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Comparison of Animal Infectivity, Excystation, and Fluorogenic Dye as Measures of *Giardia muris* Cyst Inactivation by Ozone

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***Giardia muris* cyst viability after ozonation was compared by using fluorescein diacetate-ethidium bromide staining, the C3H/HeN mouse-*G. muris* model, and in vitro excystation. Bench-scale batch experiments were conducted under laboratory conditions (pH 6.7, 22°C) in ozone-demand-free phosphate buffer. There was a significant difference between fluorogenic staining and infectivity ($P \leq 0.05$), with fluorogenic staining overestimating viability compared with infectivity estimates of viability. This suggests that viable cysts as indicated by fluorogenic dyes may not be able to complete the life cycle and produce an infection. No significant differences between infectivity and excystation and between fluorogenic staining and excystation ($P \leq 0.05$) were detected for inactivations up to 99.9%. Only animal infectivity had the sensitivity to detect inactivations greater than 99.9%. Therefore, the animal model is the best method currently available for detecting high levels of *G. muris* cyst inactivation.**

The U.S. Environmental Protection Agency Primary Disinfection Rule (20) sets mandatory disinfection requirements for all public water systems and maximum contaminant levels or treatment technique requirements for various contaminants including *Giardia lamblia*. The Surface Water Treatment Rule requires that 99.9% of *G. lamblia* cysts and 99.99% of enteric viruses be inactivated or removed for surface water supplies (20). Because of difficulties in detection and viability determination, the requirement for *G. lamblia* was defined in terms of disinfectant concentration-time products (21). Published ozonation values were based on the extrapolation of 99% inactivation of *G. lamblia* results from a single laboratory (22, 23). More recently, other work on ozone inactivation of *Giardia muris* has been performed at pilot scale by the Metropolitan Water District of Southern California (24). Both studies used in vitro excystation for viability determinations.

The methods which have been used for *Giardia* viability determination include vital dye exclusion (3) and fluorogenic dye staining (7, 9, 10), as well as determinations of cyst morphology (18), animal infectivity (1, 2, 13), and in vitro excystation (4, 15, 16). In vitro excystation is the most frequently used method and has been recommended by the U.S. Environmental Protection Agency (21) for use in chemical disinfection studies.

deRegnier et al. (5) used exclusion of propidium iodide (PI) and infectivity in non-Swiss CF-1 mice to measure the viability of *G. muris* cysts suspended in lake, river, and tap water. They concluded that *G. muris* cyst viability as determined by mouse infectivity could not be directly compared with PI exclusion since as few as 5 to 10 cysts may have been an infective dose, while the inocula used in their study may have contained from 10^2 to 10^3 cysts. Schupp and Erlandsen (17) found that cysts positively stained with fluorescein diacetate (FDA) were viable, as determined by infectivity, and that PI-positive cysts were nonviable and incapable of producing infection. By using 10^3 FDA-positive cysts as the inoculum, cysts were detected on day 5; with an inoculum size of 5×10^4, cysts were detected on day 3. Smith and Smith (19) reported that FDA staining of *G. lamblia* consistently overestimated cyst viability and PI staining underestimated viability compared with in vitro excystation estimates of viability.

A comparison of animal infectivity and excystation as measures of *G. muris* cyst inactivation by chlorine was made by Hoff et al. (8). They determined that 1 to 15 *G. muris* cysts constituted an infectious dose for outbred female Swiss CF-1 mice. Neither animal infectivity nor excystation consistently showed a higher degree of inactivation. However, only inactivations up to 99.7% were examined. Fluorogenic staining techniques have been used to assess *Giardia* cyst viability following chlorination (7) and to evaluate the efficacy of iodine- and chlorine-based backcountry water treatment devices (12). Hale et al. (7) reported that *G. lamblia* cysts ceased to stain positively with FDA only at toxic concentrations of chlorine (256 mg/liter) with a contact time of 30 min. Ongerth et al. (12) reported that fluorogenic staining indicated a slightly higher proportion of viable *G. muris* cysts than did in vitro excystation following heat inactivation. No studies investigating the use of fluorogenic staining following ozonation were found.

