

Leucine Aminopeptidase Is an Immunodominant Antigen of *Fasciola hepatica* Excretory and Secretory Products in Human Infections[▽]

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The liver fluke *Fasciola hepatica* parasitizes humans and ruminant livestock worldwide, and it is now being considered a reemerging zoonotic disease, especially in areas in which it is endemic, such as South America. This study investigates the immune response to excretory and secretory products produced by *F. hepatica* in a group of patients from the Peruvian Altiplano, where the disease is highly endemic. Using a proteomic approach and immunoblotting techniques, we have identified the enzymes leucine aminopeptidase (LAP) and phosphoenolpyruvate carboxykinase as immunodominant antigens recognized by sera from fasciolosis patients. An indirect enzyme-linked immunosorbent assay using recombinant LAP as the antigen was developed to check sera from individuals of this region. Our results demonstrate that LAP produces a specific and strong reaction, suggesting its potential use in the serologic diagnosis of *F. hepatica* infections in humans.

Fasciolosis is an important disease caused by the liver flukes *Fasciola hepatica* and *F. gigantica*, infecting several mammalian species, including cattle and sheep, and consequently leading to significant global economical losses, valued at \$3 billion annually (59). In addition, *F. hepatica* infestation recently 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 (30, 41). Those health problems are encountered especially in areas in which the disease is endemic, like the Andean region in Peru, where well-known human areas of hyperendemicity include the Puno Altiplano (18), the Cajamarca valley (26, 27), and the Mantaro valley (4, 47, 60).

Sensitive and specific diagnostic tools are necessary in order to treat patients early and to avoid the major clinical complications caused by the parasite. In this context, immunological probes are replacing direct diagnosis by detecting eggs in feces (20), although there is still a lack of consensus regarding the choice of the immunologic analysis for human fasciolosis. Among the immunological methods, enzyme-linked immunosorbent assay (ELISA) and immunoblotting are well known, while other new systems are being evaluated (35). Most of these tests use *F. hepatica* molecules present in excretory and secretory products (ESP), since they produce a more intense response from the host immune system than somatic antigens (7, 10, 16, 17, 50). The identification of such molecules could

represent specific markers for the detection of *F. hepatica*. In this context, several molecules present in ESP have been characterized, including the cathepsin family of cysteine proteases (11), CuZn superoxide dismutase (25, 43), enolase (5, 24), fatty acid binding proteins (21), glutathione *S*-transferases (9, 64), leucine aminopeptidase (LAP) (1), saposin-like molecules (15, 19, 48), and thioredoxin-associated proteins (23, 24, 29, 32, 36, 51, 52, 56, 57).

The exopeptidase LAP (EC 3.4.11.1) originally was identified in detergent-soluble extracts of *F. hepatica*, with a very low level of activity detected in ESP (1). This peptidase has been shown to induce protection against fasciolosis in sheep (44), similar to that observed with endopeptidases like cathepsins (11, 33).

In this study, we have identified the enzymes LAP and phosphoenolpyruvate carboxykinase (PEPCK) as the reactive antigens present in *F. hepatica* ESP by using a combination of immunoblotting with human antisera and mass spectrometry (MS). Recombinant *F. hepatica* LAP (rLAP_{Fh}) also was recognized by individual positive serum samples by ELISA, adding a putative diagnostic value in addition to their protective properties.

MATERIALS AND METHODS

Preparation of parasite ESP. Livers from naturally infected sheep were collected from a local slaughterhouse, and parasites were removed immediately and washed extensively with prewarmed phosphate-buffered saline, pH 7.4 (PBS). Flukes were incubated at 37°C for 12 h with slow agitation in PBS containing 100 µg/ml streptomycin (Sigma), using 1 ml per fluke (14). Thereafter, the flukes were removed and the culture medium was centrifuged at 15,000 × *g* for 15 min at 4°C. The supernatant was precipitated using equal volumes of ice-cold 20% trichloroacetic acid and solubilized in loading buffer for gel electrophoresis. The

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protein content was measured using a modified Bradford assay (Bio-Rad) and adjusted to 10 mg/ml using an ultrafiltration membrane (YM-3; Millipore).

