SHORT REPORT: IMMUNODIAGNOSIS OF HUMAN FASCIOLIASIS USING RECOMBINANT FASCIOLA HEPATICA CATHEPSIN L1 CYSTEINE PROTEINASE

SANDRA M. O’NEILL, MICHAEL PARKINSON, ANDREW J. DOWD, 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, Secretaría Nacional de Salud, La Paz, Bolivia

Abstract. Our laboratory recently developed a diagnostic test (ELISA) for human fascioliasis based on the detection of serum IgG4 antibodies reactive with Fasciola hepatica cathepsin L1 (CL1). In the present study, we have used recombinant CL1, generated by functional expression of the cDNA in Saccharomyces cerevisiae, in this immunodiagnostic test and compared its performance with native CL1. Sera obtained from 64 individuals living in Cutusuma village in the northern Altiplano of Bolivia, a region with a high prevalence of human fascioliasis, were analyzed by the IgG4-ELISA. A highly statistically significant correlation (r² = 0.751, P < 0.001) was demonstrated between the absorbances obtained using the recombinant and native proteins. These assays showed that 38 (59%) of the individuals tested were seropositive for fascioliasis, whereas only 26 of them were coprologically positive for F. hepatica eggs. All seronegative patients were also coprologically negative. Serum from individuals infected with schistosomiasis mansoni, cysticercosis, hydatidosis, and Chagas disease did not contain antibodies reactive with the recombinant or native CL1. Therefore, recombinant CL1 shows excellent potential for the development of the first standardized assay for the sensitive and specific diagnosis of human fascioliasis. Finally, our data supports earlier reports on the high prevalence of human fascioliasis in the Bolivian Altiplano, which collectively suggest that the disease has been endemic there for more than a decade.

Fascioliasis is caused by liver flukes of the genus Fasciola. Although the disease is predominantly one of domestic animals such as sheep and cattle, it is now emerging as an important chronic disease of humans. Recent estimates suggest that as many as 2.4 million people are infected with the parasite worldwide and 2.4 million are at risk. The disease is particularly prevalent among the Ayamaran people of the northern Altiplano region of Bolivia, with incidences of up to 60% being reported. Our laboratory recently developed a simple ELISA for the detection of serum IgG4 antibodies reactive with F. hepatica cathepsin L1 (CL1) proteinase. The use of purified CL1 antigen in this ELISA provided a more specific immunodiagnosis of human fascioliasis than tests that use parasite somatic or excretory/secretory (ES) antigen.

We have also recently reported the expression of functionally active recombinant F. hepatica CL1 in the yeast Saccharomyces cerevisiae. In the present study, we examined the potential of this recombinant protein as a diagnostic reagent for human fascioliasis in the IgG4-ELISA and compared the performance of the antigen with native CL1 antigen. Recombinant CL1 was purified from yeast culture medium by gel filtration chromatography on Sephacryl S200 HR and native CL1 was purified from ES products by a combination of gel filtration chromatography on Sephacryl S200 HR and ion exchange chromatography on QAE-Sephadex. The concentration of each antigen preparation was measured using a bicinchoninic acid protein assay kit (Pierce and Warner, Chester, United Kingdom).

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. Blood samples were obtained in 1986 from 64 native Aymara living in the village of Cutusuma (approximately 50 km north of La Paz, Bolivia). The serum was separated and stored at −20°C. The age of the volunteers ranged between seven and 76 years, with a mean ± SD age of 15.23 ± 12.46 years. Coprologic analysis for F. hepatica eggs was performed on fecal samples obtained from all individuals using the sedimentation method of Richet. Serum samples obtained from patients infected with schistosomiasis mansoni (20), cysticercosis (15), hydatidosis (15) and Chagas disease (15) were obtained from the Instituto Nacional de Laboratorios de Salud (INLASA) serum library and used to test for cross-reactivity in the test. Control serum samples were provided by eight volunteers at Dublin City University. The study was approved by the Human Ethics Committee of INLASA and the Department of Health of Bolivia.

The ELISAs were carried out as previously described by O’Neill and others. Briefly, 100 µl of native or recombinant CL1 antigen (5 µg/ml) was incubated overnight at 37°C in wells of microtiter plates (Nuclon, Kamstrup, Roskilde, Denmark). The wells were blocked with a solution of 2% bovine serum albumin/0.1% Tween 20 in phosphate-buffered saline for 30 min at 37°C and human sera (1:125 dilution) was then added into triplicate wells. Bound human antibodies were detected using biotin-conjugated anti-human IgG4 (1:2,000 dilution), avidin-conjugated alkaline phosphatase, and the substrate 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (all from the Sigma Chemical Co., Poole, Dorset, United Kingdom). Plates were read after 15 min on a Titerette (Helsinki, Finland) multispan ELISA plate reader at an absorbance of 405 nm.

