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CHEMICAL INACTIVATION OF *TOXOPLASMA GONDII* OOCYSTS IN WATER

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ABSTRACT: The protozoan parasite *Toxoplasma gondii* is increasingly recognized as a waterborne pathogen. Infection can be acquired by drinking contaminated water and conventional water treatments may not effectively inactivate tough, environmentally resistant oocysts. The present study was performed to assess the efficacy of 2 commonly used chemicals, sodium hypochlorite and ozone, to inactivate *T. gondii* oocysts in water. Oocysts were exposed to 100 mg/L of chlorine for 30 min, or for 2, 4, 8, 16, and 24 hr, or to 6 mg/L of ozone for 1, 2, 4, 8, or 12 min. Oocyst viability was determined by mouse bioassay. Serology, immunohistochemistry, and in vitro parasite isolation were used to evaluate mice for infection. Initially, mouse bioassay experiments were conducted to compare the analytical sensitivity of these 3 detection methods prior to completing the chemical inactivation experiments. *Toxoplasma gondii* infection was confirmed by at least 1 of the 3 detection methods in mice inoculated with all doses (10^5–10^0) of oocysts. Results of the chemical exposure experiments indicate that neither sodium hypochlorite nor ozone effectively inactivate *T. gondii* oocysts, even when used at high concentrations.

Toxoplasma gondii is a protozoan parasite whose worldwide importance as a zoonotic, food-borne pathogen is well documented (Choi et al., 1997; Mead et al., 1999). In recent years, increasing reports of major outbreaks of human toxoplasmosis associated with public water supplies in several countries have been attributed to waterborne transmission (Benenson et al., 1982; Bowie et al., 1997; American Water Works Association Research Division Microbiological Contaminants Research Committee, 1999; Aramini et al., 1999; Hall et al., 1999; Bahia-Oliveira et al., 2003; de Moura et al., 2006). Humans and other warm-blooded mammals and birds all serve as intermediate hosts for *T. gondii*. However, only wild and domestic felids are definitive hosts (Dubey and Beattie, 1988; Frenkel, 1990; Tenter et al., 2000). In felids, the parasite multiplies sexually in the intestine, producing millions of environmentally resistant oocysts that are shed in the feces (Tenter et al., 2000; Dubey, 2004). Once shed, these oocysts may sporulate and become infectious within 24 hr, are widely dispersed, and may survive in terrestrial and aquatic environments for months or years (Dubey and Beattie, 1988; Tenter et al., 2000; Lindsay et al., 2003; Dubey, 2004). Humans and livestock can be infected directly by drinking water contaminated with *T. gondii* oocysts or by ingesting vegetation irrigated with oocyst-contaminated water (Ortega et al., 1997; Slifko et al., 2000; Kniel et al., 2002; Dubey, 2004). Humans can also acquire an infection by consuming inadequately cooked meat products from infected livestock or from contact with infected cat feces (Tenter et al., 2000). In most immunocompetent people, *T. gondii* infections are mild to asymptomatic and parasites ultimately encyst in the host tissues as quiescent bradyzoite cysts. However, during periods of immunosuppression, encysted bradyzoites can transform into invasive, pathogenic tachyzoites and cause severe encephalitis (Frenkel, 1990; Luft and Remington, 1992; Montoya and Liesenfeld, 2004). The risk is also great for women who are infected during pregnancy; transplacental transmission of *T. gondii* may result in fetal death, abortion, congenital malformations, blindness, or mental retardation (Jones et al., 2001).

