Development of Two Antibody-Detection ELISAs for Serodiagnosis of Human Chronic Fascioliasis

Kimberly Cabán-Hernández, José F. Gaudier, Caleb Ruiz-Jiménez, Ana M. Espino#

Laboratory of Immunology and Molecular Parasitology, Department of Microbiology, University of Puerto Rico, School of Medicine

Running Head: ELISA for serodiagnosis of human fascioliasis

#Address correspondence to Espino, A.M. ana.espino1@upr.edu

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

KCH and JFG contributed equally to this work
ABSTRACT
Coprological examination based on egg detection in stool samples is currently used as the gold standard for diagnosis of human fascioliasis. However, this method is not effective during the acute phase of the disease and has poor sensitivity during the chronic phase. Serodiagnosis has become an excellent alternative to coprological examination in efforts to combat the impact of fascioliasis on human and animal health. Two novel recombinant Fasciola hepatica proteins, a ferritin (FhFtn-1) and a tegument-associated protein (FhTP16.5), were used as antigen to develop in-house ELISA methods. The assays were optimized and validated using 152 sera from humans with known infection status, which included healthy subjects, patients with chronic fascioliasis, and patients with other parasitic diseases. The FhFtn-1-ELISA was shown to be 96.6% sensitive and 95.7% specific. The same parameters for the FhTP16.5-ELISA were 91.4% and 92.4%, respectively. The performance of the FhFtn-1- and FhTP16.5-ELISAs was compared to that of an available commercial test (DRG-test) using a subset of sera. Our in-house tests were slightly more sensitivity than the DRG test in detecting antibodies against F. hepatica but differences were not statistically significant. In conclusion, the present study provides evidence of the potential of FhFtn-1- and FhTP16.5-ELISAs as diagnostic tools for human fascioliasis, as might be implemented in conjunction with standard assays for large-scale screenings in endemic areas and for the detection of occasional cases in clinical laboratories.
INTRODUCTION

Fascioliasis is a disease caused by liver flukes of the genus *Fasciola*, of which *F. hepatica* and *F. gigantica* are the most common representatives. The disease causes significant economic losses worldwide, approaching 2 billion USD dollars per year due to ruminant livestock infection alone (1). Human fascioliasis cases have been steadily rising since the 1970s and are now considered a re-emerging parasitic disease in humans, a phenomenon that has partly been attributed to climate change (1-3). The World Health Organization recognized fascioliasis as an important infectious disease, with an estimated 17 million people affected worldwide (1). Humans become infected after ingestion of water or aquatic vegetation contaminated with *F. hepatica* metacercariae. *F. hepatica* predominates in temperate climates, and *F. gigantica* overlaps with *F. hepatica* and is also found in the tropical regions of Asia and Africa (4). Fascioliasis has historically been severely neglected by the medical and scientific communities; however, the disease has recently been recognized as a global human concern.

Confirmatory diagnosis of *F. hepatica* infection is based on the identification of eggs in feces or bile drainage. However, there is a consensus that this method is not wholly reliable for several reasons. In non-endemic regions infections with immature flukes are not detected. Diagnosis (detection of eggs) often happens during the chronic phase and when eggs are detected much of the liver damage has already occurred (5). The eggs are released intermittently from the bile ducts, so that stool samples of infected patients may not contain eggs (2). This makes it necessary to perform serial analysis of samples using concentration techniques, which makes coprological examination a labor-intensive method of diagnosis. Often, the number of eggs shed is so low that it is
necessary to analyze up to six stool samples (6), and this can lead to unreliable results in epidemiological studies, and over burden clinical laboratories. Due to these limitations of the coprological diagnosis, other standardized tests are urgently needed for both individual patient diagnosis and epidemiological surveys in endemic areas for human fascioliasis. To date, various diagnostic techniques have been developed, including molecular techniques such as PCR, which facilitate the identification and discrimination of *Fasciola* *spp.* in endemic areas where *F. gigantica* and *F. hepatica* coexist (7, 8). Recently, evaluation of field-collected stools samples of ruminants and humans by duplex PCR revealed that this method is sensitive and able to identified *Fasciola* *spp.* (8). Other serological techniques have been studied in which specific antibodies are detected including the Dot-blot (9), lateral flow immunoassay (10) and indirect enzyme-linked immunosorbent assay (ELISA) (11-13). Detection of antibodies in serum by ELISA is a frequently used diagnostic tool and is considered a sensitive and reliable means of diagnosing acute infection and it can be used as an adjunct to fecal analysis for the diagnosis of latent and chronic infections (14).

