

IMMUNODIAGNOSIS OF EGYPTIAN HUMAN FASCIOLOSIS *GIGANTICA* USING FAS1 AND FAS2 CYSTEINE PROTEINASE ANTIGENS

By

IBRAHIM RABEE¹, NOHA AHMED MAHANA^{2*}
And ABEER MAHMOUD BADR²

Theodore Bilharz Research Institute¹, Imbaba, Giza, and Department of Zoology², Faculty of Science, Cairo University, Egypt* Correspondence: Noha Mahana; E-mail: nohamahana@gmail.com

Abstract

Fasciolosis caused by *Fasciola gigantica* is one of the major public health problems in the world including Egypt. Immunodiagnostic methods are more applicable for their better sensitivity and specificity than other methods. The present study was conducted to cysteine proteinase (CP) antigens of *F. gigantica* in IgG-ELISA to diagnose human fasciolosis. IgG-ELISA with 2 cysteine proteinases of 27 kDa (Fas1) and 29 kDa (Fas2), obtained from the regurgitated materials of adult worms, were evaluated using serum samples from 90 Egyptian patients infected with *F. gigantica*, 55 patients with other parasitic infections and 50 healthy volunteers. The diagnostic sensitivity and specificity of Fas1 for detection of *F. gigantica* infection were 91.1% and 89.1%, respectively. The positivity of the assay was 95%. The positive and negative predicted values were 91% and 86%, respectively. These data suggest that IgG-ELISA with Fas1 is highly sensitive and specific assay and could be used for the immunodiagnosis of human fasciolosis.

Keywords: *Fasciola gigantica*; serodiagnosis; IgG-ELISA; cysteine proteinases.

Introduction

Fasciolosis caused by *Fasciola hepatica* and *F. gigantica* is one of the major public health problems in the world (Rashed *et al*, 2010). While *F. hepatica* has a more cosmopolitan distribution, *F. gigantica* tends to be restricted to the more tropical regions (WHO, 1995). Estimations for all continents reach 17 million people to be infected, and this may even be an underestimation if the total lack of data concerning numerous Asian and African countries is considered (Mas-Coma *et al*, 2005). Epidemic fasciolosis has caused exten-

sive suffering and economic losses in some regions (Rokni *et al*, 2002).

Diagnosis of fasciolosis is performed either by eggs in stool samples (Lumbreras *et al*, 1962), serologic (Capron *et al*, 1964) or by ultrasonography (Haseeb *et al*, 2003a). The immunodiagnosis was improved by the development of ELISA using somatic antigens and excretory-secretory products (ESP) derived from adult worms (Khalil *et al*, 1990; Haseeb *et al*, 2003b). One dimensional gel electrophoresis indicated that the most prominent *F. gigantica* ESP are of 66, 64, 58, 54, 28 and 26-27

kDa (Sobhon *et al*, 1996). The former four molecules were shown to be derived from the worm tegument, while the 28 & 26 kDa species appeared to be released from the cells lining the gut (Dalton *et al*, 2003; Anuracpreeda *et al*, 2006). The 28 & 26-27 kDa molecules are essentially consist of cathepsin cysteine proteinases (Dixit *et al*, 2004).

Fasciola-derived ESP cysteine proteases, for diagnosis implied that these molecules elicit strong immune responses during natural infection. Naturally infected cattle and sheep mounted vigorous cellular and humoral immune responses to ESP during *F. hepatica* (Chauvin *et al*, 1995) and *F. gigantica* (Phiri *et al*, 2006) natural infection.

Fluke cysteine proteinases are sensitive and specific markers for human diagnosis (Cordova *et al*, 1997; O'Neill *et al*, 1998; Strauss *et al*, 1999). The major components of *F. hepatica* ESP are cysteine proteinases, which are termed Fas1 and Fas2. Both parasite enzymes were purified by a simple procedure (Cordova *et al*, 1999) and evaluated as markers of the infection in alpacas that were naturally exposed to liver fluke in endemic areas (Neyra *et al*, 2002).

The current study aims to examine the potential of *F. gigantica*-derived ESP (Fas1 and Fas2 cystein proteinases) in immunodiagnosis and prevention of human fasciolosis.

