Published in final edited form as:

Diagn Microbiol Infect Dis. 2011 July ; 70(3): 355–361. doi:10.1016/j.diagmicrobio.2011.03.016.

***Fasciola hepatica* saposin-like-2 protein based ELISA for the serodiagnosis of chronic human fascioliasis**

Olgary Figueroa-Santiago, Bonnibel Delgado, and Ana M. Espino*

Laboratory of Immunology and Molecular Parasitology, Department of Microbiology, University of Puerto Rico, School of Medicine, PO BOX 365067, San Juan, PR 00936-5067

Abstract

An indirect enzyme-linked immunosorbent assay (ELISA) was developed and evaluated for its diagnostic ability to detect human IgG antibodies against *Fasciola hepatica* saposin-like protein-2. The assay was compared with an indirect ELISA with excretory-secretory products (FhES) from adult *F. hepatica*. In an analysis of the sera of 37 patients infected with *F. hepatica*, 40 patients with other parasitic infections, and 50 healthy controls, the sensitivity of both ELISA assays was 100%. However, the FhSAP2-based ELISA was more specific (95.6%) than the FhES-ELISA (91.9%). These results demonstrated that FhSAP2 can be used in the serodiagnosis of chronic human fascioliasis with additional advantage that is relative cheap and easy to produce. Studies are in progress to evaluate this FhSAP2-ELISA assay in a large-scale prevalence surveys in endemic areas.

Keywords

Fasciola hepatica; immunodiagnosis; ELISA; saposin-like proteins; ES antigens

1. Introduction

The helminth parasite *Fasciola hepatica* causes liver fluke disease or fascioliasis, a major zoonotic disease that affects the health of humans and animals. Worldwide losses in agriculture due to fascioliasis are estimated at over \$2 billion dollars per year due to an increased in animal mortality and a reduction in productivity (Spithill et al., 1999, Torgerson and Claxton, 1999). Until the mid 1990s human fascioliasis was largely underestimated because it was believed to be restricted to isolated outbreaks, such as those reported in Iran (Massoud, 1990), Peru (Knobloch, 1985), and Cuba (Espino et al., 1998). However, reports of human infection are steadily increasing in many countries (Chen, 1990) and recently, it has been recognized as an emerging/reemerging zoonotic disease with an estimated prevalence of up to 17 million people infected and 180 million at risk for infection worldwide (Mas-Coma, 2005, Parkinson et al., 2006). Those health problems are encountered especially in areas in which the disease is endemic such as the Andean region in Peru, where well-known areas of hyperendemicity include the Bolivian Altiplano (Esteban et al., 2002), the Cajamarca valley (Knobloch, 1985), and the Mantaro valley (Raymundo et al., 2004).

*Corresponding author: Tel. (787) 758-2525 Ext. 1318; Fax: (787) 292-3693, ana.espino1@upr.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The clinical manifestations of fascioliasis in humans include fever, right hypochondrial pain, persistent diarrhea, and vomiting (Arjona et al., 1995). 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 for diagnosing acute infections and can also be used as an adjunct to fecal analysis for the diagnosis of latent and chronic infections (Hillyer et al., 1992).

Among the ELISA assays that detect anti-fluke antibodies in serum, the most popular are those that detect the IgG isotype. This is because 100% of persons with acute or chronic infection develop high and sustainable levels of IgG antibody against the parasite, while the IgM isotype is only detectable in patients with acute infection, and the IgE and IgA antibodies are detected in a limited number of infected patients (Demirci et al., 2009). However, even the presence of IgG antibodies does not correlate with the existence of an active infection; it indicates an exposure to the parasite (Sanchez-Andrade et al., 2001). In that sense, antigen-capture detection assays could undoubtedly be a better alternative to the antibody detection in serum (Espino et al., 1990, Mezo et al., 2004). However, these assays require highly specific monoclonal antibodies to capture the antigen in clinical samples and the preparation of monoclonal antibodies is an expensive and time consuming process. In contrast, the IgG-antibody detection assay is economic and relatively simple to optimize and for these reasons is the method of choice for the immunodiagnosis of fascioliasis (Cordova et al., 1999), permitting a rapid screening of people living in human endemic and non endemic areas.

