

# Enzyme-Linked Immunotransfer Blot Analysis of Somatic and Excretory- Secretory Antigens of *Fasciola hepatica* in Diagnosis of Human Fasciolosis

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## Abstract

The liver fluke *Fasciola hepatica* causes fascioliasis, a liver disease in most part of the world and particularly in north of Iran. Diagnosis of the diseases is anchored in coprological manner but serological methods are preferable due to some obscurities. In this study, sera obtained from human patients infected with *Fasciola hepatica* were tested by the enzyme-linked immunotransfer blot (EITB) technique with the parasite's somatic and excretory-secretory (ES) antigens in order to evaluate the diagnostic potential of the assay. The study included sera from 40 patients infected with *F. hepatica*, 20 infected with hydatidosis, 6 with toxocariasis, 10 with strongyloidiasis, 10 with amoebiasis, 5 with malaria and 30 normal controls. By this assay, most of the serum samples from humans with fascioliasis recognized two antigenic polypeptides of 27 and 29 kDa using both antigens. The sensitivity, specificity, positive and negative predictive values for somatic antigen were 91.0%, 96.2%, 95.2% and 92.7% respectively, while these parameters as for ES antigen were 95.2%, 98.0%, 97.5% and 96.2%, correspondingly. Totally, two cases of reactions for the first antigen and one for the latter were verified. The study suggests that the 27 and 29 kDa bands for two antigens in EITB test could be considered for the immunodiagnosis of human fascioliasis.

**Keywords:** *Fasciola hepatica*, SDS-PAGE, EITB, ES antigen, Somatic antigen

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## Introduction

*Fasciola hepatica*, a trematode parasite of ruminant and human, causes fascioliasis, a liver disease in most part of the world and particularly in the north of Iran. The disease is established by eating contaminated plants with infective metacercaria which are derived from an intermediate molluscan host (1).

Parasitological diagnosis is still commonly employed to diagnose human fasciolosis despite the fact that this method is not wholly reliable (2). 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.

Early diagnosis of this disease is very important in order to treat patients successfully. At present, haemagglutination (HA) (3, 4), indirect fluorescence antibody test (IFAT), immunoperoxidase (IP)(5), counter-electrophoresis (CEP) (6) and enzyme-linked immunosorbent assay (ELISA)(7-9) are being considered in diagnosing of the human fascioliasis. Enzyme-Linked Immunotransfer Blot analysis (EITB) or Western blotting is evaluated in some research centers and encompasses remarkable sensitivity and specificity in diagnoses of the disease (10-12). The purpose of this study was to test the sensitivity and specificity of the EITB assay using somatic (S) and excretory-secretory (ES) antigens of *F.hepatica* to diagnose human fascioliasis.

## Materials and Methods

**Sera** Blood samples were collected from individuals infected with *F.hepatica*, diagnosed based on stool examination and ELISA test. Only 40 individuals that were coprologically positive for merely fasciolosis (28 females, 12 males) were included in the present study. Serum samples obtained from patients infected with hydatidosis (n=20), toxocariasis (n=10), amoebiasis (n=10), strongyloidiasis (n=10) and malaria(n=5) were obtained from the Tehran School of Public Health serum blood bank whom were diagnoses based on the specific assays such as stool exam, ELISA and IFA as well as surgical operation confirmation. Control serum samples were obtained from 30 normal healthy subjects. The human's ethics committee at the School of Public Health, Tehran University of Medical Sciences, approved the study.

**Preparation of antigens** Adult *F.hepatica* was obtained from infected sheep livers collected from local abattoirs. Somatic antigen(S) was prepared by homogenizing adult worms in 0.045 M PBS/pH 7.2 using electrical homogenizator (Edmund Buhler Co., model Homo 4/A mit uhr) followed by sonication (Tommy Seiko model UP-200P, Tokyo), and then centrifugation at 15000g at 4° C for 30 minutes.

