Enzyme Immunoassay for Detection of *Giardia lamblia* Cyst Antigens in Formalin-Fixed and Unfixed Human Stool

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An antigen-capture enzyme-linked immunosorbent assay employing rabbit and mouse antisera to *Giardia lamblia* cyst antigens was developed for the diagnosis of *Giardia* infection through detection of *G. lamblia*-specific stool antigens in cell-free aqueous eluates of human stool. This is the first report of the use of anti-cyst antibodies in an enzyme immunoassay for *G. lamblia*. The assay gave a positive result with 54 of 59 stools from patients with symptomatic, clinically diagnosed giardiasis, giving the test a sensitivity of 91.5%. A negative reading was obtained with all of 25 stools from *G. lamblia*-negative control patients. The assay could detect as few as 20 sonicated cysts added to control stool eluate. The assay was more sensitive to cyst-derived antigens than to trophozoite-derived antigens. With two exceptions, the assay gave a negative result with stools from patients infected with *Entamoeba histolytica* (seven), *Cryptosporidium* sp. (four), or *Blastocystis hominis* (seven) and thus appears to be specific for *G. lamblia* antigens. Storage of stool eluates for more than 6 months at 4°C as unpreserved aqueous eluates or as formalinized eluates did not affect the ability of the assay to detect the giardial antigens. The enzyme-linked immunosorbent assay proved useful for monitoring the levels of *G. lamblia*-specific stool antigens in the stool of patients undergoing antigiardial chemotherapy.

**Giardia lamblia**, a protozoan parasite, is a worldwide agent of enteric illness and is the most commonly identified human parasite in the United States (3, 8, 14, 23). It is also the most frequently documented agent of water-borne epidemic diarrhea here (6). Chronic infection with *G. lamblia* is common; chronic cases probably outnumber acute cases (4). Despite the importance of this parasite in causing disease, clinical methods most widely used today for diagnosing human infection with *G. lamblia* are still relatively primitive; all involve a microscopic search for whole parasites in stool or in material retrieved from the small intestine by duodenal aspiration, duodenal biopsy, or the string test (2, 10–12, 19, 22). A single microscopic examination of stools from giardiasis patients will generally miss detecting the infection about 20 to 50% of the time even if concentration methods are employed (2, 22), chiefly because of the extreme variability with which the parasite is excreted in both symptomatic and asymptomatic infections (7, 15). A method that could rapidly detect soluble antigens or other products of *G. lamblia* in a single stool would obviously be preferred over the microscopic methods. Antigen-capture enzyme-linked immunosorbent assay (ELISA) methods for the detection of *G. lamblia*-specific antigens in stool have already been reported (9, 13, 20); these assays have all employed antibodies prepared against the trophozoite form of *G. lamblia*. Counterimmunoelectrophoresis for the detection of giardial antigens in stool has also been described by us and others (5, 16, 21). We report here the development of an antigen-capture ELISA for *G. lamblia* stool antigens; the ELISA employs antibodies prepared against the cyst stage of this parasite.

**MATERIALS AND METHODS**

**Antigen preparation.** Cysts of the H-2 and H-3 isolates of *G. lamblia* were used in immunizing rabbits. These isolates were acquired in infected gerbils from Charles Hibler of Colorado State University and were adapted to axenic growth in TYI-S-33 medium. Cysts were obtained from gerbils that had been infected 5 to 25 days earlier by intragastric inoculation with approximately 3 x 10⁵ trophozoites from a log-phase culture. The degree and duration of cyst excretion were enhanced by including, beginning at the time of infection, dexamethazone (Roxane Laboratories, Inc., Columbus, Ohio; solution of 1 mg/ml) in the gerbils’ drinking water at a final concentration of 1 mg/100 ml of water. Feeces were collected from gerbils by placing the animals over a sheet of hardware cloth of 1/2-in. (ca. 1.25-cm) mesh overlying a small layer of water in an otherwise empty cage with food and water provided ad libitum. Cysts were purified from the collected feeces by centrifugation at 500 x g of feeces (previously crushed, homogenized in distilled water, and gauze filtered) over a 15-ml layer of 1 M sucrose in water in a 50-ml disposable centrifuge tube, followed by centrifugation at 500 x g of the cysts (collected from the top of the sucrose layer and washed twice in distilled water) over two layers of Percoll (Sigma Chemical Co., St. Louis, Mo.) of specific gravities 1.05 and 1.09, prepared in distilled water. Cysts were harvested by aspiration from the interface between the Percoll layers with specific gravities of 1.09 and 1.05; rinsed three times with distilled water, and stored for up to 1 week at 4°C in distilled water containing penicillin and streptomycin at 100 U/ml and 100 μg/ml, respectively. Some of the cysts were frozen at −80°C for up to 3 months as cell pellets at the bottom of polystyrene centrifuge tubes.

