

Comparison of *Cryptosporidium parvum* Viability and Infectivity Assays following Ozone Treatment of Oocysts

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Several *in vitro* surrogates have been developed as convenient, user-friendly alternatives to mouse infectivity assays for determining the viability of *Cryptosporidium parvum* oocysts. Such viability assays have been used increasingly to determine oocyst inactivation following treatment with chemical, physical, or environmental stresses. Defining the relationship between *in vitro* viability assays and oocyst infectivity in susceptible hosts is critical for determining the significance of existing oocyst inactivation data for these *in vitro* assays and their suitability in future studies. In this study, four viability assays were compared with mouse infectivity assays, using neonatal CD-1 mice. Studies were conducted in the United States and United Kingdom using fresh (<1 month) or environmentally aged (3 months at 4°C) oocysts, which were partially inactivated by ozonation before viability and/or infectivity analyses. High levels of variability were noted within and between the viability and infectivity assays in the U.S. and United Kingdom studies despite rigorous control over oocyst conditions and disinfection experiments. Based on the viability analysis of oocyst subsamples from each ozonation experiment, SYTO-59 assays demonstrated minimal change in oocyst viability, whereas 4',6'-diamidino-2-phenylindole-propidium iodide assays, *in vitro* excystation, and SYTO-9 assays showed a marginal reduction in oocyst viability. In contrast, the neonatal mouse infectivity assay demonstrated significantly higher levels of oocyst inactivation in the U.S. and United Kingdom experiments. These comparisons illustrate that four *in vitro* viability assays cannot be used to reliably predict oocyst inactivation following treatment with low levels of ozone. Neonatal mouse infectivity assays should continue to be regarded as a "gold standard" until suitable alternative viability surrogates are identified for disinfection studies.

Cryptosporidium parvum oocysts, due to their robust nature, small size, and ability to withstand chlorination levels that are normally employed in water treatment processes, have been responsible for a number of waterborne outbreaks of human cryptosporidiosis worldwide (9). In the absence of viability or infectivity information on recovered oocysts, their presence in finished water samples provides no indication of their public health significance. *C. parvum* oocyst viability has conventionally been determined by using *in vitro* excystation, while mouse infectivity assays have been utilized to determine whether oocysts are infectious. Limitations of *in vitro* excystation include requirements for high numbers of organisms, usually in highly purified suspensions, and considerable microscopic expertise. This assay also poses a degree of subjectivity. For example, an oocyst which has not released all of the sporozoites would be classed as partially excysted (viable) by one microscopist but as intact with lysed contents (nonviable) by a different microscopist. Such decisions are based on the microscopist's experience and ability to differentiate confidently between the two categories. Also, oocyst enumeration during excystation is usually limited to 100 oocysts. This reduces confidence when viability values are used to extrapolate to large populations of oocysts.

Mouse infectivity assays are particularly useful where infectivity information is required for large oocyst populations and have been used by numerous investigators to measure the inactivation of oocysts following their exposure to disinfectants

(12, 13, 18). While mouse infectivity assays have become recognized as the reference method for establishing the ability of oocysts to cause infection, they too have limitations. A major concern is the variability associated with the neonatal mouse model. There are also problems with respect to using these assays to determine the infectivity of oocysts recovered from environmental samples, as the numbers of oocysts present may be insufficient to induce infection in mice. More recently, molecular evidence for exclusively human (type 1) and human and animal (type 2) isolates of *C. parvum* has also emerged (8, 19, 26), which introduces another potential problem in using mouse infectivity assays. Oocysts isolated from environmental samples may be viable and capable of inducing infections in susceptible humans but may not be infective to neonatal mice. Additionally, the expense, time-consuming nature, and need for specialized facilities and highly trained staff have limited the scope of studies that can be performed on *Cryptosporidium* oocyst inactivation using animal infectivity assays.

The *in vitro* assays offer many advantages over the animal model; they produce results in a relatively short time (hours as opposed to days) and are inexpensive and relatively easy to perform. Studies correlating various *in vitro* assays (4',6'-diamidino-2-phenylindole-propidium iodide [DAPI-PI], maximized *in vitro* excystation, SYTO-9, and SYTO-59) with animal infectivity were incomplete or inconclusive or provided conflicting results (4, 12, 13). The goal of this project was to compare four commonly used *in vitro* methods with mouse infectivity assays to determine which *in vitro* methods could serve as acceptable surrogates for animal infectivity studies. The logit dose-response model for mouse infectivity, as described by Finch et al. (11), was selected as the most suitable

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method for measuring oocyst infectivity in neonatal mice. Using this format, dose-response curves were generated in one laboratory in the United States and one laboratory in the United Kingdom and compared with the dose-response model of an independent published study (11). The three infectivity models demonstrated considerable overlap, and an analysis of covariance revealed no significant differences among the three models ($P > 0.05$) (14). This was followed by ozone disinfection experiments in both countries. At each location, the viability and infectivity of fresh and environmentally aged, ozone-treated oocysts were determined using the four standardized methods: DAPI-PI, maximized in vitro excystation, SYTO-9, and SYTO-59.

MATERIALS AND METHODS

C. parvum oocyst production and purification. The *C. parvum* oocysts used in this study were a strain maintained at the Sterling Parasitology Laboratory (Department of Veterinary Science, University of Arizona [UA], Tucson) that was originally obtained from Harley Moon (National Animal Disease Center, Ames, Iowa). Within 4 to 12 h of birth, Holstein calves were orally inoculated with 2×10^8 oocysts suspended in sterile water, and 1 to 2 h later, they were given 4 pints of colostrum, prophylactic doses of oral rota- or coronavirus, and *Escherichia coli* vaccines. Calf maintenance, fecal collection, and oocyst purification procedures have been described previously (2, 14).

