Development of Two FhSAP2 Recombinant–Based Assays for Immunodiagnosis of Human Chronic Fascioliasis

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Abstract. In the United States, infection with *Fasciola hepatica* has been identified as an emerging disease, primarily in immigrants, refugees, and travelers. The laboratory test of choice for diagnosis of fascioliasis is detection of disease specific antibodies, most commonly uses excretory-secretory antigens for detection of IgG antibodies. Recently, recombinant proteins such as *F. hepatica* antigen (FhSAP2) have been used to detect IgG antibodies. The glutathione S-transferase (GST)–FhSAP2 recombinant antigen was used to develop Western blot (WB) and fluorescent bead-based (Luminex) assays to detect *F. hepatica* total IgG and IgG₄ antibodies. The sensitivity and specificity of GST-FhSAP2 total IgG and IgG₄ WB were similar at 94% and 98%, respectively. For the IgG Luminex assay, the sensitivity and specificity were 94% and 97%, and for the IgG₄, the values were 100% and 99%, respectively. In conclusion, the GST-FhSAP2 antigen performs well in several assay formats and can be used for clinical diagnosis.

Fasciola hepatica is the causative agent of fascioliasis, a zoonosis with considerable public and veterinary health impact. It has been recognized as an emerging/reemerging zoonotic disease with an estimated 2.6 million people infected and 180 million at risk for infection worldwide.^{1,2} Autochthonous cases have been documented sporadically in the United States, mostly in Hawaii; however, most cases in the United States are restricted to travelers.³

Fascioliasis is definitively diagnosed via identification of *F. hepatica* eggs in feces; however, the prepatent period is 8–12 weeks postinfection, and so, early infections cannot be diagnosed by stool exams.^{4–6} Specific antibodies to *Fasciola* may be detectable 2–4 weeks after infection, which is 5–7 weeks before egg shedding.⁴ Thus, the detection of anti-*Fasciola* antibodies in serum (immunodiagnosis) offers a sensitive and reliable method for diagnosing acute, chronic, and latent fascioliasis.⁵ In this report, the development of two immunodiagnostic assays for fascioliasis based on a recombinant *F. hepatica* antigen (FhSAP2) are described.^{5,7}

Three sets of human sera in developing a standard Western blot (WB) and a fluorescent bead-based Luminex assay were used: 1) samples from patients with confirmed *F. hepatica* infection based on the presence of eggs in the stool (chronic fascioliasis) (WB *N* = 17; Luminex *N* = 16 for total IgG and *N* = 15 for IgG₄); 2) presumed negative samples from U.S. residents with no history of foreign travel (WB *N* = 38; Luminex *N* = 30); and 3) a convenience panel of samples from patients with various diseases other than fascioliasis, focusing mainly on helminth infections (WB *N* = 77 for total IgG and *N* = 74 for IgG₄; Luminex *N* = 58). All clinical samples used in this study were collected following written informed consent under protocols approved by the Center for Disease Control and Prevention Institutional Review Board (CDC study protocol no. 6756).

FhSAP2 with a *Schistosoma japonicum* glutathione S-transferase (GST) tag for coupling to Luminex beads was expressed, and this GST-FhSAP2 protein was incorporated into two assay formats. The coding sequence of FhSAP2 antigen (GenBank AAF88069.1) was subcloned into target vector pGS21a (GenScript, Piscataway, NJ) and transformed into *Escherichia coli* BL21 (DE3). Successful recombinant colonies were grown under selection of 100 µg/mL ampicillin at 37°C with shaking at 200 rpm. GST-FhSAP2 production was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside once cultures reached an optical density of 1.3 (at 600 nm), and were incubated overnight at 15°C with shaking at 200 rpm. Cells were collected by centrifugation at 8,000 × *g*, 4°C for 20 minutes, and the wet pellets were resuspended with 120 mL of phosphate-buffered saline (PBS), pH 7.4 + 1 mM dithiothreitol (DTT) + 1 mM phenyl methyl sulfonyl fluoride. After sonication at 500 W for 3 seconds and immersion in ice for 6 seconds for a total of 30 minutes, cell pellets were spun down at 13,000 rpm, 4°C for 30 minutes. The pellet was then resuspended with 30 mL PBS, pH 7.4 + 8 M urea and sonicated again at 500 W for 3 seconds, immersed in ice for 6 seconds for a total of 10 minutes, and centrifuged at 13,000 rpm, 4°C for 30 minutes. After centrifugation, the solubilized pellet was loaded onto 3-mL preequilibrated nickel affinity column. The protein was washed with 30 mL PBS, pH 7.4 + 20 mM imidazole + 8 M urea, and was then eluted with 30 mL PBS, pH 8.0 + 50 mM imidazole + 8 M urea and subsequently with 30 mL PBS, pH 8.0 + 500 mM imidazole + 8 M urea. The eluted proteins were then loaded onto 3-mL preequilibrated Q Sepharose Fast Flow columns (GE Life Sciences, Pittsburgh, PA, Cat. no. 17-0510-10). The flow through was pooled and proteins were refolded by dialysis against PBS, pH 7.4 + 4 mM DTT at 4°C. After dialysis, the sample was centrifuged at 13,000 rpm for 30 minutes and filtered through a 0.22-µm membrane. The GST and histidine (HIS) tags were left intact in the fusion protein. Proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and WB by using standard protocols for molecular weight and purity measurements. The primary antibody for WB was mouse-anti-GST

