IMMUNODIAGNOSIS OF HUMAN FASCIOLIASIS BY ELISA USING EXCRETORY-SECRETORY PRODUCTS AS ANTIGEN

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ABSTRACT

Background: Different immunological methods are used for the serodiagnosis of *Fasciola hepatica* infections, however, they have limited specificity and/or sensitivity.

Objective: To improve the specificity of ELISA using a partially modified method for preparation of excretory-secretory (ES) antigens.

Methods: Studied groups were as follows: 75 patients with parasitological evidence of fascioliasis; 250 samples from normal individuals from non-endemic areas with no history of fascioliasis; 50 patients who were infected with other parasites. ES was prepared by incubation of adult worms in protease inhibitor containing media. ELISA was performed using this antigen. The antigenic components of ES were revealed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Results: The sensitivity and specificity of the test were 100% and 99.7%, respectively.

Conclusion: Our results showed that the ES of adult worms of *Fasciola hepatica* is a suitable antigen for ELISA procedure.

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Key Words • Fascioliasis • ELISA • immunodiagnosis

Introduction

The trematode *Fasciola hepatica* is the causative agent of liver fluke disease. Primarily a disease of ruminants, it has a worldwide distribution and results in major economic losses in agricultural communities.[1] It is now emerging also as an important chronic disease in humans.[2-5] Research into human fascioliasis has not been extensive because it was previously believed to be restricted to isolated outbreaks, such as those reported from Iran, Peru, Cuba and Bolivia.[5-9] However, reports of infection are steadily increasing in many countries.[4,9,10] High prevalence of human fascioliasis has been reported in Iran.[6] The clinical manifestations of fascioliasis in humans include fever, right hypochondrial pain, persistent diarrhea, and vomiting.[3] Diagnosis of the disease is based on the identification of *F. hepatica* eggs in fecal samples. Since eggs appear in the feces only after the parasite has entered the bile duct and matured (approximately 12 weeks after infection), early infection can not be diagnosed coprologically.[9,11] In addition, because eggs are released sporadically from the bile ducts, an accurate diagnosis by this method requires at least two stool samples obtained at different times on consecutive days.[7,11] Immunological

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methods such as immunoelectrophoresis, hemagglutination, and counterimmuno-electrophoresis are widely used for the serodiagnosis of *F. hepatica* infections in humans and animals.[5,8,12-14] Although most of these methods are very specific, they have limited sensitivity. The detection of anti-fluke antibodies in serum by ELISA has improved the diagnosis and can be used as an adjunct to fecal analysis for the diagnosis of latent and chronic infection.[7] Previously developed ELISA methods have used crude somatic extracts, partially purified somatic antigens, excretory-secretory products (ESP) and purified or recombinant molecules such as cathepsin L.[7,11-15] In the present study, we used a partially modified method in the preparation of excretory-secretory proteins to improve the specificity of ELISA and detection of human serum antibodies and analyzed these proteins by SDS-PAGE technique.

Materials and Methods

Human sera:

Samples were obtained from 75 patients with fascioliasis. All patients had clinical signs and their stools were positive for *F.hepatica* eggs. In addition, sera were obtained from 10 patients with toxoplasmosis, 5 with amebiasis, 5 with malaria, 5 with leishmaniasis, 10 with ascariasis, 5 with hydatidosis, 5 with trichuriasis, 5 with taeniasis, and 5 with autoimmune diseases. Five hemolysed sera were also included. Two hundred and fifty sera were obtained from individuals living where the worm is not known to exist and who had no known history of exposure.

Antigens:

Adult flukes were removed from the bile ducts of naturally infected bovine livers and treated by the method ascribed by Rivera Marrero et al.[16] with some modifications. Briefly, the flukes were repeatedly washed in sterile phosphate buffer saline (PBS) (0.05 M) at room temperature and then incubated individually at 37°C in universal tubes containing 5 ml of culture medium (RPMI 1640 + 25 mM HEPES buffer + 7.5% sodium bicarbonate +100 unit/ml penicillin and 100 μg/ml streptomycin) containing 0.8 mM phenyl methyl phenyl methyl sulfonyl fluoride (PMSF) under sterile condition. After 10 hr the medium of individual tubes was pooled and centrifuged at 15,000 g for 10 min at 4 °C to remove parasite eggs. The supernatants were collected by aspiration and concentrated using an Amicon ultrafiltration unit with a UM 10 membrane. The protein content was measured by method of Lowry and adjusted at 2 mg/ml.[16] Antigens were stored at -70°C until used.