Afterwards the supernatant (S antigen) was preserved for later usage.Excretory/secretory products (ES) were prepared as described by Dalton and Heffernan (13). The concentration of each antigen preparation was measured using Bradford method (14).

**EITB** EITB was conducted essentially as previously described (15, 16).

The ES antigen was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 12.5% gel of 0.75 mm thickness at constant voltage of 100 V in the BioRad mini-gel apparatus (Bio-Rad Laboratories, Richmond, CA). The molecular mass of the antigens was estimated by comparing the migration distance of the sample to that of

known molecular mass markers (electrophoresis calibration kit; Pharmacia, Piscataway, NJ). Following electrophoresis the yielded bands were transferred onto nitrocellulose paper (Schleicher & Schuell, Keene, NH) using Akhtarian mini-transfer apparatus (Akhtarian Co., Tehran) run at 45 mA overnight. Following transfer, the nitrocellulose paper was stained with 0.2% Ponceau S stain (Sigma) to identify the lanes which were then cut out. The strips were then destained in distilled water. The strips were blocked with 3% gelatin in Tris buffer (Bio-Rad Laboratories) for 2 h and incubated in 0.05% Tween in Tris-buffered saline (Tris/T) containing primary antibody at a dilution of 1:40 for 2 h at room temperature. The strips were washed, and then incubated in a 1:1000 dilution of secondary antibody (horse-radish peroxidase conjugate rabbit antihuman IgG for 2 h at room temperature. The strips were washed and stained with DAB substrate.

**Statistical analysis** The sensitivity, specificity, and the predictive values were calculated as previously described (17).

## Results

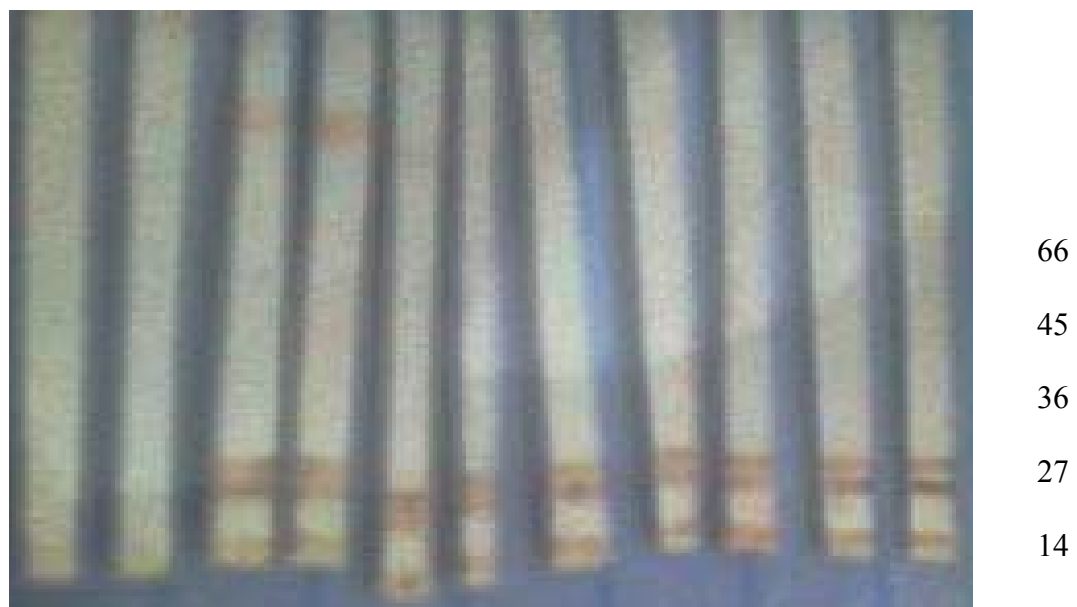
Using somatic and ES antigens, immunoblot analysis demonstrated two major regions at 27 and 29 kDa when human sera from patients with fasciolosis were examined by the test. Other than these two cases, the bands revealed in case of positive sera for *F. hepatica* were 97, 36, 29 and 17 kDa, using somatic antigen, but when the ES antigen was challenged, not mention of recognition the 27 and 29 kDa bands in most of the cases (Fig. 1), in 9 cases a protein band of 14 kDa was recognized as well (Fig. 2), so it was not detected as a specific protein band for *F. hepatica*. These 27 and 29 kDa bands were not detected in any of the 30 negative sera. Table 1 shows the cases of false positive and negative for all sera tested regarding these two protein bands and hence the cases of cross-reactions. More or less, some worthless bands were observed in nitrocellulose

