IMMUNODIAGNOSIS OF FASCIOLA HEPATICA INFECTION (FASCIOLIASIS) IN A HUMAN POPULATION IN THE BOLIVIAN ALTIPLANO USING PURIFIED CATHEPSIN L CYSTEINE PROTEINASE

SANDRA M. O'NEILL, MICHAEL PARKINSON, WILMA STRAUSS, RENE ANGLES, AND JOHN P. DALTON
School of Biological Sciences, Dublin City University, Dublin, Ireland; Unidad de Parasitología, Instituto Nacional de Laboratorios de Salud (INLASA), Secretaría Nacional de Salud, Pasaje Rafael Subieta No. 1889, Miraflores, La Paz, Bolivia

Abstract. Cathepsin L1 (CL1), an immunogenic cysteine proteinase secreted by juvenile and adult Fasciola hepatica, was assessed for its potential as a diagnostic agent for the serologic detection of human fascioliasis. Using ELISAs, we compared the ability of liver fluke homogenates (LFH), excretory/secretory (ES) products, and CL1 to discriminate between seropositive (infected) and seronegative (noninfected) individuals within a population of 95 patients from the Bolivian Altiplano. A high prevalence of human fascioliasis has been reported in this region. The division between the seropositive and seronegative individuals was poorly defined when LFH was used as the antigen. A greater discrimination between these populations was achieved with both ES and CL1. A K-means cluster analysis using the combined ES and CL1 ELISA data identified a cluster of seropositive individuals. Cathepsin L1 detected a subset (20) of these seropositive individuals while ES detected all 26; however, ES detected nine additional individuals that were in the seronegative cluster. The ratio of the mean absorbance readings between seropositive and seronegative individuals was markedly improved by using conjugated second antibodies to IgG4, the predominant isotype elicited by infection. In these IgG4-ELISAs, CL1 again identified fewer individuals as seropositive than did ES, but improved the discrimination between the seropositive and seronegative individuals and thus provided a more conclusive diagnosis. Sera obtained from patients infected with schistosomiasis mansoni, cysticercosis, hydatidosis, and Chagas’ disease were negative in these assays, which demonstrated the specificity of the IgG4-ELISA for detecting fascioliasis. Twenty of the 95 patients (21%) were seropositive for fascioliasis by the CL1 IgG4-ELISA, confirming the earlier reports of the high prevalence of disease in this region. A standardized diagnostic test for human fascioliasis, based on an ELISA that detects IgG4 responses to CL1, could be available to all diagnostic centers if sufficient quantities of recombinant CL1 can be produced.

The trematode Fasciola hepatica is a causative agent of liver fluke disease. The disease of ruminants has a worldwide distribution and results in major economic losses in agricultural communities. Research into human fascioliasis has not been extensive because it was previously believed to be restricted to isolated outbreaks, such as those reported in Iran,1 Peru,2 Cuba,3 and Bolivia.4–6 However, reports of infection are steadily increasing in many countries.7 Hillyer and others4 and Bjorland and others5 were the first to report a high prevalence of human fascioliasis in the Altiplano region of northern Bolivia where > 60% of the individuals tested showed seropositivity against liver fluke antigens.

The clinical manifestations of fascioliasis in humans include fever, right hypochondrial pain, persistent diarrhea, and vomiting.8 Diagnosis of the disease is by the identification of F. hepatica eggs in fecal samples. Since eggs appear in the feces only after the parasite has entered the bile duct and matured (approximately 12 weeks after infection), early infections cannot be diagnosed coprologically. In addition, because eggs are released sporadically from the bile ducts, an accurate diagnosis by this method requires at least two stool samples obtained at different times on consecutive days.

The detection of anti-fluke antibodies in serum by ELISA is considered a sensitive and reliable means of diagnosing acute infections and can also be used as an adjunct to fecal analysis for the diagnosis of latent and chronic infections.4 Previously developed ELISA methods have used crude somatic extracts of parasites or a preparation of molecules secreted by the parasite when cultured in vitro (excretory/secretory [ES] products) as antigen for the detection of serum antibodies.1–7,9 However, the use of complex antigen preparations can result in a reduced specificity of the assays since many parasites share similar immunogens.

