Enzyme-linked immunosorbent assay for copro-diagnosis of giardiasis and characterisation of a specific *Giardia lamblia* antigen in stools

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**Summary.** An enzyme-linked immunosorbent assay (ELISA) has been evaluated for copro-diagnosis of giardiasis with anti-trophozoite antibody to capture specific *Giardia lamblia* stool antigen (GLSA), which was then detected by specific antibody conjugated with horseradish peroxidase. GLSA was demonstrated in stool eluates from all the 24 confirmed cases of giardiasis. None of the stool eluates from apparently healthy subjects or from patients carrying intestinal parasites other than *G. lamblia* had GLSA. Of the 25 microscopy-negative clinically suspected cases of giardiasis, 17 (68%) patients had GLSA in their stool eluates; these patients responded to anti-giardial therapy. The specific antigen was isolated and affinity-purified by the use of specific antibody; it had a M, of 66 Kda, and its immunoreactivity was lost after treatment with heat or trypsin but unaltered by metaperiodate. ELISA seems to be a sensitive and specific method for copro-diagnosis of giardiasis, especially in highly suspected cases.

**Introduction**

*Giardia lamblia*, a flagellate protozoan parasite, colonises the upper gastrointestinal tract of man and is responsible for a varied spectrum of disease ranging from the asymptomatic carrier state to acute fulminating diarrhoea with or without malabsorption, especially in children. The laboratory diagnosis of giardiasis is made primarily by detecting the cysts or trophozoites of parasites in stool specimens, but this fails to detect the parasite in 30–50% of clinically suspected cases. Examination of duodenal aspirate or duodenal biopsy specimens confirms the diagnosis in a few more cases.

Anti-giardia IgG antibody has been demonstrated in patients' sera, but this fails to distinguish current from past infection. Anti-giardia IgA appears to be a useful indicator of current infection, but absence does not exclude infection because up to two-thirds of patients do not seem to produce a measureable serum IgA response. Anti-giardia IgM levels have been of some use in differentiating current from previous or asymptomatic infection, but this test requires further evaluation. Recently, counter-current immuno-electrophoresis (CIEP) and enzyme-linked immunosorbent assay (ELISA) have been used for the demonstration of parasite products in the stool. Most investigators have employed anti-trophozoite antiserum for detection, but the nature and the identity of the antigen(s) involved remain unclear. The sensitivity and specificity of the test can be enhanced by the use of antibody to specific *G. lamblia* stool antigen (GLSA). In the present study, antibody to GLSA has been used to assess ELISA for the detection of GLSA in stool eluates. Also, GLSA has been affinity-purified and partially characterised.

**Materials and methods**

**Anti-trophozoite antibody**

Axenically grown *G. lamblia* (strain Portland I) was maintained in Diamond's Trypticase Panmede Serum (TPS-1) medium. The trophozoites from 48–72-h culture were harvested, washed thrice in normal saline and then subjected to ultrasonic disintegration at 20 kc/s with six 30-s bursts in an ultrasonicator (Measuring and Scientific Equipment). The sonicated material was centrifuged at 250 g for 10 min in the cold. The supernate, crude giardia trophozoite antigen, was used for the immunisation of rabbits with three doses at weekly intervals, each dose containing 1 mg of giardial protein. The first dose was emulsified in Freund's complete adjuvant, the other two in Freund's incomplete adjuvant; 1 week after the last dose, the rabbits were bled. The pooled serum, anti-trophozoite antiserum, was subjected to diethylaminoethyl (DEAE) cellulose chromatography to obtain the IgG fraction.
Antibody to GLSA

A 24-h stool sample from a confirmed case of giardiasis, with cysts in the stools, was emulsified in distilled water and centrifuged at 7500 g for 30 min in the cold; the supernate was treated with ammonium sulphate 60%20 and the pooled precipitates were dialysed against distilled water for 24 h. With anti-trophozoite antiserum, GLSA was immuno-precipitated by CIEP.15 The antigen-antibody complex was cut from the agarose gel, emulsified in 0.15 M phosphate-buffered saline, pH 7.2 (PBS) and stored at −20°C for 3 days. Agarose particles were removed by centrifugation at 250 g for 10 min in the cold; and 0.5-ml volumes containing 400 µg of protein were injected into rabbits with the same schedule as for trophozoite antigen. The IgG fraction was obtained by DEAE cellulose chromatography,19 and this specific antibody to GLSA was stored at −20°C. Some of it was coupled to horseradish peroxidase by the periodate method.19