At present there are no specific regulations or approved methods to control *T. gondii* oocysts if present in public water supplies, and research in this area has been limited (Dubey et al., 1970; Lindsay et al., 2002, 2003). The purpose of this study was to evaluate 2 chemicals that are most commonly used to treat drinking water, i.e., chlorine (sodium hypochlorite) and ozone (Betancourt and Rose, 2004), for their ability to inactivate *T. gondii* oocysts in water. Resistance to chlorine has been demonstrated with other coccidian organisms. In prior research, 90% inactivation of *Cryptosporidium parvum* oocysts was achieved following exposure to 80 mg/L of free chlorine for 90 min (Korich et al., 1990). *Cryptosporidium parvum* oocysts treated with 5.25% aqueous sodium hypochlorite (undiluted household bleach) for a maximum of 120 min remained infectious to neonatal mice (Fayer, 1995). Additionally, oocysts from both *Cryptosporidium* and *Eimeria* species are routinely exposed to 1.75–5.25% aqueous sodium hypochlorite for 15 to 30 min to purify oocysts from bacteria and debris (Wagenbach et al., 1966; Bonnin et al., 1991). Given this high resistance of coccidian oocysts to at least chlorine, in the present study *T. gondii* oocysts were exposed to chlorine and ozone at concentrations greater than would typically be used for water disinfection. Typical chlorine concentrations used to disinfect drinking water range from 0.2 to 2 mg/L; for ozone disinfection, concentrations of ozone range from <0.1 to 1 mg/L (U.S. Environmental Protection Agency, 1999a). Thus, the chemical concentrations used in this study for *T. gondii* oocyst inactivation are ≥50 times higher than typical for chlorination and ≥6 times higher for ozonation of drinking water. Post-treatment oocyst viability was assessed using the mouse bioassay. Mouse infection was confirmed using 3 methods: serology, immunohistochemistry, and in vitro parasite isolation.

MATERIALS AND METHODS

Animals

All animal experiments were conducted with the approval and oversight of the Institutional Animal Care and Use Committee at the Uni-

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versity of California, Davis, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Female Swiss-Webster (SW) and C57BL/6 mice (Charles River Laboratories, Wilmington, Massachusetts), 20 to 22 g in weight, and 9- to 12-wk-old specific pathogen free kittens (Nutrition and Pet Care Center, Department of Molecular Biosciences, University of California, Davis, California) were used for oocyst production. An indirect fluorescent antibody test (IFAT) was used to prescreen mouse and kitten sera at 1:10 and 1:40 dilutions, respectively, for the presence of antibodies to *T. gondii* (Arkush et al., 2003; Dabritz, 2006).

Oocyst production

To produce bradyzoite cysts in mouse brains, 64 female mice (32 SW and 32 C57BL/6) were inoculated with culture-derived tachyzoites from a well-characterized type II *T. gondii* strain isolated from a southern sea otter (*Enhydra lutris nereis*) in California (Miller et al., 2004). Half of the mice (16 SW and 16 C57Bl/6) were inoculated subcutaneously (s.c.) with 10^6 tachyzoites and the remaining half (16 SW and 16 C57BL/6) were inoculated with 10^8 tachyzoites. When tissue cysts were detected by immunohistochemical examination of brains selected from randomly sampled mice, the remaining mice were killed. Their brains were removed and fed to 2 kittens that were serologically and fecally negative for *T. gondii*. Each kitten received a total of 16 brains from seropositive mice 48–49 days postinfection (PI). From the same group of mice fed to the kittens, 24 half-brain sections were also submitted for immunohistochemistry. At the time of death, blood samples were collected from all mice and screened with the IFAT at a 1:40 dilution. From the day of acquisition of the kittens until 7 days beyond the last day of oocyst detection, feces were collected from the kittens and evaluated for the presence of oocysts by microscopic examination after a zinc sulfate flotation. Once oocysts were detected PI, all feces were collected and processed daily to recover oocysts. Sodium chloride (1.2 specific gravity) was used to concentrate the oocysts by flotation. Briefly, feces were mixed with 0.1% Tween 80 to form a smooth slurry at approximately 10 ml of Tween per g of feces. This mixture was poured through a fine-mesh tea strainer into 50-ml tubes and centrifuged for 10 min at 1,000 *g* (Sorvall RT 6000D centrifuge; Rotor H-1000B, Thermo Fisher Scientific, Waltham, Massachusetts). The supernatant was decanted and sodium chloride solution was added to a final volume of 25 ml. The fecal pellet was thoroughly resuspended in the salt solution and centrifuged for 10 min at 1,250 *g*. The top 15 ml of the flotation fluid containing the oocysts was aspirated and placed into a clean 50-ml tube. The oocysts were then washed 3 times, once with 0.1% Tween 80 and twice with double-distilled water (ddH_2O). Following the final wash, the oocyst pellet was resuspended in 5 ml of 2% sulfuric acid. Oocysts were incubated and aerated for 2 wk at room temperature (~22 C) to allow for sporulation. They were then stored in the 2% sulfuric acid at 4 C and used within 3.5 mo. Prior to use, the oocysts were washed 3 times, the resultant pellet was resuspended in ddH_2O, and the proportion of sporulated oocysts was determined using a hemocytometer. All oocysts used for the chemical inactivation experiments were from a single batch of oocysts and oocyst concentrations were determined counting the proportion of sporulated (and presumed viable) oocysts.

