Propidium Iodide as an Indicator of Giardia Cyst Viability

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The use of propidium iodide, whose uptake indicates cell death or damage, was investigated to assess the viability of heat-inactivated and chemically inactivated Giardia muris cysts. This was done by comparing propidium iodide staining with excystation. We first determined that propidium iodide could be used with an immunofluorescence detection procedure by showing that the percentages of Giardia lamblia cysts stained with this dye before and after subjecting them to a fluorescence detection method were similar. G. muris cysts were then exposed to heat (56°C), 0.5 to 4 mg of chlorine per liter (pH 7.0, 5°C), 0.1 to 10 mg of a quaternary ammonium compound per liter, or 2 mg of preformed and forming monochloramine per liter (pH 7.2, 18 to 20°C). A good positive correlation between percent propidium iodide-stained cysts and lack of excystation was demonstrated for G. muris cysts exposed either to heat or to the quaternary ammonium compound. However, no significant correlation between absence of excystation and propidium iodide staining was found for cysts exposed to chlorine or monochloramines. These results demonstrate that the propidium iodide staining procedure is not satisfactory for determining the viability of G. muris cysts exposed to these two commonly used drinking water disinfectants.

Between 1965 and 1985, 95 outbreaks of waterborne giardiasis in the United States were reported (5). Unfortunately, the public health significance of Giardia cysts detected in the public water supplies is unknown, since neither the source nor the viability of these cysts can be easily and reliably determined.

Excystation and animal infectivity are indicators of viability that have been used to evaluate disinfection inactivation of Giardia cysts (12). However, these techniques are impractical to use on the small numbers of Giardia cysts found in water samples. The viability of mammalian cells is frequently determined with fluorescein diacetate (FDA) and propidium iodide (PI) (13, 26). FDA is a nonpolar ester which passes through cell membranes and is hydrolyzed by intracellular esterases to produce fluorescein. Accumulated fluorescein exhibits green fluorescence when excited by blue light, indicating a viable cell. PI, which is capable of passing through only damaged cell membranes, intercalates with the nucleic acids of injured and dead cells to form a bright red fluorescent complex.

Recently, Schupp and Erlandsen (22) compared FDA and PI staining with animal infectivity to indicate viability in normal and heat-killed Giardia muris cysts. Cysts that were stained with FDA were reported to correlate with infective cysts, whereas PI staining was reported to correlate with noninfective and presumably dead cysts. Recently, FDA and PI have been used to evaluate the viability of G. muris cysts exposed to a variety of environmental waters (7) and to determine the viability of in vitro-derived Giardia intestinalis (24) and human-source G. intestinalis cysts (25).

We have previously described an immunofluorescence method for the detection of Giardia cysts in water samples (18). The viability of cysts detected by this method is unknown. Therefore, in this study, we initially investigated the combined use of PI and the fluorescein-labeled antibody detection method and found the two tests to be compatible.

A combined detection-viability method available for use in analyzing water samples would be useful for checking the efficacy of disinfection. We therefore studied the relationship between PI staining and G. muris cysts inactivated by two commonly used drinking water disinfectants (chlorine and monochloramine), heat, and a quaternary ammonium compound.

MATERIALS AND METHODS

Giardia cysts. Giardia lamblia cysts were harvested from fecal pellets of Mongolian gerbils (2) and G. muris cysts were harvested from mouse feces (15) by flotation of the fecal slurry over 1.0 M sucrose. Cysts were purified further as previously described (17), stored at 4°C in distilled water, and used for experimentation within 2 weeks.

Excystation. G. muris cysts were excysted by a modification of the method of Schaefer et al. (21). Briefly, the initial incubation step in reducing solution (21) was followed by a final 45-min incubation in 0.5% proteose peptone (catalog no. 0120-01; Difco) prepared in pH 7 phosphate-buffered saline (19). Intact cysts, empty cyst walls, and partially excysted trophozoites were enumerated and excystation was calculated as previously described (21). Results were expressed as percent intact cysts (presumably nonviable) and derived by subtracting percent excystation from 100. For all experiments, excystation was performed on a minimum of 10⁶ cysts; at least 100 forms were counted in each of two or three replicate counts per experimental variable.

