APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1997, p. 3844–3850 Vol. 63, No. 10
0099-2240/97/$04.00+0

Assessment of a Dye Permeability Assay for Determination of Inactivation Rates of *Cryptosporidium parvum* Oocysts

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Received 21 May 1997/Accepted 30 July 1997

The ability to determine inactivation rates of *Cryptosporidium parvum* oocysts in environmental samples is critical for assessing the public health hazard of this gastrointestinal parasite in watersheds. We compared a dye permeability assay, which tests the differential uptake of the fluorochromes 4′-6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) by the oocysts (A. T. Campbell, L. J. Robertson, and H. V. Smith, Appl. Environ. Microbiol. 58:3488–3493, 1992), with an in vitro excystation assay, which tests their ability to excyst and, thus, their metabolic potential and potential for infectivity (J. B. Rose, H. Darbin, and C. P. Gerba, Water Sci. Technol. 20:271–276, 1988). Formaldehyde-fixed (killed) oocysts and untreated oocysts were permeabilized with sodium hypochlorite and subjected to both assays. The results of the dye permeability assays were the same, while the excystation assay showed that no excystation occurred in formaldehyde-fixed oocysts. This confirmed that oocyst wall permeability, rather than metabolic activity potential, was the basis of the dye permeability viability assessment. A previously developed protocol (L. J. Anguish and W. C. Ghiorse, Appl. Environ. Microbiol. 63:724–733, 1997) for determining viability of oocysts in soil and sediment was used to examine further the use of oocyst permeability status as an indicator of oocyst viability in fecal material stored at 4°C and in water at various temperatures. Most of the oocysts in fresh calf feces were found to be impermeable to the fluorochromes. They were also capable of excystation, as indicated by the in vitro excystation assay, and were infective, as indicated by a standard mouse infectivity assay. The dye permeability assay further showed that an increase in the intermediate population of oocysts permeable to DAPI but not to PI occurred over time. There was also a steady population of oocysts permeable to both dyes. Further experiments with purified oocysts suspended in distilled water showed that the shift in oocyst populations from impermeable to partially permeable to fully permeable was accelerated at temperatures above 4°C. This sequence of oocyst permeability changes was taken as an indicator of the oocyst inactivation pathway. Using the dye permeability results, inactivation rates of oocysts in two fecal pools stored in the dark at 4°C for 410 and 259 days were estimated to be 0.0040 and 0.0056 oocyst day^{-1}, respectively. The excystation assay gave similar inactivation rates of 0.0046 and 0.0079 oocyst day^{-1}. These results demonstrate the utility of the dye permeability assay as an indicator of potential viability and infectivity of oocysts, especially when combined with improved microscopic methods for detection of oocysts in soil, turbid water, and sediments (L. J. Anguish and W. C. Ghiorse, Appl. Environ. Microbiol. 63:724–733, 1997).

Little is known about the survival of *Cryptosporidium parvum* oocysts in natural environments once they are shed by their host (11, 18). Various environmental stresses, such as temperature extremes and desiccation, have been shown to inactivate oocysts under close-to-natural conditions (20), but little is known about the survival kinetics of oocysts under the actual conditions that exist in watersheds. Therefore, integrated natural disinfection processes are not usually taken into account in protective strategies for public water supplies.

The infective agent of cryptosporidiosis is the thick-walled, environmentally resistant oocyst stage of *C. parvum* (6). It is assumed that this resistance is related to the metabolic dormancy of the oocyst and to the impermeability of the oocyst wall, which confers protection against agents that may stimulate excystation or exposure to lethal chemical agents in the environment. Campbell et al. (4, 5) developed a differential fluorochrome dye uptake assay (dye permeability assay) to assess the viability of *C. parvum* oocysts. We have recently shown (1) that a slightly modified version of the dye permeability assay can be used for assessment of viability of oocysts in feces, soil, and sediment samples.

In this study, we examined the dye permeability assay in greater detail by comparing it with in vitro excystation and animal infectivity assays and by investigating the inactivation pathway of oocysts as indicated by changes in their permeability at different temperatures. The dye permeability and excystation assays yielded comparable inactivation rates for oocysts stored in feces at 4°C. The results demonstrated the utility of the dye permeability assay for determining inactivation rates and studying mechanisms of inactivation of oocysts under a variety of environmental conditions.

MATERIALS AND METHODS

Oocyst purification. Feces from 6- to 20-day-old Holstein calves with cryptosporidiosis were processed by a continuous-flow differential density flotation method similar to that described by Vetterling (22). Fecal material was mixed with an unadulterated sucrose solution (specific gravity, 1.3 g cm^{-3}), and a chemical centrifuge with a rotor (International Centrifuge model V 10867-8; International Equipment Co., Boston, Mass.) equipped with a nonperforated basket was used to separate oocysts from fecal material by continuous-flow centrifugation at room temperature. After several centrifugations using sucrose solutions with specific gravities ranging from 1.0 to 1.15, the cleaned oocysts were further purified and concentrated by using a sucrose step gradient in 50-ml centrifuge tubes. Purified oocysts were washed three times by centrifugation in

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cold distilled water at 2,100 × *g* for 10 min to remove sucrose, adjusted to a concentration of 10^7 ml^{-1}, and stored in distilled water containing 100 U of penicillin G sodium ml^{-1}, 100 μg of streptomycin sulfate ml^{-1}, and 0.25 μg of amphotericin B ml of suspension^{-1} at 4°C. Each lot of purified oocysts was tested by the dye permeability assay (see below) after purification and periodically during storage. Some lots were also tested with the in vitro excystation assay (see below).