Subjects, Materials and Methods

All serum samples from 145 individuals were collected from Theodor Bilharz Research Institute (TBRI, Giza). Coprological analysis for *Fasciola*

eggs was done on fecal samples obtained from patients (Katz *et al*, 1972), and examined by microscopy. Positive patients (n= 90) were included. They were subjected to adequate clinical chemotherapy. Serum samples from 50 healthy volunteers were used as negative controls. Also, sera from patients with *schistosomiasis mansoni* (n=10), echinococcosis *granulosus* (n=15), ancylostomiasis *duodenale* (n=15), and ascariasis *lumbricoides* (n=15) were collected from different laboratories in TBRI. All procedures related to animal experimentation met the international guiding principles for biomedical research on animals as issued by the International Organizations of Medical Sciences.

Hillyer and Santiago de Weil (1977) method was adopted to prepare crude *F. gigantica* antigen. Adults were collected from the infected livers of sheep slaughtered at the Giza governmental abattoir, washed three times with phosphate-buffered saline (PBS), homogenized and centrifuged at 5X10⁴ g for 1 hr. at 4°C. Supernatant protein content was measured (Lowry *et al*. 1951).

Adults were removed from the bile duct and washed 6 times with cold 0.01 M PBS (pH 7.4) containing 125 mM NaCl for 1 hr. to eliminate any traces of bile or blood. They were incubated for 16 hrs at 37°C in RPMI 1640 medium (pH 7.4), medium removed, centrifuged at 15000 X g for 30 min. and the supernatant containing ESP were collected. The protein content of the ESP preparations was evaluated by the Bradford method using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA,

USA), and stored at -20°C . ESP were concentrated using an Amicon 8400 ultrafiltration unit with membrane (3000 Da cut-off). The sample applied to a 120 ml Sepharcl S200 HR column (gel filtration chromatography) equilibrated in 0.1 M Tris-HCl, pH 7. Fractions of 5 ml were collected, after the void volume (110 ml) was passed. The column elute was monitored at 280 nm. Fractions are analysed for enzymatic activity. Those fractions containing the cysteine proteinases were pooled and applied to a 50 ml QAE Sephadex column equilibrated in 0.1 M Tris-HCl, pH 7. The unbound fraction (150 ml) was collected and concentrated in an amicon ultrafiltration unite to 10 ml. dialysis again-st ultra-pure water and freeze dried.

Fas1 and Fas2 were prepared (Cordova *et al*, 1997). Antigens were purified from the regurgitated material produced by *F. gigantea* adults when placed in cold water for 2 hr. The suspension was then centrifuged at $7000 \times g$ for 10 min., lyophilized and stored at -20°C . Two hundred and fifty mg of this material was resuspended in 5.5 ml of 0.2 M sodium citrate (pH 4.9) containing 0.1 mM HgCl_2 at 4°C . Chilled ethanol (98%) was added to a concentration of 60%, incubated for 15 min on ice and centrifuged $12000 \times g$ at 4°C for 10 min. Supernatant was dialyzed in 10 mM of sodium citrate (pH 4.9) containing 0.1 mM HgCl_2 at 4°C , in dialysis membranes (cut-off 12 kD) and frac-

tionated in carboxymethylsephadex C-50 column (1.8 cm \times 35 cm) in 10 mM sodium citrate (pH 4.9) containing 0.1 mM HgCl_2 with a gradient of NaCl (0-1 M). Fractions with proteolytic activity were lyophilized and stored at -20°C . Fas1 eluted before the salt gradient was applied and Fas2 eluted with 0.6 M NaCl. Crude extract and protein fractions were characterized by discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) SDS-PAGE in 12% slab gels (1 mm thick), under reducing conditions (+2-mercaptoethanol) and stained with Coomassie blue (Bio-Rad). Apparatus and chemicals were purchased from Bio-Rad and assemblies of apparatus and gel preparations were performed according to the operation manual.

ELISA test (El Shazly *et al*, 2002) was done with some modifications. Wells of polystyrene microtiter plates (Costar, Corporate Headquarters, Cambridge, MA, USA) were coated with purified Fas1 (1.5pg/ml) antigens in coating buffer (carbonate-bicarbonate buffer), then incubated overnight at 4°C . Plates were washed 5 times with buffer (PBS with 0.05% Tween 20), blocked by dispensing 200 μl /well of 1% bovine serum albumin (BSA) in PBS and left for 2 hrs at room temperature. After washing the wells 5 times, 100 μl /well a serum sample (primary antibody), previously diluted 1/100 Fas1 in washing buffer, were added to the plates and incubated for 1 hr. at 37°C . Plates were washed 5 times with buffer and 100 μl /well of goat anti-human IgG conjugated to horse radish peroxidase (HRP, secondary antibody), previously diluted

to 1-1000 in washing buffer, were added to each well and incubated for 1 hr. at 37°C. After washing 5 times, a color reaction was observed by incorporating 0-phenylene-diamine dihydrochloride (Sigma) into the reaction, stopped by adding 50µl of 2M H₂SO₄. Optical density was measured at 492 nm (OD₄₉₂) in an ELISA reader (Bio-Rad microplate reader, Richmond, Ca.).