Enzyme-linked immunosorbent assay (ELISA):

ELISA for detection of antibodies to *F. hepatica* ES was performed according to the protocol of Espino et al.[17] Briefly, 96 well microtiter Nunc (Roskilde, Denmark) plates were coated with 100 μl/well of 7.5 μg/ml antigenic solution in 0.1 M carbonate/bicarbonate buffer (pH 9.6) by incubating for 2 hr at 37°C. The plates were then washed twice with PBS containing 0.05% (V/V) Tween 20 (PBS- T); the remaining binding sites were blocked with 0.1% gelatin in PBS-T for 1 hr at 37°C. The plates were either used immediately or stored at 4°C. Human sera (diluted 1:100 in PBS-T) were dispensed into each well and the plate was incubated for 1 hr at 37°C. Then the plates were washed three times with PBS-T and peroxidase-conjugated goat anti-human IgG (Dako) (dilution 1:2500 in PBS-T) was added to each well. The plates were incubated for 1 hr at 37°C. After another washing step, bound antibodies were detected by addition of tetramethylbenzidine (TMB) in phosphate citrate buffer. After 20 min the plates were read at 450 nm on ELISA reader (Titertek, Helsinki, Finland).

Optimization:

In order to reduce non-specific reactions,

Table 1: Mean ± SD of absorbance for different groups. Serum dilution:1:100, antigen concentration: 7.5 μg/ml, conjugate dilution: 1:2500

Groups	Mean±SD	Range
Healthy individuals	0.187 ± 0.064	0.073-0.205
Patients with fascioliasis	1.83 ± 0.41	0.496-2.89
Patients with other diseases	0.197 ± 0.046	0.089-0.220

the optimal conjugate dilution was determined by titration. The conjugate was diluted from 1:500 to 1:3000 and each dilution was tested against different dilutions of pooled negative and pooled positive serum samples. Results were plotted using signal to noise (S/N) ratio against conjugate dilutions. The S/N ratio is the ratio of observed OD reading of positive serum to the observed OD reading of negative serum at the same serum dilution.[18]

Antigen was titrated, using a checkerboard titration in which each row of wells from A to H was coated with different concentrations of antigen from 1.2 μg/ml to 7.5 μg/ml of protein. Known pooled positive and negative serum samples were added in different dilutions from 1:100 to 1:500 to the coated wells, in such a way that only one combination of antigen and positive or negative serum dilution existed.

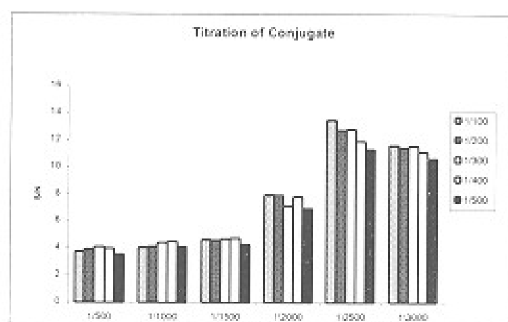

Figure 1: Signal to noise (S/N) ratio to determine optimal conjugate : Serum dilution. Conjugate was diluted from 1:500 to 1:3000 and serum was diluted from 1:100 to 1:500. Ag concentration was 7.5 μg /ml.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE):

Components of the ESP were resolved under reducing conditions on a 12% homogeneous polyacrylamide gel prepared according to the method of Laemmli.[19]

Samples containing protein were boiled for 5 min in sample buffer (10% glycerol, 5% 2-mercaptoethanol, 2% SDS, 0.0625 M Tris-HCl, pH 6.8, 0.001% bromphenol blue) before loading onto the gel. After termination of electrophoresis, which was carried out over a period of 5 hr at 100 volts for stacking gel and 200 volts for running gel, the gels were stained with Coomassie blue G250.

