Chapter 1.

Evaluation of a Monoclonal-based Enzyme Immunoassay (EIA) for the Detection of *Giardia lamblia* in Human Stool

ABSTRACT

Giardiasis is the most frequently identified parasitic infection in the U.S.. The disease is caused by a protozoan parasite known as *Giardia lamblia*. Symptoms consist of diarrhea, flatulence, weight loss and, in severe cases, malabsorption. In this study, an enzyme immunoassay (EIA) for the detection of *Giardia lamblia* in human stool was developed and its performance characteristics were determined. This EIA, given the name "*GIARDIA* TEST", utilizes an immobilized monoclonal antibody that reacts with an unidentified cyst wall antigen. Evaluations were performed in-house at TechLab and off-site at three clinical laboratories using a total of 805 formalinized stool specimens. In the clinical evaluations, we compared this EIA test to the Alexon Prospect T *Giardia* TEST, another commercial monoclonal-based EIA. Both tests were compared to routine ova and parasite examination (O&P) using 535 stool specimens. Overall correlation to O&P was 95.9% for the Alexon test and 98.5% for the *GIARDIA* TEST. There were 8 discrepant results for the *GIARDIA* TEST and 22 for the Alexon test. All discrepant results were resolved by microscopic examination using immunofluorescence (IFA). In a separate in-house study, I compared the *GIARDIA* TEST to the Remel test. Overall correlations to O&P were 94.4% for the Remel test and 96% for the *GIARDIA* TEST. The lower limit of detection for the *GIARDIA* TEST was determined using *Giardia* cysts prepared *in vivo*. My findings show that the *GIARDIA* TEST is a suitable aid for the diagnosis of giardiasis because it is sensitive, highly specific and correlates well with O&P.

INTRODUCTION

Giardia lamblia is a protozoan parasite that causes a diarrheal disease in humans. The clinical significance was not fully appreciated in the United States until the early 1970's when a large number of travelers returned from the Soviet Union with giardiasis (23). This disease now is the most commonly diagnosed parasitic infection in the U.S. (8). High risk groups include young children, immunocompromised patients and persons who have not had previous exposure to the organism. Symptoms include diarrhea, abdominal cramps, bloating, flatulence, fatigue and weight loss caused by malabsorption.

The life cycle of *Giardia* consists of growth of trophozoites, encystment and excystment (Fig.1). Infection results from the consumption of as few as 10 cysts in contaminated water or feces (16). The cysts are resistant to the acidic barrier of the stomach and pass directly into the duodenum where each cyst matures into two trophozoites. The trophozoites infect and colonize the duodenum and jejunum by attaching to enterocytes of the brush border where they multiply by binary division. The attachment to the mucosal wall occurs by way of an "adhesive disc" which is found only on the trophozoite. The trophozoites subsequently detach as they begin to encyst and pass into the large intestine where they develop into mature four-nucleated cysts with a protective cyst wall. The highly infective cysts are excreted in the feces (8).

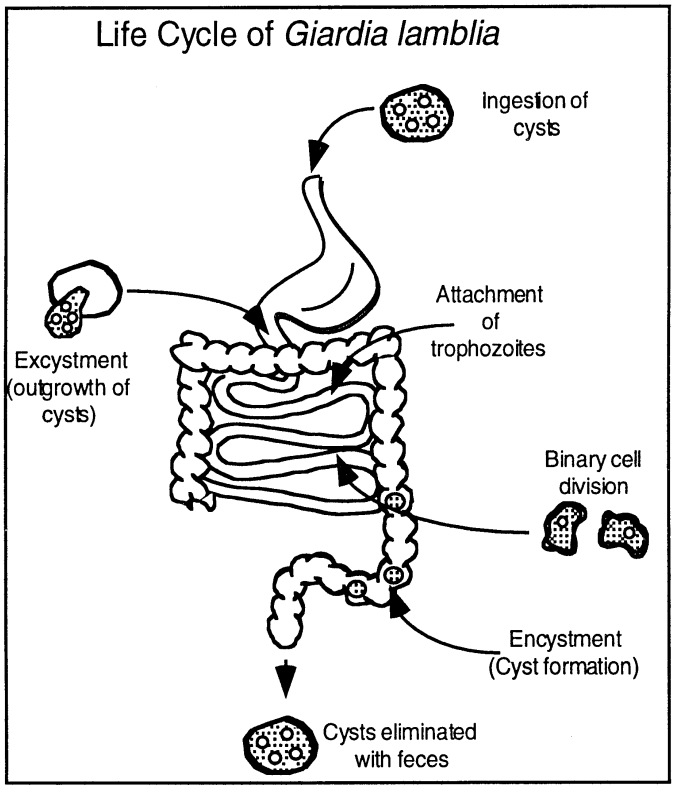

Figure 1. *Giardia* Life Cycle

Giardiasis is the most common waterborne illness in the United States. In a study reported in 1983, this parasite caused 68% of outbreaks of waterborne illness in which the infectious agent was identified (5). Outbreaks occur when public water supplies become contaminated by runoff water containing feces from infected animals. The first suspected outbreak of waterborne giardiasis in the United States occurred in Aspen, Colorado during the winter of 1965-66. Sewage-contaminated well water was the source of the organism. The first direct evidence that *Giardia* was a cause of waterborne illness came in 1975 in Rome, New York when the Centers of Disease Control identified *Giardia* cysts in the city water supply. Animals were identified as a source of infection after an outbreak during the spring of 1976 in Camas, Washington involving a filtered water supply. Upon investigation, *Giardia* cysts were identified in the feces of beaver living near the city water supply intakes (5).

Giardia trophozoites and cysts are very different in appearance. Trophozoites are tear-drop in shape and 10 to 20 μm in size (Fig. 2). Each trophozoite has eight flagella, a pair of nuclei, a pair of axonemes, a pair of median bodies and one adhesive disc. The flagella provide motility and are believed to cause negative pressure that allows the binding of the adhesive disc to the epithelial cells of the small intestine. The two nuclei contain equal amounts of DNA, together holding a diploid genome of around 12 MB (13). The function of the median bodies is not fully understood but it is hypothesized that they may store prepolymerized material for the construction of the adhesive disc. The paired flagella axonemes are visible in the trophozoites and are used to anchor the flagella. The ventral adhesive disc is used for attachment to epithelial cells of the small intestine (7). It is not clear whether this attachment is to secure nourishment or whether it simply provides a means of avoiding removal by the host.

Figure 2. *Giardia* trophozoites (Electron microscope photograph, R. Thompson, Murdoch University, Murdoch, Australia).

The mature cysts are highly infectious and differ in appearance and composition from the trophozoites. *Giardia* cysts are oval to round in shape and 10 to 15 μm in size (Fig. 3).

Each cyst has four nuclei, a pair of axonemes and a pair of claw-shaped median bodies. The outer filamentous wall is composed of proteins and glycoproteins with the primary carbohydrate being galactosamine and/or N-acetylgalactosamine. The outer cyst wall is protective against harsh conditions such as chlorine treatment and physical stress (2). Under the filamentous wall is a thin layer of cytoplasm next to the cell membrane of the two enclosed trophozoites (13). Many aspects of the *Giardia* cyst wall, such as composition and the function of cyst wall proteins, remain unknown.

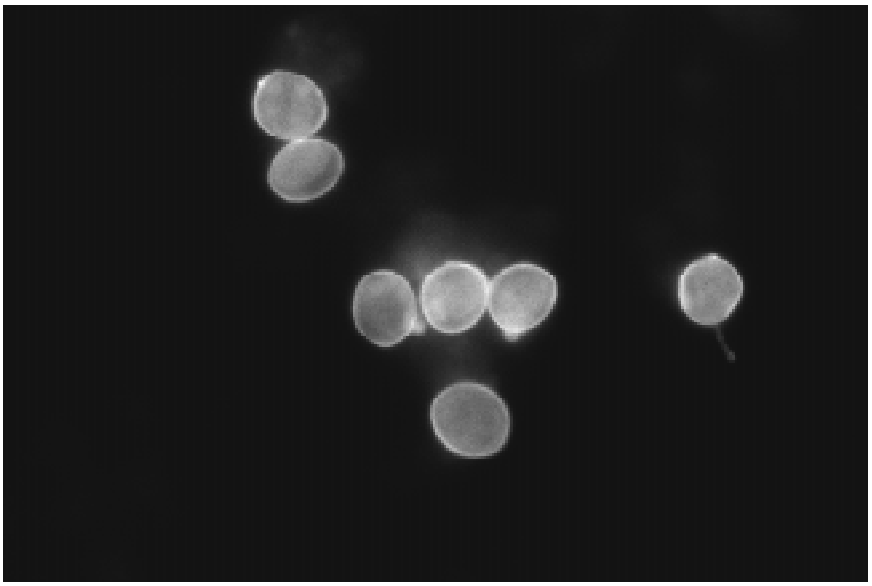

Figure 3. The *Giardia* cysts (1000 times)

The diagnosis of giardiasis is by the detection of whole cysts or antigens in the stools of infected persons. There are several commercial tests available including conventional microscopy stains, immunofluorescence antibody tests (IFA) and enzyme immunoassay (EIA). Microscopic examination involves the use of stains such as iodine and IFA tests. IFA tests consist of antibodies that are raised against cyst proteins and tagged with a fluorescent dye. The tagged antibodies bind to the *Giardia* cysts which can then be observed using a fluorescent

microscope. IFA tests are rapid and more sensitive than conventional microscopy (3, 6). Several IFA tests are commercially available (Table 1).

Table 1. Commercially available tests for the detection of *Giardia*

Method	Principle of test	Advantages	Limitations	Source of reagents
Ova/Parasite	Stains: Iodine Trichrome	Low cost	Not very sensitive Efficiency varies among different laboratories	Para Pak Wheatley's Trichrome Stain (Meridian Diagnostics, Inc.)
	Direct wet mount	Rapid Low cost Can determine motility	Difficult to read Low sensitivity Requires fresh stools	Iodine Solution Lugol's Concentrate (EMDS)
Immuno-fluorescence	Utilizes fuorescein-tagged monoclonal antibodies	Low cost Rapid Simple Sensitive Specific	Requires fluorescence microscope	MeriFluor (Meridian Diagnostics, Inc.) *Giardia*-IF TEST and *Crypto/Giardia*-IF TEST (TechLab, Inc.)
EIA	Uses monoclonal and polyclonal antibodies against *Giardia* antigens	Sample can be batched Sensitive Detects antigens when organism is no longer present	Not as specific as Immunofluorescence More expensive than staining and Immuno-fluorescence	ProspecT (Alexon) *Giardia* EIA (REMEL) LMD *Giardia* EIA (LMD Laboratories) *Giardia* TEST (TechLab, Inc.)
Flow-through membrane	Same as EIA	Rapid Simple	Low sensitivity	ProspecT (Alexon)

Another diagnostic method is the detection of *Giardia* specific antigens by EIA (11, 15, 18, 20, 21). These tests consist of antibodies (monoclonal or polyclonal) immobilized on plastic assay wells which capture the antigens. The bound antigens are then detected with antibodies in solution that have been conjugated to an enzyme such as horseradish peroxidase. The *Giardia* antigens present in the stool specimen are "sandwiched" by the capture and conjugated antibodies. The last step is the addition of an enzyme substrate that produces a colored product when antigens are present. The color reactions may be read visually and/or by a spectrophotometer. EIAs are specific for *Giardia* antigens and have been shown to be more

sensitive than conventional microscopy (1, 19). Some *Giardia* antigens detected by commercial EIA tests have not been identified, causing confusion about which antigens are detected by the various tests (1, 20, 22).

In the following study, I evaluated a new monoclonal-based EIA which I developed for the detection of *Giardia lamblia* in human stool. The assay was evaluated through a multicenter study comparing performance characteristics to routine O&P and two *Giardia*-specific EIA tests.

MATERIALS AND METHODS

Study sites and stool specimens. Four separate studies were performed in the evaluation: (i) Study 1, performed at Sacred Heart Medical Center in Spokane, WA; (ii) Study 2, performed at Dekalb Medical Center, Atlanta, GA with additional samples being supplied from SmithKline Laboratory, Atlanta, GA; (iii) Study 3, performed at Virus Reference Laboratory (VRL), Antonio, TX. (iv) Study 4, performed at TechLab with stool specimens supplied by an outside reference laboratory (Parasite Diagnostics, Snellville, GA). All specimens included in this study were preserved in formalin and submitted for routine ova and parasite examination.

