedded in paraffin wax, sectioned and stained with hematoxylin and eosin stains. Tissue samples were examined by randomly selecting 10 villi and counting the number of the parasite on each epithelium. The average number of parasites per villi was then calculated. The index of infection was determined by scoring on a scale of 0 to 3 as follows: 0 = no parasite observed; 1 = small numbers of parasites focally distributed in the tissue (< 10% of the tissue colonized); 2 = moderate numbers of parasites widely distributed throughout the tissues (10 to 50% of the tissue colonized); 3 = large numbers of parasites widely distributed throughout the tissues (> 50% of the tissue colonized).

Table 1. Viability of *Cryptosporidium* (*C.*) *parvum* oocysts exposed to commercial bleach by nucleic acid Syto-9 staining

Time (Min)	Concentration of commercial bleach (%)						
	Control	3.2	6.3	12.5	25	50	100
10	94.0 ± 1.49	91.6 ± 2.61	88.4 ± 2.61	82.8 ± 3.11	72.2 ± 5.97	72.4 ± 3.65	64.4 ± 7.92
30	94.0 ± 1.49	82.4 ± 4.77	85.0 ± 3.81	82.2 ± 3.63	69.2 ± 4.38	70.2 ± 3.49	52.4 ± 5.68
60	94.0 ± 1.49	83.6 ± 2.19	80.4 ± 5.32	78.4 ± 2.61	71.0 ± 4.95	68.0 ± 4.42	43.2 ± 3.11
120	94.0 ± 1.49	84.0 ± 1.41	85.8 ± 3.77	69.2 ± 3.63	71.6 ± 3.21	64.0 ± 3.39	41.2 ± 2.28
180	94.0 ± 1.49	88.4 ± 2.97	74.4 ± 3.51	63.0 ± 4.36	52.6 ± 4.22	32.6 ± 5.73	26.6 ± 5.55

Each value represents the mean (%) of triple repetitions of five determinations with the standard deviations.

Table 2. Patterns of oocyst shedding on days 4~16 postinoculation in mice inoculated with *C. parvum* oocysts contacted with commercial bleach

Time (Min)	Concentration of commercial bleach (%)						
	Control	3.2	6.3	12.5	25	50	100
10	3,996 ± 172.71	3,828 ± 270.68	3,644 ± 256.67	3,526 ± 290.05	3,440 ± 298.75	3,516 ± 388.95	3,106 ± 288.67
30	3,996 ± 172.71	3,688 ± 131.42	3,510 ± 230.22	3,540 ± 204.33	3,280 ± 393.06	3,350 ± 374.17	2,950 ± 173.21
60	3,996 ± 172.71	3,710 ± 125.10	3,600 ± 158.11	3,470 ± 144.05	3,148 ± 330.94	3,090 ± 281.51	1,676 ± 470.14
120	3,996 ± 172.71	3,818 ± 295.58	3,838 ± 222.98	3,116 ± 83.85	3,128 ± 80.12	2,606 ± 298.21	1,674 ± 484.13
180	3,996 ± 172.71	3,826 ± 298.13	3,256 ± 81.73	3,004 ± 121.78	2,462 ± 323.37	1,576 ± 290.74	1,048 ± 118.41

Each value represents the mean (10^3) of five determinations with the standard deviations.

Table 3. Parasite colonization of ileum on 16 day postinoculation in mice inoculated with *C. parvum* oocysts contacted with commercial bleach

Time (Min)	Concentration of commercial bleach (%)						
	Control	3.2	6.3	12.5	25	50	100
10	3	3	3	3	3	3	3
30	3	3	3	3	3	2	2
60	3	3	3	2	2	2	2
120	3	3	3	3	2	2	2
180	3	3	3	3	2	2	2

The index of infection was quantitated by scoring on a scale of 0 to 3 as follows: 0 = no parasite observed; 1 = a small number of parasites focally distributed in tissues (< 10% of villi colonized); 2 = a moderate number of parasites widely distributed throughout tissues (10 to 50% of villi colonized); 3 = a large number of parasites widely distributed throughout tissues (> 50% of villi colonized).

Results

Thirty tubes each containing 2×10^6 *C. parvum* oocysts were exposed to commercial bleach according to the concentration and exposure time, respectively. At the desired exposure time, 1×10^5 oocysts were picked up in each tube to another each tube and washed with DW to remove the commercial bleach. These tubes were added to nucleic acid dye Syto-9 solution and then incubated at 37ºC for 60 min in the dark. Every oocyst in each tube was examined by a fluorescent microscope. Table 1 shows the viability of oocysts contacted with commercial bleach, determined by nucleic acid Syto-9 staining. The viable oocysts in the fresh sample was 94.0 ± 1.49%. The longer *C. parvum* oocyst is exposed to commercial bleach, and the higher its concentration is, the more its viability decreases. The oocysts exposed to 100 or 50% commercial bleach for 180 min had the lowest scores in the viability as 26.6 ± 5.55% or 32.6 ± 5.73%. But the viability of all sample's oocysts was not enormously different each other between control and experimental groups.

