APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1991, p. 1573–1575
0099-2240/91/051573-03$02.00/0

Vol. 57, No. 5

Immunofluorescence and Morphology of *Giardia lamblia* Cysts Exposed to Chlorine

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Received 20 September 1990/Accepted 1 March 1991

***Giardia* cyst-like objects detected by immunofluorescence in chlorinated water samples often cannot be positively identified by their morphological appearance. To determine the effect of chlorine on cyst immunofluorescence and morphology, *Giardia lamblia* cysts were exposed to chlorine for 48 h. The majority of cysts exposed to chlorine concentrations of 1 to 11 mg/liter at 5 and 15°C lost their internal morphological characteristics necessary for identification, but most of them were still detectable by immunofluorescence.**

Giardia species are now recognized as significant human intestinal pathogens, capable of survival and transport in aquatic environments such as municipal water supplies. Between 1965 and 1985, 95 outbreaks of disease caused by waterborne *Giardia* species have been reported in the United States (3).

Giardia cysts are microscopically detected among contaminating microfloras and debris in water samples by specific size, shape, and characteristic internal morphological features (9). To expedite this process, immunofluorescence methods were recently developed and have been used to detect *Giardia* cysts in water samples (6, 8, 10, 11) by fluorescence, size, and shape. However, confirmation of the identity of a fluorescing *Giardia* cyst-like object still requires the observation by phase-contrast microscopy of two or more internal morphological features, such as nuclei, axonemes, or median bodies (8).

During investigations of waterborne giardiasis, immunofluorescence analyses of several water samples in our laboratory revealed brightly fluorescing *Giardia* cyst-like objects which could not be positively identified because the morphological features typical of *Giardia* cysts were absent (8). Some of these samples were from various points in the unit processes of the treatment system where the maximum chlorine exposure could have been up to 8 mg/liter for 24 h. We therefore engaged in a study to determine the effect of chlorination, under laboratory conditions, upon immunofluorescence detection and the phase-contrast identification of *Giardia* cysts.

***Giardia* cysts.** *Giardia lamblia* (CDC:0284:1) and *Giardia muris* cysts were harvested from Mongolian gerbils (2) and mice (5), respectively, by centrifugation of sieved and washed fecal slurry over 1.0 M sucrose. The cysts were purified as previously described (7), stored at 4°C in distilled water, and used for experiments within 2 weeks. Prior to exposure of these cysts to chlorine or chlorine demand-free buffer, portions of the stock suspensions were subjected to the immunofluorescence assay on membrane filters (8).

Chlorine. Chlorine solutions (1 to 11 mg/liter) were prepared by adding a laboratory-grade sodium hypochlorite solution (Fisher Scientific Co.) to 0.05 M KH_2PO_4 chlorine demand-free buffer, pH 7.0. The chlorine concentration was determined by the *N*,*N*-diethyl-*p*-phenylenediamine (DPD) colorimetric method (1). One control consisted of only 0.05 M KH_2PO_4 chlorine demand-free buffer, pH 7.0. For another control, *Giardia* cysts were incubated in 200 ml of chlorine (11 mg/liter) in phosphate buffer (pH 7.0) previously neutralized with 6.3% sodium thiosulfate. A 10-ml volume of undiluted neutralized chlorine tested with the DPD method (1) demonstrated that all of the chlorine in that control was neutralized before the cysts were added.

Chlorination. Beakers were filled with 200 ml of the appropriate chlorine solution (0, 1, 3, 9, or 11 mg/liter in

TABLE 1. Residual chlorine levels and *G. lamblia* cyst fluorescence during exposure of cysts to chlorine[a]

Contact time (h)	Result at 5°C		Result at 15°C	
	Residual chlorine (mg/liter)	% Fluorescent cysts	Residual chlorine (mg/liter)	% Fluorescent cysts
0	0.0	—[b]	0.0	—
	1.1	—	1.1	—
	3.2	—	3.1	—
	11.2	—	9.2	—
1	0.0	88	0.0	100
	0.9	100	0.9	90
	3.0	96	2.9	100
	11.7	97	9.9	98
4	0.0	99	0.0	100
	0.8	98	0.8	97
	2.8	96	2.8	100
	10.2	90	9.8	100
24	0.0	100	0.0	100
	0.6	79[c]	0.6	99
	2.4	96	2.0	100
	9.6	99	8.8	100
48	0.0	100	0.0	99
	0.4	72[c]	0.4	100
	2.2	99	2.0	100
	9.6	93	9.0	100

[a] Each value is the average of two independent experiments.
[b] —, 99 to 100% (n = 100, two experiments) of a portion of the nonchlorinated stock *G. lamblia* cyst suspension fluoresced on membrane filters.
[c] These two values are low because of the inclusion of deviant values obtained in the first experiment. These values were not reproduced and probably reflect procedural error.