We isolated one of the major molecules in the ES products of F. hepatica parasites and characterized it as a cysteine proteinase, cathepsin L1 (CL1).10 Since the molecule is highly immunogenic in infected animals, we considered it potentially useful for the sensitive and specific immunodiagnosis of human fascioliasis. In the present study, we have analyzed and compared the suitability of crude parasite extracts, ES products, and purified CL1 as antigens in ELISAs for the serologic detection of human fascioliasis. Crude parasite extracts failed to adequately separate seropositive from seronegative individuals in a population from the Bolivian Altiplano, whereas both ES and CL1 were more discriminatory. Since the predominant antibody isotype elicited by liver fluke in humans is IgG4, we developed ELISAs with a very high degree of sensitivity based on the detection of IgG4 responses to ES or CL1. The specificity of these assays for fascioliasis was demonstrated using sera obtained from individuals infected with schistosomes mansoni, cysticercosis, hydatidosis, and Chagas’ disease. Our data demonstrate that an ELISA based on the detection of IgG4 responses to F. hepatica CL1 could be developed as a standardized immunodiagnostic test for human fascioliasis.

MATERIALS AND METHODS

Reagents. Microtiter plates were obtained from Nuclon (Kamstrup, Roskilde, Denmark). Peroxidase-conjugated anti-human immunoglobulin, peroxidase-conjugated avidin, biotin-conjugated anti-human monoclonal antibodies, and the substrate 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic
adult [ABTS]) were obtained from the Sigma Chemical Company (Poole, Dorset, United Kingdom). Roswell Park Memorial Institute 1640 medium (RPMI 1640) was obtained from Gibco Life Technologies Ltd. (Paisley, Scotland, United Kingdom). The bicinchoninic acid (BCA) protein assay reagent kit was supplied by Pierce and Warriner (Chester, United Kingdom).

**Serum samples and coprologic analysis.** Serum samples were obtained on December 16, 1991 from 95 native Aymara (47 males and 48 females) living in Calysaya, a small village northwest of La Paz, Bolivia. The age of the volunteers ranged between one and 85 years, with a mean ± SD age of 31.4 ± 19.9 years. Coprologic analysis for *F. hepatica* eggs were performed on a single feces sample obtained from 58 individuals in this group using the Kato-Katz method. In addition, 91 samples were serologically analyzed for cysticercosis by ELISA using soluble extracts of *Taenia solium* as previously described. This study was approved by the Human Ethics Committee of INLASA and by the Department of Health, Bolivia. 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 carried their name and personal details.

Serum samples obtained from patients with schistosomiasis mansoni (20), cysticercosis (15), hydatidosis (15), and Chagas’ disease (15) were obtained from the INLASA serum library. Control serum samples were provided by eight volunteers at Dublin City University.

**Preparation of antigens.** Adult liver flukes were obtained from the infected livers of cattle at a local abattoir. The flukes were washed in phosphate-buffered saline (PBS), pH 7.3, and homogenized in a Thyristor Regler TR homogenizer (Janke and Kunkel, Staufen, Germany). The homogenate was centrifuged at 13,000 × g for 30 min and the supernatant (liver fluke homogenate [LFH]) was stored in PBS at −20°C. Excretory/secretory products were prepared as described by Dalton and Heffernan. Briefly, adult liver flukes were cultured for 16 hr in RPMI 1640, pH 7.3, containing 2% glucose, 30 mM HEPES, and gentamicin (25 mg/ml) at 37°C. The culture medium was then removed, centrifuged at 13,000 × g for 30 min, and the supernatant was concentrated using an Amicon (Danvers, MA) 8400 Ultrafiltration Unit with a Ym3 membrane (3,000 molecular weight cut-off). Cathepsin L1 was purified from the ES products as previously described. The concentration of each antigen preparation was measured using a BCA protein assay kit.