The oocyst shedding patterns on days 4~16 PI of mice infected with oocysts contacted with commercial bleach

were not significantly different each other, there is a slight tendency to be an inverse relationship between the oocyst shedding and the exposed time and concentration of commercial bleach (Table 2).

Scores for the index of infection on days 4~16 PI determined by parasite colonization of ileum in prolonged, exposed time of 25, 50 and 100% groups are slightly reduced, in general, compared to those of control, 3.2. 6.3 and 12.5% groups, but oocysts exposed to even undiluted commercial bleach did not eliminate infectivity for mice. Meanwhile, a great number of developmental stages of *C. parvum* was found all segment sections in control group (Table 3).

Discussion

C. parvum is an undisputed waterborne pathogen of humans and other animals. This intestinal protozoon has been responsible for outbreaks of cryptosporidiosis traced back to surface and potable water sources [11, 20]. Because low numbers of oocysts present a potential risk for infection, the challenge has been to develop immunofluorescent and flowcytometric assays capable of detecting only a few oocysts [18, 19].

C. oocysts are extremely resistant to most commonly used disinfectants. Viability was not affected by exposure to 1.05 and 3% chlorine as sodium hypochlorite for up to 18 h. Long-term exposure to 10% formalin, 5~10% ammonia and 70 to 100% bleach was deemed necessary to completely eliminate infectivity [4, 14]. Although the studies of these exposures did not specifically address the use of chlorine in drinking water treatment, it is probable that chlorine disinfection alone is not sufficient to prevent *Cryptosporidium* infection. It has been reported that oocyst viability or infectivity is not affected by exposure of oocysts to 1.05~3% sodium hypochlorite solution(equivalent to 20~60% commercial bleach) for up to 18 h at 4 or 37°C and only 70~100% commercial bleach can destroy oocyst infectivity [4, 10]. *C. parvum* oocysts were still infective in an animal model after exposure to 5.25% sodium hypochlorite for 2 h at 21℃ [5].

Survival of *C. parvum* oocysts under various environmental pressures have also been studied. Slightly longer periods (> 2 h) of air drying at room temperature resulted in 100% death of the oocysts. Using inclusion or exclusion of duo-fluorogenic vital dyes staining, it was found that snap-freezing of oocysts in liquid nitrogen resulted in 100% death and slow freezing, however, was less effective at killing oocysts. After 21 h at –22℃, only 67% of oocysts had been killed, and even though this proportion increased to over 90% after 152 h, a small proportion of oocysts were still viable even after 750 h [15]. Another study using bioassay for infectivity by detection of development-stage *C. parvum* from small intestine in neonatal mice revealed that oocysts suspended in deionized water were still infectious after 168 h at 5℃, 168 h at –10℃, 24 h at –15℃, and 5~8 h at –20℃, but became non-infectious after 168 h at –15℃, 24 h at –20℃, or 1 h at –70℃ [6]. It was concluded that there was a significant health risk as a result of the probable presence of oocysts in the water in Milwaukee, Wisconsin and therefore in the dairy sanitizer solution.

Any test for disinfectant effectiveness must ultimately depend upon some measure of oocyst viability. Unfortunately, a quick, easy, reliable and completely reproducible test is not yet available. The present study assessed inactivation by uptake of the nucleic acid dye and bioassay for mouse infectivity. The nucleic acid staining by Syto-9 was used in this study as a possible indicator of oocyst viability.

In the present study, it appears to be an inverse relationship between the viability of *C. parvum* oocysts and the exposed time and concentration of commercial bleach. Scores obtained from the present study indicated that exposure of oocysts to 100 and 50% commercial bleach for as long as 180 min at room temperature significantly reduced the number of live oocysts. But the amount of the live oocysts have enough to patent infection to animals, because Yang *et al.* [21] reported that a single oocyst can induce patent *C. parvum* infection in immunosupressed C57BL/6N mice, and *C. parvum* can induce infection with as few as 10 oocysts or less in adult human volunteers [12]. These observations in the present study indicated that the oocysts exposed to commercial bleach can produce patent infection in immunosupressed mice.

Conclusion

Infectivity of *C. parvum* oocysts for mice was the most sensitive indicator of oocyst inactivation under all the experimental conditions tested. Results of the present study show that *C. parvum* oocysts exposed to undiluted commercial bleach for as long as 180 min are infectious

for animals through the bioassay. Although commercial bleach is widely used as a bacterial and viral disinfectant, the present findings indicate that it is not an effective disinfectant for *C. parvum* oocysts under practical conditions.

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