PI. A stock solution of PI (catalog no. P4170; Sigma Chemical Co., St. Louis, Mo.) was prepared by dissolving 5 mg of PI in 50 ml of Dulbecco phosphate-buffered saline, pH 7 (22). Suspensions (about 0.02 to 0.1 ml) of 10⁵ to 10⁶ G. muris cysts were stained with 0.003 mg of PI (0.03 ml of the stock solution) for 10 min at room temperature. Stained cysts (presumably nonviable) were enumerated with a Leitz Dialux 20 epifluorescence microscope equipped with an M2 filter block (546-nm exciting filter, 580-nm beam-splitting...
mirror, and 580-nm barrier filter). Phase optics were used to obtain total numbers of cysts observed (at least 100).

**Use of PI with immunofluorescence detection method.** *G. lamblia* cysts were applied to membrane filters as previously described (18). After excess antibody-fluorescein conjugate was removed, 0.6 ml of a 1/100 dilution of stock solution of PI in Dulbecco phosphate-buffered saline was added. Evans blue counterstain, used in the original immunofluorescence detection method (18), was omitted because it interfered with PI evaluation. To determine whether the reagents used in the immunofluorescence procedure would decrease or increase PI staining of *G. lamblia* cysts, fresh cysts were enumerated for PI uptake before addition to membrane filters. Another sample from the same preparation of fresh, presumably viable unstained cysts was subjected to the immunofluorescence assay, which included PI staining. At least 100 cysts were counted for each evaluation. Phase optics were used to enumerate total cysts and to evaluate internal morphology and refractivity.

**Heat exposure.** *G. muris* cysts were suspended in distilled deionized water (DDW) at a density of $4 \times 10^6$ cysts per ml. The suspensions were placed in a 56°C water bath. Samples of 0.15 ml were removed for excystation, and samples of 25 µl were removed for PI uptake, at times corresponding to 1, 3, 5, 10, and 15 min after initial exposure. The samples were then immediately placed on ice. Controls consisted of the same population and volume of cysts suspended in DDW and kept on ice. Results shown in this article represent the averages of three independent experiments, each of which included two or three replicates at each exposure time.

**Exposure to a quaternary ammonium compound.** Benzylidimethyltetradecyl ammonium (BTA; kindly provided by Donald Lindmark, Cleveland State University) was prepared as a 10-mg/liter stock solution in DDW. About $2 \times 10^6$ *G. muris* cysts per volume were added to 5-ml volumes of 0-, 0.1-, 0.5-, 1.0-, 2.5-, 5-, and 10-mg/liter BTA in DDW. The resulting cyst suspensions were rocked for 10 min at room temperature (about 24°C) and then diluted to 15 ml with DDW. These cysts were then washed three times with DDW containing 0.01% Tween 20 (TWD) and finally suspended in 0.1 to 0.2 ml of TWD. About $2.5 \times 10^6$ to $5 \times 10^6$ cysts (25 to 50 µl) were removed from each concentration tube for PI staining, and the rest (about $1.5 \times 10^6$ cysts) were used for excystation analysis. Results shown are the averages of three independent experiments, each of which included two or three replicates at each disinfectant concentration.