Rigorous quality control methods were used to ensure that the experiments in the U.S. and United Kingdom locations were conducted using oocysts produced by the same calf and that they were the same age after shedding. This was accomplished by assigning lot numbers to each calf on the day of infection and batch numbers to the oocysts on the day they were shed. These lot and batch numbers were recorded to validate oocyst age and to ensure that trials in both laboratories were performed with the same lot number and batch of oocysts. The oocysts were shipped to and from participating laboratories in ice-cooled, insulated boxes by Federal Express.

Environmental aging of oocysts. *C. parvum* oocysts were environmentally aged by being placed in a chamber enclosed by a 1.2- μ m-pore-size Vokes membrane filter (23). The semipermeable chamber was placed in a 10-liter volume of reservoir water at 4°C for a period of 3 months. Environmental aging of the oocysts was conducted at the Scottish Parasite Diagnostic Laboratory (SPDL) with purified stock oocyst suspensions obtained from UA. The environmentally aged oocyst suspension was enumerated at the SPDL and divided into two equal portions. One half of the aged oocyst suspension was returned to UA for the U.S. disinfection experiments, while the second half was retained at the SPDL for the United Kingdom disinfection experiments.

In vitro surrogate assays. (i) Maximized in vitro excystation. Aliquots of control or disinfectant-treated oocysts ($\sim 10^5$) were acidified by incubation in acidified Hanks' balanced salt solution (HBSS) (pH 2.75, 1 h, 37°C). Each sample was washed three times with HBSS (pH 7.2) and concentrated (12,500 \times g, 30 s) to 100 μ l. Then 200 μ l of 1% bile solution (prepared in 1% Hanks' minimal essential medium) and 50 μ l of sodium bicarbonate (0.44% in deionized [DI] water) were added. Each sample was vortexed and incubated at 37°C for 30 min, and an aliquot of each sample was removed to determine sporozoite ratios. The percentage of excysted oocysts was determined after 4 h of incubation at 37°C. For each experimental regimen, the maximized in vitro excystation assay was performed in quintuplicate by using Nomarski-differential interference contrast microscopy (magnification, $\times 400$) (22).

(ii) Fluorogenic vital-dye assays. (a) DAPI-PI. Aliquots of control or disinfectant-treated oocysts ($\sim 10^5$) were acidified by incubation in acidified HBSS (pH 2.75, 1 h, 37°C). Each sample was washed three times with HBSS (pH 7.2) and concentrated (12,500 \times g, 30 s) to 100 μ l. Then 10 μ l of 2-mg ml⁻¹ DAPI and 10 μ l of 1-mg ml⁻¹ PI were added. These samples were incubated at 37°C for 2 h. Optimally diluted fluorescein isothiocyanate (FITC)-conjugated anti-*Cryptosporidium* monoclonal antibody was added after 90 min, and incubation (37°C) was continued for a further 30 min. Following 2 h of incubation with the dyes, each sample was washed three times with HBSS (pH 7.2) and concentrated to 100 μ l. For each replicate, 10- to 20- μ l aliquots were placed on glass microscope slides, covered with coverslips, and examined by fluorescence microscopy. For each experimental regimen, the fluorogenic vital-dye-based assay was performed in quintuplicate by using fluorescence microscopy (magnification, $\times 400$) (7).

(b) SYTO-9. A stock solution consisting of 3.34 mM SYTO-9 (Molecular Probes, Inc., Eugene, Ore.) was stored at -20°C in the dark and thawed immediately prior to use. For the viability assay, a suspension of approximately 10^5 oocysts was washed twice in phosphate-buffered saline (PBS) and resuspended in 98 μ l of PBS. An appropriate volume of SYTO-9 was diluted 1:10, and 2 μ l of the diluted dye was added to each 98- μ l volume of the oocyst suspension to yield a final dye concentration of 0.334 μ M. Each sample was vortexed, wrapped in aluminum foil, and incubated (37°C, 30 min). The samples were vortexed, placed on glass slides, covered with coverslips, and sealed with nail polish. A blue FITC filter was used to examine the SYTO-9-stained organisms.

Nonviable oocysts were stained green or bright yellow, whereas viable oocysts had a green halo surrounding a dark interior. For each experimental regimen, the assays were performed in quintuplicate, using fluorescence microscopy at a magnification of $\times 400$ (3).

(c) SYTO-59. A stock solution consisting of 5 mM SYTO-59 (Molecular Probes, Inc.) was stored at -20°C in the dark and thawed immediately prior to use. For the viability assay, a suspension of approximately 10^5 oocysts was washed twice in PBS and resuspended in 96 μ l of PBS. An appropriate volume of SYTO-59 was diluted 1:10, and 4 μ l of the diluted dye was added to each 96- μ l volume of oocyst suspension to yield a final dye concentration of 500 μ M. Each sample was vortexed, wrapped in aluminum foil, and incubated (37°C, 30 min). The samples were vortexed, placed on glass slides, covered with coverslips, and sealed with nail polish. A green filter was used to examine the SYTO-59-stained organisms. Nonviable oocysts were stained bright red, whereas viable oocysts were unstained. For each experimental regimen, the assays were performed in quintuplicate, using fluorescence microscopy at a magnification of $\times 400$ (3).

Neonatal mouse infectivity assays. CD-1/ICR dams at specific stages of pregnancy were obtained from Charles River Laboratories, Inc. (Wilmington, Mass.) and Charles River UK Limited (Margate, Kent, England). Infant mice were inoculated by delivering a 10- μ l dose of oocysts suspended in sterile water to the back of the throat with a calibrated pipette. Infection at both laboratories was determined by examining formalin-fixed, slide-mounted, hematoxylin- and eosin-stained sections (5 μ m by 2 to 3 cm) of the terminal ileum, removed from each animal at necropsy 7 days postinoculation (14). The proportion of animals infected at each dose was determined from the plus or minus scores of the histological sections.