Mouse bioassay experiments

Prior to conducting the oocyst inactivation experiments, the analytical sensitivity of each method of detecting infections in mice (serology, immunohistochemistry, and in vitro parasite isolation) was determined. Five replicates of each log dilution (10^5–10^0) of sporulated oocysts were suspended in 200 μl of ddH_2O water and inoculated s.c. into SW mice. Negative controls included 5 mice inoculated with processed fecal material from an uninfected, seronegative cat and 5 mice inoculated with sterile phosphate-buffered saline solution (PBS). This experiment was conducted twice, using 2 batches of oocysts from the same *T. gondii* isolate, but produced at different times. All exposed and control mice were held for 43–47 days before being killed and were evaluated for the presence of *T. gondii* infection via serology, immunohistochemistry, and parasite isolation. All evaluations were completed by persons who were blinded to the infection status of each mouse.

Chemical inactivation experiments

Chlorine: For the first set of chlorine experiments, oocysts were exposed to approximately 100 mg/L of free chlorine in a sodium hypochlorite solution (made daily from 10% sodium hypochlorite stock) for 30 min, or for 1, 2, 4, 8, 16, and 24 hr. Prior to use, the stock sodium hypochlorite solution (EMD Chemicals, Gibbstown, New Jersey) was stored at 4 C and the percentage of free chlorine was confirmed with iodometric titration (American Public Health Association, 1992). All experiments were performed at room temperature (~22 C) using ddH_2O (pH 7.25) as diluent that was previously determined to be demand-free of chlorine and organic materials (1.37 ppm TDS). All new glassware and magnetic stir rods were made demand-free by being soaked for 24 hr in Decon* Contrad* 70 liquid detergent (Fisher Scientific, Pittsburgh, Pennsylvania) and rinsed 3 times with ddH_2O. For each experiment, the starting concentration of sodium hypochlorite working solution of approximately 100 mg/L (±5 mg/L) free chlorine was measured 3 times using a LaMotte Chlorine/Bromine Titrator kit (model CL-BR, code 3624, LaMotte, Chestertown, Maryland) as directed by manufacturer's instructions.

For each time point, 50 ml of the working solution of sodium hypochlorite were added to each of 3 125-ml Erlenmeyer flasks containing a Teflon-coated magnetic stir rod. The starting temperature of the solution was recorded and 10^4 sporulated oocysts (in 100 μl of ddH_2O) were added to each flask. Each flask was sealed with Parafilm and continuously stirred at low speed by placement on a stir plate. Ten minutes prior to the end of the designated exposure time, the contents of the flasks were transferred to 50-ml tubes and centrifuged for 10 min at 1,250 *g* to pellet the oocysts and prevent contamination when sampling the supernatant to evaluate chlorine concentration. Five milliliters of the supernatant were collected to determine the end concentration of free chlorine and 3 ml of 0.1 N sodium thiosulfate were added into the remaining supernatant and oocyst pellet to quench any remaining free chlorine. The triplicate samples were pooled, mixed, washed 3 times with ddH_2O, and evenly divided for s.c. inoculation into 3 mice. Preliminary experiments revealed that replicates of 10^4 sporulated oocysts remained viable and infective to mice after exposure to 0.1 N sodium thiosulfate for 2 hr (data not shown).

The chlorine experiments were repeated twice but, based on preliminary results, only the highest (24 hr) exposure time point was retested in mice starting with either 4×10^4 or 4×10^5 sporulated oocysts. Following 24 hr of sodium hypochlorite exposure, the oocysts from each flask were washed and processed separately and the entire contents of each flask were inoculated s.c. into 1 of 4 mice. Positive control mice were inoculated with 10^4 or 10^5 sporulated oocysts suspended in ddH2O and stirred for 24 hr. In a final experiment, mice were each inoculated with 10^4 oocysts exposed to 50 ml of 5.25% aqueous sodium hypochlorite (undiluted household bleach) for 24 hr. The bleach was neutralized with 50 ml of 0.1 N sodium thiosulfate and the oocysts were washed 3 times and inoculated s.c. into 4 SW mice.