Although several recombinant proteins have been widely employed as potential vaccines against *F. hepatica* (Muro et al., 1997, Kofta et al., 2000, Dalton et al., 2003, Law et al., 2003,), their usefulness for the diagnosis of fascioliasis have only been evaluated in experimental animals (Cornelissen et al., 2001, Silva et al., 2004, Paz-Silva et al., 2005, Arias et al., 2006). In this publication the recombinant form of a novel member of the *F. hepatica* saposin-like-2 protein family (FhSAP2) was employed to detect antibodies in the sera of subjects that live in endemic areas and that had been confirmed positive to fascioliasis by the presence of *F. hepatica* egg in feces. The results were compared to those obtained in an antibody assay that uses *F. hepatica* excretory-secretory antigens (FhES).

2. Materials and methods

2.1. Serum samples

Sera were obtained from serum banks of the National University of Cajamarca, Peru; Institute of Tropical Medicine, Central University of Venezuela and from the Infectious Diseases Division, Washington University School of Medicine. Each serum sample was aliquoted and stored at -20°C until used. Samples were collected according the Human Research Ethics Committee of each University. A total of 37 sera were obtained from parasitologically confirmed cases of *F. hepatica* infection. To evaluate potential cross-reactivity, serum samples were obtained from parasitologically confirmed cases of paragonimiasis ($n=5$), schistosomiasis ($n=17$), filariasis ($n=5$), trichuriasis ($n=5$), amebiasis ($n=2$), giardiasis ($n=4$) and ascariasis ($n=2$). Negative control sera were obtained from 50 healthy subjects that during the sampling period lived in a non-endemic area of fascioliasis. Stool examinations were performed at the time of the blood collections using the formaline-ether concentration method (Erdman, 1981). No evidence for intestinal parasitic infection was found.

2.2. Preparation of ES products

Excretory-secretory products (FhES) from *F. hepatica* were prepared as previously described (Espino et al. 1987). Briefly, adult flukes were collected from cattle's bile ducts obtained from a slaughterhouse, and were washed several times in 0.01M phosphate-buffered saline (PBS), pH 7.4 to remove all traces of blood and bile. The flukes were incubated under sterile conditions at 37°C for 24 hr in RPMI-1640 medium supplemented with 25mM HEPES buffer, 7.5% sodium bicarbonate, 100µl penicillin, and 100µg/ml streptomycin at a ratio of one fluke per 5ml of media. Flukes were removed from the tubes and the remaining medium containing FhES were pooled and centrifuged ($1,500 \times g$ for 10 min at 4°C) to remove parasite eggs. The supernatants was collected and concentrated using a high flow UM 10 membrane filter (Amicon Corp., Lexington, Massachusetts).

The concentration of proteins for FhSAP2 and FhES products was measured using a BCA protein assay kit (Thermo Scientific, Rockford, USA).

2.3. Preparation of recombinant *F. hepatica* saposin-like2 protein

cDNA encoding FhSAP2 (GenBank AF286903) was cloned into the restriction sites of pBAD-His B expression vector (Invitrogen, Carlsbad, CA) and this vector was used to transform *E. coli* TOP10 to overexpress the polypeptide as a fusion protein containing six histidine residues. The expressed protein was purified using Ni^{2+} affinity column chromatography as previously described (Espino and Hillyer, 2003).

2.4. Indirect enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA assays for FhSAP2 or FhES were performed on human serum samples following a published protocol (Espino et al., 1987, Espino and Hillyer, 2003), which was optimized by checkerboard titration. Disposable polystyrene plates (Costar, Corning, USA) were coated overnight (O/N) at 4°C with 2.5µg/ml of FhSAP2 or 35µg/ml of FhES in coating buffer (0.05M carbonate-bicarbonate buffer pH 9.6). The plates were then washed 3 times with PBS containing 0.05% Tween 20 (PBST) and blocked during 1 hour at 37°C with 300µl/well of 3% albumin in PBST. The blocking solution was removed; the serum samples were diluted 1:400 in PBST (100µl/well) and were added to the plates and incubated for 1hr at 37°C. The incubation with sera was followed by another washing step and by the addition of 100µl/well of an anti-human IgG conjugated with peroxidase (diluted 1: 7,500 in PBST). Plates were incubated at 37°C for 1hr. Finally, after three consecutive washes with PBST, 100µl of substrate solution containing 20 mg *o*-phenylenediamine and 30% H_2O_2 was added to each well. After 30min incubation at RT the enzymatic reaction was stopped using 50µl/well of 12.5% sulfuric acid. Absorbance values at a wavelength of 492nm (A_{492}) were determined using a BioRad (Hercules, CA) microplate reader.

