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Inactivation of *Giardia lamblia* and *Giardia canis* Cysts by Combined and Free Chlorine

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Free chlorine and a combined organic *N*-chloramine (3-chloro-4,4-dimethyl-2-oxazolidinone, compound 1) were compared for efficacy as disinfectants against an admixture of cysts of *Giardia lamblia* and *Giardia canis* in water solution under a variety of test conditions; variables were pH, temperature, and water quality. In general, compound 1 was found to reduce the giardial excystation in the solutions at lower concentration or shorter contact time at a given total chlorine concentration than did free chlorine.

The presence of *Giardia lamblia* in potable water supplies is a serious problem throughout the world in that it causes a severe form of dysentery (3–5, 10). Prior laboratory studies have indicated that *G. lamblia* cysts are rather resistant to disinfection by free chlorine, particularly at high pH (≥8) and low temperature (5°C) (6, 11). Cysts of *Giardia muris* from mice have been shown to be even more resistant to disinfection by free chlorine (9, 11) and by ozone (13) than are cysts of *G. lamblia*, leading some workers to propose the use of *G. muris* as a model for the study of inactivation of the human parasite, *G. lamblia*. However, rather than extrapolate to *G. lamblia* the results with *G. muris*, we have chosen to use an admixture of *G. canis* and *G. lamblia* extracted from the feces of dogs either naturally infected in Auburn, Ala., by *G. canis* or infected by a laboratory strain (Portland strain) of *G. lamblia*. The feces samples were pooled, and the cysts were extracted from the pooled samples.

Recent work in our laboratories has shown that certain organic *N*-halamine compounds are very stable, persistent disinfectants in water for a variety of organisms (12, 14–16, 18) under a wide range of treatment conditions (pH, temperature, and water quality). This work will show that one of the *N*-halamine compounds (3-chloro-4,4-dimethyl-2-oxazolidinone, henceforth referred to as compound 1) at low concentration is significantly more giardiacidal than is free chlorine. Preliminary evidence in our laboratories has indicated that this observation is generally true for a variety of organic *N*-halamine compounds (17).

Compound 1 was synthesized by the method of Kaminski et al. (7), who had originally shown it to be bactericidal in water (8). Calcium hypochlorite (HTH) was used as a source of free chlorine. Disinfection assays were conducted for water samples containing admixtures of cysts of *G. lamblia* and *G. canis* and either of the two disinfectants or no disinfectant (control). The water samples were either made demand free (DFW) (15, 16) and buffered to pH 4.5, 7.0, or 9.5 and held at 4 or 22°C, or contained controlled synthetic chlorine demand (SDW) in the form of a combination of inorganic salts, bentonite clay, humic acid, heat-treated horse serum, and heat-killed yeast cells buffered to pH 9.5 and held at 4°C (15, 16). The latter samples should provide a worst case scenario for disinfection (high pH, low temperature, and heavy demand).

Cysts extracted from the feces of naturally and experimentally infected dogs were concentrated and purified with slight modification of a procedure described previously (1, 6). Cyst concentrations were determined by counting with a hemacytometer, and then cysts were suspended at a concentration of 10^6 to 10^7 per ml in distilled water. Merthiolate (1:10,000) was added to eliminate bacterial and fungal contamination during storage, and the cyst suspension was stored at 4°C. In a typical disinfection assay, 20 ml of DFW or SDW was placed in a 50-ml centrifuge tube and inoculated with purified cyst suspension to a final concentration of approximately 800 cysts per ml. This suspension of cysts was allowed to equilibrate at a given temperature (4 or 22°C) by immersion in a thermostatically controlled water bath (±1°C) for 15 min. Recently prepared stock disinfectant was added to the centrifuge tube to bring the total chlorine concentration in the mixture to a specific level (20, 10, 5, 2, 1, or 0.5 mg/liter). Samples (1 ml) were removed from the mixture after predetermined contact times (2, 5, or 10 min) and quenched by an equal volume of sterile 0.02 N sodium thiosulfate buffered at pH 7.0. The quenched samples were centrifuged at 600 × *g* for 5 min, and the resulting cyst pellets were exposed to a modified excystation procedure (1, 2, 6). Briefly, this method involved exposure of purified cysts to pH 2.0 aqueous HCl for 60 min, followed by washing cysts free of acid and resuspending them in HSP-3 growth medium (2). Cysts were then placed in chamber slides and incubated at 37°C for 1 h. Controls were cysts suspended in buffer solutions or SDW without disinfectants and otherwise treated the same as those in the disinfectant assays. The experimental protocols involved simultaneously conducting buffer and sodium thiosulfate controls at each temperature, pH, and contact time as for the disinfectant treatments.