membrane belong to other helminthic infections (data not shown). Based on the obtained observations, the sensitivity, specificity, positive and negative predictive values of 27 and 29

kDa bands for somatic antigen were 91.0%, 96.2%, 95.2% and 92.7% respectively, while these parameters as for ES antigen were 95.2%, 98.0%, 97.5% and 96.2%, correspondingly.



**Fig.1:** Detected bands in the sera of patients positive for *Fasciola hepatica* by Western blotting. Lanes 17 and 18 stand for normal control sera.



**Fig. 2:** Detected bands in the sera of patients positive for *Fasciola hepatica* by Western blotting. Lanes 1 and 2 stand for normal control sera. Protein marker index is represented on the right.

**Table 1:** Frequency of false positive and negative cases of all sera tested by EITB using somatic and ES antigens of *F.hepatica*

Disease	No.	Somatic antigen		ES antigen	
		*FN	*FP	FN	FP
Fascioliasis	40	4	-	2	-
Hydatidosis	10	0	1	-	-
Toxocariasis	10	0	1	-	1
Strongyloidiasis	5	0	-	-	-
Amoebiasis	5	0	-	-	-
Trichostroglyoidiasis	1	0	-	-	-
Normal control	30	0	-	-	-

FN= false negative    FP: false positive

## Discussion

Coprolological examination for diagnosing of fasciolosis is a regular but of a low sensitivity manner (2). Evaluating of immunodiagnostic methods such as ELISA and EITB is in the center of notification by this field researcher. The need to find and establish a new sensitive and specific method and to decrease as much as possible the cases of cross-reactions made us to evaluate the EITB test in this regard. In the present study, using EIT test and somatic as well as ES antigen, two major protein bands of 27 and 29 kDa were detected by most of the sera infected with fasciolosis. The ES antigen showed less cross-reaction cases than somatic one. In similar immunodiagnostic studies on this parasite, it was demonstrated that ES antigen was more specific than other somatic and surface antigens (18, 19). In Western blotting studies using excretory/secretory antigens in humans infected with *F. hepatica*, it was determined that bands 12, 17, 25, 27, 29, and 49.5 kDa were specific for humans (20-22). Intapan (21) accounted the sensitivity, specificity, positive and negative predictive values for a 27 kDa protein band yielded from the ES antigen as 100%, 98%, 66.7% and 100%.

Hammami demonstrated that two bands of 29 and 57 kDa could be recognized by infected sera. He reported the specificity of the test as 100% for both antigens, but a sensitivity of 93% and 79% was stated for 29 and 57 kDa antigens, in that order (11). Somatic antigen has been challenged in this test and Shaker has reported a sensitivity and specificity of 100% to diagnose the disease (23). More than human fasciolosis trials, some studies have been conducted in animals as well. Ortiz et al. (24) used E/S, somatic (SO) and surface (SU) antigens of the adult parasite for antibody response determination in dairy infected cattle. They reported that antibody responses were developed against 60-66 kDa in E/S and SU antigens and 17 kDa in SO antigen. Using E/S antigens, Qureshi et al. (25) recounted that an approximately 15 kDa *F. hepatica* E/S antigens can be used for species diagnosis in cattle. It is concluded that EITB method is a high-quality confirmation test for the diagnosis of human fasciolosis, but the need of supplementary studies remains to become aware of the most specific bands, so that the cross-reaction issue which being regarded as a major limitation in serodiagnosis of helminthic infections might be clarified.

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