

PURIFICATION AND EVALUATION OF SOMATIC, EXCRETORY-SECRETORY AND CYSTEINE PROTEINASE ANTIGENS OF FASCIOLA HEPATICA USING IgG-ELISA IN DIAGNOSING FASCIOLIASIS

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Abstract - Fasciolosis, or liver fluke disease, caused by parasites of the genus *Fasciola* is emerging as an important disease in man and animals, in the world and Iran, particularly in northern parts. The economical losses in domestic animals are considerable. In the recent decade there were two major outbreaks of human fasciolosis in the Caspian region, northern part of Iran with 7000-10000 infected cases. Since it is impossible to diagnose fasciolosis in acute phase using coprological methods and even in chronic phases its sensitivity is low, evaluating and establishing a reliable and cost-effective test is indispensable and noteworthy.

In the present survey, we produced and examined the sensitivity and specificity of liver fluke homogenate (LFH), excretory-secretory (ES) and cysteine proteinase (CP) antigens of *F. hepatica* using IgG-ELISA test. A 25-27 kilo Dalton coomassie blue-stained band was observed and using of specific inhibitors indicated that this antigen belongs to the class of cysteine proteinase. The sensitivity of LFH, ES and CP antigen in IgG-ELISA was 100% for each, while their specificity was 97.8%, 98.8% and 98.8% respectively. There was a significant difference in mean OD values between cases of proven fasciolosis and other true negative cases, including healthy control individuals and patients with other parasitic diseases.

This present report is the first to demonstrate the purification and evaluation of *F. hepatica* cysteine proteinase antigen by IgG-ELISA test for the diagnosis of fasciolosis in Iran. In conclusion, the IgG-ELISA using ES and CP show high sensitivity and specificity and would be a valuable tool to diagnose human fasciolosis in Iran, particularly in endemic areas.

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INTRODUCTION

Fasciolosis is a disease caused by liver flukes of the genus *fasciola*. Although fasciolosis is a common disease

associated with domestic livestock it is now recognized by the World Health Organization (WHO) as a serious public health problem in man (1). Humans are infected by ingestion of aquatic plants that are contaminated with encysted cercariae (metacercariae) shed from the intermediate snail host. After excysting the metacercariae in the duodenum and forming juvenile flukes, they migrate across the liver parenchyma causing the extensive hemorrhage and liver damage. Then they reach the bile ducts after 8-12 weeks and mature sexually, afterwards commence egg production. This is the latent period of infection and individuals can be asymptomatic for several months to several years (2). The chronic stage of infection proceeds when the parasite causes damage to the bile ducts and surrounding tissue. Eggs are passed into the intestine with the bile juices and reach the exterior following defecation. The eggs then hatch to release miracidia that infect snail host thus completing the life cycle (2).

Human fasciolosis is becoming increasingly recognized as a serious public health problem. A WHO review report estimate that as many as 17 million people are infected with liver flukes worldwide (1) and in countries such as Bolivia, Peru and Egypt the disease is thought to be hyper-endemic (3). Fasciolosis is now emerging as an important chronic disease of humans in the Gilan province of northern Iran (1). Over the past twelve years, local health clinics in this province diagnosed many cases of the disease annually (range 68-223) but in 1989 and 1999 the number of cases rose to over 7,000 and 10,000, respectively (4,5,6,7). It is imperative that studies are carried out to determine the extent of the disease throughout the Gilan and neighboring provinces as well as to diagnose infected people. Therefore the use of a simple but highly specific and sensitive standardized diagnostic test is highly essential. Coprological analysis is still commonly employed to diagnose human fasciolosis 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 (3). 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).

Some attempts have been made to diagnose human fasciolosis by detecting antibodies in the serum of patients by ELISA method. In these reports the sensitivity and specificity of test ranged between %89 - %100 and 56.2%-100% respectively (9-16). The purpose of the present study was to isolate the cysteine proteinase antigen from the crude ES products and its evaluation by ELISA method comparing adult somatic and ES antigens.

MATERIALS AND METHODS

Preparation of antigens

Liver fluke homogenate antigen (LFH) was prepared by homogenizing adult worms in 0.15 M PBS using hand homogenizator followed by sonication, then centrifugation at 15000 x g at 4° C for 30 minutes, afterwards the supernatant (LFH) was stored at -20° C for later analysis.