The antigens traditionally used in serological tests are crude extracts or excretory-secretory products (ESPs) of *F. hepatica* (11, 15, 16). Several *F. hepatica* purified antigens (17, 18) and recombinant antigens have been employed to enhance the specificity of the diagnostic assays (11, 19, 20). The most notable are cathepsin-L the major protease involved in the *F. hepatica* virulence (21), fatty acid binding proteins (FABPs) (22-24) and saposin-like proteins (FhSAP2) (25), which have been documented as useful immunodiagnostic antigens for serological detection of fascioliasis. In spite of the many serological methods published only a few have been commercialized. One of
these assays (Ildana Biotech) uses as antigen recombinant forms of cathepsin-L1 and has been optimized for detecting antibodies in serum and milk of cattle (26). Others methods, as the AccuDiag™ Fasciola IgG ELISA (Diagnostic Automation/Cortez Diagnostic, Inc.), Bio-X and DRG-kits (DRG Instruments GmbH, Germany) have been optimized for detecting antibodies in sera of cattle (27) and humans (28) respectively. These assays use ESPs as antigens, which could limit their usefulness due to cross-reactions with other parasites (27, 28).

Our research group recently reported the molecular cloning, purification and characterization of two novel *F. hepatica* antigens. One of these antigens is a 16.5kDa tegument-associated protein of unknown function, termed FhTP16.5 (29), and the other is a protein with ferroxidase activity classified as a member of the *F. hepatica* ferritin protein family (FhFtn-1) (30). Both molecules are differentially expressed during parasite development and have been shown to be highly reactive with sera from experimental animals with acute or chronic infection. The present study aimed to examine the potential of FhTP16.5 and FhFtn-1 as antigens to detect antibodies in humans with chronic fascioliasis and to compare the suitability of the FhFtn-1- and FhTP16.5-ELISA with a commercial ELISA method that uses crude ES antigens.

