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Diagnosis of *Giardia lamblia* Infections by Detection of Parasite-Specific Antigens

EDWARD N. JANOFF,[1,2]†* J. CARL CRAFT,[3] LARRY K. PICKERING,[4] THOMAS NOVOTNY,[5] MARTIN J. BLASER,[1,2] CATHY V. KNISLEY,[4] AND L. BARTH RELLER[2]

Infectious Disease Section, Veterans Administration Medical Center, Denver, Colorado 80220[1]; Division of Infectious Diseases, Department of Medicine, University of Colorado School of Medicine, Denver, Colorado 80262[2]; Department of Pediatrics, Tulane University School of Medicine, New Orleans, Louisiana 70112[3]; Program in Infectious Diseases and Clinical Microbiology, University of Texas Medical School, Houston, Texas 77030[4]; and Centers for Disease Control, Atlanta, Georgia 30333[5]

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Antigen detection methods may facilitate diagnosis of *Giardia lamblia* in stool specimens. As determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis and immunoblotting, *G. lamblia* cysts and trophozoites share several antigens, especially in the 65-kilodalton and 30- to 34-kilodalton regions. By using blind methods, we compared results obtained by counterimmunoelectrophoresis using cyst-immune rabbit serum and by enzyme-linked immunosorbent assay (ELISA) using trophozoite-immune rabbit serum with results obtained by microscopic examination of a preserved, concentrated, and permanently stained stool specimen. Results were similar when these three methods were used to examine 118 stool specimens from clinical microbiology laboratories (53 specimens with *G. lamblia*) and specimens from 239 day-care-center toddlers (39 specimens with *G. lamblia*). Compared with microscopy, we found, for counterimmunoelectrophoresis and ELISA, respectively: sensitivity, 88 versus 94%; specificity, 97 versus 95%; positive predictive value, 86 versus 76%; negative predictive value, 98 versus 97%; and concordance, 89%. The false-positive rate by ELISA was 24% (10 of 42) in day-care-center toddlers but only 3% (1 of 32) in healthy adults ($P < 0.04$) as corroborated by microscopy. This discrepancy suggests that the ELISA may be more sensitive than microscopy, which is considered the reference standard, and that results may be dependent, in part, on the epidemiology of the infection in the study subjects.

Reliable information on the epidemiology and natural history of *Giardia lamblia* infections has been limited because the sensitivity of microscopic diagnosis of the parasite in concentrated stool specimens varies from 46 to 95% (1, 6, 12, 13). Examination of multiple samples may be required for diagnosis, particularly from persons with early (9) or chronic infections (3). Other diagnostic methods, some of which require sampling of the small bowel, are more expensive, uncomfortable, and invasive (6, 10, 13). Stool antigen detection systems for the diagnosis of *G. lamblia* infections have been reported to be highly sensitive and specific (2, 7, 14, 23, 24). These methods may permit large numbers of stool samples to be tested rapidly and may reduce technician time and bias among observers.

Standard diagnostic methods are designed to detect the specific stages of the life cycle of *G. lamblia*, cysts and trophozoites. Microscopic examination of stool samples most often detects cysts (4, 21), whereas duodenal aspiration and biopsy primarily identify trophozoites (6). In contrast, antigen detection systems, although also designed to detect the distinct stages of the life cycle, show similar diagnostic accuracies. We identified the antigens detected by two antigen detection methods to understand the relationship between cyst and trophozoite antigens and to better define and standardize the assay systems. We performed the first blinded comparison of three noninvasive methods for diagnosis of *G. lamblia* infections from stool samples: microscopic examination, detection of cyst antigens by counterimmunoelectrophoresis (CIE), and detection of trophozoite antigens by enzyme-linked immunosorbent assay (ELISA).

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MATERIALS AND METHODS

Antigen preparation. *G. lamblia* Portland-1 trophozoites, graciously provided by Ernest Meyer, were grown and prepared as previously described (8). Purified cysts were prepared from human fecal specimens, as previously described (2), and were kindly provided, in part, by Charles Hibler. The cyst purity was assessed by microscopy (2), and the preparations were sonicated, centrifuged at 1,000 × *g* for 30 min, and preserved at −70°C.

