**EVALUATION OF ELISA FOR DETECTION OF GIARDIA LAMBLIA-SPECIFIC COPRO-ANTIGEN EMPLOYING MONOSPECIFIC ANTIBODIES**

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**SUMMARY:** An enzyme linked immunosorbent assay (ELISA) system, using monospecific antibodies for the detection of *Giardia lamblia* specific 66 kDa copro-antigen has been developed and evaluated. The assay detected the antigen in stool eluates of all the 24 microscopically confirmed cases of giardiasis and in 17 (68%) of the 25 microscopy-negative clinically suspected cases of giardiasis. None of stool eluates from 20 subjects infected with other protozoal / helminthic intestinal parasites or from 20 apparently healthy subjects had *G. lamblia*-specific copro-antigen. The ELISA employing monospecific antibodies is a sensitive and specific tool for the diagnosis of giardiasis and is especially useful for confirming microscopy-negative suspected cases of giardiasis.

**INTRODUCTION**

*Giardia lamblia*, a protozoan parasite inhabiting the upper small intestine, has been implicated in a number of water-borne epidemics (1). Infection may remain asymptomatic (2) or lead to an acute or chronic diarrhea with malabsorption (3). Repeated microscopic examinations of stool specimens failed to detect the parasite, *G. lamblia*, in a significant number of clinical cases of giardiasis (4). Examination of duodenal aspirates may confirm the diagnosis in a few more cases (5). Recently, demonstration of parasite products either by counterimmuno-electrophoresis (CIEP) or by enzyme linked immunosorbent assay (ELISA) has been employed as a rapid diagnostic procedure (6-9). In most
instances, polyclonal antibodies raised against a complex mosaic of *G. lamblia* antigens have been employed to capture specific copro-antigens in the stool eluates of clinical cases of giardiasis which led to variable degrees of sensitivity and specificity. The sensitivity and specificity of the assay can be improved by employing antibodies specific to *G. lamblia* copro-antigen present in the stool eluates. The present report describes the potential diagnostic utility of an ELISA system employing monospecific antibodies to capture the *G. lamblia*-specific 66 kDa copro-antigen present in the stool eluates of clinical cases of giardiasis.

**MATERIALS AND METHODS**

*Parasite culture:* Trophozoites of *G. lamblia* cultured axenically in TSP-1 medium (10) were washed three times in 0.15 M phosphate buffered saline, pH 7.2 (PBS) and disintegrated ultrasonically at 20 kc/sec in an MSE ultrasonic disintegrator (7). The disintegrated material was centrifuged at 1,200 × g for 30 min at 4 C and the supernatant was labeled as "Crude Giardia Extract" (CGE).

*Anti-CGE antibodies:* Rabbits were given three doses of 1 mg proteins of CGE at weekly intervals. The first dose given was emulsified in Freund's complete adjuvant and two subsequent doses were given in Freund's incomplete adjuvant. The rabbits were bled 10 days after the last injection.

*G. lamblia* copro-antigen (GCA)-specific antibodies (anti-GCA antibodies): A stool sample (100 g) from a confirmed case of giardiasis was emulsified in distilled water (200 ml) and the emulsion was filtered through cotton gauze. The filtrate was centrifuged at 7,500 × g for 30 min at 4 C. The supernatant obtained was subjected to 60% ammonium sulfate precipitation (11). The precipitates were dialyzed against distilled water for 24 hr. The GCA present in dialyzed stool eluates was isolated by CIEP employing anti-CGE antibodies (7). The precipitates containing GCA were cut from agarose gels, emulsified in PBS and stored at -20 C for 3 days. Thereafter, the agarose particles were removed by centrifugation at 250 × g for 10 min at 4 C and the supernatant obtained was used for immunizing the rabbits. The schedule for immunization was identical to that described above. Each immunization dose contained 400 μg of proteins. The sera collected from the immunized rabbits were subjected to DEAE-cellulose chromatography (12) for separation of anti-GCA-IgG. The IgG fraction was coupled to horseradish peroxidase by periodate method (12) and labeled as "HRP-anti-GCA-IgG".

*Affinity column chromatography:* Column chromatography was performed with cyanogen bromide-activated Sepharose 4B (Sigma Chemical Co., St. Louis, MO) coupled with anti GCA-IgG. The CGE was passed through the Sepharose
column and subsequently washed with PBS for removing uncoupled materials. The coupled GCA was eluted with 0.1 M glycine-HCl buffer, pH 2.5 (12). Fractions containing the antigen were pooled, dialyzed and lyophilized. With an identical affinity column, GCA was also obtained from the ammonium sulfate-precipitated and dialyzed stool eluate of a confirmed case of giardiasis. The purity of the isolated fraction was confirmed by SDS-PAGE and immunoblotting.