Production of rLAP_{Fh} and anti-rLAP_{Fh} sera. Based on published cDNA sequences of LAPs, degenerate primers were synthesized corresponding to conserved amino acid regions for metal binding (VGKG) and for the active site (NTDAEGRL). Using adult fluke cDNA as a template, a 280-bp fragment was generated by PCR with these primers. Purified PCR products were subcloned in pCR4TOPO (Invitrogen) and sequenced. New gene-specific primers were synthesized and used in rapid amplification of cDNA ends (RACE)-PCR. Products of 1.2 (5'RACE) and 0.7 kb (3'RACE) were obtained, subcloned, and sequenced. A full-length construct was generated by mixing gel-extracted 5'RACE and 3'RACE aliquots in a 100- μ l PCR that contained no primers. The full-length fragment was obtained by extension from the overlapping region. After five cycles, new primers designed from both ends of the LAP_{Fh} were added and used for an additional 25 cycles of amplification. These primers included BamHI and SalI restriction sites to facilitate subcloning in the expression vectors. The expected full-length cDNA product was obtained, subcloned, and sequenced (accession number AY64459). The full-length cDNA product was cloned in frame in BamHI and BglII sites of linearized pThio HisC *Escherichia coli* expression vector (Invitrogen), fused downstream of the *E. coli* thioredoxin (*trx*) gene, and transformed into *E. coli* Top10 cells. Fused rLAP_{Fh} was affinity purified using a bestatin-agarose column.

A New Zealand rabbit was immunized subcutaneously four times at 3-week intervals with purified rLAP_{Fh} (50 μ g) in Freund's complete or incomplete adjuvant. Anti-rLAP_{Fh} serum was obtained 10 days after the final immunization.

Source of human sera and ELISA. Human sera were obtained from school children between 5 and 14 years of age from Cajamarca (Peru). Positive sera used in this study were classified based on the presence of eggs in feces as well as the reactivity in ELISA assays against ESP by following established protocols (18, 22). Negative sera corresponded to individuals from the same area with no presence of *F. hepatica* eggs in feces and also negative by ELISA assays using ESP. ELISA tests using rLAP_{Fh} were performed essentially as described previously (61). An aliquot of 0.5 μ g of the recombinant LAP_{Fh} protein in coating buffer (5 μ g/ml) was added to the wells (100 μ l) of flat-bottomed 96-well microtiter plates (Nunc). After incubation overnight at 4°C, the plates were blocked with PBS containing 4% nonfat dry milk for 1 h at 37°C. Serum samples diluted at 1/500 in PBS containing 4% nonfat dry milk and 0.05% Tween 20 were added to the wells (100 μ l/well) and were incubated for 1 h at 37°C. After the samples were washed three times with PBS containing 0.05% Tween 20, goat anti-human immunoglobulin G (IgG) antibodies coupled to horseradish peroxidase (HRP) (Bio-Rad) were added at 1/20,000 (100 μ l/well) and incubated for 45 min at 37°C. After the samples were washed, peroxidase activity was detected by incubation for 15 min at room temperature with ortho-phenylenediamine (OPD; Sigma) at a concentration of 0.4 mg/ml in 0.05 M phosphate citrate buffer (100 μ l/well). The reaction was stopped by adding 50 μ l of 3 M hydrochloric acid, and the optical density (OD) of the reaction was determined at 490 nm in an automated plate reader (Bio-Rad). All samples were analyzed in triplicate, and final values were calculated as the medians $\pm$ three times the standard deviations. The cutoff for each ELISA was defined as the mean of the samples from the negative patients $\pm$ three times the standard deviation.

SDS-PAGE analysis, Western blot analysis, and stripping. Two-dimensional (2D) gel electrophoresis was carried out essentially as previously described (5) by solubilizing protein samples in 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (wt/vol), 20 mM dithiothreitol (DTT), 2% (vol/vol) Biolytes 5-8, and bromophenol blue (all chemicals were from Bio-Rad). The samples then were applied to a linear pH 5 to 8 ReadyStrip immobilized pH gradient strip (7 cm long; Bio-Rad). Isoelectric focusing (IEF) was performed on a Bio-Rad Protean IEF cell at 20°C using the following program: (i) passive rehydration for 16 h; (ii) 300 V for 1 h (step and hold); (iii) 4,000 V for 2 h (linear voltage ramping until reaching 4,000 V); and (iv) 4,000 V for 6.5 h (step and hold). After electrofocusing, the strips were reduced (2% DTT) and then alkylated (2.5% iodoacetamide) in equilibration buffer containing 6 M urea, 0.375 M Tris, pH 8.8, 2% sodium dodecyl sulfate (SDS), and 20% glycerol, and the second dimension was performed using 10% polyacrylamide gels. Proteins from SDS-polyacrylamide gel electrophoresis (PAGE) were stained with Coomassie blue or electroblotted onto nitrocellulose membranes.