**MATERIALS AND METHODS**

**Human sera.** The serum samples used in this study were kindly donated by collaborators from the National University of Cajamarca, Peru (approved by the Ethics Committee of the Regional Hospital of Health from Cajamarca and the General Direction of Zoonosis...
from the Ministry of Health (MINSA), Lima; the Department of Sanitary Parasitology, ANLIS “Dr. Carlos G. Malbrán”, Argentina; the Tropical Medicine Institute (IMT) of the Central University of Caracas, Venezuela (approved by the Ethical Scientific Committee of the IMT); and the Infectious Diseases Division, Washington University School of Medicine, St. Louis, MO (approved by the Institutional Review Boards at Washington University School of Medicine). Serum samples all originated from adult individuals and were collected after obtaining written informed consent from all subjects. All serum samples had been previously sent to the respective laboratories and therefore not collected specifically for this study. No personal identifiers were retained. Samples were stored frozen at -70°C for up to six months until use and the aliquots tested in this study had not been thawed prior testing. In total, 152 serum samples were analyzed. Sixty sera were from patients with a confirmed diagnosis of fascioliasis based on detection of *F. hepatica* eggs in their stools and absence of other parasitic infections (true positives). Of these, 42 were from subjects who lived in the Bolivian Altiplano, and 18 were from subjects who lived in Cajamarca, Peru, where fascioliasis is highly endemic (12, 31). Sera from 51 healthy subjects who in the sampling period lived in non-endemic areas for trematode infections (Puerto Rico, Argentina) were also obtained. The stools of these subjects were negative for *F. hepatica* and other parasites by coprological examination (CE) and used as negative controls (true negatives). To evaluate potential cross-reactivity, serum samples were also obtained from parasitologically and/or serologically confirmed cases of paragonimiasis (n=5), schistosomiasis (n=15), filariasis (n=5), visceral larva migrans (VLM) caused by *Toxocara canis* (n=4), trichinellosis (n=4), toxoplasmosis (n=4), and hydatidosis (n=4).
Preparation of recombinant FhFtn-1 and FhTP16.5 proteins. cDNA encoding FhFtn-1 (GenBank: HQ316639.1) was cloned into the pRSET A plasmid (Invitrogen, Carlsbad, CA) and used to transform E. coli BL21 (DE3) cells (Stratagene, San Clara, CA). The recombinant polypeptide was overexpressed as a fusion protein with a His-tag and purified using a HisTrap FF™ crude affinity column (GE Healthcare Biosciences, Pittsburgh, PA) as previously described (30). cDNA encoding FhTP16.5 (GenBank: AY851159) was cloned into pGEX4T-1 plasmid and expressed in the same bacterial system. The GST-tagged recombinant protein was purified by affinity chromatography using a GST-Trap HR 5/5 column (GE Healthcare) and cleaved from the GST-tag as described by Gaudier et al. (29).

Indirect enzyme-linked immunosorbent assay (ELISA). ELISA was performed following a basic protocol previously reported (15) that was optimized by checkerboard titration to maximize the sensitivity of the assay. Briefly, polystyrene ELISA plates (Costar, Cambridge, MA) were coated overnight at 4°C with 100μl/well of recombinant FhFtn-1 (5μg/ml) or FhTP16.5 (10μg/ml) in 0.1ml of 0.05M carbonate buffer (pH 9.6). After three washes with PBS containing 0.05% Tween-20 (PBST), the plates were blocked with 1% BSA in PBST (300μl/well) for 1 h, 37°C. The sera were diluted 1:800 (for FhFtn-1-ELISA) or 1:1,600 (for FhTP16.5-ELISA) in PBST (100μl/well) and incubated at 37°C for 1 h. The excess of antibody was washed with PBST, and HRP-anti-human IgG conjugate (Bio-Rad, Hercules, CA) diluted 1:5,000 was added to the wells (100μl/well) and incubated at 37°C for 1 h. After another washing step, the substrate solution was added (100μl/well) (25 ml of 0.1 M citrate-phosphate buffer pH 5.0 containing 20mg O-phenylenediamine dihydrochloride and 30% H₂O₂) and the plates
were incubated in the dark for 30 min at room temperature. The reaction was stopped with 50 μl/well of 12.5% sulfuric acid and the absorbance was measured at 490nm using an ELISA reader (Bio-Rad). Positive and negative controls were included on each plate. Each ELISA determination was performed in duplicate and the results expressed as the mean absorbance value (A_{490}) for each determination.

