Diagnosis of *Giardia duodenalis* infection in Bangladeshi infants: faecal antigen capture ELISA

Amanda J. Goldin¹, Andrew Hall²*, Rabindra Nath Sarker², David C. Warhurst¹ and Michael A. Miles¹

¹Applied Molecular Biology and Diagnostics Unit, Department of Medical Parasitology, London School of Hygiene and Tropical Medicine, Keppel Street, London, WC1E 7HT, UK; ²International Centre for Diarrhoeal Disease Research, P.O. Box 128, Dhaka 1000, Bangladesh

Abstract

An enzyme-linked immunosorbent assay (ELISA) to detect antigens of *Giardia duodenalis* in faeces was evaluated as a diagnostic tool by testing faecal samples collected during a cohort study of 229 infants living in an urban slum in Dhaka, Bangladesh. Faecal samples had been collected at enrolment, on a routine monthly basis, and repeatedly during episodes of diarrhoea and infection with *Giardia*, and a portion of all samples was frozen in saline. A direct smear of all had been examined by microscopy and again after concentrating cysts by other sedimentation. A total of 2121 of the 4936 samples stored during the 22 months study were tested by the ELISA. After excluding non-specific binding, the sensitivity of the assay was 94.2% and the specificity was 98%. The presence of other parasites, including flagellated protozoa, was not linked to false positive ELISA results. There was a correlation between the number of *Giardia* cysts present and the ELISA optical density. Assuming that the ELISA is 100% sensitive, microscopy detected 92.4% of the infections detected by the ELISA.

Introduction

Several studies have implicated the intestinal protozoan *Giardia duodenalis* (=*G. intestinalis*) as a cause of persistent diarrhoea and growth retardation among children (COLE & PARKIN, 1977; GUPTA & URRUTIA, 1982; FARTHING et al., 1986). However, the epidemiology of *Giardia* is poorly understood, partly due to the perceived lack of a simple and reliable technique to diagnose infections: stool microscopy is considered to be unreliable, usually because the organism is known to be excreted intermittently (BURKE, 1977; GARCIA & BRUCKNER, 1988). A sensitive and specific enzyme-linked immunosorbent assay (ELISA) to detect small amounts of antigen in faeces has the potential to detect infections when neither cysts nor trophozoites are being excreted in the stools, and may offer an efficient diagnostic technique for large scale epidemiological studies of infections with *Giardia*. An ELISA to detect antigens in faeces has had limited application in the diagnosis of infections with *Giardia* in hospital patients in both North America (ADISS et al., 1991) and the United Kingdom (GREEN et al., 1985; ALLISON et al., 1988), to detect experimental human infections (NASP et al., 1987), to study waterborne outbreaks of *Giardia* in England and Sweden (GREEN et al., 1990; WIENNECKA et al., 1989) and an earlier study involving 3 of us used an ELISA to estimate the prevalence of *Giardia* among schoolchildren in the city of Santiago, Chile (GOLDIN et al., 1990).

*Giardia* is endemic in Bangladesh and the reported prevalence of infection ranges from 4% to 21% depending on the methods of examining stool samples and on whether the stools came from healthy individuals or from people with diarrhoea (BLACK et al., 1980, 1982; HOSAIN et al., 1983; GILMAN et al., 1985; STANTON et al., 1989). A study of a cohort of Bangladeshi mothers and their children from birth revealed that 42% of children were infected at least once in their first year of life (ISLAM et al., 1983), while 2 scrological surveys in Bangladesh have shown that specific serum antibodies are not a reliable marker of current infection (ISLAM et al., 1983; GILMAN, 1985). All of these studies used microscopy to diagnose infections.

A prospective study by one of us (A. H.) on the impact of infections with *Giardia* on the growth of Bangladeshi infants provided an opportunity to assess the usefulness of an antigen capture ELISA as an epidemiological and diagnostic tool, and to evaluate its sensitivity and specificity under field conditions.