Enumeration of oocyst challenge doses. (i) UA. Oocyst doses for mouse infectivity assay at UA were prepared by dilution from an accurately enumerated stock suspension of ozone-treated oocysts. Before the administration of inocula in neonatal mice, actual oocyst numbers in the prepared doses were confirmed from a mean of at least five hemocytometer counts, to ensure that the dilution protocol produced the desired oocyst concentration. After the mice were inoculated, the number of oocysts in 10- μ l volumes of the remaining suspension of the UA doses was validated by using a Chemunex laser scanning instrument (known as either ChemScan [in Europe] or Scan-RDI [in the United States]) by Thames Water Utilities. Briefly, ChemScan enumeration was performed by preparing 10 10- μ l aliquots of oocyst suspensions on individual 0.2- μ m-pore-size membrane filters, which were then stained with a direct, FITC-conjugated anti-*Cryptosporidium* monoclonal antibody (Cellabs, Brookvale, New South Wales, Australia). Each individual membrane was placed into the ChemScan unit, and the entire membrane surface was scanned with a laser. Using computer software, the data were interpreted to enumerate oocysts, based on size, shape, and fluorescence intensity. Ten replicates of each sample were analyzed with ChemScan, and 2 of 10 membranes were validated manually by epifluorescence microscopy (21).

(ii) SPDL. Quintuplicate hemocytometer counts were performed on stock oocyst suspensions as described immediately above, and stock suspensions were diluted appropriately to yield 100 10- μ l inocula of preselected oocyst doses. Prepared doses were placed as 10- μ l aliquots on well slides and dried. Furthermore, during inoculation of each litter, the 5th, 10th, and last doses were also dispensed onto well slides, dried, and examined by immunofluorescence assay to ensure quality delivery of target doses. Although the methods employed to confirm oocyst doses in the U.S. (ChemScan) and United Kingdom (fluorescence microscopy) studies were different, published evidence suggests that these two methods yield statistically similar data (21).

Oocyst treatment with ozone. Compressed air was passed through a corona discharge ozone generation system (CD10/AD; Clearwater Technology Inc.) to generate ozone gas, which was bubbled through a 2-liter sidearm glass flask into approximately 1 liter of stirring DI water for predetermined time periods. Approximately 20 ml of the ozonated water was dispensed into an ozone demand-free glass beaker. The indigo trisulfonate method, with AccuVac ampoules (Hach Co., Loveland, Colo.), was utilized to determine ozone concentrations in the DI water.

Individual ozone demand-free glass beakers containing magnetic stirrers were placed on individual stir plates. Approximately 80 ml of ozonated water (pH 6.3 to 6.7, 22°C) was poured into each beaker, the beakers were covered with Parafilm, and the stir plates were switched on. The initial ozone concentration in the samples was determined, and then approximately 10^7 *C. parvum* oocysts were added to each beaker and mixed by continuous stirring. Immediately following oocyst addition, another 20-ml subsample was removed to determine the ozone concentration and to calculate the ozone demand exerted by addition of the oocyst suspension. Following predefined exposure times, another 20-ml subsample was removed to determine residual ozone concentrations. Simultaneously, 250 μ l of 0.7-mg/ml sodium thiosulfate solution was added to the oocyst suspensions in the glass beakers to neutralize the residual ozone and to terminate the disinfection experiment. Two replicate ozone disinfection experiments were conducted using fresh and aged oocysts at both the U.S. and United Kingdom locations.

Statistical analyses of in vitro viability assays. The four in vitro viability surrogates were compared to evaluate their relative response to the oocyst inactivation method. Surrogate data were also compared between laboratories, taking into account the effects of oocyst age. Two-tailed *t* tests were used to

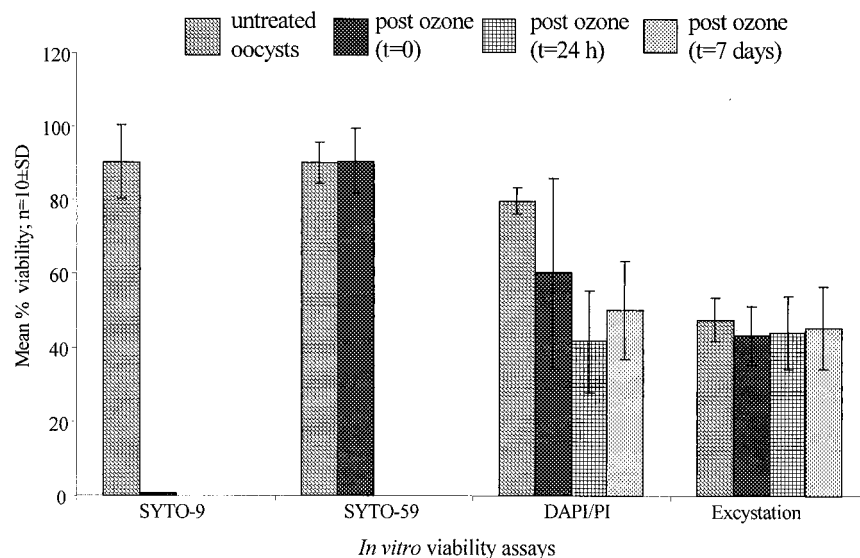


FIG. 1. Viability of fresh oocysts (age of <2 weeks) by in vitro surrogates following exposure to ozone ($0.4 \text{ mg liter}^{-1}$; 2 min) (U.S. phase). SD, standard deviation.

evaluate interactions among methods, laboratories, and oocyst ages. Pairwise comparisons were performed to compare viability at three different time periods following inactivation.