Dye permeability assay. The dye permeability assay, the details of which have been described previously (4), was modified by addition of a fluorescent antibody detection step (1). Stock solutions of 4′,6-diamidino-2-phenylindole (DAPI) (2 mg ml^{-1} in high-performance liquid chromatography-grade methanol) and propidium iodide (PI) (1 mg ml^{-1} in 0.1 M phosphate-buffered saline [PBS; 0.028 M $NaH_2PO_4 \cdot H_2O$, 0.072 M NaH_2PO_4, 0.145 M NaCl, pH 7.2]) were added to aliquots of the sample in 1.5-ml microcentrifuge tubes at a ratio of 1 to 10 (vol/vol), the contents of the tubes were mixed with a Vortex mixer, and the tubes were incubated in the dark at 37°C for 2 h. Each aliquot was stained with Hydrofluor antibody (Meridian Diagnostics, Cincinnati, Ohio, or EnSys Environmental Products, Inc., Research Triangle Park, N.C.) as described previously (1), washed twice with PBS, and resuspended to their original volume in 0.3 M 1,4-diazabicyclo[2.2.2]octane (DABCO) in 0.1 M PBS (pH 7.2) (DABCO-PBS). The samples were stored in the dark at 4°C until examined (within 72 h) under the microscope and by microscopic procedures as described previously (1) and below.

In vitro excystation assay. The acid pretreatment protocol was as described by Robertson et al. (19) (protocol M). Each aliquot of sample was centrifuged in a microcentrifuge at 11,300 × *g* for 30 s and then washed once by resuspending the pellet in 1 ml of acidified Hanks' balanced salt solution (HBSS; Sigma Chemical Co., Saint Louis, Mo.) (100 μl of 1 M HCl in 10 ml of HBSS, pH 2.5) and centrifuging again. The second pellet was resuspended in 1 ml of the acidified HBSS and incubated at 37°C for 1 h in the dark. Oocyst suspensions were then sedimented by centrifugation, and the pellets were washed three times with warm (37°C) HBSS to remove residual acid. The final pellet was resuspended in warm HBSS to a volume of 100 μl. Ten microliters of 2.2% sodium bicarbonate in HBSS and 10 μl of 1% sodium deoxycholate in Hanks' minimal essential medium (Sigma) were added to each sample. The suspensions were again mixed on a Vortex mixer and then incubated for 3.5 h at 37°C in the dark. After incubation, 10 μl of primary Hydrofluor antibody was added to each sample, which was then mixed on a Vortex mixer and returned to the incubator for 0.5 h, for a total excystation incubation time of 4 h. The samples were centrifuged again, washed in PBS (pH 7.2), stained with Hydrofluor secondary-antibody labeling reagent, washed once and then resuspended in DABCO-PBS, and stored at 4°C until examined (within 72 h) by differential interference contrast (DIC) and epifluorescence microscopy (see below).

Mouse infectivity. Neonatal CD-1 mice produced by timed-pregnant females (Charles River Laboratories, Wilmington, Mass.) were used for the infectivity assays. Mice were housed in barrier facilities of the Laboratory Animal Services Unit of the College of Veterinary Medicine, Cornell University. Two infectivity assays were performed. For the purpose of 50% infective dose (ID_{50}) calculations, the data obtained by the two assays were treated as a single data set. In both assays, 6-day-old mouse pups were inoculated with prepared dilutions of freshly purified *C. parvum* oocysts. These oral doses were prepared by serial dilution specifically to correspond to the following concentrations: 0, e^2, e^3, $e^{3.5}$, e^4, e^5, e^6, e^8, and e^{10} (or 0, 7, 20, 33, 55, 148, 403, 2,980, and 22,026 oocysts per dose, respectively). With these doses of *C. parvum*, 23, 10, 9, 28, 11, 10, 19, 12, and 10 mouse pups, respectively, were inoculated. Mouse pups were killed 6 days postinoculation by deep methoxyflurane anesthesia followed by vertebral dislocation. After the death of a pup, its cecum was excised and the fecal material was removed from it and placed in sterile 1.5-ml Eppendorf microcentrifuge tubes. The fecal material was assayed for the presence of oocysts by the ProSpect *Cryptosporidium* Rapid Assay, an enzyme immunoassay for the detection of *C. parvum* antigen in aqueous extracts of fecal specimens (Alexon, Inc., Sunnyvale, Calif.). ID_{50} determinations were made by binary logistic regression (Minitab, release 11; Minitab, Inc., State College, Pa.) as described by Finch et al. (10).

