

Comparison of Adult Somatic and Cysteine Proteinase Antigens of *Fasciola gigantica* in Enzyme Linked Immunosorbent Assay for Serodiagnosis of Human Fasciolosis

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ABSTRACT

Fasciolosis caused by *Fasciola hepatica* and *F.gigantica* is one of the major public health problems in the world and in Iran. Considering that stool examination for *Fasciola* eggs is not a sensitive method and only 25% of infected patients pass the eggs in the faeces, and immunodiagnosis methods are more applicable for this purpose, the present study was conducted to compare the somatic (S) and cysteine proteinase (CP) antigens of *F.gigantica* in IgG-ELISA to diagnose human fasciolosis. This has been the first report on this case so far in Iran. Serum samples obtained from 178 individuals collected during the fasciolosis outbreak in 1999 in the Gilan province, northern Iran, that were coprologically positive for fasciolosis, were analyzed by IgG-ELISA for total antibody responses against (S) and CP antigens from *Fasciola gigantica*. The cut-off points for (S) and CP were 0.38 and 0.33, respectively. All cases that showed clinical manifestations of fasciolosis, were also seropositive using both (S) and CP antigens whereas all 25 non-infected controls were seronegative. Therefore, the sensitivity of the test was 100% for both antigens. On the other hand the specificity of (S) and CP antigens were calculated as 96.4% and 98.1%, respectively. The positive and negative predictive values of the test regarding (S) antigen were 97.8% and 100%, whereas these values as for CP antigen were 98.9% and 100% correspondingly. Two individuals with hydatidosis and two with toxocariasis had antibodies against (S) antigen whereas concerning CP antigen, one individual with hydatidosis and another with toxocariasis showed cross reactivity against it. We have demonstrated that altogether CP antigen provide a more conclusive diagnosis as possessing lower cut-off and enabling better to discriminate between seronegative and seropositive subpopulations. This study may be useful to implement a reliable test to diagnose human fasciolosis and for seroepidmiological objectives.

INTRODUCTION

Fasciolosis caused by *Fasciola hepatica* and *F.gigantica* is one of the major public health problems in the world and in Iran (16,21). Epidemics of fasciolosis has caused extensive suffering and economic losses. Humans are infected by ingestion of aquatic plants that contain the infected metacercariae. Metacercariae excyst in the duodenum to form juvenile flukes that burrow through the gut mucosa and migrate across the liver parenchyma causing the extensive hemorrhaging and liver damage associated with the acute stage of the infection. After 8-12 weeks the juvenile flukes reach the bile ducts where they mature sexually and commence egg production. This is the latent period of infection and individuals can be asymptomatic for several months to several years (13). Fasciolosis is now emerging as an important chronic disease of humans in the Gilan province of northern Iran (21). Over the past twelve years two epidemics in 1989 and 1999 caused rising of the number of infected cases to over 7,000 and 10,000, respectively (1, 5,16).

Diagnosis of fasciolosis is based on clinical findings as well as laboratory tests. The most reliable means is the finding of eggs in the stool of infected individual despite the overwhelming consensus that this method is not wholly reliable (8). Using this method eggs are not detected until the latent period of infection when much of the liver damage has already occurred. In addition, eggs are released sporadically from the bile ducts and hence stool samples of infected patients can contain no eggs (14). Serological diagnosis is preferred particularly since anti-fasciola antibodies can be detected as early as two weeks post

infection and can thus facilitate early chemotherapeutic intervention (8).

Despite of the recent studies involving the diagnosis of fasciolosis using *Fasciola hepatica* antigens in ELISA test (3,17), the effectiveness of *F.gigantica* somatic (S) and cysteine proteinase (CP) as antigens in ELISA have not been studied adequately and specially in Iran this is the first report in this case.

The purpose of this investigation was to compare aforementioned *F.gigantica* antigens in ELISA test for serodiagnosis of human fasciolosis.

MATERIALS AND METHODS

Clinical Samples

Blood samples from *Fasciola*-infected individuals were collected during the fasciolosis outbreak in 1999 in the Central Health Clinic of Rasht, in the Gilan province, northern Iran. Coprological analysis for *Fasciola* eggs was performed on faecal samples obtained from all individuals as previously described (9). Only individuals that were coprologically positive cases and presented with a history of the disease and clinical manifestations were included in the present study. The age of these individuals ranged from 3-70 years, with a mean \pm standard deviation of 33.47 ± 16.86 years. The number of females participating in the study was 62.5%. Serum samples from patients infected with hydatidosis (45 individuals), toxocariasis (20 individuals), amoebiosis (10 individuals), malaria (5 individuals), Kala-azar (2 individuals) and 25 control serum samples were obtained from the School of Public

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Health serum blood bank, Tehran University of Medical sciences.