2.5. SDS-PAGE and Western blot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of (Laemmli, 1970). After electrophoresis, proteins were silver-stained or transferred into nitrocellulose membranes (BioRad). For immunoblot analysis, membranes were washed three times with PBST and blocked with the ELISA blocking solution for 1hr at RT. Each membrane was cut into strips and the strips were incubated O/N with either a Monoclonal antibody anti-X Press epitope (Invitrogen) diluted 1:5,000 or human sera diluted 1:200. A peroxidase immunoconjugate (BioRad) anti-mouse IgG (diluted 1:3,000) or an anti-human IgG (diluted 1:5,000) was used as the secondary antibody followed by the addition of diaminobenzidine as chromogenic substrate.

2.6. Statistical Analysis

Each ELISA determination was performed in triplicates and the results expressed as the mean absorbance at 492nm (A_{492}) of all three measurements. The cutoff point for this assay was established as the mean A_{492} plus 2 standard deviations ($A_{492} \pm 2 \text{ SD}$) of the negative controls. The results obtained from the FhSAP2-ELISA and FhES-ELISA was compared to those obtained by coprological examination CE (the gold standard) to calculate the sensitivity, and specificity for each assay using the method of Galen (1980). These values were calculated and expressed as follows: Sensitivity (S), which means the ability of a test to correctly identify the percent of samples that have the disease was equal to the $[\text{no. of true positives}/(\text{no. of true positives} + \text{no. of false negatives}) \times 100]$; specificity (Sp), which means the ability of a test to correctly identify the percentage of those who do not have the disease was equal to the $[\text{no. of true negatives}/(\text{no. of false positives} + \text{no. of true negatives}) \times 100]$; whereas accuracy that describe the degree to which measurement reflects the true status of what is being measured and it is used to express the overall performance of a diagnostic test was equal to $[\text{no. of true positives} + \text{no. of true negatives}/(\text{no. of true positives} + \text{no. of false negatives} + \text{no. of true negatives}) \times 100]$; true positive was the number of proven fascioliasis samples that were positive by the assay; true negative was the number of control samples (other parasitoses and healthy subjects) that were negative by the assay; false positive was the number of control samples that were positive by the assay; and false negative was the number of proven fascioliasis samples that were negative by the assay. A Pearson's correlation coefficient was used to identify any correlation between the results obtained using ELISA assays and CE. The *Kappa* statistic was calculated to assess the agreement between the different tests used according to Thrusfield (1997).

3. Results

3.1. Reactivity of the FhSAP2 with human sera compared to the FhES

FhSAP2 and FhES antigens were used to sensitize ELISA plates which were subsequently probed with a panel of human sera. In the FhSAP2-ELISA assay, the A_{492} values for the negative control group ranged between 0.06 to 0.63 (A_{492} mean = 0.26 ± 0.12). The cutoff point resulted in 0.50 (dashed line in Fig. 1). Sera from patients with fascioliasis showed a mean value of $1.04 \pm 0.29 \text{ AU}$, with a range between 0.54 and 1.55. Using the calculated cutoff point, the sensitivity of the assay was 100%. The specificity of the test was analyzed by measuring the reactivity of sera from negative controls and patients with other parasitic diseases (Fig. 1). Two negative controls and one schistosomiasis case showed false-positive results. No false-positives were detected among paragonimiasis and other helminthiasis sera. The average absorbance obtained for the sera from patients infected with *Schistosoma* or other helminths were not significantly different from those obtained for the negative control sera ($P=0.69$). Moreover, all samples from *F. hepatica* infected patients had significantly higher absorbance readings than those obtained from individuals infected with other parasites ($P<0.001$). According to these results, the specificity of the assay was 96.5% and its accuracy 97.6%.

For the FhES-ELISA assay, the mean A_{492} for the negative control group was 0.198 with standard deviation of 0.13. The cutoff point resulted in 0.46 (dashed line in Fig. 1). As expected, all sera samples from patients with fascioliasis tested positive for antibodies to the FhES antigen. Positive sera showed a mean value of 1.28 ± 0.52 with a range between 0.64 and 2.2. Statistical differences were also found between the mean A_{492} value of the fascioliasis sera and the negative sera ($P<0.001$). When sera from individuals with chronic schistosomiasis were tested in the FhSAP2-ELISA, absorbance values that ranged between 0.17 and 0.81, with a mean OD of 0.156 ± 0.17 were obtained. the absorbance for the

schistosomiasis sera ranged between 0.05 and 0.73, with a mean A_{492} of 0.15 ± 0.16 , the absorbance for the paragonimiasis sera ranged between 0.22 and 1.52, with a mean A_{492} of 0.58 ± 0.48 and the absorbance for the sera obtained from individuals that tested positive for other parasites ranged between 0.09 and 0.35 (mean 0.13 ± 0.89). Based on the cutoff point for FhES-ELISA assay, four false-positives were detected in the negative control group, one false-positive was detected in the schistosomiasis group and two false-positive were detected in the paragonimiasis group. Table-1 shows a statistical comparison between FhSAP2, FhES, and CE results. Using the Pearson's test, a significant positive correlation between FhSAP2 and CE and between FhES antigen and CE were obtained.