Clinical specimens

Stool specimens from 92 subjects (table I) were examined, within 1–2 h of collection, by direct smears and after formol-ether concentration.21 Confirmed cases of giardiasis: gastrointestinal symptoms such as abdominal pain, loss of weight, loss of appetite, flatulence and vomiting; stools positive for giardia cysts or trophozoites. Clinically suspected cases of giardiasis: gastrointestinal symptoms, but repeated stool examinations negative for cysts and trophozoites. Patients with other parasites: gastrointestinal symptoms, but stool microscopy positive for parasites other than G. lamblia. Apparently healthy subjects: no gastrointestinal symptoms; repeated stool examinations negative for any parasite.

Examination of specimens for GLSA

Stool specimens were prepared as described earlier.15 Briefly, c. 1 g of stool was emulsified in 1 ml of distilled water, and centrifuged at 250 g for 3 min; the cloudy supernate (stool eluate) was stored at −20°C until tested. The presence of GLSA in stool eluates was assessed by double antibody sandwich ELISA and by CIEP. CIEP was performed in agarose 1% gel in veronal buffer, pH 8.6. Faecal samples were placed near the cathode and antigiardial antiserum near the anode. A constant potential difference of 100 V and a current of 5 mA/slide was applied for 1.5 h.15 Slides were examined for precipitin bands immediately and after 24 h at 4°C. ELISA was performed basically as described by Ungar et al.18 Alternate double rows of wells in microELISA polyvinyl plates (M/S Costar Corporation, USA) were coated with optimally diluted anti-trophozoite IgG or normal rabbit IgG. Captured GLSA was detected by specific antibody conjugated with horseradish peroxidase. The specific optical density (OD) at 492 nm was calculated by subtracting the mean OD of wells coated with normal IgG from the mean OD of wells coated with anti-trophozoite IgG. A specimen was considered to contain GLSA if the specific OD value was higher than the mean +2 SD of specific OD values for stool eluates from apparently healthy subjects.

Affinity purification of GLSA

Rabbit IgG antibody to GLSA was coupled to cyanogen-bromide-activated sepharose 4B. Ammonium-sulphate-precipitated and dialysed stool eluates from a confirmed case of giardiasis were passed through the sepharose column. Unbound material was removed by washing with PBS. Elution was performed with 0.1 M glycine-HCl buffer, pH 2.5, and subsequently with dioxane 10% in the buffer.19 The fractions were pooled, dialysed and lyophilised, and they were tested for GLSA by ELISA and CIEP. Physical and immunochemical characterisation of purified antigen. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of affinity-purified antigen was performed under reducing conditions22 and its immunochemical nature was further assessed by the alteration of its immunoreactivity after treatment with heat, trypsin and metaperiodate.23 The sensitivity to heat was determined by exposure to 60°C or 100°C for 10 min before coating ELISA plates. For proteolytic digestion, antigen-coated wells of ELISA plates were treated with trypsin (Sisco Labs, India) 10, 100 or 1000 µg/ml at 37°C for 2 h. The periodate oxidation was accomplished by incubating antigen-coated wells with 0.05 M sodium metaperiodate in the dark at 4°C for 24 h in 20 mM sodium acetate buffer, pH 4.5. As a control, antigen-coated wells were incubated with acetate buffer. The plates were washed with PBS before use, and altered immunoreactivity of the treated antigen was assessed by interaction with specific antibody to GLSA. A decrease of OD in wells containing treated antigen compared with untreated controls indicated the effects of the various treatments.23

Statistical analysis

Data were analysed by Student’s t test.

Results

Evaluation of double antibody sandwich ELISA to detect GLSA

The mean specific OD values of stool eluates from 30 healthy subjects was 0.079 (SD 0.04); a specific OD of 0.159 (mean +2SD) for eluates from healthy
subjects) was used as the lower limit for considering results with clinical samples to be positive (fig. 1). All the 24 confirmed cases of giardiasis had GLSA in their stools; the specific OD ranged from 0.164 to 1.38 (mean 0.404, SD 0.261). The stools of 17 (68%) of 25 clinically suspected cases of giardiasis whose stools were negative for G. lamblia or other parasites by microscopy, contained GLSA; these 17 were relieved of their gastrointestinal symptoms by metronidazole. None of the stool eluates from 13 patients with parasites other than G. lamblia had GLSA.

