



Comparative assessment of recombinant and native immunogenic forms of *Fasciola hepatica* proteins for serodiagnosis of sheep fasciolosis

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Abstract

Laboratory diagnosis of sheep fasciolosis is commonly performed by coprological examinations; however, this method may lead to false negative results during the acute phase of the infection. Furthermore, the poor sensitivity of coprological methods is considered to be a paradox in the chronic phase of the infection. In this study, we compared the immunoreactivity of native and recombinant forms of *Fasciola hepatica* excretory/secretory antigens and determined their capabilities for the development of *F. hepatica*-specific immunoassays. Immunoreactivity and specificity of recombinant and native forms of *F. hepatica* antigens, including fatty acid binding protein (FABP), glutathione-S-transferase (GST), and cathepsin L-1 (CL1), in parallel with native forms of FABP and GST, were studied for serodiagnosis of the chronic form of sheep fasciolosis, individually or in combination with each other by enzyme-linked immunosorbent assays (ELISA). The correlation of the findings was assessed by receiver-operator characteristic (ROC); furthermore, the specificity and sensitivity were assessed by Youden's J. Serologic cross-reactivity was evaluated using samples from healthy sheep ($n = 40$), *Fasciola*-infected sheep ($n = 30$), and sheep with other parasitic infections ($n = 43$). The FABPs were determined to be greater than 95% sensitive for *F. hepatica* serodiagnosis. The most desirable diagnostic recombinant antigen was rCL1, which showed 100% sensitivity and 97% specificity in ELISA and was capable of discriminating the positive and negative samples by maximum Youden's J results. We conclude that rCL1 can be used for routine serodiagnosis of chronic fasciolosis. Thus, it could be advantageous in development of immunoassays for screening of ovine herds in fasciolosis-endemic areas and as a reliable agent for detection of fasciolosis in non-endemic regions.

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Introduction

Fasciolosis is a zoonotic disease commonly transmitted by metacercariae-infected herbs or vegetables. The causative agents, *Fasciola hepatica* and *Fasciola gigantica*, infect both livestock and humans (Mas-Coma et al. 2009). *F. hepatica*, a well-known liver fluke, is a concern worldwide, and according to estimates provided by the World Health Organization (WHO 2008), 2.4 million people are infected with *F. hepatica* and nearly 17 million are at risk for infection (WHO 2008; Mas-Coma et al. 2009). The human infection statistics correlate closely with animal infections. Thus, economic consequences will exceed diminished animal products, e.g., milk, wool, and meat (Mezo et al. 2011; Mateus Fracasso et al. 2017). Diagnosis of sheep fasciolosis is usually performed by observation of parasite eggs in the feces approximately from 7 weeks post-infection and in some cases may require multiple stool examinations; however, this method has low reliability and sensitivity during the prepatent period of the infection. For this reason, more potent diagnostic methods should be developed (Martínez-Valladares et al. 2010; Martínez-Pérez et al. 2013).

Development of sensitive and specific antibody detection methods, and rapid diagnosis of infection, may prevent the negative impact of fasciolosis on the productivity of ovine herds and prevent economic loss (Guobadia and Fagbemi 1995; Sriveny et al. 2006). The desirable properties of ELISA and other immunoassays make them the methods of choice for detection of *Fasciola*-specific antibodies in sheep sera (Sriveny et al. 2006; Anuracpreeda et al. 2009).

Recently, immunologic studies focused on characterization and production of immunogenic parasite molecules, as well as application of appropriate ones in serodiagnosis and vaccinology (Velusamy et al. 2004; Turner et al. 2016; Mokhtarian et al. 2016a, b). A combination of immunodiagnostic methods with clinical findings could likely aid in early diagnosis of ovine fasciolosis.

More recently, purified native and recombinant *F. hepatica* antigens have become available; however, evaluations of the sensitivities and specificities of those proteins in serodiagnosis are necessary. Previous studies showed that cathepsin L1 (CL1), fatty acid-binding proteins (FABPs), and glutathione-S-transferase (GST) are the major immunogenic proteins of *F. hepatica* and could be used for diagnostic purposes (Anuracpreeda et al. 2016; Mokhtarian et al. 2016a, b). The aim of this study was to compare the main immunogenic recombinant and native proteins from the *F. hepatica* excretory/secretory antigens (E/S Ags) for serodiagnosis of sheep fasciolosis.