Sodium hypochlorite permeabilization and formaldehyde fixation of oocysts. Four replicate 100-μl aliquots of fresh, purified oocysts in a distilled water-antibiotic storage mixture were centrifuged, as described above, and exposed to 5.25% sodium hypochlorite solution for 5 min on ice. This treatment has been reported to increase the permeability of the oocyst wall (17). Oocysts were then washed at least three times by centrifugation in PBS (pH 7.2) to remove the hypochlorite. The final pellet was resuspended in 100 μl of PBS. The hypochlorite-treated oocysts, as well as untreated oocysts from the original storage suspension, were fixed in 4% formaldehyde–PBS (1 part methanol-free 16% formaldehyde [Polyscience, Warrington, Pa.] in 3 parts PBS, pH 7.2) for 1 h at 4°C. Oocysts were then washed three times in PBS to remove the formaldehyde.

Effects of temperature on oocyst permeability. Duplicate or triplicate aliquots (100 μl) of a fresh suspension of purified *C. parvum* oocysts (in water plus antibiotics) were placed in 1.5-ml microcentrifuge tubes and incubated at 22, 37, 60, 70, 80, and 100°C for various durations from 5 s to 150 h. Samples were exposed to all higher temperatures (≥60°C) by incubation in a heating block (Scientific Products, Evanston, Ill.). All tubes were cooled immediately after each sampling time, the 22 and 37°C samples being cooled in a refrigerator at 4°C and the other tubes being transferred from the heating block to ice. The dye permeability assay was performed after each temperature exposure time period, and two 10-μl aliquots from each sample were examined microscopically. At least 100 oocysts in each aliquot were examined.

Preparation of fecal pools. Fecal samples were collected from 6- to 20-day-old Holstein calves at Cornell's Teaching and Research Center (Harford, N.Y.). Samples were immediately transported to the laboratory; smears (10 μl) of each were made on microscope slides, and the remainder of each sample was stored at 4°C. After being dried overnight at room temperature, the smears were stained by using a Hydrofluor monoclonal fluorescent antibody kit in accordance with the manufacturer's protocol. The smears were mounted in 10 μl of DABCO-PBS, and *C. parvum* oocysts were counted at a magnification of ×1,000 (1). Samples that showed at least 10^6 oocysts g of feces^{-1} were pooled. The fecal material was strained through a fine-mesh tea strainer to remove large particulates and stored in 500-ml glass screw-cap bottles at 4°C. Four such fecal pools (pools A to D) were prepared. Each consisted of approximately 400 ml of fecal slurry containing 40 to 95% (vol/vol) solids. Pool A was derived from the fecal material of 2 calves, pool B contained feces from 8 calves, pool C consisted of feces from 4 calves, and pool D had fecal material from 12 calves. The final concentration of oocysts in each pool was determined by counting the number of oocysts that had been stained with the Hydrofluor antibody in a 1:10 or 1:100 dilution of feces in PBS (pH 7.2), using a Neubauer-Levy-Hausser counting chamber. To count the oocysts, 10 10-μl aliquots of diluted and stained fecal sample were examined through a 40× Neofluar objective on the Zeiss LSM 210 instrument under DIC and epifluorescence optics (see reference 1 and below).

Experimental design for determining oocyst inactivation rates in fecal pools. Oocyst inactivation rates (i.e., loss of viability) at 4°C were determined by the dye permeability and in vitro excystation assays at various intervals during long-term storage periods (0 to 410 days for pool A and 0 to 259 days for pool B). At each sampling time, the fecal pool was mixed thoroughly for at least 1 min. A 5-ml sample was removed, placed in a 15-ml disposable plastic centrifuge tube, and mixed with a Vortex mixer for 1 min. Ten aliquots (100 μl each) of the mixed fecal material were then placed in 1.5-ml microcentrifuge tubes and incubated with DAPI and PI as described for the dye permeability assay. After incubation of the tubes for 2 h at 37°C, a 20-μl aliquot was removed from each and stained with the Hydrofluor antibody to complete the dye permeability assay. The remaining 80 μl was subjected to the in vitro excystation assay. Statistical analyses of preliminary results (data not shown) indicated that the results from the 10 aliquots for each sampling time would provide 95% confidence that the final estimates would be within approximately 9% of the true mean, expressed as the percentage of viable oocysts in each pool (2, 13, 21). Therefore, for each assay, a 10-μl subsample of each replicate was placed on an agar-coated microscope slide and a 22- by 22-mm coverslip was applied with slight pressure to spread the sample evenly to the edge of the coverslip. At least 100 oocysts in randomly chosen fields in each smear were identified by antibody fluorescence and categorized for differential dye uptake and structure by epifluorescence and DIC microscopy (1). At each sampling time, at least 1,000 oocysts were examined per viability assay. The relationship of dye permeability and structural appearance to the viability of each oocyst observed has been described elsewhere (1, 5, 18). We adopted the convention that the sum of impermeable (DAPI- and PI-negative [DAPI− PI−]) and semipermeable (DAPI-positive [DAPI+] PI−) oocysts was the number of viable oocysts (1).

For the in vitro excystation assay, the percentage of viable oocysts was determined by subtracting the number of empty oocysts observed by DIC and antibody fluorescence microscopy before excystation from the number of empty oocysts observed after excystation. This difference was then added to the number of partially empty oocysts to obtain the total number of excysted (viable) oocysts for each aliquot.