**DRG® Fasciola hepatica IgG ELISA (DRG-test).** FhFtn-1 and FhTP16.5 ELISA tests were compared to an ELISA test (DRG-test, DRG Instruments GmbH, Germany) that is based on excretory-secretory products (ESPs) from adult *F. hepatica* mainly containing cysteine proteases (27, 28). This test was used on a subset of 86 serum samples selected among those that had been previously used to validate the FhFtn-1- and FhTP16.5-ELISAs. To avoid any bias, the sera used in the subset were blindly selected and included those sera that were misdiagnosed with FhFtn-1- or FhTP16.5-ELISAs. The subset of sera included 10 negative control sera, 41 sera from patients infected with *F. hepatica* and 35 sera from subjects infected with other parasitic diseases. All steps, data analysis and validation were carried out according to the manufacturer’s instructions. Human sera, negative and positive controls and a cut-off control (CO) (1:100 dilution) were added into duplicate wells. Plates were read at 450nm using a Bio-Rad ELISA reader. The test run was considered valid if the substrate blank had an absorbance value below 0.1, the negative control below 0.2, the CO control between 0.25 and 0.75, and the positive control above 0.6. A serum is considered positive when its absorbance value is above 10% of CO. The results in DRG Units (DU) were calculated according to the following formula: DU = (sample mean A x 10) / CO. The results were negative if DU < 9, and positive if DU > 11.
**Data analysis.** The optimal cutoff for each ELISA method was established by Receiver Operating Characteristic (ROC) curve analysis using the EpiTools epidemiological calculator (32) considering a 95% confidence interval (CI). According to an arbitrary guideline, the area under the curve (AUC) was considered: non-informative (AUC=0.5); low accurate (0.5 < AUC ≤ 0.7); moderately accurate (0.7 < AUC ≤ 0.9); highly accurate (0.9 < AUC < 1) or perfect (AUC = 1) (33). Intra-plate repeatability was evaluated for both in-house tests by measuring the coefficient of variation (CV) of 96 repeats of 10 sera. Reproducibility was evaluated by performing 5 independent assays for each recombinant protein using 5 positive control and 5 negative control sera on separate runs. The robustness of the ELISA tests was also evaluated when different operators performed the test using two batches of each antigen. Results obtained with FhFtn-1- and FhTP16.5-ELISAs were compared to the commercial test (DRG-test) using a subset of sera. Correlations between the in-house ELISAs and the DRG-test were performed using the Pearson correlation coefficient (95% CI). To evaluate the agreement among the ELISA methods or with the CE, inter-rater agreement (kappa) was calculated according to Thrusfield (34). The Kappa values (κ) were considered as follows: poor agreement (κ<0.2); fair agreement (κ=0.21-0.4); moderate agreement (κ=0.41-0.6); good agreement (κ=0.61-0.8), or very good agreement (κ=0.81-1.0).

**RESULTS**

Using the CE method as gold standard, ROC curves were built based on the absorbance values obtained with sera from the two reference populations, those infected with *F.*
hepatica (positive to CE) and those that are healthy (negative controls). In the FhFtn-1-ELISA, absorbance values for the negative control group ranged between 0.07 and 0.31, with a mean A$_{490}$ value of 0.216 ± 0.065 (standard deviation), whereas in the FhTP16.5-ELISA the absorbance of the negative control group ranged between 0.05 and 0.46, with a mean value of 0.182 ± 0.094. The AUC was 0.999 for the FhFtn-1-ELISA and 0.981 for the FhTP16.5-ELISA, indicating that both assays provide equally accurate results (Fig. 1). The ROC optimized cut-offs were 0.48 and 0.57 for the FhFtn-1 and FhTP16.5 ELISAs, respectively. When sera of patients with confirmed fascioliasis were tested in the FhFtn-1-ELISA, the absorbance ranged between 0.37 and 1.7, with a mean absorbance value of 0.97± 0.36, whereas the absorbance in the FhTP16.5-ELISA ranged between 0.22 and 2.45, with a mean value of 1.1 ± 0.47. Based on the cutoff values established, no seropositives among the true negative population were found irrespective of the antigen used. However, 2 seronegative sera were detected among the true positive population using the FhFtn-1-ELISA, whereas with the FhTP16.5-ELISA, 5 seronegative sera were found. Thus, the FhFtn-1 and FhTP16.5 ELISAs reached a sensitivity of 96.6% (95% CI: 88.3% to 99.0%) and 91.4% (95% CI: 81.4% to 96.3%), respectively, and yielded a specificity of 100% (95% CI: 93% to 100%). The false negative sera found with FhFtn-1 were from Peruvian patients, whereas the false negative sera found with FhTP16.5 were from Bolivian patients. Therefore, when the results of both antigens were combined, the assay rendered 100% sensitivity. No statistical differences were found between absorbance values of the positives to CE based on their geographic origin with either of the antigens.
We assessed the reproducibility of the ELISAs by calculating the CV of data from 5 different assays and 96 repeats of 10 sera. The intra- and inter-assay reproducibility was 4.9% and 11.8% for the FhFtn-1-ELISA and 6% and 15% for the FhTP16.5-ELISA, respectively.

