APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1994, p. 2732–2735 Vol. 60, No. 8
0099-2240/94/$04.00+0

Effect of High Temperature on Infectivity of *Cryptosporidium parvum* Oocysts in Water

RONALD FAYER*

Zoonotic Diseases Laboratory, Livestock and Poultry Sciences Institute, USDA Agricultural Research Service, Beltsville, Maryland 20705

Received 23 February 1994/Accepted 10 May 1994

***Cryptosporidium parvum* oocysts suspended in 0.5 ml of distilled water were pipetted into plastic vials which were inserted into wells in the heated metal block of a thermal DNA cycler. Block temperatures were set at 5°C incremental temperatures from 60 to 100°C. At each temperature setting four vials containing *C. parvum* oocysts were placed into wells and held for 15 s before time was recorded as zero, and then pairs of vials were removed 1 and 5 min later. Upon removal, all vials were immediately cooled on crushed ice. Also, at each temperature interval one vial containing 0.5 ml of distilled water was placed in a well and a digital thermometer was used to record the actual water temperature at 30-s intervals. Heated oocyst suspensions as well as unheated control suspensions were orally inoculated by gavage into 7- to 10-day-old BALB/c mouse pups to test for infectivity. At 96 h after inoculation the ileum, cecum, and colon from each mouse were removed and prepared for histology. Tissue sections were examined microscopically. Developmental-stage *C. parvum* was found in all three gut segments from all mice that received oocysts in unheated water and in water that reached temperatures of 54.4, 59.9, and 67.5°C at 1 min when vials were removed from the heat source. *C. parvum* was also found in the ileum of one of six mice that received oocysts in water that reached a temperature of 59.7°C at 5 min. These data indicated that when water containing *C. parvum* oocysts reached temperatures of 72.4°C or higher within 1 min or when the temperature was held at 64.2°C or higher for 2 min of a 5-min heating cycle, infectivity was lost.**

Cryptosporidium parvum, a widespread zoonotic protozoan parasite, has been demonstrated to be present in the effluent from numerous water treatment plants in North America (7). Eighty-two finished drinking water samples from 66 surface water treatment plants in 14 states and one Canadian province were examined for *C. parvum*; *C. parvum* was observed in 22 samples (26.8%). In Carrolton (Ga.), Oregon (two outbreaks), Pennsylvania, Milwaukee (Wis.), and Ontario (Canada), water intended for drinking was implicated as the source of infection for numerous cases of human cryptosporidiosis (8–10). An estimated 13,000 cases of symptomatic infections were reported in the Carrolton outbreak, 3,000 were reported in the two Oregon outbreaks, 551 were reported in the Pennsylvania outbreak, 403,000 were reported in the Milwaukee outbreak, and 200 laboratory-confirmed cases were reported in the Ontario outbreak (8–10). To reduce the risk of infection during the period that potable water exceeds the safety limits for turbidity, some public health personnel have recommended that either commercially bottled water be substituted for tap water intended for drinking and for the preparation of food or tap water be boiled for various lengths of time before such use. Some public health officials have assumed that boiling will destroy *C. parvum* oocyst infectivity. Others have assumed that it is necessary to boil water for 10 to 30 min to achieve this end. However, only one study that provided data on heat inactivation of *C. parvum* oocysts could be found in the scientific literature (1). A review article referred to unpublished data indicating a loss of infectivity above 65°C for 30 min but provided no information on how that time-temperature relationship was determined (11). Because of the need to confirm the initial findings of Anderson (1) and to provide a series of time–high-temperature relationships found to render *C. parvum* oocysts noninfectious, the present study was undertaken.

* Mailing address: USDA, ARS, LPSI, Building 1040, Beltsville, MD 20705. Phone: (301) 504-8201. Fax: (301) 504-5306.