Preparation of Antigens

F. gigantica somatic (S) antigen was prepared by homogenizing adult worms in 0.045 M PBS/pH 7.2 using electrical homogenizer (Edmund Buhler Co., model Homo 4/A mit uhr) followed by sonication (Tomy Seiko model UP-200P, Tokyo), and then centrifugation at $15000 \times g$ at $4^{\circ}C$ for 30 minutes. Afterwards the supernatant (S antigen) was subjected to a 134 ml Sephacryle S200 HR gel-filtration column, equilibrated in 0.1 M Tris-HCL, pH 7.2. One-milliliter fractions were collected and monitored by absorbance at 280 nm for protein concentration. Each fraction was also assayed for cysteine proteinase activity using the fluorescent substrate Z-Phe-Arg-AMC (7-amino-4-methylcoumarin). The release of the fluorescent leaving group, AMC was monitored in a Perkin-Elmer Luminescence Spectrometer model LS 50, at exciter and analyzer wavelengths of 370 nm and 440 nm, respectively (19). Fractions containing proteolytic activity were pooled, and then were subjected to ion exchange chromatography on QAE-Sephadex A50 equilibrated in 0.1 M Tris-HCL, pH 7.2. The first obtained fraction, which had the highest proteolytic activity, concentrated and used as cysteine proteinase CP antigen.

The concentration of each antigen preparation was measured using Bradford method (2). Both prepared antigens were analyzed by 15% SDS-PAGE (7). In order to determine the class of these antigens specific inhibitors at final concentration of phenylmethylsulfonyl fluoride (PMSF) 10mM; EDTA 10mM; E-64 5 μ g/ml; leupeptin 5 μ g/ml and pepstatin 1 μ M were used (5).

ELISA Test

The immunodiagnostic assay was performed as previously described (18). Briefly, 100 micro liters of (S) and CP antigens (30 μ g/ml and 5 μ g/ml respectively) was dispensed into the wells of microtiter plates (Nuclon, Kamstrup, Roskilde, Denmark) and then incubated overnight at $37^{\circ}C$. Excess binding sites were blocked with 200 μ l of bovine serum albumin (2% diluted in PBS/0.1% Tween 20) and incubated for 30 minutes at $37^{\circ}C$. After the wells were washed three times with PBS /Tween 20, 100 μ l of a serum sample (diluted 1:1250) was added to each plate and incubated for 60 min at $37^{\circ}C$. Following another washing step, 100 μ l of peroxidase-conjugated goat anti-human IgG (diluted 1:6000) was added to each well and the plates incubated for a further 60 minutes at $37^{\circ}C$. Following a final washing step 100 μ l of O-phenyldiamine dihydrochloride (OPD) substrate (all from Sigma Chemical Co., Poole, Dorset, United Kingdom) was added to each well and the reaction stopped after 5 minutes by adding 50 μ l of 12.5% H_2SO_4 . The optical density (OD) of the samples was measured

at 492 nm using a Titerteck (Helsinki, Finland) multiscan ELISA plate reader.

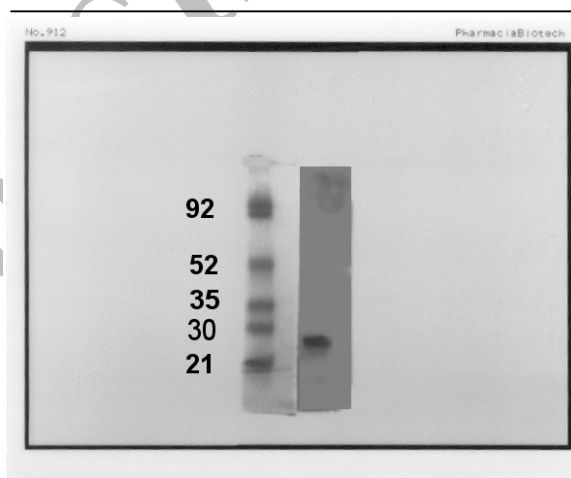
Statistical Analysis

The sensitivity, specificity, and the predictive values were calculated using the method of Galen et al(7). All assays were tested in triplicate and repeated twice. Statistical analysis was carried out using SPSS for windows, version 10.

