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Research Article

Comparison of Crude and Excretory/Secretory Antigens for the Diagnosis of *Fasciola hepatica* in Sheep by Western Blotting

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Abstract: Crude and excretory/secretory (E/S) antigens of *Fasciola hepatica* were subjected to SDS-PAGE and Western blot analysis in order to identify protein bands that would enable the specific and sensitive immunodiagnosis of sheep. Sera from 20 sheep with natural infections of *F. hepatica, Dicrocoelium dendriticum, Cyst hydatid, Cysticercus tenuicollis, Trichostrongylidae, Paramphistomum* spp., and *Trichuris* spp. and the same number of sera naturally infected with the same helminths without *F. hepatica* were tested using Western blotting, and the bands obtained from positive and negative groups were compared. According to the test results the specific protein bands for *F. hepatica* infection were 33, 39.5 and 42 kDa in E/S antigens and 24, 33 and 66 kDa in crude antigen.

Key Words: *Fasciola hepatica*, sheep, SDS-PAGE, Western blotting

Koyunlarda *Fasciola hepatica* Enfeksiyonunun Ekskresyon / Sekresyon ve Olgun Antijenleri Kullanılarak Western Blotting Yöntemi ile Karşılaştırmalı Teşhisi

Özet: Bu çalışmada, SDS-PAGE ve western blotting yöntemleri ile *Fasciola hepatica* ekskresyon/sekresyon (E/S) ve olgun antijenleri kullanılarak enfekte koyunlarda duyarlı ve özgün protein bantları tespit edilmiştir. Araştırmada, *F. hepatica, Dicrocoelium dendriticum, Cyst hydatid, Cysticercus tenuicollis, Trichostrongylidae, Paramphistomum* spp., ve *Trichuris* spp. ile doğal enfekte 20 koyun serumu ile aynı sayıda *F. hepatica* dışında diğer helmintlerle enfekte koyun serumları kullanılmıştır. Western blot yöntemi ile yapılan incelemede *F. hepatica* yönünden pozitif ve negatif gruplar arasındaki farklılıklar ortaya çıkarılmıştır. Araştırma sonuçlarına göre, E/S antijeni için 33, 39,5 ve 42 kDa, olgun antijeni için 24, 33, and 66 kDa'luk bantların spesifik olduğu belirlenmiştir.

Anahtar Sözcükler: *Fasciola hepatica*, koyun, SDS-PAGE, western blotting

Introduction

Fasciola hepatica is an important trematode parasite of ruminants and occasionally of humans. Early diagnosis of this disease is very important for succesful treatment. The diagnosis of juvenile flukes of *F. hepatica*, which migrate through the liver parenchyma, is not possible via routine laboratory procedures (1). *F. hepatica* is quite prevalent in Turkey, and surveys of cattle (2) and sheep (3) have shown the prevalence of *F. hepatica* to range from 10% to 90% and 0.5% to 90%, respectively, in different regions of Turkey.

Different immunodiagnostic tests have been used in the early immune diagnosis of fasciolosis, but they have some disadvantages, such as cross reactions with other trematodes, leading to false positive results (1,4,5). In recent years, SDS-PAGE and Western blot procedures have created a new era in immunodiagnosis, and greatly reduced cross reactions (6). In previous immunodiagnostic studies on *F. hepatica* using SDS-PAGE and Western blotting techniques, it was determined that excretory/secretory antigens were more specific than the others (somatic and surface antigens) (7). Immune responses of excretory/secretory products of adult *F. hepatica* were studied in sheep (8-11) and the bands of 17, 23-27, 29, 25-30 and 63 kDa were found to be specific. Researchers reported that *F. hepatica* infection was detected as early as 2 weeks after the infection (10).

The aim of the present work was to determine specific protein bands for fasciolosis from the sera of sheep naturally infected with *F. hepatica* using crude and E/S antigens and to compare the results within the study and with those of other studies.