EIA tests. The *GIARDIA* TEST (TechLab, Blacksburg, VA) was performed as follows. A total of 0.1 mL of formalized stool was placed into micro-titer type wells that had been coated with an IgM mouse monoclonal antibody (Ct7 MAb) against a *Giardia* cyst antigen. Wells were incubated 10 or 60 minutes at room temperature and then washed 4 times with the kit wash solution. One drop of rabbit polyclonal antibody raised against *Giardia* cysts was added to each well and incubated for 20 minutes at room temperature. The wash step was repeated and one drop of goat anti-rabbit polyclonal antibody conjugated to horse-radish peroxidase was added. Wells were incubated for 5 minutes at room temperature and washed as described above. One drop of each substrate was added to each well and wells were incubated for 5 minutes at room temperature. One drop of stop solution was added to each well and the plate was read visually and at 450 nm or 450 nm plus 620 nm as a reference.

The following EIA tests were performed and results were interpreted as indicated by the manufacturer's directions: (1) RIM® *Giardia* Antigen Detection Microwell ELISA (Remel,

Lenexa, KS), (2) Alexon Prospect T® *Giardia* Microplate Assay (Alexon, Inc., Sunnyvale, Calif.).

Immunofluorescent antibody test (IFA). The *GIARDIA* IF TEST and the Merifluor IF test (Meridian Diagnostics, Inc., Cincinnati, Ohio) were performed as described by the manufacturer.

Preparation of *Giardia* cysts. *G. lamblia* WB ATCC 30957 trophozoites were axenically cultured in Keister's modified TYI-33 medium at pH 7.1 and incubated at 37°C (8). After a confluent monolayer was formed, trophozoites were chilled on ice for 20 minutes, pelleted by centrifugation at 800 x g and suspended in TYI-33 medium containing 10 mg bovine bile / mL (Sigma; St. Louis, MS) at pH 7.8 to trigger encystment (10, 12). After 5 days, the culture was harvested by centrifugation at 800 x g. The pellet was suspended in deionized water and incubated overnight at room temperature to lyse trophozoites and pre-cysts (12, 14). Cysts were pelleted by centrifugation as previously described and washed several times with sterile water. Purified cysts were counted using trypan blue and stored in phosphate buffered saline (PBS pH 7.5) containing 0.01% sodium azide as a preservative.

Ova and parasite examination of stools positive for other intestinal parasites. Formalinized stools were submitted to Parasite Diagnostics Laboratory (Lilburn, GA) for ova and parasite analysis, concentrated using the formalin-ethyl acetate method and stained for microscopic examination (17, 24). Briefly, 5 mL of formalinized stool was strained through wet gauze and diluted to 10 mL with deionized water. The suspension was centrifuged at 500 x g for 2 minutes and the supernatant was discarded. The pellet was mixed with 9 mL of PBS containing 10% formalin. This suspension was mixed with 4 mL of ethyl-acetate and centrifuged for 2 minutes at 500 x g. The concentrated pellet was mixed with 50 µL of PBS

and 50 μL of Dobel's iodine solution for a wet mount preparation for microscopic examination except for *Cryptosporidium*-positive stools which were confirmed by acid-fast staining using the Kinyoun solution.

Determination of lower limits of detection for the *GIARDIA* TEST. To evaluate the sensitivity of the *GIARDIA* TEST, dose response curves were generated using purified *Giardia* cysts. Cysts were serially diluted in phosphate buffered saline, pH 7.4, and tested, in duplicate, in the EIA using optimal amounts of reagents. Dilutions containing low numbers of cysts were included to determine the lower limit of detection. An absorbance value ≥ 0.150 at 450 nm was used as the cutoff value between a positive and negative result. The absorbance values versus number of cysts per well were plotted on a graph.

RESULTS

Performance characteristics. The clinical performance was determined in 3 clinical laboratories using stool specimens preserved in 10% formalin. *Giardia*-positive stools were determined using routine O&P. *GIARDIA* TEST results were compared to results from the Alexon Prospect T *Giardia* TEST. All EIA results that did not match O&P (discrepants) were evaluated by IFA. Of the total 535 specimens analyzed, 96 were positive for *Giardia* by O&P. Of these, 93 were positive by the *GIARDIA* TEST and 96 were positive by the Alexon Test. A summary of sensitivities (Sens; how well the assay detects a positive result), specificities (Spec; how well the assay differentiates between a positive and negative result), predictive positive value (PPV; confidence in the positive result) and negative values (PPV; confidence in a negative result) and the overall correlation with O&P are shown in Table 2. Table 3 and 4 show the results from each clinical study.

The *GIARDIA* TEST had 8 discrepant results as compared to O&P. IFA analysis revealed 3 false-negatives, 3 false-positives and 2 true positives. The Alexon test had 22 false-positive results. Of these, 13 were negative following repeat testing. All three clinical sites noted that the Alexon false-positive results were from test wells that surrounded test wells containing strong positive-specimens. The remaining 9 were repeated and remained positives.

The assay performance of the *GIARDIA* TEST was compared to the Remel test by an in-house study at TechLab (Blacksburg, VA). Of 270 specimens, 120 were positive for *Giardia* by O&P using the conventional iodine staining procedure. Of these, 108 were positive by the *GIARDIA* TEST and 110 were positive by the Remel test. Of the 12 discrepant stools, 10 that were negative in the *GIARDIA* TEST and O&P positive were also negative by the Remel test.

All 10 of these stools were negative by IFA. The remaining 2 false-negatives had < 1 cyst per high field. There were 150 specimens were negative by O&P. All of these also were negative by the *GIARDIA* TEST and 146 were negative by the Remel test. The 4 stools that were positive in the Remel test and negative by O&P were also negative by the *GIARDIA* TEST. Sensitivities and specificities for both EIA tests as compared with O&P are shown in Table 5.

The *GIARDIA* TEST had a minimum detection limit of about 10 to 15 cysts per well (Fig. 4). Positive wells were yellow and had absorbance values of ≥ 0.150. The wells with lower dilutions of *Giardia* cysts were clear and had absorbance values ≤ 0.100 and a average standard deviation of ≤ 0.100 absorbance. The *GIARDIA* TEST did not show reactions with multiple stool specimens positive for a variety of intestinal parasites and enteric pathogens (Table 6).

To test reliability of the *GIARDIA* TEST, the inter-assay performance was evaluated. This statistical analysis shows reproducibility of test results over time. Results are expressed as the average percent difference of each absorbance value from the mean absorbance value. This analysis is referred to as the percent coefficient of variation (% CV). To examine inter-assay performance, eight positive and eight negative fecal specimens were assayed three times over a three day period (Table 7). The % CV of positive specimens ranged from 3.6 to 16.5, with an average of 11.2. The % CV of negative specimens ranged from 3.2 to 10.8, with an average of 6.9. Results are within the same range as other commercial EIA tests (Remel) which have % CV values below 20 %.

Assay reproducibility of the *GIARDIA* TEST was evaluated by the determination of intra-assay variation. The intra-assay coefficient of variation was determined by analyzing two positive and two negative specimens. Each specimen was assayed in quadruplicate over a three-

day period (Table 8). The positive specimens had an average % CV of 5.3 with a range of 2.4 to 12.3. The negative specimens had an average % CV of 10.9 with a range of 7.2 to 15.1. As with the intra-assay results, these percent values are within the same range as other commercial EIA tests (≤ 20 % CV).

TABLE 2. A summary of three independent clinical studies comparing the *GIARDIA* TEST and Alexon Test to ova and parasite examination (O&P). Both EIA tests were performed as instructed by the manufacturer. O&P was done using in-house methods implemented at each study site.

GIARDIA TEST	positive	negative
positive	93	5
negative	3	434
	Sensitivity	95.8%
	Specificity	98.9%
	PPV	96%
	PNV	99.3%
	Corr.	98.5%
	Number	535

Alexon Test	positive	negative
positive	96	22
negative	0	417
	Sensitivity	100%
	Specificity	94.9%
	PPV	81.4%
	PNV	100.0%
	Corr.	95.9%
	Number	535

TABLE 3. A summary of each clinical study comparing the *GIARDIA* TEST with O&P. Discrepant results were resolved using immunofluorescence.

GIARDIA TEST	Study 1 Sacred Heart Med. Ctr., Spokane, WA	Study 2 Dekalb Med. Ctr. Decatur, GA	Study 3 Virus Reference Lab. San Antonio, TX
Test Results	Total stools = 114 Total pos. = 31 Total neg. = 79 Total discrep. = 4	Total stools = 221 Total pos. = 46 Total neg. = 175 Total discrep. = 0	Total stools = 200 Total pos. = 16 Total neg. = 180 Total discrep. = 4
Sens	91.4%	100%	100%
Spec	100%	100%	97.8%
PPV	100%	100%	80%
PNV	96.3%	100%	100%
Corr	97.4%	100%	98%

TABLE 4. A summary of each clinical study comparing the Alexon test with O&P. Discrepant results were resolved using immunofluorescence.

Alexon Test	Study 1 Sacred Heart Med. Ctr., Spokane, WA	Study 2 Dekalb Med. Ctr. Decatur, GA	Study 3 Virus Reference Lab. San Antonio, TX
Test Results	Total stools = 114 Total pos. = 34 Total neg. = 75 Total discrep. = 5	Total stools = 221 Total pos. = 46 Total neg. = 165 Total discrep. = 10	Total stools = 200 Total pos. = 16 Total neg. = 177 Total discrep. = 7
Sens	100%	100%	100%
Spec	94.9%	94.3%	96.2%
PPV	89.7%	82.1%	69.5%
PNV	100%	100%	100%
Corr	96.5%	95.5%	96.5%

TABLE 5. A summary of results from an in-house study done at TechLab. The *GIARDIA* TEST and Remel test were compared to O&P using 270 stools preserved in 10% buffered formalin. Discrepant results were resolved using immunofluorescence.

In-house study TechLab, Inc. Blacksburg, VA	*GIARDIA* TEST	Remel *Giardia* Test
Test Results	Total stools = 270 Total pos. = 108 Total neg. = 150 Total discep. = 12	Total stools = 270 Total pos. = 108 Total neg. = 145 Total discep. = 10
Sens	90%	91.7%
Spec	100.0%	96.7%
PPV	100.0%	95.7%
PNV	92.6%	93.5%
Corr	96.0%	94.4%

TABLE 6. Microorganisms that do not react in the *GIARDIA* TEST. The specificity was determined by analyzing stool specimens that were positive for a variety of intestinal parasites and enteric pathogens as determined by conventional microscopy and EIA. The numbers in parenthesis indicate the number of specimens tested. All absorbance values were ≤ 0.100.

Blastocystis hominis (2)	*Clonorchis* (1)
Cryptosporidium (8)	*Cyclospora* (1)
Dientamoeba fragilis (3)	*Diphyllobothrium latum* (2)
Endolimax nana (13)	*Entamoeba coli* (8)
Entamoeba hartmanni (3)	*Enterobius* (1)
Hookworm (1)	*Hymenolepis nana* (3)
Iodamoeba butchlii (2)	*Microsporidium* (1)
Rotavirus (15)	*Strongyloides stercoralis* (2)
Taenia species (2)	

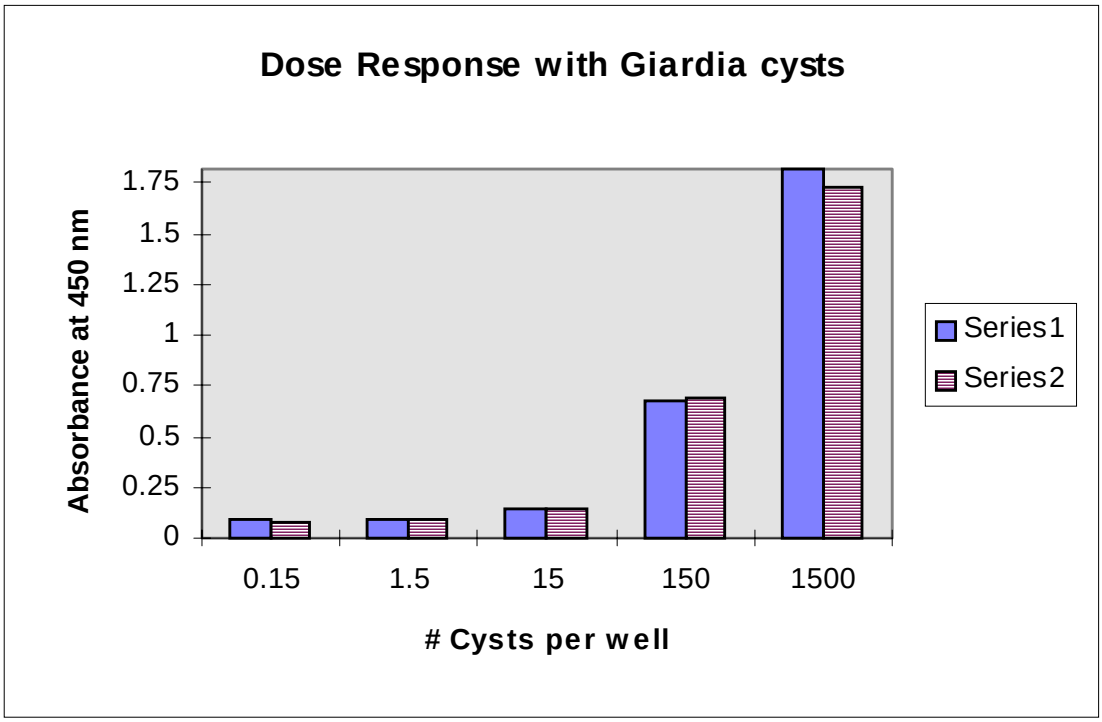

FIGURE 4. Dose response curves were generated using the *GIARDIA* TEST and purified cultured *Giardia* cysts. Cyst were serially diluted and 0.1 mL was added to each test well. Absorbance values ≥ 0.150 were considered positive results as determined by correlation of positive results to disease. The assay was repeated using test kits from 2 separate lots (series 1 & series 2).