The objective of this study was to compare *G. muris* cyst inactivation following ozonation by three viability methods using split samples. The methods used were a fluorescein diacetate-ethidium bromide (FDA-EB) staining technique, the C3H/HeN mouse-*G. muris* model, and in vitro excystation.

MATERIALS AND METHODS

Stock ozone solution. Ozone was generated from extradry oxygen by using a corona discharge generator (C2P-9C; PCI Ozone Corp.). The ozone concentration in the carrier gas was approximately 4.8% ozone (wt/wt) at the ambient temperature and pressure. The ozone-oxygen gas mixture was bubbled for a minimum of 20 min through 400 ml of 22°C Milli-Q water (OM-140; Millipore Corp.) in a 500-ml gas

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absorption flask. The stock solution concentration ranged from 18 to 20 mg of O_3 per liter.

Phosphate buffer and ozone reactor. A 0.05 M phosphate buffer (pH 6.7) was used for suspending cysts. Phosphate buffer was prepared by using potassium dihydrogen orthophosphate and disodium hydrogen orthophosphate (AnalaR grade; BDH). The bench-scale batch reaction vessel consisted of a 250-ml Erlenmeyer flask containing approximately 200 ml of test liquid. The reactor and buffer were made ozone-demand-free prior to use as described elsewhere (11). The concentration of ozone in an aqueous solution was continuously determined by using a diode array spectrophotometer (8452A; Hewlett-Packard) at 260 nm and a molar absorption coefficient of 3,300 $M^{-1} \cdot cm^{-1}$.

Cyst preparation. The strain of *G. muris* used in this study was originally isolated by Roberts-Thomson et al. (13) and was obtained from B. J. Underdown, McMaster University, Hamilton, Ontario, Canada. Specific-pathogen-free (SPF) 6-to-8-week-old male C3H/HeN mice were used and kept under SPF conditions (in a laminar flow isolator) prior to use. In order to produce highly purified cysts, feces were collected from 10 to 15 mice over a period of 2 to 3 h and cysts were isolated from the feces by using the sucrose gradient centrifugation technique described by Roberts-Thomson et al. (13) and modified by Belosevic and Faubert (1). Briefly, collected feces were soaked for 30 min in Milli-Q water, emulsified by using applicator sticks, layered on a sucrose solution (specific gravity, 1.12), and centrifuged for 15 min at 400 × *g* in a swinging-bucket centrifuge (GR4.11; Jouan) at 4°C. After centrifugation, cysts trapped at the water-sucrose interface were carefully removed with a pipette and washed in Milli-Q water by centrifugation at 600 × *g* for 10 min. Two to four additional Milli-Q water washes were performed so that the A_{260} of a cyst suspension of 10^4 cysts per ml did not exceed 0.03/cm. The final cyst preparation was resuspended to the desired concentration by using ozone-demand-free Milli-Q water. The number of cysts in the sample was determined by counting four complete grids of a hemocytometer. Cysts were stored at 4°C for 18 h and were used within 48 h of preparation.

Isolation of trophozoites. The upper 25% of the small intestine was placed in 3 ml of phosphate-buffered saline (GIBCO). Four to 10 mucosal scrapings from this segment were placed on glass microscope slides and examined for the presence of trophozoites by using a bright-field microscope.

Experimental procedure. The 200-ml volume of phosphate buffer was seeded with *G. muris* cysts to provide a cyst concentration that was typically 10^4 cysts per ml. The concentration of stock ozone solution was measured twice immediately before a calculated volume of ozone solution was added to the liquid with a mass-calibrated pipette. This was the applied ozone dose. Over the duration of the experiment, the test solution was pumped through a 35-μl, 1-cm-light-path-length flow cell at approximately 8 ml/min. Residual ozone was neutralized in the reactor at the end of the contact period by using 50 μl of 1.0 M sodium formate. Tests were conducted at 22°C. Tween 20 (Sigma Chemical Co., St. Louis, Mo.) was added to the ozone reactor (0.01%, vol/vol) after the ozone was neutralized to enhance cyst recovery. Four replicate hemocytometer counts of cyst density following ozonation were made. All disinfected liquid from the reactor was centrifuged in 175-ml plastic conical centrifuge tubes at 800 × *g* for 10 min. The supernatant was removed, leaving a 2.1-ml centrifugate, of which 1.0 ml was used for infecting mice, 0.5 ml was used for excystation, and 0.100 ml was used for FDA-EB fluorogenic staining, the remaining portion being surplus.