**Monospecific antibodies to affinity purified antigen:** Antiserum to affinity-purified antigen from the trophozoites was raised in rabbits with 100 µg of affinity-purified antigen per dose. The schedule for immunization of rabbits was identical to that described above. The IgG fraction of antiserum was obtained by DEAE-cellulose chromatography (12).

**SDS-PAGE and western blotting:** The SDS-PAGE was performed in the presence of β-mercaptoethanol (13) in 4.5% stacking gel and 10% separating gel. Antigens separated on SDS-PAGE were transferred to nitrocellulose paper in a Bio-Rad transblot apparatus (14). After blocking non-specific binding sites, the nitrocellulose strips were incubated with appropriately diluted anti-CGE antibodies or monospecific antibodies followed by reaction with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulins (15). Some of the strips were also allowed to react with HRP-anti-GCA-IgG. The enzymatic reactions were developed with freshly prepared 0.06% (w/v) 4-chloro-1-naphthol (Sigma Chemical Co.) and 0.01% hydrogen peroxide in PBS (16).

**Clinical specimens:** Stool specimens from following subjects examined by the formol ether concentration method (17) were analyzed for the presence of *G. lamblia*-specific 66 kDa copro-antigen by ELISA.

a) **Reference positive cases** (n=24): These patients had gastrointestinal symptoms such as pain in the abdomen, loss of weight, loss of appetite, flatulence, and diarrhea and their stools were positive for *G. lamblia* cysts or trophozoites.

b) **Clinically suspected cases of giardiasis** (n=25): These patients had gastrointestinal symptoms as above but three repeated microscopic examinations of their stools revealed no *G. lamblia* cysts/trophozoites or any other parasites.

c) **Reference samples from non-giardial clinical cases** (n=20): This group consisted of patients infected with *Entamoeba histolytica*, *E. coli*, *Endolimax nana*, *E. hartmanni* cysts, *Hymenolepis nana*, *Ascaris lumbricoides* or *Anacylostoma duodenale* eggs.

d) **Reference negative cases** (n=20): These were apparently healthy subjects who had no gastrointestinal symptoms and their repeated stool examinations were negative for any parasite.

**Preparation of specimens (for demonstration of copro-antigen):** Approximately 1 g of stool was emulsified in 1 ml of distilled water and the emulsion was centrifuged at 250 ×g for 3 min (7). The cloudy supernatant labeled as “stool eluate” was stored at −20 C until tested for antigen by ELISA.
Enzyme linked immunosorbent assay: A double antibody sandwich ELISA was performed by the method similar to that described by Ungar et al (18). In brief, for each test sample two wells were coated with optimally diluted monospecific IgG antibody to affinity-purified 66 kDa antigen and two with optimally diluted normal rabbit IgG. Stool eluates (from clinical cases or control subjects) were added to antibody-coated wells. For the detection of captured copro-antigen, HRP-anti-GCA-IgG was added to each of the wells. The optical density (OD) was measured in an ELISA reader (Lab. System, Uniskan I, Finland) at a wavelength of 492 nm. The specific OD was calculated by subtracting the mean OD of wells coated with normal rabbit IgG from mean OD of wells coated with monospecific IgG. A clinical specimen was considered positive for 66 kDa copro-antigen if the specific OD value was higher than the mean plus 2 SD of specific OD values of stool eluates from apparently healthy subjects.

RESULTS

SDS-PAGE and Immunoblotting

G. lamblia antigens obtained as CGE or ammonium sulfate-precipitated and dialyzed stool eluates, upon affinity purification, contained a protein of 66 kDa molecular mass as shown in Fig. 1. On western immunoblotting, the affinity-purified component reacted specifically with anti-CGE antibodies, monospecific antibodies and HRP-anti-GCA-IgG (Fig. 2). The monospecific antibodies against affinity-purified 66 kDa antigen detected a single discrete band at the 66 kDa position in western blots of CGE (Fig. 3).