Antigen analysis. Rabbit sera used in the antigen detection systems were compared by ELISA as previously described (8), except that the solid-phase antigens were whole frozen *G. lamblia* Portland-1 trophozoites (10^4 organisms per well) or purified *G. lamblia* cysts (10^3 cysts per well). The endpoint was defined as the highest dilution of rabbit serum that resulted in an optical density (OD) greater than 0.200 at 405 nm. The specificities of the antibodies detected were determined by adsorption of immune serum four times for 45 min each time at 37°C on a circular rotator with each of the following Formalin-fixed organisms (number of organisms per adsorption): *G. lamblia* Portland-1 trophozoites (6×10^6); *Trichomonas vaginalis* cultured from a symptomatic patient (6×10^6); *Cryptosporidium* oocysts and freshly excysted sporozoites (10^7), provided by Charles Sterling and

* Corresponding author.
† Present address: Infectious Disease Section 111F, Veterans Administration Medical Center, 1 Veterans Dr., Minneapolis, MN 55417.

Michael Arrowood; *Campylobacter jejuni* (10^9); enterotoxigenic *Escherichia coli* (10^9); and *Candida albicans* (10^9).

Whole-cell preparations of purified *G. lamblia* cysts and trophozoites were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (8). Immunoblots of trophozoite antigens were prepared with serum from New Zealand White rabbits immunized with *G. lamblia* cysts or trophozoites and used in the CIE and ELISA systems, respectively (2, 8).

Stool specimens. In Denver, Colo., in 1985, we obtained (i) single unpreserved stool samples from each of 239 randomly selected toddlers in day-care centers (T. Novotny, R. S. Hopkins, P. Shillam, and E. N. Janoff, Abstr. Annu. Conf. Epidemic Intelligence Service, Centers for Disease Control, 1986) and (ii) 118 samples submitted to the clinical microbiology laboratories of the University of Colorado, Veterans Administration Medical Center, and Denver General Hospital from 86 persons with diarrhea and 32 healthy adults with no history of gastrointestinal symptoms or known *G. lamblia* exposure within the past 2 months. Fresh stool specimens were preserved in 10% Formalin or in polyvinyl alcohol, and a third fraction was stored within 12 h of collection from test subjects without preservative at −70°C until processed.

Microscopy. The entire 22-mm^2 cover slip from each Formalin-ether (or Formalin-ethyl acetate) concentrate was examined at a magnification of × 10, as was each trichrome stain of polyvinyl alcohol-preserved stool specimen examined under an oil immersion lens. Each concentrated specimen was stained for *Cryptosporidium* oocysts and sporozoites with the modified Kinyoun acid-fast (cold) technique (11), and the entire slide was scanned for 5 min at a magnification of × 40. Positive specimens were confirmed by examination under an oil immersion lens. Identification of *G. lamblia* by microscopic examination of preserved concentrate or of a permanently stained smear was considered the reference standard for the antigen detection systems. The numbers of *G. lamblia* organisms detected were graded as rare to few, moderate, or many in 31 of 39 positive specimens from day-care-center toddlers.