Rabbit polyclonal anti-Fas1 IgG antisera were obtained by inoculating 200 mg of Fas1 emulsified in 0.5ml of Freund's complete adjuvant in two female New Zealand white rabbits. They received three boosters with the same dose of Fas1 in Freund's incomplete adjuvant at weekly intervals. Blood samples were collected one week after final inoculation. Serum was pooled and a fraction enriched with anti-Fas1-IgG was prepared by precipitation in 50% ammonium sulphate (Nowotny, 1979). Precipitate was further purified by 7% Caprylic acid (McKinney and Parkinson, 1987), then dialyzed in PBS pH 7.4 and stored at -70°C in 50% glycerol until needed. The IgG pAb purity after each purification step was assayed by 12% SDS-PAGE (1 mm thick), under reducing conditions.

Microtitre plate IMMULON14HBX (Dynerx Technologies Inc., USA) was coated overnight at 4°C with 30µg/ml ESP in carbonate coating buffer, blocked with 0.1% BSA in PBS then 100 µl/well of serially diluted pAb (1:50 to 1:3200) in washing buffer was added. Hundred µl/well of anti-rabbit IgG peroxidase conjugate (Sigma) diluted in washing buffer (1/1000) was dispensed. Fifty µl/well of 8 N H₂SO₄ was

added to stop the enzyme substrate reaction. Absorbance was measured at 492 nm by the ELISA reader. After each step, they were washed 5 times with buffer and incubated for 1 hr. at 4°C.

Ninety samples from patients with *F. gigantica* and samples 50 healthy controls were tested by sandwich ELISA and compared with 55 patients with *S. mansoni*, *E. granulosus*, and *A. doude-nale* and sensitivity and specificity of sandwich ELISA were evaluated.

Wells of microtitre plates were coated with 100µl/well of purified pAb IgG (10µg/ml) in 0.06 M carbonate buffer, pH 9.6, then blocked after washing with 200µl/well 2.5% fetal calf serum (FCS, Sigma) in 0.1 M PBS/T for 2 hr at 37°C. After washing 5 times, 100µl of purified Fas1 (1.5 µg/mL) antigens were added for 1 hr at room temperature. One hundred µl/well of pooled positive and negative sera diluted 1/100 were in washed buffer, added individually to each well, and incubated for 1 hr. at 37°C. Plates were washed three times with buffer, then 100µl/well of peroxidase-conjugated IgG antibodies of dilution 1/1000 were dispensed and plates were incubated for 1 hr. at 37°C. Color appearance was done by addition of 100µl/well substrate buffer and plates were kept in dark at room temperature for 30 min, then the enzyme reaction was stopped by 50µl/well of 8 N H₂SO₄. The absorbance was measured at 492 nm using ELISA reader (Bio-Rad).

Statistical analysis: Fas1- and Fas2-ELISA cut-off values were estimated as the mean (M) OD_{492 nm} plus two times the value of the standard devia-

tion (SD) of sera from healthy controls (Neyra *et al*, 2002) and considered significant at $P < 0.05$. The sensitivity, specificity and predictive values were determined by using a 2x2 contingency table.

Results

In the present study, *F. gigantica* infected group (ova count/gm stool) was subdivided into light (n= 30), moderate (n= 40) and heavy (n= 20) infection using Nucleopore technique. *Fasciola* eggs were detected in 90 (55.3%) patients and *S. mansoni*, *E. ganulosus*, *A. duodenale* and *A. lumbricoides* were in 55 patients. Protein content assays indicated that ESP preparations contained an average of 1.0 mg/ml protein, with SD ranged between 5-10% around the mean. The OD₂₈₀ profile of adult *F. gigantica* cysteine proteinase antigen fractions obtained following purification of whole *F. gigantica* ESP antigens could be represented by fractions (no. 8) with a maximum OD single peak value equal to 1.1 (Fig. 1).