The absorbance readings obtained from the ELISAs using recombinant and native CL1 were plotted as scattergrams (Figure 1). Hierarchical agglomerative cluster analysis revealed that the scatter of points divided into two clusters, one compact cluster of low absorbance readings and a diffuse cluster of high absorbance readings (Figure 1). Cut-off points between the clusters were set at 3.09 standard deviations from the mean of the low absorbance cluster and were 0.117 and 0.121 for the recombinant and native proteins, respectively. Using the cut-off points, 38 individuals (59%)...
were seropositive and 26 were seronegative by the IgG4-ELISA using both the recombinant and native CL1 antigens. Hierarchical agglomerative cluster analysis using the combined data obtained for both proteins separated the population into low absorbance (seronegative) and high absorbance (seropositive) subpopulations. The vertical and horizontal dashed lines indicate the calculated cut-off points for each antigen. The closed circles represent individuals who were coprologically positive and the open circles represent those who were coprologically negative. The diagonal line represents the regression of seropositive individuals for recombinant CL1 against native CL1.

Chi-square analysis showed that it was also seropositive in the IgG4-ELISA using the recombinant CL1 against native CL1. Cathepsin L1 was considered a potential candidate for serologic diagnosis of fascioliasis because it was secreted by all stages of the parasite that develop in the mammalian host and was highly immunogenic in infected animals (antibodies can be detected within two weeks after infection).10 O’Neill and others7 demonstrated that this defined antigen discriminated between seropositive and seronegative individuals for fascioliasis markedly better than crude antigens such as whole worm extracts of total ES products. Both the present study and that of O’Neill and others7 have shown that all patients who were coprologically positive for F. hepatica eggs were also serologically positive in the IgG4-ELISA that used CL1 as antigen. The fact that all seronegative patients were also coprologically negative indicated that CL1 could be used for the sensitive diagnosis of human fascioliasis, and reassured us that those coprologically negative individuals that were seropositive were indeed infected.

Roche and others5 showed that recombinant CL1 produced in yeast was not only functionally active but also exhibited similar physiochemical properties as the native CL1, sharing similar molecular size, pH profile of activity, stability, and substrate specificity. Therefore, it is not overly surprising that the recombinant antigen performed similarly (as shown by a regression line slope of 0.797, Figure 1) to the native antigen in the IgG-ELISAs described in this study. However, the important development in this study was the demonstration that the possibility of producing sufficient cercos, hydatidosis, and Chagas disease were negative by IgG4-ELISAs that used either recombinant or native CL1. Although schistosomes also express cathepsin L proteinases,12 we have shown that antibodies prepared against F. hepatica cathepsin L proteinases do not cross-react with these (Brady C, Dalton JP, unpublished data).

Human fascioliasis is becoming increasingly recognized as a serious public health problem, particularly in regions of Bolivia and Peru. Regional climatic factors coupled with the rural lifestyle and dietary habits of the indigenous population contribute to transmission of the disease year round.6,8,13 Clinical manifestations of human fascioliasis include fever, right hypochondrial pain, anorexia, weight loss, persistent diarrhea, and vomiting. However, many individuals are asymptomatic or present vague symptoms rendering clinical diagnosis problematic.1 Accordingly, the development of a simple, sensitive, and cost-effective diagnostic assay is imperative. While coprologic analysis may fulfill some of these requirements, it is clear that it provides a definitive parasitologic diagnosis only when eggs are found in the patient’s feces, and that early infections cannot be detected using this method.

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**Figure 1.** Scattergram of the data obtained with the IgG4-ELISA using recombinant cathepsin L1 (CL1) and native CL1 antigens. Hierarchical agglomerative cluster analysis using the combined data obtained for both proteins separated the population into low absorbance (seronegative) and high absorbance (seropositive) subpopulations. The vertical and horizontal dashed lines indicate the calculated cut-off points for each antigen. The closed circles represent individuals who were coprologically positive and the open circles represent those who were coprologically negative. The diagonal line represents the regression of seropositive individuals for recombinant CL1 against native CL1.
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quantities of antigen to provide all diagnostic centers with a standardized serologic test for human fascioliasis exists.

In this study, we have confirmed the high prevalence of human fascioliasis in the northern Altiplano region of Bolivia. A large-scale regional survey is required to fully establish the prevalence of the disease throughout the entire region. Studies carried out to date have indicated a prevalence of human fascioliasis of approximately 59% in the village of Cutusuma in 1986 (Table 1, this study), 49% in Calasaya in 1991, 67.53% in Coropata in 1992, and 38.2% in Huacullani in 1993. Collectively, these reports indicate that this disease has been endemic in the region for more than a decade.

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

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