Materials and methods

Sheep sera

All experiments were approved by the Ethics Committee of the Faculty of Iran University of Medical Science. *F. hepatica*-positive serum samples were collected from 30 *F. hepatica*-infected sheep slaughtered at Kahrizak slaughterhouse, Tehran, Iran. Samples were diagnosed by observation of adult *Fasciola* flukes in the sheep livers and bile ducts in chronic phase of the infection. The infection-negative serum samples were collected from 40 healthy sheep whose stool exams were negative and showed no evidence of intestinal or liver parasite infections. The positive and negative sera aided in immunoassay development. Subsequently, antibody cross-reactivity was determined using 43 serum samples from sheep with other parasitic infections including hydatidosis ($N = 10$), cysticercosis ($N = 7$), coenurosis ($N = 8$), dicrocoeliasis ($N = 8$), and brucellosis (10), all of which were purchased from the Research Center of Veterinary, Tehran University, Tehran, Iran. Clinical diagnosis of the mentioned infections was performed by observation of parasites in special organs of the sheep and also molecular confirmatory methods. Since these common ovine parasites may show cross-reactivity with *Fasciola* and interfere with results of the immunoassays, we applied them in this study.

Preparation of *F. hepatica* E/S Ags

F. hepatica were collected from infected sheep livers from the Kahrizak abattoir in Tehran, Iran. Adult flukes were removed from the bile ducts and washed several times with 150 mM phosphate-buffered saline (PBS), pH 7.4, to remove blood and bile. Finally, *F. hepatica* E/S Ags were prepared as previously described (Mokhtarian et al. 2016a, b). The E/S products were centrifuged at $10,000\times g$ for 60 min at 4 °C. The clear supernatant was then concentrated by ultrafiltration using 4 kDa membrane filters (Eppendorf, Germany). The protein concentration was measured by the Bradford method and the product stored at -80 °C (Bradford 1976).

Purification of *F. hepatica* native fatty acid-binding protein and native glutathione-S-transferase

Native FABP of E/S Ags was separated by precipitation with 70% saturated ammonium sulfate solution. The supernatant containing the desired proteins was dialyzed against 20 mM Tris-HCl, pH 8, at 4 °C overnight and subjected to anion

exchange chromatography (DEAE sepharose 6B, Amersham, Sweden) and size exclusion gel chromatography columns, respectively, as previously described (Timanova-Atanasova et al. 2004; Mokhtarian et al. 2016a, b).

Native GST was purified from *F. hepatica* E/S Ags on a glutathione-affinity agarose matrix (Sigma-Aldrich, USA) using a wash-batch method, as previously described by Brophy et al. (1995), and the purified protein was stored at $-80\text{ }^{\circ}\text{C}$.

Molecular cloning and expression of recombinant proteins

The genes encoding CL1, FABP, and GST were cloned and their proteins expressed previously (Mokhtarian et al. 2016a, b). In brief, the cDNA encoding FhCL-1 (GenBank accession no. U62288.2) was cloned into pET-21 plasmid (Invitrogen, USA), and the cDNAs encoding FhFABP and FhGST (GenBank accession nos. AJ250098.1 and HM584608.1, respectively) were cloned into pET-28 and expressed in *Escherichia coli* BL21 (DE3) cells (Invitrogen, USA). In this study, the recombinant polypeptides were purified using nickel affinity chromatography with a His Trap column (Ni-IDA Resin, Parstous, Iran).

The purified fractions were analyzed by SDS-PAGE and Coomassie Blue staining, and the protein concentrations were determined by the Bradford assay.

Enzyme-linked immunosorbent assay

The immunological interactions of the native and recombinant proteins with the infected and healthy ovine sera were determined by indirect ELISA as described by Espino et al., with minor modifications (Espino and Hillyer 2004). Following determination of the optimal concentrations of the antigens by a preliminary checkerboard titration test, polysorb microtitration plates (Nunc, Denmark) were coated with 100 μl of a 10 $\mu\text{g}/\text{ml}$ solution of each native or recombinant protein in 0.1 M bicarbonate buffer, pH 9.6, at $4\text{ }^{\circ}\text{C}$ overnight. After washing with PBS containing 0.05% Tween 20 (PBS-T), wells were blocked with 2% BSA for 2 h at $37\text{ }^{\circ}\text{C}$. After a second wash, 100 μl of 1:500 diluted sheep sera (from healthy sheep or sheep with fasciolosis or other parasitic infections) was added and the plates were incubated for 2 h at $37\text{ }^{\circ}\text{C}$. Following another wash, 100 μl of 1:5000 diluted horseradish peroxidase-labeled polyclonal rabbit anti-sheep IgG (Sigma-Aldrich, Germany) was added and the plates incubated for 1 h at $37\text{ }^{\circ}\text{C}$. After an extensive wash, 100 μl of tetramethylbenzidine/ H_2O_2 mixture was added as chromogen/substrate solution. Following a 15-min incubation in the dark, the enzymatic reaction was

stopped by adding 100 μl of 2 N H_2SO_4 and the optical density was measured at 450/630 nm.

