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Identification of Algae Which Interfere with the Detection of *Giardia* Cysts and *Cryptosporidium* Oocysts and a Method for Alleviating This Interference

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Fifty-four algal species were tested for cross-reaction in the American Society for Testing and Materials *Giardia*/*Cryptosporidium* indirect immunofluorescence assay, and 24 showed some degree of fluorescence. Two species, *Navicula minima* and *Synechococcus elongatus*, exhibited a bright apple green fluorescence. The addition of goat serum to the assay mixture blocked the fluorescence of most nontarget organisms tested and also decreased the background fluorescence. Goat serum did not interfere with the fluorescence of *Giardia* cysts or *Cryptosporidium* oocysts or the identification of cyst and oocyst internal structures.

Giardia and *Cryptosporidium* spp. are protozoan parasites which cause gastrointestinal disease in humans. The environmentally resistant forms of *Giardia* and *Cryptosporidium* spp. cysts and oocysts, respectively, are ubiquitous in surface waters (7). Although their concentrations may be low, cysts and oocysts are capable of surviving for weeks in surface waters (2, 4, 11) and the infective dose for humans for both parasites is low, being 10 *Giardia lamblia* cysts and 30 *Cryptosporidium parvum* oocysts (5, 10). These two parasites are responsible for large outbreaks of waterborne disease. *Giardia* spp. were implicated in 95 outbreaks of waterborne disease between 1965 and 1985 (3). In April 1993, over 400,000 people in Milwaukee, Wis., contracted cryptosporidosis, making this the single largest outbreak of waterborne disease in U.S. history (9).

The method currently used to detect these parasites in surface waters is an indirect immunofluorescence assay (IFA) based on the American Society for Testing and Materials (ASTM) proposed standard (1). This method has limitations in that it does not provide information on the viability or species of cysts and oocysts and recovery is affected by water turbidity. In addition, a filtered surface water sample, even after Percoll-sucrose flotation purification, contains debris and a myriad of microorganisms, including protozoans, bacteria, and algae. Some of these nontarget objects exhibit a green fluorescence, similar to *Giardia* and *Cryptosporidium* fluorescence, after being stained with the IFA reagents. The presence of fluorescent organisms similar in size and shape to *Giardia* and *Cryptosporidium* organisms increases the likelihood of false-positive results. To gain a better understanding of the contribution of algae to this problem, cultures of freshwater algae were obtained and tested for cross-reaction by the ASTM IFA procedure, with the exception that Evan's blue was not used as a counterstain. The cultures used represented 4 algal divisions and 19 families, and all cultures were less than 2 months old when tested. The Hydrofluor-Combo (HC) immunofluorescence kit (Meridian Diagnostics, Cincinnati, Ohio) was used for all IFA assays (this kit is currently available from EnSys Environmental Products, Inc., Research Triangle Park, N.C.). The kit consisted of three reagents: the primary antibodies, the labeling reagent, and a positive control antigen. The primary antibodies were two different mouse-derived monoclonal antibodies, one of which recognizes a *Giardia* cyst wall antigen and the other recognizes a *Cryptosporidium* oocyst wall antigen. The anti-*Giardia* antibody was immunoglobulin G (IgG), and the anti-*Cryptosporidium* antibody was IgM. The labeling reagent consisted of goat-derived anti-mouse IgG and anti-mouse IgM molecules conjugated to fluorescein. A Zeiss Axiophot epifluorescence microscope with a 450- to 490-nm excitation filter and a 520-nm barrier filter was used for all analyses.

Fifty-four species of algae were tested, and 24 were found to exhibit some degree of nonspecific green fluorescence (Table 1). The fluorescence of most algal species which cross-reacted was limited to a dull-medium intensity, except for that of two species, *Navicula minima*, a diatom, and *Synechococcus elongatus*, a cyanobacterium, which fluoresced a bright apple green, similar to that seen with *Giardia* and *Cryptosporidium* spp. (Fig. 1A). *N. minima* was most similar to cysts and oocysts in shape and size, typically appearing oval (10 to 17 μm long and 4 to 7 μm wide) (Fig. 1B). *N. minima* is a freshwater diatom with a wide geographical distribution and is found in both flowing and standing eutrophic, alkaline waters (8). Both *N. minima* and *S. elongatus* were maintained as growing cultures. While the *N. minima* cells consistently bound the HC reagents and fluoresced green, the *S. elongatus* cells did not always appear bright green after being stained with the HC reagents. This inconsistency of fluorescence was also observed with *Cylindrospermum* spp., *Pandorina morum*, and *Anacystis nidulans* and may have been due to differences between cells in different phases of growth.