**Production of antisera.** Rabbit anti-*G. lamblia* cyst serum was obtained by immunizing two female 6- to 7-lb (ca. 2.7- to 3.2-kg) New Zealand White rabbits with cysts of a mixture of the H-2 and H-3 isolates. A total of approximately 55 x 10⁵ cysts, representing approximately 2.2 mg of protein

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as measured by the Bradford method (1), was given to each rabbit in the entire course of immunization. Each rabbit received an initial intradermal injection of $25 \times 10^6$ cysts suspended in distilled water and mixed 1:1 in Freund complete adjuvant at six sites on the back. Beginning 3 weeks later, each rabbit received a series of three intramuscular inoculations (each boost separated by a period of 2 to 3 weeks) of cysts ($10 \times 10^6$ cysts per boost) suspended in incomplete Freund adjuvant. Rabbits were bled out by cardiac puncture under anesthesia 2.5 months after the first injection and 10 days after the last boost. Serum was collected after the blood was allowed to clot at 4°C for 48 h. The titer of this immune serum was determined after serial 10-fold dilution by indirect immunofluorescence on 1- by 3-in. (ca. 2.5- by 7.5-cm) microscope slides with air-dried and acetone-fixed spots of fresh human feces containing abundant G. lamblia cysts and was found to be approximately 1:500 (the highest dilution giving readily observable fluorescence). The immunoglobulin G (IgG) fraction of the immune serum and of preimmune serum collected from the rabbits was isolated by the caprylyl acid method (18). IgG was dialyzed with dialysis tubing (12,000- to 14,000-molecular-weight cutoff) into 0.0175 M phosphate-buffered saline (pH 7.4) (PBS) and stored at a concentration of 0.6 mg of protein per ml at −20°C (protein measured by the Bradford method [1]).

Mouse anti-G. lamblia cyst serum was prepared by the repeated intraperitoneal inoculation of BALB/c female mice with purified live cysts; $1 \times 10^6$ to $2 \times 10^6$ cysts suspended in distilled water were given to each mouse biweekly for 8 to 10 weeks. After the intraperitoneal injections, an intravenous injection of $3 \times 10^6$ to $2 \times 10^6$ cysts suspended in sterile normal saline was given to each mouse via the tail vein. At 4 to 5 days after the final boost, mice were bled from the heart under chloroform anesthesia. Whole mouse serum, consisting of the pooled serum from eight mice, was used. The immunofluorescence titer of this pooled serum for G. lamblia cysts was determined to be 3,200.

**Patient specimens.** Stool specimens from 59 patients with confirmed, symptomatic giardiasis were provided by local clinical laboratories (see Acknowledgments). In every case a positive microscopic diagnosis of G. lamblia, either by direct wet mount or after ethyl acetate-Formalin concentration, had been made by professional personnel at these laboratories before the specimens were donated to us. These specimens were not reported to contain any other parasites. A number of specimens from patients infected with Entamoeba histolytica (seven), Cryptosporidium sp. (four), and Blastocystis hominis (seven) were also provided to us. A G. lamblia-negative control stool specimen was obtained from each of 25 healthy, paid volunteers who claimed to have had no recent history of symptoms resembling those typical of giardiasis. Each specimen was checked microscopically for cysts of G. lamblia or other parasites after ethyl acetate-formalin concentration (24); all were confirmed as G. lamblia negative.