Approximately 15mg from ESP and the eluted purified protein fraction (no. 8) preparation were resolved by SDS-PAGE, 12% gel, under reducing (+2-mercapto-ethanol) conditions, stained by Coomassie blue and photographed (Fig. 2), which showed different bands ranged from 17-116 kDa, and Fas1 and Fas2 were visualized as 27 & 29 kDa bands in stained 12% SDS-PAGE.

The antigenicity of the purified cysteine proteinase Fas1 was tested by indirect ELISA, and sera from *F. gigantica* infected was equal to 2.09 nm and approximately without reactivity with sera of patients infected with other par-

asites (Tab. 2). The antigenicity of Fas2 was lower than Fas1, and cross reacted with other parasitic patients.

The purity of IgG Fas1 pAb after each purification step was assayed by 12% SDS-PAGE under reducing conditions (Fig. 3). Analysis of 50% ammonium sulfate-precipitated proteins revealed several bands. The purified IgG pAb after 7% Caprylic acid was represented by 2 bands, L and H-chain bands at 31 and 53 kDa, respectively. The pAb appears free from other proteins (Fig. 3).

Sera of rabbit injected with *F. gigantica* Fas1 were tested for specific anti-*F. gigantica* ESP by indirect ELISA showed increasing antibody level started one week after the 1st booster dose. Three days after 2nd booster dose, immune sera gave a high titer against *F. gigantica* ESP with OD of 2.97 at 1/100 dilution (Fig. 4).

The *F. gigantica* cysteine proteinase antigen in human sera by Sandwich ELISA (Tab. 3) was 100% negative from *Fasciola* cystein proteinase antigens. The OD values of *Fasciola* infected patients were significantly higher than both negative controls and other parasitic patients. Eighty two out of 90 cases were positive samples of *F. gigantica* patients, the remaining 8 were among the light infection followed by moderate and heavy subgroups, respectively. The assay positivity was 95% in moderate and heavy infected groups followed by 83.3% in light ones.

Regarding patients with other parasites, there was a degree of cross reactivity as schistosomiasis showed the highest one, followed by echinococcosis, ancylostomiasis and ascariasis,

respectively. The detection of serum cysteine proteinase antigens by sandwich ELISA gave sensitivity of 91.1% and specificity of 89.1%. The positive and negative predictive values (PPV & NPV) were calculated coprologically as a reference test for diagnosis of human fasciolosis. Fas1 IgG-ELISA gave PPV= 91.1% and NPV= 86%.

Discussion

In fasciolosis and other gastrointestinal tract helminthes of livestock, the eggs in fecal samples proved the most reliable diagnostic test. But, the inability of coprologic test to detect prepatent helminthes and less sensitivity in light infections pave the way to immunodiagnosis (Goubadia and Fagbemi, 1995), despite their satisfactory sensitivity, immunodiagnostic assays are hampered by lack of specificity attributed to the possession of common antigens by different helminthes (Fagbemi and Obarisiagbon, 1991). Fasciolosis diagnosis based on antibody detection did not differentiate between past and present infections due to antibodies persistence for prolonged time. This limits the advantages of antibody based diagnostic assays over antigen based assays in practice (Sriveny *et al*, 2006). Thus, antigen detection assays was tried to avoid problems of animals' past infections (Velusamy *et al*, 2004).

The identification of parasite antigens that elicit a humoral response in human fasciolosis is important for better diagnosis (Demirci *et al*, 2009). The enzymes of adult helminthes were widely used as antigens in immunodiagnostic assays to improve quality and such macromolecules include alkaline phosphatase

(Efthimiou *et al*, 2010) and host proteases (Zorina and Zorin, 2013). Cysteine proteinases are predominant ESP proteolytic enzymes released by the midgut epithelial cells of the parasite (Smith *et al*, 1993). Several protease genes constituting a cysteine proteinase gene family have been identified in both *F. hepatica* and *F. gigantica*, and regulate the development of the parasite in its host environment (Grams *et al*, 2001). These protease molecules were assayed in immunodiagnosis of human and animal fasciolosis (Carnavale *et al*, 2001; Dixit *et al.*, 2004; El Sibaei *et al*, 2013) and show promise as an aid in the diagnosis of early prepatent infection.

Two cysteine proteinases, referred to as Fas1 and Fas2, were evaluated as antigens for diagnosis of *F. hepatica* infection man, detected in patients' sera (Cordova *et al*, 1997). Fas1 and Fas2 shared common epitopes, which was confirmed by partial identity pattern in double immune-diffusion assays (Cordova *et al*, 1999). Thus, the current study used 27 (Fas1) & 29 kDa (Fas2) cysteine proteases of adult *F. gigantica* ESP and evaluated as immunodiagnostic antigens for human fasciolosis.