3.2. Western blot analysis

The recombinant protein FhSAP2 was analyzed by SDS-PAGE and after silver staining the gel, a strong band was visible at 14.6kDa. This size corresponds to the expected molecular weight of the fusion protein based on the amino-acid sequence. FhSAP2 contains 101 amino acids with a predicted MW of 11.5kDa, but when expressed in *E. coli* as a fusion protein, the MW is slightly enlarged to 14.6kDa due to the six histidine residues, the X-Press epitope (Asp-Leu-Tyr-Asp-Asp-Asp-Lys), and the enterokinase site at the N-terminal (Fig. 2A). After Western blotting, purified FhSAP2 was probed against an anti-X-Press monoclonal antibody, against negative control sera, as well as against sera from individuals with fascioliasis or schistosomiasis. FhSAP2 polypeptide band was detected in all fascioliasis positive sera, but not in the negative control sera. The FhSAP2 polypeptide band was undetectable in the two control sera that had been recorded as sero-positive in the FhSAP2-ELISA assay. FhSAP2 polypeptide band was undetected in 11 of the 17 schistosomiasis positive samples, but was detected in a proven schistosomiasis positive sample that also tested positive in the FhSAP2-ELISA assay (Fig. 2B).

4. Discussion

The antigens most commonly used in ELISA assays are the ES products (Knobloch, 1985, Espino et al., 1998, Carnevale et al., 2001). It has been demonstrated that in animals experimentally infected with *F. hepatica* metacercariae, IgG against crude ES- products are detected between the second and third week after infection (Salimi-Bejestani et al., 2005). Nevertheless, the use of complex antigens such as the ES-products reduces the specificity of the test due to potential cross-reactivity with parasites sharing similar immunogens (Hillyer and Serrano, 1983, Cornelissen et al., 1999,), which was corroborated in the current study.

We have developed and evaluated a diagnostic-antibody detection ELISA for *F. hepatica* infection in humans. The test is based on the recombinant protein FhSAP2, which has shown to be a good immunogen capable of inducing protective immune responses to *F. hepatica* challenge infection in mice and rabbits (Espino et al. 2010, Espino and Rivera, 2010). Moreover, FhSAP2 is one of the molecules contained in the ES-products of *F. hepatica* (Espino and Hillyer, 2003).

A new diagnostic test should ideally be evaluated against a gold standard. To this end, we used a panel of human sera of known infection status. The positive sera belonged to persons that lived in Cajamarca Peru, where the prevalence of human and animal infection is considered to be very high (Claxton, 1997, Espinoza et al., 2010). All these persons had positive fecal egg counts indicating that they were currently infected. The negative samples consisted from persons that were not currently infected with *F. hepatica* and from persons infected with other parasites. Using these groups of serum samples, the diagnostic sensitivity, specificity and accuracy of the FhSAP2-ELISA were 100%, 96.5% and 97.6% respectively. Our results indicate that FhSAP2 is more specific than FhES products as target antigen for ELISA-based serodiagnosis of human fascioliasis since the best results for

specificity and accuracy were achieved with the recombinant protein. However, we noticed that the mean value for negative controls and for other groups of samples is higher for the FhSAP2-ELISA than for FhES-ELISA, which leads to a higher cut-off value; and that the mean value for the positive control samples in the FhSAP2-ELISA is slightly lower than the mean value obtained for these samples in the FhES-ELISA. This may result in the risk of a lower specificity and or sensitivity when a larger group of patients is being tested. It must be taken in consideration that, the sensitivity and specificity of tests will vary according to the population on which they are tested (Whiting et al., 2004, Leeflang and Bossuyt, 2005). Furthermore, no tests currently available can be considered as having both 100% sensitivity and 100% specificity.

Although, sera from humans with acute fascioliasis were not available at the moment of this study, the detection of anti-FhSAP2 antibodies in sera from animals experimentally infected and with more than 4 week of infection (Espino and Hillyer, 2003, Espino and Hillyer, 2004, Espino and Rivera, 2010) suggests that this antigen could be useful for the serological detection of fascioliasis at early stages in humans.

