The Value of micro-ELISA Test in the Diagnosis of *Fasciola hepatica* Infection

Fasciola hepatica Enfeksiyonunun Tanısında micro-ELISA Testinin Değeri

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ABSTRACT

Objective: In sero-diagnosis of parasitic infection, it is essential to inspect cross-reactivity between the target parasite and other parasites in order to assess diagnostic performance. The aim of this study was to determine the cut-off value of antibody titer for diagnosis of *F. hepatica* (FH) infection by using the micro-ELISA and diagnostic performance of this test.
Methods: The study population consisted of the following groups: FH group (n=42), *Echinococcus granulosus* (EG) group (n=27) and control group (n=33). The micro-ELISA test for detection of anti-*F. hepatica* antibody was performed in all groups.
Results: The test was positive in all patients with FH, in 3 out of 27 (11%) patients with EG and in none of the control group. Mean antibody titer was significantly higher in the FH group compared to the EG group (23.8±0.9 DU vs. 5.7±1.2 DU; p<0.001) and compared to the control group (23.8±0.9 DU vs. 2.4±0.2 DU; p<0.001). When we used 11,5 DU as a cut-off value for sero-diagnosis of FH, the positive predictive value was 93.3%, negative predictive value was 100%, sensitivity was 100%, and specificity was 95%.
Conclusion: Cross-reactions are an important issue in serological diagnosis of parasitic infections. The micro-ELISA test for FH antibody can not definitely discriminate fascioliasis from hydatid disease. *(Turkiye Parazitol Derg 2013; 37: 23-7)*

Key Words: *Fasciola hepatica*, *Echinococcus granulosus*, micro-ELISA test

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ÖZET

Amaç: Paraziter hastalıkların serolojik tanısında kullanılan testin güvenirliliğini saptayabilmek için, hedef parazit ile diğer parazitler arasındaki çapraz reaksiyonun göz önünde bulundurulması gerekir. Çalışmamızın amacı *F. hepatica* (FH) tanısında kullanılan antikor titresinin eşik değerini saptamak ve testin güvenirliliğini ortaya koymaktır.
Yöntemler: Çalışmaya aşağıdaki gruplar alındı: FH grubu (n=42), *Echinococcus granulosus* (EG) grubu (n=27) ve kontrol grubu (n=33). FH antikorlarının saptanması için micro-ELISA testi tüm gruplarda çalışıldı.
Bulgular: Micro-ELISA testi FH grubundaki tüm hastalarda ve EG grubundaki 27 hastanın 3'ünde (%11) pozitif saptanırken, kontrol grubundaki hiç bir hastada pozitif saptanmadı. Ortalama antikor titresi FH grubunda EG grubuna göre (23.8±0.9 DU vs. 5.7±1.2 DU; p<0.001) ve kontrol grubuna gore (23.8±0.9 DU vs. 2.4±0.2 DU; p<0.001) anlamlı olarak daha yüksek saptandı. Fascioliasisin serolojik tanısı için 11.5 DU eşik değer olarak alındığında, micro-ELISA testinin pozitif prediktif değeri %93.3 ve negative prediktif değeri %100; duyarlılığı %100 ve özgüllüğü %95 olarak saptandı.
Sonuç: Paraziter enfeksiyonlarda çapraz reaksiyon önemli bir sorundur. FH enfeksiyonunun serolojik tanısında kullanılan micro-ELISA testi fascioliasisi hydatik hastalıktan ayırmada tek başına yeterli bir yöntem değildir. *(Turkiye Parazitol Derg 2013; 37: 23-7)*

Anahtar Sözcükler: *Fasciola hepatica*, *Echinococcus granulosus*, micro-ELISA testi

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INTRODUCTION

Fascioliasis is an infection caused by a trematode of the liver, *F. hepatica,* that particularly affects sheep, goats and cattle. The disease is transmitted to humans via ingestion of metacercaria from contaminated plants and after 3 to 4 months, the parasite is lodged in the biliary ducts of the liver. Afterwards, the final host releases the parasite eggs through the feces. Liver infection involves two stages- hepatic and biliary (1, 2). Common signs and symptoms of the hepatic phase are abdominal pain, fever, eosinophilia and abnormal liver function tests (1-4). The biliary phase usually presents with intermittent right upper quadrant pain with or without cholangitis or cholestasis (4, 5). Diagnosis may be delayed because of the wide spectrum of the differential diagnosis and low incidence of *F. hepatica* infection (1).