Statistical analysis:

Each assay was done in duplicate and the results were expressed as mean absorbance value for each determination. We used the mean plus three standard deviation (mean + 3 SD) OD value of the healthy group sera as the lower limit of the positivity. All data were analyzed using student's t-test.

Results

In order to find the optimum dilution of conjugate, different dilutions were tested from 1:500 to 1:3000. The highest S/N ratio value

was obtained at 1:2500 of conjugate dilution (Fig.1).

Antigen was titrated, using checkerboard titration. Known pooled positive and negative serum samples were added in different dilutions from 1:100 to 1:500 to the coated wells. The results showed that antigen concentrations of 1.25, 2.5, and 5 μg/ml were inadequate to saturate the wells. The best results were obtained when the plate was coated with 7.5 μg/ml of antigen (Fig. 2), and the optimum serum dilution was 1:100. These dilutions were used to evaluate all serum samples.

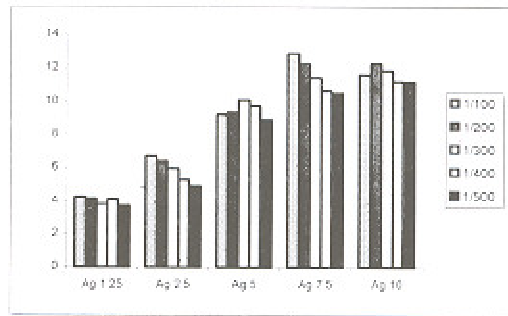

Figure 2: Checkerboard titration of antigen (1.25-10 μg/ml). Conjugate dilution : 1:2500, Serum dillution 1:100 - 1:500, S/N : Signal to noise

Serum samples of different groups were analyzed by ELISA. The mean ± SD absorbance of control group was (0.187 ± 0.064). Sera from patients with fascioliasis showed a mean value of (1.83 ± 0.41), whereas the mean absorbance value in sera from the patients with other parasitic infections, autoimmune diseases, and hemolysed sera was 0.197 ± 0.046 (Table 1). Mean ELISA values showed a significant difference between fascioliasis and other groups ($p < 0.001$). The mean plus three standard deviation of absorbance value of the healthy group sera (0.250) was considered as cut-off absorbance (Fig. 3). The specificity and sensitivity of ELISA test were 99.7% and

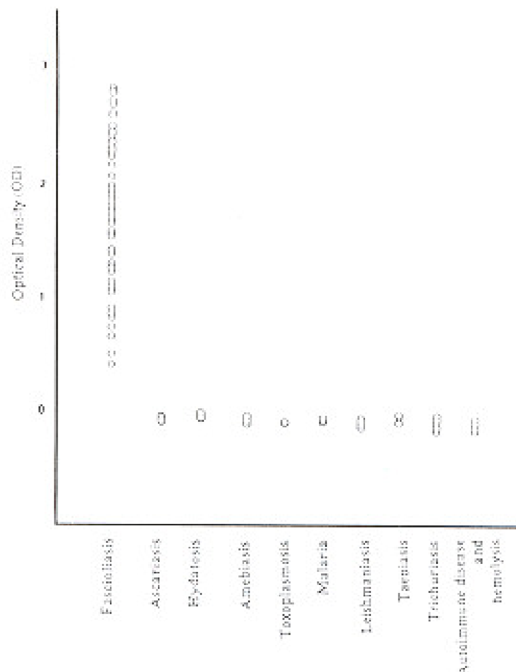

Figure 3: Absorbance value of serum from different groups. Cut-off OD is the mean + 3SD of OD value of the healthy group sera.