Fluorogenic staining. A modified FDA-EB staining technique originally described by Jackson et al. (9) was used. FDA stock solution (5 mg/ml in acetone; ICN Biochemicals Corp.) and EB stock solution (20 μg/ml) in phosphate-buffered saline (Sigma) were stored at −30°C prior to use. A working dilution of FDA was made by adding 20 μl of stock FDA to 10 ml of Milli-Q water. A 0.1-ml aliquot of *G. muris* cysts was added to a 1:1 solution of diluted FDA (50 μl) and stock EB (50 μl). The mixture was incubated at room temperature for 5 min, and the reaction was then stopped by adding 200 μl of Milli-Q water. A hemocytometer was loaded with a well-mixed, stained cyst suspension, and the cysts were allowed to settle for 60 s. The cysts were counted first under incident light and then under UV light by using an epifluorescence microscope. A minimum of four complete grids of the hemocytometer were counted for each sample, and counting was completed within 1 h.

Excystation. A procedure described by Schaefer et al. (16) was used to determine excystation. Between 10^5 and 5×10^5 purified *G. muris* cysts contained in 0.5 ml of solution were placed into a 15-ml plastic, screw-cap, graduated conical centrifuge tube. First 5 ml of prewarmed (37°C) reducing solution was added, and then 5 ml of prewarmed (37°C) freshly prepared 0.1 M $NaHCO_3$ was added. The tube was vortexed vigorously, incubated in a 37°C water bath for 30 min, and centrifuged for 2 min at 400 × *g* in a swinging-bucket centrifuge, and the supernatant was aspirated down to 0.2 ml. The pellet was resuspended in 10 ml of chilled (4°C) trypsin-Tyrode's solution (16) and vortexed vigorously. The tube was centrifuged for 2 min at 400 × *g* in a swinging-bucket centrifuge, and the supernatant was aspirated down to 0.2 ml. Three-tenths of a milliliter of prewarmed (37°C) trypsin-Tyrode's solution was added to the tube, and the pellet was resuspended. Three to 4 drops of the suspension was placed in a chamber slide, the coverslip was sealed with a petrolatum-paraffin mixture, and the slide was incubated in a 37°C air incubator for 30 min. It was then examined with a phase-contrast microscope and a 40× phase objective lens. Empty cyst walls (ECW) and partially excysted trophozoites (PET) were counted as positive excystation; intact cysts (IC) showed no signs of excystation. The total number of ECW, PET, and IC examined was 1,000, or the number present in the entire slide area if it was not possible to find 1,000. The excystation procedure was completed within 3 h.

Infectivity. A minimum of four hemocytometer counts were made on the 1.0-ml portion of the split sample in order to determine the total number of cysts. Each one of a group of five C3H/HeN mice was orally inoculated with 0.200 ml. The size of the inoculum was typically 10^5 cysts per mouse. During experiments, mice were individually housed in filter-top cages (0.22-μm-pore-size filters) in an SPF isolation room. The feces were checked daily for *G. muris* cysts from day 3 to day 8 or until they became positive. On day 8, necropsy was performed on all mice which were negative for *G. muris* cysts and the small intestine was examined for the presence of trophozoites.