Materials and Methods

This study was carried out in sheep slaughtered in the municipal slaughterhouses of Kazan, Ankara. Two groups of sheep were formed: positive (20 sheep infected with *F. hepatica*) and negative groups (20 sheep not infected with *F. hepatica*). Blood samples were taken from positive and negative sheep and sera obtained from these sheep were stored at –20 °C before analysis. For the detection of *F. hepatica*, cysticercosis and hydatidosis, the livers and other organs of the sheep were examined carefully. At the same time, fecal samples from these sheep were obtained and examined microscopically for fasciolosis, dicrocoeliosis, paramphistomiosis and trichostrongylosis in the laboratory.

Preparation of crude antigen

Adult *F. hepatica* flukes were placed in a small pestle and mortar. Liquid nitrogen was added and the flukes were frozen until solid. The parasites were pulverized with the pestle while liquid nitrogen was still present. The nitrogen was allowed to evaporate. Two milliliters of PBS plus protease inhibitors per gram of wet tissue were added to the mortar and mixed well. The antigen source was placed into Eppendorf tubes and spun at 13,000 rpm for 10 min. The pellets were discarded and the antigen was stored in aliquots at –80 °C until use.

Preparation of excretory-secretory (E/S) antigen

F. hepatica were collected from the bile ducts and washed 6 times in 0.01 M phosphate buffered saline (PBS) at 37 °C. The flukes were then left for 2-3 h in PBS at 37 °C to allow regurgitation of the cecal contents. The viability of flukes after this incubation was normally 100%. The flukes were then harvested and used to prepare excretory/secretory (E/S) extracts as follows. E/S product isolated parasites were washed 6 times with 0.01 M (PBS), pH 7.2, and a further 6 times with RPMI-1640 medium. The worms were then incubated in cell-cultured flasks in RPMI-1640 medium with 100 IU of penicillin and 100 µg of streptomycin per milliliter of medium (1 fluke per 3 ml) at 37 °C in a 5% CO_2 incubator for 24 h. After the incubation, the supernatant was collected and centrifuged at 5000 g for 30 min at 4 °C. The samples were aliquoted and stored at –70 °C.

Polypeptide analysis

E/S and crude antigens obtained from *F. hepatica* were separated by SDS-PAGE. Proteins were visualized with silver staining and their molecular weights were determined by comparing with molecular weight standards (Sigma wide molecular weight range- M-4038, Sigma Chemical Co., USA). To determine the most appropriate amount of antigen, a gel (5% stacking + 15% separating) was prepared. Then 10, 15, 20, 25, 30 and 35 µl of antigen were loaded onto this gel and 30 µl of antigen was determined to be the best amount for this study.

Antigenic Analysis

Antigenically active components among the resolved bands in SDS-PAGE were detected by Western blotting. After SDS-PAGE, the proteins were transferred electrophoretically onto a nitrocellulose sheet using a transfer blot apparatus. Nitrocellulose containing transferred samples was incubated overnight at 4 ºC in 3% nonfat dried milk, and then rinsed in PBS before 2 h incubation with sera containing test antibodies. Following 3 PBS washes to remove unbound antibodies, the nitrocellulose sheets were incubated for 1 h in horseradish peroxidase conjugate anti-IgG (A-8917, Sigma). Unbound conjugate was removed by 3 PBS washes before the addition of substrate solution containing DAB (3,3'- Diaminobenzidine, D-4293, Sigma). Bands were visible within 15 min and development was stopped by removing the substrate with distilled water and air drying the nitrocellulose.

Results

In the macroscopic examination of the sheep, hydatid cysts were seen in 2 animals in the positive group and in 3 in the negative group. *Cysticercus tenuicollis* was seen in 5 animals in the positive group and in 4 in the negative group (Table 1). Fecal examinations of positive and negative control group animals were carried out using sedimentation and flotation procedures. According to these examinations in positive group animals, the eggs of Fasciola spp. were found in all of the sheep, Trichostrongylidae spp. in 15, *Dicrocoelium dendriticum* in 9, Paramphistomum spp. in 4 and Trichuris spp. in 1. (Table 1). During the fecal examinations of the 20

Table 1. Worm burden and detected specific protein bands in crude antigens in positive and negative groups for *Fasciola hepatica*.