Rationale for comparing in vitro surrogates with neonatal mouse infectivity assays. Following ozonation, in vitro surrogate methods provided information on the percentages of viable and nonviable oocysts in each sample. Mouse infectivity assays yielded infection values for groups of mice. In order to compare mouse infectivity data with in vitro viability data, it was necessary to determine the percentage of infective oocysts in each dose administered to the mice. First, the 10 standard curves (i.e., three replicates using fresh, untreated oocysts and two replicates using aged, untreated oocysts at both UA and the SPDL) were transposed individually to generate 10 logit dose-response curves as described earlier (14). They were analyzed using analysis of covariance to determine if differences existed in the slopes and intercept lines. These statistical analyses indicated that the UA and SPDL logistic dose-response curves were homogeneous, enabling percent infection data for a group of mice receiving each inoculum to be placed on each of the 10 standard curves (percent infection, y axis; administered inoculum, x axis) in order to extrapolate the number of infective oocysts. Thus, each infection value (between 1 and 99%) for a group of mice led to the generation of 10 individual values for the number of infective oocysts present in the inoculum administered to that group of mice. A ratio for each of the 10 values of the number of infective oocysts was determined with respect to the original inoculum given to that group of mice. Each of the 10 ratio values was multiplied by 100 to yield 10 infective-oocyst (or infectivity) values per inoculum, which in turn were then used to calculate a mean and standard deviation for percent infectivity of oocysts in the given inoculum.

RESULTS

Preliminary ozonation experiments. Preliminary disinfection experiments were performed to identify the ozone concentrations and contact times (CT) necessary to render approximately 50% of the fresh and aged oocyst populations nonviable. The same investigators conducted preliminary ozonation experiments at both the U.S. and United Kingdom laboratories, to determine the ozone CT for 50% inactivation of oocysts. Measuring the outcome of preliminary disinfection experiments with mouse infectivity assays was not within the scope of the project. Based on previously published data (7, 22), it was considered prudent to utilize DAPI-PI and/or in vitro excystation as the in vitro surrogates for the preliminary viability measurements. These preliminary experiments indicated that for fresh oocysts, 0.4 mg of ozone per liter at a CT of 2 min reduced oocyst viability to 50% by DAPI-PI and/or in vitro excystation. In contrast, for environmentally aged oocysts, a reduction in viability to 50% required an ozone concentration of 0.3 mg/liter at a CT of 2 min (data not shown).

Determination of oocyst viability with in vitro surrogates following ozonation. (i) Fresh oocysts. (a) U.S. phase. Initial viability results from the fresh, untreated oocyst suspensions demonstrated considerable interassay variability. While the lowest viabilities were recorded with maximized in vitro excystation (64%), the three vital-dye-based assays revealed mean viabilities between 80 and 90% (Fig. 1). Immediately following ozone treatment, large differences were noted in oocyst viability values among the four viability assays. The highest level of inactivation was predicted by SYTO-9, whereas SYTO-59 indicated no change in oocyst viability. DAPI-PI and excystation demonstrated marginal reductions in oocyst viability immediately following ozonation, but viability values did not show a further significant decline with these two assays at intervals of 24 h and 7 days postozonation (Fig. 1).

(b) United Kingdom phase. The initial viability values of the fresh oocyst suspensions exhibited considerable interassay variability; however, the overall patterns were very similar to those observed in the U.S. phase. Immediately following ozonation, in contrast to the U.S. data, SYTO-9 demonstrated no significant change in oocyst viability in the United Kingdom study (Fig. 2). The two additional vital-dye-based assays (SYTO-59 and DAPI-PI) also showed low levels of oocyst inactivation, whereas excystation demonstrated a significant reduction in oocyst viability immediately following ozonation (Fig. 2).

(ii) Environmentally aged oocysts. (a) U.S. phase. Assessment of untreated-oocyst viability with the four in vitro viability assays indicated similar viability values for SYTO-9 and SYTO-59 (approximately 90% viable) and for DAPI-PI and excystation (approximately 60% viable). Immediately following ozonation, SYTO-9 showed the largest reduction in oocyst viability, whereas SYTO-59 demonstrated no significant change in oocyst viability. Both DAPI-PI and excystation indicated reductions between 40 and 50% in oocyst viability, as well as a consistent decline in oocyst viability at 24 h and 7 days post-ozonation (Fig. 3).

(b) United Kingdom phase. Untreated-oocyst viability values obtained with SYTO-9 and SYTO-59 were similar and agreed closely with the U.S. viability data for the same vital-dye-based assays. Untreated-oocyst viability values obtained with DAPI-PI were considerably higher than those obtained in

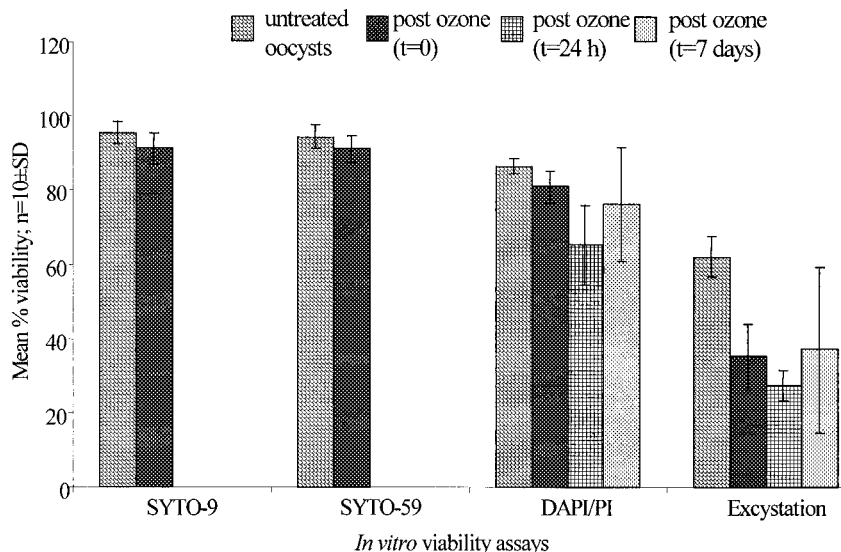


FIG. 2. Viability of fresh oocysts (age of <2 weeks) by in vitro surrogates following exposure to ozone (0.4 mg liter⁻¹; 2 min) (United Kingdom phase) SD, standard deviation.