Analysis. (i) Fluorogenic staining. The equation used for determining inactivation (N/N_0) from FDA-EB data was

$$\frac{N}{N_0} = \frac{\%\text{ stained cysts that are FDA-positive ozonated}}{\%\text{ stained cysts that are FDA-positive control}} \quad (1)$$

where FDA-positive cysts are viable and have a green

TABLE 1. C3H/HeN mice infected by *G. muris* as a function of days after infection and inoculum size

Inoculum (cysts/mouse)	No. of mice	No. of mice positive for cysts on postinfection day[a]:									
		1	2	3	4	5	6	7	8	9	10
10^5	9	0	0	9	9	9	9				
10^4	10	0	0	0	10	10	10	10			
10^3	10	0	0	0	3	10	10	10	10		
10^2	9	0	0	0	0	5	9	9	9	9	
10	9	0	0	0	0	3	6	6	9	9	9
1	12	0	0	0	0	0	0	2	3	5	5[b]

[a] Data were collected until day 10 or for 3 days after all mice in the group became positive.
[b] Trophozoites were detected in 9 of the 12 mice in this group.

perimeter when viewed under UV light by using an epifluorescence microscope. EB-positive cysts are nonviable and fluoresce red by using epifluorescence microscopy.

(ii) Excystation. The equation used for determining excystation from *G. muris* excystation data (15, 21) was

$$\% \text{ excystation} = \frac{\text{ECW} + \text{PET}}{\text{ECW} + \text{PET} + \text{IC}} \times 100 \quad (2)$$

Inactivation (N/N_0) is calculated from

$$\frac{N}{N_0} = \frac{\% \text{ excystation ozonated}}{\% \text{ excystation control}} \quad (3)$$

(iii) Infectivity and the C3H/HeN mouse model. The animal model used was proposed by Roberts-Thomson et al. (13) and further refined by Belosevic and Faubert (1). Data on the effects of different doses of untreated cysts on the duration of the latent period in SPF male C3H/HeN mice are presented in Table 1. The data show a significant correlation between the size of the inoculum (cysts per mouse) and the time (days) when all mice in a given dose group were passing cysts. The discriminating variable is not the beginning of the latent period but rather the time when all mice in a group were releasing cysts in the feces. To date, there have been 11 replications of the 10^5 cyst dose; 13 replications of the 10^4 cyst dose; and 2 replications for each of the 10^3, 10^2, and 10 cyst doses, all with identical results. The equation used for determining inactivation (N/N_0) from mouse model data was

$$\frac{N}{N_0} = \frac{n \times I}{n_0 \times I_0} \quad (4)$$

where n is the number of positive mice in an ozonated trial, I is the number of infectious cysts per positive mouse after ozonation, as determined by using the latent period (if the inoculum had 10^5, 10^4, 10^3, 10^2, or 10 infectious cysts, mice became positive on day 3, 4, 5, 6, or 8, respectively), n_0 is the number of positive mice in the control trial, and I_0 is the inoculum size in the control, as determined by the hemocytometer count.

In equation 4, the latent period definition was the time when all mice became positive. The term n was required in equation 4 since, on occasion, not all of the mice in a cohort became positive. If not all of the mice in an ozonated trial became positive, the last day which showed an increase in the number of positive mice was used as the latent period. The term n_0 was required in equation 4 since an occasional mouse in the control cohort died before day 8, such as in trial B2, and could not be included in estimating the initial number of viable cysts.

TABLE 2. Experimental conditions and bench-scale ozone inactivations estimated by three viability methods for *G. muris* in ozone-demand-free phosphate buffer (pH 6.7, 22°C)

Trial	Contact time (min)	Residual ozone (mg/liter)	Inactivation (log N/N_0)[a]		
			FDA-EB staining	In vitro excystation	C3H/HeN mouse model
A1	5	0.3	>2.1	>2.8	4.2
A2	5	0.4	>2.2	>2.7	4.2
B1	5	0.1	2.3	3.0	3.1
B2	5	0.1	2.7	>3.0	3.1
C1	5	1.3	>2.8	>2.9	4.4
C2	0.58	0.04	0.2	0.5	1.2
C3	0.25	0.2	0.006	0.7	ND
C4	0.33	0.1	0.004	0.2	ND
D1	0.58	0.2	0.5	1.8	2.2
D2	1	0.1	0.7	2.1	ND
D3	2	0.1	2.4	2.0	ND
D4	1.53	0.02	0.5	2.4	ND

[a] >, detection limit; ND, not determined.