Diagnosis of *F. hepatica* infection has traditionally relied on detecting the presence of eggs in fecal samples, but this method is unreliable and complicated (6, 7). At present, the routine diagnosis of human fascioliasis is based on the detection of antifluke antibodies in serum. Methods such as immunoelectrophoresis and counterimmunoelectrophoresis, although they are very specific, have limited sensitivity (1, 8). Diagnosis was improved by the development enzyme-linked immunosorbant assay (ELISA) (9-11). The cross-reactivity between *Echinococcus granulosus* (EG) and *F. hepatica* infection has been reported previously (12-14). Parasitic helminths express some antigen which often accounts for serological cross-reactions. In serodiagnosis, it is essential to inspect cross-reactivity between the target parasite and other parasites in order to assess diagnostic performance (14).

The aim of this study was: to determine the cut-off value of antibody titer by using micro-ELISA for diagnosis of *F. hepatica* infection, to determine the positivity rate of micro-ELISA test for EG in patients with *F. hepatica* infection, to determine the diagnostic performance of micro-ELISA test for *F. hepatica* infection (1-3).

METHODS

Study Population

This prospective study was conducted in the department of Gastroenterology and General Surgery of Dicle University Hospital between February 2010 and April 2012. The study population consisted of the following groups: *F. hepatica* group, Hydatid disease group and control group. All patients gave written informed consent and the study was approved by the local Ethics Committee.

In all subjects, initial complete clinical history, physical examination findings, routine laboratory results including complete blood count and routine biochemical analysis were recorded. Contrast enhancement abdominal computerized tomographic (CT) examination was performed in all patients with *F. hepatica* infection and hydatid disease. Abdominal ultrasound (US) examination was performed in all patients in the control group. All the CT scans were obtained using a 4 channel multislice CT scanner (Sensation 4, Siemens Medical Solutions, Erlargen, Germany). A 3.75-MHz convex probe (Toshiba SSA-270 A, Tokyo, Japan) was used for US of the abdomen.

Micro-ELISA test using *F. hepatica* antigen from adult liver fluke [DRG Instrument GmbH, Germany; cut-off: 11.5 DRG Units (DU)] was used for serological diagnosis of fascioliasis. Titer of antibody was calculated according to the manufacturer's instruction.

The diagnosis of *F. hepatica* infection with the hepatic phase was based on: (a) The presence of previously described characteristic findings on the abdominal CT examination and exclusion of all known disease that cause hepatic lesions seen on CT examination; (b) and/or the presence of eggs of *F. hepatica* in the fecal examination (3, 4). The diagnosis of *F. hepatica* infection with the biliary phase was based on the extraction of living *F. hepatica* during endoscopic retrograde cholangio pancreaticography (ERCP).

The diagnosis of Hydatid disease was confirmed by characteristic CT findings before surgery and typical hydatid cystic appearance during surgery (15, 16).

Patients who were followed in the routine check-up department and without any disease were included in the study as the control group.

Statistical Analysis

Mean and standard deviation (SD) were calculated for continuous variables. The normality of the variables was analyzed by the Kolmogorov-Smirnov test. The Chi-square (χ^2) test was used for categorical variables. The one-way ANOVA test was used for normal distributed numerical values. The Kruskal-Wallis test was used for non-normal distributed numerical values. Two-sided p values were considered statistically significant at $p \leq 0.05$. Analyses of nonparametric receiver operating characteristic (ROC) curves were performed to calculate the cut-off values. Statistical analyses were carried out by using the statistical packages for SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Table 1 shows demographic features and laboratory results of all groups. During the study period, 42 patients were diagnosed as *F. hepatica* infection. In all patients, the diagnosis of fascioliasis was based on positive micro-ELISA test (titers >11.5 DU) and characteristic abdominal CT findings. The mean titer of micro-ELISA for *F. hepatica* 23.85±0.99 (range: 13-38) DU. Forty-one out of 42 patients were accepted as hepatic phase of fascioliasis. The remaining one patient was accepted as biliary phase of fascioliasis. The diagnosis of biliary phase of fascioliasis was confirmed by extraction of living mobile *F. hepatica* from extrahepatic biliary ducts during the ERCP procedure. Microscopic examination of fecal specimen for eggs of *F. hepatica* revealed positive results only in two out of 42 patients with the hepatic phase of fascioliasis. After confirmation of fascioliais, triclabendazole was administered at a dose of 10-12 mg/kg for 1 day in all patients. Six months after treatment, there was significant clinical, laboratory and tomographic improvement in all patients.

