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Fas2-ELISA in the detection of human infection by *Fasciola hepatica*

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Abstract

Fasciola hepatica has recently emerged as a major pathogen of humans from reports on areas of endemicity and hyper-endemicity for fascioliasis. This situation is aggravated by the lack of standard assays for the screen diagnosis of *F. hepatica* infection in humans living in endemic areas. Our laboratory has developed an enzyme-linked immunosorbent assay (Fas2-ELISA) based on the capture of IgG antibody by a purified protein Fas2, which is an adult fluke cysteine proteinase. Fas2-ELISA exhibited 95% sensitivity and 100% specificity in 38 individuals infected with *F. hepatica* diagnosed by finding eggs in stools and 46 serum samples from healthy volunteers. No cross-reaction was observed with 54 serum samples from patients with ten different parasitic infections including the trematodes *Paragonimus westermani* and *Schistosoma mansoni*. The high antigenicity of Fas2 is suggested by the fact that antibodies to Fas2 rise rapidly by 1–2 weeks of infection and rise until patency at 8 weeks of infection in experimentally infected alpacas. Field screening for human fascioliasis using Fas2-ELISA and coprology in three endemic locations of the Peruvian Andes resulted in 95.5% sensitivity, 86.6% specificity in a population of 664 children in an age range of 1 to 16 years old. These results provide evidence of the clinical potential of Fas2-ELISA to diagnose fascioliasis in humans exposed to liver fluke infection in endemic areas for this parasite. Fas2-ELISA is currently developed as a standard assay for both field screening for fascioliasis in people living in endemic areas and detecting occasionally *F. hepatica* infected patients in clinical laboratories.

Introduction

Fascioliasis is a chronic helminthic disease with a worldwide distribution. Humans may acquire the infection by ingestion of raw vegetables or water contaminated with metacercariae (Hillyer, 1999). In recent years, this infection emerged as a public health problem of major importance as a consequence of the increasing number of reported clinical cases and the identification of areas of endemicity and hyper-endemicity for human infection (Mas Coma *et al.*, 1999). Human infection has been reported from Europe, Latin America, North Africa, Asia and the Western Pacific (Chen & Mott, 1990). Recent estimates suggest that up to 17 million people are infected with the liver fluke worldwide (Mas-Coma *et al.*, 1999). This parasitic disease is also a major cause of morbidity and mortality in domestic herbivores, causing serious economic losses due to the decrease in productivity of infected cattle, sheep, goats and camelids (Spithill *et al.*, 1999).

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Parasitological diagnosis of human fascioliasis

Until recently the definitive diagnosis of fascioliasis was the finding of *F. hepatica* eggs in faeces of infected individuals (Hillyer, 1999; Mas-Coma *et al.*, 1999).

However, this assay, even though highly specific, has serious limitations in sensitivity because acute or erratic infections pass undetected, since eggs are produced when sexually mature flukes are residing in the host biliary ducts, which takes 3 to 4 months after infection or even longer (Hillyer, 1999). In addition, difficulties in obtaining and manipulating stool samples in the field, intermittence in egg shedding and false positives by ingestion of infected raw liver contribute to the inaccuracy of the parasitological diagnosis (Hillyer, 1999). The application of control measures that curb human infection in endemic areas depends critically on the detection of infected people. If this is solely based on parasitological diagnosis, the lack of sensitivity of this assay may overlook a considerable number of infected individuals, as already suggested in several reports from field studies (Hillyer *et al.*, 1992; Mas-Coma *et al.*, 1999; Strauss *et al.*, 1999; Raymundo *et al.*, 2004).

Cysteine proteinases as markers of *F. hepatica* infection

Excretory/secretory products (E/S) from *F. hepatica* have proved to be a most valuable source of antigens for diagnosis of the disease in humans, as well as in naturally and experimentally infected animals. As E/S is a rich source of numerous proteins, among which cysteine proteinases are probably the most abundant (Dalton & Heffernan, 1989), its immunogenicity could be explained, at least partially, by the presence of these enzymes (Cordova *et al.*, 1997). Cysteine proteinases are expressed at different stages of the development of *Fasciola* and they are secreted by both adult (Dalton *et al.*, 2003) and juvenile forms (Law *et al.*, 2003). These enzymes are highly antigenic in infected humans with *F. hepatica* (Cordova *et al.*, 1997) as well as in other susceptible species (Cornelissen *et al.*, 1999, 2001; Neyra *et al.*, 2002; Ruiz *et al.*, 2003). These proteinases are used by the parasite to penetrate through the host tissue (Halton, 1967; Berasain *et al.*, 1997, 2000), to evade the host immune system (Chapman *et al.*, 1982; Smith *et al.*, 1993a; Carmona *et al.*, 1993; Berasain *et al.*, 2000) and are involved in the degradation of bile ducts and liver parenchymatous tissue in chronic infected hosts (Berasain *et al.*, 1997; Timoteo *et al.*, 2005).