Ozone: The first ozone experiments were conducted to test the ability of 6 mg/L of ozone to inactivate oocysts, when exposed for 1, 2, 4, 8, or 12 min at room temperature. Ozone was generated by passing pure oxygen across tungsten points, as part of a high-voltage discharge and analysis system (Ozone Generator BMT 803 and Ozone Analyzer BMT 964, BMT Messtechnik, Berlin, Germany), and was bubbled continuously at a concentration of 40,000 to 50,000 ppm ozone to oxygen into 48 ml of ddH2O in demand-free 125-ml Erlenmeyer flasks. Initial concentrations of ozone in each flask were measured with a test kit (ozone test kit 20644-00, Hach Company, Loveland, Colorado). Following each exposure period, 3 ml of 0.1 N sodium thiosulfate were added to neutralize the ozone. Triplicate samples of 10^4 sporulated oocysts were treated and the oocysts from the 3 flasks were pooled, mixed, washed 3 times, and separated into equal doses for s.c. inoculation into 3 mice. For positive controls, 3×10^4 sporulated oocysts were added to 48 ml of ddH2O into which pure oxygen was bubbled for 12 min.

Duplicate ozone experiments were conducted with either 10^4 or 10^5 sporulated oocysts, but only the 8-min ozone exposure was tested in mice. Concentrations of 4×10^4 or 4×10^5 oocysts were added to each of the 3 flasks of dd H_2O into which ozone was continually bubbled for the 8-min period. After 3 ml of 0.1 N sodium thiosulfate were added to quench the reaction, the contents of each individual flask were washed and inoculated s.c. into each of 4 mice. Positive controls were

TABLE I. Evaluation of the analytical sensitivity of mouse bioassay (experiment 1) at 43–47 days post-inoculation of *Toxoplasma gondii* oocysts or at time of death.

Oocyst dose	No. of mice	IFAT*	Immunohistochemistry		Parasite isolation
			Reader 1	Reader 2	
10^5	5	4†	5	5	5
10^4	5	4†	5	5	5
10^3	5	5	1	2	5
10^2	5	5	2	3	5
10^1‡	5	4	1	2	4
10^0	5	0	0	0	0
Negative controls					
Feces	3	0	0	0	0
Saline	3	0	0	0	0

* IFAT: Indirect fluorescent antibody test with positive cut-off at 1:40.
† One mouse died ≤11 days post inoculation and a serologic response was not detectable.
‡ One mouse was negative by all 3 detection methods.

TABLE II. Evaluation of the analytical sensitivity of mouse bioassay (experiment 2) at 43–47 days post-inoculation of *Toxoplasma gondii* oocysts.

Oocyst dose	No. of mice	IFAT*	Immunohistochemistry		Parasite isolation
			Reader 1	Reader 2	
10^5	5	5	5	5	5
10^4	5	5	5	5	5
10^3	5	5	5	5	5
10^2	5	5	5	5	5
10^1	5	5	5	5	5
10^0†	5	4	3	4	4
Negative controls					
Feces	3	0	0	0	0
Saline	3	0	0	0	0

* IFAT: Indirect fluorescent antibody test with positive cut-off at 1:40.
† One mouse was negative by all three detection methods.

performed as previously described, with each of 4 mice being inoculated s.c. with 10^4 sporulated oocysts.

Evaluation of oocyst viability

For all inactivation experiments, the chemically treated oocysts were inoculated s.c. into 20- to 22-g female SW mice (Charles River Laboratories) that had been prescreened for the presence of *T. gondii* antibodies using the IFAT (1:10) before use. Prior to initiation of the chemical inactivation experiments, we evaluated oocyst viability and compared 3 methods (serology, immunohistochemistry, and in vitro cultivation of parasites from brain tissue) of detection of *T. gondii* infection in mice, when killed 46 to 49 days PI. Samples recovered from mice that died prematurely were also evaluated using the same methods. Serum obtained from blood samples from each mouse at death was evaluated for the presence and concentration of antibodies to *T. gondii* tachyzoite-specific antigens by 2 independent readers (K.W. and A.P.) using the IFAT at a 1:40 positive cut-off dilution, as previously described (Miller et al., 2001; Arkush et al., 2003). For histological evaluation, the spleen, 1 lung lobe, the heart, and half of the brain were fixed in 10% phosphate-buffered formalin for at least 24 hr, but no longer than 72 hr. With the first mouse bioassay experiment, all tissues were cut lengthwise into 2 equal sections for immunohistochemical evaluation. For mouse bioassay experiment 2 and all of the chemical inactivation experiments, the lung lobe and heart were cut lengthwise into 2 equal sections; the spleen was cut in half and then lengthwise to yield 4 equal sections; and the half brain was thinly sliced to yield 6 cross-sections of the cerebrum and cerebellum. These formalin-fixed tissues were submitted to the California Animal Health and Food Safety Laboratory, University of California–Davis, California, for sectioning and preparation for immunohistochemical stains, as previously described (Miller et al., 2001; Arkush et al., 2003). Two experienced pathologists (M.M. and B.B.) independently examined 2 slides from each mouse for evidence of positively stained parasites.