Gels were transferred to nitrocellulose membranes in 20 mM Tris, 192 mM glycine, 20% (vol/vol) methanol, pH 8.3. Filters were stained with 0.1% Ponceau S (Sigma) for 10 min and blocked for 2 h in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) containing 3% bovine serum albumin (BSA) (Roche). After being extensively washed with TBS containing 0.05% Tween-20 (TBST), blots were incubated overnight at 4°C with pooled human sera (1:200 in TBS plus 3% BSA). Bound antibodies were detected by incubating blots for 1 h at room temperature

with HRP-conjugated goat anti-human IgG (Bio-Rad) in TBST-1% BSA. After being washed, immune complexes were visualized using Lumilight Western blotting substrate (Roche) by following the manufacturer's instructions and were exposed to X-OMAT film (Kodak).

Filters were stripped at 50°C for 30 min in a solution containing 100 mM of 2-mercaptoethanol and 2% SDS in 62.5 mM Tris-HCl, pH 6.7, followed by extensive washing and another blocking step prior to reprobing, either with human antisera (1:200 in TBS plus 3% BSA) or with rabbit serum against rLAP_{Fh} (1:1,000 in TBS plus 1% BSA), followed by extensive washing and incubation with secondary antibodies coupled to HRP (1:10,000 in TBS plus 1% BSA).

MS and protein identification. Each gel spot (2D) was manually excised from the gel, washed twice with double-distilled water, and digested with sequencing-grade trypsin (Roche Molecular Biochemicals) as described previously (6, 55). After digestion, 1 μ l of the supernatant was spotted onto a Teflon-coated plate (PerSeptive Biosystems, Framingham, MA) and allowed to air dry for 10 min at room temperature. A volume of 0.4 μ l of matrix (3 mg/ml alpha-cyano-4-hydroxycinnamic acid [Sigma]) diluted in 0.1% trifluoroacetic acid-acetonitrile/H₂O (1:1, vol/vol) was added to the dried digested peptides and allowed to air dry for another 10 min at room temperature. Matrix-assisted laser desorption/ionization-MS (MALDI-MS) was carried out with a Voyager DE STR instrument fitted with a 337-nm nitrogen laser that was shot under threshold irradiance (PerSeptive Biosystems) with an accelerating voltage of 20,000 V. All MALDI spectra were calibrated externally using a standard peptide mixture of angiotensin I (1,296.48 Da), adrenocorticotrophic hormone fragment 18-39 (2,465.19 Da), and adrenocorticotrophic hormone fragment 1-17 (2,093.15 Da) (Sigma). Peptides from the trypsin autodigestion were used for the internal calibration. The analysis by MALDI-MS produced peptide mass fingerprints (PMF). MS/MS sequencing analyses were carried out using a MALDI-tandem time of flight mass spectrometer 4700 Proteomics analyzer (Applied Biosystems, Framingham, MA).

Database search. The monoisotopic PMF data obtained from MALDI-MS were used to search for protein candidates in the Swiss-Prot/TrEMBL nonredundant protein database (www.expasy.ch/sprot) using the MS-fit (www.prospector.ucsf.edu), ProFound (www.prowl.rockefeller.edu), and MASCOT (www.matrixscience.com) software. Peptide modifications included in the search were carbamidomethyl (C) (fixed) and oxidation (M) (variable), allowing for one missed cleavage site. A restriction was placed on pI 5 to 8, and a protein molecular mass range from 10 to 100 kDa was accepted. Positive identifications were accepted when at least 20% of the peptide coverage of the theoretical sequences matched within a mass accuracy of 50 ppm. For individual peptide sequences obtained from MS/MS, BLAST searches (2) were carried out.

RESULTS

Identification of *F. hepatica* immunogenic proteins in ESP detected by human sera from an area in which fasciolosis is endemic. To investigate the antigenicity of the ESP proteins, we used sera from patients diagnosed with fasciolosis from Cajamarca (Peru), an area where the disease is highly endemic (63). To identify immunogenic spots, a pool of positive sera (from patients eliminating parasite eggs in feces) were used to blot ESP subjected to 2D gels in the pH range of 5 to 8. Five major reactive spots were detected by Western blotting, ranging from a pI of 5.0 to 7.6 and having molecular masses between 35 and 120 kDa (Fig. 1A). After the filters were stripped and reprobed with sera obtained from healthy individuals from the same area in which the disease is endemic, two spots, previously immunodetected by positive sera, were detected (Fig. 1A and B). No other signal was obtained when negative sera were used, indicating the specificity of the detection (Fig. 1B). Matching immunoblots with their corresponding Coomassie-stained gels (Fig. 1C) allowed for the excision of the corresponding protein spots. The proteins contained in these spots were subjected to trypsin digestion and MS analysis. As shown in Table 1, spot 1 was identified as *F. hepatica* LAP (EC 3.4.11.1; NCBI accession no. AAV59016), with the