**Stool eluate preparation.** Aqueous eluates were made of a portion of each stool specimen by adding approximately two parts of distilled water to one part stool in a centrifuge tube, stirring well with a thin wooden applicator stick or split tongue depressor, and then centrifuging at 900 g for 10 min, after which the supernatant fluid was aspirated and saved and the pellet was discarded. The main portion of each supernatant was stored in a coded, 1- dram (ca. 3.7-ml) vial at −20°C; in addition, a smaller sample of 12 of the G. lamblia-positive and 12 of the G. lamblia-negative control stool eluates was stored at 4°C without preservatives to determine the relative stability of the giardial antigens at these two temperatures. Moreover, 30 of the G. lamblia-positive and 30 of the negative control stools were also eluted (as above) in 10% Formalin in PBS to determine stability of the giardial antigens in the presence of formaldehyde (final concentration, 2.46%) and were stored at 4°C. The aqueous eluates stored at 4°C and the formalinized eluates had been stored for a period of 6 to 24 months before testing. The aqueous eluates stored at −20°C had also been stored for this range of time.

**Antigen-capture ELISA protocol.** Alternating columns of 96-well, flat-bottom, polystyrene plates (no. 3915; Falcon Plastics, Becton Dickinson and Co., Lincoln Park, N.J.) were coated with 100 µl of the IgG fraction of either rabbit anti-cyst or preimmune serum diluted to 2 µg of protein per ml in 0.05 M carbonate buffer (pH 9.6) (protein measured by the Bradford method [1]). Coating was carried out by incubation at 4°C overnight. Afterward, plates were rinsed twice with distilled water, and then nonspecific binding sites were blocked by adding 100 µl of a solution of 2% bovine serum albumin in PBS to each well and incubating the plates at 37°C for 30 min. The plates were then rinsed twice with distilled water, shaken dry, wrapped in plastic food wrap, and stored for 1 to 3 days at 4°C. Stool eluates (100-µl volumes), diluted 1:60 in PBS-0.05% Tween 20 (PBS-T) relative to the volume of the specimen sample from which the eluate was made, were then added to two wells coated with immune antibody and to two wells coated with preimmune IgG antibody. The eluates were incubated in the plates for 2 h at 37°C and then rinsed four times with PBS-T. Then 100 µl of mouse anti-G. lamblia cyst antisemur, diluted 1:1,000 in PBS-T, was added; the plates were incubated again for 2 h at 37°C and then rinsed as before. At this point, 100 µl of horseradish peroxidase-labeled goat anti-mouse immunoglobulins (no. 3211-0231, heavy and light chain specific; Cooper Biomedical, Inc., Malvern, Pa.) diluted 1:1,000 in PBS-T containing 5% normal goat serum was added, and the plates were incubated for 1 h at 37°C. After rinsing as before, 100 µl of enzyme substrate, consisting of 40 µg of o-phenylenediamine (Sigma) per 100 ml of PBS containing 40 µl of 30% hydrogen peroxide (Sigma) per 100 ml, was added, and the plates were incubated in ambient temperature in the dark for 30 min. The chromogenic reaction was terminated by adding 50 µl of 2.5 N sulfuric acid to each well. Absorbance was measured at 492 nm with a Titertek Multiskan automatic ELISA plate reader (Flow Laboratories, Inc., McLean Va.). The adjusted absorbance value for each specimen calculated by subtracting the mean absorbance in the two wells coated with preimmune IgG from the mean of the wells with immune IgG.

The utility of the assay for monitoring the levels of Giardia stool antigens in the stool of patients undergoing metronidazole therapy was also evaluated in four patients.