Antigen detection. The CIE assay was performed as previously described (2), with serum from cyst-immune rabbits. For the ELISA system, New Zealand White rabbits were immunized with axenically grown *G. lamblia* Portland-1 trophozoites. A crude serum immunoglobulin fraction was precipitated with ammonium sulfate and dialyzed, and a portion was conjugated to horseradish peroxidase (22). Wells of microdilution plates (Immulon II; Dynatech Laboratories, Inc., Alexandria, Va.) were coated with unlabeled rabbit anti-*G. lamblia* antibody in 100 mM carbonate buffer (pH 9.0) overnight at 4°C. Freshly thawed fecal specimens were diluted 1:2 with 50% fetal calf serum–0.01 M phosphate-buffered saline (pH 7.2) plus 2 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.) and 0.1% thimerosal, vortexed, and allowed to settle for 30 min. The supernatant was incubated for 1 h at 37°C in duplicate wells. After wells were washed three times by using a multichannel pipette, peroxidase-conjugated rabbit anti-*G. lamblia* antibody was applied, incubated for 1 h at 37°C, and developed with *o*-phenylenediamine in 53 mM citrate–94 mM phosphate buffer (pH 5.0) for 15 min. The reaction was stopped with 2.5 N H_2SO_4, and the A_{490} was measured on a microELISA plate reader (MR580; Dynatech). Positive and negative controls were included on each plate. Specimens which produced an OD of less than 0.100 were considered negative, those with an OD between 0.100 and 0.250 were considered equivocal, and those which produced an OD above 0.250 were considered positive on the basis of results previously obtained from specimens characterized by microscopy (C. V. Knisley, P. G. Englekirk, L. K. Pickering, S. West, and E. N. Janoff, Am. J. Clin. Pathol., in press). Preliminary studies showed that an OD of 0.250 resulted from the addition of 250 trophozoites (1 ng of protein) to a test well. We decided in advance to consider any specimens which produced equivocal results upon CIE or ELISA to be negative for the purpose of analysis.

We evaluated each method on the basis of the number of specimens tested by that method; all 357 specimens were tested by microscopic examination, 327 (92%) were tested by CIE, and 312 (87%) were tested by ELISA. We compared the three assays by using results from the 282 (79%) specimens tested by all three methods. To investigate the reproducibility of test results, nine stool samples were tested in a blind manner in duplicate by CIE, as were six by ELISA. ODs by ELISA were compared for 20 samples tested twice on separate days to establish the test-retest reliability.

Statistics. Results were evaluated by the chi-square and Fisher exact tests as indicated.

RESULTS

Antigen analysis. The trophozoite-immune rabbit serum used in the ELISA system showed a titer of 1:25,600 with the trophozoites and a titer of 1:400 with the cysts as the solid-phase capture antigens. The cyst-immune rabbit serum used in the CIE system showed a titer of 1:200 with the trophozoites and a titer of 1:3,200 with the cysts as the solid-phase capture antigens. Preimmune rabbit serum showed a titer of <1:100, the lowest dilution tested, with both antigens. Trophozoite-immune serum showed the greatest decrease in titer following adsorption with *G. lamblia* trophozoites, from 1:25,600 to 1:3,200. Titers after adsorption with other protozoal, bacterial, and fungal organisms remained 1:12,800.

Eight distinct bands, with major bands at 88, 60, 55, 48, 33, and 31 kilodaltons (kDa), were resolved from the soluble *G. lamblia* cyst preparation (Fig. 1). Silver staining of solubilized trophozoites also produced bands of 189, 88, 75, 68, 55, 47, and 34 kDa (13). Immunoblotting of whole trophozoites with cyst-immune and trophozoite-immune rabbit sera revealed common trophozoite antigens of 185, 65, and 57 kDa and produced the strongest reactivity with heavy bands of 33 and 30 kDa (Fig. 2).

Antigen detection in stool samples. The sensitivities and specificities of the two antigen detection methods (CIE and ELISA) were similar to results obtained by microscopy with stool specimens from day-care-center toddlers and with specimens submitted to clinical laboratories (Table 1). The numbers of organisms identified by microscopy in 31 of 39 day-care-center samples examined in which *G. lamblia* was identified were rare to few in 48%, moderate in 16%, and many in 35%. Both CIE and ELISA showed negative results for two specimens in which *G. lamblia* was detected by microscopy. Two stool samples taken from patients 2 weeks after they started therapy for documented *G. lamblia* infections were negative by microscopy; one sample was positive by CIE, as was the one sample tested by ELISA. Thirteen stool samples with other parasites, including *Entamoeba histolytica* ($n = 2$), *Cryptosporidium* spp. ($n = 7$), *Blastocystis hominis* ($n = 3$), and others (*Entamoeba coli*, *Entamoeba nana*, *Trichuris trichuris*) showed negative results by both methods. Nine samples tested in a blind manner in duplicate by CIE gave concordant results (seven positive and two