**Enzyme-linked immunosorbent assay.** One hundred microliters of LFH, ES or CL1 antigen (50 μg/ml, 20 μg/ml, and 5 μg/ml, respectively) was dispensed into the wells of microtiter plates that were then incubated overnight at 37°C. Excess protein binding sites were blocked at 37°C for 30 min by adding 200 μl of 2% bovine serum albumin diluted in PBS/0.1% Tween 20 to each well. After the wells were washed three times with PBS/0.1% Tween 20, human sera (diluted 1:250) was added and the plates were incubated for 30 min at 37°C. Following another wash, 200 μl of peroxidase-conjugated anti-human IgG (diluted 1:8,000) was added to each well and the plates were incubated for an additional 30 min at 37°C. After another washing step, bound antibodies were detected by the addition of 100 μl of the ABTS (10 mg/100 ml) in phosphate citrate buffer, pH 5.0. After the color had developed for 10 min, the plates were read on a Titereck multiscan (EFLAB, Helsinki, Finland) at 405 nm.

Sera from 26 individuals that showed seropositivity for *F. hepatica* infection by the above ELISA method were selected for an analysis of the isotypic responses of humans to liver fluke ES and CL1 antigens. In this assay, bound antibodies were detected by adding 200 μl of biotin-conjugated anti-human IgG1, IgG2, IgG3, or IgG4 monoclonal antibodies (diluted 1:1,000, 1:4,000, 1:4,000, and 1:1,000, respectively) to each well for 30 min at 37°C. The binding of the secondary antibody was visualized with 200 μl of avidin-conjugated peroxidase (diluted 1:8,000) and the substrate ABTS. All assays were tested in triplicate.

**Statistical analysis.** All statistical analysis was carried out using SPSS for Windows® (SPSS, Inc., Chicago, IL). The K-means cluster analysis was carried out to separate the ELISA data into two subpopulations. Euclidean distances between pairs of data points were first calculated using the combined, unweighted absorbances due to two antigen preparations (ES and CL1). Two initial cluster centers were derived from the Euclidian distances and class assigned to clusters by a process of iteration and classification using a maximum of 10 iterations, and a convergence criterion of 0.02. These parameters correspond to the default settings for the process in SPSS.

**RESULTS**

**Analysis of human serum samples by ELISA.** The serum samples obtained from 95 Aymarans were analyzed by ELISA for total antibody responses against LFH, ES, and CL1. The population was then examined by plotting the frequency of absorbancy measurements, grouped into blocks of 0.05 absorbance units, as histograms (Figure 1). The frequency distribution of the absorbances obtained with all three antigens was bimodal. The largest mode situated to the left of the histograms represents the seronegative population and the second mode located to the right represents the seropositive population. The histogram displaying LFH absorbance shows a single broad peak at 0.175 absorbance units (A) and a long tail stretching up to 0.7 A (Figure 1A). There is a second, poorly defined peak at approximately 0.5 A. In this histogram, it is difficult to distinguish between the seronegative and seropositive population because there is no clear division between the peaks in the histogram. In contrast, the frequency distribution obtained with the ELISA using ES as antigen exhibits two peaks, at 0.175 A and 0.675 A, although a small peak at 0.375 A may represent the points where these seronegative and seropositive populations cross (Figure 1B). The ELISAs performed with purified CL1 as antigen further discriminated between the seronegative and seropositive populations, with absorbance peaks at 0.15 A and 0.475 A (Figure 1C). For both ES and CL1 antigens, the low absorbance peak follows a bell-shaped curve characteristic of a normal distribution. For normally distributed populations, 99.9% of all individuals should lie within 3.09 standard deviations of the mean. Therefore, a cut-off point that separates the seronegative from the seropositive population can be calculated. The calculated values for ES and CL1 antigens are 0.35 A and 0.32 A, respectively (dashed line in Figure 1B and C).
FIGURE 1. Analysis of sera obtained from 95 Aymarans by total IgG-ELISA using Fasciola hepatica A, liver fluke homogenate, B, excretory/secretory (ES) products, and C, purified cathepsin L1 (CL1) antigen. The frequency of the absorbance readings obtained were grouped into 0.05 absorbance units and plotted as histograms. The vertical dashed lines in B and C represent the cut-off point between the seronegative and seropositive populations, which were calculated as 3.09 standard deviations from the mean of the seronegative population.