RESULTS

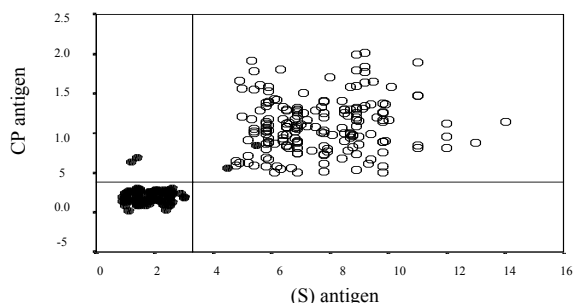
Analysing the first peak gained from ion-exchange chromatography, i.e., CP antigen, by SDS-PAGE, a protein of about 27-28 kDa was identified (Fig.1). Using specific inhibitors the class of this antigen was detected as cysteine proteinase.

Fig. 1. Reducing SDS-PAGE of purified *F. gigantica* cysteine proteinase in a 15% polyacrylamide gel stained with coomassie blue. On the left shows the protein marker



Serum samples obtained from positive group were analyzed by ELISA for total antibody responses against somatic (S) and CP antigens. The absorbance readings obtained for (S) and CP antigens were plotted against each other on scattergrams, which revealed a compact cluster of individuals with low absorbance readings and a second cluster with high absorbance readings (Fig. 2). Using hierarchical agglomerative cluster analysis the cut-off points between clusters were 0.38 and 0.33 for (S) and CP, respectively. Therefore absorbance readings greater than the cut off value were considered to be seropositive for fasciolosis. Accordingly, all 178 cases that showed clinical manifestations of fasciolosis, were also seropositive using both (S) and CP antigens (Fig. 2, indicated by open circles) whereas all 25 non-infected controls clustered together as seronegative (Fig. 2, indicated by closed circles). Therefore, the sensitivity of the test was 100% for both antigens.

Fig. 2. Scatter graph of the combined data for 178 *Fasciola*-infected and 25 non-infected volunteers obtained in the total IgG-ELISA using somatic (S) and cysteine proteinase (CP) antigens of *F.gigantica* antigens



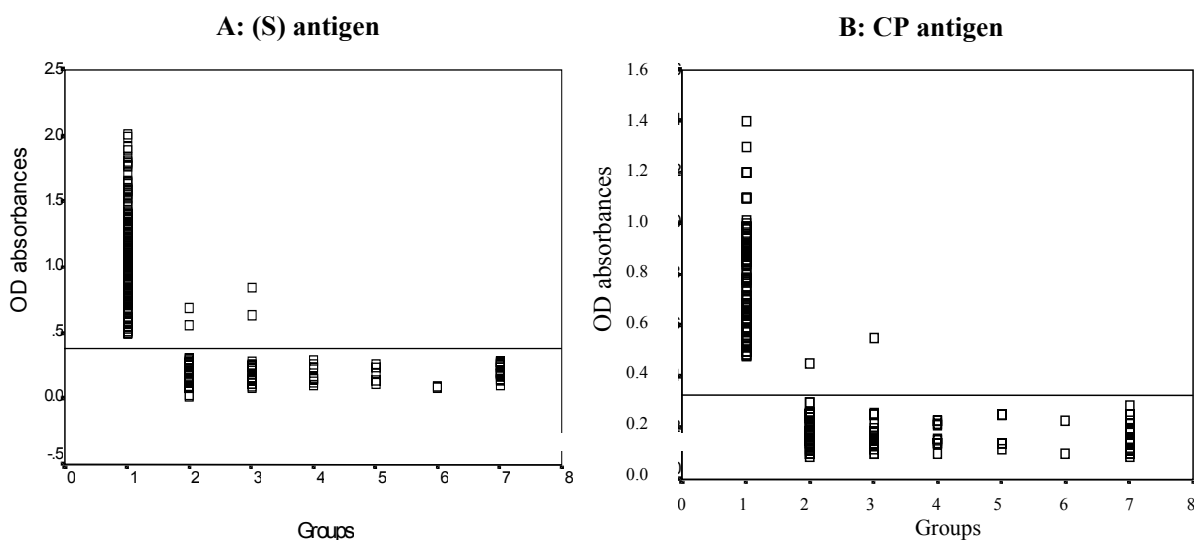
The K-means cluster analysis using the combined data obtained for (S) and CP in the total IgG-ELISA separated the population into seronegative (indicated by closed circles) and seropositive (indicated by open circles) subpopulations. The vertical and horizontal lines in the Figure indicate the calculated cut-off points for each antigen in the total IgG-ELISA.

