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

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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 extensive 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 5X10^4 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 Sepharcyl 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 un-bound 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. gigantica* adults when placed in cold water for 2 hr. The suspension was then centrifuged at 7000 × 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 HgCl₂ at 4°C. Chilled ethanol (98%) was added to a concentration of 60%, incubated for 15 min on ice and centrifuged 12000×g at 4°C for 10 min. Supernatant was dialyzed in 10 mM of sodium citrate (pH 4.9) containing 0.1 mM HgCl₂ at 4°C, in dialysis membranes (cut-off 12 kD) and fractionated in carboxymethylsephadex C-50 column (1.8 cm×35 cm) in 10 mM sodium citrate (pH 4.9) containing 0.1 mM HgCl₂ 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-mercaptopethanol) 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 200µl/well of 1% bovine serum albumin (BSA) in PBS and left for 2 hrs at room temperature. After washing the wells 5 times, 100µl/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 100µl/ 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 H$_2$SO$_4$. Optical density was measured at 492 nm (OD$_{492}$) in an ELISA reader (Bio-Rad microplate reader, Richomond, Ca.). Rabbit polyclonal anti-Fas1 IgG antisera were obtained by inoculating 200 mg of Fas1 emulsified in 0.5 ml 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% Caprilic 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 (Dynex 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 H$_2$SO$_4$ 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. douzcnale 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 Fasl (1.5 pg/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 trice 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 NH$_2$SO$_4$. 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$_{492}$ mm 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 OD$_{280}$ 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 parasites (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 pre-patent 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 (Carnevale et al, 2001; Dixit et al., 2004; El Sibaei et al, 2013) and show promise as an aid in the diagnosis of early pre-patent infection.

Two cysteine proteinases, referred to as Fasl and Fas2, were evaluated as antigens for diagnosis of F. hepatica infection man, detected in patients’ sera (Cordova et al, 1997). Fasl 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 IgG-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. Rotki 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**

<table>
<thead>
<tr>
<th>Fasciolosis</th>
<th>No.</th>
<th>Ova count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light (&lt; 30)</td>
<td>30</td>
<td>2 ± 8.8</td>
</tr>
<tr>
<td>Moderate (30-70)</td>
<td>40</td>
<td>18 ± 11.91</td>
</tr>
<tr>
<td>Heavy (&gt; 70)</td>
<td>20</td>
<td>22 ± 3.31</td>
</tr>
</tbody>
</table>

M= Mean, SD= Standard deviation

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

<table>
<thead>
<tr>
<th>Sera parasites</th>
<th>OD readings at 492 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasciola gigantica</td>
<td>2.092 ± 0.201</td>
</tr>
<tr>
<td>Schistosoma mansoni</td>
<td>0.209 ± 0.342</td>
</tr>
<tr>
<td>Echinococcus</td>
<td>0.106 ± 0.094</td>
</tr>
<tr>
<td>Ancylostoma</td>
<td>0.182 ± 0.082</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Groups</th>
<th>Positive cases</th>
<th>Negative cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M ± SD</td>
<td>M ± SD</td>
</tr>
<tr>
<td>Healthy (n= 50)</td>
<td>No.</td>
<td>percent</td>
</tr>
<tr>
<td>Fasciola light infection(n= 30)</td>
<td>0.52 ± 0.20</td>
<td>25</td>
</tr>
<tr>
<td>Moderate infection (n=40)</td>
<td>0.79 ± 0.32</td>
<td>38</td>
</tr>
<tr>
<td>Heavy infection (n=20)</td>
<td>1.02 ± 0.21</td>
<td>19</td>
</tr>
<tr>
<td>Schistosomiasis (n=10)</td>
<td>0.74 ± 0.22</td>
<td>2</td>
</tr>
<tr>
<td>Echinococcosis (n=15)</td>
<td>0.71 ± 0.22</td>
<td>2</td>
</tr>
<tr>
<td>Ancylostomiasis (n=15)</td>
<td>0.82±0.11</td>
<td>1</td>
</tr>
<tr>
<td>Ascariasis (n=15)</td>
<td>0.65±0.15</td>
<td>1</td>
</tr>
</tbody>
</table>

Cut off value: 0.312

**References**


Haseeb, AN, El Shazly, AM, Arafia, MAS, Morsy, ATA, 2003b: Evaluation of excretory secretory Fasciola


Smith, AM, Dowd, AJ, Heffernan,


**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.