Data analysis

The results of the immunoreactivity assays were compared between groups using the Tukey's or Dunn's multiple comparison tests. The optimal cutoff value for each ELISA method, with a 95% confidence interval (CI), was established by receiver-operating characteristic (ROC) curve analysis using Tukey's multiple comparison tests. All statistics were analyzed and graphs plotted using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA). *P* values < 0.05 were considered statistically significant. According to arbitrary guidelines, the area under the curve (AUC) values were considered as follows: $\text{AUC} < 0.5$, non-informative; $0.5 < \text{AUC} < 0.7$, low accuracy; $0.7 < \text{AUC} < 0.9$, moderate accuracy; and $0.9 < \text{AUC} < 1$, high accuracy (Swets 1988).

In the ROC analysis, cutoff values for the following conditions were derived according to cutoff for the maximum specificity (SP) where the sensitivity (SE) was still 100%. Cutoff for the maximum value for Youden's J was measured by the formula $(\text{SE} + \text{SP} - 1)$.

Results

Distribution of absorbance values (at 450 nm)

As expected, the distribution of absorbance values varied significantly between positive and negative samples; however, it also varied between different assays and different optimal cutoff values for all five ELISA tests (Fig. 1; see the "ROC analyses" section).

ROC analyses

The immunoreactivities of the recombinant and native forms of *F. hepatica* antigens were compared with each other. The optimal cutoff value for each ELISA result was derived from four different conditions according to the following: (a) maximum specificity at which the sensitivity was still 100%, (b) maximum sensitivity at which the specificity was still 100%, (c) maximum value for Youden's index J $(\text{SE} + \text{SP} - 1)$, and (d) maximum accuracy (maximum true positive and negative results) (Table 1). Receiver-operating characteristic analyses determined that the AUC values of native fatty acid-binding protein (nFABP), recombinant fatty acid-binding protein (rFABP), and recombinant cathepsin L1 (rCL1) were 0.96, 0.97, and 0.99, respectively. The AUC values for recombinant glutathione-S-transferase (rGST) and native glutathione-S-transferase (nGST) were 0.96 and 0.90, respectively (Fig. 2; Table 1).