Subjects and Methods

The subjects

The 229 children taking part in this study were mainly Bihari refugees (see WHITAKER et al., 1982) living in a slum in Mirpur, a suburb of Dhaka, Bangladesh. Participation was by informed consent given by each child's mother, and the study had been approved by the Ethical Review Committee of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B).

All the subjects were reported to be between 2 and 8 months old when they were recruited in December 1987 for the study, which lasted 22 months. Stool samples were collected from children for microscopic examination as follows: at enrolment; routinely once a month if the child was healthy; 48 h after the onset of diarrhoea, when a sample was subjected to microbiological culture; 14 days after the onset of diarrhoea if it persisted, also for microbiological culture and, if *Giardia* had been diagnosed, once every 2 weeks until treatment was given. Faecal samples collected because children had diarrhoea were cultured for species of *Shigella*, *Salmonella* and *Vibrio*, and for enterotoxigenic *Escherichia coli*, using standard techniques (WHO, 1987).

Each faecal sample was collected freshly in a plastic bottle and its consistency was recorded. The bottle was sent to the laboratory of the ICDDR,B about 8 km away where each stool was examined microscopically within 6 h of collection by direct smear and concentration methods. A direct smear was prepared in saline (0.9% w/v aqueous NaCl) and examined microscopically; the number of cysts or trophozoites of *Giardia* was estimated semi-quantitatively as few, moderate or many. About 1 g of faeces was also fixed in a weighed bottle containing 10 ml of 10% v/v formalin in saline and weighed again in order to estimate the weight of faeces collected. The sample was then processed by a quantitative ether sedimentation technique and the sediment was examined microscopically for the eggs and larvae of helminths and for the cysts of protozoa (HALL, 1981). The number of cysts of *Giardia* was also estimated semi-quantitatively as few, moderate or many. Stools in which *Giardia* was seen are termed microscopy positive (+), while stools in which *Giardia* was not seen are termed microscopy negative (-).

Most infections with *Giardia* were asymptomatic and stool samples were collected every 2 weeks to monitor the course of the infection. Children were treated with metronidazole (375 mg/d in 3 divided doses for 5 d) for any of the following reasons: if the mother was concerned about her child's health, if the project physician considered that the child's clinical condition warranted treatment because, for example, the child was mald-
nourished; or if, in the long term, the child failed to grow. All other diseases and complaints, such as diarrhoea and respiratory tract infections, were treated appropriately. The details of this part of the study will be reported elsewhere (A. Hall, paper in publication).

The stool specimens

In addition to the microscopical examination of stools, a suspension of about 10% faeces in 0.9% saline was prepared from each fresh stool sample collected during the study. These suspensions of unfiltered faeces were placed in screw-topped cryotubes (Nunc, Denmark) labelled with the stool serial number and the child’s identification number, and stored at -20°C.

Because of limited amounts of reagents available, not all faecal samples collected during the study could be tested by the ELISA, so the following categories of samples were selected in the following order of priority for the cases in which the categories overlapped.

(i) Samples in which Giardia was seen by microscopy, in order to assess the sensitivity of the ELISA.
(ii) Stools collected from each child before it was treated with metronidazole, in order to determine whether the infection was still present before treatment.
(iii) One or 2 stools collected from each child after it had been treated with metronidazole, in order to confirm that organisms were undetectable.
(iv) All samples containing other species of protozoa, such as Chilomastix mesnili or Trichomonas hominis, in order to assess the specificity of the ELISA.
(v) All stools collected from children because they had diarrhoea, in order to assess whether microscopy missed infections with Giardia that caused diarrhoea.
(vi) All stools collected from 5 children who never had diarrhoea during the study and were never found to be infected with Giardia, in order to assess whether microscopy had missed infection.
(vii) A random sample of the remaining stools, chosen using a table of random numbers.

All the stools were identified only by their serial numbers when tested, and collectively they provided a test of the sensitivity and specificity of the ELISA compared with careful microscopy, and vice versa, as well as answering specific questions about the assay and the subjects from which the samples were collected.