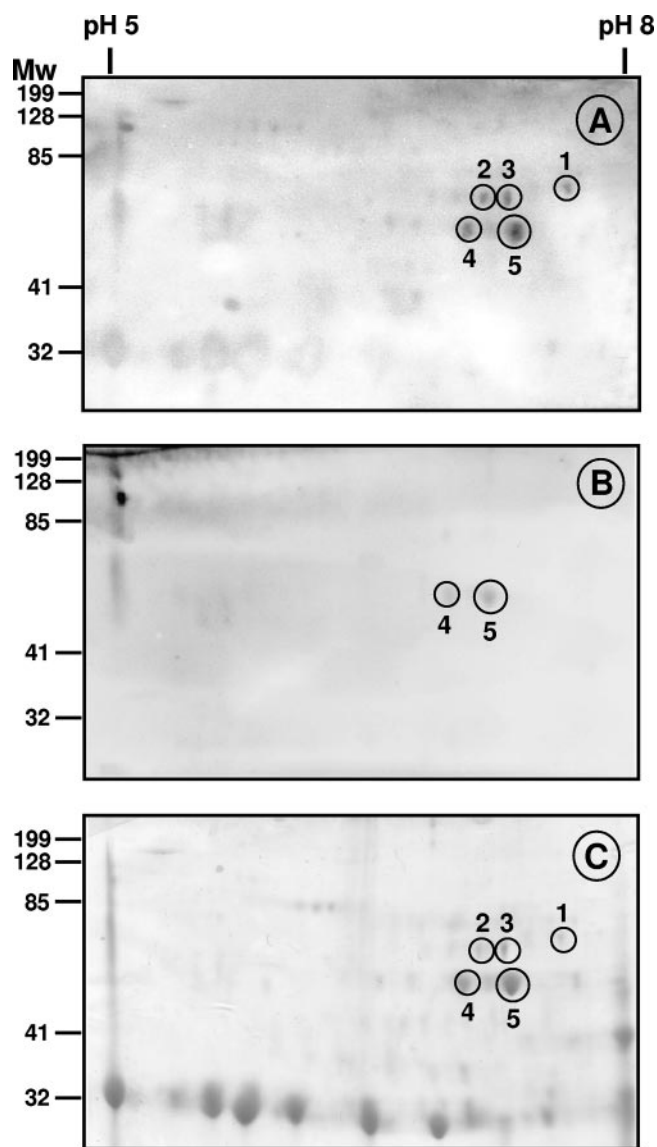


FIG. 1. Immunodetection of major antigens in ESP by human sera from fasciolosis patients. (A) Representative 2D electrophoresis gels of adult *F. hepatica* ESP, separated across a linear pH range of 5 to 8 using IEF in the first dimension and an SDS-10% PAGE in the second dimension. Following 2D electrophoresis, proteins were transferred to nitrocellulose, incubated with a pool of four positive sera from Cajamarca (Peru), incubated with secondary antibodies, and developed with Lumilight Western blotting substrate (Roche). Spots corresponding to major reactive proteins are circled and numbered according to the identified proteins in Table 1. (B) Immunoreaction of ESP by negative sera. The filters were identical to those used for panel A and contained *F. hepatica* ESP, and they were probed with a pool of six negative sera from patients from the same area of Cajamarca (Peru). Reactive spots also observed using sera from infected individuals are circled. (C) Representative 2D electrophoresis gels of adult *F. hepatica* ESP stained with Coomassie blue; the corresponding identified spots are circled. Mw, molecular mass markers, in kilodaltons.

peptides identified by PMF covering 45% of the molecule. Spots 2 and 3 corresponded to two peptides each, which were assigned to PEPCK (EC 4.1.1.32) with 100% homology to the peptides from the *Ascaris suum* molecule (accession no.

Q05893) (Table 1). Spots 4 and 5, immunodetected by both positive and negative sera (Fig. 1A and B), also corresponded to *F. hepatica* phosphopyruvate hydratase (enolase) (EC 4.2.1.11; accession no. A53665), with coverages of 69 and 43%, respectively (Table 1).