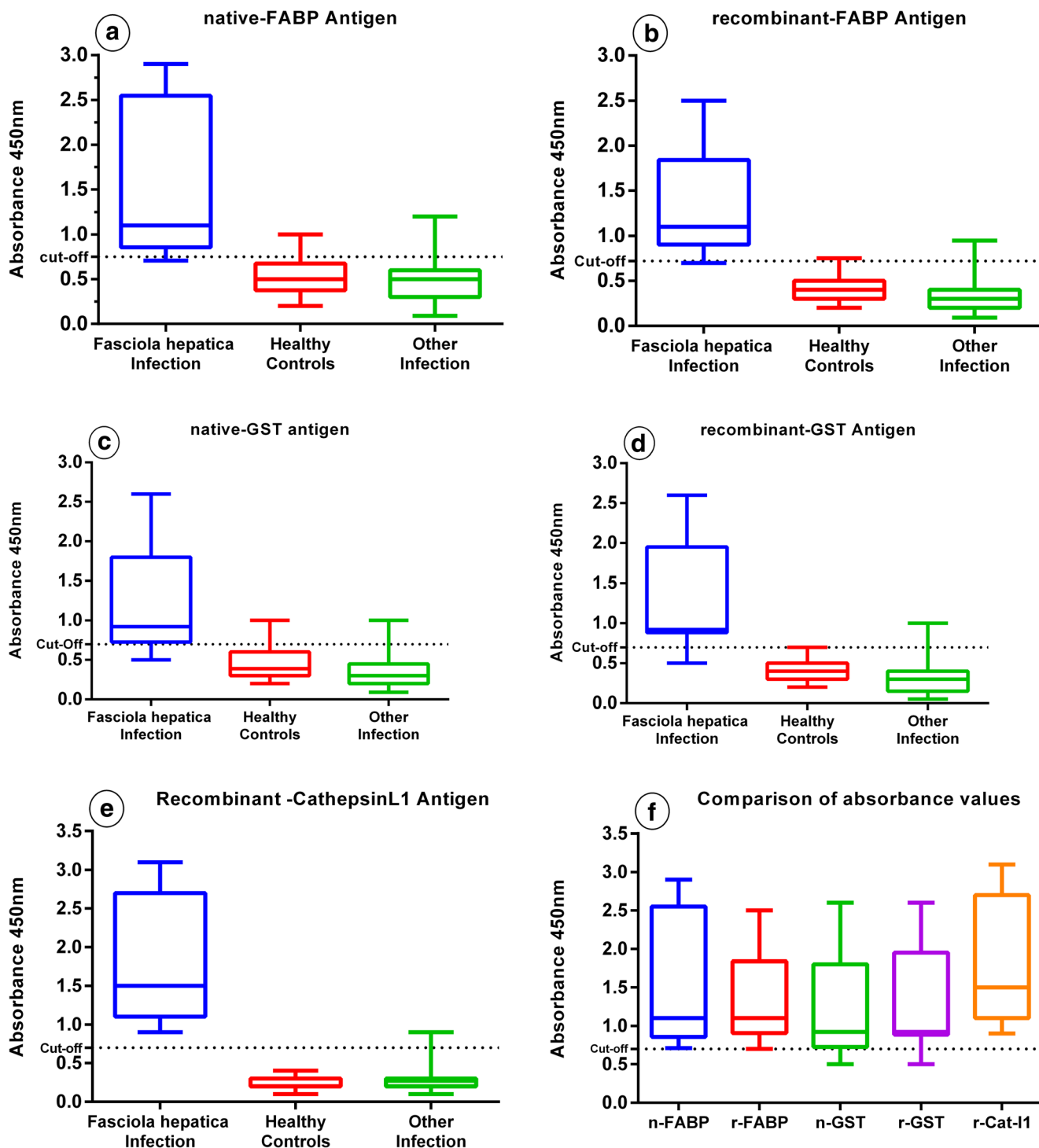


Fig. 1 Box plots showing the absorbance values (450 nm) of ELISA results for discrimination of the immunoreactivity of native and recombinant *Fasciola hepatica* antigens in *Fasciola*-infected sheep versus healthy and other parasite-infected sheep (a–e). Absorbance values

of ELISA results for native and recombinant *F. hepatica* antigens (f). nFABP native fatty acid-binding protein, nGST native glutathione-S-transferase, rCL1 recombinant cathepsin L1, rFABP recombinant fatty acid-binding protein, rGST recombinant glutathione-S-transferase

The ROC-optimized cutoff values for nFABP and rFABP were 0.7 and 0.72 in ELISA, respectively. Sensitivities for nFABP and rFABP were both 97%, and specificities were 92 and 96% in ELISA, respectively.

The ROC-optimized cutoff values for nGST and rGST were 0.72 and 0.71, respectively, and their sensitivities were 75 and 86%, which were the lowest sensitivities of the proteins studied. Their specificity values were 91 and 97%, respectively.

Table 1 Specificity and sensitivity of immunoreactivity of native and recombinant *Fasciola hepatica* proteins based on cutoff values and Youden's J results obtained from *Fasciola*-infected sheep sera. The table includes also the ROC AUC for the five different *Fasciola*-specific ELISAs

Antigen	Cutoff	Sensitivity (%)	Specificity (%)	Youden's J	AUC
Native FABP (nFABP)	> 0.47	100	40	0.40	0.96
	> 0.65	100	75	0.75	
	> 0.70	97	92	0.89*	
	> 0.72	96	92	0.88	
Recombinant FABP (rFABP)	> 0.48	100	62.5	0.63	0.97
	> 0.67	100	92.5	0.92	
	> 0.72	97	96	0.93*	
	> 0.77	93	98	0.91	
Recombinant cathepsin L1 (rCL1)	> 0.25	100	52	0.52	0.99**
	> 0.35	100	92	0.93	
	> 0.60	100	97	0.97*	
	> 0.80	99	97	0.96	
Native GST (nGST)	> 0.45	100	65	0.65	0.90
	> 0.67	79	83	0.62	
	> 0.72	75	91	0.66*	
	> 0.77	72	91	0.63	
Recombinant GST (rGST)	> 0.25	100	23	0.23	0.96
	> 0.45	100	72	0.72	
	> 0.71	86	97	0.83*	
	> 0.80	79	97	0.76	

Based on the established cutoff value of 0.6 for rCL1, no seropositive samples were found in the true-negative population. Specificity and sensitivity for rCL1 were 100 and 97%, respectively.

According to the arbitrary guidelines, the AUC values of 0.9–1.0 indicated high accuracy for discrimination between positive and negative results.