Groups	Helminth Species	The number of infected sheep (%)	Bands (kDa)				
			24	33	35	46-55	66
Positive (20 sheep)	*F. hepatica*, *Trichostrongylidae* spp.	4 (20)	+	+	+	+	+
	F. hepatica, *Trichostrongylidae* spp. *D. dendriticum*	4 (20)	+	+	+	+	+
	F. hepatica, *Trichostrongylidae* spp., *Cyst hydatid*	2 (10)	+	+	+	+	+
	F. hepatica, *Trichostrongylidae* spp., *C. tenuicollis*	5 (25)	+	+	+	+	+
	F. hepatica, *D. dendriticum*, *Trichuris* spp.	1 (5)	+	+	+	+	+
	F. hepatica, *Paramphistomum* spp., *D. dendriticum*	4 (20)	+	+	+	+	+
Negative (20 sheep)	Trichostrongylidae spp., *C. tenuicollis*,	6 (30)	-	-	+	+	-
	Trichostrongylidae spp., *D. dendriticum*	8 (20)	-	-	+	+	-
	D. dendriticum, *Paramphistomum* spp.	3 (20)	-	-	+	+	-
	Trichostrongylidae spp., *Cyst hydatid*	2 (20)	-	-	+	+	-
	Trichostrongylidae spp., *Cyst hydatid, Trichuris* spp.	1 (20)	-	-	+	+	-

negative controls, the eggs of Trichostrongylidae spp., *D. dendriticum*, Paramphistomum spp. and Trichuris spp. were seen in 17, 11, 3 and 1 sheep, respectively.

SDS-PAGE and Western blotting analysis of crude antigen

Seven protein bands were detected between 6.5 and 205 kDa in polyacrylamide gel cast as separating and stacking gel (Figure 1) in crude antigen. The bands revealed in the sera of positive animals for *F. hepatica* were as follows: 24, 33, 35, 44-55, and 66 kDa (Figure 2). However, bands of 35 and 44-55 kDa were also revealed in the sera of negative animals (Figure 3). Therefore, we conclude that these bands were not specific for *F. hepatica* in crude antigen. According to the results, the specific bands were 24, 33 and 66 kDa determined by Western blotting using the crude antigen prepared. These bands were also revealed in the sera of all the sheep infected with *F. hepatica*. These bands were not detected in any of the 20 sera negative for *F. hepatica* (Table 1).

