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Use of Indirect Excretory/Secretory Enzyme-Linked Immunosorbent Assay (ES-ELISA) for the Diagnosis of Natural *Fasciola hepatica* Infection in Eosinophilic and Non-Eosinophilic Cattle from Eastern Turkey

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Abstract: The aim of this study was to investigate fasciolosis in a group of eosinophilic and non-eosinophilic cattle in eastern Turkey. All the cattle were examined for antibodies against *F. hepatica* by modified indirect excretory/secretory enzyme-linked immunosorbent assay (ES-ELISA). Of the 1200 cattle tested, 164 (13.6%) were found to be eosinophilic by absolute eosinophil counts. Out of the 164 eosinophilic cattle, 121 (73.7%) were found to be positive for fasciolosis by indirect ES-ELISA, while the rate was only 35% among the non-eosinophilic group ($P < 0.001$). Our data indicate that parasitic infections should be considered an important cause of eosinophilia, particularly in fasciolosis, and the indirect ES-ELISA is useful as a screening test to examine anti-*F. hepatica* antibodies for the diagnosis of fasciolosis in cattle.

Key Words: *Fasciola hepatica*, eosinophilia, ES-ELISA, cattle, Turkey

Türkiye'nin Doğusunda Eozinofilik ve Eozinofilik Olmayan Sığırlarda Doğal *Fasciola hepatica* Enfeksiyonunun Tanısında İndirekt Excretory/Secretory Enzyme-Linked Immunosorbent Assay (ES-ELISA)'in Kullanımı

Özet: Bu çalışmanın amacı, Türkiye'nin doğusunda eozinofilik ve eozinofilik olmayan sığırlarda fasciolosisin araştırılmasıdır. Bu amaçla, çalışmaya dahil edilen sığırlarda *Fasciola hepatica*'ya karşı oluşmuş antikor yanıtı indirekt ES-ELISA ile araştırılmıştır. Absolut eozinofil sayımı yapılan 1200 sığırın 164 (% 13,6)'ü eozinofilik olarak bulunmuş ve bu 164 sığırın 121 (% 73,7)'inde indirekt ES-ELISA ile *F. hepatica*'ya karşı oluşmuş antikor cevabı tespit edilmiştir. Eozinofilik olmayan sığırlarda ise bu oran % 35 olarak belirlenmiştir ($P < 0,001$). Bu bulgular, özellikle fasciolosis gibi paraziter enfeksiyonların eozinofilinin önemli bir nedeni olduğuna ve sığır fasciolosisinin tanısında indirekt ES-ELISA'nın kullanışlı bir test olduğuna işaret etmektedir.

Anahtar Sözcükler: *Fasciola hepatica*, eozinofili, ES-ELISA, sığır, Türkiye

Introduction

Fasciolosis is a disease of cattle and sheep that has a worldwide distribution and causes serious economic damage. The economic importance of *Fasciola hepatica* to the livestock industry has stimulated research on many aspects of this parasite (1). Diagnosis of fasciolosis is based on clinical findings and laboratory tests. The most reliable means is the finding of eggs in the stool of infected individuals despite overwhelming consensus that this method is not wholly reliable (2). With this method,

eggs cannot be detected until the latent period of infection, when much of the liver damage has already occurred. Moreover, weak infections can pass undetected and parasitised animals that are considered negative may be sources of new infections. Sensitive early diagnostic methods are needed in order to adopt control methods for reducing the negative economic effects of fasciolosis (1). Serology has the advantage of being able to indicate infection much earlier (around 4-5 weeks) than coprological methods, which tend to detect the presence

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of fasciola eggs in the faeces at about 10-11 weeks post-infection (3). An additional drawback of the faecal egg counting technique is an estimated low sensitivity of only 30% (4), which might result in under diagnosis of liver fluke infestations.

Techniques of indirect immunoenzymatic diagnosis represent an important contribution to the early detection of infection with *F. hepatica* by means of the indirect-ELISA. Based on the excretory/secretory (ES) antigen, it has been possible to detect experimental infections in cattle from the third to the fifth week after infection (5).

Eosinophilia in the developing world should first suggest the possible presence of helminthic infection (6). Although eosinophilia implicates, in particular, the presence of a helminth, the absence of eosinophilia cannot exclude these parasites. The diagnosis and treatment of parasitic diseases with eosinophilia are easy (7). It is the most frequent laboratory abnormality in fasciolosis (2). Eosinophilia in the presence of a helminthic infection may be considered an adaptive measure to damage the parasite (6).

This study was undertaken to investigate fasciolosis in eosinophilic and non-eosinophilic cattle with indirect ES-ELISA in eastern Turkey, and to investigate the seroprevalence of fasciolosis in the cattle of the same area.

