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ORIGINAL ARTICLE

Serologic detection of antibodies against *Fasciola hepatica* in sheep in the middle Black Sea region of Turkey

Mustafa Acici [a,*], Ozlem Buyuktanir [b], Cenk Soner Bolukbas [a], Gokmen Zafer Pekmezci [c], Ali Tumay Gurler [a], Sinasi Umur [a]

[a] *Department of Parasitology, Faculty of Veterinary Medicine, Ondokuz Mayıs University, Samsun, Turkey*
[b] *Department of Microbiology, Faculty of Veterinary Medicine, Ondokuz Mayıs University, Samsun, Turkey*
[c] *Department of Preclinical Sciences, Faculty of Veterinary Medicine, Ondokuz Mayıs University, Samsun, Turkey*

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Abstract *Background/Purpose:* The aim of the present study was to estimate the prevalence of *Fasciola hepatica* infection in sheep in the Black Sea region of Turkey.
Methods: Samples from 213 sheep were collected randomly in Samsun, Tokat, and Sinop from September 2005 to January 2007 and tested by indirect enzyme-linked immunosorbent assay (ELISA) and Western blot analysis using *F. hepatica* excretory-secretory (E/S) antigens.
Results: The distribution of ELISA-positive samples for *F. hepatica* infections out of a total of 213 sheep serum samples was 23/71 (32.4%), 15/59 (25.4%), and 29/83 (34.9%) in Samsun, Sinop, and Tokat, respectively. The immunodominant proteins were determined by Western blot analysis using molecular weight markers of 14 kDa, 20 kDa, 24 kDa, 27 kDa, 33 kDa, 45 kDa, and 66 kDa and extracted from sera of sheep that were positive for *Fasciola* spp. eggs and also hyperimmune sera from rabbits immunized with E/S antigens.
Conclusion: The ELISA-positive results were confirmed by Western blot analysis. As a result, seroprevalence of *F. hepatica* infection was found in 31.4% of sheep from the Karayaka breed in the Middle Black sea region of Turkey.

* Corresponding author. Department of Parasitology, Faculty of Veterinary Medicine, Ondokuz Mayıs University, 55139 Samsun, Turkey.
E-mail address: acicim@omu.edu.tr (M. Acici).

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Introduction

Fasciolosis is an economically important disease in domestic livestock, particularly cattle and sheep, and occasionally in humans. The disease is caused by digenean trematodes of the genus *Fasciola*, commonly referred to as liver flukes.[1] *Fasciola hepatica* has a worldwide distribution, but predominates in temperate zones, while *Fasciola gigantica* is found primarily in tropical regions.[1,2] Additionally, *F. hepatica* infection in sheep is prevalent in many parts of Turkey.[3–5] In a previous study, prevalence of *F. hepatica* was 3.99% in sheep and ranged from 0.48% to 2.65% in cattle in the Trakya region of Turkey.[6] In another study based on fecal examination in Samsun, *Fasciola* spp. was found in 20.99% of sheep.[7] The assays based on antibody detection are overwhelmingly the preferred method for immune diagnosis of fasciolosis. The reasons include the relative simplicity of the assays and early seroconversion during primary infections. Consequently, most investigators today use enzyme-linked immunosorbent assay (ELISA) and immunoblots for the immune diagnosis of fasciolosis. Antigenic preparations used have been primarily derived from adult worm extracts, excretion–secretion products of adult worms, or the partially purified fractions.[8–11] Although *F. hepatica* is endemic in many rural areas of Turkey, there have been few serodiagnostic studies by ELISA and Western blotting undertaken in sheep far.[3–6,12,13]

The aim of the present study was to estimate the prevalence of fasciolosis in sheep by indirect ELISA and Western blot tests using *F. hepatica* excretory–secretory (E/S) antigens in the Middle Black sea region, Turkey.

Methods

Sera and feces

Serum samples from 213 Karayaka sheep randomly collected in Samsun, Sinop, and Tokat from September 2005 to January 2007 were tested for antibodies against *F. hepatica*.