Several cysteine proteinases were purified and characterized by biochemical procedures from the *F. hepatica* E/S (Smith *et al.*, 1993b; Cordova *et al.*, 1997). Fas2 and Fas1, two of the major cysteine proteinases of the E/S of *F. hepatica* adult worms were formerly found to be highly antigenic in the human infection by using active-site affinity radio-labelled antigens and sera from *F. hepatica* infected individuals (Cordova *et al.*, 1997). Fas2 turned out to be a sensitive and specific antigen for the diagnosis of human fascioliasis (Cordova *et al.*, 1997, 1999; J.R. Espinoza *et al.*, unpublished). A cathepsin L-like enzyme purified from E/S of adult worms by analogous procedures was evaluated as a marker of human infection in Bolivia (Smith *et al.*, 1993b; O'Neill *et al.*, 1998; Strauss *et al.*, 1999), and recently in Iran (Rokni *et al.*, 2002) with similar satisfactory results. A cathepsin B-like enzyme, present in juvenile forms, was purified and expressed as recombinant protein that was recognized by the sera of experimentally infected sheep and rats (Law *et al.*, 2003); further evaluation of the cathepsin B-like antigen as marker of the human infection is expected.

Recently, ELISAs using recombinant cysteine proteinases from *F. hepatica* produced in yeast (O'Neill *et al.*, 1999), in *E. coli* (Carnevale *et al.*, 2001) were evaluated in the diagnosis of human infection displaying similar performance characteristics as native antigens.

Fas2-ELISA for diagnosis of human infection

Fas2-ELISA is an indirect serological technique based on the capture of circulating IgG by a 25 kDa cysteine proteinase Fas2 (Cordova *et al.*, 1999). *Fasciola hepatica*-infected humans and animals elicit an early strong humoral immune response against the parasite by raising circulating specific anti-Fas2 IgG antibodies. In an animal model, the immunogenic capacity of this antigen allows detection of the disease as early as 10 days post-infection by Fas2-ELISA (fig. 1). This result suggests that the assay can also detect human infection in its acute phase, thus an early drug treatment will diminish liver damage caused by migrating flukes.

Fas2-ELISA was initially evaluated with sera from 38 patients infected with *F. hepatica* diagnosed by finding eggs in stools, 54 serum samples from patients with other parasitic infections and 46 serum samples from healthy volunteers (fig. 2). Fas2-ELISA was performed with 95% sensitivity, 100% specificity. No cross-reaction was observed with sera from patients infected with other parasites such as *Echinococcus granulosus*, *Taenia solium*, *Trypanosoma cruzi*, *Hymenolepis nana*, *Strongyloides stercoralis*, *Trichuris trichiura*, *Ascaris lumbricoides*, *Toxocara canis*, *Paragonimus mexicanus* (*peruvianus*) and *Schistosoma mansoni* (Cordova *et al.*, 1999). In addition, Fas2-ELISA was shown to be more specific and sensitive than ELISA using

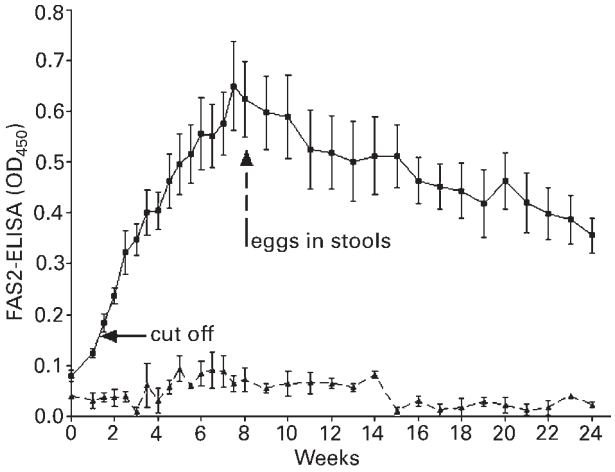

Fig. 1. Total IgG circulating antibodies against Fas2 detected by ELISA. *Fasciola hepatica* experimentally infected alpacas ($n = 6$) and controls ($n = 3$) were evaluated by ELISA with Fas1. Infected alpacas (■) and controls (▲). (From Timoteo *et al.*, 2005.)