Materials and Methods

Preparation of *F. hepatica* excretory/secretory products

The use of excretory-secretory antigen adult *F. hepatica* was based on prior reports of the successful use of this antigen (1). This antigen was obtained by collecting adult liver flukes from cattle bile ducts at the slaughterhouse. Specimens were washed several times in 0.01 M phosphate-buffered saline (PBS) (pH 7.4) and incubated in the PBS at 37 °C for 6 h to remove intestinal contents. Eggs were removed by sieving and 10,000 rpm centrifugation at 4 °C for 30 min, and then the supernatant containing products were filtered from a 0.2 µm pore size filter. Subsequently, these products were dialysed against distilled water for 24 h. After dialysing, 5 mM EDTA (ethylenediaminetetraacetic acid) and 5 mM sodium azide were added to the antigen for protein

protection. During the last step, the samples were aliquoted and stored at -20 °C until assayed. The protein concentration was estimated by the method described by Lowry et al. (8).

Sera

Şanlıurfa, Erzurum, Muş, Van, Kars, Erzincan, Bingöl, and Elazığ provinces in eastern Turkey are the major animal (mainly cattle and sheep) breeding areas of the country and have the largest pastures. Blood samples were randomly collected from 1200 cattle from these 8 provinces, and the sera were kept at -20 °C until used. The majority of the cattle were selected from farms that grazed pastures from March to October. Peripheral blood smears were taken from ear tips of the cattle and the smears were stained with 5% Giemsa solution in the laboratory. All of the peripheral blood smears were inspected microscopically and absolute eosinophil counts were determined. An eosinophil level < 5% was considered normal, a level of 6%-10% eosinophils was regarded as moderate eosinophilia, and ≥ 11% eosinophils was noted as significant eosinophilia (9). The eosinophilic group comprised 126 females and 38 males. The distribution of their ages were as follows: < 1 year old (n = 9); 1 year old (n = 13); 2 years old (n = 47); 3 years old (n = 19); 4 years old (n = 18); 5 years old (n = 12); ≥ 6 years old (n = 46). The non-eosinophilic group included 107 females and 107 males with an age distribution as follows: < 1 year old (n = 64); 1 year old (n = 36); 2 years old (n = 57); 3 years old (n = 12); 4 years old (n = 6); 5 years old (n = 10); ≥ 6 years old (n = 29).

Indirect ES-ELISA

This technique was performed according to Ibarra et al. (10) with little modification. ELISA plates (Dynatech Laboratories, IA, USA) were coated with 100 µl of 5 µg/ml E/S antigens in 0.1 M carbonate/bicarbonate buffer (pH 9.6) per well. Following overnight incubation the plates were washed twice with PBS containing 0.01% Tween-20 (PBS/Tween) and blocked with 130 µl per well of a solution containing 5% skimmed-milk powder in 0.01 M PBS (pH 7.4) for 1.5 h at 37 °C. After blocking, the plates were washed 3 times with PBS/Tween. Then 100 µl of sera diluted 1:50 in PBS containing 0.05% Tween-20 was added to the wells, followed by incubation for 1.5 h at 37 °C. The plates were again washed (5 times) and 100 µl of 1:1000 anti-bovine IgG conjugated to rabbit

peroxidase (Sigma Co. St. Louis, MO, USA, Cat no: A7414) was added to the wells and the plates were incubated at 37 °C for 2 h. Finally, after another 5 washes, 100 µl of substrate containing O-phenylenediamine (OPD) and hydrogen peroxide (H₂O₂) in citrate/phosphate buffer were added to each well, and conveniently readable results were obtained after 15 min incubation at room temperature. The enzymatic reaction was stopped with 50 µl per well of 1 N sulphuric acid and the plates were read at 450 nm on an ELISA reader (Medispec ESR 200). The results were expressed as the mean of the optical density (OD). All samples were studied in duplicate. When the difference in OD between same 2 samples exceeded 10%, they were re-tested. The cut-off value was calculated as the mean of the negative control sera absorbance values plus 3 standard deviations.

Sensitivity and specificity of the test

Positive control sera (n = 20) from experimentally infected cattle (infected with 500 metacercariae each) were obtained from Novartis Animal Health R & D Centre (Yarrandoo, Australia) (11), and another 20 sera from naturally infected cattle (selected on the basis of faecal egg count and autopsy) were obtained from a local abattoir in Elazığ province.

Negative control sera (n = 20) were obtained from calves that were maintained indoors since birth at the Firat University, Faculty of Veterinary Medicine Investigation and Application Farm (Elazığ); these calves were found by repeated coprological examinations to be free of fasciolosis. Additional negative control sera (n = 20) (based on epidemiological information and faecal egg count) were obtained from Novartis Animal Health R & D

Centre (11). Positive and negative control sera were used in each ELISA.

Chi-square test was performed to confirm the difference between groups.

Results

In the eosinophilic cattle, eosinophil counts were between 6% and 50%. In non-eosinophilic cattle, eosinophil counts were < 6%. In this study, out of 1200 cattle, 164 (13.6%) were found to be eosinophilic by absolute eosinophil count. Of these 164 eosinophilic cattle, 121 (73.7%) were found to be positive for fasciolosis by indirect ES-ELISA, while the rate was only 35% among the non-eosinophilic group, a statistically significant difference (χ^2 55.78; $P < 0.001$). The results are shown in the Table.