TABLE 7. Inter-assay testing using the *GIARDIA* TEST. To examine inter-assay performance, eight positive and eight negative fecal specimens were assayed a total of three times over a three day period. The percent coefficient of variation (% CV) is defined as the average percent difference of each absorbance value from the mean absorbance value.

Sample	Day 1	Day 2	Day 3	Average	Standard deviation	% CV
Positive # 1	1.099	1.108	1.036	1.081	0.039	3.63
Positive # 2	1.330	1.104	1.396	1.277	0.153	11.99
Positive # 3	1.377	1.110	1.533	1.340	0.214	15.96
Positive # 4	1.771	1.515	1.595	1.627	0.131	8.05
Positive # 5	1.443	1.337	1.567	1.449	0.115	7.95
Positive # 6	1.250	0.931	0.973	1.051	0.173	16.48
Positive # 7	1.295	0.995	1.106	1.132	0.152	13.40
Positive # 8	1.522	1.234	1.568	1.441	0.181	12.56
Negative # 1	0.079	0.064	0.069	0.071	0.008	10.81
Negative # 2	0.070	0.061	0.06	0.064	0.006	8.65
Negative # 3	0.053	0.061	0.050	0.055	0.006	10.42
Negative # 4	0.050	0.048	0.047	0.048	0.002	3.16
Negative # 5	0.056	0.052	0.054	0.054	0.002	3.86
Negative # 6	0.049	0.052	0.047	0.049	0.003	5.10
Negative # 7	0.052	0.057	0.045	0.051	0.006	11.76
Negative # 8	0.059	0.063	0.048	0.057	0.008	13.63

TABLE 8. Intra-assay testing using the *GIARDIA* TEST. The intra-assay coefficient of variation was determined by analyzing two positive and two negative specimens. Each specimen was assayed in quadruplicate over a three-day period.

Specimen	Day	result 1	result 2	result 3	result 4	Average	Standard deviation	% CV
Sample	1	2.185	2.089	2.114	2.194	2.145	0.051	2.39
# 1	2	1.874	1.934	1.592	1.802	1.801	0.149	8.28
(positive)	3	1.622	1.735	1.711	1.72	1.697	0.051	3.00
Sample	1	1.592	1.562	1.492	1.552	1.550	0.042	2.70
# 2	2	1.557	1.565	1.465	1.574	1.540	0.051	3.28
(positive)	3	1.461	1.456	1.146	1.219	1.321	0.162	12.28
Sample	1	0.057	0.057	0.048	0.048	0.053	0.005	9.897
# 3	2	0.076	0.062	0.058	0.057	0.063	0.009	13.87
(negative)	3	0.065	0.058	0.055	0.049	0.057	0.007	11.72
Sample	1	0.057	0.052	0.058	0.062	0.057	0.004	7.18
# 4	2	0.078	0.078	0.059	0.061	0.069	0.01	15.12
(negative)	3	0.063	0.068	0.061	0.073	0.066	0.005	8.12

DISCUSSION

In this study, the *GIARDIA* TEST had a high correlation (98.5 %) to conventional microscopy which was similar to results of the Remel test and Alexon test. The *GIARDIA* TEST and the Alexon test had the highest specificity and predictive negative values. This is not surprising since both of these EIA tests are monoclonal-based whereas the Remel test uses polyclonal antibodies. All three EIA tests had sensitivity values of 90 to 91 %. The Alexon test consistently showed more false-positive results for O&P negative stool samples. Other studies of the Alexon test have noted similar results, but reported the specimens as positive, explaining that the detected antigen in the Alexon test is a secreted antigen and may be present when cysts are not seen. In this study, the majority of these specimens were negative by the Alexon test following repeat testing. This indicates nonspecific reactions rather than true positive results. In addition, these false-positives surrounded test wells containing strong positive-samples, further suggesting that they were false-positives resulting from wash-over.

All of the EIAs have quick and easy to use formats but the *GIARDIA* TEST and the Remel test can be completed with in 40 minutes whereas the Alexon test requires 2 hours. EIAs are easier and faster than microscopy and stool specimens can be batched and tested rapidly. The *GIARDIA* TEST has an added feature of a 10 to 60 minute variable incubation time for the specimen in the well. This range for the initial incubation allows for easier batching of large numbers of samples which is an advantage for large reference labs trying to save time and money. Testing of large number of stool specimens by conventional microscopy is tedious and can lead to unreliable results (6).

The *GIARDIA* TEST showed a high sensitivity for detecting cultured encysting *Giardia*. The lower limits of detection of approximately 15 cysts per well (150 cysts per mL) and high EIA sensitivity for a secreted antigen, shows the potential for detecting *Giardia* antigen in stool specimens where cysts can not be seen by microscopy. Relying entirely on conventional microscopy could lead to confusion between other illnesses such as Crohn's disease and ulcerative colitis and patients that don't shed intact organisms (4).

This data supports other research that has shown EIA tests are a sensitive and specific alternative method for diagnosing giardiasis (11, 15, 18). Recent evaluations suggest the implementation of both microscopy and EIAs for optimizing the diagnosis of giardiasis (9). A practical approach analyzing stools for *Giardia* may be the screening of stools by EIA in addition to or prior to a O&P microscopic exam. The use of both testing methods appears logical after considering that giardiasis is the most common parasitic infection diagnosed in the U.S. and that the sensitivity of EIAs are much greater than microscopy-sensitivity.

In conclusion, my results show that the *GIARDIA* TEST is a highly specific and sensitive EIA test that correlates well with O&P for the diagnosis of *Giardia lamblia.* In addition, the test is rapid and offers a unique variable specimen incubation time for the analysis of large numbers of samples.

REFERENCES

1. Addis, D. G., Mathews, H. M., Stewart, J. M., Wahquist, S. P., Williams, R. M., Finton, R. J., Spencer, H. C. and D. D. Juranek. 1991. Evaluation of a commercially available enzyme-linked immunosorbant assay for *Giardia lamblia* antigen in stool. J. Clin. Microbiol. 29:1137-1142.

2. Aley, S. B., and Frances D. Gillin. 1995. Specialized surface adaptations of *Giardia lamblia*. Infect Agents and Disease. 4:161-166.

3. Alles, A. J., Waldron, M. A., Sierra, L.S., and A. R. Mattia. 1995. Prospective comparison of direct immunofluorescence and conventional staining methods for the detection of *Giardia lamblia* and *Cryptosoridium spp.* in human fecal specimens. J. Clin. Microbiol. 33:1632-1634.

4. Allison, M. C., E. L. Green, D. N. Bhattacharya, A. Smith, and R. E. Pounder. 1988. A microscopic and immunodiagnostic search for giardiasis in patients with gastrointestinal disorders. Scan. J. Gastroenterol. 23:209-212.

5. Craun, G. F. 1979. Waterborne outbreaks of giardiasis. In Jakubowski, W., J. Hoff (ED)., Waterborne transmission of giardiasis. U.S. Environmental Protection Agency, Washington, D.C., pp. 127-149.

6. Dixon, B. R., Monique, P., Martineau, C, and Jocelyn Fournier. 1997. A comparison of conventional microscopy and flow cytometry in the detection of *Giardia lamblia* in beaver fecal samples. J. Immunol. Methods. 202:27-33.

7. Feely, D. E., S. Erlandsen, and D. Chase. 1984. Structure of trophozoite and cyst. In S. Erlandsen and Ernest Meyer (ed), *Giardia* and Giardiasis. Plenum Press, New York and London.

8. Garcia, L. S., and D. Bruckner. 1993. Intestinal protozoa: Flagellates and ciliates. In L. Garcia and D. Bruckner (ed.), Diagnostic Medical Parasitology. American Society for Microbiology, Washington, D.C.

9. Garcia, L. S. and Robin Shimizu. 1997. Evaluation of nine immunoassay kits (Enzyme immunoassay and direct fluorescence) for detection of *Giardia lamblia* and *Cryptosporidium* in human fecal specimens. J. Clin. Microbiol. 35:1526-1529.

10. Gillin, F.D., Reiner, D.S., Gault, M.J., Douglas, H., Das, S., Wunderlich, A., and J.F. Sauch. 1987. Encystation and expression of cyst antigens by *Giardia lamblia* in vitro. Science. 235: 1040-1043.

11. Janoff, E. N., J. C. Croft, L. K. Pickering, T. Novotny, M. J. Blaser, C. V. Knisley, and L. B. Reller. 1989. Diagnosis of *Giardia lamblia* infections by detection of parasite antigens. J. Clin. Microbiol. 27:431-435.

12. Keister, D.B. 1983. Axenic culture of *Giardia lamblia* in TYI-33 Medium supplemented with bile. Trans. R. Soc. Trop. Med. Hyg. 77:487-488.

13. Kulda, J., and E. Nohynkova. 1995. *Giardia* in Humans and Animals. In J. Kreier (ed.), Parasitic Protozoa. Academic Press, San Diego, CA.

14. Meng, T., M. Hetsko, and F. Gillin. 1996. Inhibition of *Giardia lamblia* excystation by antibodies against cyst walls and by wheat germ agglutinin. Infect. Immun. 64:2151-2157.

15. Nash, T.E., D. A. Herrington, and M. M. Levine. 1987. Usefulness of an enzyme-linked immunosorbant assay for detection of *Giardia* antigen in faeces. J. Clin. Microbiol. 27:1169-1171.

16. Rendtroff, R. C. 1954. The experimentation transmission of human intestinal protozoa parasites. II. *Giardia lamblia* Cysts Giben in Capsules. Am. J. Hyg. 59:209-220.

17. Ritchie, L.S. 1948. An ether sedimentation technique for routine stool examination. Bull. U.S. Army Med. Dept. 81326.

18. Rosoff, J. D., C. Sanders, S. Sonnad, P. De lay, W. Hadley, F. Vincenzi, D. Yajko, and P. O'Hanley. 1989. Stool diagnosis of giardiasis using a commercially available enzyme immunoassay to detect *Giardia*-specific antigen 65 (GSA65). J. Clin. Microbiol. 27:1997-2002.

19. Scheffler, E.H. and L.L. Van Etta. 1994. Evaluation of rapid commercial enzyme immunoassay for detection of *Giardia lamblia* in formalin-preserved stool specimens. J. Clin. Microbiol. 32:1807-1808.

20. Stibbs, H. 1989. Monoclonal antibody-based immunoassay for *Giardia lamblia* in human Stool. J. Clin. Microbiol. 27:2582-2588.

21 Stibbs, H. H., Samadpour, M. and Manning, J.F.. 1988. Enzyme immunoasssay for the detection of *Giardia* cyst antigens in formalin-fixed and unfixed human stool. J. Clin. Microbiol. 26:1665-1669.

22 Stibbs, H. H., Samadpour, M. and Ongerth, J.E.. 1990. Identification of *Giardia lamlia*-specific antigens in infected human and gerbil feces by western immunoblotting. J. Clin. Microbiol. 28:2340-2346

23. Wolfe, M. 1992. Giardiasis. Clin. Microbiol. Rev. 5:93-100.

24. Young, K.H., S.L. Bullock, D.M. Melvin, and C.L. Spruill. 1979. Ethyl acetate as a substitute for diethyl ether in the formalin-ether sedimentation technique. J. Clin. Microbiol. 10:852-853.