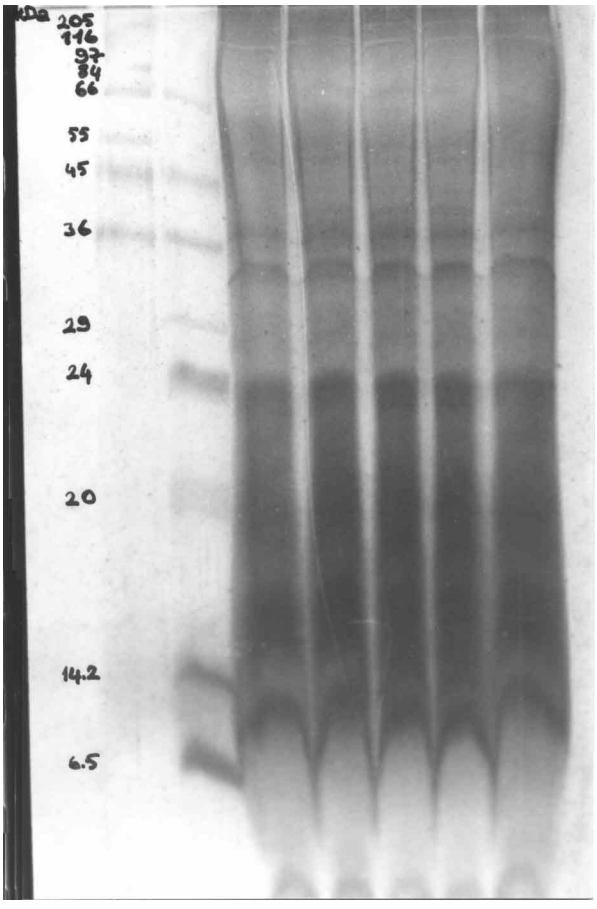

Figure 1. Detected protein bands in crude antigen of *Fasciola hepatica* using SDS-PAGE.

SDS-PAGE and Western blotting analysis of E/S antigen

Twenty-three protein bands were detected between 6.5 and 205 kDa in polyacrylamide gel cast as separating and stacking gel (Figure 4) in E/S antigen. The bands revealed in the sera of positive animals for *F. hepatica* were as follows: 24, 33, 39.5, 42 and 44-55 kDa (Figure

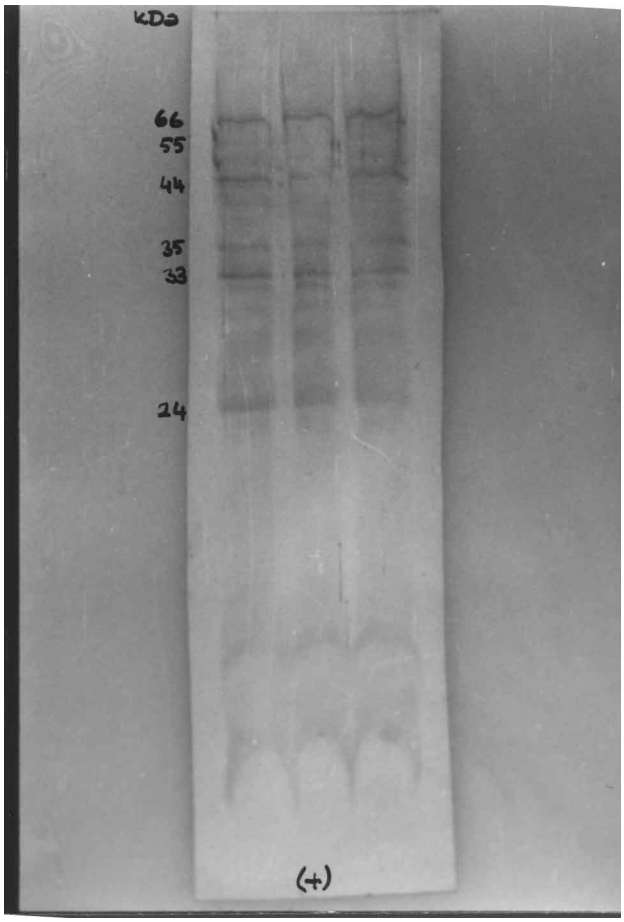

Figure 2. Detected bands in the sera of sheep positive for *Fasciola hepatica* by Western blotting in crude antigen.