The dye permeability and excystation assays were run in conjunction with one another. Neither exposure of a sample to DAPI and PI before acid pretreatment nor the incubation period of the excystation assay affected excystation. Thus, the dye permeability of each intact or partially intact oocyst could be observed by examining fluorescence after the excystation assay. Using this technique, statistical comparisons of the results of both assays done on the same sample could be made.

Microscopy. All samples were examined with a Zeiss LSM-210 microscope (1), in the conventional DIC and epifluorescence mode, equipped with a triple excitation-emission filter set (catalog no. 61001; Chroma Technology Corp., Brattleboro, Vt.) with excitation bands at 390 to 410, 485 to 510, and 555 to 585 nm and emission bands at 450 to 475, 510 to 550, and 595 to 660 nm. A separate UV filter combination (excitation bands at 310 to 395 nm) was used for DAPI fluorescence. A Zeiss 100×/1.3 Plan-Neofluar DIC objective combined with 10× eyepieces was used for all microscopy procedures except for enumerations, which were done with a Zeiss 40×/0.85 Plan-Neofluar DIC dry objective.

Statistical analysis. Except when otherwise stated, all statistical analyses of data were performed with Minitab statistical software.

RESULTS

Dependence of dye uptake on oocyst wall permeability. The mechanism of the dye permeability assay was tested to confirm the hypothesis (1, 4) that differential uptake of the fluoro-

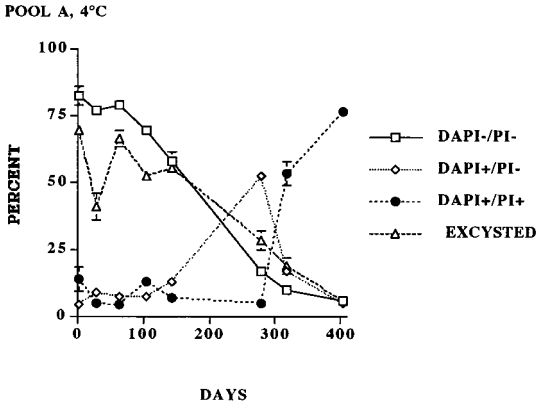

FIG. 1. Dynamics of dye permeability and viability of *C. parvum* oocysts as determined by the dye permeability assay compared to the in vitro excystation assay for fecal pool A stored at 4°C for 400 days. Each point corresponds to one in Fig. 3 (pool A); SE bars that are not visible are smaller than the symbols in the graph.

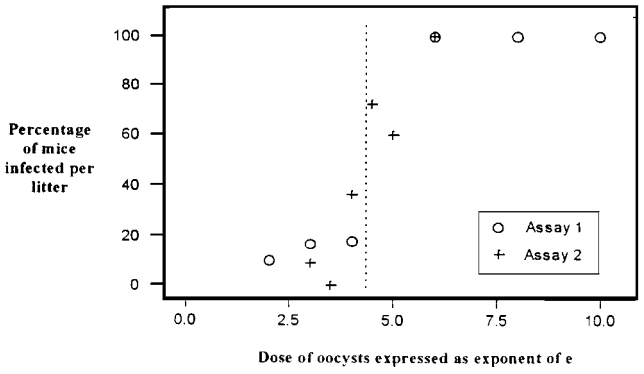

FIG. 2. Dose response of mouse pups inoculated with oocysts of *C. parvum*. The vertical line represents the ID_{50} calculated by binary logistic regression and represents $e^{4.4}$ or 81.5 oocysts. From left to right, the indicated values along the *x* axis are equal to 1, 12, 148, 1,808, and 22,026 oocysts.

chromes (DAPI and PI) by oocysts was attributable to differences in the permeability of the oocyst wall and not linked to the metabolic activity of the sporozoites. We reasoned that if the assay results were dependent on wall permeability and not on sporozoite activity, oocysts killed with formaldehyde and then permeabilized by treatment with sodium hypochlorite should take up DAPI but not PI and should not excyst. Untreated and permeabilized oocysts should also take up DAPI, but not PI, and they should excyst. When this experiment was done with freshly purified oocysts, the expected results were obtained: at first all oocysts were impermeable (i.e., 97 and 99% of the fixed and untreated oocysts, respectively, did not take up either dye [data not shown]). In vitro excystation of the fixed and untreated oocysts showed that none of the formaldehyde-fixed oocysts excysted whereas the untreated oocysts showed a normal proportion of excystation (approximately 90%). As predicted, after hypochlorite permeabilization, all fixed and untreated oocysts showed a decrease in DAPI− PI− oocysts and a corresponding increase in DAPI+ PI− oocysts (data not shown). We concluded from these results that excystability (i.e., sporozoite metabolic activity) per se did not control dye uptake by the oocysts. The dye permeability assay should be viewed as a test of oocyst wall permeability rather than one of oocyst metabolic activity.