Chapter 2.

Identification of *Giardia lamblia* Cyst Wall Protein 1 Detected by the Ct7 Monoclonal Antibody Used in the *GIARDIA* TEST.

ABSTRACT

Giardiasis can be diagnosed by the microscopic detection of cysts using immunofluorescence, differential staining methods and by immunological detection of antigens in stool. The purpose of this study was to identify and characterize the cyst antigen that reacts with a monoclonal antibody (Ct7 MAb) which is used for the detection of *Giardia* in feces and water. The antigen that reacts with this MAb (Ct7 Ag) was affinity purified from supernatant fluids of encystment cultures using immobilized MAb. Two proteins of 22 and 26 kDa were eluted from this column. Both proteins reacted intensely with the Ct7 MAb by Western blot analysis. Two minor protein bands of 32 and 39 kDa also were present which did not react with the Ct7 MAb. The N-terminal amino acid sequences of the 22 and 26 kDa bands were identical to cyst wall protein 1 (CWP1) whereas the 32 and 39 kDa bands had sequences identical to cyst wall protein 2 (CWP2). In encysting cultures, the 26 kDa CWP1 was detected at 18 hours, followed by the appearance of the 22 kDa form at 42 hours. Both proteins were stable throughout a 30-day period in encysting cultures. The two forms of CWP1 were resistant to heating at 100°C, proteolysis by trypsin, chymotrypsin or Pronase and stable in human stool specimens from persons with giardiasis. In addition, both forms were resistant to N- and O-glycanases and oxidation by sodium periodate. Purified recombinant CWP1 had a molecular weight of 26 kDa, a perfect match to the native protein which also suggests that this protein is not glycosylated. In culture filtrate, CWP1 appears to form a predominant homodimer and a less abundant heterodimer with CWP2.

INTRODUCTION

Giardiasis is the most commonly diagnosed parasitic infection of humans in the U.S. (5). The protozoan parasite, *Giardia lamblia*, causes this diarrheal disease. In the 1970's, the clinical significance was recognized when a large number of travelers returned from the Soviet Union with giardiasis (30). Infection results from the consumption of cysts in contaminated water or feces, with a minimum infectious dose of approximately 10 cysts (23). Symptoms include diarrhea, abdominal cramps, bloating, flatulence, fatigue, and weight loss caused by malabsorption.

Monoclonal antibodies raised against cyst walls have been used to characterize and evaluate the function of cyst wall antigens (2, 17, 27, 29). Several *Giardia* antigens have been characterized and identified. GSA 65 is a cyst glycoprotein that is present both in trophozoites and cysts and has been isolated from the stools of infected persons (26). GSA 65 is resistant to proteolysis and heat, and is sensitive to oxidation, indicating that the antigenic site is a carbohydrate. GSA 65 reportedly is detected by a commercial MAb-based immunoassay (Alexon; Sunnyvale, CA) as a marker of giardiasis (25, 26).

Cyst wall protein 1 (CWP1) and cyst wall protein 2 (CWP2) are cyst wall proteins that were identified using monoclonal antibodies. The deduced amino acid sequence of both proteins has been determined from sequencing cDNA prepared from total mRNA of encysting trophozoites, and shown to contain leucine rich repeats (19, 15). The molecular weights of CWP1 and CWP2 were estimated to be 26 kDa and 39 kDa, respectively using the gene sequences. These novel proteins form a stable CWP1/CWP2 65 kDa heterodimer as determined

by immunoprecipitation of purified encystment secretatory vesicles. CWP1 and CWP2 are not produced by growing trophozoites (15).

Other *Giardia* antigens identified using monoclonal antibodies include group 1 and group 2 antigens. Group 1 antigens were detected by MAb GCSA-1 and 8C5 early in encystment at 4 hours and range in size from 26 to 46 kDa (29, 2). The antigenic epitopes for these antigens appear to be protein and not carbohydrate since they are resistant to oxidation with periodate and the binding of MAbs was reduced following digestion with Pronase. Group 2 antigens have molecular weights of 66, 85, 120 and 140 kDa and appear later in encystment at 24 hours. The group 1 antigens are distinct from group 2 antigens since they bind wheat germ agglutinin, which is specific for N-acetylglucosamine or sialic acid (17). Both groups of antigens were shown to be different from the previously described GSA 65 as determined by their absence in trophozoites and sensitivities to proteases (2, 22, 29).

In this study, I describe the isolation, by use of immuno-affinity chromatography, of the cyst wall antigen that reacts with Ct7 MAb. This IgM MAb is the basis of several commercial diagnostic assays for giardiasis including immunofluorescence and enzyme immunoassay (10, 21). Until now, the marker antigen(s) detected by this MAb has been unknown.

MATERIALS AND METHODS

Preparation of *Giardia* cysts. *G. lamblia* WB ATCC 30957 trophozoites were axenically cultured in Keister's modified TYI-33 medium at pH 7.1 and incubated at 37°C as previously described (9). After a confluent monolayer was formed (3 days), trophozoites were chilled on ice for 20 minutes, pelleted by centrifugation at 800 x g and suspended in TYI-33 medium containing 10 mg/mL of bovine bile (Sigma Chemical Co., St. Louis, MO.) at pH 7.8 to trigger encystment (12, 9). After 5 days, the cysts were harvested by centrifugation at 800 x g. Cysts were suspended in deionized water and incubated overnight at room temperature to lyse residual trophozoites (12). Cysts were pelleted by centrifugation as described above and washed three times with sterile deionized water. Purified cysts were counted using trypan blue exclusion and stored in phosphate buffered saline (PBS, pH 7.5) containing 0.01 % sodium azide as a preservative.

Purification of the Ct7 monoclonal antibody (MAb). Mouse ascites fluid containing a hybidoma generated using *Giardia* cysts and BALB C mice (CeLLabs Inc., Sydney, Australia) was filtered through a 0.2 micron serum acrodisc (Gelman Sciences, Ann Arbor, MI.) to remove cells. The filtrate was dialyzed against a 2% boric acid solution in water, pH 6.0, for 48 hours (7). The precipitated Ct7 MAb was pelleted by centrifugation at 8000 x g. The pellet was dissolved in PBS, pH 7.5 and dialyzed for 72 hours against PBS to remove residual boric acid. The protein concentration was determined by the Coomassie® Plus Protein assay (Pierce Chemical, Rockford, IL) using bovine IgG as the standard protein (1).

Preparation of polyclonal antisera. Female New Zealand rabbits (5-lb) were injected with purified *Giardia* cysts suspended in sterile saline. Each rabbit received initial

subcutaneous injections (0.5mL/injection) of 10^7 purified *Giardia* cysts diluted 1:2 with Freund's Complete Adjuvant. Booster injections were administered every 7 days using 10^7 cysts diluted 1:2 with Freund's Incomplete Adjuvant. Pre-bleed sera were collected 3 days prior to the first injection. Test bleed sera were obtained at two week intervals beginning at week 3. IgG polyclonal antibodies against *Giardia* cysts were purified from antisera by chromatography on ImmunoPure® Immobilized Protein A (Pierce Chemical). Purified IgG antibody was stored in PBS containing 25% glycerol and 0.02% sodium azide.

Immunofluorescent antibody test (IFA). Immunofluorescence was performed using the *GIARDIA* IF TEST (TechLab, Inc., Blacksburg, VA) as recommended by the manufacturer. The IFA stain consisted of fluorescein isothiocynate-conjugated Ct7 MAb. The negative and positive controls consisted of human feces diluted (1:25) with PBS containing 10% formalin. The positive control was spiked with purified *Giardia* cysts at a final concentration of 10^5 cysts/mL. Cysts were observed by use of a fluorescence microscope. Ten to 15 fields were examined with each specimen.

Enzyme immunoassay (EIA) analysis. The *GIARDIA* TEST (TechLab, Inc.) was performed as recommended by the manufacturer. Briefly, 0.1 mL of sample was placed into wells containing immobilized MAb against a *Giardia* cyst wall antigen. Wells were incubated for 10 minutes at room temperature and washed 4 times with the wash solution. One drop (0.05 mL) of rabbit polyclonal antibody raised against *Giardia* cysts was added to each well and incubated for 20 minutes at room temperature. The wash step was repeated and one drop of anti-rabbit polyclonal antibody conjugated to horse-radish peroxidase was added. Wells were incubated for 5 minutes at room temperature and washed as described above. One drop (0.05mL) of each substrate was added to each well and wells were incubated for 5 minutes at

room temperature. One drop (0.05 mL) of stop solution was added to each well and the plate was read visually and at 450 nm using a EIA reader (EIA 400 AT; Whittaker, Walkersville. MA.). All reagents were used as standardized solutions in the *GIARDIA* TEST.

Purification of the Ct7 MAb Antigen. (i) Preparation of the immunoaffinity column. A total of 4 ml (50% solution) of AFFI-GEL 15 (Bio-Rad Laboratories, Melville, NY.) specific for primary amine groups was incubated for 3 hours at 4°C with 6.25 mg of Ct7 MAb purified as described above. The remaining N-hydroxysuccimide active sites were blocked by the addition of 0.1 mL of 1 M Tris buffer, pH 6.8, to the gel for 1 hour at room temperature. The 2 mL gel bed was washed with 50 ml of sterile PBS to remove unbound Ct7 MAb. Total protein estimations of the starting coating solution and of the excess antibody were used to determine the binding efficiency of the AFFI-GEL 15. The coated gel matrix contained 2.75 mg of Ct7 MAb per ml of gel resulting in a 87.2 % binding efficiency.

(ii) Purification of the Ct7 MAb antigen. Culture filtrate (268 mL) from 2 x 10^8 encysting *Giardia* was passed through the 2 mL bed of the immunoaffinity column. The culture filtrate was separated into 6-45 mL volumes and passed separately though the column at 22°C for a total of 3 passes to optimize absorption by the Ct7 Ag. The column was then washed with 40 mL of sterile PBS and the antigen was eluted using 100 mM glycine buffer, pH 2.5, containing 10% ethylene glycol. A total of 12-1 mL fractions was collected into 1 mL tubes containing 0.1 mL of 10 X PBS, pH 7.5, to neutralize the pH. All fractions were serially diluted and screened for the Ct7 antigen by EIA. The antigen containing fractions (a total volume of 32 mL) were pooled and concentrated to 1.7 mL at 4°C by centrifugation in a Centri-plus concentrator (Amicon, Beverly, MA) with a membrane molecular weight cut-off of 10,000 kDa.

Electrophoretic techniques. The molecular weight was determined by sodium deodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), essentially as described by Laemmli (12). Protein staining of the gels was done using Coomassie® Brilliant Blue R-250 and SYPRO™ Orange Protein Stain (Bio-Rad Laboratories) as instructed by the manufacturer.

Western blot analysis. Western blot analysis was performed by the method of Towbin (28). Briefly, the electrophoretic transfer was done using a 38 mM Tris-HCL buffer transfer buffer containing 2.9% w/v glycine and 40% methanol at a constant voltage of 100 volts for 2 hours. Nitrocellulose membranes were rinsed with 25 mM Tris-buffered saline containing 0.15 M sodium chloride, pH 7.5 (TBS) three times. Membranes were blocked for 30 minutes using 0.5% casein dissolved in TBS, pH 7.5. The primary detecting antibody consisted of one of the following antibodies: purified Ct7 MAb, ascites fluid containing 7D2 MAb (Laboratory of Parasitic Diseases, NIAID, Bethesda, Maryland) or polyclonal antibody raised against *Giardia* cysts as described above. Blocked membranes were incubated with primary antibody diluted in TBS (15 μg/ml Ct7 MAb, 1:500 7D2 ascites fluid, 2.5 ug/ml rabbit antisera) overnight at 22°C. Membranes were then washed 3 times with TBS and incubated for 1 hour with anti-mouse whole IgG-HRP or anti-rabbit antibody whole IgG-HRP conjugate (Sigma Chemical Co.) diluted 1:500 and 1:1000 in TBS, respectively. Membranes were washed with deionized water and soaked in a tetramethylbenzidine-sodium peroxide substrate solution (TMB- Membrane Peroxidase substrate reagent; KPL; Gaithersburg, MD). Once bands were visible, the color development was stopped using several washes of deionized water. Nonreducing SDS-PAGE was done as described above using sample buffer that did not contain 2-β-mercaptoethanol.