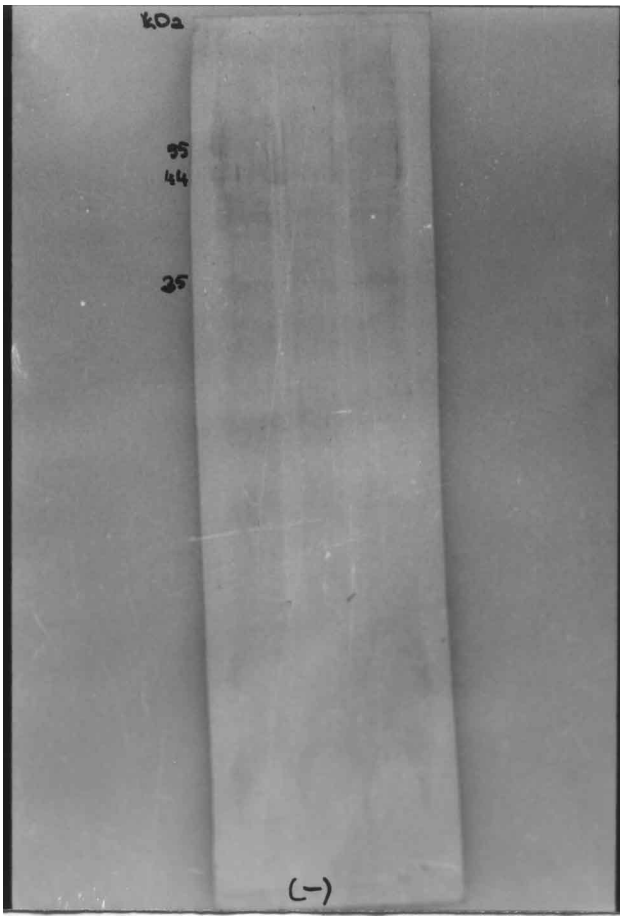

Figure 3. Detected bands in the sera of sheep negative for *Fasciola hepatica* by Western blotting in crude antigen.

5). However, bands of 24 and 44-55 kDa were also revealed in the sera of negative animals (Figure 6). Therefore, we conclude that these bands were not specific for *F. hepatica* in E/S antigen. According to the results, the specific bands were 33, 39.5 and 42 kDa determined by Western blotting using the E/S antigen prepared. These bands were revealed in the sera of all the sheep infected with *F. hepatica*. These bands were not detected in any of the 20 sera negative for *F. hepatica* (Table 2).

Discussion

In recent years, Western blotting has greatly decreased the risk of cross reactions in studies carried out in humans and animals with fasciolosis (8-10). Ruiz-Navarrete et al. (10) studied the immune response of sheep to somatic components and E/S products of adult *F. hepatica*, and they determined that the bands of 20-23 kDa obtained from somatic antigens and 23-27 kDa obtained from E/S antigens could be used for diagnosis. A molecular weight of 24 kDa was observed in our somatic antigen. We think that this band is the same band (20-23 kDa) observed in the study by Ruiz-Navarrete et al. (10).

Hillyer and Soler De Galanes (9) obtained sera from human patients, calves, sheep and rabbits infected with *F. hepatica* and tested the enzyme-linked immunoelectrotransfer blot (Western blot) techniques with *F. hepatica* E/S antigens in order to evaluate their immunodiagnostic potential. Researchers (9) reported that serum samples from humans, rabbits, cattle, and sheep with fascioliosis recognized 2 antigenic polypeptides of 17 and 63 kDa in the form of sharp bands. It is thought that 63 kDa might be the same polypeptide observed at 66 kDa in our somatic antigen.