Comparison of dye permeability with in vitro excystation and mouse infectivity. The dye permeability assay was performed on 24 batches of *C. parvum* oocysts purified from infected calf feces, and the results for each permeability category were expressed (mean percentages ± standard error [SE]). The vast majority of oocysts (84.8% ± 2.3%) were impermeable to DAPI and PI (DAPI− PI−). A smaller fraction (8.2% ± 1.3%) was permeable to DAPI but not to PI (DAPI+ PI−). The sum of these two categories was considered to be the proportion of potentially viable oocysts (93.6% ± 1.4%) in the purified batch. Dead oocysts were either permeable to both dyes (5.1% ± 1.3%) or empty (1.8% ± 0.7%). A similar distribution of oocysts was observed when fecal material was pooled before purification. Again, most of the oocysts in the pooled calf feces were impermeable to both DAPI and PI (Fig. 1). These impermeable oocysts displayed distinct internal structures under DIC optics and showed distinct outlines of sporozoites and oocyst walls, suggesting their potential viability (1). As seen in Fig. 1, the oocysts in each pool became more permeable, first to DAPI and then to PI, during storage at 4°C. An initial decline in impermeable oocysts was followed by an increase and then a decline in DAPI+ PI− oocysts, followed by an increase in DAPI+ PI+ and empty oocysts.

To determine the infectivity of the freshly purified oocysts, a standard mouse infectivity assay was performed on one batch of purified oocysts. The ID_{50} for one batch of purified oocysts was 81.5 (or $e^{4.4}$) oocysts as determined by binary logistic regression (Fig. 2). All mouse pups receiving ≥400 (or e^{6}) oocysts were infected. None of the nine mouse pups receiving 33 (or $e^{3.5}$) oocysts developed patent infections. One of 10 mouse pups receiving 7 (or e^{2}) oocysts developed a patent infection. Two litters of mouse pups that were used as negative controls were completely free of infection. Results of the dye permeability assay that was performed on the purified oocysts used in the infectivity experiment indicated that 94% were viable (85% DAPI− PI−, 9% DAPI+ PI−) and 6% were nonviable (DAPI+ PI+) or empty.

To determine the stability of oocyst numbers in fecal pools stored at 4°C, the concentrations of oocysts in three of the four fecal pools were determined at several time points after they were prepared. Oocyst concentrations within each fecal pool were compared by using the two-sample *t* test with 95% confidence. The results showed that oocyst numbers did not change significantly in any pool during storage at 4°C. Times between sampling ranged from 292 to 356 days, and densities ranged from 5×10^{-6} to 1×10^{-7} oocysts ml of feces^{-1}. There was an increase in oocyst numbers recorded for pool A on day 491; this increase was, however, attributed to analytical error because oocysts would be expected not to replicate under the storage conditions employed.

Dye permeability of oocysts before and after excystation. Comparison of the dye permeability results obtained before and after excystation indicated that the main source of excysted oocysts originated from the DAPI− PI− fraction (compare the data in Fig. 1 with the results for pool A in Fig. 3). The correlation coefficient (*r* value) between DAPI− PI− oocysts and excysted oocysts in pool A was 0.91. The mean percentage (± SE) of DAPI− PI− oocysts excysting at each sampling period for pool A was 93.3 (± 7.9). Only on sampling day 278 did the results of the in vitro excystation assay indicate that a fraction of the DAPI+ PI− oocysts in pool A had excysted. The number of DAPI+ PI− oocysts after in vitro excystation was greater than the number before excystation, indicating an accumulation of nonexcysted DAPI+ PI− oocysts (data not shown). Pool B showed a similar pattern. The distribution of DAPI+ PI+ oocysts before excystation for pools A and B did

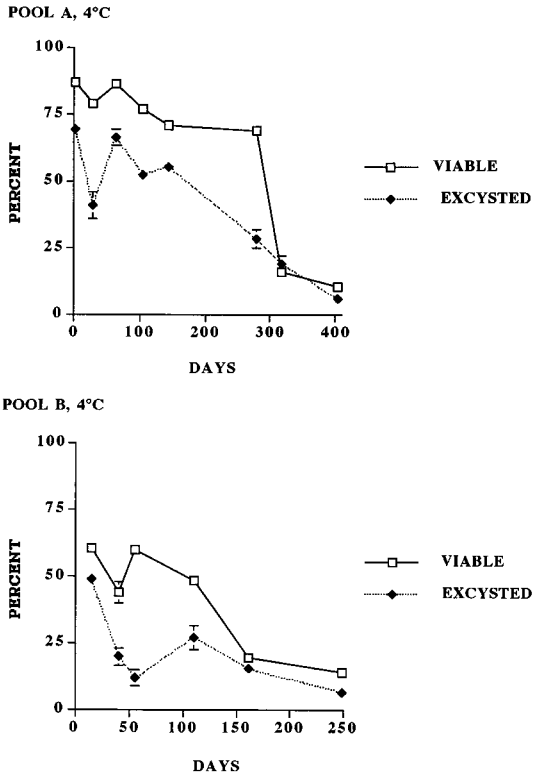

FIG. 3. Mean percent viability of *C. parvum* oocysts in two pools (A and B) of infected calf feces stored at 4°C. Each point was determined at a designated time by both the fluorochrome dye permeability and in vitro excystation assays as described in Materials and Methods. The sum of DAPI− PI− and DAPI+ PI− oocysts (Fig. 1) is the number reported as viable. Each point represents the mean ± the SE for 10 replicate samples in which at least 100 oocysts per sample per assay were counted and characterized at random as viable or nonviable by the two assays. SE bars that are not visible are smaller than the symbols in the graph. Net percent excystation was determined by subtracting the percentage of excysted oocysts present before excystation from the percentage present after excystation.

not significantly differ from the fraction of DAPI+ PI+ oocysts after excystation. The inverse correspondence between the decrease in number of excysted oocysts and the increase in the percentage of DAPI+ PI+ oocysts further indicated the loss of excystability by DAPI+ PI+ oocysts.