Experimental design. A single control trial and two or more ozonated trials were performed on each off our cyst preparations (AtoD). Cysts from each control and ozonated trial were concentrated, with the resulting concentrate split three ways to provide cysts for the different viability determination methods. This design permitted a comparison of differences in the viability methods while eliminating run-to-run variations in the test water and ozonation. Trials B1 and B2 were replicates.

RESULTS

The final centrifugate in controls contained approximately 1.4×10^6 *G. muris* cysts in 2.1 ml, and the mean recovery of control cysts was 68% ± 9%. It was possible to determine cyst losses after each step in the process since multiple hemocytometer counts were made. Cyst losses totalled approximately 30%, with the greatest loss (15%) accounted for by pumping during the reaction. Excystation values in controls were 96.3, 99.1, 95.8, and 93.0% for preparations A, B, C, and D, respectively. The mean control excystation was 96.1% ± 2.5%, indicating that cyst preparations were of high quality. After fluorogenic staining, three populations of cysts were observed: green (FDA positive), red (EB positive), and black (unstained). It was easier to observe black cysts under bright-field microscopy than with epifluorescence microscopy. The fraction of control cysts that were EB positive ranged from 9 to 12%. Forty to 60% of *G. muris* cysts in the controls were unstained. However, this fraction decreased to 6.9 ± 5.5% after ozonation, with a range of 1.9 to 16.1%.

Experimental conditions and inactivation results for the three viability methods are summarized in Table 2. Trials A1, A2, and C1 had ozone residuals of 0.3 mg/liter or higher, and although the entire slide area was examined in the excystation method it was only possible to count 669, 546, and 782 cysts, respectively, with all cysts being intact cysts. The corresponding counts in the fluorogenic dye method were 143, 169, and 675, all cysts being either EB positive or unstained. These trials were at the detection limit since there was no positive excystation or any FDA-positive cysts. None of the mouse model results were at the detection limit since at least one mouse became positive in all trials.

TABLE 3. One-way ANOVA for three viability determination methods performed on split samples following ozone inactivation of *G. muris*

Source	Sum of squares[a]	Degrees of freedom[b]	Mean square
Between viability methods[c]	1.97	2	0.986
Error	1.48	9	0.164

[a] Total sum of squares was 3.45.
[b] Total degrees of freedom was 11.
[c] F ratio and P were 6.005 and 0.022, respectively.

The three viability methods were compared by a one-way analysis of variance (ANOVA). Results are summarized in Table 3. In the analysis, trials A1, A2, and C1 were excluded since they were beyond the detection limit of all methods except infectivity. Fluorogenic staining was used as a reference method since the difference between all of the other methods could be calculated. The ANOVA was performed by using differences from the reference method. The underlying assumptions of the ANOVA were tested and found to be satisfied. The Duncan multiple range test (6) was used to determine if there was a significant difference between the viability methods. It was determined that there was a significant difference between infectivity and fluorogenic staining ($P \leq 0.05$). There was no significant difference between infectivity and excystation or between excystation and fluorogenic staining ($P \leq 0.05$).

DISCUSSION

General. Fluorogenic staining significantly underestimated inactivations determined by using infectivity ($P \leq 0.05$). This would suggest that not all FDA-positive cysts were capable of producing an infection. In contrast, the difference between infectivity and excystation and between fluorogenic staining and excystation was not statistically significant ($P \leq 0.05$).

In Table 2, it can be seen that fluorogenic staining gave the highest viabilities, or smallest inactivations, and infectivity provided the lowest viabilities; excystation provided results intermediate to the other two methods. It can also be seen that there was a greater difference between fluorogenic staining and the other methods at lower inactivations than at higher ones by comparing those trials which had an ozone residual of 0.1 to 0.2 mg/liter. However, the animal model is a measure of the potential of the cysts to complete their life cycle in the host. In contrast, fluorogenic staining measures the presence or absence of metabolic activity. It is possible that cysts which show some metabolic potential will not cause an infection in the animal host, thereby causing an apparent overestimation of viability.