With the aim of obtaining ES and CP antigens, live *F. hepatica* adult worms were obtained from infected sheep livers and washed 6 times in PBS 0.15M, Ph7.3, then maintained in RPMI-1640, Ph 7.3 culture medium, containing 25 mg/L Gentamicin overnight. The culture medium, i.e., excretory - secretory products (ES), were concentrated and applied to a 320 ml Sephaeryl S200 HR gel-filtration column, equilibrated in 0.1 M Tris-Hel, ph 7.2. Three milliliters fractions were collected and monitored by absorbance at 280 nm for protein content. Each fraction was also assayed for cysteine proteinase activity using the fluorogenic substrate Z-Phe- Arg-AMC. The release of the fluorescent leaving group, 7-amino-4-methylcomarin (AMC) was monitored in a Perkin-Elmer Luminescence Spectrometer model LS 50, at exciter and analyzer wavelengths of 370 nm and 440 nm, respectively (17). Fractions containing proteolytic activity again were tested using Gelatin-SDS-PAGE (18) for more accuracy and were pooled, after that used as the source of cysteine proteinase (CP) antigen. Semi purified CP were analyzed by 15% SDS-PAGE (19).

The concentration of each antigen preparation was measured using Bradford method (20).

Clinical samples

Serum samples from fasciola-infected individuals were collected during the fasciolosis outbreak in 1999 in the Gilan province of Northern Iran.

Totally 178 patients diagnosed with fasciolosis, based on coprological analysis for *F. hepatica* eggs, performed on faecal samples as previously described (21); clinical presentation; Indirect Fluorescent Antibody test (IFA) using frozen sections of somatic antigen of *F. hepatica* performed in the laboratory of helminthological serology, School of Public Health, Tehran University of Medical Sciences. This test (IFA) gives a lightly inferior percentage of positivity to that obtained by ELISA (22); furthermore all the sera later were examined by JgG-ELISA using cathepsin L1 (CL1) antigen in JPD lab, School of Biotechnology, Dublin City University, Dublin, Ireland and all of them showed seropositive results. The Sensitivity and specificity of this test are reported as 100% for each (11,12).

The age of the patients ranged from 3-70 years, with a mean \pm standard deviation of 33.51 ± 16.95 years. The number of females participating in the study was 62.5%. Serum samples from patients infected with hydatidosis (45 individuals), toxocarasis (20 individuals), amoebiasis (10 individuals), malaria (5 individuals), and kalaazar (2 individuals) were obtained from the School of Public Health, Tehran University of Medical Sciences using a collection of methods (23) and preparing from different laboratories. Control serum samples were obtained from 50 healthy individuals, which were examined for all related parasitic diseases.

Inhibitor studies

In order to determine the class of protease enzyme some inhibitors were used and were added to the enzyme before assaying the proteolytic activity with substrate Z-Phe-Arg-AMC (17). These inhibitors were used at final concentration of phenylmethylsulfonyl fluoride (PMSF) 10mM; EDTA 10 mM; E-64 5 μ g/ml; leupeptin, 5 μ g/ml and pepstatin, 1 μ M (18).

Enzyme-linked immunosorbent assay (ELISA)

The Immunodiagnosis assay was performed as previously described by O'Neill et al(12). Briefly, 100 micro liters of LFH, ES or CP antigen (20, 15 and 10 μ g/ml respectively) was dispensed into the wells of micro titer plates (Nuclon, Kamstrup, Roskilde, Denmark) and then incubated overnight at 37° 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°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°C. Following another washing step, 100 μ l of peroxidase-conjugated goat anti-human IgG (diluted 1:7000) was added to

each well and the plates incubated for a further 60 minutes at 37°C. Following a final washing step 100 µl of O-phenylenediamine 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 µl of 12.5% H₂SO₄. The optical density (OD) of the samples was measured at 492 nm using a Titertec (Helsinki, Finland) multiscan ELISA plate reader. All assays were tested in triplicate and repeated twice. The experiments were conducted in Dept. of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences and JPD Lab, School of Biotechnology, DCU, Dublin, Ireland

Statistical analysis

All statistical analysis was carried out using SPSS for windows version 10. The parameters of sensitivity, specificity and predictive values were determined according to the method of Galen (24) as follows: SE = TP/(TP + FN) × 100; SP = TN/(TN + FP) × 100; Positive Predictive Value = TP / (TP + FP) × 100 and Negative Predictive Value = TN / (TN + FN) × 100, where "TN" stands for true negative (number of control samples, i.e., other parasitosis and healthy controls) that had an extinction value of the sera, at 1:1250 dilution in ELISA test lower or higher than the cut-off value respectively; "FN" for false negative (number of proven fasciolosis samples that were negative by ELISA and "FP" for false positive (number of control samples, positive by ELISA).