The second halves of these same mice brains (removed aseptically) were placed in antibiotic saline at 4 C for 24 hr, homogenized, and incubated in 0.25% trypsin solution at 37 C for 1 hr. The trypsinized homogenate was then layered over monkey kidney cells (MA104, BioWhittaker, Walkersville, Maryland) and incubated at 37 C for 2 hr. After incubation, the homogenate and media were removed and the feeder layer washed once with fresh media (Dulbecco's modified essential medium supplemented with L-glutamine, penicillin G, streptomycin, HEPES buffer, 2-mercaptoethanol, and fetal bovine serum). Fresh medium was added and replaced every 2 to 3 days and the cultures were microscopically assessed each time for the presence of *T. gondii* tachyzoites. Cultures were considered negative and discarded if no parasites were observed after 30 days PI.

RESULTS

Mouse bioassay demonstrated that untreated oocysts remained viable and infective after inoculation in the 2 selected strains of mice. In experiment 1 (Table I), *T. gondii* infection was detected by serology, immunohistochemistry, and/or parasite isolation in all 20 mice inoculated with 10^5–10^2 oocysts and in 4 of 5 mice inoculated with 10^1 oocysts. The fifth mouse tested negative for *T. gondii* by all 3 detection methods. Parasite isolation had high analytical sensitivity in detecting infections in all other mice. Serology was also reliable for detecting infections, except in 2 mice, i.e., 1 mouse inoculated with 10^5 oocysts (PID = 10) and 1 inoculated with 10^4 oocysts (PID = 11). Since both of these mice died within 11 days of exposure, the negative results on IFAT are likely due to insufficient time to achieve a measurable antibody response. However, both of these mice tested positive for *T. gondii* by immunohistochemistry and parasite isolation. For IFAT interpretation, the 2 readers were in agreement within 1 dilution of each other 100% of the time and were 100% consistent in determining the positive or negative status of mice based on IFAT results. Immunohistochemistry was the least sensitive detection method in experiment 1, with 100% of infections, as determined by the other 2 tests, detected only in mice inoculated with $\geq 10^4$ oocysts. The 2 pathologists had 92% agreement on positive samples and only disagreed on the positive status of 3 mice inoculated with low doses of oocysts (10^3–10^1). In experiment 2 (Table II), infections were detected by all 3 methods in all of the mice except for 1 mouse inoculated with a single oocyst based on serial dilution. The pathologists were in 97% agreement on samples positive by immunohistochemistry, with 1 disagreement on a mouse inoculated with 10^1 oocysts. For experiment 2, using the more complete tissue trimming protocol, results of immunohistochemistry were the same or nearly the same as those for serology or parasite isolation, depending on which reader's results were considered. For both bioassay experiments, none of the negative control mice was found to be infected with *T. gondii*.

All mice inoculated with oocysts exposed to approximately 100 mg/L of free chlorine for up to 24 hr became infected with *T. gondii*, demonstrating that under these experimental conditions, sodium hypochlorite did not inactivate *T. gondii* oocysts

TABLE III. Results of mouse bioassay (46–49 days post-inoculation or at time of death) to assess viability of *Toxoplasma gondii* oocysts after exposure to 100 mg/L chlorine.

Oocyst dose	Chlorine exposure time	No. of mice	IFAT*	Immunohistochemistry		Parasite isolation	Average initial chlorine (mg/L)	Average final chlorine (mg/L)	Ct values (mg × min/L)†
				Reader 1	Reader 2				
Experiment 1									
10^4	0 min	3	3	3	3	3	NA‡	NA	NA
10^4	30 min	3	3	3	3	3	103	102	3,075
10^4	2 hr	3	3	3	3	2/2§	101	99	12,000
10^4	4 hr	3	3	2	2	3	100	100	24,000
10^4	8 hr‖	2	2	2	2	2	101	94	46,800
10^4	16 hr	3	3	3	3	3	101	81	87,360
10^4	24 hr	3	3	3	3	3	104	82	133,920
Experiment 2									
10^4	0 min	4	4	4	4	4	NA	NA	NA
10^5	0 min	4	4	4	4	4	NA	NA	NA
10^4	24 hr	12	12	11	12	11	107.5	39.6	105,912
10^5	24 hr‖	11	10#	11	11	11	108.5	15.3	89,165