Cross-reactivity

Cross-reactivity, as the discrimination of positive and negative results, was measured based on the designated cutoff value

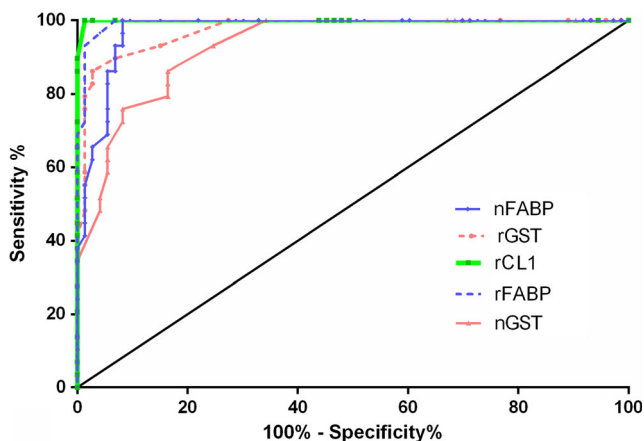


Fig. 2 Receiver-operator characteristic (ROC) curve for five *Fasciola hepatica* antigens. The values for the area under the ROC curve (accuracy) were 0.96 for the nFABP, 0.97 for the nFABP, 0.99 for the rCL1, 0.90 for the nGST, and 0.96 for the rGST in ELISA

and presented according to the parasitic disease investigated (Table 2). Sera from hydatidosis cases displayed considerable cross-reactivity in all five ELISA reactions, whereas all other cross-reactions were individually scattered among individual ELISAs. The highest specificity score of 97% was obtained by the rCL1-based ELISA, with two instances of cross-reactivity as described above (Table 2). nFABP cross-reacted with six sera out of 43 samples with parasitic diseases with 86.04% specificity.

Discussion

In this study, we focused on improving the diagnosis of fasciolosis using native and recombinant proteins and a panel of sheep positive and negative control serum samples. A critical point for the evaluation of a new immunodiagnostic test is the establishment of cutoff points. We determined these points with control sera. Using ROC curve and Youden's J analyses, we selected the cutoff values and found the best balance of sensitivity and specificity for the ELISA (Zweig and Campbell 1993).

In ELISA, the highest sensitivities were obtained with rCL1 and rFABP, and the lowest with nGST. High antibody titers during active sheep infections indicate that these molecules are repeatedly and effectively exposed to the host immune system.

Our findings support previous studies indicating high sensitivity of rCL1 as a prominent secretory enzyme of adult liver

Table 2 Potential cross-reactivity of *Fasciola*-infected sheep sera with other parasites

Parasitic disease	rFABP	nFABP	rGST	nGST	rCL1	Total
Hydatidosis	2	3	3	3	2	10
Cysticercosis	0	0	0	0	0	7
Coenurosis	0	2	0	0	0	8
Dicrocoeliasis	0	1	0	1	0	8
Brucellosis	0	0	0	0	0	10
Total	2	6	3	4	2	43
Relative specificity (%)	95.5	86	93	90.7	95.5	–

flukes for serodiagnosis of fasciolosis (Cordova et al. 1999; Tantrawatpan et al. 2007; Valero et al. 2012; Gonzales Santana et al. 2013; Kuerpick et al. 2013; Anuracpreeda et al. 2016). In the present study, similar to the study of Mezo et al., distinct cutoff values were used to discriminate between the sera of fasciolosis cattle and healthy ones with 100% sensitivity and 97% specificity (Mezo et al. 2011). Cornelissen et al. and Kuerpick et al. used rCL1 for determination of cattle fasciolosis and achieved 100% sensitivity and 88.6–94.6% specificity (Cornelissen et al. 1992; Kuerpick et al. 2013).

Anuracpreeda et al. used an anti-CL1 monoclonal antibody-based immunochromatography method to identify the acute and chronic phases of *F. gigantica*-caused fasciolosis in cattle and achieved 96.7% sensitivity with 100% specificity (Anuracpreeda et al. 2016). Our results were similar.

Interestingly, more than 95% sensitivity was achieved with both rFABP and nFABP, with cutoff values of 0.7, indicating their applicability in differentiating healthy from infected ovine sera.

Notably, a 14.7-kDa FABP was previously recognized in *F. hepatica* and its recombinant analogue has been studied due to its ability to stimulate an anti-parasite host immune response (Hillyer et al. 1987; Muro et al. 1997). Several studies suggest that FABP can induce protection against *F. hepatica* in small and large laboratory animal models and could be a candidate for immunodiagnosis of fasciolosis (Sobhorn et al. 1998; Dalton et al. 2003; Wedrychowicz and Wisniewski 2003). *Fasciola*-infected ovine sera have specific antibodies against this immunogenic protein. Overall, our findings agree with studies in cattle and sheep (Casanueva et al. 2001; Timanova-Atanasova et al. 2004; Rabia et al. 2007; Allam et al. 2012; Teofanova et al. 2012; Anuracpreeda et al. 2016) and other animal species, but disagree with results achieved by (Aly et al. 2014), which could be due to specific characteristics of the studied antigens, the genotypic diversity of various *F. hepatica* isolated, status and phase of the infection, and the applied diagnostic techniques.