The OD absorbance at 492 nm cut-off values for the IgG-ELISA with each antigen was determined as the mean OD 492 nm of the total number of serum samples from healthy people + 3.09 standard deviation.

RESULTS

Quality of cysteine proteinase (CP) antigen

The quality of the CP was checked using 15% SDS-PAGE and Gelatin SDS-PAGE. A 25-27 kilo

Dalton coomasie blue-stained band was observed and used as CP antigen in IgG-ELISA. Its proteolytic activity was completely decreased using E-64 (specific inhibitor of cysteine proteinase), whereas other inhibitors, EDTA, pepstatin and PMSF (inhibitors of metalloproteinase, aspartic protease and serine protease in that order) showed no effect or little inhibitory effect on it. These findings overall indicated that this antigen belongs to the class of cysteine proteinase.

Sensitivity, specificity and predictive values of ELISA test

The optimal concentration of LFH, ES and CP antigens for IgG-ELISA was 20,15 and 10 µg/ml in that order, at a serum dilution of 1:1250 and peroxidase-conjugated goat anti-human IgG conjugate dilution of 1:7000.

There were no significant differences in absorbance readings between genders and no age group within the sample population demonstrated a tendency to have a higher incidence of infection.

The OD absorbances at 492 nm for the 178 patients infected with fasciolosis and those of other parasitic infections as well as healthy controls are summarized in table 1. The cut-off value calculated as described earlier was 0.38, 0.28 and 0.27 for LFH, ES and CP correspondingly. Absorbance readings greater than the cut-off value were considered to be seropositive for fasciolosis. Accordingly all 178 individuals that showed clinical manifestations of fasciolosis were also seropositive using three antigens (Figure 1, lane 1) whereas all 50 non-infected controls were seronegative (Fig 1, lane 7). Consequently, the sensitivity of the test was 100% for all three antigens (table 2). The mean absorbance and standard deviation for each group of individuals that were infected with diseases other than fasciolosis was verified and shown to be not significantly different from those for negative control sera (table 1).

Table 1. Absorbance values of various sera in an indirect ELISA test using homogenate (LFH), excretory / secretory (ES) and cysteine proteinase (CP) antigens of *F. hepatica*

| Group | No. of Individuals | LFH STd. | | ES | | CP | |
|--------------|--------------------|----------|-----------|-------|----------------|-------|----------------|
| | | Mean | Deviation | Mean | Std. Deviation | Mean | Std. Deviation |
| Fasciolosis | 178 | 1.364 | 0.30 | 1.158 | 0.23 | 0.944 | 0.250 |
| Hydatidosis | 45 | 0.193 | 0.16 | 0.123 | 0.09 | 0.085 | 0.079 |
| Toxocariasis | 20 | 0.253 | 0.19 | 0.161 | 0.13 | 0.118 | 0.143 |
| Amoebiasis | 10 | 0.157 | 0.09 | 0.094 | 0.04 | 0.045 | 0.01 |
| Malaria | 5 | 0.142 | 0.049 | 0.114 | 0.04 | 0.100 | 0.06 |
| Kalaazar | 2 | 0.09 | 0.03 | 0.062 | 0.01 | 0.029 | 0.006 |
| Control | 50 | 0.155 | 0.07 | 0.129 | 0.05 | 0.122 | 0.05 |

Table 2. Sensitivity, specificity and predictive values of liver fluke homogenate (LFH), excretory-secretory (ES) and cysteine proteinase (CP) antigens of *F. hepatica* in IgG-ELISA test

| Antigen | Sensitivity | Specificity | Positive predictive value | Negative predictive value |
|---------|-------------|-------------|---------------------------|---------------------------|
| LFH | 100% | 97% | 97.8% | 100% |
| ES | 100% | 98.5% | 98.8% | 100% |
| CP | 100% | 98.5% | 98.8% | 100% |