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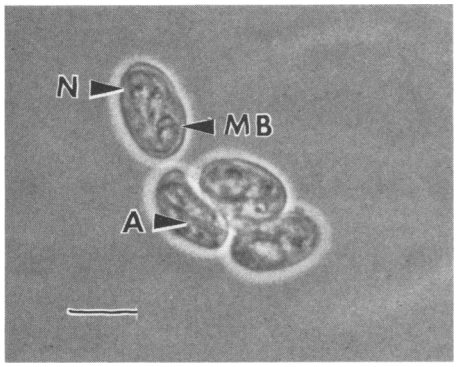

FIG. 1. Phase-contrast micrograph of *G. lamblia* cysts exposed to phosphate buffer for 48 h. Note the presence of axonemes (A), nuclei (N), and median bodies (MB), used to confirm the cystlike object as a *Giardia* cyst. Bar = 10 μm.

phosphate buffer) and then placed in a 5 or 15°C water bath. After temperature equilibration, stirring was started and *Giardia* cysts were added to a final density of 5×10^3 cysts per ml in each beaker. At timed intervals (1, 4, 24, and 48 h), samples were removed for chlorine determination, immunofluorescence assay, and cyst morphological observations. The temperature and pH of the chlorine solution were also determined at these time intervals.

Immunofluorescence and phase-contrast observations. At appropriate time intervals, cysts were transferred from the reaction beakers, concentrated by membrane filtration, deposited on membrane filters, and then submitted to the immunofluorescence assay as previously described (8). Briefly, they were incubated with anti-*G. lamblia* whole-cyst rabbit sera (prepared in our laboratory), subsequently with fluorescein-conjugated goat anti-rabbit immunoglobulin G (heavy and light chains) (no. 65173-2; ICN Biomedicals, Inc., Costa Mesa, Calif.), and finally with Evans blue counterstain. Membrane filters were cleared in glycerol, mounted on slides, and then examined at 625× magnification with a Leitz Dialux 20 microscope equipped for epifluorescence and phase-contrast observations. One hundred cysts from each chlorine level at each time interval were examined for the presence or absence of fluorescence and for the presence of nuclei, axonemes, and median bodies.

Cysts were scored as being detectable by fluorescence if they exhibited bright apple green fluorescence typically concentrated on the periphery of the cyst. Identification was confirmed if these same cysts exhibited two or more internal morphological features characteristic of *Giardia* cysts. Results are the average of two independent experiments and were read at random by the same observer.

Residual chlorine levels for each chlorine concentration tested dropped within the 48-h contact time (Table 1), probably because of chlorine volatilization and increased chlorine demand from deteriorated cyst debris. The pH (mean ± standard deviation) remained stable at 7.1 ± 0.06 or 7.01 ± 0.01 during the course of exposure to chlorine at 5°C (4.5 to 6.9°C) and 15°C (no temperature fluctuations), respectively.

The majority of *G. lamblia* cysts (Table 1) exposed to chlorine demand-free phosphate buffer (pH 7.0) for 1 to 48 h showed typical bright apple green fluorescence concentrated primarily on the periphery of the cysts. Although more than 90% of chlorine-exposed cysts also fluoresced after 48 h of exposure to the highest chlorine residuals (9.6 and 9.0 mg/liter), the fluorescence of these cysts appeared to be patchy yellow-green compared with the normal overall apple green of the controls and of the cysts exposed to lower chlorine concentrations. However, this alteration did not