* IFAT: Indirect fluorescent antibody test with positive cut-off at 1:40.
† mg/L used to calculate Ct (disinfectant concentration × contact time) is the average of initial and final chlorine concentration.
‡ NA: not applicable.
§ One culture became contaminated with bacteria and was discarded.
‖ Mouse lost because of early death and cannibalism.
One mouse died at PID = 11 and a serologic response was not detectable.

(Table III). The infections were detectable by all 3 methods, with the exception of a single mouse inoculated with 10^4 oocysts exposed to chlorine for 24 hr (Table III, experiment 2) and another mouse inoculated with 10^5 oocysts exposed to chlorine for 24 hr (Table III, experiment 2). For the first mouse, no parasites were observed in culture after 30 days but infection was detected with serology and immunohistochemistry. The second mouse died only 11 days PI before a detectable antibody response developed, but parasites were observed in culture and by immunohistochemistry. For both experiments 1 and 2, the pathologists had 98% agreement on positive samples, disagreeing on only 1 mouse inoculated with 10^4 oocysts exposed to sodium hypochlorite for 24 hr (Table III, experiment 2). Oocysts also remained viable and infectious to mice following exposure to undiluted bleach for 24 hr.

Oocysts remained infectious following exposure to 6 mg/L of ozone for up to 12 min (Table IV). Infections were detected by all 3 detection methods in all of the inoculated mice, showing that under these conditions ozone was ineffective in inactivating oocysts. The pathologists had 96% agreement on positive samples, with 1 disagreement in each of experiments 1 and 2. *Toxoplasma gondii* infections were also detected in all positive control mice in both the chlorine and ozone experiments.

DISCUSSION

Both chlorine and ozone are strong oxidizing agents. Chlorine can cause cell death through inhibition of enzymatic activity, alterations in cell permeability, or damage to DNA and RNA (U.S. Environmental Protection Agency, 1999b). In general, chlorination is cost effective as a water treatment and efficiently inactivates a range of microbes, has a residual effect after the initial treatment, and is easy to use in sanitizing high volumes of water (Moore and Payne, 2004). In our study, the maximal test dose of 100 mg/L applied to oocysts in water was 4 times the concentration currently recommended to treat raw sewage and ≥50 times greater than that used to disinfect drinking water (Palin, 1983; U.S. Environmental Protection Agency, 1999a; Cheremisinoff, 2002). As previously described, aqueous sodium hypochlorite solutions are used to purify coocidian oocysts. However, oocyst exposure to the chlorine is generally limited to 15–30 min. In this study, *T. gondii* oocysts were exposed to 5.25% aqueous sodium hypochlorite for 24 hr without resulting in oocyst inactivation. To our knowledge, no other coccidian organism has remained viable under comparable experimental conditions. Another research group reported exposing cat feces containing *T. gondii* oocysts to 5.25% aqueous sodium hypochlorite for 24 hr, also with no success in killing the oocysts (Dubey et al., 1970). The present study differs because the oocysts were purified from the cat feces before the exposure to bleach. This would allow for an increased interaction to occur between the chlorine and the oocyst, instead of chlorine chemically reacting with the organic components of the cat feces itself. Overall, our findings suggest that *T. gondii* oocysts present in either sewage or drinking water that is disinfected with chlorine at standard dosages will likely remain viable.

Ozone is an allotrope of oxygen that forms a highly unstable gas, which can damage cells by modifying cellular components (U.S. Environmental Protection Agency, 1999c). Potential advantages of using ozone to treat drinking water include high efficacy for inactivating a range of microbes, low production of toxic byproducts, and ease of treating large volumes of water (Betancourt and Rose, 2004). For our ozone experiments, a dose of 6 mg/L of ozone was tested because this was the highest concentration of ozone that could be passively bubbled into the ddH_2O. This concentration is ≥6 times that used at many facilities to disinfect drinking water. Previous studies have shown

TABLE IV. Results of mouse bioassay (46–49 days post-inoculation) to assess viability of *Toxoplasma gondii* oocysts after exposure to 6 mg/L ozone.