Our results confirmed that both CL1 and FABP have similar AUCs, thus to choose the most suitable protein for

differential diagnosis of fasciolosis we used Youden's J index. Similar to our previous finding in human fasciolosis (Mokhtarian et al. 2016a, b), in this study, CL1, with Youden's J = 0.97, was determined to be the preferred immunogenic protein for serodiagnosis of sheep fasciolosis.

Another immunogenic protein we studied was GST. Both rGST and nGST showed low specificity and sensitivity with cutoff values of 0.7. Youden's J was calculated as 0.83 and 0.66, respectively, for rGST and nGST; thus, both forms showed low immunological affinity to positive control sera. Glutathione-S-transferases belong to a well-known family of cytosolic enzymes involved in detoxification. Glutathione-S-transferases in adult flukes are related with gut lamellae and may play a role in absorptive function of adult gut. They are therefore tempting targets for both chemotherapy and vaccine development (Meyer and Thomas 1995; Rossjohn et al. 1997).

Fasciolosis is endemic in Iran; therefore, it is possible that a dose of infectious metacercariae could affect the development of a detectable antibody response to particular antigens (Cornelissen et al. 1999). Due to availability of naturally *F. hepatica* infected sheep, we used their sera to determine immunogenic proteins of this parasite. It means that the results are generalizable to the rest of the sheep population in the region.

In the present study, we found significant cross-reactivity between fasciolosis and echinococcosis, especially with GST and FABP. This finding is consistent with those of several other studies (Intapan et al. 2003; Rabia et al. 2007; Allam et al. 2012; Aly et al. 2014). This cross-reactivity may be due to common antigenic lipoprotein components or epitopes of *Echinococcus multilocularis*, *Echinococcus granulosus*, and *Fasciola* species (Yamano et al. 2009). Previous reports demonstrated cross-reactivity of *F. hepatica* Ags with schistosomiasis, paragonimiasis, and clonorchiasis. Cornelissen et al. showed cross-reactivity between infected ovine sera with *Fasciola* and *Eimeria* species, as well as *E. granulosus*; however, they also reported 100% sensitivity and 96% specificity. Interestingly, infections with *Schistosoma* spp., *Paragonimus* spp., and *Clonorchis* spp. have not been reported in some countries, including Iran, which is commonly due to the absence of the specific carrier in those areas. Thus, it remains necessary to design a larger confirmatory study with more positive serum samples during both acute and chronic phases of fasciolosis and determine the cross-reactivity with endemic helminths, particularly *Taenia*, *Echinococcus*, and *Strongyloides stercoralis*.

In conclusion, in this study, we developed sensitive and specific ELISA, employing rCL1 and rFABP, to detect *F. hepatica*-specific antibodies in ovine sera. Application of recombinant proteins will allow scaling up of the procedure for mass screening. Also, sufficient antigen quantities are needed for applications in acute, chronic, and post-treatment studies to gain more consistent results and adjust the established ELISA methods for commercialization and further justification in areas where the disease is endemic.

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Compliance with ethical standards All experiments were approved by the Ethics Committee of the Faculty of Iran University of Medical Science.