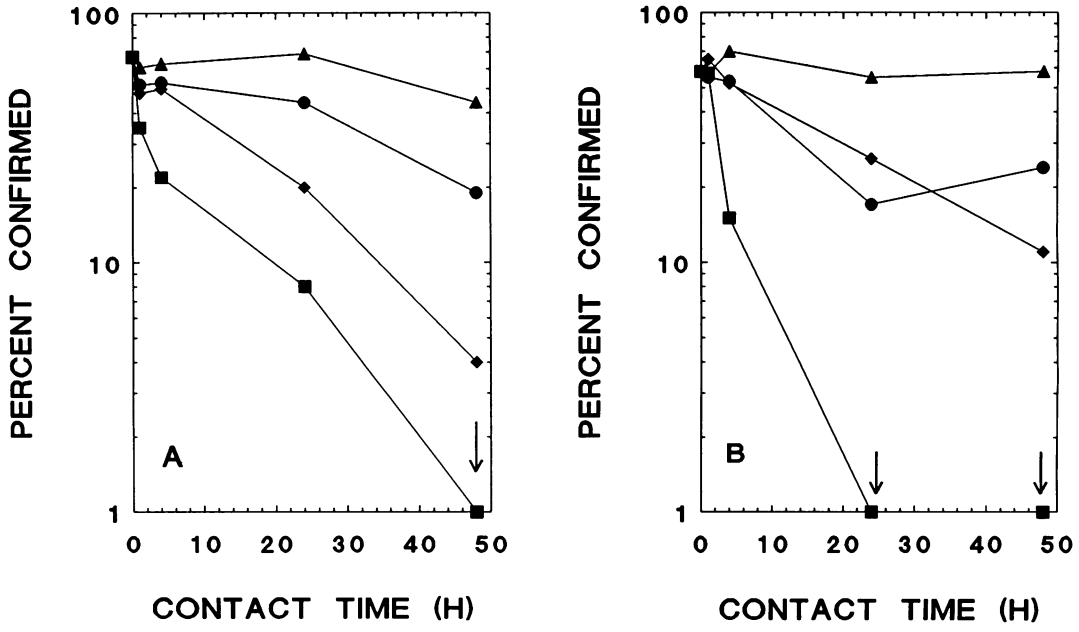

FIG. 2. Effects of chlorination temperature and chlorine concentration on the morphological identification of *G. lamblia* cysts. (A) Chlorination performed at 5°C with 0 (▲), 1 (●), 3 (◆), and 11 (■) mg of chlorine per liter. (B) Chlorination performed at 15°C with 0 (▲), 1 (●), 3 (◆), and 9 (■) mg of chlorine per liter. All datum points are the average of two experiments. Arrows indicate samples in which all cysts were detected (by fluorescence) but in which none were identified by morphological characteristics.

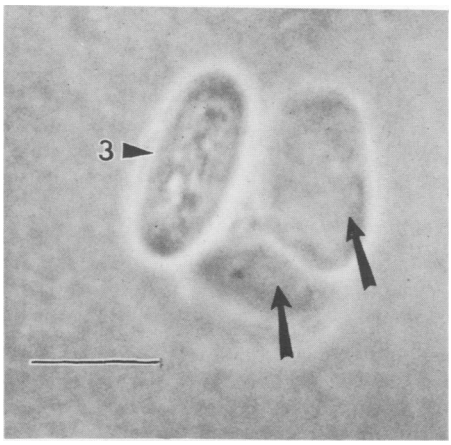

FIG. 3. Phase-contrast micrograph of *G. lamblia* cysts exposed to 3 mg of chlorine per liter for 48 h. Note the dissolution of all internal morphological features (arrows) within two of the cysts. The third cyst (cyst 3) could not be positively identified because of deterioration of internal features. All three, however, were fluorescent (not shown) when observed under UV light. Bar = 10 μm.

prevent the detection of these cysts by fluorescence, size, and shape. Virtually all the *G. lamblia* cysts exposed to chlorine residuals of 1 to 9.6 mg/liter for up to 48 h could still be detected by immunofluorescence (Table 1).

The presence of two or more morphological characteristics (Fig. 1) that have been traditionally used (9) to confirm the identity of *Giardia* cyst-like objects (clearly visible nuclei, axonemes, or median bodies) decreased with chlorine exposure time. These data are presented in Fig. 2A and B. The rate of morphological destruction also increased with increasing chlorine concentrations; in contrast, the majority of control cysts were identified during the same time period. After 1 h, 11.7 mg of chlorine per liter (residual level as shown in Table 1) at 5°C appeared to significantly affect cyst morphology. After 4 h at both 5 and 15°C, almost 50% of the cysts exposed to 0.8 and 2.8 mg of chlorine residuals per liter and >50% exposed to 9.8 and 10.2 mg of residuals per liter had no visible internal features, although a cyst wall and trophozoite form were clearly visible. After 24 and 48 h at 5 and 15°C, cysts exposed to 2.4 or 9.6 mg of residual chlorine per liter were detectable by fluorescence, but they could not be morphologically confirmed since phase-contrast examination revealed cyst shapes that lacked all internal morphological detail (Fig. 3, arrows). *G. muris* cysts showed a similar pattern of deterioration (data not shown). Others have reported similar morphological alterations of *G. muris* cysts after exposure to chlorine (4).

G. lamblia cysts exposed to neutralized chlorine for 48 h did not exhibit the type of morphological destruction that was shown by cysts in nonneutralized chlorine (Fig. 2A and B). About 40 to 50% of the cysts incubated in phosphate buffer only were easily identified at both temperatures (Fig. 2A and B), whereas 64% (5°C) and 66% (15°C) of cysts exposed to neutralized chlorine for 48 h could be identified, demonstrating a protective effect of sodium thiosulfate on the morphology of the cysts.

These results clearly imply that if chlorinated (>1 mg/liter) water samples are to be used for the detection and confirmation of *Giardia* cysts and if they are collected, transported, and stored for more than 4 h, the majority of *Giardia* cysts present will lack the internal morphology necessary to confirm the sample as positive. Complicating this issue, however, is the fact that some utilities have chlorine contact times equal to or greater than 4 h in their treatment train. Samples taken at the treatment plant effluent for *Giardia* analysis may have already been exposed to chlorine. Storage and time of transport to the laboratory can also add to chlorine exposure time. Therefore, it seems prudent to treat chlorinated water samples at the time of collection to neutralize the residual chlorine and to minimize the morphological destruction of *Giardia* cysts. In this way, morphological identification of *Giardia* cysts, detected by whatever method, may be feasible.

We thank Walter Jakubowski, Frank W. Schaefer III, Ronald Stetler, and Eugene W. Rice for critical review of the manuscript and Debbie Flanigan for technical assistance.

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