MATERIALS AND METHODS

Source of oocysts. Two holstein-friesian male calves were taken from their dams within an hour of birth and were housed in an isolated building containing pens (1.85 by 2.46 m) with concrete floors and separated by 2.00-m-high cinderblock walls. At 1 day of age the calves received injections of BoSe selenium; vitamins A, D, and E; Quatracom 2X; and iron dextran complex at the manufacturers' recommended dosages. Twice daily the calves were fed 750 ml of Calfmaker milk replacer supplemented with MicroVet VMS vitamins and minerals. Fresh water and pelleted grain were always available. Clean sawdust bedding replaced soiled or wet sawdust daily. Calves were orally infected with 1.5×10^6 *C. parvum* oocysts (AUB-1 strain). Feces were collected daily directly from the calves for 21 consecutive days. Feces found to contain oocysts were stored at 5°C in 2.5% aqueous potassium dichromate until use, less than 1 month later. Oocysts were cleaned of debris by passage through a graded series of sieves down to a pore size of 45 μm followed by density gradient centrifugation over cesium chloride as previously described (5). Residual cesium chloride was removed by centrifugation at $1,000 \times g$ for 10 min, aspiration of the supernatant, and resuspension of the pelleted oocysts in distilled water. This procedure was repeated three times.

Heat treatment. Oocysts suspended in distilled water were counted with the aid of a hemacytometer, diluted with distilled water to a concentration of 10^6 per ml, and pipetted in 0.5-ml aliquots into 40 plastic conical vials (Microtube PCR Thermal, model no. T53-300; LabSource, Chicago, Ill.) which were sealed with snap closure lids. Each of an additional nine vials containing 0.5 ml of distilled water had a hole bored in the lid just large enough to insert the probe from a Barnant model 90

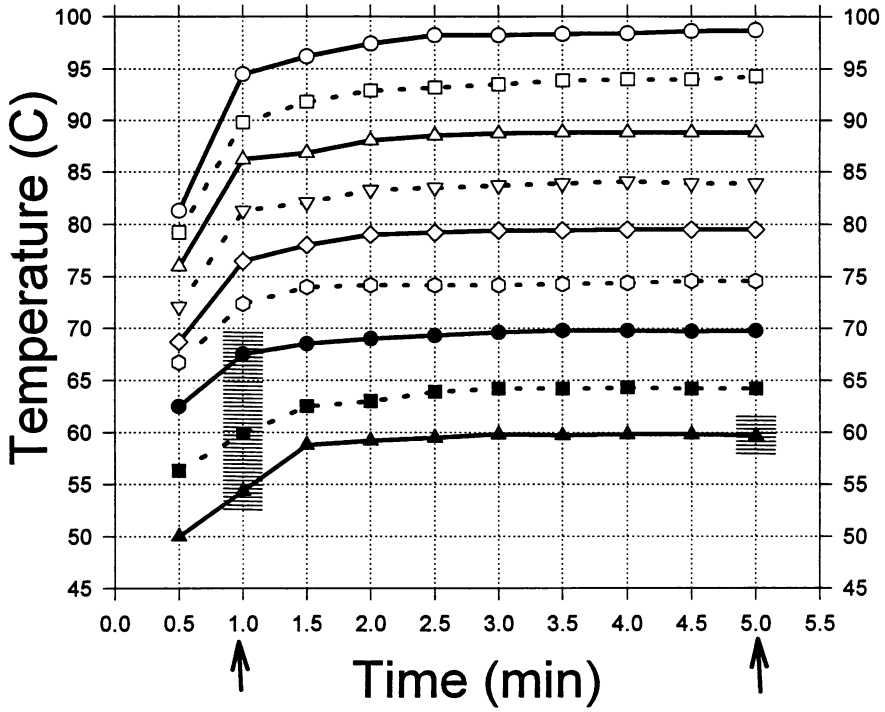

FIG. 1. Plot of time versus actual temperature of water in vials placed in wells of a DNA thermocycler preset at temperatures from 60 to 100°C at 5°C increments. Arrows designate the 1- and 5-min time intervals after which tubes containing aqueous suspensions of *C. parvum* oocysts were removed from wells. Shaded bars indicate oocyst suspensions that were infective for mice. Preset temperatures (in degrees Celsius) were 60 (▲), 65 (■), 70 (●), 75 (○), 80 (◇), 85 (▽), 90 (△), 95 (□), and 100 (○).

digital thermometer fitted with a type J thermocouple (Scientific Sales and Service, Ft. Worth, Tex.).