Enzyme-linked immunosorbent assay

Anti-Giardia antibodies were raised in rabbits against axenically grown trophozoites and cysts purified from faeces and were affinity-purified as described previously (GOLDIN et al., 1990). Horseradish peroxidase (HRP)-conjugated anti-Giardia antibodies and non-immune rabbit immunoglobulin G (IgG) were also prepared and all reagents were transported to Bangladesh on ice. All other solutions were prepared freshly in Bangladesh at the time the samples were tested, in March 1990.

In order to assess the contribution of non-specific binding to the immunoglobulins used in the assay, rather than IgG antibodies, all samples in duplicate using immune serum (IS) and non-immune serum (NIS) capture wells, all samples were first tested in duplicate using only IS capture wells. Any sample considered to be positive by visual inspection of the wells or by spectrophotometry, in comparison with the controls on each plate, was retested by adding each sample and controls to one IS coated well and one NIS coated well. Dynatech Immulon 1® ELISA plates were coated with 200 μL of affinity-purified pooled anti-Giardia serum or 200 μL of non-immune rabbit IgG, both prepared at a concentration of 10 μg/mL in carbonate coating buffer (pH 9.6). The plates were incubated overnight at 4°C, washed 3 times in phosphate-buffered saline (pH 7.2) coating (0.05%) Tween 20® (PBST), and then blocked for 1 h at 37°C with 300 μL of 50% foetal calf serum (FCS) in coating buffer (GOLDIN et al., 1990). After being washed 3 times with PBST, equal volumes of FCS and the stool suspensions were added to a well coated with IS and/or a well coated with NIS and incubated for 2 h at room temperature. Controls were provided by a stool sample known to contain Giardia (positive control) and one known to be free of Giardia (negative control), as well as controls of 50% FCS in PBS, 50% FCS in saline, and trophozoite antigen. Each plate was then washed 3 times in PBST, affinity-purified rabbit anti-Giardia HRP conjugate (1:50 in 50% FCS/PBST) was added, and the plates were incubated for 2 h at room temperature. The assays were developed as described by GOLDIN et al. (1990) and the result was assessed visually and spectrophotometrically using a Titertek Multispan Plus® plate reader at a wavelength of 492 nm to determine optical density (OD).

Data processing

The cut-off point for all samples tested for Giardia using only the IS capture wells was calculated by finding the standard deviation (SD) of the duplicate PBS controls: any observations 3 standard deviations or more above this point were considered to be positive. For the repeated tests using IS and NIS capture wells an OD ratio was calculated for each sample by dividing OD values for IS capture wells by OD values for NIS capture wells. The cut-off point was taken to be 3 times the SD above the mean ratio of PBSIS and PBSNIS controls. Stools in which Giardia was detected by the ELISA are termed ELISA positive (E+), while stools in which Gia-

The predictive value of the ELISA was estimated (VEC CHIO, 1966) using the mean monthly prevalence of infection with Giardia of 9.9%. The predictive value was also estimated using the f index (YOUDEN, 1950), which does not require an accurate prevalence.

Results

In all, 4936 stools from 229 children were stored frozen during the study, and 2212 samples (43.0%) from 227 children were tested 'blindly' by the capture ELISA.
Table. The results of microscopical examination of different categories of faecal samples selected for testing in an antigen capture ELISA for *Giardia duodenalis*

<table>
<thead>
<tr>
<th>Category</th>
<th>No. of samples tested</th>
<th>True positive M+E+</th>
<th>True negative M+E-</th>
<th>False positive M+E+</th>
<th>False negative M+E-</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Giardia</em> seen by microscopy</td>
<td>483</td>
<td>455</td>
<td>0</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>Stools collected before treatment but M-</td>
<td>240</td>
<td>0</td>
<td>220</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Stools collected after treatment and M-</td>
<td>419</td>
<td>0</td>
<td>410</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Other species of protozoa seen by microscopy</td>
<td>57</td>
<td>0</td>
<td>57</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Stools from children with diarrhoea but M-</td>
<td>685</td>
<td>0</td>
<td>682</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Stools from children who never had diarrhoea or <em>Giardia</em></td>
<td>114</td>
<td>0</td>
<td>113</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Random selection of the remaining stools</td>
<td>123</td>
<td>0</td>
<td>123</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Totals</td>
<td>2121</td>
<td>455</td>
<td>1605</td>
<td>33</td>
<td>28</td>
</tr>
</tbody>
</table>

*M+ =* *Giardia* seen by microscopy; *M- =* *Giardia* not seen by microscopy; *E+ =* positive ELISA; *E- =* negative ELISA.