References

- Allam G, Bauomy IR, Hemyeda ZM, Sakran TF (2012) Evaluation of a 14.5 kDa-Fasciola gigantica fatty acid binding protein as a diagnostic antigen for human fascioliasis. *J Parasitol Res* 110(5):1863–1871
- Aly I, Gehan E, Ismail M, Mohamed H, Ashraf E, Azza F et al (2014) Evaluation of a fatty acid binding protein and cysteine proteinase antigens of *Fasciola gigantica* for serodiagnosis of fasciolosis. *WJMS* 11(4):590–599
- Anuracpreeda P, Wanichanon C, Chawengkirtikul R, Chaithirayanon K, Sobhon P (2009) *Fasciola gigantica*: immunodiagnosis of fasciolosis by detection of circulating 28.5 kDa tegumental antigen. *Exp Parasitol* 123:334–340
- Anuracpreeda P, Chawengkirtikul R, Sobhon P (2016) Immunodiagnosis of *Fasciola gigantica* infection using monoclonal antibody-based sandwich ELISA and immunochromatographic assay for detection of circulating cathepsin L1 protease. *PLoS One*. <https://doi.org/10.1371/journal.pone.0145650> 5
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72(1–2):248–254
- Brophy P, Patterson L, Brown A, Pritchard D (1995) Glutathione S-transferase (GST) expression in the human hookworm *Necator americanus*: potential roles for excretory-secretory forms of GST. *Acta Trop* 59(3):259–263
- Casanueva R, Hillyer GV, Ramajo V, Oleaga A, Espinoza EY, Muro A (2001) Immunoprophylaxis against *Fasciola hepatica* in rabbits using a recombinant Fh15 fatty acid-binding protein. *J Parasitol* 87(3):697–700
- Cordova M, Reategui L, Espinoza J (1999) Immunodiagnosis of human fascioliasis with *Fasciola hepatica* cysteine proteinases. *Trans R Soc Trop Med Hyg* 93(1):54–57
- Cornelissen JB, De Leeuw WA, Vander Heijden PJ (1992) Comparison of and indirect haemagglutination assay and an ELISA for diagnosing *Fasciola hepatica* in experimentally and naturally infected sheep. *Vet Q* 14:152–156
- Cornelissen JB, Gaasenbeek CP, Boersma W, Borgsteede FH, van Milligen FJ (1999) Use of a pre-selected epitope of cathepsin-L 1 in a highly specific peptide-based immunoassay for the diagnosis of *Fasciola hepatica* infections in cattle. *Int J Parasitol* 29(5):685–696
- Dalton JP, Neill SO, Stack C, Collins P, Walshe A, Sekiya M et al (2003) *Fasciola hepatica* cathepsin L-like proteases: biology, function, and potential in the development of first generation liver fluke vaccines. *Int J Parasitol* 33(11):1173–1181
- Espino AM, Hillyer GV (2004) A novel *Fasciola hepatica* saposin like recombinant protein with immunoprophylactic potential. *J Parasitol* 90(4):876–879
- Fracasso M, Da Silva AS, Baldissera MD, Bottari NB, Gabriel ME, Piva MM, Stedille FA et al (2017) Activities of ectonucleotidases and adenosine deaminase in platelets of cattle experimentally infected by *Fasciola hepatica*. *Exp Parasitol* 176:16e20
- Gonzales Santana B, Dalton JP, Camargo FV, Parkinson M, Ndao M (2013) The diagnosis of human fasciolosis by enzyme-linked immunosorbent assay (ELISA) using recombinant cathepsin L protease. *PLoS Negl Trop Dis* 7(9):e2414
- Guobadia EE, Fagbemi BO (1995) Immunodiagnosis of fasciolosis in ruminants using a 28-kDa cysteine protease of *Fasciola gigantica* adult worms. *Vet Parasitol* 57:309–318
- Hillyer GV, Tahir EI, Haroun M, Galenes D, Maricelis S (1987) Acquired resistance to *Fasciola hepatica* in cattle using a purified adult worm antigen. *Am J Trop Med Hyg* 37(2):362–369
- Intapan PM, Maleewong W, Nateeworanart S, Wongkham C, Pipitgool V, Sukolapong V et al (2003) Immunodiagnosis of human fascioliasis using an antigen of *Fasciola gigantica* adult worm with the molecular mass of 27 kDa by a dot-ELISA. *Southeast Asian J Trop Med Public Health* 34(4):713–717
- Kuerpick B, Schnieder T, Strube C (2013) Evaluation of a recombinant cathepsin L1 ELISA and comparison with the Pourquier and ES ELISA for the detection of antibodies against *Fasciola hepatica*. *Vet Parasitol* 193:206–213
- Martínez-Pérez JM, Robles-Pérez D, Valcárcel-Sancho F, González-Guirado AM, de Castro IC, Nieto-Martínez JM (2013) Effect of lipopolysaccharide (LPS) from *Ochrobactrum intermedium* on sheep experimentally infected with *Fasciola hepatica*. *Parasitol Res* 112:2913–2923
- Martínez-Valladares M, Cordero-Pérez C, Castañón-Ordóñez L, Famularo MR, Fernández-Pato N, Rojo-Vázquez FA (2010) Efficacy of a moxidectin/triclabendazole oral formulation against mixed infections of *Fasciola hepatica* and gastrointestinal nematodes in sheep. *Vet Parasitol* 174:166–169
- Mas-Coma S, Valero MA, Bargues MD (2009) *Fasciola*, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. *Adv Parasitol* 69:41–146
- Meyer DJ, Thomas M (1995) Characterization of rat spleen prostaglandin H D-isomerase as a sigma-class GSH transferase. *Biochem J* 311(3):739–742
- Mezo M, Gonzalez-Warleta M, Castro-Hermida JA, Muino L, Ubeira FM (2011) Association between anti-F. hepatica antibody levels in milk and production losses in dairy cows. *Vet Parasitol* 180:237–242
- Mokhtarian K, Akhlaghi L, Meamar AR et al (2016a) Serodiagnosis of fasciolosis by fast protein liquid chromatography fractionated excretory/secretory antigens. *Parasitol Res* 15:1–9
- Mokhtarian K, Akhlaghi L, Mohamadi M, Meamar AM, Razmjou E, Khoshmirsafa M, Falak R (2016b) Evaluation of anti-Cathepsin L1: a more reliable method for serodiagnosis of human fasciolosis. *Trans R Soc Trop Med Hyg* 110:542–550
- Muro A, Ramajo V, López L, Simón F, Hillyer GV (1997) *Fasciola hepatica*: vaccination of rabbits with native and recombinant antigens related to fatty acid binding proteins. *Vet Parasitol* 69(3):219–229
- Rabia I, Salah F, Neamat M, Raafat A (2007) Evaluation of different antigens extracted from *Fasciola gigantica* for effective specific diagnosis of fascioliasis. *New Egypt J Med* 36:40–47
- Rosjohn J, Feil SC, Wilce MC, Sexton JL, Spithill TW, Parker MW (1997) Crystallization, structural determination and analysis of a novel parasite vaccine candidate: *Fasciola hepatica* glutathione S-transferase. *J Mol Biol* 273(4):857–872
- Sobhorn P, Anantavara S, Dangprasert T, Viyanant V, Krailas D, Upatham E, Wanichanon C, Kusamran T (1998) *Fasciola gigantica*: studies of the tegument as a basis for the developments of immunodiagnosis and vaccine. *Southeast Asian J Trop Med Public Health* 29:387–400
- Sriveny D, Raina OK, Yadav SC, Chandra D, Jayraw AK, Singh M et al (2006) Cathepsin L cysteine proteinase in the diagnosis of bovine *Fasciola gigantica* infection. *Vet Parasitol* 135:25–31