It was of interest to determine which component(s) of the Hydrofluor kit was responsible for the fluorescence of the *Navicula* cells. We found that the algal cells fluoresced brightly when stained with just the fluorescein-conjugated anti-Ig mixture, as well as when stained with the components of this mixture separately. These results indicated that molecules on these cells were binding Ig in much the same way that protein A, the staphylococcal cell wall protein, binds the Fc portion of mammalian IgG molecules (6). If this were the case, it should be possible to block this binding by saturating the algal cells with an excess of nonspecific Igs. Figure 1C shows the results of staining *Navicula* cells by using the proposed ASTM protocol with the addition of 0.5 ml of goat serum (no. 9023; Sigma

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TABLE 1. Algal species tested and their appearance after staining with the *Giardia*/*Cryptosporidium* HC reagents

Organism[a]	Size (μm)	Shape	Intensity of green fluorescence[b]
Division Chlorophyta			
Class Chlorophyceae			
Order Chlorellales			
Family Chlorellaceae			
Ankistrodesmus falcatus EPA-28	44 × 3	Sickle	−
Chlorella ellipsoidea EPA-95	10	Round	−
Chlorella pyrenoidosa EPA-121	5	Round	−
Chlorella vulgaris EPA-263	9 × 6	Oval	−
Oocystis alpina UT-2541	9 × 6	Oval	−
Oocystis crassa LC-CH68	8 × 6	Oval	2+
Oocystis lacustris LC-CH70	20 × 15	Oval	−
Oocystis minuta UT-2071	9 × 5	Oval	−
Oocystis solitaria LC-CH67	16 × 13	Oval	−
Selenastrum capricornutum EPA-255	8	U shape	2+
Family Scenedesmaceae			
Scenedesmus basiliensis EPA-83	11 × 5	Oval	−
Scenedesmus bijugatus UT-413	6 × 4	Oval	1+
Scenedesmus obliquus EPA-92	10 × 5	Oval	1+
Pectodictyon cubicum LC-CH31	6	Round	1+
Order Chlorococcales			
Family Chlorococcaceae			
Chlorococcum botryoides EPA-91	9 × 6	Oval	1+
Chlorococcum macrostigmatum EPA-109	44 × 70	Rod	−
Family Dictyosphaeriaceae			
Botryococcus braunii LC-CH29	8	Round	1+
Dictyosphaerium pulchellum LC-CH37	4	Round	1+
Family Hydrodictyaceae			
Pediastrum boryanum UT-471	12 × 9	Rod	−
Family Micractinaceae			
Micractinium pusillum LC-CH36	5	Round	−
Order Tetrasporales			
Family Palmellaceae			
Gloeocystis ampla LC-GR39	7	Round	−
Family Tetrasporaceae			
Tetraspora lubrica LC-GR19	5	Round	2+
Order Ulotrichales			
Family Ulotrichaceae			
Stichococcus bacillaris EPA-86	10 × 3	Rod	−
Order Volvocales			
Family Chlamydomonadaceae			
Chlamydomonas minuta UT-2439	9 × 5	Oval	1+
Family Volvocaceae			
Eudorina unicocca UT-737	19	Round	1+
Pandorina morum EPA-18	52 × 42	Oval	2+
Division Chrysophyta			
Class Bacillariophyceae			
Order Centrales			
Family Thalassiosiraceae			
Cyclostephanos invisitatus LC-L310	6	Round	−
Cyclotella pseudostelligera LC-A14	7 × 3	Rod	1+
Stephanodiscus hantzschii LC-L371	10	Round	−
Thalassiosira pseudonana LC-L494	5 × 2	Rod	2+
Order Pennales			
Family Achnanthaceae			
Achnanthidium lanceolatum LC-L224	14 × 5	Oval	2+