The results obtained by FhSAP2-ELISA were confirmed with the results obtained by Western blot and both demonstrated that during the natural infection, humans elicit antibodies against FhSAP2 as indicated by the presence of the typical polypeptide band at the molecular weight predicted for the fusion protein in all the fascioliasis sera samples. The two seropositives detected in the negative control group could be attributed to the persistence of very low antibodies against *F. hepatica*, as these persons could have been previously exposed to this parasite, although at the time of sample collection they were living in a non-endemic area. Moreover, based on the low absorbance values obtained for these sera ($A_{492} = 0.63$), it was not surprising that the FhSAP2 polypeptide was not detected in Western blot because this assay is less sensitive than the ELISA. Such ambiguities associated with the antibody based ELISA can be resolved using an antigen detection ELISA with monoclonal antibodies (Ubeira et al. 2009).

Cross-reaction with the serum of a case with schistosomiasis was demonstrated. This could either due to a subclinical infection with *F. hepatica* (the authors had no history of watercress consumption for the schistosomiasis patients and the prevalence of fascioliasis in the region where these patients lived was also unknown) or because FhSAP2 shares some epitope with *Schistosoma*, which has been previously demonstrated in previous studies (Torres and Espino, 2006). It will be necessary a larger scale validation test that include sera from acute infections and a larger number of sera from other helminthiasis, particularly schistosomiasis, and paragonimiasis which are major causes of cross-reactivity (Ikeda et al., 1996).

Another important element of this study was the ability to demonstrate that, contrary to FhES products that depend on the collection of the trematode at the slaughterhouse, recombinant FhSAP2 could be easily obtained and produced at large and homogeneous amounts to produce a standardized serological test for human fascioliasis. The comparison of results between different laboratories might also be easier if the same antigen is used in all laboratories.

Studies are in progress to analyze the usefulness of FhSAP2 in seroepidemiological studies of human fascioliasis. Moreover, studies are also in progress to develop monoclonal antibodies against those regions of the FhSAP2 protein moiety that are known not to contain *Fasciola/Schistosoma* cross-reactive epitopes (Torres and Espino, 2006). This strategy could provide two FhSAP2-based immunodiagnosis tools (antibody and antigen detection assays) with high potential for the immunodiagnosis of fascioliasis.

Acknowledgments

This work was supported by a research grant from the NIH/NIAID 1SC1AI096108-01A2 and the sub-award from the RCMI-RTRN program 10-11-KN-GR020000-UPR (Prime Award No. 3 U54 RR022762-0351). We are indebted to Dr. Pedro Orbitas (Universidad de Cajamarca, Facultad de Ciencias Veterinarias), Dr. Oscar Noya (Instituto de Medicina Tropical, Universidad Central de Venezuela) and Dr. Gary J. Weil (Infectious Diseases Division, Washington University School of Medicine) for kindly providing sera used in the current study. Authors also thank Dr. Lizzie Santiago-Santiago for editing the manuscript.