The Fas1 proved to be a good antigen than either crude *Fasciola* antigen or other ESP (Fas 2), as Fas1 did not cross-react with schistosomiasis patients' sera and the IgG ELISA cutoff value was lower than that of either ESP or crude antigen, and thus proved specific in immunodiagnostic tests. Studies found that Fas2 was a good marker than Fas1 with significant sensitivity,

specificity, PPV and NPV (Cordova *et al.*, 1997; 1999; Neyra *et al.*, 2002).

Yamasaki *et al.* (1989) reported high sensitivity and specificity in Igs-ELISA by *Fasciola* ESP antigens with one schistosomiasis *japonicum* cross reaction. Rodriguez-Perez and Hiller (1995) found that ESP antigens were more specific than somatic and surface antigens for sheep fasciolosis. Rokni *et al.* (2004) reported sensitivity and specificity values for somatic antigens 91.0% & 96.2%, and for ESP antigens 95.2%

& 98.0%, respectively. These agreed with the present data as antigens from ESP gave sensitive (91.1%) and specific (89.1%) with two cross reactions with schistosomiasis and echinococcosis and one case with ancylostomiasis and ascariasis.

Conclusion

The outcome results showed that IgG-ELISA with Fas1 proved to be an adequate test to diagnose fasciolosis and to assess its prevalence in humans living in endemic areas.

Table 1: Intensity of fascioliasis (N=90) by ova count

Fasciolosis	No.	Ova count
Light(< 30)	30	2±8.8
Moderate (30- 70)	40	18±11.91
Heavy(> 70)	20	22±3.31

M= Mean, SD= Standard deviation

Table 2: Purified *F. gigantica* cysteine proteinase antigen by indirect ELISA

Sera parasites	OD readings at 492 nm
<i>Fasciola gigantica</i>	2.092±0.201
<i>Schistosoma mansoni</i>	0.209±0.342
<i>Echinococcus</i>	0.106±0.094
<i>Ancylostoma</i>	0.182±0.082

Table 3: *Fasciola* antigen in patients' sera (n=90) and other parasites (n=35) by sandwich ELISA

Groups	Positive cases			Negative cases		
	M± SD	No.	percent	M±SD	No.	percent
Healthy (n= 50)	-	-	-	0.29 ± 0.01	50	100%
<i>Fasciola</i> light infection(n= 30)	0.52 ± 0.20	25	83.3 %	0.32 ± 0.11	5	16.7 %
Moderate infection (n=40)	0.79 ± 0.32	38	95 %	0.40 ± 0.23	2	5 %
Heavy infection(n= 20)	1.02 ± 0.21	19	95 %	0.42 ± 0.18	1	5 %
Schistosomiasis (n=10)	0.74 ± 0.22	2	20 %	0.22 ± 0.19	8	80 %
Echinococcosis(n=15)	0.71 ± 0.22	2	13.2 %	0.15 ± 0.21	14	82.8 %
Ancylostomiasis(n= 15)	0.82±0.11	1	6.6 %	0.24 ± 0.22	14	93.4 %
Ascariasis(n= 15)	0.65±0.15	1	6.6 %	0.21 ± 0.11	14	% 93.4

Cut off value: 0.312

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Explanation of figures

Fig. 1: OD₂₈₀ profile of cysteine proteinase antigen fractions from purification of whole *F. gigantica* worm antigen.

Fig. 2: SDS-PAGE (12%, 1mm, under reduction) of *F. gigantica* ESP before and after elution from affinity chromatography columns. Lane 1: Low molecular weight standard; Lane 2: Crude *Fasciola* antigen; Lane 3: Purified ESP antigen; lane 4: Purified cysteine proteinase by DEAE; Lane 5: Fas 1 and Fas 2.

Fig. 3: 12% gel (1mm) under reduction of anti-*F. gigantica* Fas1 IgG antibody before and after pAb purification with commassie blue stain. Lane 1: Molecular weight of standard protein; Lane 2: Crude anti-*F. gigantica* Fas1 IgG pAb; Lane 3: Precipitated proteins after 50% ammonium sulfate treatment; Lane 4: Purified IgG antibodies after 7% caprylic acid treatment; Lane 5: More purified IgG by column chromatography.

Fig. 4: Reactivity of immunized rabbit anti-*F. gigantica* Fas1 antisera against *F. gigantica* ESP by indirect ELISA.

