Detection of *Giardia duodenalis* Antigen in Human Fecal Eluates by Enzyme-linked Immunosorosbent Assay Using Polyclonal Antibodies

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The present study developed and standardized an enzyme-linked immunosorbent assay (ELISA) to detect Giardia antigen in feces using rabbit polyclonal antibodies. Giardia cysts were purified from human fecal samples by sucrose and percoll gradients. Gerbils (Meriones unguiculatus) were infected to obtain trophozoites. Rabbits were inoculated with either cyst or trophozoite antigens of 14 Colombian Giardia isolates to develop antibodies against the respective stages. The IgG anti-Giardia were purified by sequential caprylic acid and ammonium sulfate precipitation. A portion of these polyclonal antibodies was linked to alkaline phosphatase (conjugate). One hundred and ninety six samples of human feces, from different patients, were tested by parasitologic diagnosis: 69 were positive for Giardia cysts, 56 had no Giardia parasites, and 71 revealed parasites other than Giardia. The optimal concentration of polyclonal antibodies for antigen capture was 40 µg/ml and the optimal conjugate dilution was 1:100. The absorbance cut-off value was 0.24. The parameters of the ELISA test for Giardia antigen detection were: sensitivity, 100% (95% CI: 93.4-100%); specificity, 95% (95% CI: 88.6-97.6%); positive predictive value, 91% (95% CI: 81.4-95.9%); and negative predictive value, 100% (95% CI: 96.1-100%). This ELISA will improve the diagnosis of Giardia infections in Colombia and will be useful in following patients after treatment.

Key words: *Giardia* - antigen - enzyme-linked immunosorbent assay (ELISA) - feces - Colombia

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detection in eluates of human fecal samples, using specific polyclonal antibodies raised against endemic parasite isolates of Colombia.

MATERIALS AND METHODS

Parasitological diagnosis - Fecal specimens, collected from 196 different patients, were submitted to direct wet mount and formol-ether concentration methods in order to identify *Giardia* trophozoites or cysts (Truant et al. 1981).

Production of polyclonal anti-*Giardia* cyst and trophozoite antibodies

Preparation of *Giardia* cyst and/or trophozoite inocula - *Giardia* cysts were purified from human fecal samples. Trophozoites were obtained from experimentally infected gerbils with 14 different *Giardia* human isolates following the procedure described by Belosevic et al. (1983). Their concentration was independently adjusted to 1x10^6 per ml saline (Green et al. 1985). Freeze (-196°C) and thaw (4°C) cycles were carried out. These solutions were submitted to ultrasound sonication (Harlow & Lane 1988).

Immunization scheme - Rabbits were used to produce polyclonal antibodies against *Giardia* parasites. All procedures were carried out according to international guidelines (CIOMS 1985). Rabbits were inoculated via intradermal injection with *Giardia* cyst and trophozoite antigen, independently (Harlow & Lane 1988) on days 1, 15, 30, 60 and 120. Rabbits which were inoculated intradermally with cyst antigen were also immunized intravenously on day 30 (Harlow & Lane 1988).

Purification of polyclonal anti-*Giardia* cyst and trophozoite antibodies - Rabbit polyclonal anti-*Giardia* cyst and trophozoite antibodies were purified from hyperimmune rabbit sera by sequential caprylic acid and ammonium sulfate precipitation (Arévalo 1999).

Detection of *Giardia* antigen in human fecal samples using an antigen-capture ELISA