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TABLE 1
Performance of GST-FhSAP2 WB and Luminex

Assay characteristics	GST-FhSAP2 WB				GST-FhSAP2 Luminex			
	Total IgG	95% CI	IgG ₄	95% CI	Total IgG	95% CI	IgG ₄	95% CI
Cutoff point					27.8 MFI units		7.2 MFI units	
J-index					0.90		0.99	
Sensitivity <i>N</i> positive/ <i>N</i> tested (%)	16/17 (94)	84–100	16/17 (94)	84–100	15/16 (94)	82–100	15/15 (100)	100
Specificity <i>N</i> negative/ <i>N</i> tested (%)	113/115 (98)	96–100	111/112 (98)	96–100	87/90 (97)	93–100	89/90 (99)	96–100

CI = confidence interval; GST-FhSAP2 = *Fasciola hepatica* antigen; MFI = mean fluorescence intensity; WB = Western blot.

monoclonal antibody (GenScript, Cat. no. A00186). The concentration was determined by Bradford protein assay with bovine serum albumin (BSA) as a standard.

After titrating the optimum antigen concentration (Figure 1), GST-FhSAP2 recombinant protein was electrophoretically separated using Criterion TGX (Bio-Rad, Hercules, CA, Cat. no. 567-1092) at 6.25 ng/mm (for total IgG detection) and 12.5 ng/mm (for IgG₄ detection) and then transferred to nitrocellulose membranes (Whatman Protran BA83, GE Life Sciences, Cat. no. 10 541 103, 0.2-μm pore size). The blots were cut into 2.5-mm strips and stored in PBS + 0.1% sodium azide (NaN₃) at 4°C before use. Sera were tested and total IgG and IgG₄-specific antibodies were detected as described previously.^{8,9} A positive reaction was defined as the presence of a band at ~38 kDa.

For the Luminex format, coupling of protein to MagPlex Magnetic Microspheres (Luminex, Austin, TX) was carried out using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride-*N*-hydroxysulfosuccinimide protocol,^{10,11} with 12.5 μg of GST-FhSAP2 for 1.25 × 10⁶ beads that were used in the coupling reaction. The antibody detection was also carried out following the procedure previously described.^{10,11} The complex of antibody and coupled beads was detected using 50 μL of biotin-XX mouse antihuman IgG₄ (Cat. no.

A10663, Life Technologies, Carlsbad, CA, Cat. no. A10663) diluted 1:200 in PBS + 1% BSA + 0.05% NaN₃ or 50 μL of mouse antihuman IgG (Fc)-BIOT (Cat. no. 9042-08, Southern Biotech) of the same dilution.

Data were tabulated and analyzed using Microsoft Excel (Microsoft Corp., Redmond, WA). Determination of the cutoff value and assay performance was obtained by using R statistical software version 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria) and pROC package.¹²

The purity of the protein was ~75% as estimated by densitometry analysis of the Coomassie Blue-stained SDS-PAGE gel, and the optimum concentration for detection of total IgG and IgG₄ was at 6.25 ng/mm and at 12.5 ng/mm, respectively. At these concentrations, the performance of GST-FhSAP2 WB total IgG and IgG₄ was similar, with a sensitivity of 94% (Table 1). Both assays cross-reacted with the same hookworm sample. In addition, the total IgG assay cross-reacted with toxocariasis samples (Table 2), but due to the difference in the number of samples included for specificity testing of the two assays, the specificity calculated was 98% for both.

The GST-FhSAP2 protein was successfully coupled to the MagPlex microspheres. Using a cutoff value of 27.8 mean fluorescence intensity (MFI), the sensitivity and specificity of

TABLE 2
Cross-reactivity of GST-FhSAP2 WB and Luminex

Conditions represented by sera	GST-FhSAP2 WB				GST-FhSAP2 Luminex			
	Total IgG		IgG ₄		Total IgG		IgG ₄	
	No. of sera tested	Cross-reactivity (%)	No. of sera tested	Cross-reactivity (%)	No. of sera tested	Cross-reactivity (%)	No. of sera tested	Cross-reactivity (%)
U.S. negatives	38	0	38	0	30	0	30	0
Ascariasis	5	0	5	0	2	0	2	0
Baylisascariasis	3	0	3	0	NT		NT	
Cysticercosis	6	0	5	0	9	0	9	0
Cryptococcosis	1	0	1	0	NT		NT	
Echinococcosis	6	0	6	0	16	0	16	0
<i>Endolimax nana</i>	1	0	1	0	NT		NT	
Gnathostomiasis	2	0	2	0	NT		NT	
Hookworm	10	10	9	11	8	0	8	13
(<i>Necator americanus</i>)								
Hymenolepiasis	5	0	5	0	1	0	1	0
Paragonimiasis	2	0	2	0	2	0	2	0
Schistosomiasis	4	0	3	0	9	22	9	0
Strongyloidiasis	5	0	5	0	1	0	1	0
Taeniasis	5	0	5	0	4	0	4	0
(<i>Taenia solium</i>)								
<i>Taenia saginata</i>	1	0	1	0	NT		NT	
Toxocariasis	8	13	8	0	2	50	2	0
Toxoplasmosis	5	0	5	0	NT		NT	
Trichinellosis	5	0	5	0	4	0	4	0
Trichuriasis	3	0	3	0	NT		NT	