References

- Arias M, Hillyer GV, Sanchez-Andrade R, Suarez JL, Pedreira J, Lomba C, Diaz P, Morrondo P, Diez-Banos P, Paz-Silva A. A 2.9 kDa *Fasciola hepatica*-recombinant protein based ELISA test for the detection of current-ovine fasciolosis trickle infected. *Vet Parasitol.* 2006; 137:67–73. [PubMed: 16417971]
- Arjona R, Riancho JA, Aguado JM, Salesa R, Gonzalez-Macias J. Fascioliasis in developed countries: a review of classic and aberrant forms of the disease. *Medicine (Baltimore).* 1995; 74:13–23. [PubMed: 7837967]
- Carnevale S, Rodriguez MI, Santillan G, Labbe JH, Cabrera MG, Bellegarde EJ, Velasquez JN, Trgovcic JE, Guarnera EA. Immunodiagnosis of human fascioliasis by an enzyme-linked immunosorbent assay (ELISA) and a micro-ELISA. *Clin Diagn Lab Immunol.* 2001; 8:174–177. [PubMed: 11139214]
- Chen MG, Mott KE. Progress in assessment of morbidity due to *Fasciola hepatica* infection. A review of recent literature. *Trop Dis Bull.* 1990; 87:1–38.
- Claxton JR, Zambrano H, Ortiz P, Amoros C, Delgado E, Escurra E, Clarkson MJ. The epidemiology of fasciolosis in the inter-Andean valley of Cajamarca, Peru. *Parasitol Int.* 1997; 46:281–288.
- Cordova M, Reategui L, Espinoza JR. Immunodiagnosis of human fascioliasis with *Fasciola hepatica* cysteine proteinases. *Trans R Soc Trop Med Hyg.* 1999; 93:54–57. [PubMed: 10492791]
- Cornelissen JB, Gaasenbeek CP, Boersma W, Borgsteede FH, van Milligen FJ. Use of a pre-selected epitope of cathepsin-L1 in a highly specific peptide-based immunoassay for the diagnosis of *Fasciola hepatica* infections in cattle. *Int J Parasitol.* 1999; 29:685–696. [PubMed: 10404262]
- Cornelissen JB, Gaasenbeek CP, Borgsteede FH, Holland WG, Harmsen MM, Boersma WJ. Early immunodiagnosis of fasciolosis in ruminants using recombinant *Fasciola hepatica* cathepsin L-like protease. *Int J Parasitol.* 2001; 31:728–737. [PubMed: 11336755]
- Dalton JP, Brindley PJ, Knox DP, Brady CP, Hotez PJ, Donnelly S, O'Neill SM, Mulcahy G, Loukas A. Helminth vaccines: from mining genomic information for vaccine targets to systems used for protein expression. *Int J Parasitol.* 2003; 33:621–640. [PubMed: 12782060]
- Demirci M, Tanyel TT, Kaya S. Investigation of immunoglobulin isotypes in patients with fasciolosis. *Mikrobiyol Bul.* 2009; 43:471–475. [PubMed: 19795623]
- Erdman DD. Clinical comparison of ethyl acetate and diethyl ether in the formalin-ether sedimentation technique. *J Clin Microbiol.* 1981; 14:483–485. [PubMed: 7309845]
- Espino AM, Dumenigo BE, Fernandez R, Finlay CM. Immunodiagnosis of human fascioliasis by enzyme-linked immunosorbent assay using excretory-secretory products. *Am J Trop Med Hyg.* 1987; 37:605–608. [PubMed: 3688313]
- Espino AM, Marcet R, Finlay CM. Detection of circulating excretory secretory antigens in human fascioliasis by sandwich enzyme-linked immunosorbent assay. *J Clin Microbiol.* 1990; 28:2637–2640. [PubMed: 2279994]
- Espino AM, Diaz A, Perez A, Finlay CM. Dynamics of antigenemia and coproantigens during a human *Fasciola hepatica* outbreak. *J Clin Microbiol.* 1998; 36:2723–2726. [PubMed: 9705421]
- Espino AM, Hillyer GV. Molecular cloning of a member of the *Fasciola hepatica* saposin-like protein family. *J Parasitol.* 2003; 89:545–552. [PubMed: 12880256]
- Espino AM, Hillyer GV. A novel *Fasciola hepatica* saposin-like recombinant protein with immunoprophylactic potential. *J Parasitol.* 2004; 90:876–879. [PubMed: 15357089]