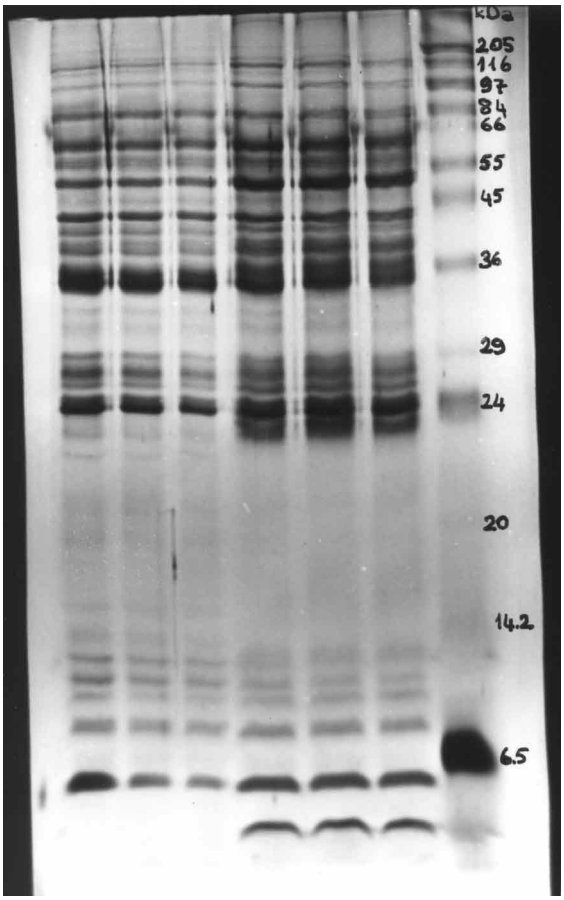

Figure 4. Detected protein bands in E/S antigens of *Fasciola hepatica* using SDS-PAGE.

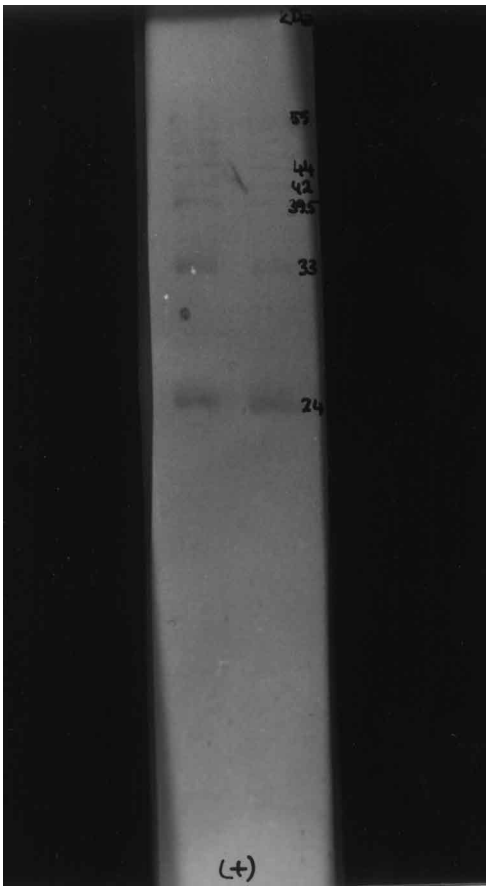

Figure 5. Detected bands in the sera of sheep positive for *Fasciola hepatica* by Western blotting in E/S antigens.

Rivera-Marrero et al. (8) used Western blotting and reported that the bands of 25-30 kDa in E/S antigens were specific for acute and chronic fasciolosis in rabbits, cows and sheep. Fredes et al. (11) analyzed the antigenic components of E/S products of adult *F. hepatica* by SDS-PAGE followed by Western blotting. SDS-PAGE and Western blotting results using serum samples from infected sheep, pigs and horses showed that the 400, 150, 29 and <29 kDa fractions contained polypeptides, specifically recognized only by infected animals. When these fractions were evaluated with serum samples from sheep, horses and pigs by means of ELISA the most efficient fraction was the 29 kDa, exhibiting a mean sensitivity and specificity of 94.5% and 93.5%, respectively. They concluded that the 29 kDa fraction has the potential for the development of a specific ELISA test for the mass screening of fasciolosis. It is seen that the bands at different molecular weights were reported in fasciola studies in sheep as in our study. The sheep used in our study were of the Akkaraman breed, the most common breed in Turkey. It has been reported that genetic variances within different sheep breeds can influence the recognition of different antigens (12). Although the breeds were not defined in the studies given above, the different molecular weights may be due to genetic variances in the sheep.