N-terminal sequencing. The N-terminal peptide sequence of the purified antigen was determined on an Applied Biosystems Procise Sequencer (Perkins-Elmer Corp., Norwalk, CT) at

the University of Virginia (Charlottesville, VA). Samples were prepared by using 1 μg of immunoaffinity purified Ct7 Ag separated by SDS-PAGE as described above. Proteins bands were transferred to PVDF membrane as described above. The membrane was stained using Coomassie® Brilliant Blue R-250 and destained as described above. Visible bands were excised and stored separately at -20°C. Prior to sequencing, membranes were rinsed with a methanol-chloroform solution to remove bound Coomassie® R-250 stain. A total of 10 amino acids were identified per protein using 10 consecutive Edman degradation cycles. Sequence homologies with known sequences were determined by the FASTA PROGRAM (19).

Assessment of the stability of Ct7 Ag. (i) Determination of heat stability. Immunoaffinity purified Ct7 Ag was diluted in PBS to a final concentration of 210 ng/mL and boiled for 5 minutes. Boiled antigen was serially diluted 1:10 and each dilution was tested by EIA. Results were compared with a standard curve generated from untreated Ct7 antigen. Boiled Ct7 antigen was further analyzed by Western blot using the Ct7 MAb.

(ii) Stability to proteases. Sensitivity to proteolysis was determined by incubating purified Ct7 antigen (30 μL) at 210 ng/mL with a trypsin / chymotrypsin stock solution (30 μL) of 200 μg/mL in 0.1 M Tris-HCL buffer, pH 8.0 at 200 as described by Rossoff (26) and Pronase dissolved at 2 mg/mL in 0.1 M Tris-HCL, pH 7.5 containing 0.01 M EDTA and 0.5% SDS. All incubations were done at 37°C. Following incubations, proteases were inactivated by boiling the mixture for 5 minutes. The mixtures were then analyzed for residual immunoreactivity and changes in molecular weight using the Ct7 MAb by EIA and Western blotting, respectively as described above. Negative Controls consisted of untreated Ct7 Ag diluted in the appropriate reaction buffer. Positive controls included azo-casein and enzymatic

activity was assessed using the method of Charney and Tomarelli (27). Briefly, the trypsin/chymotrypsin (50 μL) or Pronase was combined with azo-casein (80 μL, Sigma Chemical Co.) dissolved in 0.1 M Tris-HCL buffer, pH 8.0 at 25 mg/mL. The solution was incubated as described above and undigested azo-casein was precipitated with 5% trichloroacetic acid solution (600 μL). The precipitated protein was pelleted by centrifugation at 10,000 x g. The supernatant fluid (200 μL) was mixed with a 0.5 M NaOH solution (50μL) and absorbance values were determined using a EIA reader (Whittaker).

(iii) Determination of stability to glycanases. Purified Ct7 antigen (1.3 μg) was denatured using 0.1 % SDS solution in water and boiling at 100°C for 3 minutes. The excess of SDS was bound using a 10% Nonidet P 40 solution in water (10 μL). Two samples of denatured antigen (640 ng) were combined separately with two glycanase solutions. N-linked glycanase treatments consisted of 1.2 U of Peptide-N-Glycosidase F (Oxford Glycosciences Inc., Wakefield, MA) dissolved in 20 mM sodium phosphate buffer, pH 7.5 containing 50 mM EDTA. O-linked glycanase treatments included 6 mU of Endo-α-N-acetylgalactosamidase (Oxford Glycosciences Inc.), dissolved in 100 mM sodium citrate phosphate buffer, pH 6 containing 100 μg/ml bovine serum albumin (BSA). All treatments were done at 37°C for 24 hours. Cleavage of glycans were determined by a decrease in molecular weight as compared to untreated antigen controls using SDS-PAGE and Western blot analysis. Bovine fetuin was used as a positive control for N-glycanase and O-glycanase reactions. Glycans were visualized using the Glyco-Track Test kit, a carbohydrate biotinylation assay (Oxford Glycociences Inc.). Briefly, PVDF membranes were rinsed three times with PBS, pH 7.5, and soaked for 30 minutes with 100 mM sodium acetate buffer, pH 5.5, containing 0.2% EDTA and 10 mM sodium

periodate. Aldehyde groups generated by oxidation were labeled with biotin-hydrazine at room temperature and developed using the streptavidin-alkaline phosphatase conjugate system. Glycoproteins appeared as dark bands against a white background.

(iv) Stability to oxidation. The effect of oxidation was determined by using SDS-PAGE and Western blotting. Immunoaffinity purified Ct7 Ag (630 ng) was separated by SDS-PAGE under reducing and nonreducing conditions as described above. The antigen was transferred to a 0.2 micron nitrocellulose membrane at a constant voltage of 100 volts for 2 h. The membrane was rinsed three times using PBS, pH 7.4. Immobilized antigen was soaked in the dark for 4 h in 100 mM sodium acetate buffer, pH 5.5, containing 0.2% EDTA and 10 mM sodium periodate. The nitrocellulose membrane were rinsed three times with TBS and blocked with TBS containing 0.5% casein for 30 min. and rinsed. The residual immunoreactivity and molecular weight were assessed by Western blot analysis as described above.

Detection of the Ct7 Ag in stool specimens. Formalinized *Giardia*-positive and *Giardia*-negative stool specimens as determined by microscopy, were kindly supplied by Parasite Diagnostics (Lilburn, GA.). Formalinized stools submitted for ova and parasite analysis were concentrated by using the formalin-ethyl acetate method (24, 31). Briefly, 5 ml of formalinized stool was strained through wet gauze and diluted to 10 ml with deionized water. The suspension was centrifuged at 500 x g for 2 minutes and the supernatant was discarded. The pellet was mixed with 9 ml of PBS containing 10% formalin. This suspension was subsequently mixed with 4 ml of ethyl-acetate and centrifuged for 2 minutes at 500 x g. The pellet was suspended in PBS (50 µl) and Dobel's iodine solution (50 µl) of for a wet mount preparation. Samples were considered *Giardia*-positive if cysts or trophozoites were observed. All microscopic *Giardia*-positive stools were screened by EIA. All stools with absorbance

readings of ≥ 0.15 were considered positive. Supernatant fluids from each stool specimen were subsequently tested by SDS-PAGE under reducing conditions followed by Western analysis as describe above. The molecular weight of visible bands were compared to a purified Ct7 Ag control.

Expression of recombinant CWP1 (rCWP1). The CWP1 gene was obtained previously by Mowatt from a cDNA expression library using total RNA extracted from two cell types: cysts purified from feces of infected gerbils and cultured encysting trophozoites (19). The clone including the CWP1 gene was obtained for this study. This clone included the CWP1 gene, starting with nucleotide 46 of the published sequence, that was placed into the BamH1 site of a GST gene fusion vector containing a thrombin protease recognition site and a *tac* promoter (pGEX-4T-1, Pharmacia Biotech, Piscataway, NJ). The host *E. coli* BL21 strain was maintained on Terrific Broth (TB; Sigma) agar plates supplemented with ampicillin. *E. coli* cultures were started in TB liquid media containing 100 μg/mL of ampicillin. Cultures were grown at 37°C with shaking (200 rpm) for 6h. The expression of the GST fusion protein was induced during mid log phase using isopropyl β-D-thiogalactoside (IPTG) at a final concentration of 200 μM.

Purification of rCWP1. *E. coli* cultures containing recombinant protein (as determined by EIA) were chilled to 4°C and lysed by sonication. The *E. coli* lysate was centrifuged for 10 minutes at 10,000 x g and the supernatant was removed. The cell pellet and supernatant were analyzed by EIA for the presence of rCWP1. The cell supernatant containing the GST-CWP1 fusion protein was passed through a Glutathione Sepharose 4B-column (0.5ml; Pharmacia Biotech) twice and the gel matrix was washed with 20 ml of sterile PBS, pH 7.5. The washed Glutathione Sepharose 4B was suspended in PBS, pH 7.5 containing 25 U of bovine thrombin

(Pharmacia Biotech) and incubated overnight at room temperature with gentle shaking. Partially purified rCWP1 was eluted from the column and analyzed by EIA and Western blotting.

RESULTS

Immunoaffinity purification of the Ct7 MAb Ag. The amounts of Ct7 antigen in culture filtrates and cell pellets of encysting *Giardia* were determined by EIA. Culture filtrates contained much higher levels of soluble Ct7 Ag than the cell pellet preparations as seen in Table 1. A summary of the antigen purification is shown in Table 2. Recoveries from various steps of the purification scheme are shown in Table 3. Analysis of the antigen eluted from the immunoaffinity column by SDS-PAGE revealed two proteins of 22 and 26 kDa (Fig. 1). We could not detect any other bands by staining with Coomassie Blue-staining but a more sensitive, fluorescent protein stain, showed the presence of two additional minor bands of 32 and 39 kDa (gel not shown).

Western blot analysis. The Ct7 MAb reacted intensely with the 22 and 26 kDa bands from *Giardia* cysts, encystment culture filtrate and the eluate from the immunoaffinity column (Fig. 2). An additional minor band of 20 kDa was observed in the cyst lysate. The MAb did not react with nonencysting trophozoites. Western blot analysis of nonreducing SDS-PAGE gels using purified Ct7 Ag showed bands of 50 and 65 kDa. The 7D2 MAb, which binds to CWP2, reacted with the minor bands of 32 and 39 kDa under reducing conditions and both MAb's reacted with the 65 kDa complex formed under nonreducing conditions (Fig. 3).

N-terminal sequencing. The N-terminal amino acid sequences of the 22 and 26 kDa proteins were identical with the N-terminus of cyst wall protein 1 (CWP1) whereas the N-terminal sequences of the 32 and 39 kDa proteins were identical to the N-terminus of cyst wall protein 2 (CWP2). The N-terminal sequences of native CWP1 and CWP2 began with L^{15} and A^{16} of the sequenced genes respectively (Fig. 4). This analysis identified and confirmed

signaling peptides of 14 and 15 aa for both CWP1 and CWP2 respectively. The Ct7 MAb reacted with the purified recombinant CWP1. Both native and recombinant CWP1 showed an identical molecular weight of 26 kDa.

Assessment of CWP1 stability. CWP1 was stable and immunoreactive by EIA and Western blot analysis following boiling for 10 minutes and treatments using trypsin, chymotrypsin and Pronase.

In addition, oxidation using sodium periodate also had no effect on the immunoreactivity and molecular weight of CWP1. Further analysis for carbohydrate using N-linked and O-linked specific enzymes showed no change in molecular weight and immunoreactivity as determined by Western blot analysis (membrane not shown).

Assessing multiple forms of CWP1. The EIA, Western blot analysis and immunofluorescence were used to evaluate the expression of CWP1 at timed intervals prior to, during and after the encystment process. The EIA analysis showed an initial positive result at 6 hours with an absorbance reading of 0.175. The absorbance values increased to 2.2 by 50 hours into the encystment process. Western blot analysis using the Ct7 MAb showed the 26 kDa CWP1 band appearing at 18 h and the 22 kDa CWP1 band clearly visible at 42 h (Fig. 5a). Both bands were stable throughout a 30-day period in encystment media (blot not shown).

Immunofluorescence of encysting trophozoites using the fluorescein labeled Ct7 MAb conjugate showed the appearance of the CWP1 along the trophozoite cell membrane after 48 h into the encystment process as seen in Fig. 5b. After 72 h of encystment, the mature cysts showed bright fluorescence of the cyst wall and cyst tail (Fig. 5c). Nonencysting trophozoites showed no fluorescence following IFA staining.

Detection of Ct7 antigen in stool specimens. Nine each of microscopy *Giardia*-positive and *Giardia*-negative stools were evaluated by Western blot analysis using the Ct7 MAb. The CWP1 antigen was seen as a smeared band of about 26 kDa in 6 of the 9 *Giardia*-positive stools tested. Two *Giardia*-positive stools that were analyzed by Western botting are shown in Fig. 6. In addition, 3 stools had both the 26 and 50 kDa bands following concentration of stool supernatant. There were no bands observed in *Giardia*-negative stools (data not shown).

TABLE 1. Assessment of the starting material for the immunoaffinity purification of the Ct7 Ag from culture filtrate of *Giardia lamblia*. The starting culture filtrate was prepared by growing *Giardia lamblia* WB ATCC 30957 in TYI-33 medium by the method of Keister (12) for 5 days at 37°C followed by encystment (7,19). Cysts and residual trophozoites were removed by centrifugation and the supernatant fluid was filtered through a 0.20 μm membrane. Filtrates and pellets were serially diluted and tested by EIA to determine units of activity.