the U.S. study, whereas excystation demonstrated considerably lower viability values than excystation and DAPI-PI did in the U.S. study. Immediately following ozonation, a marginal reduction in oocyst viability was noted with SYTO-9 and SYTO-59. In contrast, both DAPI-PI and excystation demonstrated between 30 and 50% reduction in oocyst viability (Fig. 4). Examination of oocyst viability at 24 h and 7 days postozonation showed higher variations in viability values determined with DAPI-PI than with excystation. Unlike in the U.S. study, these assays failed to demonstrate declining viability with increases in time following ozonation (Fig. 4).

Statistical analyses of in vitro viability assays. SYTO-9 and SYTO-59 assays consistently demonstrated higher oocyst viability than did in vitro excystation and DAPI-PI. Following oocyst exposure to ozone, the examination of viability values at various time intervals (up to 7 days) failed to reveal consistent results. A summary of two-tailed *t* test analyses is presented in Table 1.

Neonatal mouse infectivity assays. (i) Fresh oocysts. For mean inoculum sizes between 31 and 138 for untreated, fresh oocysts, percent infection in CD-1 neonates ranged between 13 and 58.3% in the U.S. study; percent infection ranged between 13.6 and 33.3% in the United Kingdom study when untreated, fresh oocyst inocula between 25 and 137 were used. Following ozonation, oocyst inocula of 250, 2,500, and 25,000 were administered to groups of mice in both studies. In the U.S. study, infectivity ranged from 0 to 30% for the dose of 250 and from 0 to 100% for the dose of 2,500. The dose of 25,000 demonstrated 100% infectivity in both replicates (Table 2). In the United Kingdom study, infectivity ranged from 37.5 to 50% for the dose of 250 and from 87.5 to 91.7% for the dose of 2,500. The dose of 25,000 demonstrated 100% infectivity in both replicates (Table 2).

(ii) Environmentally aged oocysts. For inoculum sizes similar to those of the fresh, untreated oocysts and prior to ozonation, the environmentally aged oocysts yielded 13.6 to 73%

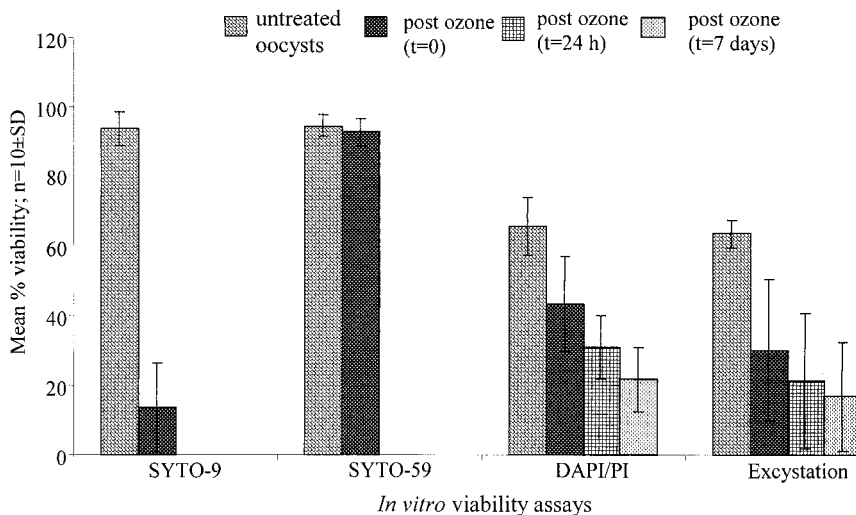


FIG. 3. Viability of environmentally aged oocysts (age of 3 months) by in vitro surrogates following exposure to ozone (0.3 mg liter⁻¹; 2 min) (U.S. phase). SD, standard deviation.

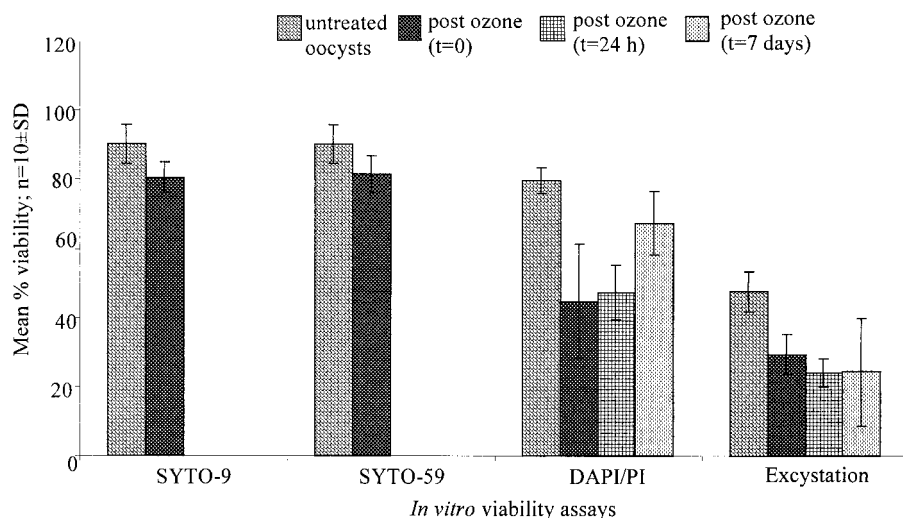


FIG. 4. Viability of environmentally aged oocysts (age of 3 months) by in vitro surrogates following exposure to ozone (0.3 mg liter⁻¹; 2 min) (United Kingdom phase). SD, standard deviation.