- Espino AM, Morales A, Delgado B, Rivera F, Figueroa O, Suárez E. Partial immunity to *Fasciola hepatica* in mice vaccinated with a DNA construct encoding for FhSAP2. *J Eth Dis*. 2010; 20:S1-17–23.
- Espino AM, Rivera F. Quantitation of cytokine mRNA by real-time RT-PCR during a vaccination trial in the rabbit model of fascioliasis. *Vet Parasitol*. 2010; 169:82–92. [PubMed: 20056331]
- Espinoza JR, Terashima A, Herrera-Velít P, Marcos LA. Human and animal fascioliasis in Peru: impact in the economy of endemic zones. *Rev Peru Med Exp Salud Publica*. 2010; 27:604–612. [PubMed: 21308203]
- Esteban JG, Gonzalez C, Bagues MD, Angles R, Sanchez C, Naquira C, Mas-Coma S. High fascioliasis infection in children linked to a man-made irrigation zone in Peru. *Trop Med Int Health*. 2002; 7:339–348. [PubMed: 11952950]
- Galen RS. Predictive value and efficiency of laboratory testing. *Pediatr Clin North Am*. 1980; 27:861–869. [PubMed: 7454413]
- Hillyer GV, Serrano AE. The antigens of *Paragonimus westermani*, *Schistosoma mansoni*, and *Fasciola hepatica* adult worms. Evidence for the presence of cross-reactive antigens and for cross-protection to *Schistosoma mansoni* infection using antigens of *Paragonimus westermani*. *Am J Trop Med Hyg*. 1983; 32:350–358. [PubMed: 6404188]
- Hillyer GV, Soler de Galanes M, Rodriguez-Perez J, Bjorland J, Silva de Lagrava M, Ramirez Guzman S, Bryan RT. Use of the Falcon assay screening test--enzyme-linked immunosorbent assay (FAST-ELISA) and the enzyme-linked immunoelectrotransfer blot (EITB) to determine the prevalence of human fascioliasis in the Bolivian Altiplano. *Am J Trop Med Hyg*. 1992; 46:603–609. [PubMed: 1599055]
- Ikedo T, Oikawa Y, Nishiyama T. Enzyme-linked immunosorbent assay using cysteine proteinase antigens for immunodiagnosis of human paragonimiasis. *Am J Trop Med Hyg*. 1996; 55:435–437. [PubMed: 8916802]
- Knobloch J. Human fascioliasis in Cajamarca/Peru. II. Humoral antibody response and antigenaemia. *Trop Med Parasitol*. 1985; 36:91–93. [PubMed: 4023558]
- Kofta W, Mieszczynek J, Plucienniczak G, Wedrychowicz H. Successful DNA immunisation of rats against fasciolosis. *Vaccine*. 2000; 18:2985–2990. [PubMed: 10825600]
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970; 227:680–685. [PubMed: 5432063]
- Law RH, Smooker PM, Irving JA, Piedrafita D, Ponting R, Kennedy NJ, Whisstock JC, Pike RN, Spithill TW. Cloning and expression of the major secreted cathepsin B-like protein from juvenile *Fasciola hepatica* and analysis of immunogenicity following liver fluke infection. *Infect Immun*. 2003; 71:6921–6932. [PubMed: 14638781]
- Leeftang MG, Bossuyt PMM. Test accuracy is likely to vary depending on the population it is based on. *Vet Parasitol*. 2005; 134:189. [PubMed: 16154707]
- Mas-Coma S. Epidemiology of fascioliasis in human endemic areas. *J Helminthol*. 2005; 79:207–216. [PubMed: 16153314]
- Massoud J. Fascioliasis outbreak in man and drug test (triclabendazole) in Caspian littoral, northern part of Iran, 1989. *Bull Soc Fran Parasitol*. 1990; 8:438.
- Mezo M, Gonzalez-Warleta M, Carro C, Ubeira FM. An ultrasensitive capture ELISA for detection of *Fasciola hepatica* coproantigens in sheep and cattle using a new monoclonal antibody (MM3). *J Parasitol*. 2004; 90:845–852. [PubMed: 15357080]
- Muro A, Ramajo V, Lopez J, Simon F, Hillyer GV. *Fasciola hepatica*: vaccination of rabbits with native and recombinant antigens related to fatty acid binding proteins. *Vet Parasitol*. 1997; 69:219–229. [PubMed: 9195732]
- Parkinson M, O'Neill SM, Dalton JP. Endemic human fasciolosis in the Bolivian Altiplano. *Epidemiol Infect*. 2006; 135:669–674. [PubMed: 17064455]
- Paz-Silva A, Hillyer GV, Sanchez-Andrade R, Rodriguez-Medina JR, Arias M, Morrondo P, Diez-Banos P. Isolation, identification and expression of a *Fasciola hepatica* cDNA encoding a 2.9-kDa recombinant protein for the diagnosis of ovine fasciolosis. *Parasitol Res*. 2005; 95:129–135. [PubMed: 15609063]