The mean absorbance readings and standard deviation from the ELISAs for each group that were infected with diseases other than fasciolosis was determined and shown to be not significantly different from those of the negative control sera (Table 1). Moreover, the absorbance readings from all samples from the *Fasciola* seropositive individuals were significantly higher than those obtained from patients that were seronegative or were infected with other parasites ($P < 0.001$) (Fig. 3). However two individuals with hydatidosis and two with toxocariasis had antibodies against *F.gigantica* (S) antigen whereas concerning CP antigen, one individual with Hydatidosis and another with toxocariasis showed cross reactivity against it (Fig. 3, lanes 2,3). Consequently the specificity of (S) and CP antigens were calculated as 96.4% and 98.1%, respectively. The positive and negative predictive values of the test regarding (S) antigen were 97.8% and 100% respectively, while as for CP antigen were 98.9% and 100% correspondingly.

Table 1. Absorbance values of various sera in an indirect ELISA test using somatic (S) and cysteine proteinase (CP) antigens of *F.gigantica*

Group	No. of Individuals	S		CP	
		Mean	Std. Deviation	Mean	Std. Deviation
Fasciolosis	178	1.10	0.33	0.76	0.18
Hydatidosis	45	0.20	0.12	0.20	0.07
Toxocariasis	20	0.24	0.19	0.19	0.09
Amoebiasis	10	0.19	0.06	0.17	0.05
Malaria	5	0.19	0.06	0.18	0.06
Kala-azar	2	0.09	0.01	0.16	0.09
Control	25	0.21	0.05	0.16	0.05

Fig. 3. Analysis of sera from patients with various single infections by IgG-ELISA using *Fasciola gigantica* somatic (S) and cysteine proteinase (CP) as antigens



Serum samples obtained from patients with fasciolosis (178, lanes 1), hydatidosis (45, lanes 2), toxocariasis (20, lanes 3), amoebiasis (10, lanes 4), malaria (5, lanes 5), kala-azar (2, lanes 6) and control human sera (25, lanes 7).

DISCUSSION

Stool examination for *Fasciola* eggs is a reliable method, but the sensitivity is very low and only 25% of infected patients pass the eggs in the faeces (8). The infected individuals during prepatent period failed to release the eggs in the stool as well, so Immunodiagnosis methods are more applicable for this purpose (8,17). Recently more emphasis is on the antigenic product of cysteine proteinase from *Fasciola hepatica* (17,18) but as far as we know there is no report of using *F.gigantica* cysteine proteinase as antigen in ELISA test to diagnose human fasciolosis. The purification of a cysteine proteinase from *F.gigantica* adult worms has been reported (6). Cysteine proteinase is immunogenic at all stages of liver fluke development in the definitive host and can be used to diagnose both the acute and chronic stages of infections.

In an attempt to compare the somatic (S) and CP antigens of *F.gigantica* in IgG-ELISA the present study was conducted. As is shown in figure 3, antibodies in the sera of four patients infected with hydatidosis and toxocariasis were reactive with *F.gigantica* (S) antigen, whereas the number of cross reaction cases decreased to two sera infected with CP antigen. This finding suggests that using of more purified antigen than (S) improves the output. There is possibility that an antigen other than the protease in the *F.gigantica* (S) and CP shares epitope(s) with a hydatid or toxocara immunogen. Besides, subclinical *Fasciola* infection may be involved. In some previous studies the sensitivity and specificity of *F.gigantica* somatic antigen has been reported as 100% and 98% (12), 100% and 82.5% (22) respectively. Using immunoblotting technique one antigenic component of *Fasciola gigantica* somatic extract, i.e. 38 kDa M_r , was found to give a consistent reaction with sera of patients with fasciolosis (100% sensitivity and 96.7% specificity) (11).

The present report is the first to demonstrate the use of *F.gigantica* somatic (S) and CP antigens for the diagnosis of fasciolosis in Iran. To date there is no data which indicates species of *Fasciola* is responsible for fasciolosis in Iran (15). It is demonstrated that using of *F.gigantica* purified antigens could be used as antigen in diagnose of infections with *Fasciola hepatica* (20). This study shows a higher specificity of CP than (S) antigen. We have demonstrated that altogether CP antigen provides a more conclusive diagnosis as possessing lower cut-off and also enabling better to discriminate between seronegative and seropositive subpopulations.

Based on coprological tests acute and chronic fasciolosis have the same diagnostic dilemma for the physician since in acute phase there is no eggs in stool and in chronic phase the sensitivity is merely 25%. So it is essential to establish a reliable test like ELISA aiming at diagnosis and seroepidemiology of the disease. We hope this study may be functional to implement control measures against the spread of the infection.