All vials were held at room temperature (22.1°C) before exposure to elevated temperatures in a DNA thermal cycler (model 480; Perkin-Elmer Cetus, Norwalk, Conn.). Nine temperatures were programmed into the thermal cycler: 60, 65, 70, 75, 80, 85, 90, 95, and 100°C. At each temperature setting four vials containing *C. parvum* oocysts were each inserted into 1 of 48 wells in the heated metal block of the thermal cycler. These wells had a few drops of mineral oil placed in them to facilitate the transfer of heat from the block to the vial. For each temperature setting, after 15 s was allowed for the initial heat transfer, two vials marked with the preset temperature, the time, and the letter A or B were removed at 1 min. The two remaining vials, similarly marked, were removed at 5 min. Immediately after removal from the wells in the heated block, the vials were held in crushed ice until mice were inoculated. Although the temperature setting of the heated block was displayed on the thermal cycler, the actual temperatures of the water inside the vials were recorded separately at 30-s intervals for 5 min. For each temperature setting a fresh vial containing 0.5 ml of distilled water at room temperature (22.1°C) was selected and the probe from the digital thermometer was inserted through the hole in the lid (Fig. 1). Four additional vials containing *C. parvum* oocysts in water were held at room temperature as controls and then held on ice with the previously heated vials until used for inoculation of mice.

Bioassay for infectivity. Twenty-one litters of 1- to 3-day-old BALB/c mice obtained from a commercial supplier (National Cancer Institute, Frederick, Md.) were each housed in separate rat cages on a rack that provided filtered air to each cage. Fresh water and pelleted feed were available at all times. At 7 to 10 days of age, within 15 min after oocysts were heat treated, mouse pups were orally inoculated with a 150-μl aqueous suspension containing 150,000 *C. parvum* oocysts. Each litter received oocysts treated at a different temperature-time interval. Two litters received oocysts held at room temperature. One litter received only distilled water. In each litter of six pups, three pups received oocysts from vial A and three received oocysts from vial B. Those receiving oocysts from vial B were distinguished from their littermates by staining a small spot of hair with picric acid. At 96 h after inoculation with oocysts or water alone all mice were killed by exposure to CO_2, and 3- to 5-mm segments of ileum at the ileocecal junction, of cecum, and of colon at the cecal-colonic junction were removed from each mouse and were fixed in 10% neutral buffered formalin for histology. Tissue sections stained with hematoxylin-eosin were examined by light microscopy for developmental-stage *C. parvum*.

RESULTS

Time zero was defined as 15 s after the vials were placed in the wells of a preheated metal block; the data for the actual water temperatures within the vials, taken at 30-s intervals for 5 min at each preset temperature from 60 to 100°C, are presented in Fig. 1.

At the 1-min interval, the actual water temperatures in the vials at the preset temperatures of 60, 65, 70, 75, 80, 85, 90, 95, and 100°C were 54.4, 59.9, 67.5, 72.4, 76.5, 81.3, 86.2, 89.8, and 94.5°C, respectively (Table 1). Of the six vials (two for each temperature) removed upon reaching 54.4, 59.9, and 67.5°C, all contained *C. parvum* oocysts that were still infectious for mice as determined by microscopic examination of tissues for

TABLE 1. Effect of temperature on *C. parvum* oocyst infectivity

Set temp (°C)	Actual temp (°C) at removal time[a]:	
	1 min	5 min
100	94.5	98.7
95	89.8	94.3
90	86.2	88.8
85	81.3	83.9
80	76.5	79.5
75	72.4	74.6
70	67.5[b]	69.8
65	59.9[b]	64.2
60	54.4[b]	59.7[b]

[a] Time of removal of vials from heated wells.
[b] Temperature at which *C. parvum* oocysts were infectious.

developmental-stage *C. parvum* (Fig. 1). Tissue sections from the ilea, ceca, and colons from all three mice that received the contents of vial A and the same tissues from the three mice that received the contents of vial B were all infected. No developmental-stage *C. parvum* was found in any tissues from mice that received oocysts heated to 72.4°C or higher (Fig. 1).

