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Effects of Low Temperatures on Viability of *Cryptosporidium parvum* Oocysts

RONALD FAYER* AND THOMAS NERAD

Parasite Immunobiology Laboratory, Livestock and Poultry Sciences Institute, Agricultural Research Service, United States Department of Agriculture, Beltsville, Maryland 20705 and American Type Culture Collection, Rockville, Maryland 20852

Received 24 October 1995/Accepted 18 January 1996

Microcentrifuge tubes containing 8×10^6 purified oocysts of *Cryptosporidium parvum* suspended in 400 μl of deionized water were stored at 5°C for 168 h or frozen at −10, −15, −20, and −70°C for 1 h to 168 h and then thawed at room temperature (21°C). Fifty microliters containing 10^6 oocysts was administered to each of five to seven neonatal BALB/c mice by gastric intubation. Segments of ileum, cecum, and colon were taken for histology from each mouse 72 or 96 h later. Freeze-thawed oocysts were considered viable and infectious only when developmental-stage *C. parvum* organisms were found microscopically in the tissue sections. Developmental-stage parasites were not found in tissues from any mice that received oocysts frozen at −70°C for 1, 8, or 24 h. All mice that received oocysts frozen at −20°C for 1, 3, and 5 h had developmental-stage *C. parvum*; one of 6 mice that received oocysts frozen at −20°C for 8 h had a few developmental-stage parasites; mice that received oocysts frozen at −20°C for 24 and 168 h had no parasites. All mice that received oocysts frozen at −15°C for 8 and 24 h had developmental-stage parasites; mice that received oocysts frozen at −15°C for 168 h had no parasites. All mice that received oocysts frozen at −10°C for 8, 24, and 168 h and those that received oocysts stored at 5°C for 168 h had developmental-stage parasites. These findings demonstrate for the first time that oocysts of *C. parvum* in water can retain viability and infectivity after freezing and that oocysts survive longer at higher freezing temperatures.

Knowledge of factors affecting the survival and infectivity of oocysts of *Cryptosporidium parvum* are important with respect to the epidemiology and control of infection as well as to the storage of organisms for research purposes. A major difficulty for researchers attempting to solve biological and medical problems associated with cryptosporidiosis has been the lack of ability to acquire and store a stock of viable, infectious organisms for use over an extended period. Complete loss of infectivity was observed within 5 days at 37°C and 2 weeks at 15 to 20°C (7). Oocysts stored at 4°C underwent a progressive loss of infectivity: no infectivity was detected after 60 days in distilled water, 80 days in 5% bovine serum albumin, 100 days in phosphate-buffered saline, and 190 days in aqueous 2.5% potassium dichromate (7). Few investigators have attempted to prolong infectivity by freezing. Despite the use of cryoprotectants and numerous protocols for controlled freezing rates, three studies reported that oocysts or sporozoites were not infectious after thawing (1, 4, 7). One study reported infectivity of oocysts after thawing (6), but a coauthor found that hamsters used to test for infectivity had acquired accidental infections (5). From these studies it appeared that freezing, rather than prolonging infectivity of oocysts and sporozoites, had destroyed infectivity. In nearly all the protocols tested, organisms had been frozen to −70°C or lower temperatures. Organisms were frozen at −20°C in only three protocols, in each case for 3 days or longer (7). The present study was undertaken to determine if oocysts of *C. parvum* would retain infectivity when frozen at −20°C for shorter periods or if they retained infectivity when frozen at −15 or −10°C. The practical considerations leading to this study included the need to know if ice produced from contaminated potable water in a home or commercial freezer could serve as a source of infection if ingested shortly after freezing and if oocysts in the environment could remain infectious after longer exposure to temperatures slightly below freezing.

Source of oocysts. Oocysts of *C. parvum* (AUCP-1 isolate) were collected in feces of an experimentally infected 2-week-old calf and cleaned of fecal debris as previously described (3). Oocysts stored at 4°C in 2.5% aqueous potassium dichromate were less than 2 months old when used.