Finally, the sensitivity of the assay in terms of the minimum number of sonically disrupted cysts detectable was measured by adding known, decreasing numbers of purified, sonicated cysts to four different negative control stool eluates and then diluting in PBS-T to a final dilution of 1:60 as before. This experiment was performed to determine the sensitivity of the assay for giardial antigens but was not intended to imply that the presence of whole cysts in stool would be necessary for the test to detect the presence of infection. The relative sensitivity of the assay for trophozoite- versus cyst-derived antigens was also measured by determining whether the assay could detect the antigens of
FIG. 1. Adjusted absorbance values obtained by antigen-capture ELISA on aqueous stool eluates from 59 symptomatic *G. lamblia*-positive patients and 25 *G. lamblia*-negative control patients. Each specimen was assayed in duplicate. The cutoff point for positive readings was an optical density of 0.0803 (see text). The mean optical density of the *G. lamblia*-positive specimens was 0.2731, and that of the negative specimens was 0.0061.

10^1 to 10^5 sonicated trophozoites or cysts of the H-2 isolate per well (each diluted in PBS-T).

The statistical significance between the means of the groups of specimens was calculated by using a two-tailed Student t test. Differences giving a P of <0.05 were regarded as significant.

**RESULTS**

The adjusted absorbance values of 59 *G. lamblia*-positive and of 25 *G. lamblia*-negative control specimens are shown in Fig. 1. The optimal coating IgG concentration proved to be 2 μg/ml; above and below this concentration the adjusted absorbance values diminished. The mean adjusted absorbance of the *G. lamblia*-positive specimens was 0.2731 (standard deviation, 0.1940); that of the controls was 0.0061 (standard deviation, 0.0121). In the manner of Ungar et al. (20), specimens were considered positive when their adjusted absorbance values exceeded the value of the negative control mean plus two standard deviations plus 0.05; the total value was 0.0803. Using this criterion, 54 of the 59 *G. lamblia*-positive specimens were ELISA positive, and all 25 controls were negative. The sensitivity of the test was therefore 91.5% for specimens already known to contain *G. lamblia*. Of the five false-negatives obtained, three were specimens for which the clinical report stated that cysts were rare; they were the only specimens of the whole group for which this had been stated. One other false-negative value came from a specimen containing a large number of trophozoites and no cysts. However, another specimen like this gave a positive reading of 0.33. The last false-negative specimen contained what was called "many" cysts.

In general, there was not a clear correlation between the ELISA absorbance value and the level of *G. lamblia* cysts or trophozoites as noted by the clinics. The reason for this apparent absence of correlation may lie in the fact that the observations of parasite numbers in the stools examined by the clinical technicians were recorded in subjective or at best semiquantitative language, e.g., "2+", "4+", "many", "moderate," etc. (language which suffices for clinical diagnostic purposes); therefore it was virtually impossible for us to convert these observations to reliable cyst or trophozoite concentrations.

Of seven specimens known to contain only *E. histolytica*, one was positive (absorbance of 0.116) by the ELISA. One of four specimens from patients with *Cryptosporidium* infections was also positive (absorbance of 0.212), whereas none of seven specimens from patients with *B. hominis* infections and none of three specimens from patients with *Endolimax nana* infections (one had *E. nana* and *E. hartmanni*) was positive. All of these patients had been symptomatic with gastrointestinal complaints. It is probably reasonable to assume, given the insensitivity of the single microscopic examination for parasites that had been performed on these specimens, that the two ELISA-positive results obtained with these stools represented patients who had undiagnosed, perhaps cryptic, *G. lamblia* infections. The assay appears to be specific for antigens of *G. lamblia*, although at this point specimens containing other protozoa, fungi, bacteria, viruses, and helminths, some of which might liberate antigens that cross-react with those of *G. lamblia*, have not been tested. The assay gave a negative result with a sonicated preparation of axenically cultured *Penatrichomonas hominis* (Diamond strain).