Effect of temperature on dye permeability. Purified oocysts incubated in distilled water at 22°C showed a decrease over time in the number of DAPI− PI− oocysts and a corresponding increase in the number of DAPI+ PI− oocysts; there was also an increase in the proportion of DAPI+ PI+ oocysts, reflecting the overall decline in viability of the oocysts incubated at 22°C (Fig. 4). Similar experiments in which freshly purified oocysts were incubated at 37, 60 (not illustrated), 70, 80, and 100°C (not illustrated) indicated the same changes in permeability of the oocysts from DAPI− PI− to DAPI+ PI− to DAPI+ PI+. Comparisons of this change from DAPI− PI− to DAPI+ PI− oocysts in several different experiments, e.g., fecal pool A held at 4°C (Fig. 1) and purified oocysts exposed to temperatures of 70 and 80°C (Fig. 4), indicated the development of a maximum intermediate level of approximately 50% DAPI+ PI− oocysts before the final shift to nonviable DAPI+ PI+ oocysts occurred.

DISCUSSION

We concluded from these studies that while the dye permeability assay does not test viability directly, it can be used as a reasonable indicator of potential viability (excystability). Our results also show that the majority of impermeable oocysts in fresh feces are infective and that the impermeable oocysts may pass through an intermediate, partly permeable (DAPI+ PI−) stage before they become fully permeable to both dyes (inactive).

The percentage of potentially viable oocysts in the pooled calf feces held at 4°C decreased over time, as indicated by their dye permeability (Fig. 3). In pool A, 10% of the oocysts were still viable after 410 days of storage; in pool B, 14% of the oocysts were still viable after 259 days. In vitro excystation of the same pooled oocysts showed a similar rate of decline in excystability (Fig. 3). However, the proportion of excystable

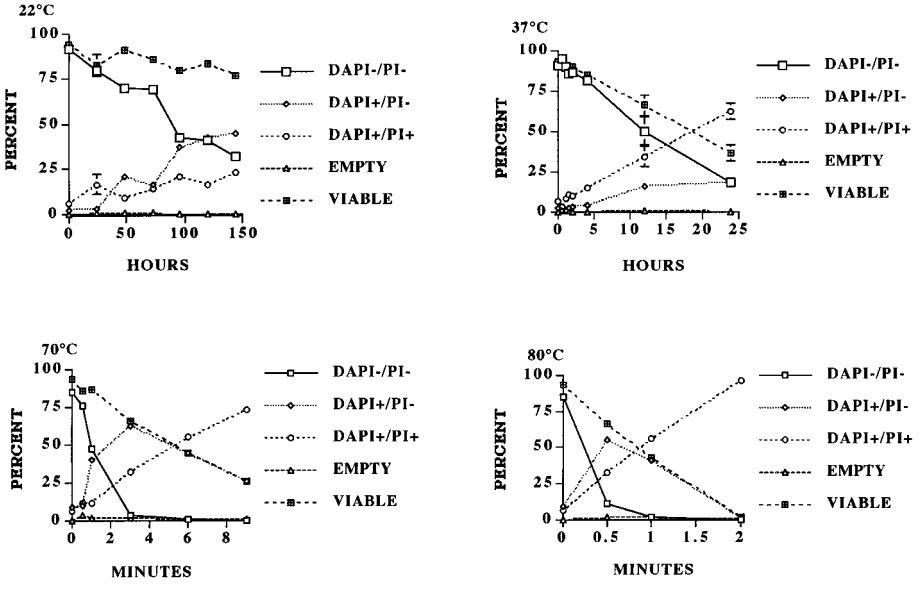

FIG. 4. Dynamics of dye permeability of fresh purified oocysts exposed to temperatures of 22, 37, 70, and 80°C. The sum of DAPI− PI− and DAPI+ PI− oocysts is the number reported as viable. Each point represents the mean percentage ± the SE for at least duplicate samples in which 100 oocysts were characterized at random.