**Exposure to chlorine.** A stock solution of about 10 mg of chlorine per liter was prepared by adding purified-grade sodium hypochlorite solution (catalog no. SS290-1; Fisher Scientific) to 0.05 M KH$_2$PO$_4$ chlorine-demand-free (CDF) buffer, pH 7.0. About $5 \times 10^5$ *G. muris* cysts were washed three times by centrifugation in CDF buffer and finally suspended in 5 ml of CDF buffer containing 0.02% Tween 20. One milliliter of cysts (10$^7$) was added to 200 ml each of CDF buffer (the control) and CDF buffer containing 0.5, 2.0, or 4.0 mg of chlorine per liter (with the buffer previously cooled to 5°C). The cysts were exposed to chlorine and buffer for 30 min at 5°C, and then the reaction was stopped by adding 2 ml of 6.3% sodium thiosulfate solution to 200 ml of reaction mixture. One milliliter of the sodium thiosulfate solution was added to 100 ml of the cyst control in CDF buffer. The remaining volume of the control (100 ml) was not exposed to sodium thiosulfate. Cysts were recovered by filtration of the entire content of each reaction container through 1.0-µm pore-size Nuclepore membrane filters, which were then washed with TWD; cysts were washed off the filters into a test tube by squirting the filters with TWD, centrifuged to reduce the volume in the test tubes, and finally suspended in 1 ml of TWD. For each exposure concentration, 50 µl of cysts (about $5 \times 10^6$ cysts) was stained with PI, and about $1 \times 10^5$ cysts were subjected to the excystation procedure. Results shown are the averages of three experiments. The free chlorine residual of each chlorine concentration was determined by the N,N-diethyl-phenylenediamine (DPD) colorimetric method (1) before the addition of cysts, immediately after the addition of cysts, and just before the termination of the reaction with sodium thiosulfate.

**Exposure to monochloramines.** Monochloramine was prepared from solutions of ammonium sulfate and chlorine. The chlorine-to-ammonia ratio was 5:1 on a weight basis. In each case, the indicated amounts of ammonium sulfate were dissolved in 1 liter of 0.05 M KH$_2$PO$_4$ buffer at pH 7. The method for preparation of the buffer, the method to make it demand free, and the DPD monochloramine assay method have been described elsewhere (3). About 2 mg of forming monochloramine was obtained per liter by adding 0.5 ml of 800-mg/liter buffered chlorine (pH 7.0) to 200 ml of 1,860-mg/liter buffered ammonium sulfate (pH 7.0) at the time when cysts were added. Two portions of preformed monochloramine were added to each sample of each experiment. One portion was then neutralized with the required amount of 6.3% sodium thiosulfate to serve as a control. Other controls were pH 7 CDF buffer and pH 7 CDF buffered ammonium sulfate. All solutions were equilibrated at 18 to 19°C and held at that temperature throughout the exposure period. About $10^7$ *G. muris* cysts, previously washed and suspended in CDF buffer (pH 7) containing 0.02% Tween 20, were added to the 200 ml of reagent in each reaction container. Monochloramine was assayed prior to the addition of cysts and at all sampling times. At 1, 3, and 24 h after the addition of cysts, 50-ml samples were removed from each container, neutralized with 0.5 ml of 6.3% sodium thiosulfate (except cysts in the CDF buffer), filtered as in the chlorine experiments, and washed with Hanks' balanced salt solution. The final suspension of cysts was divided into 25-µl portions (about $1 \times 10^5$ cysts each) for PI staining and portions of about 0.18 ml ($9 \times 10^6$ cysts each) for excystation. Results presented are the averages of two experiments, both of which included two replicates for all conditions at each time point.

**RESULTS**

Initially, a fresh harvest of *G. lamblia* cysts was stained with PI in suspension as previously described (22); PI-positive cells were enumerated with an epifluorescence microscope, and the total number of cysts was determined by phase optics. In this suspension, 23% ± 8% (mean ± standard deviation; n = 10 counts of 100 cells each) were PI positive. Another portion of the same suspension (not stained with PI) was subjected to the immunofluorescence detection method and PI. Counts of at least 100 fluorescein-labeled cysts on each of nine membrane filters revealed that an average of 29% ± 8% of these cysts also stained bright red with PI when examined with the epifluorescence microscope. Thus, we found, as have others for mammalian cells (13), that the PI stain was compatible with an immunofluorescence method that utilizes a fluorescein label for detection of *Giardia* cysts in water samples.

When we heat treated *G. muris* cysts at 56°C for various times, the percentage of cysts not excysting (intact cysts presumed nonviable) and the percentage of cysts staining
with PI both increased with time (Fig. 1A). Although this positive correlation was significant (Kendall’s rank coefficient = 0.74 [Fig. 1B]), the percentage of cysts stained with PI (presumed nonviable) was lower than the percentage of intact cysts, especially at 3 and 5 min. This can also be seen on a scatter plot (Fig. 1B), which does not appear to be linear. When only the data for the longest exposure period are examined, it is seen that 100% of the cysts are intact (not capable of excysting) and >99% of the cysts in these samples are stained with PI. Thus, given sufficient exposure to heat, the correlation between inability to excyst and propidium iodide staining is almost perfect. It may be possible that the greater number of intact cysts observed at 3 and 5 min were viable cysts that could not excyst (i.e., the organisms may have been injured rather than killed during these time periods).