The ELISA was useful for monitoring the level of *G. lamblia* antigens in stool during the course of chemotherapy with metronidazole (250 mg three times daily for 1 week in each case) in four patients with clinical and diagnosed giardiasis. Results obtained with one of the patients are shown in Fig. 2. This patient, an adult male, was administered a 7-day course of metronidazole, beginning at day 0. Cyst excretion had ceased by day 4 but antigen was still detectable, and the patient remained mildly symptomatic. The patient had a relapse of acute symptoms and of cyst excretion by day 16 and was put back on metronidazole for another week. The ELISA detected the reappearance of
**Giardia** stool antigen at the time of the relapse (day 16), and antigen levels became undetectable by days 20 and 22, at which time cyst excretion and all symptoms had also ceased. The ELISA was unable to detect the presence of infection 2 days after the end of the first course of chemotherapy (i.e., day 9). Apparently, the very low numbers of trophozoites that survive the chemotherapy in cases of therapy failures such as this one do not liberate enough antigen to be detectable by this assay. Results with three other patients showed that again levels decreased almost immediately after the commencement of metronidazole therapy (data not shown). With one of the four patients, there also existed a point when **Giardia** stool antigen was detectable through the ELISA but when cysts were not detectable microscopically even after stool concentration.

Tests to determine the minimum amount of cyst antigen detectable in stool by adding cyst antigen to control stool eluates revealed that the test could detect the antigens present in as few as 20 sonicated cysts (equivalent to the antigens of 200 cysts per ml). The assay could not detect the antigens of 100 or 1,000 sonicated trophozoites per well suspended in PBS-T; however, larger numbers of trophozoites (10,000 per well and above) were detectable. The antigens of 20 and 50 sonicated cysts in PBS-T were readily detectable.

Experiments aimed at determining the stability in 10% Formalin and in water at 4°C of the **Giardia** stool antigen(s) being detected by the assay showed that the antigens were, with two exceptions, completely stable for periods of up to 2 years under both of these conditions. In fact, a number of formalized stool eluates gave considerably higher absorbance values than their aqueous, paired counterparts stored at −20°C; Formalin seemed to enhance the availability of the **Giardia** antigens, especially in samples which, like the aqueous eluates, were only weakly ELISA positive. The mean adjusted absorbance of 30 formalinized eluates was 0.3996, whereas that of their aqueous counterparts stored at −20°C was 0.291. The values for formalinized specimens were therefore 56.6% higher on the average than those for the aqueous specimens stored at −20°C; this difference was significant by the one-tailed Student t test but not by the two-tailed test (for the latter, 0.10 > P > 0.05). Formalin did not effect the readings of 10 *G. lamblia*-negative control specimens that were also tested as paired samples. Only one formalinized specimen of the 30 *G. lamblia*-positive specimens showed a complete loss of antigen. Twelve *G. lamblia*-positive stool eluates stored in water at 4°C without preservatives gave a mean adjusted absorbance that was only 8% lower than the mean of 12 paired specimens stored at −20°C, a difference that was not statistically significant. Only one of the 4°C specimens showed a complete loss of antigen.

**DISCUSSION**

This represents the first reported ELISA for **Giardia** stool antigen in which antibodies against the cyst form of the parasite are employed. We have already described the use of counterimmunolectrophoresis in detecting cyst antigens and, in particular, the stable GSA-65 antigen, in human stool (16, 17). We have also isolated and partially characterized the latter antigen (17). Craft and Nelson (5) had previously found that antisera prepared against a mixture of cysts and trophozoites could detect giardial antigens in human stool eluates by counterimmunolectrophoresis. Vinayak et al. (21) have reported the use of counterimmunolectrophoresis employing antitrophozoite serum for the detection of **Giardia** stool antigens. Ungar et al. (20), Green et al. (9), and Nash et al. (13) have reported the use of antitrophozoite serum or IgG as the capture antibody in the design of antigen-capture ELISA for detection of **Giardia** stool antigens. Green et al. (9) claimed to have an assay that could be read visually and also claimed to have been able to detect antigen in stool from a patient undergoing tinidazole therapy 2 days longer than cysts or trophozoites could be detected by microscopy. The only one of the above-mentioned studies to have examined the effects of Formalin or of lengthy storage of unprocessed aqueous stool eluates on the detectability of **Giardia** stool antigens was that of Ungar et al. (20), who found, as observed here, that the antigens are stable for long periods in water alone at 4°C but, in contrast to the results reported in this paper, found that the presence of formaldehyde in the extracts or eluates gave poor results. Our use of anti-cyst antibodies for the first time makes it possible to detect **Giardia** antigens that are stable in the presence of formaldehyde. Stool can be eluted with 10% Formalin or water alone—tapwater is probably sufficient—and stored for at least a month and probably for years at 4°C. The formalinized stools can probably be stored at 25°C, although this was not specifically studied here.