Oocyst dose	Ozone exposure time	No. of mice	IFAT*	Immunohistochemistry		Parasite isolation	Average initial ozone (mg/L)	Ct values (mg × min/L)†
				Reader 1	Reader 2			
Experiment 1								
10^4	0 min	3	3	3	3	3	NA‡	NA
10^4	1 min	3	3	3	3	3	6.00	6.00
10^4	2 min	3	3	3	3	3	6.00	12.00
10^4	4 min	3	3	2	3	3	6.33	25.32
10^4	8 min	3	3	3	3	3	6.17	49.34
10^4	12 min	3	3	3	3	3	5.83	69.96
Experiment 2								
10^4	0 min	4	4	4	4	4	NA	NA
10^4	8 min	12	12	11	12	12	6.00	48.00
10^5	8 min	12	11§	12	12	12	6.00	48.00

* IFAT: Indirect fluorescent antibody test with positive cut-off at 1:40.
† mg/L used to calculate Ct (disinfectant concentration × contact time) is the average of initial ozone concentrations.
‡ NA: not applicable.
§ One serum sample was missing.

that oocysts of a related chlorine-resistant parasitic protozoan, *Cryptosporidum parvum*, are inactivated (≥99%) following exposure to 3 or 4 mg × 1 min/L of ozone (Finch et al., 1993). However, our studies revealed that *T. gondii* oocysts remained viable and infectious for mice at all times when subjected to 6 mg/L of ozone for up to 12 min. Differences in ozone susceptibility between *T. gondii* and *C. parvum* may be attributed to differences in oocyst wall composition. The oocyst wall of *T. gondii* is comprised of 3 distinct layers: a 20- to 50-nm thick, electron-dense outer layer, a 8- to 10-nm thick, electron-lucent middle layer, and a 50- to 90-nm thick moderately electron-dense inner layer (Speer et al., 1998). The *C. parvum* oocyst wall is comprised of 2 or 3 layers, ranging from 40 to 49.7 nm in total width (Reduker et al., 1985; Harris and Petry, 1999). In addition to having a thicker oocyst wall, *T. gondii* also has a sporocyst wall surrounding the sporocysts that is not present in *C. parvum* (Dubey et al., 1998; Harris and Petry, 1999). Both of these characteristics may contribute additional protection to the *T. gondii* sporozoite.

Potential health risks of human exposure to *T. gondii* and reduction in oocyst numbers due to sampling and purification methods following chemical exposure precluded determination of final ozone concentrations after oocyst treatment. Moreover, prior to the addition of oocysts, continuous flow of ozone into the test flasks was shown to maintain a consistent ozone concentration of 6 mg/L. Since there was no evidence of oocyst inactivation at this concentration, no recommendations can be made for the application of this method to treat *T. gondii* oocysts in drinking water.

For both chlorine and ozone experiments, flasks of treated sporulated oocysts were initially pooled by treatment group prior to mouse inoculation to increase the likelihood that each mouse would be inoculated with an equivalent dose of treated oocysts. In subsequent experiments, replicates were obtained by increasing the number of mice and increasing the oocysts per flask, with the contents of each individual flask being inoculated into 4 mice. However, no significant differences were detected between these experimental approaches and the number of mouse infections that resulted.

Different properties of water, including pH, temperature, turbidity, or organic compound concentration, could affect the ability of both sodium hypochlorite and ozone to inactivate oocysts. Cost and time constraints of oocyst production and application of the mouse bioassay precluded testing a broader range of water conditions. Our chlorine and ozone experiments were conducted at room temperature (22 C) with pH neutral (7.2) ddH_2O. These parameters were selected to limit any additive effects of temperature and pH on oocyst inactivation, as well as effects on the efficacy of the chemicals tested. The disinfection properties of chlorine are affected by both pH and temperature, i.e., lower temperatures and higher pH both reduce disinfection efficacy. At pH levels of <8, free chlorine is predominately present as hypochlorous acid, the most effective disinfectant form of chlorine (Cheremisinoff, 2002; American Water Works Association, 2003). The disinfection rate of ozone is less sensitive to changes in pH and temperature levels than that of chlorine. Ozone is less soluble and stable with increasing water temperatures, but this does not affect disinfection efficiency (U.S. Environmental Protection Agency, 1999a).