Sampaio-Silva et al. (13) reported that 25-27 kDa components were recognized by all 20 fasciolosis sera from humans using E/S products of adult worms of *F. hepatica*. Guobadia and Fagbemi (14) found that 17, 21, 57 and 69 kDa protein bands were specific for *F. gigantica* infection in sheep. Even if it seems inappropriate to compare specific antigens tested using the sera from other animals or using antigens from different fasciola species, it is interesting to note that the molecular weights of those antigens are very close to the

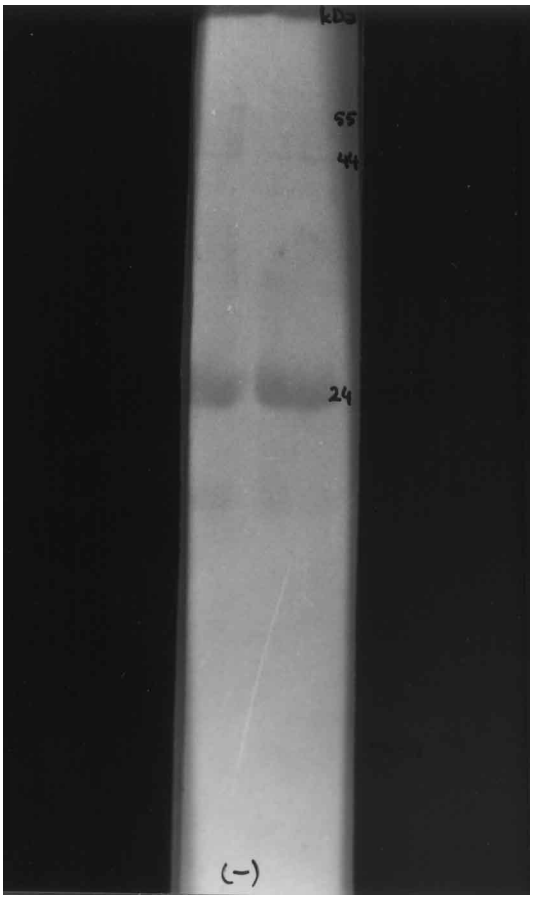

Figure 6. Detected bands in the sera of sheep negative for *Fasciola hepatica* by Western blotting in E/S antigens.

molecular weights of antigens tested with sheep sera in our study. The closeness in the molecular weights of antigens can be seen in the studies by Rivera-Marrero et al. (8), Hillyer and Soler De Galanes (9), Ruiz-Navarrete et al. (10) and ours when compared to each other.

According to the test results in our study, the specific protein bands for *F. hepatica* infection were 33, 39.5 and 42 kDa in E/S antigens and 24, 33 and 66 kDa in crude antigen. There was no animal to animal variation in the recognition by antibodies of some bands, and there were also no major differences in antigen recognition between animals in our study. The 33 kDa band is the common immune reactive band, and it is the most appropriate for use with this antigen. Since this polypeptide was recognized by all infected animals using both E/S and crude antigens, this could play an important role in the diagnosis of sheep fasciolosis.

We conclude that specific protein bands were determined in E/S antigens obtained from *F. hepatica* in this study. Using E/S antigens in Western blotting gave reliable results. The common immune reactive band (33 kDa) between E/S and somatic antigens can be purified using some modern equipment such as Prep-Cell, Rotofor-Cell, and Gel Eluter to use in the diagnosis of *F. hepatica* in sheep.

Table 2. Worm burden and detected specific protein bands in excretory/secretory antigens in positive and negative groups for *Fasciola hepatica*.

Groups	Helminth Species	The number of infected sheep (%)	Bands (kDa)				
			24	33	39.5	42	44-55
Positive (20 sheep)	*F. hepatica*, *Trichostrongylidae* spp.	4 (20)	+	+	+	+	+
	F. hepatica, *Trichostrongylidae* spp. *D. dendriticum*	4 (20)	+	+	+	+	+
	F. hepatica, *Trichostrongylidae* spp., *Cyst hydatid*	2 (10)	+	+	+	+	+
	F. hepatica, *Trichostrongylidae* spp., *C. tenuicollis*	5 (25)	+	+	+	+	+
	F. hepatica, *D. dendriticum*, *Trichuris* spp.	1 (5)	+	+	+	+	+
	F. hepatica, *Paramphistomum* spp., *D. dendriticum*	4 (20)	+	+	+	+	+
Negative (20 sheep)	*Trichostrongylidae* spp., *C. tenuicollis*	6 (30)	+	-	-	-	+
	Trichostrongylidae spp., *D. dendriticum*	8 (20)	+	-	-	-	+
	D. dendriticum, *Paramphistomum* spp.	3 (20)	+	-	-	-	+
	Trichostrongylidae spp., *Cyst hydatid*	2 (20)	+	-	-	-	+
	Trichostrongylidae spp., *Cyst hydatid, Trichuris* spp.	1 (20)	+	-	-	-	+