The identity and physicochemical nature of the **Giardia** stool antigens detected by the anti-cyst IgG and the secondary mouse antiserum in this assay are unknown. It is clear, however, that they are stable in formaldehyde and resistant to proteases existing in stool. The GSA-65 antigen described by us previously (16, 17) is very likely one of the major antigens among those captured. A strong antibody titer against this antigen exists in the rabbit anti-cyst serum employed here, since in crossed immunolectrophoresis anti-cyst serum precipitates only one antigen from *G. lamblia*-positive human stools, and we have shown that this antigen is GSA-65 (16). GSA-65 has a molecular weight of approximately 65,000, is stable in Formalin, and is resistant to proteases and to boiling. It has a carbohydrate moiety that is sensitive to periodate oxidation and appears to be the antigenic portion of the molecule. Immunofluorescence tests have indicated that this antigen is probably present in far greater quantities in the cyst than in the trophozoite of *G. lamblia* and that it appears to be a component of the cyst wall (16, 17). The cellular origins and function of this molecule and the reason why it appears in a cell-free, water-soluble form in the stool of giardiasis patients, even when the cyst excretion rate is low, are not understood. It is possible that the GSA-65 antigen is produced at a somewhat constant rate by a subpopulation of the trophozoites that is preparing to encyst, and that the conditions for perfect and complete encystation come and go as they are disturbed by chemical and immunological changes in the intestinal lumen. Antigen may be liberated from forms that are unable to form perfect cysts and are lysed during transit down the intestine. This may help explain why there was no clear correlation between ELISA value and cyst or trophozoite numbers in the stool. Our observations that the ELISA can detect the antigens of as few as 20 cysts and that it is clearly more sensitive for cyst-derived than trophozoite-derived antigens suggest that the primary antigens being detected in the assay are antigens produced by the **Giardia** cell during encystation.

The ELISA has been shown here to be useful in monitoring the levels of **Giardia** antigens excreted by patients during and after antigiardial chemotherapy with metronidazole. From the fact that on two occasions **Giardia** antigen was...
detectable by ELISA when cysts could not be found microscopically, it would appear that the test may be more sensitive than a single microscopic examination. However, a meaningful comparison of the sensitivity of this test for detecting *Giardia* infection with that of microscopy can only be made through applying it and microscopic examination simultaneously to a sizeable number of consecutive, unscreened stool specimens submitted to clinics. Nevertheless, it is apparent that the ELISA is a practical and sensitive assay for detecting giardial infection. Because it allows for extended storage of Formalin-fixed stools before testing, it may be a more useful assay than the ELISA as performed with antiparathozone capturing antibodies, in that specimens sent to state health and other central laboratories are often mailed and need to be formalinized. The test should be easily adaptable to simpler methodologies such as dip-stick or dot ELISA, magnetic bead ELISA, etc. We believe that the most important application of this test will lie in cases where a patient has been repeatedly negative by microscopic parasite exam (ova and parasites) but continues to have symptoms specifically suggestive of giardiasis, either of an acute or chronic type. Because in acute cases a relatively high rate of cyst excretion usually exists, the presence of *Giardia* cysts or trophozoites would usually be picked up on the standard microscopic ova and parasites exam; an antigen-capture assay for *Giardia* stool antigens in these cases would be unnecessary. It is instead the chronic and cryptic cases of giardiasis that demand a more sensitive test for infection and for which this type of assay would be most useful. As Chester et al. have shown (4), more than half of all giardial infections are chronic. Thus, the test may serve as an important adjunct method to microscopic ova and parasites analysis, to be resorted to after repeated microscopic analysis has failed to detect *G. lamblia* but the patient continues to experience typical giardial-like symptoms.

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**LITERATURE CITED**