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Application of Western Blotting for the Immunodiagnosis of *Fasciola hepatica* in Cattle Using Excretory/Secretory Antigens

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Abstract: In this study, protein bands of excretory/secretory antigens of *Fasciola hepatica* were determined using SDS-PAGE and Western blotting. Blood and faecal samples were obtained from cattle brought to Kazan slaughterhouse. After examining the organs and faecal samples of these cattle for *Fasciola hepatica* and other helminths, serum samples were divided into two groups as positive (10 cattle) and negative (5 cattle) for *F. hepatica*. The sera of these two groups were tested using Western blotting. The bands obtained from the sera of positive and negative groups were compared and the specific protein bands for *F. hepatica* infection were 36, 29 and 17 kDa.

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

Sığırlarda Ekskretsiyon/Sekresyon (ES) Antijeni Kullanılarak *Fasciola hepatica*'nin Western Blotting Yöntemi ile Serolojik Teşhisi

Özet: Bu çalışmada, SDS-PAGE ve Western blotting yöntemleri kullanılarak *Fasciola hepatica* ekskretsiyon/sekresyon antijeninin protein bantları ve bu bantlardan spesifik olanlar ortaya çıkarılmıştır. Kazan Mezbahasına getirilen sığırların *F. hepatica* ve diğer helmint enfeksiyonları ile doğal enfekte olup olmadıklarının belirlenmesi amacıyla organ ve dışkı muayeneleri yapılmıştır. Sığırlar *F. hepatica* enfeksiyonu bakımından organ ve dışkı bakıları göz önüne alınarak negative (5 sığır) ve pozitif (10 sığır) olmak üzere iki gruba ayrılmıştır. *Fasciola hepatica* bakımından pozitif ve negatif olan sığırlardan elde edilen serumlar Western blotting yöntemi kullanılarak incelenmiş, elde edilen bantlar karşılaştırılarak *F. hepatica* enfeksiyonu için spesifik protein bantlarının 36, 29 ve 17 kDa olduğu belirlenmiştir.

Anahtar Sözcükler: *Fasciola hepatica*, SDS-PAGE, sığır, Western blotting

Introduction

The liver fluke *Fasciola hepatica* lives in bile ducts and has a wide variety of mammalian hosts from ruminants through to man. The life cycle of the liver fluke is complex including passage through an intermediate molluscan host from which the infective metacercariae are derived. The final host can be infected by eating contaminated plants with metacercariae (1). *F. hepatica* is a cosmopolitan species and surveys of cattle (2) and sheep (3) in different regions of Turkey have shown the prevalence of *F. hepatica* to range from 10 to 90% and 0.5 to 90%, respectively.

The diagnosis of juvenile flukes of *F. hepatica*, which migrate through the liver parenchyma, is not possible via routine laboratory procedures. Early diagnosis of this disease is very important in order to treat patients

successfully. Currently, haemagglutination (HA) (4,5) indirect fluorescence antibody test (IFAT), immunoperoxidase (IP) (6), counterelectrophoresis (CEP) (7) and enzyme-linked immunosorbent assay (ELISA) (8-12) are used in the early diagnosis of this disease, but they have some disadvantages such as cross-reactions with other trematodes leading to false positive results. Therefore, the reliability of these tests is not high.

In recent years, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting procedures have initiated a new era in immunodiagnosis which greatly reduced cross-reactions (13). These techniques were used as a verifying test in the diagnosis of viral and bacterial infections at first, but lately these techniques have been used in the field of parasitology (14,15).