A qualitative comparison of the three viability methods in terms of the basis of the method, equipment, and time required and theoretical and observed detection limits following ozonation is provided in Table 4. It can be seen that observed detection limits do not always equal their theoretical values and that infectivity requires considerably more time than the other methods. The time given in Table 4 for the mouse model was that required for a test trial latent period of up to 5 days greater than that of the control. To demonstrate 3 log inactivation, it would only be necessary to check control mice on day 3 and test mice on day 6. In the numerical comparison of inactivation estimates following ozonation, it should be remembered that the basis of each method, as indicated in Table 4, is different. The fact that two in vitro methods are being compared with one in vivo method adds an additional dimension of uncertainty.

The observed fluorogenic staining and in vitro excystation detection limits were 2.1 to 3.0 log inactivation depending on the number of cysts counted or found. In three of the twelve excystation trials, there were fewer than 1,000 cysts available for counting. FDA-EB inactivation estimates were based on stained cysts only, and the number counted ranged from 140 to 966 in the 12 trials. The detection limit for both of these methods based on the microscopic examination of 1,000 cysts is 3.0 log inactivation. The detection limit for the C3H/HeN mouse-*G. muris* model is based on the size of the inoculum in the control (10^5 cysts) and is 5 log inactivation. The observed mouse model detection limit was >4.4 log inactivation.

Fluorogenic staining. A potential problem with fluorogenic dyes is that they are sometimes affected by the particular disinfectant used (14). This did not appear to be the case with ozone and FDA-EB. Unstained cysts were not used in calculating the ozone and FDA-EB results. Unstained cysts were not used in calculating inactivation since their numbers decreased markedly following ozonation and since assumptions would have been required regarding the unstained cyst viability. Schupp and Erlandsen (17) found that unstained *G. muris* cysts were infective to mice in a study that did not involve disinfectants. In studies using *G. lamblia*, Hale et al. (7) found that 21% of *G. lamblia* cysts were unstained with FDA-EB after 30 min of exposure to 1 mg of chlorine per liter, and Smith and Smith (19) found one of four *G. lamblia* isolates was 100% unstained by using FDA-PI. Unstained cysts decrease the efficiency of fluorogenic staining procedures in determining inactivation since 1,000 stained cysts rather than 1,000 total cysts must be counted to determine 99.9% inactivation.

Excystation. In vitro excystation is based on chemical induction. Care must be taken to determine whether the

TABLE 4. Generic comparison of three viability methods in terms of basis of method, equipment, and time required and theoretical and observed detection limits following ozonation

Method	Basis of method	Detection limit (log N/N_0)		Time required	Specialized equipment required
		Theoretical	Observed		
FDA-EB	Biochemical metabolism or exclusion	3.0	2.1 to 2.8	1 h	Epifluorescence microscope
In vitro excystation	Chemical induction	3.0	2.7 to 3.0	3 h	Phase-contrast microscope
C3H/HeN mouse model	Infectivity	5.0	>4.4	8 days	Animal care facilities

excysted trophozoites are alive or dead (16). Schupp and Erlandsen (17) noted that the method is subjective, since aborted attempts at trophozoite emergence might be included. Results from excystation replicates B1 and B2 agreed, even though one of the values was at the detection limit. In trials A1, A2, and C1, which had ozone residuals of 0.3 mg/liter or higher, it was not possible to find 1,000 cysts by using the normal in vitro excystation procedure. Therefore, it was suspected that one of the modes of action of ozone was to lyse cysts, since all factors except the ozone residual were the same as in other trials in which 1,000 cysts were found.

Infectivity. Animal infectivity provided a direct measure of the ability of cysts to cause infection. Results from C3H/HeN mouse model replicates B1 and B2 agreed. Infectivity provided information on groups of cysts rather than on individual cysts and consequently detected high inactivations. No other viability method was capable of detecting inactivations with ozone residuals of 0.3 mg/liter or higher in the demand-free phosphate buffer.