The mouse bioassay was selected to evaluate oocyst viability after chlorine or ozone treatment because of the high analytical sensitivity of this assay in previous studies (Hitt and Filice, 1992; Garcia et al., 2006). Mice were held on average for 45 days PI to ensure dissemination of infection and development of *T. gondii* tissue cysts in the brains of infected mice. Preliminary studies (data not shown) demonstrated that the type II *T. gondii* strain selected for oocyst production yielded moderate to high tissue cyst numbers in the brains of the mice strains selected for our experiments. In mouse bioassay experiment 1 (Table I), *T. gondii* infections could be detected with IFAT and parasite isolation in mice inoculated with $\geq 10^1$ oocysts. Analytical sensitivity increased with both of these detection methods and infections were detected in mice inoculated with $\geq 10^0$ oocysts in the second mouse bioassay experiment (Table II).

The reason(s) for this increase in the sensitivity of detection has not been determined. Analytical sensitivity of immunohistochemistry also increased between the 2 mouse bioassay experiments: infections were detected in mice inoculated with $\geq 10^4$ oocysts in the experiment 1 (Table I), whereas infections were detected in mice inoculated with $\geq 10^0$ oocysts in experiment 2 (Table II). Increasing the total surface area of tissue examined on the microscope between the first and second mouse bioassays could have significantly contributed to the increased sensitivity of immunohistochemical evaluation.

Multiple detection methods were employed to increase the likelihood of correctly determining the true infection status of inoculated mice after chemical treatment of the oocysts. Each of the 3 detection methods offers different advantages and disadvantages. The IFAT is rapid and "user-friendly," with high sensitivity in determining infection. However, this test can result in false negative results if an animal dies prematurely before a measurable antibody response can develop and, because it is a fluorescent test, results may vary by 1 or more dilutions when results are evaluated by >1 independent readers. Immunohistochemistry is rapid and sensitive and can also provide visual evidence of tissue distribution of parasites and inflammation, as well as pathologic changes resulting from a *T. gondii* infection. The drawbacks of this test include potential false negatives if insufficient tissue surface area is trimmed, stained, and evaluated; false positives if artifacts from antibody staining are present; the requirement for technical expertise to process and examine tissue samples; and an inherent level of subjectivity, as seen with the IFAT. For the mouse bioassay experiments, we were able to substantially increase analytical sensitivity of immunohistochemistry in experiment 2 (Table II) by increasing the number of tissue sections cut and evaluated from each tissue sample. In both mouse bioassay experiments, the high agreement (92% in experiment 1 and 97% in experiment 2) between the 2 pathologists indicates that individual subjectivity was not problematic in this study. In vitro parasite isolation is also a sensitive detection method and can be advantageous to definitively establish the presence and viability of parasites from sampled tissues. Potential limits include expense and labor in maintaining tissue cultures, technical expertise required to safely handle and maintain parasite and cell lines, increased time required to obtain results, and the potential for bacteria or mold to contaminate and destroy culture samples.

Partial oocyst inactivation may have occurred with chlorine or ozone treatments, but would be undetectable with the 3 detection methods employed. The infectious dose of *T. gondii* oocysts for humans is not known, but animal infectivity studies indicate that such doses could be as low as a single oocyst (Dubey, 1996; Dubey et al., 1996, 1997). Therefore, the aim of our study was to evaluate the efficacy of sodium hypochlorite and ozone for complete inactivation of oocysts under our experimental conditions.

Treatment of drinking water for human consumption is a safety issue of global concern. In 1995, in Vancouver, British Columbia, waterborne transmission of *T. gondii* infected an estimated 2,894 to 7,718 individuals, including 100 individuals identified with acute cases of toxoplasmosis. In this largest known outbreak of water-transmitted *T. gondii*, the contaminated drinking water was chlorinated, but not filtered (Bowie et al., 1997). Filtering water is ideal for physical removal of many biological contaminants or pathogens, including *T. gondii* oocysts, which are 10×13 μm in size (Dubey et al., 1998). However, filtration is not always available in water treatment facilities serving small communities or with sources of water that do not contain sufficient particulate matter for conventional coagulation and sedimentation processes (Bowie et al., 1997; Betancourt and Rose, 2004; Goh et al., 2005). Therefore, most water supplies are treated with chemicals, most notably chlorine, to inactivate potential waterborne pathogens. Given the findings of this study, current chemical water treatment practices are insufficient to inactivate *T. gondii* oocysts, if present, in public water supplies. The biochemical properties of the oocyst wall that impart such strong chemical resistance for *T. gondii* oocysts remain unknown. Such information would be useful to determine effective means of inactivating oocysts.

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