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Evaluation of Enzyme-linked Immunotransfer Blot for the Immunodiagnosis of Human Fascioliasis Using Cysteine Proteinase Antigen

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Abstract: The present study was targeted as examining sera obtaining from patients infected with *Fasciola* sp. by the Enzyme-linked Immunotransfer Blot (EITB) technique using the parasite's Cysteine Proteinase (CE) antigen in order to evaluate the diagnostic potential of the assay. Altogether, a sort of sera including 80 cases of fasciolosis, 80 with other parasitosis other than fasciolosis and 30 normal control sera were enrolled in the trial. Hinge on the collected results, 78 fasciolosis serum samples recognized two antigenic polypeptides of 27 and 29 kDa. The sensitivity, specificity and positive and negative predictive values for CP antigen were 97.5, 98.8, 98.7 and 97.7%, respectively. Utterly, one case of cross-reaction was verified with a toxocariasis case. Concluding remark suggests that the 27 and 29 kDa bands in EITB test could be imperative in the immunodiagnosis of human fascioliasis.

Key words: *Fasciola* sp., SDS-page, EITB, cysteine proteinase

INTRODUCTION

Fasciolosis, caused by the liver flukes of *Fasciola hepatica* and *gigantica*, is widespeared in most part of the world and including the northern parts of Iran. Eating contaminated plants with infective metacercaria, derived from an intermediate molluscan host, results to ascertain the disease[1].

Evaluating and setting up an authentic diagnostic method using a putative antigen, prior to establishing the disease, i.e., during prepatency, is of major significance. A category of serological diagnostic techniques, including haemaglutination (HA)[2,3], Indirect Fluorescence Antibody Test (IFAT), immunoperoxidase (IP)[4], counterelectrophoresis (CEP)[5], enzyme-linked Immunosorbant assay (ELISA)[6-8] and Enzyme-Linked Immunoelectrotransfer Blot analysis (EITB) or Western blotting[9-11] have been appraised so far to diagnose human and animal fasciolosis. In the present study, the latter method, owing to enclosing high validity was challenged to diagnose human fasciolosis using CP antigen. This antigen has been assessed in ELISA test to diagnose human fasciolosis and in sum a sensitivity of >97% and specificity of > 98% has been confirmed[9-11], but thus far, its utilization in EITB method had not been studied comprehensively in this regard.

MATERIALS AND METHODS

A sort of 80 cases of fasciolosis, diagnosed anchored in the stool examination and ELISA test were involved in the trial from Nov. 2004 to July 2005. To resolve the specificity and relevant diagnostic parameters of the test, serum samples obtained from patients infected with hydatidosis (20), toxocariasis (10), amoebiasis (10), strongyloidiasis (10) and malaria (5) were acquired from the Tehran School of Public Health serum blood bank whom were diagnosed founded 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.

Adults *F. hepatica*s were obtained from infected bovine livers collected from local abattoirs. After washing the worms for 6 times with PBS, they were homogenized in 0.045 M PBS, pH 7.2 using electrical homogenizator (Edmund Buhler Co., model Homo 4/A mit uhr) followed by sonication (Tomy Seiko model UP-200P, Tokyo) and then centrifugation at 15000 g at 48°C for 30 min. Afterwards the supernatant was subjected to a 134 mL Sephacryle S 200 HR gel filtration column, equilibrated in 0.1 M Tris-HCl, pH 7.2. One-milliliter fractions were collected and monitored by absorbance

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at 280 nm for protein concentration. Each fraction was also assayed for CP activity using the flurogenic substrate Z-Phe-Arg-AMC (7-amino-4-methylcomarin). 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 and 440 nm, respectively[12]. Fractions containing proteolytic activity were pooled and then were subjected to ion exchange chromatography on QAE-Sephadex A 50, equilibrated in 0.1 M Tris-HCl, pH 7.2. The first obtained fraction, which had the highest proteolytic activity, concentrated and used as CP antigen. The concentration of each antigen preparation was measured using Bradford method[13].