There were 27 patients in the hydatid disease group. Hydatid cyst was located with the right lobe of the liver in 18 patients, left lobe in 8 patients, and both lobes in 1 patient. The number of cysts was one in 18 patients, two in 6 patients and three in 3 patients. According to Gharbi's classification (16), there were type II cysts in 5 patients, type III in 15 patients and type IV in 7

Table 1. Shows initial demographic and laboratory features of all groups

	Fasciola hepatica group (F)	Hydatid disease group (H)	Control group (C)	p value
Gender (M/F) Age (range)	12/30 41.9 (18-72)	9/18 41.7 (17-79)	21/12 31.4 (15-65)	NS F-H: 0.726 H-C: 0.043 F-C: 0.001
Hb (g/dL) WBC (n/mm^3)	12.4±0.26 10990±297	12.5±0.3 8420±552	13.1±0.3 6947±297	NS F-H: 0.028 H-C: 0.05 F-C: p<0.001
Eosinophil (n/mm^3)	3276±628	406±189	154 ±18	F-H: <0.001 H-C: 0.103 F-C: <0.001
ALT (U/L)	36±17	60±28	20±2.4	F-H: <0.001 H-C: 0.011 F-C: 0.001
AST (U/L)	30±5.3	43±13	19±2.4	F-H: 0.267 H-C: <0.003 F-C: 0.031
GGT (U/L)	64±13	83±30	27±4.6	F-H: 0.856 H-C: <0.001 F-C: <0.001
T. bilirubin (mg/dL)	0.47±0.03	0.9±0.18	0.61±0.05	F-H: 0.039 H-C: 0.898 F-C: 0.039
ESR (mm/h)	38±6	12±1	20±3	F-H: <0.001 H-C: 0.258 F-C: 0.014
Micro-ELISA (DU)	23.8±0.9	5.7±1.2	2.4±0.2	F-H: <0.001 H-C: <0.001 F-C: <0.001

ALT: Alanine aminotransferase (range: 10-40 U/L), AST: Aspartat aminotransferase (range: 10-35 U/L), GGT: Gamma glutamyl transferase (range: 9-64U/L), ALP: Alkaline phosphatase (range: 40-150 U/L), T. bilirubin: Total bilirubin (range: 0.2-1.2 mg/dL), ESR: Erythrocyte sedimentation rate, NS: Not-significant, F-H: Fasciola hepatica group vs. Hydatid disease group, H-C: Hydatid disease group vs. control group, F-C: Fasciola hepatica group vs. control group. DU: DRG unite

patients. The mean cyst diameter was 8.35±0.68 (range: 34-122) mm. The mean titer of micro-ELISA for *F. hepatica* was 5.7±1.2 (range 1-32). The micro-ELISA test was positive in 3 out of 27 (11%) patients. Of patients with positive anti- *F. hepatica* antibody, two patients were female, and all patients had one cyst located in the right lobe of liver.

There were 33 subjects in the control group. Abdominal US showed no mass lesion in any of the patients. The mean titer of micro-ELISA for *F. hepatica* was 2.4±0.25 (range: 1-6) DU. The micro-ELISA test was negative in all patients.

Comparison of Groups

The positivity rate of micro-ELISA was significanltly higher in the fascioliasis group compared to the hydatid disease group (100% vs. 11%; p<0.001) and compared to the control group (100% vs. 0%; p<0.001). Mean micro-ELISA titers were significantly higher in the fascioliasis group compared to the hydatid disease group (23.8±0.9 DU vs. 5.7±1.2 DU; p<0.001) and compared to the control group (23.8±0.9 DU vs. 2.4±0.2 DU; p<0.001). When we used 11.5 DU as a cut-off value for sero-diagnosis of *F. hepatica*, the positive predictive value was 93.3%, negative predictive value was 100%, sensitivity was 100%, specificity was 95%.

There was no significant difference between the three groups regarding mean haemoglobin level. Mean eosinophil count was significantly elevated in the *F. hepatica* group compared to the hydatid disease group (p<0.001) and the control group (p<0.001). Serum alanine aminotransferase (ALT) level was significantly

lower in the fascioliasis group compared to the hydatid disease group (p<0.001) and significantly higher in the fascioliasis group compared to the control group (p=0.001). There were no significant differences between the fascioliasis group and hydatid disease group regarding serum aspartate aminotransferase (AST) and gama glutamyl transferase (GGT) levels. Serum total bilirubin level was significantly higher in the fascioliasis group compared to the hydatid disease group and the control group (p=0.039). Erythrocyte sedimentation rate was significantly higher in the fascioliasis group compared to the hydatid disease group (p<0.001) and the control group (p=0.014).