Bioassay for infectivity. Twenty-four BALB/c mouse dams, each with five to seven pups 1 to 3 days old, were purchased from the National Cancer Institute, Frederick, Md. Each litter was housed separately and had fresh water and mouse chow (Agway Prolab Diet 3000; Agway, Inc., Syracuse, N.Y.) at all times. Litters were allowed to acclimate for 1 week before pups were utilized for bioassay. Each pup received 10^6 oocysts in 50 μl of water or water alone by gastric intubation with a 24-gauge gavage needle and was killed 72 to 96 h later by CO_2 overexposure. Although 1,000 oocysts were previously found to infect 100% of 16 neonatal mice (2), the objective of the present study was to detect any oocyst survival by subsequent infectivity, so that even if only 0.1% of the oocysts were infectious after freezing, 100% of the recipients should be found to be infected in the present study. A control group received oocysts that were stored at 5°C for 168 h, the longest period for which other oocysts were stored frozen.

Histology. Segments of ileum, cecum, and colon from each mouse were fixed in 10% neutral buffered formalin, stained, and examined as previously described (3). Briefly, five or more microscopic fields of 100 epithelial cells each were examined in each segment. Scores of 0, 1, 2, 3, or 4 (0%, ≤1%, 2 to 33%, 34 to 66%, and ≥67%, respectively) designated the percent parasitized epithelial cells. Scores for the three segments for each mouse were added, and mean cumulative scores were calculated for each group (Table 1).

Freezing equipment. For freezing at −10, −15, −20, and −70°C, respectively, the following freezers were used:

* Corresponding author.

TABLE 1. Histologic scores for mice that received *C. parvum* oocysts stored unfrozen or frozen

Storage time (h)	Mean cumulative histologic score for mice receiving oocysts stored at[a]:				
	5°C	−10°C	−15°C	−20°C	−70°C
1	ND	ND	ND	4.7	0
3	ND	ND	ND	8.7	ND
5	ND	ND	ND	8.9	ND
8	ND	10.3, 10.1	7.7, 7.8	0.2	0
24	ND	10.7, 11.1	8.8, 9.3	0, 0, 0	0
168	11.2	6.5, 4.0	0, 0	0	ND

[a] Segments of ileum, cecum, and colon from each mouse were examined and scored according to percent parasitized epithelial cells as described in the text; each score is cumulative for one group of mice.

Cryomed freezing apparatus model 1010 (Cryomed, Mt. Clemens, Mich.) and Cryomed freezing apparatus model 900, Whirlpool model EHT-171HK, and So-Low model A18-100 (Environmental Equipment Co., Cincinnati, Ohio).

Experimental design. Twenty-three polypropylene microcentrifuge tubes (USA Scientific Plastics, Ocala, Fla.), each containing 8×10^6 oocysts in 400 μl of deionized water, were placed in freezers at −10 and −15°C for 8, 24, and 168 h (two tubes at each interval), −20°C for 1, 3, 5, 8, 24, and 168 h, and at −70°C for 1, 8, and 24 h (one tube at each interval, except that −20°C for 24 h had three tubes). They were thawed at room temperature and administered to mice. One microcentrifuge tube was stored in a laboratory refrigeration cabinet at 5°C for 168 h. Each litter of neonatal mice received oocysts from a single microcentrifuge tube. All microcentrifuge tubes that were removed from freezers were visually examined and found to contain ice pellets, although it could not be ascertained if the contents were completely and solidly frozen.

Phase-contrast, interference-contrast, and fluorescence microscopy were used to examine the morphology of oocysts before and after freezing. For fluorescence microscopy, a drop of the aqueous oocyst suspension dried on a treated microscope slide provided in the Merifluor Cryptosporidium/Giardia test kit (catalog no. 250050; Meridian Diagnostics, Inc., Cincinnati, Ohio) was treated with reagents also provided in the kit.

Oocyst morphology. Oocysts frozen at −70°C appeared similar whether thawed 1, 8, or 24 h after freezing. When examined by phase-contrast or interference-contrast (Fig. 1A) microscopy, some appeared to have no contents, others had partial contents, and still others appeared similar to unfrozen controls (Fig. 2A and C). Oocysts frozen at −10, −15, and −20°C for all periods up to and including 168 h were not morphologically distinguishable from unfrozen controls when examined by phase-contrast or interference-contrast microscopy (Fig. 2).