EITB was conducted essentially as previously described[14,15]. The CP 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 (Sclileichcr and 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 (horseradish peroxidase conjugate rabbit antihuman IgG) for 2 h at room temperature. Unbound conjugate was removed by three PBS washes before addition of substrate solution containing 3,3'- Diaminobenzidine (DAB) (Sigma Chemical Co.). Bands were perceptible within 10 min and development was stopped by removing substrate with distilled water and air drying the nitrocellulose.

Statistical analysis: The sensitivity, specificity and the predictive values were calculated as previously described[16].

RESULTS AND DISCUSSION

Obtained results demonstrated that when infected sera with fasciolosis, were examined by EITB method using prepared Cysteine Proteinase (CP) antigen, two protein bands of 27 and 29 kDa were specific in diagnosing them (Fig. 1). These bands were not identified in any of the 30 negative sera and when the other sera infected with parasitic disease except than fasciolosis, were tested, only one case of cross-reaction was verified, which belonged to a toxocariasis one.

On the whole, the sensitivity, specificity, positive and negative predictive values were calculated as 93.5, 96.1, 72.9 and 99.2%, respectively.

Serological methods, in particular antibody detection tests, are considered the preferred method for immunodiagnosis of fasciolosis. The reasons include the relative simplicity of the assays and early seroconversion (usually 1-2 weeks) during primary infection as compared to late patency (2-3 months)[17]. A variety of different antigens obtained from *Fasciola* sp. have been evaluated using different serological tests to diagnose human and animal fasciolosis[9-11]. Two *F. hepatica* CP, isolated from the parasite ES products, are believed to be a part of the mosaic of antigens that form the arc5 percipitin band which was used in early studies to diagnoses fasciolosis[17]. The cysteine proteinases comprise

Fig. 1: EITB analysis of *F. hepatica* cysteine proteinase probed with serum infected with fasciolosis. Two lanes demonstrating two bands of CP antigen as 27 and 29 kDa

a large family with a number of classes and cathepsin L and B in particular have been studied in relation to parasite invasion, feeding, immune evasion, vaccine potential and diagnosing[18].

Early diagnosis of fasciolosis, due to some obscurities in its diagnosis by coprological methods, sounds important. The present study, undertook to evaluate CP antigen by EITB method in this regard. Analyzing obtained protein bands demonstrated that two bands of 27 and 29 kDa were detected by most of the sera (78 cases) infected with fasciolosis.

Reviewing present literatures, shows a few studies conducted based on using CP antigen in diagnosis of human fasciolosis by EITB method[19]. But, of course, in animal fasciolosis this antigen has been challenged in a similar manner[20,21].

Dixit *et al*.,[21] reported that specific antigen of 28 kDa could recognize homologues antifluke antibodies by EITB method as early as 2nd week post infection with 100% sensitivity in ovine fasciolosis. In Western blotting studies using excretory/secretory (ES) antigens in humans infected with *F. hepatica*, it was verified that bands 12, 17, 25, 27, 29 and 49.5 kDa were specific for detecting the disease[21-24]. Intapan *et al.*[23] using Western blotting technique accounted the sensitivity, specificity, positive and negative predictive values for a 27 kDa protein band yielded from the ES antigen of *F. hepatica* as 100, 98, 66.7 and 100%, respectively, which were one way or another identical to our results, not mention of sensitivity value. Somatic antigen also has been challenged in this test and Shaker has reported sensitivity and specificity of 100% to diagnose the disease[25]. It is worth mentioning that differentiation in various strains of *F. hepatica* in diverse countries can result to variable results in similar studies and evidently the nature of used antigen play an important role in this regards.

EITB, despite the fact that give the impression of being time consuming and expensive in comparison with the other diagnostic methods, could be regarded as a complimentary test with satisfactory strength. The obtained results in this study, shows that a usual encumber in diagnosing helminthic infections, i.e., cross-reaction, might be clarified using this method and CP antigen as well.

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