- Swets JA (1988) Measuring the accuracy of diagnostic systems. *Science* 240(4857):1285–1293
- Tantrawatpan C, Maleewong W, Wongkham C, Wongkham S, Intapan PM, Nakashima K (2007) Evaluation of immunoglobulin G4 subclass antibody in a peptide-based enzyme-linked immunosorbent assay for the serodiagnosis of human fascioliasis. *Parasitology* 134:2021–2026
- Teofanova D, Hristov P, Yoveva A, Radoslavov G (2012) Native and Recombinant Fatty Acid Binding Protein 3 from *Fasciola hepatica* as a Potential Antigen. *Biotechnol Biotechnol Equip* 26(sup1):60–64
- Timanova-Atanasova A, Jordanova R, Radoslavov G, Deevska G, Bankov I, Barrett J (2004) A native 13-kDa fatty acid binding protein from the liver fluke *Fasciola hepatica*. *Biochim Biophys Acta Gen Subj* 1674(2):200–204
- Turner J, Howell A, McCann C, Caminade C, Bowers RG, Williams D et al (2016) A model to assess the efficacy of vaccines for control of liver fluke infection. *Sci Rep* 6:23345
- Valero MA, Periago MV, Pérez-Crespo I, Rodríguez E, Perteguer MJ, Gárate T, González-Barberá EM, Mas-Coma S (2012) Assessing the validity of an ELISA test for the serological diagnosis of human fascioliasis in different epidemiological situations. *Tropical Med Int Health* 17(5):630–636
- Velusamy R, Singh BP, Sharma RL, Chandra D (2004) Detection of circulating 54 kDa antigen in sera of bovine calves experimentally infected with *F. gigantica*. *Vet Parasitol* 119:187–195
- Wedrychowicz H, Wisniewski M (2003) Progress in development of vaccines against most important gastrointestinal helminth parasites of humans and animals. *Acta Parasitol* 48(4):239–245
- WHO (2008) Fact sheet on fascioliasis. In: Action against worms, World Health Organization, Headquarters. Newsletter, Geneva, 10:1–8
- Yamano K, Goto A, Nakamura-uchiyama F, Nawa Y, Hada N, Takeda T (2009) Gal β 1-6Gal, antigenic epitope which accounts for serological cross-reaction in diagnosis of *Echinococcus multilocularis* infection. *Parasite Immunol* 31(8):481–487
- Zweig MH, Campbell G (1993) Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clin Chem* 39(4):561–577