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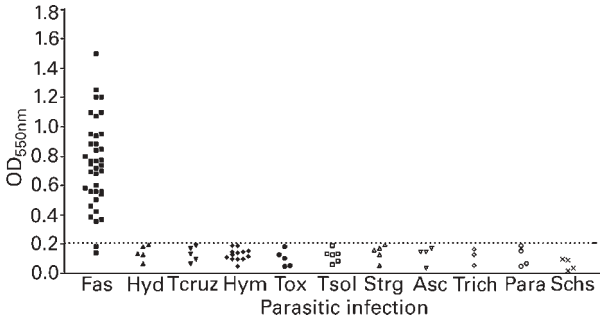

Fig. 2. Fas2-ELISA in groups of patients with a proven parasite infection: fascioliasis (Fas), toxocariasis (Tox), hydatidosis (Hyd), strongyloidiasis (Strg), hymenolepiasis (Hym), *Trypanosoma cruzi* infection (Tcruz), ascariasis (Asc), cysticercosis (Tsol), trichuriasis (Trich), paragonimiasis (Para) and schistosomiasis (Schs) The dotted line represents the cut-off value. (From Cordova *et al.*, 1999.)

E/S antigen preparation (Cordova *et al.*, 1999) as found in a similar study in Iran (Rokni *et al.*, 2002).

The evaluation of the performance characteristics of Fas2-ELISA in the diagnosis of *F. hepatica* infection was recently reported in children living in areas of high endemicity for fascioliasis in the Peruvian Andes (Raymundo *et al.*, 2004; J.R. Espinoza *et al.*, unpublished). The study was conducted in three Andean localities from Cajamarca, Junin and Puno. A total population of 664 children in an age range of 1 to 16 years old resulted in a prevalence of 24% in Cajamarca, 21.1% in Junin and 25.7% in Puno estimated by parasitological diagnosis of the infection by the inspection of a single stool specimen (table 1). The children are infected with pathogenic intestinal parasites, helminths and protozoa as a result of the unhealthy living conditions prevailing in these localities. However, Fas2-ELISA displayed no cross-reaction with other helminth or protozoan infection present in the population under study. The seroprevalence of *F. hepatica* infection, determined by Fas2-ELISA, was 29.1% in Cajamarca, 27.7% in Junin and 42.2% in Puno. The overall sensitivity of Fas2-ELISA was 95.5%, the specificity, 86.6% and the negative predictive value 98.3% (fig. 3).

The human population in a fascioliasis endemic area can be grouped as result of parasitological and Fas2-ELISA serological evaluation as follows: copro-negatives/sero-negatives; copronegatives/seropositives; copropositives/sero-positives and copropositives/seronegatives (table 1). As reported by others, coprological detection appears to underscore the real prevalence of *F. hepatica* infection assessed by serological procedures (Hillyer *et al.*, 1992; Strauss *et al.*, 1999).

Other *F. hepatica* antigens as markers of infection

Fasciola hepatica proteins different from proteases are actively searched through biochemical or molecular biology approaches as potential markers of infection. For instance, an O-deglycosylated fraction of 7–40 kDa derived from *F. hepatica* E/S antigens has been used for the generation of monoclonal antibodies (mAb). These mAb were employed for the detection of infection in sheep and cattle faeces by capture ELISA assay (Mezo *et al.*, 2004). The antibodies were able to detect coproantigens even preceding the presence of eggs in faeces by 1–5 weeks and converted to negative after treatment. Silva *et al.* (2004) reported the detection of antibodies against a recombinant antigen, rFh8, in experimentally infected animals (rabbits, rat, cattle and sheep), as well as naturally infected hosts.

Kim *et al.* (2003) reported the isolation of an 8 kDa protein from *F. hepatica* extracts that reacted positively with human fascioliasis sera, whereas sera from healthy individuals or patients infected with other trematodiasis (paragonimiasis, clonorchiasis and schistosomiasis) showed no reactivity. The biochemical nature and function of this protein has not yet been described.

Another low molecular weight protein with potential application in diagnosis is a 2.9 kDa recombinant protein obtained from a 400-bp cDNA. The recombinant (APS) has been evaluated by Paz-Silva *et al.* (2005) in its ability to be detected by sera from experimentally infected sheep. Interestingly, after treatment of fascioliasis, the IgG response against APS seroconverts to negative values, making this antigen a good candidate for the detection of active infections.