GST-FhSAP2 = *Fasciola hepatica* antigen; NT = not tested; WB = Western blot.

A against total IgG **B against mouse anti Human IgG₄**

FIGURE 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of the *Fasciola hepatica* antigen (GST-FhSAP2) recombinant protein. Titration of GST-FhSAP2 antigen concentration against (A) total IgG and (B) mouse antihuman IgG₄. The recombinant protein samples at concentrations of: 25 ng/mm (lane 1), 12.5 ng/mm (lane 2), 6.25 ng/mm (lane 3), 3.125 ng/mm (lane 4), 1.6 ng/mm (lane 5), and 0.8 ng/mm (lane 6) were treated with SDS and heated at 65°C for 15 minutes, separated, and analyzed using SDS gel electrophoresis and Western blotting. Gels were transferred to nitrocellulose membranes and probed with a pooled positive *F. hepatica* human sera (fascioliasis+), a pooled negative human sera (negative human sera), and a pooled strong enzyme-linked immunosorbent assay–positive *Toxocara* human sera (toxocariasis+), all of which were diluted 1:100 in phosphate-buffered saline (PBS) + 0.3% tween + 5% milk. The membrane was exposed to goat antihuman IgG–horse-radish peroxidase (HRP) labeled at dilution of 1:8,000 in PBS + 0.3% tween (A); another gel was transferred and got the same treatment as (A), but was probed by mouse antihuman IgG₄–HRP labeled at dilution of 1:1,000 in PBS + 0.3% tween (B). M = Precision Plus Protein Dual Xtra Standards (Bio-Rad, Cat. no. 161-0377).

the total IgG Luminex assay were 94% and 97%, respectively (Table 1). For the IgG₄ Luminex assay, the sensitivity and specificity were 100% and 99% at a cutoff value of 7.2 MFI (Table 1). As with the GST-FhSAP2 WB described above, the specificity among U.S. negative controls was 100% (Table 2). Cross-reactivity for the total IgG assay was observed among sera from two schistosomiasis specimens (22%) and one toxocariasis specimen (50%). The IgG₄ assay cross-reacted with one hookworm sample (13%).

The sensitivity of GST-FhSAP2 assays reported here are comparable to the *F. hepatica* excretory-secretory antigen (FhES)–enzyme-linked immunosorbent assay (ELISA) and to the immunoblot using 12-, 17-, and 63-kDa antigens. Cross-reactivity to schistosomiasis (6% on the FhES-ELISA)⁵

was only observed using the GST-FhSAP2 Luminex total IgG. A recent study determined FhSAP2 has a specificity of 99% without cross-reactivity to schistosomiasis samples,¹³ whereas our previous FhSAP2-ELISA has 100% sensitivity and 95.6% specificity.⁵ Our assays also demonstrated that FhSAP2 has an excellent specificity and minimal cross-reactivity to schistosomiasis samples.

As polyparasitism is common in the fascioliasis-endemic area, it is important to maximize assay specificity in detecting fascioliasis. The previously published FhSAP2-ELISA detects total IgG, which may contribute to observed specificity problems.^{4,5} IgG₄ has been detected in other parasitic infections, and selectively detecting IgG₄ can improve specificity.^{14–17} This is supported in our study, where the IgG₄

assay (in both WB and Luminex) appeared to have a higher specificity compared with assays based on total IgG detection. In addition, using a different tag when expressing the FhSAP2 protein might help to decrease the cross-reactivity and facilitate purification.

Immunologic tests to detect antibodies against parasite antigens are highly sensitive and can successfully diagnose acute fascioliasis.^{5,7,18} In this study, we observed that the diagnostic performance of the GST-FhSAP2 recombinant antigen in WB and Luminex formats is comparable to the performance of previously reported tests for fascioliasis.^{5,7,13} This format should allow for simultaneous determination of antibody responses to multiple infections in a single assay run.¹¹

To summarize, we used a recombinant antigen in two novel high-performance assays for detecting *F. hepatica*-specific antibodies. Because these assays use recombinant proteins, negating the need for native parasite materials, they can be more readily adapted for use in public health laboratories. The cost- and time-effective Luminex assay format may prove especially useful in future studies on fascioliasis epidemiology.^{19,20}

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