infection in the U.S. study and 16.7 to 68.6% infection in the United Kingdom study. Following ozonation, oocyst inocula of 250, 2,500, and 25,000 were administered to groups of mice in both studies. In the U.S. study, infectivity ranged from 0 to 20% for the dose of 250, from 11 to 74% for the dose of 2,500, and from 56 to 100% for the dose of 25,000 (Table 3). In the

United Kingdom study, infectivity ranged from 12.5 to 45.8% for the dose of 250 and from 25 to 100% for the dose of 2,500 and was 100% in both replicates for the dose of 25,000 (Table 3).

Comparison of in vitro surrogates with neonatal mouse infectivity assays. The infectivity values for ozone-treated oo-

TABLE 1. Comparison of in vitro viability assays using two-tailed *t* tests

Assay comparison	Excystation		DAPI-PI		SYTO-59		SYTO-9	
Oocyst parameters								
U.S. fresh control vs U.K. ^g fresh control	- ^d		-		-		-	
U.S. aged control vs U.K. aged control	+ ^e		+		-		-	
Sample type/sample source^h								
	U.S.	U.K.	U.S.	U.K.	U.S.	U.K.	U.S.	U.K.
Fresh oocysts/replicate 1								
Fresh 1 (<i>t</i> = 0) ^a vs fresh 2 (<i>t</i> = 0)	-	+	+	+	+	+	-	+
Fresh control vs fresh 1 (<i>t</i> = 0)	+	+	+	-	-	+	+	-
Fresh control vs fresh 1 (<i>t</i> = 24) ^b	+	+	+	+	ND ^f	ND	ND	ND
Fresh control vs fresh 1 (<i>t</i> = 7) ^c	+	+	+	+	ND	ND	ND	ND
Fresh oocysts/replicate 2								
Fresh control vs fresh 2 (<i>t</i> = 0)	+	+	-	+	+	-	+	+
Fresh control vs fresh 2 (<i>t</i> = 24)	+	+	+	+	ND	ND	ND	ND
Fresh control vs fresh 2 (<i>t</i> = 7)	+	-	+	-	ND	ND	ND	ND
Aged oocysts/replicate 1								
Aged 1 (<i>t</i> = 0) vs aged 2 (<i>t</i> = 0)	+	-	-	+	+	-	+	-
Aged control vs aged 1 (<i>t</i> = 0)	+	+	-	+	-	+	+	+
Aged control vs aged 1 (<i>t</i> = 24)	+	+	+	+	ND	ND	ND	ND
Aged control vs aged 1 (<i>t</i> = 7)	+	+	+	+	ND	ND	ND	ND
Aged oocysts/replicate 2								
Aged control vs aged 2 (<i>t</i> = 0)	+	+	+	+	-	+	+	+
Aged control vs aged 2 (<i>t</i> = 24)	+	+	+	+	ND	ND	ND	ND
Aged control vs aged 2 (<i>t</i> = 7)	+	+	+	+	ND	ND	ND	ND

^a *t* = 0, immediately postdisinfection.

^b *t* = 24, 24 h postdisinfection.

^c *t* = 7, 7 days postdisinfection.

^d -, no significant differences (*P* ≥ 0.05).

^e +, significant differences (*P* ≤ 0.05).

^f ND, not done.

^g U.K., United Kingdom.

^h Designations consist of oocyst type and replicate number.

TABLE 2. Infectivity of fresh (age of <2 weeks) oocysts in neonatal CD-1 mice following ozone disinfection experiments

Location and fresh oocyst type	Replicate 1		Replicate 2	
	Mean inoculum size	% Infection (no. infected/no. challenged)	Mean inoculum size	% Infection (no. infected/no. challenged)
U.S.				
Untreated	138 91 31	58.3 (14/24) 44 (11/25) 13 (3/23)	Not done	Not done
Ozone treated (0.4 mg liter ⁻¹ , 2 min)	22,970 2,292 239	100 (26/26) 0 (0/20) 0 (0/25)	26,685 2,570 249	100 (23/23) 100 (17/17) 30.4 (7/23)
U.K. ^a				
Untreated	137 59 25	33.3 (12/36) 13.6 (3/22) 25 (9/36)	Not done	Not done
Ozone treated (0.4 mg liter ⁻¹ , 2 min)	21,000 2,683 268	100 (24/24) 87.5 (21/24) 50 (11/22)	25,950 2,383.3 242	100 (22/22) 91.7 (22/24) 37.5 (9/24)

^a U.K., United Kingdom.

cysts are presented in Table 4. A comparison of the oocyst infectivity values (Table 4) for various oocyst suspensions and their respective viability values (as determined by the in vitro assays) is presented in Fig. 5. Two-tailed *t* tests an α value of 0.05 were used, and the degrees of freedom for the test were calculated as $(n_1 + n_2 - 2)$, where n_1 and n_2 are the sample sizes of the two methods being compared (e.g., when testing animal infectivity versus excystation, $n_1 = 10$, $n_2 = 5$, and degrees of freedom = 13). Consistent differences were detected between mouse infectivity assays and in vitro viability assays. In vitro assays provided higher viability values for ozone-treated oocysts than did the animal infectivity assays. Following ozone treatment of oocysts, in vitro assays overestimated viability compared to mouse infectivity assays. Determination of correlation coefficients between each in vitro assay and mouse infectivity assay indicated the greatest disparity

between SYTO-59 and mouse infectivity assays ($r = -0.40$), followed by that between DAPI-PI and mouse infectivity assays ($r = 0.21$). While in vitro excystation demonstrated a marginally higher correlation with the mouse infectivity assay than did the DAPI-PI assay ($r = 0.23$), following ozonation, the highest correlation between an in vitro surrogate and the mouse infectivity assay was demonstrated by SYTO-9 ($r = 0.39$).