When the absorbances obtained for ES and CL1 antigens were plotted against each other, a scatter of points centered at a low absorbance and another at a high absorbance was observed (Figure 2A). The data was sorted into two clusters using K-means cluster analysis. This analysis provides an objective and unbiased method for separating the data into two subpopulations based on a range of measured parameters (in this case, antibody responses to ES and CL1). This analysis designated the 26 individuals in the high absorbance cluster as seropositive and the 69 individuals in the low absorbance cluster as seronegative (Figure 2A, indicated by solid and open circles, respectively). While all of these 26 individuals were seropositive when ES alone was used for detecting antibody, 20 were seropositive when CL1 was used (above horizontal dashed line in Figure 2A). However, nine individuals who were determined to be seronegative by cluster analysis were positive when ES was used as antigen in ELISA (open circles to the right of the vertical dashed line, Figure 2A).

Coprologic analysis was performed on 58 individuals in this population. Six individuals were diagnosed as coprologically positive for F. hepatica by the Kato-Katz technique. Chi-square statistical analysis of the observed and expected numbers revealed a highly significant ($P < 0.001$) over-representation of individuals with fluke eggs in the seropositive cluster (all six grouped with the seropositive cluster). Seven individuals that were coprologically negative were among the 26 serologically positive individuals. All remaining coprologically negative individuals were among the seronegative subpopulation.

Ninety-one samples were analyzed for cysticercosis by ELISA and 15 were positive. These positive individuals showed an even distribution throughout the population, and chi-square statistical analysis of the observed and expected numbers of these showed no significant over-representation in either cluster.
Analysis of IgG4 responses of humans. The 26 patients that were seropositive by cluster analysis were selected for analysis of the IgG1, IgG2, IgG3, and IgG4 antibody responses to ES and CL1 antigens by ELISA (Figure 3A and B, respectively). For both antigens, the highest mean absorbances were obtained for IgG4. High absorbances were also obtained for IgG1 but the ratio between the mean absorbances for the seropositive and seronegative controls were lower (particularly for the CL1 antigen) than that observed for IgG4. Both IgG2 and IgG3 reactions had low absorbances when compared with the negative controls.

The IgG4 responses of all 95 patients to ES and CL1 were then analyzed by ELISA and the absorbance frequencies were plotted as a histogram. For both antigens, the seronegative subpopulation was represented by a bell-shaped curve with a sharp single peak (Figure 4A and B). The cut-off point between the seronegative and seropositive subpopulations was calculated as above (3.09 standard deviations of the mean) and values of 0.202 A and 0.169 A were obtained for ES and CL1, respectively (vertical lines in Figure 4). These cut-off values were much lower than those calculated for the total IgG ELISA (see above and Figure 1A and B). From this analysis, it was observed that CL1 showed a more defined separation between the two populations than ES.

A comparison of scatter graphs of the absorbances obtained for total IgG-ELISA and IgG4-ELISA responses to ES and CL1 revealed that there is a better defined difference between the seronegative and seropositive subpopulation in the IgG4-ELISA (Figure 2, compare A and B). In the IgG4-ELISA, the seronegative subpopulation formed a tight cluster in the low absorbancy region (Figure 2B). Therefore, in ELISAs using ES as antigen, 29 individuals were seropositive (to the right of the vertical dashed line in Figure 2B). However, five individuals of the 26 identified as seropositive in the total IgG-ELISA were seronegative by this analysis, and eight of the previously identified seronegative individuals were now seropositive. When CL1 was used as the antigen for the IgG4-ELISA, 20 individuals were seropositive. Eighteen of these were also positive by total IgG-ELISA, while two had been previously seronegative (above the horizontal dashed line, Figure 2B). Also, two individuals previously identified as seropositive were now seronegative.

Linear regression analysis of the ELISA results obtained for all the seropositive individuals detected with ES or CL1 showed a linear relationship between both antigens (Figure 2B, dotted line indicates regression).