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REFERENCES

1. Bahonar A and Poya B (2000): A descriptive epidemiology of patients infected with fasciolosis in Rasht and Bandar Anzali in 1999. 3rd National Congress of Medical Parasitology, Sari, Iran, April, PP:9-11.
2. Bradford MM (1976): A rapid and sensitive method for the quantitation of microgram quantitative protein utilizing the principle of protein-dye binding. *Anal Biochem*, **72**: 248-54.
3. Cordova M, Reategui L and Espinoza R(1999): Immunodiagnosis of human fasciolosis with *Fasciola hepatica* cysteine proteinase. *Tran Roy Soc Med Par Hyg*, **93**:54-7.
4. Dalton JP and Heffernan M (1989): Thiol protease released in vitro by *Fasciola hepatica* .*Mol Biochem Parasitol*, **35**:161-6.
5. Delkhosh J, Noorsalehi and Sarshad (1997): Report of human fasciolosis in Gilan Province (1984-1995). 2nd National Congress of Parasitic Diseases, Tehran, Iran. October 17.
6. Fagbemi BO and Hillyer GV (1992): The purification and characterization of a cysteine proteinase of *F.gigantica* adult worms. *Vet Parasitol*, **43**:223-32.
7. Galen RS (1980): Predictive values and efficiency of laboratory testing. *Pediatr. Clin North Am*, **27**: 661-89.
8. Hillyer GV (1998): Immunodiagnosis of human and animal fasciolosis. In: Fasciolosis. Ed, JP Dalton. CABI Publishing, Oxon, Wallingford, UK, PP: 435-48.
9. Katz N, Chaves A and Pellegrin J (1972): A simple device for quantitative stool thick-smear technique in schistosomiasis mansoni. *Revista. Instit. Med Trop De Sao Paulo*, **14**: 397-402.
10. Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680-85.
11. Maleewong W, Intapan PM, Tomanakarn B and Wongkham C (1997): Antigenic components of somatic extract from adult *Fasciola gigantica* recognized by infected human sera. *Asian Pac J Allergy Immunol*, **15**(4): 213-8.
12. Maleewong W, Intapan PM, Wongkham C and Tomanakan K (1996): Comparison of adult somatic and ES antigen in ELISA for the serodiagnosis of human infection with *F.gigantica*. Southeast. *Asian J Trop Med Public Health*, Sep, **27**: 566-9.
13. Markell EK and Voge M (1999): Medical Parasitology, 8th edition. Saunders Company Publication. PP:185-8.
14. Mas-Coma S, Bargues MD and Esteban JG (1998): Human fasciolosis. In: Fasciolosis. Ed, JP Dalton. CABI Publishing, Oxon, Wallingford, UK, PP: 411-34.
15. Massoud J(1989): Fasciolosis outbreak in man & drug test (Triclabendazole) in Caspian littoral, northern part of Iran. *Bull Soc Fran Parasitol*, PP: 8-438.
16. Massoud J (1997). The importance of helminthic diseases in Iran. 2nd national congress of parasitic diseases in Iran. Tehran, Iran October 19-22. P. 74.
17. O'Neill SM, Parkinson M, Dowd AJ, Strauss W, Angles R and Dalton JP (1999): Immunodiagnosis of human fasciolosis using recombinant *Fasciola hepatica* cathepsin L1 cysteine proteinase. *Am J Trop Med Hyg*, **60**(5): 749-51.
18. O'Neill SM, Parkinson M, Strauss W, Angles R and Dalton JP (1998): Immunodiagnosis of *Fasciola hepatica* infection (fasciolosis) in a human population in the Bolivian Altiplano using purified cathepsin L cysteine proteinase. *Am J Trop Med Hyg*, **58**(4): 417-23.
19. Smith AM, Dowd A, McGonigle SM, Keegan P, Brennan G, Trudget A and Dalton JP (1993): Purification of a cathepsin L-like proteinase secreted by

- adult *Fasciola hepatica*. *Mol Biochem Parasitol*, **62**:1-8.
20. Weiyi H, Zhang W and Tian E (1997): Analysis of *F. gigantica* ES antigens. *J Guax Agricultural University*, PP:115-24.
21. World health organization (1995): Control of food born trematode infection. Report of a WHO study group, Geneva.
22. Youssef FG and Mansour NS (1991): A purified *Fasciola gigantica* worm antigen for the serodiagnosis of human fasciolosis. *Trans Roy Soc Trop Med Hyg*, **85**:535-7.

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