Evaluation of Antigen Capture ELISA in Clinical Cases of Giardiasis

A specific OD of 0.081 (mean + 2 SD of specific OD of stool eluates from apparently healthy subjects) was used as a cut-off value for clinical specimens. The stool eluates from all the confirmed cases of giardiasis showed a specific OD higher than the cut-off value (Fig. 4). None of the stool eluates from patients with parasites other than G. lamblia had G. lamblia-specific 66 kDa copro-antigen. However, 17 (68%) of the 25 microscopy-negative highly suspected cases of giardiasis contained detectable levels of G. lamblia-specific 66 kDa copro-antigen (Fig. 4). Repeated stool examinations of five microscopy-negative highly suspected cases of giardiasis on follow up revealed the presence of G. lamblia
Fig. 1. SDS-PAGE analysis of *G. lamblia*-specific copro-antigen chromato-graphically purified from CGE (Lane B) or from ammonium sulfate-precipitated stool eluates of *G. lamblia*-positive case (Lane C). Lane A shows the molecular weight markers.

Fig. 2. Western blotting of affinity-purified *G. lamblia*-specific copro-antigen. Strips A, B and D show reaction with rabbit anti-CGE IgG, monospecific IgG and normal rabbit IgG, respectively. Strip C shows reaction with HRP-anti-GCA-IgG.

Fig. 3. Western blotting of CGE. Strip A, stained with amido black 0.1%, Strips B, C and D show reaction with anti-CGE IgG, monospecific IgG and normal rabbit IgG, respectively. Strip E shows reaction with HRP-anti GCA-IgG.
cysts. All the suspected cases were relieved of their clinical symptoms following metronidazole therapy (7.5 mg/kg body weight three times a day for 7-10 days).

DISCUSSION

Diagnosis of giardiasis depends on multiple stool examinations. However, in 30-50% cases of giardiasis, the stool examinations remain negative (4). Examination of duodenal aspirates or imprint smears of biopsy detects few more cases (5). The demonstration of *G. lamblia*-specific antigen either by CIEP or ELISA has been suggested to be an ideal diagnostic procedure because these are rapid and easy-to-perform techniques on many specimens (6-9). Investigators have often employed polyclonal antibodies either against *G. lamblia* cyst or trophozoite antigens in the ELISA system to capture as well as to detect the specific antigens. We employed monospecific antibodies against *G. lamblia*-
specific 66 kDa antigen present in stool eluates. Such a testing system provided a very sensitive and specific tool for confirming giardiasis. All the microcopy-confirmed positive cases could be detected. Neither the patients infected with parasites other than *G. lamblia* nor apparently healthy subjects had any detectable level of antigen. Stool eluates from 17 (68%) of 25 highly suspected cases were also found to contain *G. lamblia*-specific 66 kDa antigen. The follow up of five such patients by repeated stool examinations confirmed the presence of *G. lamblia* cysts. It also suggested that *G. lamblia*-specific antigen appeared earlier than stool becoming positive for *G. lamblia* cysts. Thus, the assay system is useful in making possible an early diagnosis of giardiasis. In eight of the 25 clinically suspected cases of giardiasis where *G. lamblia* specific 66 kDa copro-antigen was not detectable by ELISA, the possibility of subclinical infection with other enteropathogens (bacteria/viruses) could not be ruled out. We have earlier shown employing CIEP that the copro-antigen disappeared in 80% of the treated patients within 1-2 weeks following the completion of antigiardial therapy (7). The rest of 20% cases still had positive stool examination for *G. lamblia* cysts after treatment and were also positive for *G. lamblia* copro-antigen. Thus, it appears that the demonstration of copro-antigen may be useful in monitoring the effectiveness of therapy.

We have earlier observed that immunoreactivity of 66 kDa antigen to specific antibodies is lost upon exposure to heat (100 °C) or trypsin treatment (19). However, the immunoreactivity of 66 kDa antigen was not affected by metaperiodate treatment. Recently, isolation and identification of a heat and trypsin-resistant but metaperiodate-sensitive *G. lamblia*-specific antigen (GSA 65) present in the stool has been reported (20). The immunochemical properties of 66 kDa copro-antigen detected by us appears to be different from those of the GSA 65 molecule reported earlier (20). We tend to feel that stool eluates from the patients with giardiasis may contain more than one immunoreactive antigens with identical molecular weights which may differ chemically.

On the assumption that all ELISA-positive cases were true cases of giardiasis, the microscopic examination was only 71% sensitive. We feel that, the present ELISA system will prove a useful and easy tool to investigate giardial epidemics and also for confirming diagnosis of microscopy-negative but clinically suspected cases of giardiasis.
REFERENCES


