SHORT REPORT: DIAGNOSIS OF HUMAN FASCIOLIASIS: DETECTION OF ANTI-CATHEPSIN L ANTIBODIES IN BLOOD SAMPLES COLLECTED ON FILTER PAPER

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Abstract. We have developed an ELISA for the diagnosis of human fascioliasis based on the detection of IgG4 antibodies to Fasciola hepatica cathepsin L1. Using this diagnostic assay, we confirmed earlier reports of a high incidence (22% of the population analyzed) of human fascioliasis in Calasaya, a village in the northern Altiplano of Bolivia. Further studies to examine the extent of this disease in the Altiplano region were hampered by the reluctance of the indigenous Aymaran population to provide blood. To overcome this problem, we have investigated the method of collecting small quantities of blood from the finger onto filter paper, followed by the elution of antibodies for use in the diagnostic assay. Serum samples and blood samples collected onto filter paper were obtained from 57 individuals living in the village of Cutusuma in 1987 and from 11 individuals in Chijipata in 1996. Analysis of the IgG4-ELISA results revealed that there is highly significant linear relationship ($P < 0.001$) between the two methods of sampling. Most importantly, a reliable diagnosis was made with the blood-filter samples from Cutusuma, which had been stored for 10 years at 4°C. While some deterioration of the blood-filter samples from Cutusuma had occurred over the 10-year storage period, no deterioration occurred with the Chijipata samples, which were stored for one year. Therefore, the method of collecting blood onto filter paper should prove useful for large-scale epidemiologic studies on human fascioliasis in the Bolivian Altiplano and in other regions where this disease is prevalent.

Recently, our laboratory reported the use of an ELISA for diagnosing human fascioliasis. The assay detects serum IgG4 antibodies reactive with Fasciola hepatica cathepsin L1. Using this diagnostic assay, we confirmed earlier reports of a high incidence (22% of the population analyzed) of human fascioliasis in Calasaya, a village in the northern Altiplano of Bolivia. Further studies to examine the extent of this disease in the Altiplano region were hampered by the reluctance of the indigenous Aymaran population to provide blood by venipuncture. This difficulty prompted us to explore the simpler and less invasive method of collecting samples onto filter paper after lancet-pricking of the finger. The method had already been successfully used at the Nacional de Laboratorios de Salud (INLASA) for obtaining blood for the serologic diagnosis of Chagas disease. In the present report, we have investigated the use of this method for obtaining samples for the diagnosis of human fascioliasis.

Serum was obtained from 57 individuals living in Cutusuma (a village approximately 10 km from Calasaya) during a survey in August 1987 and stored frozen in the serum library of INLASA. Samples of blood were also collected onto filter paper and stored at 4°C. During a field trip in February 1996, samples of blood were obtained from 11 individuals residing in Chijipata Alto (1 km from Cutusuma) by venipuncture (from which serum was later obtained) and spotted onto Whatman filter paper no. 1 (Whatman International Ltd., Maidstone, United Kingdom). Coprologic analysis was performed on fecal samples obtained from the 57 individuals from Cutusuma using a sedimentation technique and on samples obtained from eight of the individuals from Chijipata Alto using the Kato-Katz method. The nature of the study was explained to each individual, and prior to volunteering to provide blood and feces, each person signed a document of consent that also included their name and personal details. The study was approved by the Human Ethics Committee of INLASA and the Department of Health of Bolivia.

Two circles 5 mm in diameter were cut from the blood-stained filter paper and antibodies were eluted by soaking these in 250 μl of phosphate-buffered saline. A soaking time of 1 hr at room temperature was sufficient to elute the antibodies from the filter paper in the case of the Chijipata Alto samples. However, in the case of the Cutusuma samples, which had been stored for 10 years, a longer period of soaking was necessary; the optimum recovery of antibodies was obtained after three or more days at 4°C. An antibody dilution of 1:100 was assumed for these eluates. The IgG4-ELISA was performed as described by O’Neill and others with serum dilutions of 1:125 and blood-filter eluate dilutions of 1:100. The within-assay mean coefficients of variation for the ELISA using serum or blood-filter samples were 8.86% and 9.5%, respectively. The between-assay variations was always less than 15% in both cases. The cathepsin L1 proteinase used as antigen was purified as outlined by Smith and others. The statistical analysis of the IgG4-ELISA results was carried out using SPSS for Windows (SPSS Inc., Chicago, IL).

To show that our IgG4-ELISA could be used as a reliable assay for the detection of human fascioliasis, we initially tested gold standard positive and negative serum samples. Serum from parasitologically proven positive individuals (egg positive) from Cutusuma and Chijipata were compared with risk-free negative controls (eight volunteers from Dublin City University and INLASA). Two egg-positive individuals with unusually low serum absorbance were omitted from this analysis since it was highly likely that they were misdiagnosed. The absorbance readings of the gold standards are shown in Figure 1a. For the gold standards, the difference in serum absorbance between the positive and negative samples was highly significant ($P < 0.01$) with no overlap between them; the maximum absorbance of the uninfected was 0.146 while the minimum absorbance of the infected was 0.34. Therefore, the sensitivity of the assay was 100%, which strongly validates this serologic method for detection of infection. Since the seronegative samples represent a normally distributed subpopulation, 99.9% of them will be incorporated below 3.09 standard deviations from the mean absorbance value for these (0.197). All samples that are above this cut-off point would therefore be considered
seropositive and thus represent infections (dashed horizontal line in Figure 1a).