At the 5-min interval, the actual water temperatures in vials removed at the preset temperatures of 60, 65, 70, 75, 80, 85, 90, 95, and 100°C were 59.7, 64.2, 69.8, 74.6, 79.5, 83.9, 88.8, 94.3, and 98.7°C, respectively (Table 1). Of the tissues from the six mice that received *C. parvum* oocysts from the two vials removed upon reaching the temperature of 59.7°C, the only tissue found to be infected was that of the ileum from one of the three mice that received oocysts from vial A (Fig. 1). The tissues from all mice inoculated with *C. parvum* oocysts suspended in water that reached a temperature of 64.2°C or higher at 5 min contained no stages of *C. parvum* (Fig. 1).

The actual water temperature of the four unheated control vials was 22.1°C. The tissue sections from the ilea, ceca, and colons from the 12 mice (two litters; groups A and B in each) that received *C. parvum* oocysts from these vials were all infected.

Of the six mice that received only distilled water, none were infected.

DISCUSSION

The finding of developmental-stage *C. parvum* by microscopic examination of tissue sections of ilea, ceca, and colons from mice orally inoculated with *C. parvum* oocysts indicated that oocysts remained infectious when exposed to water temperatures that rose from ambient temperature to 67.5°C in 1 min but were rendered noninfectious upon exposure to temperatures of 72.4°C or higher. *C. parvum* oocysts were also rendered noninfectious when temperatures reached 64.2°C or higher for 2 min or longer (Fig. 1).

Because microscopic examination of tissue sections from the ileum, cecum, and colon revealed developmental-stage *C. parvum* in all such tissues from all mice that received unheated *C. parvum* oocysts, the batch of oocysts used in the present study was determined to be uniformly and highly infectious. The absence of any developmental-stage *C. parvum* in tissues from mice that received only distilled water indicated the lack of transmission of *C. parvum* oocysts between litters during the handling and housing of the mice.

C. parvum oocyst dose-response characteristics have been examined by using different strains of mice and parasites. Of 19 strains of mice tested, only BALB/c neonates and C57BL/6J *bg*^{j} (beige) adults were susceptible to infection with 10^6 *C. parvum* oocysts (3). A 50% infective dose of 60 oocysts was reported for BALB/c neonates, without supporting data (6). A 50% infective dose of 79 oocysts and a minimal infectious dose of 25 oocysts (in 2 of 25 mice) were reported for CD-1 neonates (4). Among five neonatal BALB/c mice orally inoculated by gavage with 10 *C. parvum* oocysts less than 1 month of age from the same isolate used in the present study, no developmental-stage *C. parvum* could be found in tissue sections of ilea, ceca, or colons obtained 96 h after inoculation (unpublished data). On the basis of these published and unpublished results it is estimated that heating to 72.4°C or higher at 1 min and to 64.2°C or higher at 5 min reduced the number of infective oocysts from 150,000 to a number greater than 0 and less than 25. It is likewise estimated that heating to 59.7°C at 5 min reduced the number of infective oocysts from 150,000 to between 25 and 79.

Differences in the preparation of *C. parvum* oocyst inoculum, in the heating methods, and in the time-temperature intervals tested to render *C. parvum* oocysts noninfectious may account for some differences between the neutralization of *C. parvum* oocysts in the present study and neutralization results obtained by Anderson (1). However, an overall trend was apparent. Unknown numbers of *C. parvum* oocysts in bovine ileal scrapings, feces, and cecal contents were heated and fed to mice, and tissue sections of ilea, ceca, and colons from the mice were examined microscopically for developmental-stage *C. parvum* (1). In two experiments oocyst infectivity was lost when ileal scrapings were held at 45°C for 5 min and when cecal contents were held at 45°C for 20 min, although infectivity was not lost in three other experiments when ileal scrapings or feces were held at 45°C for 2 and 4 min or at 50°C for 2 min (1). Infectivity was lost when ileal scrapings were held at 55°C or higher for 2 min or longer and when feces were held at 60°C or higher for 6 min (1). On the basis of these findings, Anderson (1) concluded that the lower requirements for milk pasteurization would neutralize *C. parvum* infectivity, but he indicated a need to test higher temperatures and shorter time intervals.