DISCUSSION

Present knowledge indicates that *Cryptosporidium* oocysts occur commonly in wild and domestic animals and that zoono-throponotic infections can occur by various routes, including the transmission of human disease by the waterborne route. Oocysts are capable of withstanding normally employed disin-

TABLE 3. Infectivity of environmentally aged (3 months in reservoir water) oocysts in neonatal CD-1 mice following ozone disinfection experiments

Location and aged oocyst type	Replicate 1		Replicate 2	
	Mean inoculum size	% Infection (no. infected/no. challenged)	Mean inoculum size	% Infection (no. infected/no. challenged)
U.S.				
Untreated	159 91 34	73 (27/37) 34.5 (10/29) 13.6 (3/22)	Not done	Not done
Ozone treated (0.3 mg liter ⁻¹ , 2 min)	25,625 2,633.3 301	100 (23/23) 74.1 (20/27) 20 (4/20)	24,717 2,480.9 280.2	56 (14/25) 11.1 (3/27) 0 (0/27)
U.K. ^a				
Untreated	136.9 76 24	68.6 (24/35) 41.7 (10/24) 16.7 (6/36)	Not done	Not done
Ozone treated (0.3 mg liter ⁻¹ , 2 min)	24,450 2,224 246	100 (23/23) 100 (24/24) 45.8 (11/24)	23,580 2,316 196	100 (22/22) 25 (6/24) 12.5 (3/24)

^a U.K., United Kingdom.

TABLE 4. Determination of percent infectivity of ozone-treated oocysts using neonatal mouse infectivity assays

Location	Oocyst type and replicate no.	Mean oocyst inoculum per mouse	% Infection per group of mice	No. of infectious oocysts per inoculum ^b	% Oocyst infectivity ^c
U.S.	Fresh, 2	249	30.4	67 ± 23	27 ± 9
	Aged, 1	2,633.3	74.1	184 ± 69	7 ± 3
		301	20.0	47 ± 27	16 ± 9
	Aged, 2	24,717	56.0	99 ± 74	0.4 ± 0.3
2,480.9		11.1	70	2.8	
U.K. ^a	Fresh, 1	2,683	87.5	217 ± 8	8 ± 0.3
		268	50.0	108 ± 61	40 ± 23
	Fresh, 2	2,383.3	91.7	219 ± 7	9 ± 0.3
		242	37.5	74 ± 29	31 ± 12
	Aged, 1	246	45.8	95 ± 47	39 ± 19
		Aged, 2	2,316	25.0	58 ± 23
	196		12.5	53 ± 28	27 ± 14

^a U.K., United Kingdom.

^b Number of infectious oocysts per inoculum calculated by extrapolation of percent infection on U.S.-U.K. logit dose-response curve (14).

^c Percent infectivity = (number of infectious oocysts per inoculum/mean oocyst inoculum per mouse) × 100.

fection regimens in water treatment processes, and considerable effort has been expended in identifying alternative, more efficacious disinfectants. An underlying barrier to accruing meaningful data from disinfection experiments has been the selection of appropriate assays for measuring oocyst inactivation following their treatment with disinfectants.

A number of different vital-dye-based viability assays have been developed as alternatives to mouse infectivity assays and are recognized to be more user-friendly than in vitro excystation, a conventional viability assay for coccidia. Various investigators have used either the vital-dye-based assays alone (17) or in conjunction with in vitro excystation (20) or in vitro excystation alone (1) or in conjunction with mouse infectivity assays (12, 13, 16) to measure oocyst inactivation following ozone treatment. Some of these assays provide an indication of viability by measuring changes in oocyst permeability or determining oocyst response to biological stimuli, whereas others measure the ability of treated oocysts to excyst, invade, and multiply within the enterocytes of susceptible animal hosts. This has created confusion regarding the significance of data obtained by these various assays. In order to identify the rela-

tionship between three vital-dye-based assays (SYTO-9, SYTO-59, and DAPI-PI) with maximized in vitro excystation and neonatal mouse infectivity assays, fresh and environmentally aged *C. parvum* oocysts were exposed to preselected ozone concentrations for times that would lead to their partial inactivation. Following their exposure, oocysts were subjected to viability and infectivity analysis with selected in vitro surrogates and neonatal mouse infectivity assays.

Our data indicate that in animal infectivity assays, untreated oocysts displayed greater variability both within and between laboratories than they did in the in vitro surrogates. Following ozonation, with the exception of SYTO-59, which was the least responsive, the in vitro surrogates demonstrated both increased variability and consistently higher viability values than did infectivity assays. Also, in these trials the viability assessment of oocysts with SYTO-9 exhibited the greatest variability between laboratories for both fresh and aged oocysts.