Disinfection experiments were performed in duplicate, and at least 500 cysts were counted microscopically from each contact time sample per replicate. The percentage of excystation was determined by counting microscopically the number of intact cysts (IC), partially excysted trophozoites (PET), and totally excysted trophozoites (TET) and applying the following formula (6). % excystation = (TET/2 + PET)/(TET/2 + PET + IC) × 100.

Mean values for the excystation of control cultures (buffer and sodium thiosulfate added to the cyst suspension) are presented in Table 1. Mean excystation of controls over all treatments (combinations of pH, temperature, and water quality) ranged from 42.8 to 47.8%, depending on the treatment conditions, and the excystation was quite consistent

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TABLE 1. Excystation following treatment

pH	Temp (°C)	Water quality	% Excystation (n = 12)		
			Mean	SD	SE
4.5	4	DFW	44.3	2.25	0.65
	22	DFW	43.3	2.29	0.66
7.0	4	DFW	42.8	3.64	1.05
	22	DFW	44.7	3.16	0.91
9.5	4	DFW	47.4	2.76	0.80
	22	DFW	43.8	3.06	0.88
	4	SDW	47.8	2.87	0.83

within treatments. The treatment variables were tested for their effect on excystation by using the analysis of variance procedure of the Statistical Analysis System (SAS Institute, Cary, N.C.). The Scheffé procedure was used for multiple means comparisons. The results of these analyses indicated that there was an interaction between pH and the temperature resulting in a significant effect on excystation ($P < 0.05$). The combination of pH 9.5 and 4°C exposure resulted in excystation percentages that were different ($P < 0.05$; minimum significant difference = 4.22%) from the excystation found following the other treatments. The reason for this enhancement of excystation by the low-temperature–high-pH treatments is not known, but perhaps the drastic change in both pH and temperature when the cysts are subjected to the excystation procedure may be partially responsible. Length of exposure of the cysts to a given treatment was not a significant factor in subsequent excystation.

The effects of compound 1 and HTH on the viability of cysts are presented in Table 2. The data represent mean values of two or more assays done with either compound 1 or HTH at each specific assay condition, *e.g.*, at 22°C in pH 4.5 chlorine-demand-free buffer (DFW) or at 4°C in pH 9.5 synthetic-demand water (SDW). Based on the range of excystation of controls, detection of >0.4% cyst survival was the lower limit of sensitivity for the counting procedure. The Statistical Analysis System was used to perform the data analysis, and Tukey's Studentized range procedure (Tukey's honestly significant difference) was used for the pairwise comparisons. An asterisk has been placed between the $Ca(OCl)_2$ and compound 1 values in Table 2 where there was a significant difference ($P < 0.05$) between the results of cysticidal assays with $Ca(OCl)_2$ and compound 1. In all instances where a significant difference existed, compound 1 was more effective than free chlorine as a giardial cysticide.

At pH 4.5, 22°C, and concentrations of 1 and 0.5 mg of total chlorine per liter, compound 1 was significantly ($P < 0.05$) more effective than $Ca(OCl)_2$ at inactivating cysts of *G. lamblia* and *G. canis*. Compound 1 and free chlorine were not significantly different in their cysticidal activity at concentrations of 2, 5, and 10 mg of total chlorine per liter. At pH 7.0, 22°C, and concentrations of 5, 2, 1, and 0.5 mg/liter of total chlorine, compound 1 was significantly ($P < 0.05$) more effective than $Ca(OCl)_2$ as a cysticide. At pH 9.5 and 22°C, compound 1 was significantly ($P < 0.05$) more effective than free chlorine at inactivating giardial cysts at most concentrations tested. At 4°C, both disinfectants were less effective than at 22°C, but compound 1 was usually more effective than $Ca(OCl)_2$; significant ($P < 0.05$) differences were more frequent at 1 and 2 mg of total chlorine per liter.

The results from the studies of assays done in SDW at 4°C showed that neither compound 1 nor $Ca(OCl)_2$ inactivated the giardial cysts as efficiently as in chlorine-demand-free buffers. However, after a 10-min contact time, compound 1 was significantly ($P < 0.05$) more effective than $Ca(OCl)_2$ as a cysticide at concentrations of 5, 2, and 1 mg of total chlorine per liter in SDW.