Culture Material Analysis	Total Number of Cells	EIA Specific Activity (U/mg protein)	Total Volume (ml)	Total Protein (mg)	Total Units of Activity (U)
Giardia cyst pellet	2×10^8	1.3×10^3	1.5	92	1.2×10^5
Culture filtrate	0	3.2×10^4	600	1494	4.8×10^7
Total Culture Material	2×10^8	1.6×10^5	602	1588	4.8×10^7

Protein concentration was determined by the method of Bradford (1) using BSA as the standard.

One unit (U) is defined as a single increment of a serial doubling dilution required to give an A450 value between 0.200 and 0.400 as determined by EIA.

TABLE 2. Immunoaffinity purification of Ct Ag from culture filtrate of *Giardia lamblia*. Ct7 MAb was purified from mouse ascites fluid and colvalently coupled to AFFI-GEL 15. Culture filtrate from 5-day *Giardia* encystment cultures in TYI-33 medium was applied to the immobilized Ct7 MAb-AFFI-GEL 15 and unbound material was washed through the column. The captured protein was eluted with 0.1 M glycine buffer, pH 2.5, containing 0.15 M NaCl and 10% ethylene glycol. The information in the table represents a single Ct7 Ag purification.

	Total Volume (ml)	Protein (mg/ml)	Total Protein (mg)	EIA Specific Activity (Units/mg)	Total (Units)	Recovery (%)	Fold Purification
Culture Filtrate	268	2.5	667	3.2×10^4	2.1×10^7		
Eluted Ct7 Antigen	1.7	0.02	0.036	7.6×10^7	2.7×10^6	13	2375

Protein concentration was determined by the method of Bradford (1) using BSA as the standard.

One unit (U) is defined as a single increment of a serial doubling dilution required to give an A450 value between 0.200 and 0.400 as determined by EIA.

TABLE 3. Recoveries for various steps of the Ct7 Ag purification process determined by EIA. Culture filtrate was passed through an immunoaffinity column and bound protein was eluted as described previously. Eluates from 3 separate absorption steps were pooled and concentrated using an centrifuge concentrator. Total protein was determined by the method of Bradford. Amounts of Ct7 Ag were estimated by standard curves generated using the EIA and purified Ct7 Ag.

Immunoabsorbtion #	Total Protein (mg)	Total Antigen (ug)	Total Absorbed (ug)	Total Eluted (ug)	Column Recovery (%)
1	224	166.5	134.56	48.96	36.4
2	222	164.7	124.67	51.89	41.6
3	222	164.7	144.37	54.61	37.8
Total Pooled (ug)	155.46			Average	38.6
Total Post Concentrator (ug)	35.7				
Total Recovery (%)	7.2				

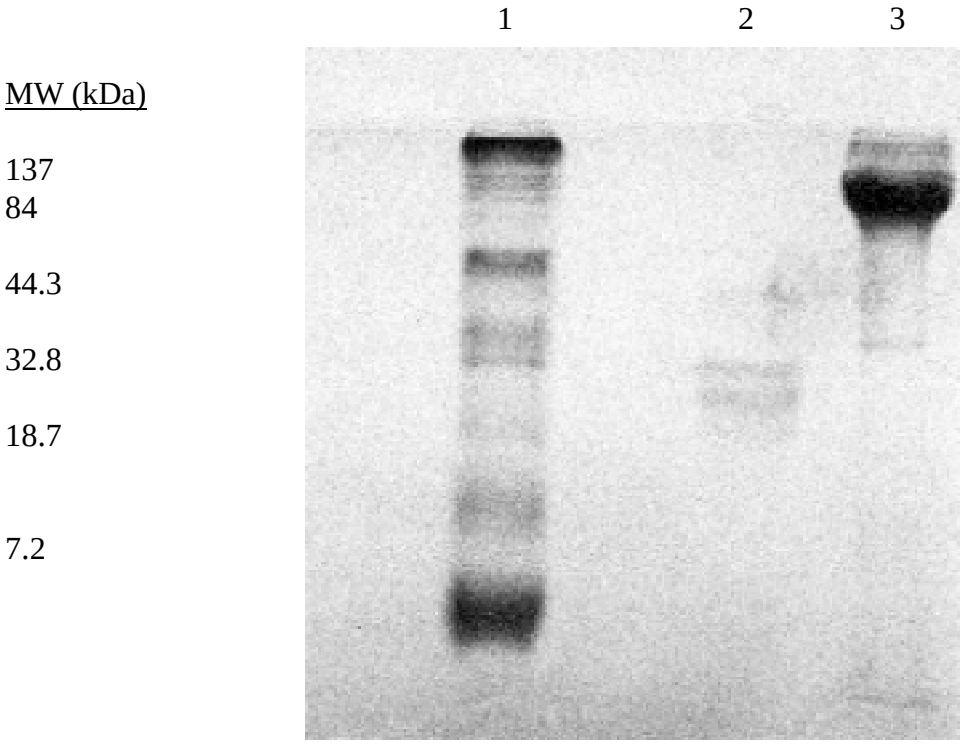

FIG. 1. Reducing SDS-PAGE analysis of the Ct7 Ag purified by immunoaffinity chromatography using immobilized Ct7 MAb. SDS-PAGE was performed in a 4% stacking-15% resolving gel at 30 mA and 4°C. Proteins were stained using Coomassie®. Lane 1: Molecular weight markers. Lane 2: Purified Ct7 Ag (1 ug). Lane 2: Starting Culture filtrate (5 ug).

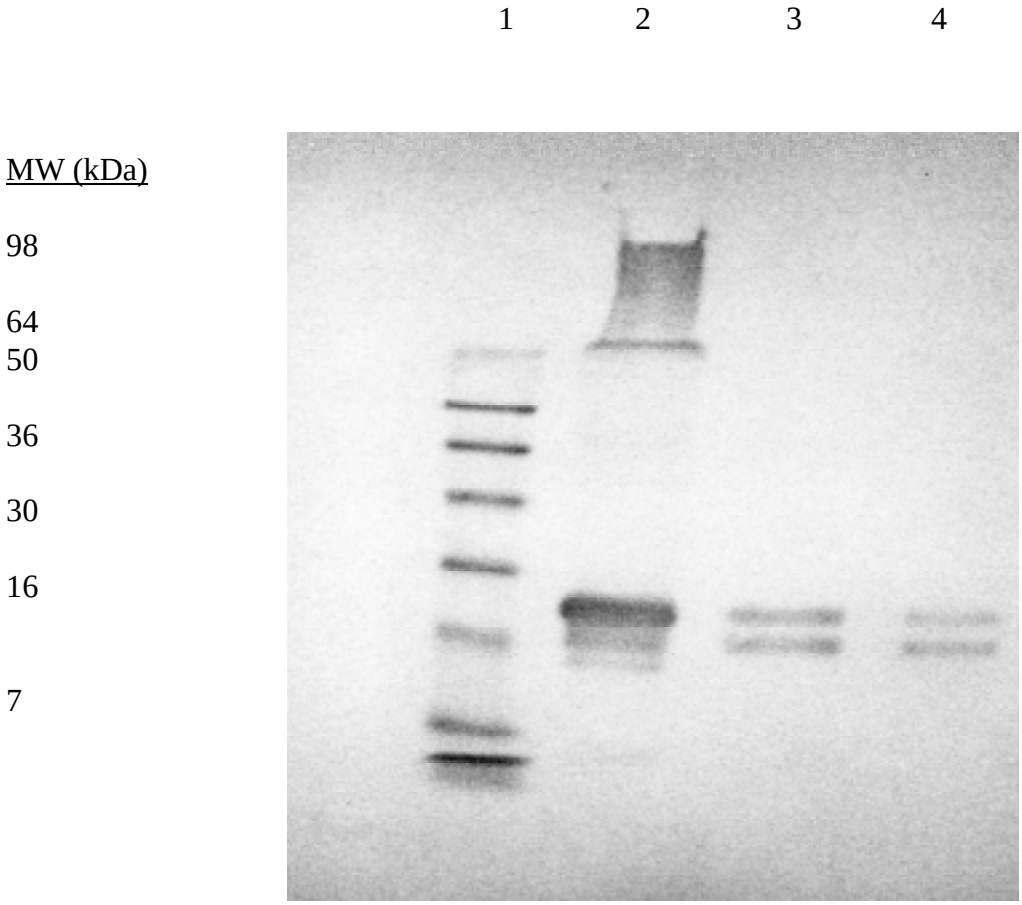

FIG. 2. Western blot analysis of *Giardia* cyst lysate, culture filtrate and immunoaffinity purified Ct7 Ag. Reducing SDS-PAGE and Western blot analysis were done as previously described. Lanes 1: Molecular weight markers. Lane 2: *Giardia* cyst lysate. Lane 3: *Giardia* encystment culture filtrate. Lane 4: Immunoaffinity purified Ct7 Ag.

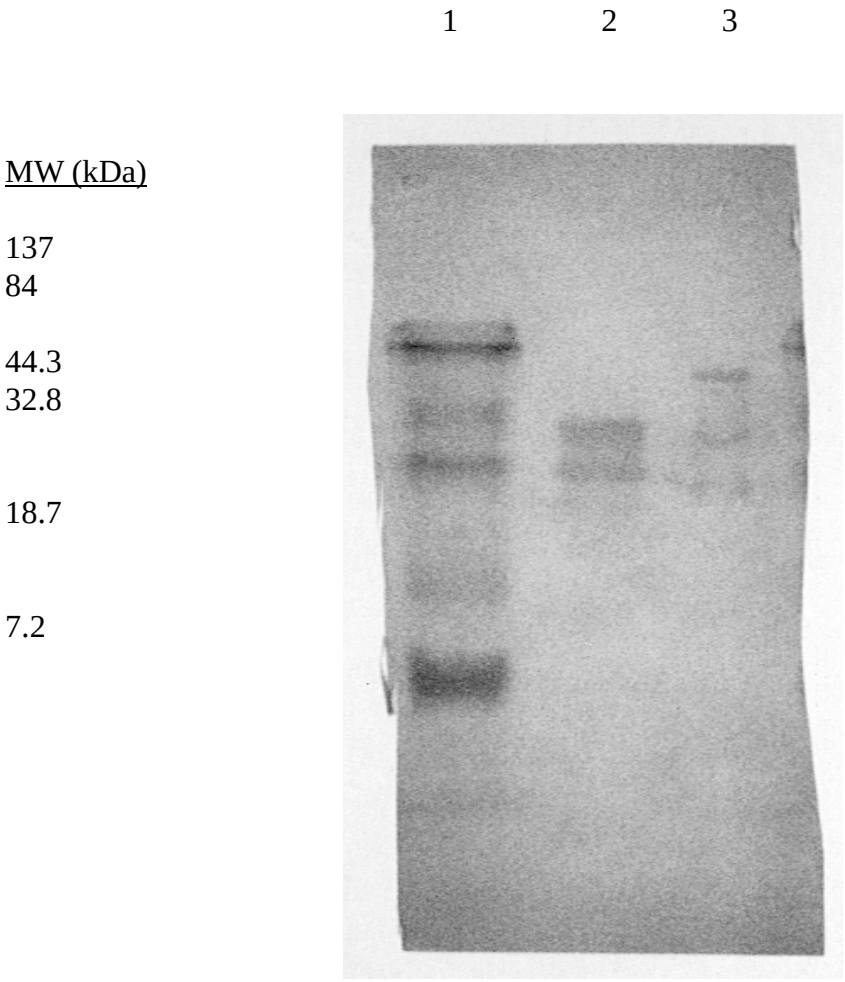

FIG. 3. Western blot analysis of immunoaffinity purified Ct7 Ag using the Ct7 and 7D2 MAbs. Western blots were performed following SDS-PAGE using a 4% stacking-12% resolving gel at 100V for 2 hours at 4°C. Lanes 1: Molecular weights markers. Lane 2: Bands of about 32 and 39 kDa detected by the 7D2 MAb under reduced conditions. Lanes 3: 65 kDa complex detected by the 7D2 MAb (specific for CWP2) under nonreducing conditions.