The results of treating cysts with a quaternary ammonium compound (BTA) are shown in Fig. 2A. BTA is a cationic surfactant which is readily adsorbed to negatively charged surfaces, and it is known to increase the permeability of cell membranes (16). Results similar to those found with heat were obtained. There was a strong positive correlation (Kendall’s rank correlation coefficient = 0.76 [Fig. 2B]) between percentage of intact cysts and percentage of cysts that were stained with PI. Examination of Fig. 2B reveals, as with the heat exposure, that the percentage of cysts stained with PI tended to be lower than the percentage not excysting. Examination of a scatter plot (Fig. 2B) also reveals the nonlinearity of the data. However, this difference disappeared once the point at which all of the cysts failed to excyst was reached.

There appeared to be no correlation (Kendall’s rank correlation coefficient = 0.28) between the percentage of intact cysts and the percent staining with PI when G. muris cysts were exposed to chlorine (Fig. 3). Cysts exposed to 0.35 mg of chlorine per liter showed normal internal morphology; those exposed to higher chlorine concentrations lacked the typical morphology associated with Giardia cysts and would not be expected to be viable on the basis of infectivity studies (8). At 1.54 and 3.06 mg of chlorine per
liter, all cysts, after 30 min, failed to excyst. However, no more than 10% of G. muris cysts exposed to any concentration of chlorine stained with PI (Fig. 3). During the course of chlorine exposure, the residual chlorine concentration remained fairly stable (Table 1). It is possible that since chlorine is a strong oxidizing agent, it masked or destroyed the sites into which the PI stain intercalates.

We also exposed G. muris cysts to preformed monochloramine and forming monochloramine, which are other commonly used disinfectants, and to the following controls: neutralized preformed monochloramine, CDF buffer (pH 7.0), and CDF buffered ammonium sulfate (pH 7.0) for 1, 3, and 24 h. Residual monochloramine concentrations (Table 2) were determined during this period. Because the concentrations of intact cysts and PI staining were similar at all time points for both preformed and forming monochloramine exposure, as were the results for all of the controls, only the results for the preformed monochloramine exposure and neutralized control are shown in Fig. 4.

As in the chlorine exposure experiments, G. muris cysts exposed to preformed monochloramine (Fig. 4) showed little correlation (Kendall’s rank correlation coefficient = 0.16) between the percentage of cysts stained with PI and the percentage of intact cysts. Unlike the case of chlorine-treated cysts, which showed about 10% staining with PI at all chlorine concentrations tested, there was an increase in the percentage of cysts (from 10 to 38%) that stained with PI as exposure time to preformed monochloramine increased to 24 h. However, the percentage of PI-stained cysts remained low in comparison with the percentage of intact cysts (about 99%) after 24 h of exposure to preformed monochloramine. Unlike the appearance of cysts exposed to chlorine, cysts exposed to preformed monochloramine appeared to retain typical internal morphology, even under conditions previously shown by others to be lethal (8). Observation of monochloramine-treated cysts after 24 h by phase optics revealed unusually bright, refractile cysts with greater refractivity than the controls. This suggests that monochloramines alter the permeability of the cyst wall, perhaps making the cyst wall less permeable. The residual monochloramine concentration decreased from 1.99 mg/liter at zero time to 0.99 mg/liter after 24 h (Table 2). The decrease might have been due to demand produced by deteriorating cysts, the presence of bacteria, or evaporation of monochloramine from the reaction container.