TABLE 1. Inactivation of *C. parvum* oocysts stored in fecal pools or water at various temperatures

Oocyst storage medium	Viability test used	Incubation time (days)	Incubation temp (°C)	K day^{-1}[a]	95% confidence interval	Reference
Distilled water with antibiotics	Dye permeability assay	667	4	0.0025	0.0020–0.0030	This paper
PBS	Dye permeability assay	6	22	0.0320	0.0214–0.0426	This paper
	Dye permeability assay	1	37	0.918	0.780–1.056	This paper
Fecal pool A	Dye permeability assay	410	4	0.0040	0.0023–0.0059	This paper
	In vitro excystation	410	4	0.0046	0.0032–0.0061	This paper
Fecal pool B	Dye permeability assay	259	4	0.0056	0.0038–0.0074	This paper
	In vitro excystation	259	4	0.0079	0.0047–0.0113	This paper
Sterile RO water[b]	Dye permeability assay	176	4	0.0042	0.0034–0.0048	18
Sterile distilled water	Animal infectivity	147	15	0.0355	0.0315–0.0395	14
	In vitro excystation	147	15	0.0211	0.0180–0.0242	14
	Animal infectivity	28	15	0.1555	0.1071–0.2039	14
	In vitro excystation	28	15	0.0907	0.0553–0.1261	14

[a] Assuming that oocyst inactivation is a first-order process, the coefficient of inactivation, K, was determined by regressing $\ln(P_0/P_t)$ against time (derived from the equation $P_t = P_0 \times e^{-Kt}$), where P_0 is the initial percentage of viable oocysts and P_t is the percentage of viable oocysts at time t [in days]). The 95% confidence intervals were determined by multiplying the Student t values at the appropriate degrees of freedom and confidence intervals of $\alpha/2 = 0.025$ by the standard deviations of K.
[b] RO, reverse osmosis.

oocysts was consistently lower than the proportion of potentially viable oocysts indicated by dye permeability (Fig. 3). For pools A and B, the correlation coefficients (r values) relating the two methods were 0.89 and 0.61, respectively; the value for pool A is significant but the value for pool B is not at $P = 0.05$.

Using the dye permeability assay data in Fig. 3, as well as data published in the literature, we can also estimate coefficients of oocyst inactivation (K [Table 1]). These estimated values of K are based on experiments with in vitro excystation, dye permeability, and animal infectivity. Pairwise comparison of the coefficients for the dye permeability and excystation assays of fecal pools A and B showed that these coefficients were not significantly different (at $P = 0.05$). They were also not significantly different from inactivation rates for purified oocysts stored at 4°C for nearly 2 years. Table 1 also shows that on the basis of data of Korich et al. (14), inactivation rates based on animal infectivity were significantly higher than rates based on in vitro excystation at 15°C whereas the inactivation rates estimated at 25°C were not significantly different (at $P = 0.05$). Others have found that the rates of oocyst inactivation increase at higher temperatures (9, 12). The dye permeability assay showed the same effects of higher temperatures up to 100°C. Inactivation indicated by dye permeability occurred over the course of several hours at 22°C, while at 70 and 80°C, inactivation took place in a few minutes (Fig. 4). Inactivation rates (K values) for purified oocysts subjected to temperatures of 70, 80, and 100°C were (per minute) 0.140, 1.830, and 6.04, respectively. Compared to the results reported by Harp et al. (12), these K values predict somewhat longer exposure times to reach 99.99% inactivation, i.e., 66, 5, and 2 min for 70, 80, and 100°C, respectively.

Our data demonstrated that naturally occurring oocysts in untreated calf feces could remain potentially infective for many months when stored at 4°C, although the proportion of potentially infective oocysts decreased with time. The rates of inactivation that we have reported (Table 1) are comparable to those based on data from Robertson et al. (18), and Korich et al. (14). Various studies on *Cryptosporidium* oocysts have indicated their capacity for long-term survival outside of a host. Although an ID_{50} was not indicated, Moon and Bemrick (16) reported that infectedcalf feces stored in 2.5% potassium dichromate at 20 to 25°C remained infective for 8 weeks. Oocysts in whole-gut homogenates that were stored in either distilled water, PBS, 5% bovine serum albumin, or 2.5% potassium dichromate remained infective for 6 months when stored at 4°C, although titers decreased significantly during this period, indicating a significant loss of viability (18). To obtain complete development of cryptosporidia in cell culture, Current and Haynes (7) purified oocysts from infected human feces that had been stored in 2.5% potassium dichromate at 4°C for a year.

Using the dye permeability assay (4), Robertson et al. (18) found that rates of survival of *Cryptosporidium* oocysts in infected human feces that were stored at 4°C differed, with viability ranging from 0 to 59% after 178 days. In other survival studies, Robertson et al. (18) showed that approximately 39% of viable, purified bovine oocysts survived 176 days of incubation in cow feces at 4°C, but that 176 days of incubation at 4°C in river water or tap water resulted in 99% nonviability.

As oocysts aged in feces at 4°C, or in a purified state at higher temperatures, they became more permeable, as indicated by the increased percentage of DAPI+ PI− and DAPI+ PI+ oocysts over time. Temperature appeared to have a direct effect on the permeability of the oocyst wall. This was also evidenced by the increase in inactivation rates with increased incubation temperature (Table 1). Our data indicated the existence of a direct relationship between rates of the shift from DAPI− PI− to DAPI+ PI− oocysts and temperature. By using animal infectivity for determining viability of oocysts, Fayer (9) found that a proportion of oocysts initially subjected to temperatures of >50°C remained infective after 5 min at 60°C, but exposure to temperatures of >60°C for 5 min resulted in the complete loss of infectivity. Our data indicated that after 9 min at 70°C, 25% of the oocysts were DAPI+ PI− and apparently viable. A 2-min exposure to a temperature of 80°C resulted in 100% DAPI+ PI+ nonviable oocysts, in agreement with Fayer's results (9). What the dye permeability assay can show that neither animal infectivity nor in vitro excystation can are changes in oocyst wall permeability, which

appears to be directly correlated to the ability of oocysts to excyst and infect animals.