There are problems, however, associated with surveys in regions endemic for fascioliasis, such as the Bolivian Altiplano, since coprologic methods are incapable of detecting the early acute stage of infection. We therefore analyzed the sera of all coprologically negative individuals from Cutusuma and Chijipata by the IgG4-ELISA. We then used a statistical method, hierarchical agglomerative cluster analysis,\(^{1,6}\) to identify subpopulations that may represent individuals that were exposed or not exposed to fasciolosis (Figure 1b). This analysis separated the population into a low absorbance group, which represents the nonexposed individuals, and a high absorbance group, which represents the exposed individuals. The cut-off values are then set at 3.09 standard deviations from the mean absorbance value of the statistically determined nonexposed individuals. This cut-off point (0.2035) was indistinguishable from the value determined by our gold standard negative samples and thus confirmed the validity of our statistical method for setting the cut-off values. It should be noted that all coprologically positive individuals were correctly identified by this statistical method.

The absorbance readings obtained by the IgG4-ELISA using the serum samples and blood-filter samples for each individual from Cutusuma were plotted against each other on a scattergram (Figure 2a). The cut-off points determined by the statistical method were 0.2035 and 0.173 for the serum and blood-filter assays, respectively. Using these cut-off points, 41 individuals were seropositive for fascioliasis and 16 were seronegative by analysis of serum and seronegative by analysis of blood-filter samples. Figure 2b, Scattergrams of the data obtained by the IgG4-ELISA using serum and blood-filter samples obtained from individuals from the village of Chijipata Alto (1996). The vertical and horizontal lines indicate the calculated cut-off points for the serum and blood-filter samples, respectively. Closed circles represent individuals who were coprologically positive and open circles represent those who were coprologically negative. The squares represent the three individuals for which no coprologic analysis was available.
lowest readings of the group that were seropositive by analysis of serum and could be better described as borderline cases (Figure 2a). A highly significant relationship was observed between the results obtained with both sampling methods ($r^2 = 0.848$, $P < 0.001$). Chi-square analysis showed that it is highly unlikely that such agreement between these two methods occurred by chance ($P < 0.001$).

All copropositively positive individuals, with the exception of two, were associated with the copropositive cluster (Figure 2, solid circles). These two individuals were $3.61 \text{ standard}$ deviation away from the mean serum absorbance of the other copropositively positive individuals and are highly likely to have been misdiagnosed. Chi-square analysis verified that the individuals with a positive coprologic diagnosis were significantly ($P < 0.001$) concentrated in the high absorbance cluster, which reinforced the designation of this group as the infected individuals. On the other hand, 22 (38.6%) and 17 (29.8%) individuals that were copropositively negative were positive by the IgG4-ELISA using the serum and blood-filter samples, respectively (Table 1). Several factors may explain this high proportion of seropositive/copropositively-negative individuals. First, it is possible that these individuals harbored early, prepatent, infections; thus, they were not always found in the feces of chronically infected individuals. Of the six coprologically negative individuals, two (3.5%) were also coprologically positive for $F. \text{hepatica}$ eggs, but no feces sample was obtained from the remaining two individuals. Of the six copropositively negative individuals, five were also serologically negative and the remaining individual did not provide a fecal sample (Table 1). It is clear from a comparison of the slope of the regression lines that some deterioration of the blood-filter samples from Cutusuma had occurred over the 10-year storage period. In contrast, the absorbances obtained with the serum samples from both Cutusuma and Chijipata were similar (Figure 2).

The results presented in this report supports the usefulness of the IgG4-ELISA, which uses $F. \text{hepatica}$ cathepsin L1 as antigen, as a diagnostic test for the sensitive detection of human fascioliasis. Moreover, we have demonstrated that there is no significant statistical difference between the results obtained with serum derived from whole blood samples or eluates from blood samples dried onto filter paper. Our analysis of the samples from Cutusuma shows that blood samples collected onto filter paper can be stored for up to 10 years at $4^\circ C$ and still provide a reliable means of detection. Therefore, the filter assay is an excellent innovation and could be a useful tool for a large-scale epidemiologic study to determine the extent of human fascioliasis in the Bolivian Altiplano and in other regions where the disease is suspected to exist. This method is not only cost-effective, an important consideration for carrying out such studies, but also facilitates large-scale screening within a relatively short time. Finally, we have shown that hierarchical agglomerative cluster analysis is a useful statistical method for the analysis of results obtained using our serologic assay for human fascioliasis.

Acknowledgment: We thank Dr. A. Dowd for providing cathepsin L1.

Financial support: This research was funded by Dublin City University, the Irish-American Partnership, and Commission of the European Community Program in Life Sciences and Technologies for the Developing Countries (STDC) (contract no. TS3-CT94-0294).

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REFERENCES

**Table 1**

Summary of coprologic and serologic (serum and blood filter samples) data for individuals from Chijipata and Cutusuma, Bolivia

<table>
<thead>
<tr>
<th>Group</th>
<th>Chijipata*</th>
<th>Cutusuma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum Filter</td>
<td>Serum Filter</td>
</tr>
<tr>
<td>Seropositive/egg+</td>
<td>3/3</td>
<td>19 (33.3%)</td>
</tr>
<tr>
<td>Seropositive/egg−</td>
<td>0/0</td>
<td>22 (38.6%)</td>
</tr>
<tr>
<td>Seronegative/egg+</td>
<td>0/0</td>
<td>2 (3.5%)</td>
</tr>
<tr>
<td>Seronegative/egg−</td>
<td>5/5</td>
<td>14 (24.6%)</td>
</tr>
<tr>
<td>Total</td>
<td>8/8</td>
<td>57</td>
</tr>
</tbody>
</table>

* Only eight of the 11 individuals at Chijipata provided serum, blood filter, and fecal samples.