All preparations of freeze-thawed oocysts examined by fluorescence microscopy were nearly indistinguishable from fresh oocysts. Contents could not be clearly observed, and walls of all oocyst preparations fluoresced alike; however, more oocysts with cracked walls were found in specimens frozen to −70°C than in other specimens (Fig. 1B to D).

Histologic findings. The lack of detectable parasite in any stage of development in histological sections from mice intubated with *C. parvum* oocysts frozen at −15°C for 168 h, at −20°C for 24 and 168 h, and at −70°C for 1, 8, and 24 h suggested that all oocysts had been rendered noninfectious (Table 2). Developmental-stage cryptosporidia were detected in all segments of intestine examined from mice intubated with

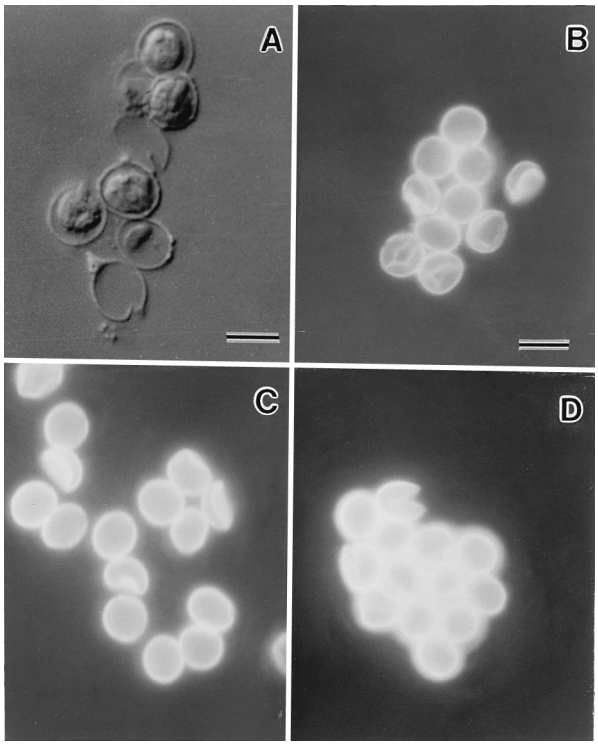

FIG. 1. Photomicrographs of *C. parvum* oocysts. (A) Interference-contrast microscopy of oocysts frozen at −70°C for 1 h; (B to D) fluorescence microscopy of fresh, unfrozen oocysts (B), oocysts frozen at −20°C for 1 h (C), and oocysts frozen at −70°C for 1 h (D). Bar, 5 μm.

oocysts frozen at −10°C for 8, 24, and 168 h, at −15°C for 8 and 24 h, and at −20°C for 1, 3, and 5 h. Mean cumulative histologic scores for groups of mice intubated with oocysts frozen for these periods appear in Table 1 and indicate that numerous oocysts had survived freezing and had initiated development of large numbers of endogenous developmental-stage parasites. For example, scores for mice receiving oocysts frozen at −10°C and −15°C for 8 and 24 h and at −20°C for 3 and 5 h reflected the fact that over 67% of the epithelial cells of the ileum of every mouse in the group were infected with developmental-stage *C. parvum*. Of six mice intubated with oocysts frozen at −20°C for 8 h, only one was infected, and only a single small cluster of developmental-stage cryptosporidia was found in the ileum of that mouse. In contrast, control mice that received unfrozen oocysts had histological scores indicating that nearly every segment of intestine from all six mice had over 67% of the epithelial cells infected. The higher scores in the control mice reflect a higher percentage of infected epithelial cells in the ceca and colons of these mice than in mice that received previously frozen oocysts. This finding suggests that freezing at any of the temperatures tested for even the shortest period rendered a portion of the oocysts noninfectious.