Test standardization - Purified rabbit polyclonal anti-*Giardia* antibodies were mixed in a 1:2 proportion of anticyst to anti-trophozoite antibodies, and the mixture then diluted in 0.05 M carbonate-bicarbonate coating buffer, pH 9.6, to final concentrations of 0.1, 0.5, 1, 2.5, 5, 10, 20, 40, 80, 160 and 320 µg/ml. Coproantigen samples were prepared by dissolving 1 g of each sample in 10 ml of PBS, pH 7.4 (Green et al. 1985). A positive human coproantigen fecal sample (eluate of a fecal sample which was diagnosed as positive for *Giardia* upon microscopical examination) and a negative coproantigen sample (sample negative for *Giardia* upon microscopical examination) were selected. An enzyme-linked anti-*Giardia* antibody conjugate was diluted to 1:300, 1:400, 1:500, 1:600, 1:800 and 1:1000 in PBS containing 0.05% Tween 20 (PBS-T). The conjugate had been prepared previously with anti-cyst and anti-trophozoite antibodies, each adjusted to 2.4 mg/ml and mixed in a 1:2 proportion, respectively. Four milligrams of alkaline phosphatase were added in the presence of 1% glutaraldehyde (EM grade) and 1 M glycine. The enzyme conjugate was stored at -20°C until used (Arévalo 1999). Finally, capture and detecting antibody concentrations were titrated in a checkerboard format using an ELISA procedure described by McLaren et al. (1981).

The optimal concentration of polyclonal anti-*Giardia* antibody necessary to capture *Giardia* antigen contained in human fecal eluates, defined as the minimum concentration which distinguished between positive and negative reference samples, was 40 µg/ml (Fig. 1) and the optimal dilution of the anti-*Giardia* enzyme conjugate, defined as that which allowed the best differentiation between positive and negative samples, was 1:400 (Fig. 2).

The following procedural steps were considered optimal upon test standardization: Dynatech Immulon 1® micro-ELISA polystyrene plates were coated with polyclonal anti-*Giardia* antibody (100 µl/well) diluted to a concentration of 40 µg/ml in 0.05 M carbonate-bicarbonate buffer, pH 9.6. The plates were incubated in a humidified chamber at room temperature (18°C) for 3 h. After incubation, plates were then washed three times with PBS-T, 5 min each. Fecal eluates were each added in triplicate, 100 µl per well; eluates were diluted to a final concentration of 1:10 in gelatin which had been previously dissolved in a 1:1 proportion with PBS-T. The fecal antigen samples were allowed to incubate for 2 h as described above. The plates were then washed for three times with PBS-T, 5 min each. The alkaline phosphatase-linked anti-*Giardia* antibody conjugate was diluted in PBS-T at a final concentration of 1:400, and 100 µl placed in each well. The plates were allowed to incubate in a humidified chamber at 4°C overnight. After incubation, all plates were washed as above in PBS-T. The reactions were developed by adding 100 µl p-nitrophenyl phosphate (1 mg/ml) in 0.1 M diethano-
lamine buffer, pH 9.8, to each well. After 30 min, color development was stopped by adding a 25 µl volume of 3N NaOH to each well. The absorbance value was determined by reading the optical density at a 405 nm wavelength using a MultiSkan MS® colorimeter.

**Evaluation of the ELISA test for antigen detection**

**Validation: statistical analysis** - Validation of the ELISA for *Giardia* antigen detection was carried out by establishing the cut-off value (Kurstak 1985) and the test parameters (Griner et al. 1981). The cut-off value, defined as the absorbance value from which a negative sample can be clearly distinguished from a positive sample, was determined by adding twice the standard deviation to the mean absorbance value of the negative samples. This provides 95% reliability (Kurstak 1985). Sensitivity, specificity, positive and negative predictive values (PPV and NPV, respectively) and their 95% confidence intervals (95% CI) were determined using a 2 x 2 contingency table (Griner et al. 1981).

**RESULTS**

The results of the parasitological examination were: 69 samples were positive for *Giardia* cysts or trophozoites and 127 were negative. In 71 samples parasites other than *Giardia* were found upon microscopic examination. The ELISA test showed 76 samples positive for *Giardia* antigen and 120 negative (Table).