When sera from subjects infected with parasitic infections other than fascioliasis were tested, 4 seropositive sera were found with FhFtn-1 and 7 were found with FhTP16.5 (Fig. 2). Therefore, when the cross-reactions were analyzed the specificity dropped to 95.7% (95% CI: 89.3% to 98.3%) for FhFtn-1 and to 92.4% for FhTP16.5 (95% CI: 85.0% to 96.3%) as summarized in Table 1. There was high agreement between both ELISA methods (κ = 0.84) and between the FhFtn-1- or FhTP16.5-ELISA and CE, with κ values of 0.92 and 0.84, respectively; a Kappa value >0.81 indicates very good agreement between two tests (34).

Table 2 shows the comparison of the FhFtn-1- and FhTP16.5-ELISAs with the DRG-test using a sub-set of the sera. No seropositives were found in the true negative population tested irrespectively of the test used; however, using the DRG test 6 positive control sera had readings below the cut-off value (DU= 10) and 4 sera from patients with other parasitic infections, namely schistosomiasis (n=1) and paragonimiasis (n=3), were recorded as seropositives. Thus, the DRG-test had a sensitivity of 87.2%, specificity of 91.8% and good agreement with the CE (κ = 0.77). The FhFtn-1-ELISA detected 39 of the 41 fascioliasis sera compared to the 35 detected by the DRG-test. Thus, FhFtn-1-ELISA was slightly more sensitive than the DRG-test, but differences were not statistically significant (P=0.26). Moreover, in terms of specificity the performance of both assays was similar. Although the sensitivity of FhTP16.5-ELISA was slightly higher...
than the DRG-test, FhTP16.5-ELISA also detected a larger number of false positives compared to DRG-test or FhFtn-1-ELISA. We found a moderate positive correlation ($r=0.68$) and good agreement ($\kappa=0.79$) when the DRG-test and FhFtn-1-ELISA were compared. The results of the comparison between the DRG-test and the FhTP16.5-ELISA also demonstrated a moderate positive correlation ($r=0.61$) and good agreement ($\kappa=0.79$) between both tests, using a 95% CI (Fig. 3).

**DISCUSSION**

Several ELISA techniques have been described for serodiagnosis of fascioliasis, and most rely on excretory-secretory products (11, 15) or recombinant proteases (11, 35) that are the major components of ESPs. In the present study, we demonstrated that a cytosolic ferritin-like protein (FhFtn-1) and a tegument-associated protein (FhTP16.5) are also excellent antigens for serodiagnosis of chronic fascioliasis. Although the observation of parasite eggs in stools by microscopical examination has multiple drawbacks, it is still used for confirmatory diagnosis of fascioliasis. In an effort to improve the methods of fascioliasis diagnosis, we validated the FhFtn-1- and FhTP16.5-ELISAs using a panel of sera obtained from humans with known infectious status confirmed by stool analysis. A new diagnostic test can be evaluated by a number of different parameters, including sensitivity, specificity, accuracy, efficiency and positive and negative predictive values (36). A critical point for this evaluation is how the cut-off point is established; therefore, we employed in the validation of our assays two well-characterized set of sera with a sample size large enough to minimize the stochastic uncertainty in cut-off selection (32).
By using ROC curve analysis, we were able to select the cut-off value that gives the best balance of sensitivity and specificity for the developed ELISAs (37).