In the present study, when a virtually pure aqueous suspension of *C. parvum* oocysts free of extraneous biological debris was used, infectivity remained high when oocysts were exposed to temperatures as high as 67.5°C with a heat-up time of 1 min. Infectivity was greatly reduced but still was not eliminated when oocysts were held in water that reached 59.7°C at 5 min. However, when oocysts were exposed to temperatures of 72.4°C or higher with a heat-up time of 1 min, infectivity could not be detected in any of 108 tissues from 36 mice.

These findings indicate that *C. parvum* oocysts in water can be rendered noninfectious when held for a relatively short time at temperatures far below boiling. However, the conditions used for testing in the present study eliminated the potentially limitless number of variables that might be expected in water that contains mineral or vegetable sediment. Other complex solutions, such as milk, with a variety of proteins, fats, vitamins, and minerals might also yield results slightly different from those obtained in the present study. The extremely short pasteurization times permitted for milk under the *Grade "A" Pasteurized Milk Ordinance* (2), which designates temperatures of 72, 89, 90, 94, 96, and 100°C for 15, 1.0, 0.5, 0.1, 0.05, and 0.01 s, respectively, have not been tested for efficacy against *C. parvum* oocysts in either water or milk. Although the present study presents clear safety guidelines for heat inactivation of *C. parvum* oocysts, future studies should be conducted to determine if the specific milk pasteurization requirements will guard against *C. parvum* oocyst infectivity.

ACKNOWLEDGMENTS

I thank Eva Kovacs for technical assistance, M. C. Jenkins for technical suggestions, and M. Fleming for graphics.

REFERENCES

1. **Anderson, B. C.** 1985. Moist heat inactivation of *Cryptosporidium* sp. Am. J. Public Health **75**:1433–1434.
2. **Anonymous.** 1989. Grade "A" pasteurized milk ordinance. Public Health Service, Food and Drug Administration, publication 229, p. 78. U.S. Government Printing Office, Washington, D.C.
3. **Enriquez, F. J., and C. R. Sterling.** 1991. *Cryptosporidium* infections in inbred strains of mice. J. Protozool. **38**:100S–102S.
4. **Finch, G. R., C. W. Daniels, E. K. Black, F. W. Schaeffer III, and M. Belosevic.** 1993. Dose response of *Cryptosporidium parvum* in outbred neonatal CD-1 mice. Appl. Environ. Microbiol. **59**:3661–3665.
5. **Kilani, R. T., and L. Sekla.** 1987. Purification of *Cryptosporidium* oocysts and sporozoites by cesium chloride and percoll gradients. Am. J. Trop. Med. Hyg. **36**:505–508.
6. **Korich, D. G., J. R. Mead, M. S. Madore, N. A. Sinclair, and C. R. Sterling.** 1990. Effects of ozone, chlorine dioxide, chlorine, and monochloramine on *Cryptosporidium parvum* oocyst viability. Appl. Environ. Microbiol. **56**:1423–1428.
7. **LeChevallier, M. W., R. G. Lee, and J. B. Rose.** 1991. *Giardia* and *Cryptosporidium* in water supplies. AWWA Research Foundation, American Water Works Association, Denver, Colo.
8. **Levine, W. C., and G. F. Craun.** 1990. Waterborne disease outbreaks, 1986–1988. Morbid. Mortal. Weekly Rep. **39**(SS-1):1–13.
9. **Moore, A. C., B. L. Herwaldt, G. F. Craun, R. L. Calderon, A. K. Highsmith, and D. D. Juranek.** 1993. Surveillance for waterborne disease outbreaks—United States, 1991–1992. Morbid. Mortal. Weekly Rep. **42**(SS-5):1–22.
10. **Stone, P. (Waterloo Regional Health Unit, Waterloo, Ontario, Canada).** 1994. Personal communication.
11. **Tzipori, S.** 1983. Crytosporidiosis in animals and humans. Microbiol. Rev. **47**:84–96.