<table>
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<tr>
<th>TABLE</th>
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<tr>
<td>Validation of the ELISA for detection of <em>Giardia duodenalis</em> antigen in fecal eluates using rabbit polyclonal antibodies</td>
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<table>
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<tr>
<th>Parasitological diagnosis</th>
<th>ELISA test</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>(OD ≥ 0.25)</td>
<td></td>
<td>69</td>
<td>7</td>
<td>76</td>
</tr>
<tr>
<td>(OD &lt; 0.24)</td>
<td></td>
<td>0</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>69</td>
<td>127</td>
<td>196</td>
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The cut-off value for positive reactions was established at 0.240, and the parameters of the ELISA for *Giardia* antigen detection were: sensitivity, 100% (95% CI: 93.4-100%); specificity, 95% (95% CI: 88.6-97.6%); positive predictive value, 91% (95% CI: 81.4-95.9%); and negative predictive value, 100% (95% CI: 96.1-100%) (Table).

The production of anti-cyst and anti trophozoite anti-*Giardia* polyclonal antibodies in independent fashion increased the reactivity of antibodies after each successive immunization as evidenced by an increasing intensity of the antigen-antibody precipitin bands as immunizations progressed.

**DISCUSSION**

The immunodiagnostic test developed and described herein for detection of Colombian *Giardia* isolates antigen in fecal eluates shows differences with similar assays developed previously. Enzyme immunoassays use either the direct (Green et al. 1985, Knisley et al. 1989, Dutt et al. 1991, Torres et al. 1997) or indirect methods. The test described herein is based on the direct method with the advantage of using polyclonal anti-*Giardia* antibodies as capture reagent, and the same antibodies linked to an enzyme as conjugate for detecting the reaction. These features allow greater specificity in the antigen-antibody reaction and also simplify the test procedure.

It is possible that optimization of the assay changes with each lot of antibody production. However, the standard conditions for the ELISA test can be maintained by titrating each new polyclonal antibody lot, which can be achieved by determining the optimal antibody concentration which allows the correct discrimination between a positive and a negative sample.

The ELISA for *Giardia* antigen detection is easy to perform, inexpensive, and allows simultaneous processing of multiple samples. The cost of giardiasis parasitological diagnosis by means of examination of multiple fecal samples is similar to that of the ELISA antigen detection assay. However, the ELISA assay developed in the current study will improve the diagnosis of *Giardia* infection because the sensitivity increases from 85% to 100%. In addition, it should improve *Giardia* diagnosis by eliminating false negative results caused by lack of training and experience of the laboratory personnel responsible for microscopic examination, untimely processing of samples, and intermittent excretion of *Giardia* cysts and trophozoites in feces.

This is the first *Giardia* antigen ELISA test developed in Colombia using polyclonal antibodies raised against endemic, homologous isolates of *G. duodenalis*, isolated from Colombian patients with parasitologically confirmed giardiasis. *G. duodenalis* isolates from other geographic regions do not necessarily share the same antigenic determinants. Because of antigenic variations or polymorphisms, antibodies raised against a particular isolate of *Giardia* may not necessarily cross-react with antigens of heterologous *Giardia* isolates from different geographic origin (Nash 1992).

The analytical sensitivity of the assay was not established; however, a previous study showed little correlation between the numbers of cysts or trophozoites observed in fecal samples and the optical density values obtained by the ELISA test for detecting *Giardia* antigens in fecal eluates, possibly because the assay detects free antigen in feces rather than whole organisms (Green et al. 1985).

The ELISA for antigen detection in human fecal eluates will improve diagnosis of *Giardia* infection. When applied under field conditions the test will be useful in determining the prevalence of *Giardia* infection and in making a timely diagnosis of infected individuals, particularly children who suffer from recurrent diarrhea, chronic abdominal pain, malabsorption and stunting as a consequence of infection. Currently, studies concerning the relationship between ELISA results, severity of disease and gastrointestinal symptoms as well as the application of the test in clinical follow-up, are being planned. Future studies should target the extent and degree of het-
erological cross-reactivity of the polyclonal antibodies used in the test with *Giardia* isolates from different geographical origins, because of the antigenic variability which might exist among them.

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