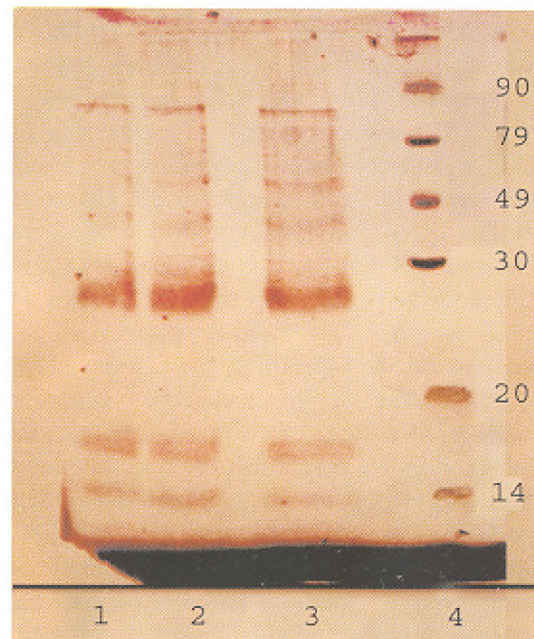

Figure 4: Lane 1-3 Excretory-Secretory products of *F. hepatica*. Lane 4 molecular weight marker. Bands of 17-19 KD and 27 KD are more prominant.

100%, respectively .

We performed SDS-PAGE to identify the protein contents of ESP. After staining, at least 9 major bands with molecular weights ranging from 12 to 90 kD were detected. Two of these bands with molecular weights of 17-19 kD and 27 kD were more prominent (Fig. 4).

Discussion

For immunodiagnostic purpose, the ESP of adult worms of *F.hepatica* showed excellent results in our immunoenzymatic assay. We studied a group of patients with parasitological evidence of fascioliasis as well as groups of patients with other parasitic diseases, and a healthy control group with no parasitic and systemic diseases. Our data showed 100% sensitivity and 99.7% specificity. Ascariasis, hydatidosis, amebiasis, toxoplasmosis, malaria, leishmaniasis, taeniasis, and trichuriasis are common parasitic infections in Iran where a high prevalence of human fascioliasis has been reported.[6] Serum of patients infected with these parasites showed no cross-reactivity with ESP antigen in our assay. Different methods of preparation of ESP, and various assay conditions may affect the sensitivity and specificity of the test. Daveau et al. reported cross-reactivity with filariasis and opisthorchis infection by using ESP collected after one week incubation of adult parasites.[20] Whereas Espino et al. reported more specificity by using ESP collected after 24 hr incubation.[12] Different methods of antigen preparation and various assay conditions may affect the sensitivity and specificity of the test. It has been shown that specificity of crude antigens is low[20] and purified antigens identify fewer individuals as seropositive.[11] Although partially purified ES antigens improve sensitivity and specificity, the procedure of antigen preparation is time-consuming and not economical. High sensitivity and specificity of our test could be due to incubation of adult parasites for 10 hr and treatment with protease inhibitor. Excretory-secretory products contain cystein protease, which may degrade proteins into cross-reactive components.[7,11,21] Furthermore, ES obtained after longer incubation may contain other metabolic molecules that may be responsible for the cross-reactions.

The high degree of sensitivity and specificity of our ELISA test shows that this test is reliable, useful, and economical and negates the need for further antigen purification.

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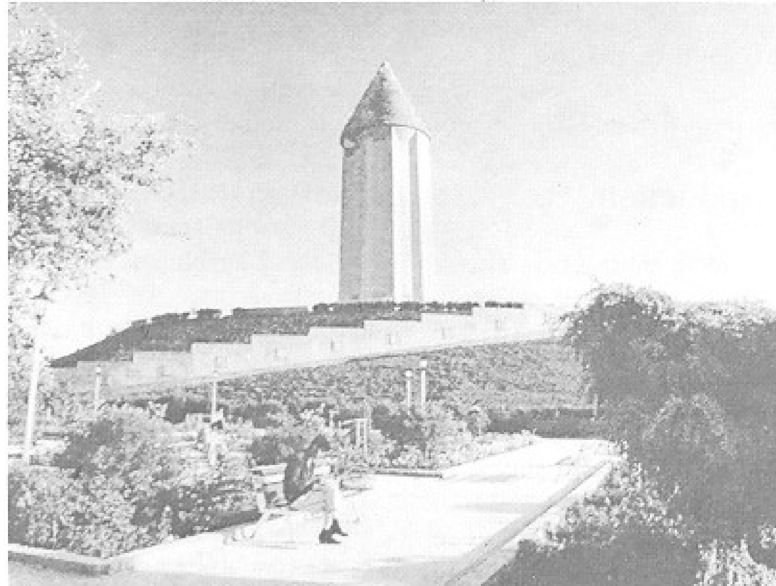

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