Continued on following page

TABLE 1—*Continued*

Organism[a]	Size (μm)	Shape	Intensity of green fluorescence[b]
Achnanthidium rostratum LC-L589	13 × 5	Oval	2+
Family Naviculaceae			
Navicula minima EPA-391	17 × 7	Oval	3+
Navicula seminuloides LC-L680	10 × 4	Oval	1+
Navicula seminulum LC-L583	13 × 4	Oval	1+
Navicula tantula LC-L340	11 × 3	Oval	−
Sellaphora pupula LC-L517	24 × 7	Rod	−
Class Chrysophyceae			
Order Ochromonadales			
Family Dinobryaceae			
Dinobryon sp. strain UT-2267	42 × 7	Tubular	1+
Division Cyanophyta			
Order Chroococcales			
Family Chroococcaceae			
Anacystis nidulans EPA-625	4 × 1	Rod	2+
Eucapsis sp. strain EPA-1519	6	Round	−
Gloeocapsa alpicola EPA-589	2	Round	−
Gomphosphaeria lacustris LC-I548	26 × 8	Rod	−
Microcystis aeruginosa EPA-1036	4	Round	−
Synechococcus cedrorum EPA-1191	4 × 1	Rod	1+
Synechococcus elongatus EPA-563	2 × 1	Rod	3+
Synechocystis parvula EPA-193	1	Round	−
Order Osillatoriales			
Family Nostocaceae			
Anabaena catenula EPA-375	13 × 5	Oval	−
Anabaena flos-aquae EPA-1444	14 × 3	Oval	−
Anabaenopsis sp. strain EPA-137	15 × 4	Oval	−
Cylindrospermum sp. strain EPA-942	2	Round	1+
Nodularia sphaerocarpa EPA-583	32 × 4	Tubular	−
Nostoc muscorum EPA-486	2	Round	−
Family Rivulariaceae			
Gloeotrichia echinulata EPA-1052	2	Round	−
Division Euglenophyta			
Class Euglenophyceae			
Order Euglenales			
Family Euglenaceae			
Strombomonas sp. strain LC-EU11	3	Round	−

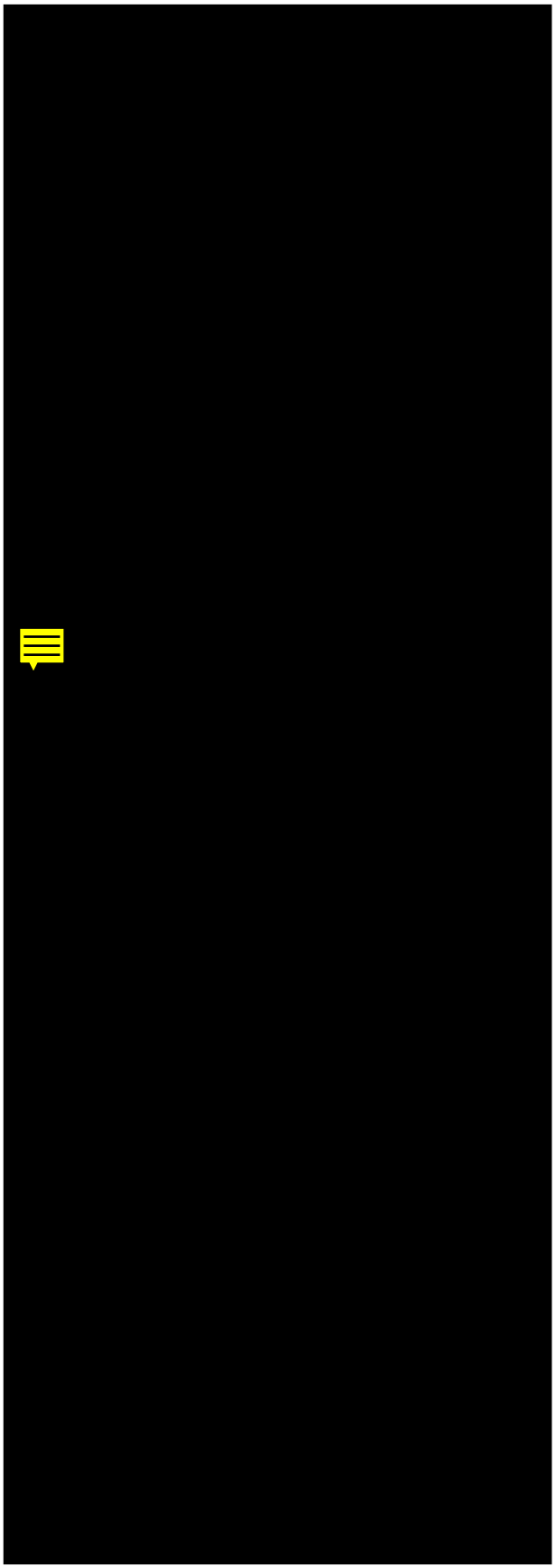
[a] Cultures with the EPA designation were obtained from the EPA algal collection. Cultures with the UT designation were obtained from the University of Texas algal collection. Cultures with the LC designation were obtained from the Loras College diatom collection.

[b] Fluorescein isothiocyanate epifluorescence, 450 to 490 nm excitation filter, 520-nm barrier filter; 1+, dull-green fluorescence; 2+, medium-green fluorescence; 3+, bright-green fluorescence; −, no green fluorescence.