Other available infectivity methods include the 50% infective dose (ID_{50}) technique and the most-probable-number method. Although ID_{50} has superior statistical properties, it was not used in the present study since the latent period approach avoided the expense of a cohort of mice at each dilution level, as required with the ID_{50} and most-probable-number methods.

Comparison with other studies. deRegnier et al. (5) noted that all *Giardia* cysts capable of producing infection show viability by fluorogenic dyes or excystation, but not vice versa. An inspection of Table 2 reveals that, in general, infectivity indicated the highest inactivations, in vitro excystation showed lower inactivation, and inactivation estimates from fluorogenic staining were the lowest. Smith and Smith (19), working with *G. lamblia*, reported that FDA staining consistently overestimated cyst viability compared with in vitro excystation estimates of viability. This is in agreement with the results reported here for *G. muris* (i.e., smaller FDA-EB inactivations in Table 2). It is also consistent with the results of Ongerth et al. (12), who reported that fluorogenic staining indicated a slightly higher proportion of viable *G. muris* cysts than did in vitro excystation after heat inactivation.

The latent periods reported by Schupp and Erlandsen (17) for non-Swiss CF-1 mice were similar to those reported here for C3H/HeN mice (Table 1). The results of this study by using a course of infection-based animal model and ozonation agree with Hoff et al. (8), who used an ID_{50} technique in which there was little difference between infectivity and excystation up to 99% inactivation by using chlorine. However, in the present study, only animal infectivity had the sensitivity to detect inactivations of >99.9%.

There were no significant differences ($P \leq 0.05$) between infectivity and in vitro excystation and between fluorogenic staining and excystation for up to 99.9% inactivation of *G. muris* cysts. Fluorogenic staining overestimated viability compared with infectivity ($P \leq 0.05$). However, the animal model is a measure of the potential of the cysts to complete their life cycle in the host. In contrast, fluorogenic staining measures the presence or absence of metabolic activity. It is possible that cysts which show some metabolic potential will not cause an infection in the animal host, thereby causing an apparent overestimation of viability. While fluorogenic staining and excystation are in vitro methods, the animal model provides an in vivo determination of viable cyst infectivity potential. The C3H/HeN mouse-*G. muris* model was highly sensitive and capable of measuring 1.5 to 2 log inactivation higher than the other two methods. Should *Giardia* inactivation requirements be made more rigorous, infectivity will provide the means for evaluating disinfection requirements.