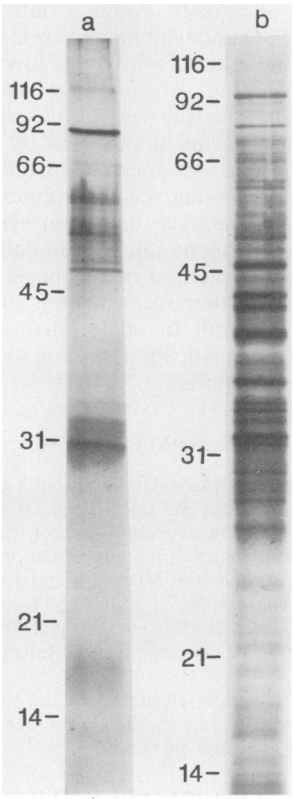

FIG. 1. Silver-stained 12% polyacrylamide SDS-PAGE preparations of solubilized *G. lamblia*. Lanes: a, cysts from an infected patient; b, axenically grown Portland-1 strain trophozoites.

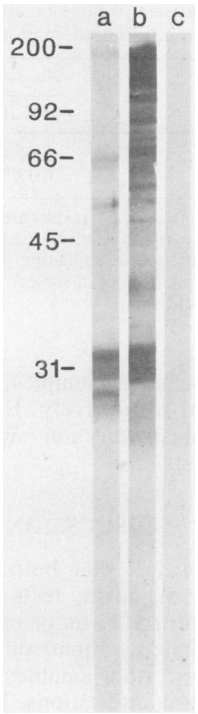

FIG. 2. Immunoblots of *G. lamblia* Portland-1 trophozoites resolved on 12% polyacrylamide SDS-PAGE gels. Nitrocellulose strips were reacted with serum from rabbits immunized with *G. lamblia* cysts (lane a), *G. lamblia* trophozoites (lane b), and preimmune serum (lane c).

negative), as did five of six samples tested by ELISA (three of four positive and two negative). The test-retest reliability of ODs produced with 20 samples tested on two occasions by ELISA was high (correlation coefficient = 0.953), and the mean values did not differ by the paired *t* test. The presence or absence of symptoms, such as diarrhea and abdominal pain, in the patients from whom the specimens were obtained did not affect the sensitivities or specificities of the two methods (data not shown). The concordance rates between the two antigen detection methods was 92% (196 of 214) for samples without *G. lamblia* and 81% (55 of 68) for samples with *G. lamblia* as corroborated by microscopy.

The predictive value of a positive result was lower in samples from day-care-center toddlers than from routine samples from clinical microbiology laboratories. When microscopy results were considered the reference standard, false-positive rates were higher among day-care-center toddlers, among whom rates of exposure to and carriage of *G. lamblia* are often higher (1, 16; Novotny et. al., Abstr. Annu. Conf. Epidemic Intelligence Service, 1986) than among healthy adults with no known *G. lamblia* exposure, who are less likely to be infected (Table 2).

Of the samples tested by CIE, 5% (16 of 327) showed equivocal results (grey zones); 13% of those tested by ELISA (41 of 312) showed equivocal results. As part of our protocol prior to testing, any equivocal result was defined as a negative test result for the purposes of analysis. In the clinical setting, these results would have been called equivocal and a second specimen would have been requested. No *G. lamblia* was identified by microscopy in 81% (13 of 16)

TABLE 1. Comparison of antigen detection methods with microscopy for diagnosis of *G. lamblia* infections

Source of specimens (diagnostic test)[a]	No. tested	No. (%) with *G. lamblia*	Sensitivity (%)[b]	Specificity (%)[b]	Positive predictive value (%)	Negative predictive value (%)
Day-care-center toddlers						
MICRO	239	39 (16)	100	100		
CIE	212	35 (17)	88	97	86	98
ELISA	218	42 (19)	94	95	76	99
Clinical microbiology laboratory						
MICRO	118	53 (45)	100	100		
CIE	115	48 (42)	96	97	96	93
ELISA	94	42 (45)	90	91	88	92

[a] MICRO, Microscopic examination of a single preserved, concentrated, and permanently stained specimen; CIE, CIE assay for cyst antigen; ELISA, ELISA for trophozoite antigen.