The fact that no developmental-stage parasites were found in mouse tissues in this study suggested that all oocysts frozen at −70°C were rendered noninfectious. This finding is supported by previous studies. Intact oocysts in the presence of cryoprotectants such as polyvinyl chloride, glycerol, and dimethyl sulfoxide were frozen and stored in liquid nitrogen for only 10 min and frozen and stored at −70°C for 20 h and 14 days (6). Upon thawing, none were infectious for mice. Sporozoites excysted from oocysts, suspended in dimethyl sulfoxide,

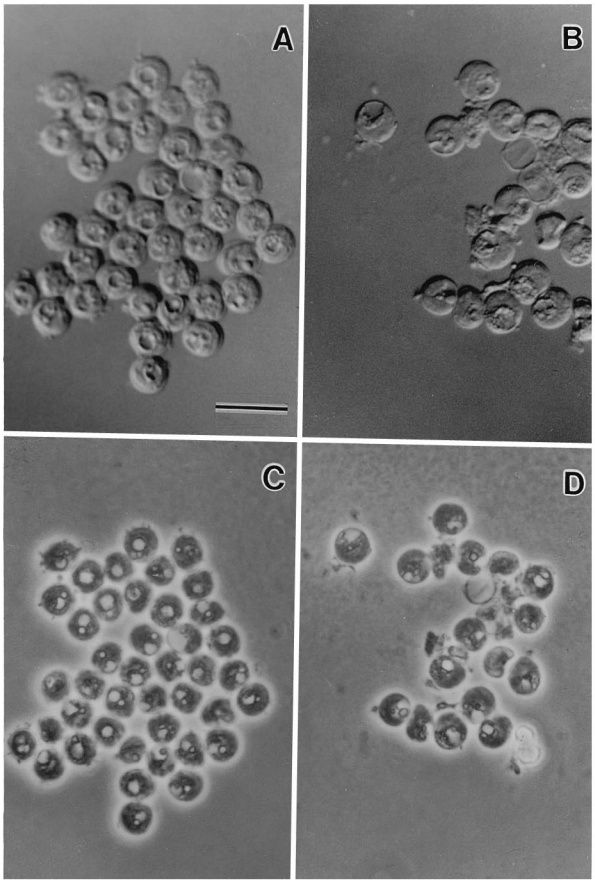

FIG. 2. Photomicrographs of *C. parvum* oocysts. (A and B) Interference-contrast microscopy of fresh, unfrozen oocysts (A) and oocysts frozen at −20°C for 18 h (B); (C and D) phase-contrast microscopy of fresh, unfrozen oocysts (C) and oocysts frozen at −20°C for 18 h (D). Bar, 10 μm.

glycerol, and polyvinylpyrrolidone, and frozen in liquid nitrogen appeared similar in morphology and movement to unfrozen sporozoites when thawed but failed to produce developmental stages in chicken embryos (1). Intact oocysts and excysted sporozoites in balanced salt solution alone or containing dimethyl sulfoxide or glycerol were frozen and stored at −70°C for 24 h or 1 week, but upon thawing they were not infectious for mice (4).

The finding of developmental-stage *C. parvum* in tissues of mice intubated with oocysts frozen at −10, −15, and −20°C is new and of epidemiologic importance. On the basis of the findings from the present study it cannot be assumed that water containing *C. parvum* oocysts is rendered noninfectious by being frozen. Furthermore, because oocysts were shown to survive longer at higher freezing temperatures, ice cubes made in the freezing compartment of a home refrigerator cannot be relied upon to be free of viable oocysts even if they have been frozen for 24 h. Although freezing compartments are supposed to maintain a temperature of −20°C, as they age the temperature tends to rise and could approach −15°C or higher. In nature, survival of oocysts might be extended beyond the times observed in the present study when water containing minerals or organic material is frozen or when oocysts are frozen at temperatures above −10°C. Such conditions might be found in areas where, after ground temperatures fall to just below freezing, a layer of snow insulates that surface from much colder air temperatures, enabling oocysts to survive perhaps for weeks or months.

TABLE 2. Viability of *C. parvum* oocysts stored frozen or unfrozen

Storage time (h)	No. of mice with *C. parvum*/no. exposed to oocysts stored at[a]:				
	5°C	−10°C	−15°C	−20°C	−70°C
1	ND	ND	ND	6/6	0/5
3	ND	ND	ND	7/7	ND
5	ND	ND	ND	6/6	ND
8	ND	6/6, 6/6	6/6, 6/6	1/6	0/6
24	ND	5/5, 6/6	6/6, 6/6	0/5, 0/6, 0/6	0/6
168	6/6	6/6, 6/6	0/6, 0/6	0/6	ND

[a] Mice were exposed to oocysts by gastric intubation, and the number of mice harboring developmental-stage cryptosporidia was determined histologically. ND, not determined.

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