The Table shows the categories of samples and the results of both microscopy and the ELISA. Assuming that microscopy does not give false positive results, the ELISA failed to detect 28 infections, results which may be defined as false negatives. These samples were more likely to contain fewer cysts after ether sedimentation than true positive samples (Fisher’s exact test, *P* = 0.009). The ELISA detected 33 infections which had not been diagnosed by microscopy; these results may be defined as false positives, but may in fact comprise samples in which *Giardia* was correctly detected by the ELISA although not seen by microscopy. The Table shows that 20 of these false positive samples (61%) were associated with an episode of infection, indicating that *Giardia* was detected by the ELISA before the infection was diagnosed by microscopy. Nine other samples (27%) were collected after treating a case of infection and probably reflect treatment failures. The remaining 4 samples were not associated with infections and may be considered to be true false positive results. The presence of helminths such as *Ascaris lumbricoides* and *Trichuris trichura*, of red blood corpuscles, of bacterial pathogens such as species of *Shigella*, *Vibrio* or *Salmonella*, and of parasitic protozoa such as *Entamoeba histolytica*, *E. coli* and the flagellate *Trichomonas hominis* (*n* = 32), were not found to be associated with either false positive or false negative results in the ELISA. However, yeast cells were found more frequently in false negative samples than in both true positive (*P* < 0.05) and true negative samples (*P* < 0.01). There was no association between the proportions of false positive or false negative results and the duration that samples had been stored. This indicates both that there was no significant deterioration in the antigen with time and that there was no change in the likelihood of detecting an infection by microscopy as a result of experience in diagnosing *Giardia*.

**ELISA performance**

There was no significant difference in the variation of results between the 3 types of negative control. There was, however, significant variation between controls run on different plates (plate-to-plate variation) and on different days (batch-to-batch variation) (*P* < 0.01). The variation was taken into account when calculating cut-off values.

Using antigen capture with IS only, the ELISA showed a sensitivity of 94.8% and a specificity of 70%. When non-specific binding had been accounted for by repeating the ELISA using IS and NIS capture wells, the assay gave a sensitivity of 94.2% and a specificity of 98.0%. In comparison, the sensitivity of a visual assessment was 93.8% and the specificity was 97.7%.

The distribution of the values for the OD ratio (sample OD for IS capture wells divided by the sample OD for NIS capture wells) for all retested samples is shown in Fig. 1. The relationship between the mean OD ratio and the semi-quantitative estimate of the number of cysts in faeces assessed by direct smear and concentration techniques is shown in Fig. 2.

The predictive value of a positive test (Vechio, 1966) was calculated as 83.8% and that of a negative test as 99.4%. The 7 index (Youden, 1950) gave a predictive value of 0.922.

**Sensitivity of microscopy**

Assuming the ELISA to be 100% sensitive and specific,
Discussion

This study has shown that an antigen capture ELISA using affinity-purified polyvalent antiserum raised to both cysts and trophozoites was a sensitive and specific diagnostic test to detect antigens of Giardia duodenalis in stools collected from children in Bangladesh. The microscopic examination of faeces detected Giardia in 483 of the 2121 faecal samples tested; the ELISA did not detect Giardia antigen in 28 of these specimens, all of which had been found by microscopy to contain only a few cysts or trophozoites. An earlier study in Chile had also shown that false negative stools also tended to contain few cysts (GOLDIN et al., 1990). The correlation between the semi-quantitative assessment of the number of cysts and the OD ratio suggests a relationship between the number of cysts and the antigen captured by the assay, but also suggests that there may be stools in which cysts are present but there may be insufficient antigen to give a positive result by the ELISA. An alternative explanation may lie in the possibility that there are differences between the strains of Giardia used to generate antibodies and those present in Bangladeshi children; there is evidence of marked heterogeneity in the genetic and antigenic characteristics of different strains (NASR, 1992). Significantly more false negative samples than true positives were seen to contain yeast cells (P<0.05).