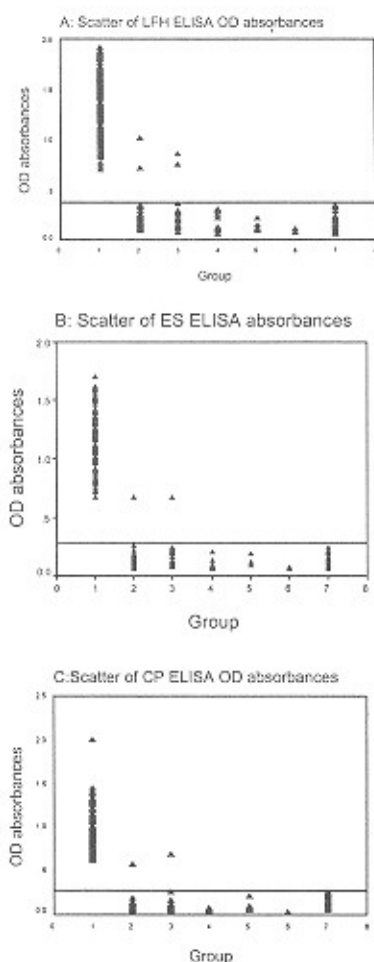


Fig. 1. Analysis of sera from patients with various single infections by IgG-ELISA using *F. hepatica* liver fluke homogenate (LFH), excretory-secretory (ES) and cysteine proteinase (CP) as antigens. Serum samples obtained from patients with fasciolosis (178 cases, lane 1), hydatidosis (45, lane 2), toxocariasis (20, lane 3), amoebiasis (10, lane 4), malaria (5, lane 5), kalaazar (2, lane 6) and control human sera (50, lane 7)

Furthermore, the absorbance readings from the *F. hepatica* seropositive individuals were significantly

higher than those obtained from patients that were seronegative or were infected with other parasites ($P < 0.001$) (Fig 1). However two individuals with hydatidosis and two with toxocariasis had antibodies that were reactive against *F. hepatica* LFH antigen whereas concerning ES and CP antigens each, one individual with hydatidosis and another with toxocariasis showed cross reactivity against them (Fig 1, lanes 2,3).

The diagnostic sensitivity, specificity and predictive values are contained in table 2.

DISCUSSION

This present report is the first in Iran to demonstrate the purification and evaluation of *F. hepatica* cysteine proteinase antigen by IgG-ELISA test for the diagnosis of fasciolosis in Iran.

Stool examination for *F. hepatica* 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 (8,11,25). Recently more emphasis is on the antigenic product of cysteine proteinase especially cathepsin L1 (12,17). Cysteine proteinase is a major component of the ES products and because it is immunogenic at all stages of liver fluke development in the definitive host it can be used to diagnose both the acute and chronic stages of infection.

In an attempt to compare the LFH, ES and CP antigens of *F. hepatica* in IgG-ELISA the present study was conducted. As is shown in figure 1, antibodies in the sera of four patients infected with hydatidosis and toxocariasis were reactive with *F. hepatica* LFH antigen, whereas the number of cross reaction cases decreased to two sera infected with those diseases. This finding suggests that using more purified antigen than LFH improves the output. There is a possibility that an antigen other than the protease in the *F. hepatica* ES and CP shares epitope(s) with a hydatid or toxocara immunogen. Cordova and co-workers (14) reported that IgG-ELISA using LFH antigen with human sera had a sensitivity and specificity of 100% and 56.2% respectively. O'Neill et al (12) concluded that the ELISA using LFH did not adequately discriminate between seronegative and seropositive individuals within the population, besides she didn't find any cross-reactivity of *F. hepatica* ES with sera from 15 patients with hydatidosis in the Bolivian Altiplano. Asmar and co-workers (9) used ES antigen in ELISA test to determine the prevalence of fasciolosis in Gilan province in Iran. In previous studies the sensitivity and specificity of ES has been reported between %89 -

%100 and %56.2 -%100 respectively (9-16).

Yamasaki and co-workers (13) have reported a sensitivity and specificity of 100% and 98% as for ELISA test using semi-purified cysteine proteinase antigen extracted from adult *F. hepatica*.

Our study shows higher specificity of ES and CP than LFH. However using the semi purified CP as an antigen revealed no advantage in sensitivity and specificity over the ES. We have demonstrated that both ES and CP antigens could be used for the sensitive and specific diagnosis of human fasciolosis using an IgG-ELISA test, but in all CP antigen provides a more conclusive diagnosis as possessing lower cut-off and enabling better to discriminate between seronegative and seropositive subpopulations.

The need for testing in humans is obvious in seroepidemiology for the study of populations; there is also a real need in individual patients either in endemic areas or after visiting such areas, before specific chemotherapy can be prescribed as well as to predict success or failure of this chemotherapy. Besides, in our country it is essential to establish a reliable test like ELISA, since IFA and coprological tests are not sensitive enough (3,8,22). Somatic antigen must be used to run the IFA test, which having a twisted nature of immunogen antigens couldn't be trustworthy enough. We hope this study may be useful to implement control measures against the spread of this infection.

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