[b] Microscopic examination is considered the reference standard for this analysis.

TABLE 2. Comparison of false-positive rates using antigen detection methods in two epidemiologically distinct groups

Group	No. false-positive/total no. (%) by[a]:	
	CIE	ELISA
Day-care-center toddlers	5/35 (14)	10/42 (24)
Healthy adults	0/32[b] (0)	1/32[b] (3)

[a] For the purposes of this analysis, results are considered as false-positives if the sample produced a positive result by ELISA or CIE but no organisms were identified by microscopic examination of a single concentrated and permanently stained sample from each subject.
[b] $P < 0.05$, Fisher exact test.

and 90% (37 of 41) of these samples, which were equivocal by CIE and ELISA, respectively. Exclusion of these samples from the analysis did not significantly change the evaluation of the tests.

DISCUSSION

This study establishes that both the CIE and ELISA systems are reliable diagnostic tests for the diagnosis of *G. lamblia* infections on the basis of established criteria (18). We performed a blinded comparison with a reference standard of diagnosis on stool samples from patients with a spectrum of clinical manifestations. We described the clinical setting and exact methods for the study, the precision of the tests, and variation among observers. The advantage of antigen detection systems is that they provide rapid, sensitive, and reproducible results for large numbers of samples; they may be less labor-intensive, less expensive, and subject to less test-to-test variation than diagnosis of *G. lamblia* infections by microscopy. These assays serve as a useful adjunct to microscopic examination, which is directed to detect a spectrum of enteric infections.

CIE and ELISA antigen detection systems showed results similar to those of microscopic examination in a large, prospective, blinded comparison for the diagnosis of *G. lamblia* in stool specimens. Although cysts are usually identified five times more often than trophozoites in stool by microscopy (4, 21), these assays, which used rabbit sera hyperimmune to two distinct phases of the life cycle of *G. lamblia*, gave comparable results. The recognition of common antigens by these sera, especially those bands of 65 kDa, as first described by Rosoff and Stibbs (17) and Stibbs et al. (19), and those between 30 and 34 kDa, which are also recognized by humans infected with *G. lamblia* (8, 20), may in part explain this similarity. These cross-reactive antigens, which are recognized by the cyst-immune serum, are probably derived from trophozoites within the cysts. This cross-reactivity was not described by another group, perhaps because of differences in immunization technique or in the method of antigen preparation (5). The CIE assay may well detect other protein or nonprotein cyst wall antigens (5, 25).

Antigen detection may be more sensitive, rather than less specific, than the reference standard, microscopic examination (14). The specificity of microscopic diagnosis in these university-affiliated and state laboratories is high in Denver, probably because of the frequency of diagnosis of the parasite and the long-term interest in *G. lamblia* in Colorado. We demonstrated the specificity of the trophozoite-immune serum in vitro by adsorption studies and by the lack of cross-reactivity with other protozoa in clinical samples. The false-positive rate was higher among day-care-center toddlers, among whom carriage rates of *G. lamblia* are high (1, 16; Novotny et al., Abstr. Annu. Conf. Epidemic Intelligence Service, 1986) and who may excrete few organisms (3, 9, 15), than among adults, who are at low risk of infection. False-negative samples were uncommon with both antigen detection methods. The true sensitivities of these tests could be determined best during an outbreak by testing acutely ill persons, with multiple specimens being taken from the same subjects (14), and by using seeding studies.

We conclude that antigen detection systems provide an acceptable and convenient adjunct to microscopic examination for noninvasive method of diagnosis of *G. lamblia* in stool specimens. Further identification of parasite-specific antigens may be useful to standardize and improve the clinical utility of diagnostic methods and to define the natural history of these infections.

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