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REFERENCES

1. **Belosevic, M., and G. M. Faubert.** 1983. *Giardia muris*: correlation between oral dosage, course of infection and trophozoite distribution in mouse small intestine. Exp. Parasitol. **56**:93–100.
2. **Belosevic, M., G. M. Faubert, J. D. MacLean, C. Law, and N. A. Croll.** 1983. *Giardia lamblia* infections in Mongolian gerbils: an animal model. J. Infect. Dis. **147**:222–226.
3. **Bingham, A. K., E. L. Jarroll, Jr., E. A. Meyer, and S. Radulescu.** 1979. Induction of *Giardia* excystation and the effect of temperature on cyst viability as compared by eosin-exclusion and *in vitro* excystation, p. 217–229. *In* W. Jakubowski and J. C. Hoff (ed.), Waterborne transmission of giardiasis, report no. EPA-600/9-79-001. U.S. Environmental Protection Agency, Cincinnati, Ohio.
4. **Bingham, A. K., and E. A. Meyer.** 1979. *Giardia* excystation can be induced *in vitro* in acidic solutions. Nature (London) **277**: 301–302.
5. **deRegnier, D. P., L. Cole, D. G. Schupp, and S. L. Erlandsen.** 1989. Viability of *Giardia* cysts suspended in lake, river, and tap water. Appl. Environ. Microbiol. **55**:1223–1229.
6. **Duncan, D. B.** 1955. Multiple range and multiple F tests. Biometrics **11**:1–42.
7. **Hale, D. C., C. C. Johnson, and M. D. Kirkham.** 1985. *In vitro Giardia* cyst evaluation by fluorescent dyes. Mikrooekol. Ther. **15**:141–148.
8. **Hoff, J. C., E. W. Rice, and F. W. Schaefer III.** 1985. Comparison of animal infectivity and excystation as measures of *Giardia muris* cyst inactivation by chlorine. Appl. Environ. Microbiol. **50**:1115–1117.
9. **Jackson, P. R., M. G. Pappas, and B. D. Hansen.** 1985. Fluorogenic substrate detection of viable intracellular and extracellular pathogenic protozoa. Science **227**:435–437.
10. **Jones, K. H., and J. A. Senft.** 1985. An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide. J. Histochem. Cytochem. **33**: 77–79.
11. **Labatiuk, C. W., M. Belosevic, and G. R. Finch.** Evaluation of high level ozone inactivation of *Giardia muris* using an animal infectivity model. Ozone Sci. Eng., in press.
12. **Ongerth, J. E., R. L. Johnson, S. C. Macdonald, F. Frost, and H. H. Stibbs.** 1989. Backcountry water treatment to prevent giardiasis. Am. J. Public Health **79**:1633–1637.
13. **Roberts-Thomson, I. C., D. P. Stevens, A. A. F. Mahmoud, and K. S. Warren.** 1976. Giardiasis in the mouse: an animal model. Gastroenterology **71**:57–61.
14. **Sauch, J., D. Flanigan, M. Galvin, and D. Berman.** 1988. Propidium iodide as an indicator of *Giardia* cyst viability, abstr. Q48, p. 291. Abstr. 88th Annu. Meet. Am. Soc. Microbiol. 1988. American Society for Microbiology, Washington, D.C.
15. **Schaefer, F. W., III.** 1990. Methods for excystation of *Giardia*, p. 111–136. *In* E. A. Meyer (ed.), Giardiasis. Elsevier Science Publishers, Amsterdam.
16. **Schaefer, F. W., III, E. W. Rice, and J. C. Hoff.** 1984. Factors promoting *in vitro* excystation of *Giardia muris* cysts. Trans. R. Soc. Trop. Med. Hyg. **78**:795–800.
17. **Schupp, D. G., and S. L. Erlandsen.** 1987. A new method to determine *Giardia* cyst viability: correlation of fluorescein diacetate and propidium iodide staining with animal infectivity. Appl. Environ. Microbiol. **53**:704–707.

18. **Schupp, D. G., and S. L. Erlandsen.** 1987. Determination of *Giardia muris* cyst viability by differential interference contrast, phase, or brightfield microscopy. Parasitology **73**:723–729.
19. **Smith, A. L., and H. V. Smith.** 1989. A comparison of fluorescein diacetate and propidium iodide staining and *in vitro* excystation for determining *Giardia intestinalis* cyst viability. Parasitology **99**:329–331.
20. **U.S. Environmental Protection Agency.** 1989. Forty CFR parts 141 and 142 drinking water; national primary drinking water regulations: filtration, disinfection, turbidity, *Giardia lamblia*, viruses, *Legionella*, and heterotrophic bacteria, final rule. Fed. Regist. **54**(124):27486–27541.
21. **U.S. Environmental Protection Agency.** 1991. Guidance manual for compliance with the filtration and disinfection requirements for public water systems using surface water sources. American Water Works Association, Denver, Col.
22. **Wickramanayake, G. B., A. J. Rubin, and O. J. Sproul.** 1984. Inactivation of *Naegleria* and *Giardia* cysts in water by ozonation. J. Water Pollut. Control Fed. **56**:983–988.
23. **Wickramanayake, G. B., A. J. Rubin, and O. J. Sproul.** 1984. Inactivation of *Giardia lamblia* cysts with ozone. Appl. Environ. Microbiol. **48**:671–672.
24. **Wolfe, R. L., M. H. Stewart, K. N. Scott, and M. J. McGuire.** 1989. Inactivation of *Giardia muris* and indicator organisms seeded in surface water supplies by peroxone and ozone. Environ. Sci. Technol. **23**:744–745.