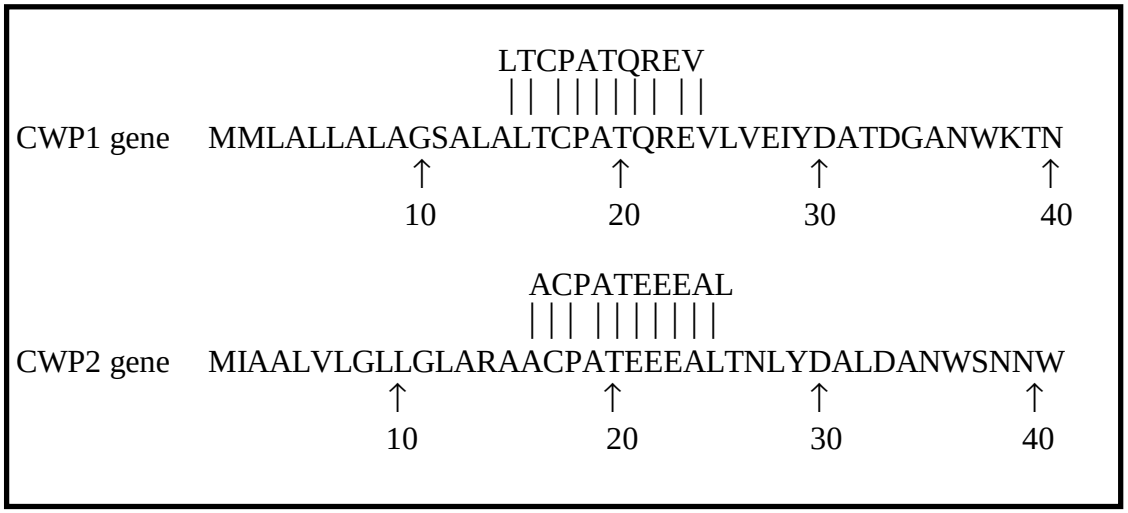

FIG. 4. The 10 N-terminal amino acids of the immunoaffinity purified Ct7 Ag were identified using the Edman degradation cycle as detected by a Applied Biosystems Procise Sequencer. Sequence homologies with known sequences were determined by the FASTA PROGRAM (19). (A) Sequence homologies showed a 100% match for the first 10 N-terminal amino acids of the 22 and 26 kDa bands with CWP1, beginning with L^{15} of the CWP1 gene. (B) The first 10 amino acid sequences for bands 32 and 39 kDa were identical with CWP2, starting with A^{16} of the CWP2 gene. Signaling peptides for native CWP1 and CWP2 of 14aa and 15 aa, respectively, were observed.

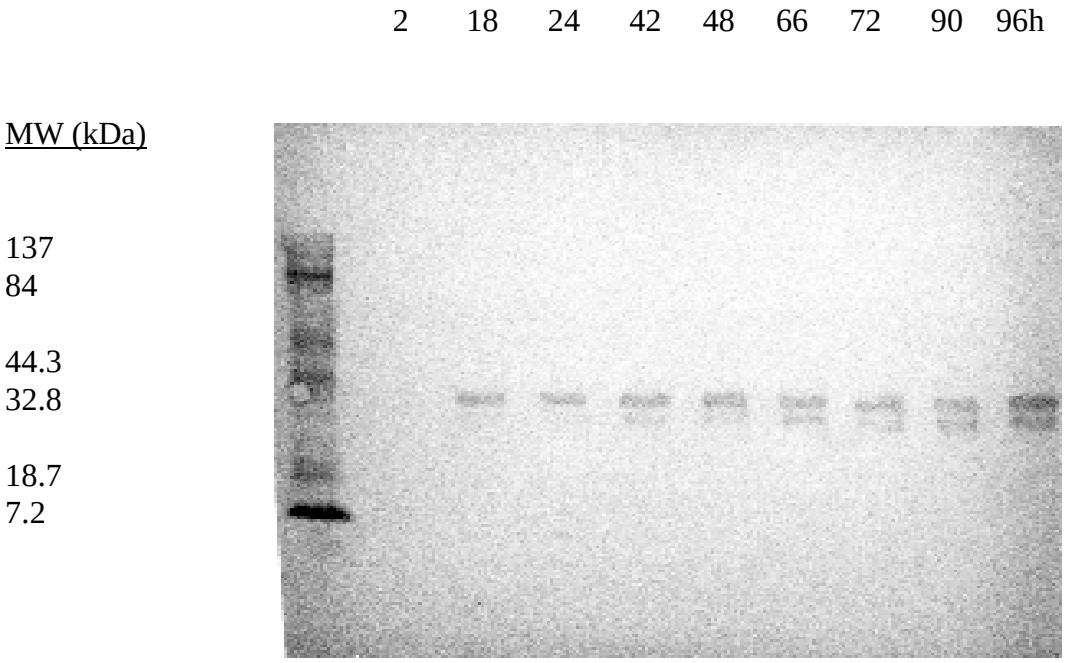

FIG. 5a. Western blot analysis of the Ct7 MAb antigen in culture filtrate during the *Giardia* encystment process. Reducing SDS-PAGE and the Western blot were done as previously described. The 26 kDa band appears at 18 hour of encystment and the 22 kDa band is visible at 42 h. Nonreducing SDS-PAGE followed by Western blot analysis showed only a 50 kDa band appearing at 18 hour. No other bands were observed.

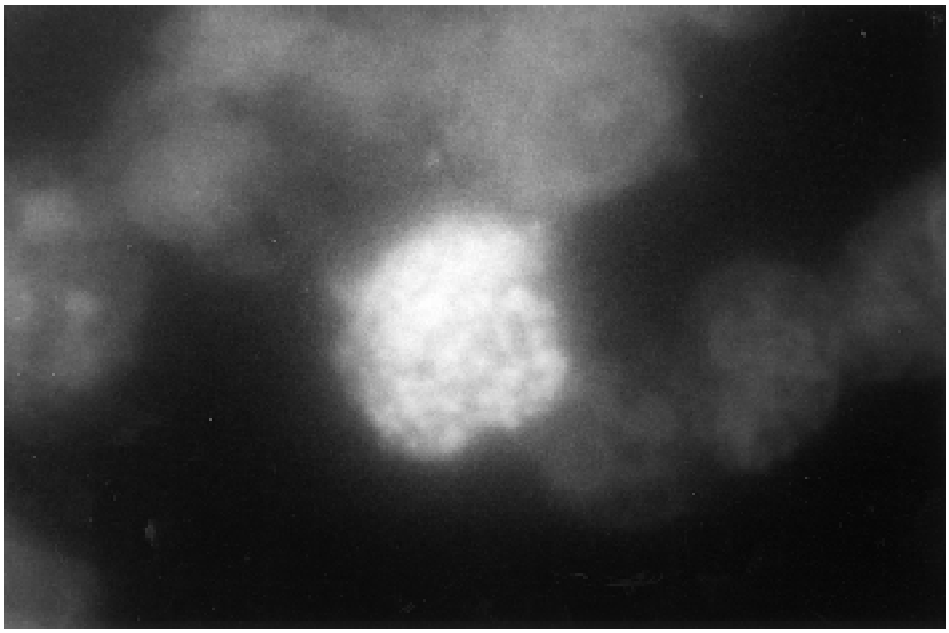

FIG. 5b. Immunofluorescent antibody staining of an encysting trophozoite by IFA using fluorescein labeled Ct7 MAb. Forty-eight hours into the encystment process, the outer portion of the cyst wall showed the appearance of CWP1.

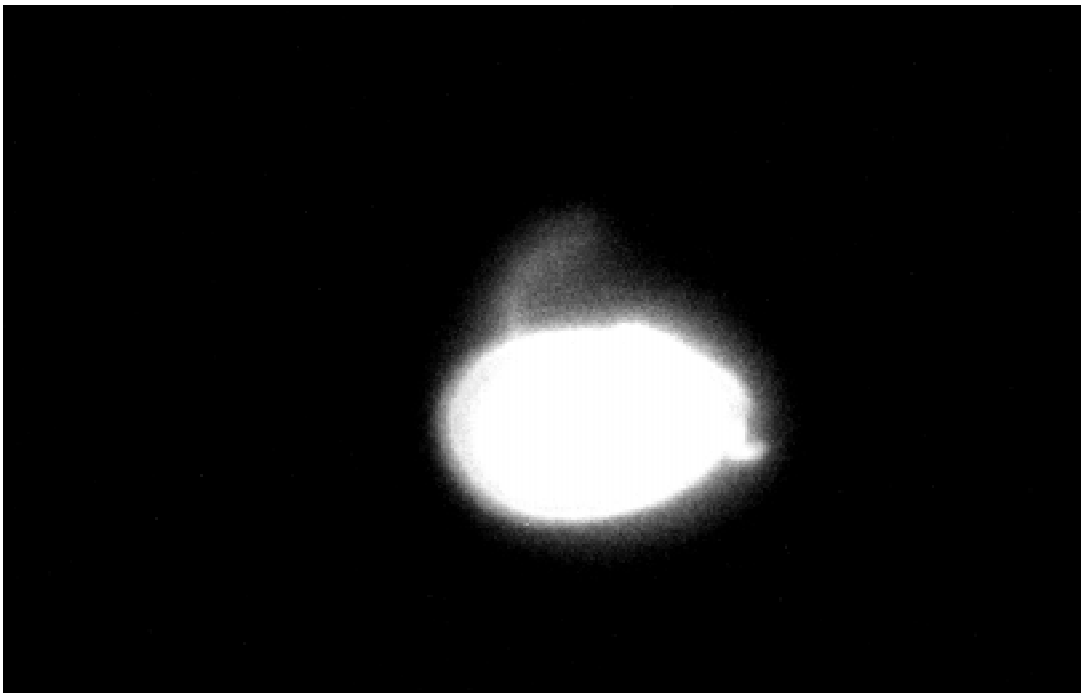

FIG. 5c. Immunofluorescence antibody staining of mature *Giardia* cysts. *Giardia* cysts were stained using a fluorescein labeled Ct7 MAb as described in Fig. 5b following 72 hours of encystment. The fluorescence of the cyst wall is continuous and shows a cyst tail.

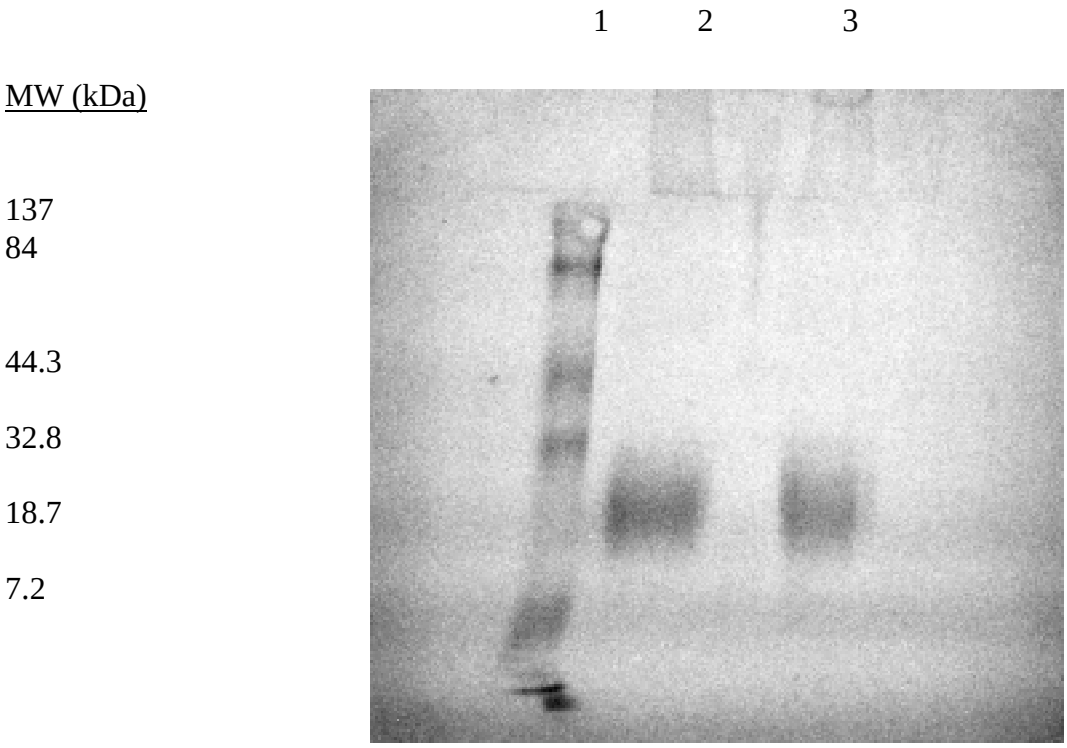

FIG. 6. Western blot analysis of the Ct7 Ag in human feces. SDS-PAGE and Western blot analysis using the Ct7 MAb were done as previously described. *Giardia*-positive specimens were determined by microscopy using iodine staining and the *GIARDIA* TEST. Lane 1. Molecular weight markers. Lanes 2 and 3: A smeared band of about 26 kDa is present in *Giardia*-positive stool specimens.