The high sensitivity shown by the FhFtn-1- and FhTP16.5-ELISAs (96.6% and 91.4%, respectively) demonstrates that, during active infection, humans elicit high antibody levels against these proteins, which indicates that these molecules are exposed to the host immune system. This finding supports our previous studies using experimentally infected rabbits, in which antibodies were detected between 2 to 4 weeks after infection using recombinant FhFtn-1 and FhTP16.5 proteins (29, 30). Unfortunately, for the present study, we were not able to obtain serum samples from humans with acute fascioliasis. It is worth mentioning that proper diagnosis of acute fascioliasis remains a challenge because the infection is often asymptomatic and evolves to chronic disease without early treatment. However, based on the performance of the recombinant proteins using sera from animals with acute infection, it is likely that the in-house ELISAs will be similarly useful for diagnosis at early stages of human infection. Moreover, very interesting studies have identified ferritin-like and tegument-associated proteins in the vomitus and exosome-like vesicles of trematodes (38-41). These extracellular vesicles account for 52% of the *F. hepatica* secretome (40-43), providing another explanation for the secretion of atypical proteins in helminths and their exposure to the host interface.

In our study, only 2 (3.3%) true positive (positive to CE) fell below the cut-off value with FhFtn-1, whereas that with FhTP16.5, 5 (8.3%) true positive were recorded as negatives. Since all sera were properly preserved at -70°C from their collection until use, it is possible to speculate that the sensitivity of both assays was influenced by factors such as the intensity of infection, persistence of antibodies post-infection, and parasite
senescence (21). Northern Peru and Bolivian Altiplano are two regions highly endemic for human fascioliasis (3, 6, 44-47). Therefore, it is also possible that the size of the dose of infectious metacercariae could affect the development of a detectable antibody response against a particular antigen (48), as humans exposed to *F. hepatica* infection are likely to ingest low doses of metacercariae over a prolonged period.

When sera from persons with other parasites were tested, the specificity of both ELISA tests dropped since 4 sera from persons carrying other infections were positive with FhFtn-1 and 7 were positive with FhTP16.5, most of them with absorbance values very close to the cut-off points. Nevertheless, the diagnostic sensitivity and specificity of the FhFtn-1- and FhTP16.5-ELISAs are similar to the results obtained with other in-house ELISAs: 92.4% and 83.6% (12), 97.2% and 100% (49), 100% and 98.9% (18, 50, 51). The cross-reactions observed in this study could be due to the existence of common epitopes between FhFtn-1 and FhTP16.5 and proteins of other parasites (52). Cross-reactivity is a significant problem, mainly for helminths, because there are many molecules such as cathepsins, hormones, receptors, etc., that have been conserved during evolution and share common epitopes (52). However, other possible explanations could also be considered: 1) the persistence of low antibody levels as these persons could have been previously exposed to *F. hepatica*, although at the time of sample collection they did not have eggs in their feces (we are unaware of whether these persons had lived in fascioliasis endemic regions prior to sample collection); or 2) cases with resolved fascioliasis infection (we had no history of possible previous treatment with triclabendazole). We further evaluated the false positive sera with two others ELISA methods previously validated in our laboratory utilizing as antigens recombinant *F.
hepatica saposin-2 (25) and F. hepatica tegumental proteins (53), which have showed diagnostic specificity higher than 95%. The results from these two additional ELISAs indicated that 5 of the 11 false positive sera (2 shistosomiasis serum, 1 paragonimiasis serum and 2 hydatidosis sera) could be true positives that were misdiagnosed by the microscopic examination. It should be also noted that the number of sera with some of the parasitic infections tested in this study was relatively low and that sera from patients infected with other food-borne trematodes (e.g. Clonorchis sinensis and Opisthorchis viverrini) were not available for study, which prevented us from establishing whether these pathogens may show cross-reactions. Therefore, it will be necessary to conduct a larger scale validation study including a larger panel of sera from other helminthiases, particularly schistosomiasis, paragonimiasis and clonorchiasis, which are major causes of cross-reactivity (54).