In our experiments, the majority of oocysts in both fresh calf feces and freshly purified suspensions were impermeable to both DAPI and PI. Campbell et al. (4) reported a higher excystation efficiency for purified suspension of oocysts with larger fractions of partially permeable DAPI+ PI− oocysts. The larger number of DAPI+ PI− oocysts observed in the purified oocyst samples used by Campbell et al. (4) in their experiments may be a result of some steps in the purification process, viz., incubation in 1% sodium dodecyl sulfate (a surfactant), acid sedimentation (acidity may permeabilize oocyst walls), and the use of ethyl acetate. Oocyst suspensions that contained a larger number of impermeable DAPI− PI− cysts showed low excystation efficiencies. Thus, Campbell et al. (4) hypothesized that the high proportion of impermeable DAPI− PI− oocysts may have accounted for the differences they observed between the dye permeability and in vitro excystation assays. Their data indicated that there was a strong correlation between DAPI+ PI− oocysts and excystation efficiency. In contrast, our data suggest strongly that the majority of DAPI− PI− oocysts in our experiments were capable of excystation. There was a strong correlation between DAPI− PI− oocysts and excystation (Fig. 2 and 3). Our mouse infectivity assays produced an ID_{50} that was virtually identical to that observed by Finch et al. (10). In both cases, 100% of the mouse pups became infected when they were inoculated with ≥400 oocysts. However, a more important result of our infectivity experiment was the unequivocal evidence of a connection between DAPI− PI− oocysts and infectivity. Campbell et al. (4) only correlated their results of the fluorescent dye assay with in vitro excystation. Furthermore, they speculated that impermeable oocysts that displayed internal structures were not viable because they appeared not to excyst. Results of our study indicate that not only did these impermeable oocysts excyst; they also were capable of infecting mice.

Detailed analysis of the dye permeability assay results showed that DAPI will stain intact sporozoite chromosomes, which appear as discrete blue dots when observed under a fluorescent microscope (1, 4). Such oocysts are characterized as DAPI+ PI− and counted as viable, but one may also observe a diffuse DAPI fluorescence in oocysts, suggesting that disruption of the nucleus of the sporozoite has occurred. Such DAPI+ PI− oocysts should not be counted as viable. Based on the observation of these two forms of DAPI+ PI− oocysts, Korich et al. (14) maintained that DAPI was a better indicator of nonviable oocysts than of viable ones. Given that there may be a transition between the viable DAPI+ PI− oocysts with distinct nuclei and oocysts showing the diffuse DAPI staining indicative of nuclear disruption and nonviability, counting rules for the nonviable category need to be established based on the degree of diffuse staining.

In a recent criticism of the dye permeability assay, Black et al. (3) assumed that uptake of DAPI measured the presence or absence of metabolic activity and asserted that there may be no connection between metabolic activity and infectivity. Indeed, some viable (and metabolically sound) oocysts may not establish an infection. It is a misconception, however, to assume that uptake of DAPI is connected to a metabolic process. Unlike the vital stain fluorescein diacetate, which requires enzymatic cleavage before fluorescence occurs (8), DAPI, a nuclear stain, fluoresces most readily only when it is bound to DNA (15). Before it binds with the DNA of sporozoites, it must, however, first pass through the oocyst wall.

The dye permeability assay may overestimate the proportion of oocysts in a sample that are capable of initiating infection. Our results confirm this general characteristic but suggest that the degree of overestimation decreases with age of the oocysts and is not excessive when compared with excystation. For environmental samples such as soil and sediments, the numbers of oocysts recovered are likely to be too few to conduct extensive tests to demonstrate animal infectivity. Anguish and Ghiorse (1) have demonstrated the applicability of the dye permeability assay in fluorescence microscopy studies of oocysts in highly turbid environmental soil, sediment, and fecal samples. The dye permeability assay provides an economical method to estimate viability and potential infectivity, although the estimate may be conservative. It can provide information on the effects of environmental stresses on the oocyst wall over time and thus may increase our understanding of the dynamics of oocyst degradation in the environment.

ACKNOWLEDGMENTS

This work was supported in part by funds from the USDA Competitive Research Grant Program; the U.S. Department of Interior through the New York State Water Resources Institute at Cornell University; the Center for Advanced Technology (CAT) at Cornell in Biotechnology, sponsored by the New York State Science and Technology Foundation, a consortium of industries and the National Science Foundation; the New York State Watershed Whole Farm Program through the New York State Water Resources Institute; and the College of Agriculture and Life Sciences at Cornell.

We are grateful to D. Graham, L. Anthony, and J. Mitchell for laboratory assistance. The expert secretarial assistance of Patti Lisk is gratefully acknowledged.

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