Chemical Co., St. Louis, Mo.) per filter to 0.5 ml of diluted primary antibody mixture (50%, vol/vol). The results show that the serum eliminated the green fluorescence from around the algal cells. The red fluorescence visible inside the individual *Navicula* cells is the result of autofluorescent chloroplast pigments and was not affected by the serum.

To determine if the goat serum also blocked the binding of the HC reagents to *Giardia lamblia* and *Cryptosporidium parvum*, purified cysts and oocysts were mixed and six replicate aliquots were filtered through 0.2-μm-pore-size cellulose acetate membrane filters. Three filters were processed by the proposed ASTM method, while the other three were processed with goat serum added to the diluted primary-antibody mixture. The six filters were examined microscopically in a random blind fashion; i.e., the analyst did not know which filters had been processed with goat serum. The total numbers of cysts and oocysts on each filter were counted and recorded. For *Giardia* cysts, 982 ± 93 and 894 ± 135 (mean ± standard deviation) were counted per filter with and without goat serum, respectively. For *Cryptosporidium* oocysts, 170 ± 19 and 170 ± 18 were counted per filter with and without goat serum, respectively. For both cysts and oocysts, the differences in the mean values with and without goat serum were not significant at the 99% confidence level (Student's *t* test). These results clearly showed that the goat serum did not interfere with the detection and quantitation of the cysts and oocysts. The fluorescence intensity appeared to be identical in both cases. Furthermore, the use of goat serum did not interfere with the identification of specific internal structures in the cysts and oocysts (data not shown).

In addition to the nonspecific fluorescence associated with various microorganisms, there is background fluorescence as-



sociated with debris in the sample and with the membrane filter itself. Background fluorescence can be a significant hindrance in detecting cysts and oocysts, in that cysts and oocysts can be found on more than one focal plane and fluorescence in one plane can mask the presence of fluorescing objects in a different plane. The effect of serum on background fluorescence was measured by processing an Ohio River water sample by the proposed ASTM procedure (1) and filtering two 1-ml aliquots of the pelleted material, obtained after Percoll-sucrose flotation, through membrane filters. Both filters were processed in a manner identical to that of the IFA reagents, except that goat serum was included in the processing of one filter. The Zeiss Axiophot microscope used was equipped with an integrated camera which automatically sets the proper exposure time by measuring the brightness of the sample to be photographed. The exposure times calculated by the camera for 50 random microscopic fields were recorded for each filter. The mean exposure time for the filter without serum was 35.8 $\pm$ 7.7 s (mean $\pm$ standard deviation), while the mean exposure time for the filter with serum was 59.3 $\pm$ 11.0 s. The mean values were significantly different by the *t* test at the 99% confidence level, showing that the addition of goat serum diminished the background fluorescence. Goat serum had a similar effect on the background fluorescence in water samples from Texas, Pennsylvania, Missouri, and New Jersey.

Goat serum has recently been used successfully to block nonspecific binding of the HC reagents to 95 to 98% of unidentified *Giardia*-like and *Cryptosporidium*-like organisms in several surface water samples (12). It is important to note that the use of goat serum did not block all nontarget fluorescence seen in any given sample. Several of the cultures listed in Table 1 were retested with the goat serum-amended procedure. In addition to *N. minima*, serum blocked the fluorescence of *Chlorococcum botryoides* and *Synechococcus cedrorum*. However, it had no effect on the fluorescence of *Selenastrum capricornutum* or *Scenedesmus obliques*. These two species did not autofluoresce green. In contrast to *N. minima*, both the primary and the labeling reagents were necessary for fluorescence, and further experiments showed that separately both the anti-*Giardia* IgG and the anti-*Cryptosporidium* IgM antibodies, when used with their respective conjugated labeling antibody, stained these cells. Our results indicate that goat serum should have a significant positive effect on the analysis of water samples for pathogenic protozoa by decreasing the level of nonspecific fluorescence. Goat serum is commercially available and relatively inexpensive and does not interfere with the fluorescence detection of *Giardia* cysts or *Cryptosporidium* oocysts.

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FIG. 1. Fluorescein isothiocyanate epifluorescence of cells stained with the HC reagents. (A) *Giardia lamblia* cysts (G) and *Cryptosporidium parvum* oocysts (C) from Meridian Diagnostics HC positive control antigen preparation. Bar, 10 μm. (B) *N. minima* cultured cells. Bar, 10 μm. (C) *N. minima* cultured cells with goat serum added during staining. Bar, 10 μm.

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a
C
G
b
N
c
N