Comparison of ELISA and CIEP indicated that ELISA was more efficient in detecting GLSA (table I): tests on samples from five clinically suspected symptomatic cases (20%) were positive by ELISA alone, whereas none was positive by CIEP alone.

**Physical and immunochemical characterisation of affinity-purified GLSA**

The M, of the affinity-purified antigen under reducing conditions was 66 Kda (fig. 2). Heat treatment, even at 60°C for 10 min, reduced its immunoreactivity significantly (table II). Proteolytic digestion with trypsin reduced the immunoreactivity progressively at concentrations of 10, 100 and 1000 µg/ml. However, the immunoreactivity was not affected by metaperiodate.

**Discussion**

Laboratory diagnosis of giardiasis is based on demonstration by microscopy of the parasite in clinical samples like stool, duodenal aspirate or duodenal imprint smears; but in a majority of clinically suspected cases the organism may not be found. Detection of GLSA in the stool, by either CIEP or ELISA, offered an alternative to microscopy or the demonstration of circulating antibody in the patient's serum. Most investigators have employed antitrophozoite antibody to capture antigen and also to

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**Table I. Comparison of ELISA and CIEP in the detection of specific GLSA**

<table>
<thead>
<tr>
<th>Clinical status of subjects</th>
<th>Number investigated</th>
<th>Number (%) positive by</th>
</tr>
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<tbody>
<tr>
<td>Microscopically confirmed giardiasis</td>
<td>24</td>
<td>both CIEP and ELISA 24 (100) CIEP alone 12 (48) ELISA alone 0 0</td>
</tr>
<tr>
<td>Clinically suspected giardiasis</td>
<td>25</td>
<td>0 5 (20) 8 (32)</td>
</tr>
<tr>
<td>Cases with other parasites</td>
<td>13</td>
<td>0 0 13 (100)</td>
</tr>
<tr>
<td>Apparently healthy subjects</td>
<td>30</td>
<td>0 0 30 (100)</td>
</tr>
</tbody>
</table>
detect the captured antigen. However, we have used anti-trophozoite antibody to capture GLSA, but conjugated IgG antibody to GLSA for its detection. Compared with healthy controls, all microscopy-positive cases had detectable levels of GLSA in their stools, giving an ELISA sensitivity of 100%. None of the apparently healthy subjects had a specific OD greater than the chosen limit (mean specificity of this assay were better than those reported from patients whose stools contained parasites other than G. lamblia had GLSA. The sensitivity and specificity of this assay were better than those reported earlier. Of the 25 clinically suspected cases whose repeated stool examinations revealed no giardia, 17 (68%) had GLSA in their stool eluates; and these patients responded to anti-giardial therapy. Previously, with CIEP for detection of antigen in the stool, we have shown that GLSA in stool eluates disappeared after anti-giardial therapy, and others had similar findings. Therefore, detection of antigen allows the efficacy of anti-giardial therapy to be monitored. Also, we showed previously that GLSA appeared before parasites were detectable microscopically. Thus, detection of GLSA can replace invasive diagnostic methods like duodenal intubation.

The present study indicated that GLSA has a M, of 66 Kda. This antigen was partially sensitive to heat at 60°C and highly sensitive at 100°C, but was resistant to metaperiodate treatment. Also, trypsin treatment reduced its immunoreactivity. Recently, by the use of giardia-cyst antiserum, isolation and characterisation of a 65-Kda copro-antigen from a case of giardiasis has been reported however, that antigen was shown by western blotting to be sensitive to metaperiodate treatment but resistant to heat and proteolytic digestion. The difference in immunochemical nature between that antigen and our GLSA may be the result of differences in the antibody employed to isolate the antigen and the technique of affinity purification. We believe that stool eluates from patients with giardiasis may contain more than one immunoreactive antigen with the same M, but differing immunochemically. Our data indicate that the detection of antigen by double antibody sandwich ELISA is a sensitive and specific tool for the copro-diagnosis of giardiasis; its routine use is recommended especially in clinically suspected cases of giardiasis.

References