Antibodies against fatty acid binding proteins from *F. hepatica* adult worms have been detected in experimentally infected rabbits, although their use as diagnostic tools remains to be established (Espino *et al.*, 2001).

Concluding remarks

There seems to be a wide acceptance that parasite 24–28 kDa cysteine proteinases isolated from E/S are sensitive and specific markers for the serodiagnosis of human infection by *F. hepatica*, (Cordova *et al.*, 1997, 1999; O'Neill *et al.*, 1998; Strauss *et al.*, 1999; Rokni *et al.*, 2002). These parasite proteins are now well characterized, their

Table 1. Population distribution in endemic areas in Peru by Fas2-ELISA and coprology.

Site and population size	Group 1 *n* (%)	Group 2 *n* (%)	Group 3 *n* (%)	Group 4 *n* (%)
Junin ($n = 144$)	103 (71.5)	10 (6.9)	30 (20.8)	1 (0.7)
Cajamarca ($n = 237$)	166 (70)	14 (5.9)	55 (23.2)	2 (0.8)
Puno ($n = 232$)	130 (56)	42 (18.1)	56 (24.1)	4 (1.7)
Three sites ($n = 613$)	399 (65)	66 (10.7)	141 (23)	7 (1.1)

Group 1, copronegative/seronegative; group 2, copronegative/seropositive; group 3, copropositive/seropositive; group 4, copropositive/seronegative.

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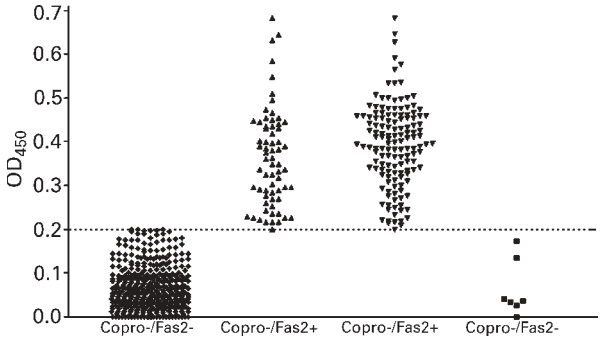

Fig. 3. Fas2-ELISA with sera from children that provided at least one stool and blood sample ($n = 664$) from Junin, Cajamarca and Puno, Peru. Data points represent the mean absorbance at 450 nm obtained from three replicates of each serum tested. The dotted line represents the cut-off value 0.2 units of OD at 450 nm.

antigenicity is proven in human and animal infections and recombinant proteins are also available, which makes the scenario ripe enough for the development of a standard screen test of antibody-detection for this infection.

However, antibody-detection methods are criticized by the limitations in discriminating between active and resolved infections, particularly frequent in endemic locations (Doenhoff *et al.*, 2004). In our field study using Fas2-ELISA and coprology, we observed a small number of copropositive/seronegative cases, which may represent infected individuals that lack antibodies to Fas2 or false negatives due to ingestion of eggs in infected livers. A high proportion, 7–18% of the population, was copronegative/seropositive, whether they are individuals bearing an active infection, cases of flawed diagnosis by microscopy, or serology deserves further attention (table 1). Copronegative/seropositive individuals pose serious challenge to the clinician in deciding either to immediately proceed with chemotherapeutic intervention or to wait for an uncertain confirmatory diagnosis based on finding eggs in repeated stool samples.

Alternative approaches to the diagnosis such as direct antigen detection in stools (Youssef *et al.*, 1991; Espino *et al.*, 1998) and in sera (Espino *et al.*, 1990) or Western blot assay (Hillyer *et al.*, 1992) showed promising results when evaluated in small groups of infected individuals. Eventually, these assays have to be assessed in endemic locations to decide if used either in the front line as a screen test or as a confirmatory assay in individuals positive to first line diagnostic tests.

The excellent performance of Fas2-ELISA reported here has precedents in previous reports on human infection detected by cysteine proteinase-based assays (Cordova *et al.*, 1997, 1999; O'Neill *et al.*, 1998; Strauss *et al.*, 1999; Carnevale *et al.*, 2001). Field screening of *F. hepatica* infection using Fas2-ELISA provides evidence of the clinical potential of this assay to diagnose fascioliasis in humans exposed to the liver fluke infection in endemic areas. Fas2-ELISA is currently developed as a simple, cheap, sensitive and specific test for the routine diagnosis of human fascioliasis. This standard assay is expected to be useful as a first line test for field screening for fascioliasis in people living in endemic areas and for detecting occasionally *F. hepatica* infected patients in clinical laboratories.

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