- Raymundo LA, Flores VM, Terashima A, Samalvides F, Miranda E, Tantalean M, Espinoza JR, Gotuzzo E. Hyperendemicity of human fasciolosis in the Mantaro Valley, Peru: factors for infection with *Fasciola hepatica*. *Rev Gastroenterol Peru*. 2004; 24:158–164. [PubMed: 15241494]
- Salimi-Bejestani MR, McGarry JW, Felstead S, Ortiz P, Akca A, Williams DJ. Development of an antibody-detection ELISA for *Fasciola hepatica* and its evaluation against a commercially available test. *Res Vet Sci*. 2005; 78:177–181. [PubMed: 15563926]
- Sanchez-Andrade R, Paz-Silva A, Suarez JL, Panadero R, Pedreira J, Diez-Baños P, Morrondo P. Effect of fasciolicides on the anigenemia in sheep naturally infected with *Fasciola hepatica*. *Parasitol Res*. 2001; 87:609–614. [PubMed: 11510995]
- Silva E, Castro A, Lopes A, Rodrigues A, Dias C, Conceicao A, Alonso J, Correia da Costa JM, Bastos M, Parra F, Moradas-Ferreira P, Silva M. A recombinant antigen recognized by *Fasciola hepatica*-infected hosts. *J Parasitol*. 2004; 90:746–751. [PubMed: 15357063]
- Spithill, TW.; Smooker, PM.; Copeman, DB. *Fasciola gigantica*: Epidemiology, Control, Immunology and Molecular Biology. In: Dalton, JP., editor. Fascioliasis. CABI Publishing; Oxon, UK: 1999. p. 465-525.
- Thrusfield, M. *Veterinary Epidemiology*. Blackwell Science; Oxford, U: 1997. (Second revised reprint)
- Torgerson, P.; Claxton, J. Epidemiology and control. In: Dalton, JP., editor. Fascioliasis. CAB International; Oxford: 1999. p. 113-150.
- Torres D, Espino AM. Mapping of B-cell epitopes on a novel 11.5-kilodalton *Fasciola hepatica*-*Schistosoma mansoni* cross-reactive antigen belonging to a member of the *F. hepatica* saposin-like protein family. *Infect Immun*. 2006; 74:4932–4938. [PubMed: 16861685]
- Ubeira FM, Muiño L, Valero MA, Periago MV, Pérez-Crespo I, Mezo M, González-Warleta M, Romarís F, Paniagua E, Cortizo S, Lovo J, Más-Coma S. MM3-ELISA detection of *Fasciola hepatica* coproantigens in preserved human stool samples. *Am J Trop Med Hyg*. 2009; 81:156–162. [PubMed: 19556582]
- Whiting P, Rutjes AW, Reitsman JB, Glas AS, Bossuyt PM, Kleijnen J. Sources of variation and bias in studies of diagnostic accuracy: a systematic review. *Ann Int Med*. 2004; 140:189–202. [PubMed: 14757617]

Figure 1. ELISA assay showing the reactivity of FhSAP2 and FhES with 50 sera from normal human subjects (NHS), 40 sera from persons infected with other parasites (OP) including *Trichuris trichiura*, *Giardia lamblia*, *Entamoeba histolytica*, *Ascaris lumbricoides*, and *Filaria*, 37 sera from patients with chronic fascioliasis (Fh), 17 sera from individuals with chronic schistosomiasis mansoni (Sm), and 5 sera from patients with chronic paragonimiasis *westermani/kellicotti* (Pw/k). Bars showing the mean and standard error of the mean are shown with lines in each column. ** indicate statistically significant differences between groups ($P < 0.001$). Horizontal dashed line indicates the cutoff value established in both assays.

Figure 2.

Purified fusion protein (FhSAP2) resolved by SDS-PAGE on 12.5% polyacrylamide gel was electrotransferred into nitrocellulose membranes and incubated with the monoclonal antibody against the X-Press epitope. (A) Immunoblot revealing the polypeptide band at 14.6kDa typical of the FhSAP2 fusion protein. The estimated MW for FhSAP2 (11.5kDa) is slightly enlarged by the (His)₆-tag plus the X-Press epitope and the enterokinase site (3.1kDa) at the amino end of protein. (B) Immunoblots of 3 µg of fusion protein FhSAP2 with a pool of normal human sera (lane 1), in which the polypeptide band of 14.6kDa is not revealed. Lanes 2–7 represent sera from persons with chronic fascioliasis confirmed by coprological examination, which were also sero-positive by FhSAP2-ELISA and FhES-ELISA. Lane 8 represents serum from a patient with chronic schistosomiasis that resulted sero-positive by FhSAP2- and FhES-ELISA. Lane 9 represents a pool of control sera that were sero-positive by the FhSAP2-ELISA and Lane 10 represents a pool of sera from patients with chronic schistosomiasis that were sero-negative by the FhSAP2- and FhES-ELISA. Molecular weights are shown on the left. The predicted FhSAP2 fusion protein (14.6kDa) is marked with arrow.

Table 1

Statistical analysis among the results obtained with the FhSAP2-ELISA and FhES-ELISA and those from coprological examination (CE) for *F. hepatica* eggs used as gold standard.

ELISA	CE +	CE -	Total	ELISA	CE +	CE -	Total
FhSAP2 +	37	3	40	FhES +	37	7	44
FhSAP2 -	0	84	84	FhES -	0	80	80
Total	37	87	124	Total	37	87	124
S	100.0%			S	100.0%		
Sp	96.5%			Sp	91.9%		
Accuracy	97.6%			Accuracy	94.3%		
Kappa	0.949			Kappa	0.85		
<i>p</i> -Value	0.005			<i>p</i> -Value	0.005		