Confirmation of the identity of LAP was achieved by Western blotting using rabbit serum raised against the recombinant protein (rLAP_{Fh}) produced in *E. coli*. As shown in Fig. 2, these antibodies strongly reacted with the native LAP, detecting various spots of about 70 kDa ranging from pH 7.0 to 8.0. The recombinant LAP_{Fh} fused to the His patch-thioredoxin (used as a control) was detected with lower mobility by the rabbit sera (Fig. 2).

Immunological detection of rLAP_{Fh} by ELISA. To evaluate the possible diagnostic potential of the rLAP_{Fh} protein, we next evaluated human serum samples individually for their reactivity against this antigen, establishing an indirect ELISA. Preliminary assays were performed to ascertain both the proper amount of rLAP_{Fh} protein (ranging from 0.05 to 1.0 µg) to cover the ELISA plates (determined as 0.5 µg per well) and the dilution of sera employed (from 1/100 to 1/2,000; optimal results were obtained with the 1/500 dilution). The results from the negative sera ($n = 6$) indicated that the cutoff point for differentiating negative from positive sera was an OD value of 0.22. All of the confirmed *F. hepatica*-infected serum samples ($n = 4$) exhibited positive values greater than 0.398 (Fig. 3).

DISCUSSION

The control of fasciolosis historically has been limited by the lack of accurate and practical tests for the early diagnosis of *F. hepatica* infection in humans and other animals. Microscopic demonstration of parasite eggs in feces, the traditional diagnostic technique still widely used, is highly specific but has limitations in sensitivity. This limitation is due to the fact that it takes at least 4 to 10 weeks after infection for flukes to mature sexually and produce eggs (20). 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 coprological diagnosis (20).

Alternatively, immunological assays can detect the infection in the initiation of egg release by analyzing antigens present in blood or released in stools (13, 34). In contrast to antigen detection, the investigation of circulating antibodies can be carried out easily, allowing earlier detection of infection and revealing current and past infections. Different indirect and capture ELISAs that are based on ESP (35, 53) or individual molecules from ESP, like Fas (10, 16) and cathepsins (8, 39), have been described. However, there is still a need for the identification of new specific markers for the detection of *F. hepatica* antibodies in serum.

The present study constitutes the first report of the proteomic identification of *F. hepatica* immunogens in humans from an area where the disease is highly endemic. A recent report has shown a preliminary antigenic characterization of adult *F. hepatica* vomit, detecting several proteins ranging from 8 to 85 kDa by using 1D electrophoresis and immunoblotting of those antigens with infected human sera (12). Here, we

TABLE 1. Putative identification of major immunogenic proteins of *F. hepatica* ESP by PMF and MS/MS^a

Spot no.	MASCOT value	Description	Species	NCBI protein accession no.	Protein coverage (%)	No. of matched peptides	Molecular mass (kDa; theoretical value/exptl value)	pI (theoretical value/exptl value)
1	208	LAP	<i>F. hepatica</i>	AAV59016	45	13	57/70	7.0/7.7
2	ND	PEPCK	<i>A. suum</i>	Q05893		2	72/65	6.3/7.2
3	ND	PEPCK	<i>A. suum</i>	Q05893		2	72/65	6.3/7.3
4	341	Enolase	<i>F. hepatica</i>	A53665	69	24	47/50	6.5/7.1
5	178	Enolase	<i>F. hepatica</i>	A53665	43	14	47/50	6.5/7.4

^a All spot identification numbers correlate with results shown in Fig. 1. Peptide sequences were used to search the Swiss-Prot/TrEMBLnr databases by MSfit (Protein Prospector) and BLASTp (NCBI). ND, not determined.

identify two specific antigens in the ESP: the enzymes LAP and PEPCK. In addition, we identified some enolase spots that also were reactive against negative sera (Fig. 2). Using a similar proteomic approach, Morphew et al. (36) have very recently described cathepsin L proteases as the most abundant and immunogenic molecules in infected sheep bile (36). In this context, previous studies have shown the potential of cathepsin L1 (either purified or produced as a recombinant protein in yeast or bacteria) as a diagnostic marker for human infections by techniques like ELISA and immunoblotting (8, 39, 40). Interestingly, here we show that even though cathepsin L proteases seem to be abundant in *F. hepatica* ESP (58) (Fig. 1C), the human sera used in this study do not seem to react strongly against them (Fig. 1A). In recent studies with infected goats, no protein spots could be detected in the low-molecular-mass range (where cathepsin L proteases appear), and no reaction was observed by immunoblotting detecting major immunoreactive areas between pI 5 to 8.5 with infected goat antisera (38).