DISCUSSION

The encystment process of *Giardia lamblia* remains one of the least understood phases of its life cycle, but during the past decade, researchers have begun to answer some of the questions about the *Giardia* cyst. A study using electron microscopic examination of purified cysts revealed a network of different sized filaments beginning at multiple points on the outer membrane of encysting trophozoites (4). These cyst filaments appeared to elongate and proceed to fully encase the trophozoite. Other studies have provided information concerning the composition of the cyst wall. Both gas chromatographic and mass spectrometric analysis of the cyst wall showed the presence of both protein and carbohydrate (16, 11). Galactosamine was the primary amino sugar. Although some progress has been made, actually very little is known about the composition of the cyst wall, the mechanism of construction and the function of known cyst wall proteins.

In this study, I identified the cyst antigen that reacts with the Ct7 MAb as Cyst Wall Protein 1 (CWP1). I also describe the first immunoaffinity purification of cyst wall proteins from culture supernatants of encysting *Giardia*. This approach enabled us to isolate secreted cyst antigens without solublizing the cyst wall with detergents; this simplified the purification process and reduced the likelihood of denaturing the antigens. Although the majority of the cyst wall proteins are in the cyst itself, there are sufficient amounts in the culture filtrate to allow purification.

Assessment of the immunoaffinity purified material revealed small amounts of a co-purifying protein. I identified this protein as Cyst Wall Protein 2 (CWP2). CWP2 did not react with the Ct7 MAb by Western blot analysis. Previous observations by Lujan indicated that

CWP1 and CWP2 form heterodimers prior to their incorporation into the cyst wall (15). Even though the majority of the purified protein was CWP1, the presence of low levels of CWP2 supports this idea. In addition, I observed a CWP1 homodimer of 50 kDa that was present in the affinity-purified preparations and some *Giardia*-positive stools. Since these complexes are dissociated only in SDS-PAGE gels containing β-mercaptoethanol, it is likely that the homo- and heterodimers may be bound by disulfide bonds.

Recent studies have shown the reaction of MAb's with antigens of different molecular weights (23, 27, 29). It was not clear in these studies whether these represent multiple forms of the same antigen or if they are complexed with other components of the cyst wall. In this study, two forms of CWP1 and CWP2 were observed in the immunopurified material. These different molecular weight sizes of the cyst wall proteins appeared in both the culture filtrate and purified cysts. The sequenced gene of CWP1 predicts a protein of 26 kDa and this was the only size produced in transformed *E. coli* containing this gene. This suggests to us that the 22 and 26 kDa forms of CWP1 seen in culture supernatants may be the result of proteolytic cleaving by a *Giardia* protease of the C-terminal end of the protein. This same mechanism was proposed by Lujan for CWP2 (15). In addition, I observed a third CWP1 form of 20 kDa present only in purified cysts. This smaller form may represent further proteolytic processing of CWP1 following the insertion into the cyst wall. Another possibility for the 22 kDa CWP1 form is a second gene product of a smaller molecular weight. Regardless of how the multiple forms are produced, CWP1 and CWP2 appear to represent building blocks for the protein portion of previously described cyst wall filaments.

I compared the N-terminal sequences of native CWP1 and CWP2 and identified signaling peptides. These peptides were the sizes previously predicted by Mowatt and Lujan

from the CWP1 and CWP2 gene sequences (15). Currently, there is a controversy in the literature on whether or not *Giardia* have a typical endoplasmic reticulum and golgi (14). Presence of the signaling peptides for these secreted cyst wall proteins provides additional evidence for these organelles.

I found that native CWP1 migrated in SDS-PAGE gels identically to the recombinant form and the molecular weight of native CWP1 was not changed by reaction with N- and O-glycanases. Thus, there are no attached glycans. This was also indicated by the resistance of CWP1 to oxidation by sodium periodate. In addition, CWP1 was not degraded by treatment with trypsin, chymotrypsin or Pronase. We also observed the 26 kDa form in human feces which is evidence that CWP1 is resistant to the digestive and bacterial enzymes of the gastrointestinal tract. The stability of CWP1 in feces is one reason why CWP1 is a useful diagnostic marker for giardiasis.

In conclusion, I identified the cyst antigen that reacts with the Ct7 MAb as CWP1 and describe a method for the immunoaffinity purification of this soluble protein from culture supernatants of encysting *Giardia*. This highly stable protein is released into the culture supernatant initially as a 26 kDa protein followed by the conversion into or expression of a second 22 kDa form. CWP1 appears to form a predominant homodimer and a less abundant heterodimer with CWP2. In addition, I suggest that CWP1 is not a glycoprotein based on the matching molecular weight of native and recombinant CWP1 and the resistance of the native form to glycanases.

REFERENCES

1. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem.,72:248-254.

2. Campbell, J. D., G. M. Faubert. 1994. Recognition of *Giardia lamblia* Cyst-Specific antigens by monoclonal antibodies. Parasite Immunol. 16:211-219.

3. Charney, J. and R. M. Tomarelli. 1947. J. Biol. Chem. 171:501-505.

4. Erlandsen, S. L., P. T. Macechko, H. Van Keulen, E. L. Jarroll. 1996. Formation of the *Giardia* cyst wall: Studies on extracellular assembly using immunogold labeling and high resolution field emission SEM. J. Euk. Microbiol. 43:416-429.

5. Feely, D. E., S. Erlandsen, D. Chase. 1984. Structure of the trophozoite and cyst. In S. Erlandsen and Ernest Meyer (ed), *Giardia* and Giardiasis. Plenum Press, New York and London.

6. Garcia, L. S., D. Bruckner. 1993 Intestinal Protozoa: Flagellates and Ciliates. In L. Garcia and Bruckner(ed.), Diagnostic Medical Parasitology. American Society for Microbiology, Washington, D.C.

7. Garvey J. S., Cremer, N. E. and D. H. Sussdof. 1977. In W. A. Benjamin (ed), Methods in immunology; 3 rd edn. Reading MA.

8. Gillin, F. D., D. S. Reiner. 1996. Cell biology of the Primitive eukaryote *Giardia lamblia*. Annu. Rev. Microbiol. 50:679-705.

9. Gillin, F. D., D. S. Reiner, M. J. Gault, H. Douglas, Das S A. Wunderlich, J. F. Sauch. 1987. Encystation and expression of cyst antigens by *Giardia lamblia* in vitro. Science. 235:1040-1043.

10. Hopkins, R. M., P. Deplazes, B. Meloni, J. Reynoldson, R. Thompson. 1993. A Field and Laboratory evaluation of a commercial ELISA for the detection of *Giardia* coproantigens in humans and dogs. Trans. Med. Hyg. 87:39-41.

11. Jarroll, E.L., P. Manning, D. G. Lindmark, J.R. Coggins, S. L. Erlandsen. 1989. *Giardia* cysts wall-specific carbohydrate: evidence for the presence of galactosamine. Mol. Biochem. Parasit. 32:121-132.

12. Keister, D. B. 1983. Axenic culture of *Giardia lamblia* in TYI-33 medium supplemented with bile. Trans. R. Soc. Trop. Med. Hyg. 77:487-488.

13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. (London). 227:680-685.

14. Lujan, H. D., A. Marotta, M. R. Mowatt, N. Sciaky, J. Lippincott-Schartz, T.E. Nash. 1995. Developmental induction of golgi structure and function in the primitive eukaryote *Giardia lamblia*. J. Biol. Chem. 270:4612-4618.

15. Lujan, H.D., M. R. Mowatt, J.T. Conrad, B. Bowers, T.E. Nash. 1995. Identification of a novel *Giardia lamblia* cyst wall protein with Leucine-rich repeats. J. Biol. Chem. 270:29307-29313.

16. Manning, P., S. L. Erlandsen, E. L. Jarroll. 1992. Carbohydrate and amino acid analysis of *Giardia muris* cysts. J. Protozoology. 39:290-296.

17. Meng, T., M. Hetsko, F. Gillin. 1996. Inhibition of *Giardia lamblia* excystation by antibodies against cyst walls and by wheat germ agglutin. Infect. Immun. 64:2151-2157.

18. Moss, D.M., H. Mathews, G. Visvesvara, J. Dickerson, E. Walker. 1991. Purification and characterization of *Giardia lamblia* antigens in the feces of Mongolian gerbils. J. Clin. Microbiol. 29:21-26.

19. Mowatt, M.R., H. D. Lujan, D. B. Cotten, B. Bower, J. Yee, T.E. Nash, H. H. Stibbs. 1995. Developmentally regulated expression of a *Giardia lamblia* cyst wall protein gene. Molecular Microbiology. 15:9555-963.

20. Pearson, W.R., D. J. Lipman. 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA. 85:2444-2448.

21. Perez, M.J., R. Aretio, E. Martin. 1994. *Giardia* tests: An evaluation of a commercial immunofluorescence assay. Abstract. In R. Thompson, J. Reynoldson, and A. Lymbery (eds), *Giardia* From Molecules to Disease. CAB International, Wallingford,,UK.

22. Reiner, D.S., H. Douglas, F. Gillin. 1989. Identification and localization of cyst-specific antigens of *Giardia lamblia*. Infection and Immunity. 57:963-968.

23. Rendtroff, R.C., 1954. The Experimental Transmission of human intestinal protozoan parasites. II. *Giardia lamblia* Cysts given in Capsules. Am. J. Hyg. 59:209-220.

24. Ritchie, L.S., 1948. An ether sedimentation technique for routine stool examination. Bull. U.S. Army Med. Dept. 81326.

25. Rosoff, J., C. Sanders, S. Sonnad, P. De lay, W. Hadley, F. Vincenzi, D. Yajko, P. O'Hanley. 1989. Stool diagnosis of giardiasis using a commercially Available Enzyme Immunoassay to detect *Giardia*-Specific antigen 65 (GSA65). J. Clin. Microbiol. 27:1997-2002.

26. Rosoff, J. H. H. Stibbs. 1986. Physical and chemical characterizations of a *Giardia lamblia*-specific antigen useful in the coprodiagnosis of giardiasis. J. Clin. Microbiol. 24:1079-1083.

27. Stibbs, H.H. 1989. Monoclonal antibody-Based enzyme immunoassay for *Giardia lamblia* antigen in human stool. J. Clin. Microbiol. 27:2582-2588.

28. Towbin, H., T. Staehlin, J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76:607-610.

29. Ward, H.D., A. V. Kane, E. Ortega-Barria, G. T. Keusch, M. E. Pereira. 1990. Identification of developmentally-regulated *Giardia lamblia* cyst antigens using GCSA-1,a cyst-specific monoclonal antibody. Mol. Microbiol. 4:2095- 2102.

30. Wolfe, M. 1992. Giardiasis. Clin. Microbiol. Rev. 5:93-100.

31. Young, K.H., S. L. Bullock, D. M. Melvin, C.L. Spruill. 1979. Ethyl acetate as a substitute for diethyl ether in the formalin-ether sedimentation technique. J. Clin. Microbiol. 10:852-853.

JAMES H. BOONE

2131 Childress Rd
Christiansburg, Virginia 24073
Home: (703) 382-8336 Work: (703) 231-3943 E-mail - jaboone2@vt.edu

OBJECTIVE: Research and Development.

EDUCATION: May, 1990: B.S. Biochemistry; Minor: Chemistry;
May, 1998: M.S., Virginia Polytechnic Institute & State University, Department of Biology.

RELATED EXPERIENCE: Familiar with study design and preparation of final reports.

Extensive experience with many aspects of commercial immunoassays (enzyme immunoassays, immunofluorescent antibody tests and latex agglutination assays) - research and development, investigational plan design and management, site inspections, trouble shooting, product production, sales and technical assistance.

Familiar with culturing of bacteria - clinical specimens, bioburden testing, large scale production and protein purification.

Familiar with writing and presenting scientific data (final reports for contract research, FDA submissions for new products, seminars, poster presentations and training workshops for national scientific meetings).

Experience with common laboratory safety procedures - development of a safety program and implementation of procedures, familiar with OSHA regulations.

EXPERIENCE: 10/91 - Present; Research Scientist, TechLab Inc., VPI Corporate Research Center, Blacksburg, Virginia.

9/89 - 10/91; Research Technician, VPI Veterinary Microbiology Research Center, Blacksburg, Virginia.

SCIENTIFIC PUBLICATIONS: Available upon request