![FIG. 3. Effect of a 30-min exposure to chlorine (5°C, pH 7.0) on G. muris cysts. The increase in the percentage of nonviable cysts is expressed for intact cysts (□) and PI-stained cysts (■) for increasing chlorine concentrations. The nonparametric Kendall’s rank correlation coefficient was 0.28 (P = 0.05). Buff+Thio, buffer plus sodium thiosulfate solution.](image)

![FIG. 4. Effect of exposure to 2 mg of preformed monochloramine per liter (18 to 19°C, pH 7.0) on G. muris cysts. The increase in the percentage of nonviable cysts with increased exposure time is expressed for intact cysts (□) and PI-stained cysts (■) in the presence of neutralized preformed monochloramine and for intact cysts (■) and PI-stained cysts (□) in the presence of preformed monochloramine. The nonparametric Kendall’s rank correlation coefficient was 0.16 (P = 0.17).](image)

<table>
<thead>
<tr>
<th>Target chlorine concn (mg/liter)</th>
<th>Measured chlorine concn (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
</tr>
<tr>
<td>0.5</td>
<td>0.35 ± 0.07</td>
</tr>
<tr>
<td>2.0</td>
<td>1.47 ± 0.32</td>
</tr>
<tr>
<td>4.0</td>
<td>2.91 ± 0.34</td>
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</table>

a Each result shown is an average of three experiments ± standard deviation.

b Measured immediately after G. muris cysts were added to the chlorine solution.

c Measured after 30 min.

**TABLE 2.** Residual monochloramine concentrations determined during exposure of G. muris cysts to preformed and forming monochloramine

<table>
<thead>
<tr>
<th>Material tested</th>
<th>Exposure time (h)</th>
<th>Final concn (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preformed NH₂Cl</td>
<td>0</td>
<td>1.99</td>
</tr>
<tr>
<td>Neutralized NH₂Cl</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Preformed NH₃Cl</td>
<td>1</td>
<td>1.88</td>
</tr>
<tr>
<td>Forming NH₃Cl</td>
<td>1</td>
<td>1.99</td>
</tr>
<tr>
<td>Preformed NH₃Cl</td>
<td>3</td>
<td>1.75</td>
</tr>
<tr>
<td>Forming NH₃Cl</td>
<td>3</td>
<td>1.83</td>
</tr>
<tr>
<td>Preformed NH₂Cl</td>
<td>24</td>
<td>0.99</td>
</tr>
<tr>
<td>Forming NH₂Cl</td>
<td>24</td>
<td>0.99</td>
</tr>
</tbody>
</table>

a Each measurement is the average of two experiments.
DISCUSSION

Giardia cyst viability has been evaluated by such methods as in vivo infectivity (8, 10), in vitro excystation (4, 10, 21, 25), the presence or absence of specific internal morphological features (23), and dye exclusion (4, 7, 9, 11, 14, 22, 24). In vitro excystation and in vivo infectivity are impractical to use on the reported small numbers (0.01 to 179 cysts per 100 liters) of Giardia cysts usually found in water samples (6). Previously, we had analyzed several disinfectant-treated water samples and were unable to morphologically confirm the identity (i.e., by the presence of nuclei, median bodies, or axonemes) of fluorescing Giardia cyst-like objects by phase-contrast microscopy. Our subsequent laboratory studies (20) showed that chlorine destroyed the cyst internal morphology.

In the present study, our results suggest that PI staining is a suitable indicator of G. muris cyst viability when cysts are inactivated by heat or by the quaternary ammonium compound (BTA). However, when chlorine or monochloramine was used to treat cysts, PI did not stain the majority of inactivated cysts. Thus, it seems that the PI exclusion test should not be used to determine the efficacy of chlorine or monochloramine disinfection of G. muris cysts. However, the results for all of the controls suggested that PI might be of value only in specific instances (e.g., in determining the viability of cysts in raw water supplies not subject to sewage contamination or in treated supplies prior to the application of chlorine or monochloramine). If sewage has contaminated the source water, the cysts may have been inactivated by chlorine or monochloramine or perhaps other chemicals that might interfere with the PI test. Thus, cysts that have been truly inactivated by these chemicals would not take up the PI, and they would be scored incorrectly as viable.

We suggest that in the future, any newly proposed tests for Giardia cyst viability should be evaluated with cysts inactivated by a variety of agents, especially those relevant to water supply disinfection.

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REFERENCES