Both in house ELISAs were compared to the DRG-test, an ELISA for antibody detection based on ESPs. Our results demonstrate that the FhFtn-1-ELISA was slightly more sensitive than FhTP16.5-ELISA or DRG-test and similar sensitivities were found between FhTP16.5-ELISA and DRG-test. The low sensitivity obtained herein with the DRG-test is in contrast with the results reported by Valero et al. (28), who validated the DRG test in populations of different epidemiological status, obtaining sensitivity and specificity of >95% compared to CE. Since the DRG-test uses ESPs presumably obtained from European isolates, we could speculate that the sensitivity of the DRG-test was impacted by using sera samples from individuals exposed to South American isolates of F. hepatica. This presumption is supported by the fact that most of the fascioliasis sera analyzed here had been evaluated for us in a previous study employing locally produced
ESPs, obtaining 100% positivity (25). However, other authors who validated the Bio-X ELISA, a cattle version of the commercial test, in animals from farms in the Cajamarca region of Northern Peru, obtained 98% sensitivity and 96% specificity (27). Based on these observations, we suppose that the differences in the performance of this assay could be related to differences in the quality of the ESP preparation rather than to problems associated with the genotypic diversity of various isolates of *F. hepatica*. Thus, different laboratories that have obtained ESPs from American or European isolates to test sera from persons or animals exposed to the same isolates report sensitivities and specificities ranging from 52% to 100% (11, 15, 25, 48, 55, 56). Despite these differences, both the FhFtn-1- and FhTP16.5- ELISAs showed good agreement with the DRG-test. However, we believe that ELISAs employing recombinant proteins instead of crude ESPs could improve the possibilities for standardization of a test for fascioliasis. Also the use of recombinant protein will be an advantage in scaling up production for mass screening. Comparison of results between different laboratories might also be easier if the same antigen is used in all laboratories. Since the precision of an immunoassay is defined as the reproducibility of results within, and between assays. The low CV (<20%) values obtained for both intra- and inter-assay precision of the in-house ELISAs ensure that the results obtained will be reproducible, and instill confidence about assay performance.

In conclusion, we have developed two sensitive and specific ELISAs that employ a recombinant ferritin and a tegument-associated protein for the detection of *F. hepatica* antibodies in humans, which have been validated using sera from humans of known infection status. These assays are part of the arsenal of immunodiagnostic tools that our laboratory is developing with the purpose of improving serodiagnosis of human
fascioliasis. Due to the concern that a positive result in antibody detection tests does not necessarily indicate a current infection, but rather a history of exposure we are working on alternatives as using an antigen detection ELISA with monoclonal antibodies. Also, further studies are in progress to adapt our in-house ELISA methods to more simple and reliable formats such as immunochromatography or Dot-ELISA to facilitate their possible commercialization and validation in endemic areas.

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Figure 1. Receiver-operator characteristic (ROC) curve. The ROC curve was built for 51 sera from healthy subjects and 60 sera from patients with confirmed diagnosis of fascioliasis by finding *F. hepatica* eggs in stool samples. The area under the ROC curve (accuracy) was 0.999 for the FhFtn-1-ELISA and 0.981 for the FhTP16.5-ELISA.

Figure 2. Analysis of sera obtained from subjects with other parasitoses tested by FhFtn-1- and FhTP16.5-ELISAs. Sera from individuals carrying parasitic infections other than *F. hepatica* were studied. The study included 15 sera from patients with *Schistosomiasis mansoni* (Sch), 5 sera with lymphatic filariasis (Fil), 5 with *Paragonimiasis westermani / kelicotti* (Psp), 4 with trichinellosis (Tri), 4 with visceral larva migrans (VLM), 4 with toxoplasmosis (Tox) and 4 with hydatidosis (Hyd). Dashed lines represent the cut-off point that gives the best balance between sensitivity and specificity according to the ROC curve.