Analysis of serum from humans infected with other parasites. Sera obtained from patients with schistosomiasis mansoni, cysticercosis, hydatidosis, and Chagas' disease were analyzed by IgG4-ELISA using F. hepatica ES and CL1 antigens (Figure 5). The mean absorbances obtained for all these sera were not significantly different from those obtained for the negative control sera. Moreover, all samples

FIGURE 3. Analysis of the isotypic responses of the 26 Aymaran individuals seropositive by the K-means cluster analysis. Sera were analyzed by ELISA using Fasciola hepatica excretory/secretory (ES) products (A) and cathepsin L1 (CL1) (B) as antigens, and isotype responses were detected with anti-human IgG1 (lanes 1), anti-human IgG2 (lanes 3), anti-human IgG3 (lanes 5), and anti-human IgG4 (lanes 7). Sera obtained from eight volunteers at Dublin City University served as background controls for IgG1, IgG2, IgG3, and IgG4 and are presented in lanes 2, 4, 6, and 8, respectively.

FIGURE 4. Analysis of sera obtained from 95 Aymarans by IgG4-ELISA using Fasciola hepatica excretory/secretory products (ES) (A) and cathepsin L1 (CL1) (B) as antigens. The frequency of the absorbance readings obtained were plotted as histograms. The horizontal lines represent the cut-off points between the seronegative and seropositive populations, which were calculated as 3.09 standard deviations from the mean of the seronegative population.
blocking antibody-mediated host immune responses.

The postulated functions of the enzyme are in assisting the migration of the maturing fluke through the liver tissue and vesicles synthesized by the intestinal cells of the liver fluke. Cathepsin L1 cysteine proteinase was initially purified from ES antigens by Smith and others, and seropositive individuals had significantly higher absorbance readings than those obtained from patients infected with the other parasites.

Additionally, the enzyme elicits a humoral response in cattle because it is secreted by all stages of fluke development in the bile ducts. Alternatively, these seven patients may have been coprologically misdiagnosed. Rigorous evidence for infection requires at least two fecal samples to be taken on separate days; however, in the present study, it was only possible to obtain a single stool sample from each patient. The remaining 45 individuals who did not have eggs in the fecal sample were associated with the seronegative cluster.

When using an ELISA for diagnosing infections in a population, it is often difficult to determine the exact cut-off point in the absorbance readings that separates the seropositive from seronegative individuals. For that reason, the ELISA data obtained for ES and CL1 were combined and sorted by K-means cluster analysis, a method now widely used to identify distinct subpopulations within whole populations. This analysis revealed that 26 of 95 patients clearly separated into a distinct high absorbance cluster (27.3%). All six individuals that were diagnosed as coprologically positive for fascioliasis were associated with this seropositive population, which was highly unlikely to have occurred by chance (P > 0.001) and supports the use of this method for the sensitive diagnosis of individuals that harbored latent or chronic-stage infections. Seven patients who were coprologically negative also associated with this seropositive population. These individuals may have harbored acute infections (that is, parasites that had not yet entered and matured in the bile ducts). Alternatively, these seven patients may have been coprologically misdiagnosed. Rigorous evidence for infection requires at least two fecal samples to be taken on separate days; however, in the present study, it was only possible to obtain a single stool sample from each patient. The remaining 45 individuals who did not have eggs in the fecal sample were associated with the seronegative cluster.

The K-means cluster analysis shows that the ES detected all 26 individuals in the seropositive cluster. However, this antigen also detected nine additional individuals who were in the seronegative cluster. In comparison, CL1 identified 20 of the 26 individuals in the seropositive cluster, all of who were well separated from the seronegative cluster. The higher proportion of individuals reacting to ES possibly reflects its more complex antigenic makeup. Moreover, it is not surprising that CL1 detects a subset of the individuals identified by the ES since CL1 is one of the major components of ES.