Enolase appears to be a major antigenic protein in several organisms, including parasitic protozoa like *Eimeria tenella* and *Plasmodium falciparum* (28, 54), yeasts like *Candida albicans* (45, 46), nematodes like *Trichinella spiralis* (37), and also trematodes like *Schistosoma bovis* (42), but its immunogenicity is controversial. A recent study was unable to detect anti-enolase IgE in sera from human patients infected with *Anisakis simplex*, suggesting an insufficient antigen presentation to induce anti-enolase antibodies in natural *Anisakis* infections (49). Moreover, Morphew et al. (36) have observed that eno-

lase accumulates in the ESP of *F. hepatica* during the incubation process, even when the trematode is dead (36). The reactivity of negative sera against two spots corresponding to enolase, which presents at least seven isoforms when anti-enolase antibodies are used (5), could be due either to cross-reacting antibodies against the molecule from other pathogens present in those patients or to nonspecific detection by the human sera studied.

In contrast to enolase, we observed clear and specific recognition of LAP and PEPCK by the Peruvian sera studied (Fig. 1A). By following a similar proteomic approach, a recent report has identified PEPCK and enolase as immunogenic proteins in the related trematode *S. bovis* (42). PEPCK previously was identified as a major immunogen protein in eggs of *S. mansoni* and was identified as being responsible for T-cell responses (3).

LAP originally was isolated from an *F. hepatica* detergent-soluble extract, and histochemical methods, detecting a very low level of activity in ESP, showed that LAP activity was associated mainly with the epithelial cells that line the digestive tract of the parasite (1). Here, we clearly demonstrate the presence of LAP in ESP of *F. hepatica* by immunoblotting, either with human sera or with rabbit sera against the recombinant protein. Our results suggest the existence of distinct



FIG. 2. Immunodetection of LAP in *F. hepatica* ESP using rabbit antisera raised against rLAP_{Fh}. Proteins were subjected to IEF in a pH range of 5 to 8, followed by SDS-PAGE in 10% acrylamide gels. Following 2D electrophoresis, proteins were transferred to nitrocellulose and incubated with rabbit sera raised against the recombinant LAP_{Fh}. The recombinant protein (rLAP) was included in the second dimension as a control.

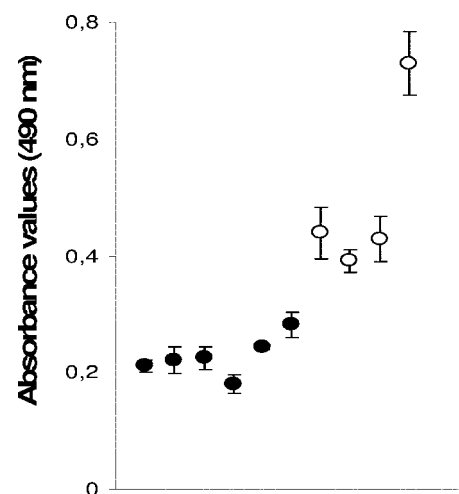


FIG. 3. Individual serum ELISA detection using rLAP_{Fh} as the antigen. Median values from individual negative human sera (closed circles) and positive human sera (open circles) are shown. Values were defined as the mean OD values at 490 nm $\pm$ three times the standard deviations. Vertical bars represent the standard deviations.

enzyme isoforms, with only one of them being detected by human sera (Fig. 1A and 2). Since this enzyme most likely functions in the final stages of the catabolism of peptides that are generated by the degradation of host tissue by endoproteases, such as the cathepsin L proteinases, and are absorbed by the epithelial cells (1), it is possible that the molecule loses activity when secreted. This enzyme also has been detected in the tegument of *S. mansoni* (31, 62), and its presence in the ESP could be related to turnover processes.

The efficiency of LAP as an immunogen has been well documented, rendering protection in animals and confirming its recognition by the host immune system (44). Our assays use dilutions of human sera (1/500) in accordance with what has been previously published for the *F. hepatica* antigen Fas-2 (10, 17), being less concentrated than the samples used in other assays for either procathespsins or cathepsin molecules, which required dilutions of 1/200 (8, 39).

In the present study, we further suggest LAP as a promising diagnostic marker in immunological tests, since it is specific and strongly detected by sera of infected individuals. Future studies with a larger number of samples will address the confirmation of its diagnostic potential.

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