Initially, fecal, serum samples, and liver flukes were collected from sheep ($n = 40$) at the local slaughterhouse in Samsun. All of the animals were not given anthelmintic, were $\geq$1 year old, and had grazed in the pasture for at least spring, summer and autumn. The sera were stored at $-20^{\circ}C$ until use and *Fasciola* spp. eggs per gram (EPG $\geq$ 50) of feces were counted by a standard sedimentation method using McMaster chambers.[14]

Preparation of E/S antigens

F. hepatica E/S antigens were obtained from adult *F. hepatica* as described by Zimmerman et al[15] Briefly, viable *F. hepatica* adult flukes collected from the bile duct of sheep were washed several times in 0.15M NaCl (pH 7.2), then incubated at 37°C (1 fluke/mL) for 17 hours in phosphate-buffered saline [PBS (pH 7.2)] with penicillin (100 IU/mL) and streptomycin (100 mg/mL). After the incubation, the supernatant was collected and centrifuged at 5000*g* for 30 minutes at 4°C. The supernatant fluids were filtered and then dialysed against distilled water. The total protein concentration of the antigen was measured as described by Bradford.[16]

Production of hyperimmune serum

To obtain anti-*F. hepatica* E/S antibodies, two New Zealand rabbits (2 kg) were immunized with *F. hepatica* E/S antigens according to Almazan et al.[17] On day zero, 1 mL (0.8 mg/mL) *F. hepatica* E/S antigen emulsified with an equal quantity of Freund's complete adjuvant (Sigma-Aldrich, USA) was administered subcutaneously. Five additional immunizations were given in equal doses of the antigen in Freund's incomplete adjuvant (Sigma-Aldrich, USA) at 15-day intervals subcutaneously. In the 10th week, the rabbits had an antibody titer >100,000 as determined by Dot blot assay. The hyperimmune rabbit sera were used for confirmation of the immunogenicity of *F. hepatica* E/S antigens and also for optimization of the ELISA and Western blot tests along with the infected and negative sheep sera. Preimmune sera were used as a negative control.

Indirect ELISA

ELISA was performed on microtiter plates as described by Guobadia and Fagbemi[18] and Ferre et al[19] with some modifications. Briefly, polystyrene microtiter plates (Nunc Maxisorp, Thermo Fisher, Denmark) were coated with 100 μL of the E/S antigen (5 μg/mL) diluted in coating buffer (pH 9.6) per well, and incubated overnight at 4°C. The plates washed with PBS + 0.1% Tween-20 (PBST) were blocked with 200 μL 1% non-fat dry milk in PBST and incubated for 1 hour at 37°C. Subsequently, 100 μL rabbit and sheep sera (1:1000 and 1:100, respectively) diluted in 1% non-fat dry milk were added and incubated for 1 hour at 37°C.

After 100 μL of anti-sheep and anti-rabbit immunoglobulin IgG alkaline-phosphatase conjugate (1:10,000 and 1:15,000, respectively) were added, the plates were incubated for 1 hour at 37°C.

The plates were washed with PBST between each step. The paranitrophenyl phosphate substrate (1 mg/mL) was added and the optical density measured at 405 nm using an ELISA reader.[20]

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses were carried out according to Laemmli[21] and Towbin et al[22] Briefly, *F. hepatica* E/S antigen was separated by 12% SDS-PAGE and then transferred to nitrocellulose (NC) membrane (0.2 μm pore size, Sigma-Aldrich) using a semi-dry system with a 25mM Tris and 192mM glycine buffer. The NC membranes were cut into strips and washed with PBST (0.05%) between each step.

The NC strips were blocked with 1% non-fat dry milk in PBST (also used as dilution buffer) incubated overnight at 4°C, followed by incubation with 1:1000 rabbit and 1:100 sheep serum sample dilutions for 1 hour at 37°C. The strips