An analysis of the isotypic responses of the patients to liver fluke CL1 and ES revealed that the predominant antibodies elicited are IgG1 and IgG4. In the present study, we exploited this observation to develop an ELISA to detect anti-IgG4 responses of humans to ES and CL1. The IgG4 subtype was chosen over IgG1 because it resulted in a higher mean seropositive:mean seronegative ratio. Histograms of

![Figure 5](image-url)
the frequency of OD values obtained using ES and CL1 as antigens showed that the seronegative population formed a sharper bell-shaped curve in the IgG4-ELISAs compared with total IgG-ELISAs, and the definition between the seronegative and seropositive populations was far more distinct. This difference was particularly obvious when the data were compared in scatter graphs (Figure 2A and B). In the IgG4-ELISAs the seronegative subpopulation were found to cluster tightly together at the low absorbance readings, while the individuals in the seropositive subpopulation were dispersed in a wide range of high absorbance readings. Consequently, the calculated cut-off points were much lower and the ratio of the positive/negative absorbance readings were higher in the IgG4-ELISA compared with the total IgG-ELISA.

Five of the 26 individuals seropositive by total IgG-ELISA employing ES as antigen were seronegative by the IgG4-ELISA. Conversely, eight of the seronegative individuals by total IgG-ELISA were seropositive by the IgG4-ELISA. The differences in the composition of the seropositive population observed in the two ELISA methods may again reflect the complexity of the ES because different immunogens would elicit different isotypic responses. In contrast, the results obtained in both ELISA methods that used CL1 antigen were more consistent. Eighteen of the 20 patients seropositive by total IgG-ELISA were also seropositive by the IgG4-ELISA. These 18 individuals were always found within in the seropositive subpopulation, even when LFH was used as antigen. However, the clearest distinction between these and the seronegative population was obtained when CL1 was used in the IgG4-ELISA. Therefore, while CL1 identifies fewer individuals as seropositive compared with ES, this antigen provides a more reliable and confident discrimination between seropositive and seronegative individuals.

Fifteen patients in our sample population were serologically positive for cysticercosis, a disease caused by *T. solium* and also highly prevalent in the Bolivian Altiplano. Chi-square analysis revealed that these patients did not cluster with those that were serologically positive for liver fluke disease, but were randomly distributed throughout the population. Thus, our ELISA using liver fluke ES or CL1 does not detect false-positive samples due to cross-reactivity with antibodies induced by cysticercosis infections. However, to examine the issue of specificity further, we analyzed sera obtained from patients diagnosed with single infections, including schistosomiasis mansoni, cystercerosis, hydatidosis, and Chagas’ disease, by the IgG4-ELISA. None of the sera cross-reacted with ES or CL1 in these assays, which provides support for their use in the specific diagnosis of fascioliasis.

In summary, we have demonstrated that both ES and CL1 antigens can be used for the sensitive and specific diagnosis of human fascioliasis using an ELISA. The ratio of the mean absorbance readings between seropositive and seronegative individuals was greatly improved by the use of conjugated second antibodies that detect IgG4 rather than total serum antibodies. Cathepsin L1 provided a more conclusive diagnosis since compared with ES it discriminated more clearly between the seropositive and seronegative subpopulations. The fact that a single antigen, CL1, is useful for the diagnosis of human fascioliasis allows the development of a standardized ELISA method using recombinant CL1 protein. For this purpose, we are producing functionally active recombinant CL1 in the yeast *Saccharomyces cerevisiae* in an effort to obtain sufficient quantities of material to supply diagnostic centers in regions where human fascioliasis is prevalent. Finally, this study, which identifies 20 of the 95 patients (21%, by CL1 IgG4-ELISA) confirms the earlier reports of the high prevalence of human fascioliasis in the Bolivian Altiplano.

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Authors’ addresses: Sandra M. O’Neill, Michael Parkinson, and John P. Dalton, School of Biological Sciences, Dublin City University, Dublin 9, Ireland. Wilma Strauss, School of Biological Sciences, Dublin City University, Dublin 9, Ireland and Unidad de Parasitología, Instituto Nacional de Laboratorios de Salud, Secretaría Nacional de Salud, Pasaje Rafael Subieta No. 1889, Miraflores, Casilla 10019, La Paz, Bolivia. Rene Angles, Unidad de Parasitología, Instituto Nacional de Laboratorios de Salud, Secretaría Nacional de Salud, Pasaje Rafael Subieta No. 1889, Casilla M-10019, Miraflores, La Paz, Bolivia.

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