Figure 3. Correlation between the FhFtn-1- and FhTP16.5-ELISAs and the DRG commercial test. A subset of 86 serum samples was blindly selected from the panel used to validate the FhFtn-1- and FhTP16.5-ELISAs and tested with the DRG-test for detection of antibodies. The Pearson correlation coefficient between the DRG-test and the FhFtn-1-ELISA was 0.68 and the *Kappa* value was 0.79. The correlation coefficient
between the DRG-test and the FhTP16.5-ELISA was 0.61 and the Kappa value was 0.79.

The results of both tests are in good agreement with those of the DRG-test.
Table 1. Diagnostic sensitivity and specificity (95% CI) for serum samples from patients with a diagnosis of *Fasciola hepatica* infection (confirmed by detection of *F. hepatica* eggs in patient stools), serum samples from healthy subjects and serum samples from persons with other parasitic infections.

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<th>Uninfected</th>
<th>n</th>
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*FhFtn-1*  

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<th>Uninfected</th>
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<td>Positive</td>
<td>True positive</td>
<td>55</td>
<td>False positive</td>
<td>7</td>
<td>62</td>
</tr>
<tr>
<td>Negative</td>
<td>False negative</td>
<td>5</td>
<td>True negative</td>
<td>85</td>
<td>90</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>60</td>
<td></td>
<td>92</td>
<td>152</td>
</tr>
</tbody>
</table>

*a* Sensitivity = 96.6% (95% CI: 88.5% to 99.1%), calculated as TP / (TP + FN) x 100  
Specificity = 95.7 % (95% CI: 89.5% to 98.3%), calculated as TN / (TN + FP) x 100  

*b* Sensitivity = 91.4% (95% CI: 81.4% to 96.3%), calculated as TP / (TP + FN) x 100  
Specificity = 92.4% (95% CI: 85.3% to 96.3%), calculated as TN / (TN + FN) x 100  

TP: true positive (positive to CE), TN: true negative (negative to CE), FN: false negative (positive to CE but negative by serological assays), FP: false positive (negative to CE but positive by serological assays). CE: coprological examination used as gold standard.
Table 2. Testing of serum samples of patients with *F. hepatica* or other parasite infections and healthy donors by the DRG-test and FhFtn-1-ELISA or FhTP16.5-ELISA

<table>
<thead>
<tr>
<th>Subjects</th>
<th>No. of serum samples</th>
<th>No. (%) of positive detection by different assays</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DRG-test</td>
</tr>
<tr>
<td>Fascioliasis*</td>
<td>41</td>
<td>35 (85.4%)</td>
</tr>
<tr>
<td>Schistosomiasis</td>
<td>10</td>
<td>1 (10%)</td>
</tr>
<tr>
<td>Paragonimiasis</td>
<td>5</td>
<td>3 (60%)</td>
</tr>
<tr>
<td>Hydatidosis</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Other parasites**</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Healthy donors</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

*Cases diagnosed by coprological examination (100% positive) ** Include filariasis (4), visceral larva migrans (4), trichinellosis (4), toxoplasmosis (4).
ROC Curve-FhFtn-1

AUC = 0.999

False positive rate (1-Specificity)

0.0                   0.2                   0.4                 0.6                  0.8                  1.0

True positive rate (Sensitivity)

ROC Curve-FhTP16.5

AUC = 0.981

False positive rate (1-Specificity)

0.0                   0.2                   0.4                 0.6                  0.8                  1.0

True positive rate (Sensitivity)

FIG-1
FIG. 2
FIG. 3

FhFtn-1-ELISA (A 490nm)

DRG-test (DU)

0.0 0.5 1.0 1.5 2.0

0 20 40 60 80 100

FhTP16.5-ELISA (A 490nm)

DRG-test (DU)

0.0 0.5 1.0 1.5 2.0 2.5 3.0

0 20 40 60 80 100