Seropositivity was determined in 93 females and 28 males in the eosinophilic group and the difference between the sexes was statistically significant. Out of 378 cattle for which serological examinations were done, 196 were positive for fasciolosis, and the seroprevalence of fasciolosis in cattle of this region was 51.8%.

Sensitivity and specificity values for indirect ES-ELISA were both 95%.

The age distribution of seropositive cattle in the eosinophilic group was as follows: < 1 year old (n = 4; 44.4%); 1 year old (n = 6; 46.1%); 2 years old (n = 35; 76.1%); 3 years old (n = 15; 78.9%); 4 years old (n = 12; 66.6%); 5 years old (n = 9; 75%); ≥ 6 years old (n = 40; 86.9%). The prevalence of seropositivity was higher in the cattle ≥ 6 years old than in the younger ones.

Table. Fasciolosis in eosinophilic and non-eosinophilic groups.

Eosinophil count (%)	Eosinophilia		Fasciolosis	
	n	%	n	%
6-10	82	50.0	49	59.7
≥ 11	82	50.0	72	87.8
Total	164	100.0	121	73.7 ^a
Negative	214	100.0	75	35.0 ^b

a,b: Significant statistical difference between groups ($P < 0.001$)

Discussion

Eosinophilia in the developing world should first suggest the possible presence of helminthic infection (6). Although eosinophilia, in particular, indicates the presence of a helminth, the absence of eosinophilia cannot exclude these parasites. The diagnosis and treatment of parasitic infections with eosinophilia is easy (7). Eosinophilia in the presence of a helminthic infection may be considered an adaptive measure to damage the parasite (6) and it is the most frequent laboratory abnormality in fasciolosis (2). Şimşek et al. (12) detected the seroprevalence of hydatidosis in cattle with eosinophilia as 78.4% by ELISA and 60.7% by IFAT. The prevalence rates were 48.1% and 43.1% by ELISA and IFAT, respectively, in the non-eosinophilic group. This indicates that hydatidosis is also one of the causes of eosinophilia.

The ELISA has been widely used in serological studies of fasciolosis (13) and has been suggested as suitable for epidemiological studies (14). The ELISA enables particularly clear discrimination between negative and positive populations, which would further safeguard against false positive or negative classification (11). Indirect ES-ELISA has been reported to be a rapid and sensitive test (sensitivity 92%, specificity 94.4%) (1). In the present study, the sensitivity and specificity of indirect ES-ELISA were both 95%.

Definitive diagnosis of fasciolosis depends on serologic tests and/or demonstration of *F. hepatica* eggs in stool samples. Serological studies are now the main diagnostic method and allow diagnosis of the disease, even in the acute stage, and before the parasite eggs can be identified in faeces. However, the serological methods, especially the ELISA test, are highly sensitive and specific when compared to diagnosing *F. hepatica* by coprological means (10). Thus, coprological examinations were not used in this study.

To the best of our knowledge there have been no previous seroepidemiological studies on cattle fasciolosis in eastern Turkey. In the present study, the seroprevalence of fasciolosis among all the cattle

examined (eosinophilic and non-eosinophilic) by indirect ES-ELISA was 51.8%, while the rate was 73.3% among the eosinophilic cattle.

Blood samples were collected in the spring and autumn from over 6000 cows in Slovenia and eosinophilia was found in 51.8% of them. Faecal examinations revealed *F. hepatica* in 36.4% of the cows (15). We found a 13.6% eosinophilia prevalence rate in 1200 cattle (164/1200) and a 73.3% *F. hepatica* seropositivity rate in the eosinophilic cattle.

In a herd of cattle in Switzerland with sub-acute fasciolosis, all animals were eosinophilic (16). In another study, Furmaga and Gundlach (17) reported that eosinophilia occurred both in acute and chronic stages in calves that were experimentally infected with *F. hepatica* metacercariae. In the present study, the rate of seropositivity of fasciolosis was 51.8% in healthy-appearing cattle.

Livers of 300 Korean cattle slaughtered at Pusan were examined and 90.7% were infected with *F. hepatica*. The incidence was higher in cattle aged 6 to 9 years than in younger cattle. It was reported that immature flukes caused massive eosinophilia (18). In the present study, the seropositivity was higher in cattle ≥ 6 years old than in younger ones. The seropositivity rates in the eosinophilic group were as follows: < 1 year old, 44.4%; 1 year old, 46.1%; 2 years old, 76.1%; 3 years old, 78.9%; 4 years old, 66.6%; 5 years old, 75%; ≥ 6 years old, 86.9%.

In conclusion, our data show that fasciolosis is endemic in eastern Turkey. The indirect ES-ELISA is useful as a screening test to examine anti-*Fasciola hepatica* antibodies for the diagnosis of fasciolosis in cattle. We suggest that parasitic infections should be considered an important cause of eosinophilia, particularly in fasciolosis.

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