After non-specific binding had been accounted for, the ELISA also gave positive results with 33 of the 1638 samples in which Giardia had not been seen by microscopy. Twenty-nine of these samples were associated with Giardia infections, either because previous samples had been seen to contain Giardia and the subjects had not yet been treated or because a known Giardia infection had just been treated. Giardia antigen is excreted for a few days after treatment (GREEN et al., 1985; NASH et al., 1987). These results suggest that there were only 4 truly false positive results among the 2121 samples tested, and that the ELISA was able to detect Giardia antigen even when cysts or trophozoites had not been seen by careful microscopy of fresh and concentrated stool specimens. There was no evidence of cross-reaction between the capture antibody and other organisms, including other protozoa such as the flagellate T. hominis.

The microscopic examination of faeces is generally considered to be an unreliable means of diagnosing Giardia: it has been reported the cysts or trophozoites may not be found in the stools of between 10% and 50% of infected individuals (BURKE, 1977), perhaps because they are excreted intermittently. If it is assumed that an ELISA can detect very small amounts of soluble or particulate antigen when cysts or trophozoites are either extremely scanty or are not being shed, then this study indicates that careful microscopic examination of both fresh and concentrated faecal samples can detect all but a few infections. In fact, if the assay is looked at in terms of the efficiency of microscopy compared with the ELISA, microscopy detected 92.4% of the infections detected by the ELISA (sensitivity) and should have been 100% specific, because Giardia has a characteristic, unique morphology. The sensitivity of the diagnosis depends largely on the skill of the microscopist, which is probably related to experience and the frequency with which the parasite is encountered. The fact that there was no relationship between false results and the age of the specimen suggests that the microscopist (R.N.S.) was consistent in detecting Giardia throughout the 22 months study.

Two types of error may affect the performance of an ELISA: operator error and variations due to changes in ambient conditions such as temperature. The ELISA showed good precision with duplicate samples tested on the same plate, but significant variation between plates and from batch to batch. This variation was probably due to differences in exposure to light during incubation of the photosensitive substrate used in the assay, using a substrate insensitive to light might reduce this variability. Plate-to-plate and batch-to-batch variation was considered when calculating the values to establish cut-off points, but applying simpler procedures (see GOLDIN et al., 1990) gave similar values for sensitivity (94.4%) and specificity (97.6%) and, more importantly, the results of the assay on individual samples were not changed.

Previous studies with an antigen capture ELISA for Giardia have shown the degree of non-specific binding to IgG to be insignificant (ALLISON et al., 1988; GOLDIN et al., 1990). In the present study, however, 490 of 1638 of microscopically negative samples (30%) gave false positive ELISA results before non-specific binding had been accounted for by retesting samples with NIS as well as IS capture wells, a procedure which considerably improved the specificity from 70% to 98%. The causes of the non-specific binding are uncertain but stool samples may contain a variety of factors which can generate it (YOLKEN & UNGAR, 1985), while the specificity may decline with the duration of storage (GOLDIN et al., 1991). In the present study no relationship was observed between the duration of storage and the number of false results.
Both the techniques used to diagnose infections with *Giardia* in the present study have advantages and disadvantages. Microscopy is quick and relatively simple and gives a precise and reliable diagnosis, although infections may be missed; an antigen capture ELISA is an efficient way to test a large number of samples for the purpose of screening and it may sometimes be able to detect antigen when no cysts or trophozoites are seen in faeces. A further valuable simplification of the capture ELISA would be adaptation to a dip-stick format. The techniques of microscopy and an antigen capture ELISA, used here in a large epidemiological study in a developing country, suggest that the combination of methods could prove to be a useful means of studying the natural history of infections with *Giardia*.

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