Compound 1 clearly has high potential as a water disinfectant to control giardial contamination. Its ability to inactivate cysts of *G. lamblia* and *G. canis* within 2 min at 22°C

TABLE 2. Excystation following treatment with compound 1 or HTH

Treatment conditions			Mean % excystation[a] at total chlorine concn (mg/liter):																	
			10			5			2			1			0.5					
pH	Temp (°C)	Time (min)	HTH		1	HTH		1	HTH		1	HTH		1	HTH		1	HTH		1
4.5	4	2	0.6		0.0	9.4	*	4.0	31.5		24.5	35.9	*	30.1	41.5		36.9			
		5	0.0		0.0	2.0		0.9	14.8		11.3	23.5		20.2	35.3		33.4			
		10	0.0		0.0	0.2		0.2	8.7	*	1.7	21.8	*	15.1	37.0	*	31.3			
	22	2	0.0		0.0	0.7		0.0	4.1		0.0	12.0	*	2.1	28.3	*	4.8			
		5	0.0		0.0	0.0		0.0	1.2	*	0.0	9.8	*	0.8	26.3	*	5.0			
		10	0.0		0.0	0.0		0.0	0.0		0.0	3.9		0.0	12.7	*	0.5			
7.0	4	2	0.0		0.0	10.7	*	6.0	34.2		31.6	38.5		37.5	46.2		43.5			
		5	0.0		0.0	2.7	*	1.9	17.9		16.5	30.8		29.9	44.2		40.3			
		10	0.0		0.0	0.7		<0.4	11.2	*	1.7	28.6	*	20.7	39.3		36.8			
	22	2	0.0		0.0	2.5	*	0.0	5.0		0.6	17.1	*	3.0	33.7	*	9.1			
		5	0.0		0.0	0.6	*	0.0	2.7	*	0.0	10.1	*	1.0	33.5	*	4.2			
		10	0.0		0.0	0.0		0.0	0.9	*	0.0	8.0	*	0.0	30.4	*	0.6			
9.5	4	2	4.3		0.6	15.6	*	9.9	36.9		33.4	42.0		38.5	47.1		46.9			
		5	3.1		0.5	5.6		3.6	19.1		18.1	32.5		29.0	43.4		41.0			
		10	0.8		<0.4	6.4		1.1	14.9	*	2.9	30.8	*	21.4	37.3		36.5			
	22	2	<0.4		0.0	9.5	*	0.0	9.7	*	1.1	23.1	*	3.8	35.1	*	9.2			
		5	0.0		0.0	3.2	*	0.0	2.8		<0.4	15.5	*	1.5	33.2	*	6.0			
		10	0.0		0.0	0.7	*	0.0	0.7	*	0.0	10.7	*	0.0	34.7	*	1.9			
9.5 (SDW)	4	2	6.1		1.8	17.0	*	12.9	38.3	*	35.9	42.7		41.6	49.0		48.9			
		5	2.9		1.0	13.1	*	7.0	23.2		21.6	35.8		30.5	46.0		45.1			
		10	1.3		0.6	9.4	*	3.3	22.4	*	5.9	34.1	*	24.1	43.4		40.2			

[a] An asterisk placed between the HTH and compound 1 values indicates a significant difference ($P < 0.05$) between the two means (Tukey's Studentized range procedure).

over a pH range of 4.5 to 9.5 with a total chlorine concentration of 5 mg/liter and within 10 min at 4°C over the same pH range is better than has been reported from studies with free chlorine (6). It was reported that 10 min of contact time was required for chlorine at 8 mg/liter from sodium hypochlorite to kill cysts (650 cysts per ml) at pH 6 and 7, 5°C, and that 30 min of contact time was required for complete killing of cysts at pH 8 (6). Of course, it must be realized that compound 1 will be more costly than free chlorine to produce, but it is hoped that the enhanced stability in water of compound 1 relative to free chlorine will make the former cost-effective.

It is clear from these studies that the mechanisms of action of compound 1 and free chlorine, at least against giardial cysts, are different. Free chlorine kills most other microorganisms (12, 14–17) more rapidly in DFW than does compound 1. It was originally thought that the small amount of free chlorine produced upon hydrolysis of compound 1 (the hydrolysis equilibrium constant has been estimated to be ca. 10^{-9}) might be the active disinfectant. This cannot possibly be the case for disinfection of cysts of *G. lamblia* and *G. canis*, because compound 1 is more efficient than free chlorine at the same concentration against this organism. Work aimed toward elucidation of the mechanism(s) of action of compound 1 and other organic *N*-halamine compounds is in progress in our laboratories. Preliminary indications are that the mode of action of the *N*-halamines involves inactivation of active sulfhydryl residues on enzymes. It is possible that the *N*-halamines, being weaker oxidants than free halogen, are able to penetrate the thick walls of the giardial cysts so as to oxidize crucial membrane enzymes rather than less crucial ones on the walls of the cysts which may react more readily with free chlorine.

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The possible mode of action by compound 1 regarding penetration of the cyst walls was suggested by a reviewer.

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