References

1. Levieux D., Levieux, A., Mage, C., Venien, A.: Early immunodiagnosis of bovine fascioliasis using the specific antigen f2 in a passive hemagglutination test. Vet. Parasitol., 1992; 44: 77-86.
2. Öge, S., Doğanay, A.: Türkiye'de sığır ve mandalarda görülen helmintler. T. Parazitol. Derg., 1997; 21: 435-441.
3. Öge, S., Doğanay, A.: Türkiye'de koyun ve keçilerde görülen helmintler. Kafkas Üniv. Vet. Fak. Derg., 1997; 3: 97-114.
4. Hillyer, G.V., Sanchez, Z., De Leon, D.: Immunodiagnosis of bovine fascioliasis by enzyme-linked immunosorbent assay and immunoprecipitation methods. J. Parasitol., 1985; 71: 449-454.
5. Hillyer, G.V., Soler De Galanes, M.: Identification of a 17-kilodalton *Fasciola hepatica* immunodiagnostic antigen by the enzyme-linked immunoelectrotransfer blot technique. J. Clin. Microbiol., 1988; 26: 2048-2053.
6. Sharma, S.D., Mullenax, J., Araujo, F.G.: Western blot analysis of the antigens of *T. gondii* recognized by human IgM antibodies. J. Immunol., 1987; 131: 977-978.
7. Rodriguez-Perez, J., Hillyer, G.V.: Detection of excretory-secretory circulating antigens in sheep infected with *Fasciola hepatica* and with *Schistosoma mansoni* and *F. hepatica*. Vet. Parasitol., 1995; 56: 57-66.
8. Rivera-Marrero, C.A., Santiago, N., Hillyer, G.V.: Evaluation of immunodiagnostic antigens in the excretory-secretory products of *Fasciola hepatica*. J. Parasitol., 1988; 74: 646-652.
9. Hillyer, G.V., Soler De Galanes, M.: Initial feasibility studies of the fast-ELISA for the immunodiagnosis of fascioliasis. J. Parasitol., 1991; 77: 362-365.
10. Ruiz-Navarrete, M.A., Arriaga, C., Bautista, C.R., Morilla, A.: *Fasciola hepatica*: characterization of somatic and excretory-secretory antigens of adult flukes recognized by infected sheep. Rev. Latinoam. Microbiol., 1993; 35: 301-307.
11. Fredes, F., Gorman, T., Silva, M., Alcaino, H.: Diagnostic evaluation of chromatographic fractions of *Fasciola hepatica* using Western Blot and ELISA in infected animals. Archivos de Med. Vet., 1997; 29: 283-294.
12. Akca, A.: Cellular and Humoral Immune Responses to Recombinant Toxoplasma-antigen, GRA-1 in Sheep Experimentally Infected with Toxoplasma gondii, Master's Thesis. Liverpool School of Tropical Medicine, University of Liverpool, U.K., 1995; p: 116.
13. Sampaio-Silva, M.L., Da Costa, J.M., Da Costa, A.M., Pires, M.A., Lopes, S.A., Castro, A.M., Monjour, L.: Antigenic components of excretory-secretory products of adult *Fasciola hepatica* recognized in human infections. Am. J. Trop. Med. Hyg., 1996; 54: 146-148.
14. Guobadia, E.E., Fagbemi, B.O.: Time-course analysis of antibody response by EITB, and ELISA before and after chemotherapy in sheep infected with *Fasciola hepatica*. Vet. Parasitol., 1995; 58: 247-253.