Our findings that in vitro excystation and DAPI-PI overestimate oocyst viability in comparison with neonatal mouse infectivity assays are in agreement with earlier studies (4, 12, 13). Previously published reports (3, 16) have indicated that

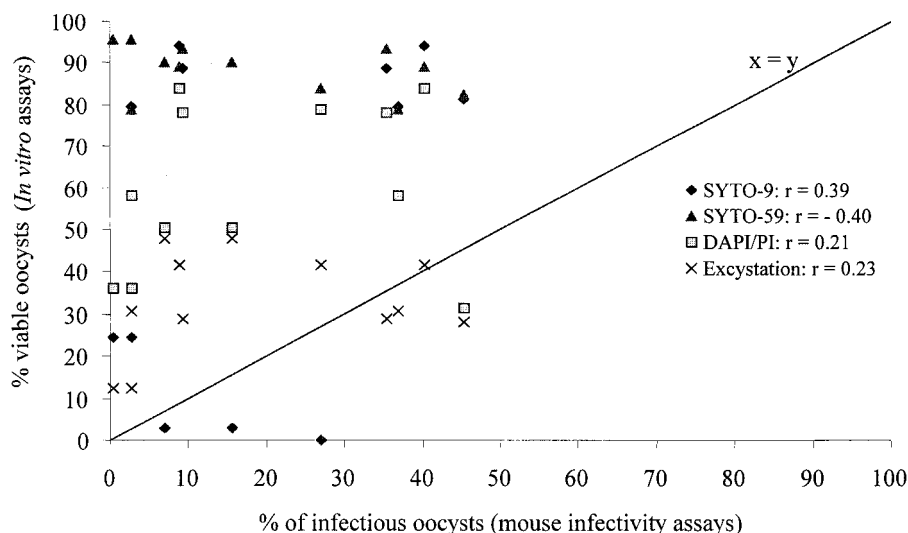


FIG. 5. Comparison of in vitro viability assays with neonatal mouse infectivity assays.

SYTO-9 and SYTO-59 correlate well with neonatal mouse infectivity assays. In contrast, our study indicated that these SYTO dye-based assays overestimate oocyst viability compared to infectivity assays of ozone-treated oocysts. Perhaps these differences were due to the fact that one of these cited studies (16) limited its evaluations to fresh, untreated oocysts.

Some investigators have attributed differences between in vitro and in vivo surrogates to the higher sensitivity of the mouse infectivity assay (12), which would suggest that the use of automated enumeration procedures for in vitro analyses could help to overcome this disparity in the number of organisms that can be analyzed by in vitro and in vivo assays. Following ozonation, the inoculum sizes in our study were selected judiciously to ensure the presence of sufficient infectious oocysts to yield a response in recipient mice. However, the administered inoculum sizes were deliberately "capped" to ensure that the enumeration of in vitro viability data remained comparable to that of infectivity data. Despite this, significant differences existed between the in vitro and in vivo assays. Possible explanations for the disparity between in vitro and in vivo assays follow. (i) Differences in the number of oocysts utilized for the enumeration of viability and infectivity values were significantly large to create method sensitivity issues. (ii) Perhaps the low ozone concentrations and contact times caused insufficient structural damage to the outer wall. Although this would not change the permeability of the outer wall to vital dyes, the ozone concentrations may have been sufficient to cause changes in either the apical complex organelles of sporozoites (preventing the attachment or invasion of enterocytes) or the structure of sporozoite DNA (preventing replication).

During the disinfection experiments, it was noted that while the vital-dye-based in vitro surrogates were the easiest to use for classifying oocysts as viable or nonviable, the in vitro excystation procedure was more problematic. Following a short oocyst exposure (3 to 4 min) to low concentrations of ozone (0.4 mg liter⁻¹), the analysis of subsamples at 30 min after incubation of the ozone-exposed oocysts in excystation fluids revealed sporozoite ratios (number of free sporozoites/number of excysted + number of partially excysted oocysts) in excess of 10 in some samples. Theoretically, a maximum sporozoite ratio of four can be expected from each excysted oocyst, as a single oocyst is known to accommodate a maximum of four sporozoites. The oocyst suspensions under examination were highly purified, and the occlusion of excysted (empty) oocysts was an unlikely factor in the peculiarly high sporozoite ratios. Our unpublished observations following oocyst exposure to low ozone concentrations indicate the promotion of the oocyst-clumping phenomenon, and our published data have also shown dead oocysts demonstrating greater adhesion to each other and to debris (5). Based on such evidence, there is a high likelihood that oocyst clumps formed after ozonation consist largely of dead and excysted oocysts. Should this have occurred, subsample analyses postexcystation could have yielded highly skewed counts of excysted and nonexcysted oocysts in the excystation fluid. While distribution of the excysted and nonexcysted oocysts may be uneven, the sporozoites could continue to be evenly distributed in the excystation fluid. This would explain why bizarre sporozoite ratios were observed postozonation and would also explain why the viability values obtained with the in vitro surrogates were significantly higher than those obtained with mouse infectivity assays.

More recently, several molecular viability markers, such as the heat shock protein 70 gene (24) and the β -tubulin gene (27), have been identified. Furthermore, refinements have been made to in vitro cultivation techniques (10, 25) to im-

prove reproducibility and sensitivity, thus facilitating the assessment of oocyst viability. Clearly, there is an urgent need to standardize and evaluate these potential viability assays with respect to mouse infectivity, in order to overcome the ethical and financial constraints of using neonatal mouse infectivity assays to measure disinfection efficacy or to determine the infectivity of environmentally derived oocysts.

In conclusion, these comparisons have illustrated that three vital-dye-based assays and maximized in vitro excystation all significantly overestimate *C. parvum* oocyst viability postozonation, compared to neonatal mouse infectivity assays. A similar disparity between in vitro and in vivo assays has also been reported for UV-treated oocysts (6). The factors responsible for these differences are unclear and may be associated with differences in assay sensitivity, mechanisms of oocyst inactivation, or the uneven distribution of the organisms posttreatment. Animal infectivity assays will continue to be regarded as a "gold standard" for the foreseeable future, and their use is advocated for disinfection studies measuring the inactivation of *Cryptosporidium* oocysts.

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