DISCUSSION

Parasitic helminths express various antigenic carbohydrates which often account for serological cross-reactions. In serodiagnosis, it is essential to inspect cross-reactivity between the target parasite and other parasites in order to assess diagnostic performance. Terminal Gal (β1-6) Gal1-motifs have previously been shown to represent antigenic epitopes of neogala-series glycosphingolipids from tape worms (17). The Gal (β1-6) Gal sequence is a common epitope between EG and *F. hepatica* (14). Wuhrer et al. (17) reported that *F. hepatica* exhibits mammalian-type glycolipids as well as Gal (β1-6) Gal-terminating glycolipids that account for cestode serological cross-reactivity. Sera with *F. hepatica* infection have cross-reacted at the highest frequency (71.4%) against *Echinococcus multiloculari* antigen. In patients with other parasitic infections, sera showed cross-reaction against *F. hepatica* antigen bound to *Echinococcus multilocularis* antigen with a high frequency (23.7%) (14). In our previous study, we identified increased incidence of anti-*Echinococcus granulosus* antibody positivity using indirect immunofluorescence assay (IFA) in patients with *F. hepatica* infection (18). Şakru et al. (19) reported that 5 out of 226 (2,2%) *Echinococcus granulosus* suspected cases were found seropositive for *F. hepatica* antibodies by an excretory secretory ELISA (ES-ELISA) test. In this study, we showed that micro-ELISA test for *F. hepatica* is positive in 11% of patients with EG infection and it is negative in healthy control. The commercial DRG test was evaluated in cattle, obtaining a sensitivity and specificity of 98% and 96% at a cut-off value of 15% positivity, respectively. The sensitivity and specificity of ELISA in-house assays using DRG test for *F. hepatica* IgG antibody have been reported as 92.6% to 100% and 83.6% to 100% respectively (20-22). This IgG antibody may be in as 32 DU at high titers. The sensitivity of micro-ELISA test in our study is 100%, but the specificity is significantly lower (50%) compared to a previously reported study. Therefore, our findings are partially compatible with previously reported results. The false positive results of micro-ELISA test for *F. hepatica* in patients with hydatid disease may be related to antigenic similarity between *F. hepatica* and EG. The presence of cross-reactivity between parasites can suggest that serological tests without additional confirmative tests such as characteristic radiological findings are not reliable methods for diagnosis of these infections.

Typical organ lesion(s) detected by imaging technique (e.g. ultrasonography, computed tomography), specific serum antibodies assessed by high-sensitivity serological tests, histopathology or parasitology compatible with EG and detection of pathognomonic macroscopic morphology of cyst(s) in surgical specimens, confirm the diagnosis of EG (16). Routine laboratory tests are not specific for diagnosis of Hydatid disease and may reveal normal or abnormal values. Screening tests such as indirect hemagglutination, enzyme-linked immunosorbent assay (ELISA) and latex agglutination use crude antigens and are associated with a high incidence of false-negative and false-positive results. The parasitic antigens of major diagnostic value are antigen 5 (arc-5) and antigen B (15). Purified fractions enriched in antigens 5 and B and glycoproteins from hydatid fluid yielded a sensitivity rate of 95%, with a specificity rate of 100% (23). The diagnosis of Hydatid disease was confirmed in all our patients by positive IFA test, computed tomography findings and pathognomonic surgical findings.

Diagnosis of fascioliasis may be delayed because of the wide spectrum of the differential diagnosis and low incidence of *F. hepatica* infection (3). Similar abnormal laboratory and radiological findings may represent viral hepatitis, liver abscess, malignancy, cholecystitis, sclerosan cholangitis, and AIDS-related cholangitis, ruptured hydatic cyst and parasites such as ascariasis and clonorchiasis (1, 3). The sensitivity and specificity of ELISA in-house assays using the DRG test for *F. hepatica* IgG antibody are between 92.6% to 100% and 83.6% to 100%; respectively (20-22).

Diagnosis is confirmed only by demonstrating the parasites or its egg in the bile or feces (1, 3). Negative stool examinations do not rule out the disease (3). A high index of suspicion and specific radiological findings including tunnel-like tracts extending towards the capsule and multiple, hypodense, linear or branching lesions on CT are very helpful in the diagnosis of fascioliasis (4). We suspected the possibility of fascioliasis in all patients with hepatic phase because of eosinophilia and characteristic CT findings. We found eggs in stool samples of two out of 42 (4.7%) patients with hepatic phase. Complete clinical, laboratory and radiological response after triclabendazole administration, associated with positive result in high titer of micro-ELISA against *F. hepatica,* confirmed the diagnosis in patients with hepatic phase of *F. hepatica* infection. Diagnosis in the patients with biliary phase was confirmed by extraction of living *F. hepatica* form bile ducts. We can suggest that stool examination for eggs is not a reliable method and both serological test and extraction of living parasites from the bile ducts are very reliable methods for diagnosis of fascioliasis.

CONCLUSION

Cross-reactions are an important issue in serological diagnosis of parasitic infections. The micro-ELISA test for *F. hepatica* IgG antibody is positive in a minority of patients with hydatid disease and negative in healthy people. In clinical practice, the micro-ELISA test for *F. hepatica* IgG antibody cannot reliably discriminate fascioliasis from hydatid disease

Conflict of Interest

No conflict of interest was declared by the authors.

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