A variety of antigens are secreted and excreted by parasites present in the blood, faeces, urine and other fluids of the infected host. These antigens have potential for use in immunodiagnosis and vaccine development (16). In previous immunodiagnostic studies on *F. hepatica* using SDS-PAGE and Western blotting, it was determined that excretory/secretory antigens were more specific than other somatic and surface antigens (10,11). The immune response of excretory/secretory products of adult *F. hepatica* was studied in cattle (17-20) and bands 15, 17, 25-30, 63 and 60-66 kDa were found to be specific. Researchers reported that the diagnosis of *F. hepatica* infection was detected as early as two weeks after infection (21). In Western blotting studies using excretory/secretory antigens in humans and other animals (sheep, deer, horses, pigs) infected with *F. hepatica*, it was determined that bands 25, 27, 29, 57 kDa were specific for humans (22,23), bands 17, 23-27, 25-30 and 63 kDa for sheep (19-21), band 15 kDa for deer (18), and bands 22-30 kDa for horses and pigs (24).

Western blotting is a good confirmation test for the diagnosis of fasciolosis, but further studies should be carried out to detect the most specific bands. The aim of the present work was to determine specific protein bands for fasciolosis from the sera of sheep naturally infected with *F. hepatica*. After obtaining these research results, further studies will be carried out in the future to improve ready-made kits for the diagnosis of acute fasciolosis in human and animals.

Materials and Methods

This study was carried out in cattle slaughtered in the municipal slaughterhouses of Kazan. Blood and faecal samples were obtained from cattle brought in for slaughtering. After examining the organs and faecal samples of the cattle for *F. hepatica* and other helminths carefully, serum samples were divided into two groups as positive and negative groups for *F. hepatica*.

Preparation of antigens *F. hepatica* were collected from bile ducts and washed six 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 caecal 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:

Excretory-secretory products (E/S) isolated parasites were washed six times with 0.01 M PBS, (pH 7.2) and a further six 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 ml of medium (1 fluke per 3 ml) at 37 °C in a 5% CO₂ incubator for 24 h. After incubation the supernatant was collected and centrifuged at 5000 g for 30 min at 4 °C. The samples aliquoted and stored at -70 °C.

Polypeptide analysis Excretory-secretory antigens were obtained from *F. hepatica* and separated by SDS-PAGE. Proteins were visualised with silver stain technique and their molecular weights were determined by comparison with molecular weight standards. 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 found to be the best amount. One protein standard was used, and this was the Sigma wide molecular weight range (Sigma Chemical Co., M-4038, St. Louis, MO, USA). SDS-PAGE, Western blotting and the preparation of solutions were performed as described by Sambrook et al. (25).

Antigenic Analysis Antigenically active components among SDS-PAGE resolved bands were detected by the Western blotting method. After SDS-PAGE, the proteins were transferred electrophoretically onto nitrocellulose sheets using transfer blot apparatus. Gels were fixed and stained with Panceau-S to determine the molecular weights of the proteins. Nitrocellulose containing transferred samples was incubated overnight at 4 °C in 3% nonfat dried milk, and rinsed in PBS prior to 2 h incubation with sera containing test antibodies. Following three PBS washes to remove unbound antibodies, the nitrocellulose sheets were incubated for 1 h in horseradish peroxidase conjugated anti-IgG (Sigma Chemical Co., A-8917, St. Louis, MO, USA). Unbound conjugate was removed by three PBS washes before addition of substrate solution containing 3,3'- Diaminobenzidine (DAB) (Sigma Chemical Co., D-4293, St. Louis, MO, USA). Bands were visible within 15 min and development was stopped by removing substrate with distilled water and air drying the nitrocellulose.

Results

In the macroscopic examination of the organs and carcasses of cattle, *F. hepatica* was seen in 10 out of 15 cattle, and hydatid cysts were seen in three of these animals. Faecal examinations of the animals were carried out by using sedimentation and flotation procedures. According to these examinations, *F. hepatica* were seen in 10, *Dicrocoelium dendriticum* in 5, *Paramphistomum* spp. in 2 and *Trichostrongyloides* spp. in 10 cattle. Positive and negative sera for *Fasciola hepatica* determined according to examinations of organs and faeces were tested by SDS-PAGE and Western blotting (Table).

In this study, 19 protein bands were detected between 6.5 and 205 kDa in polyacrylamide gel casted as separating and stacking gel (Figure 1). The bands revealed in the sera of positive animals for *F. hepatica* were 97, 36, 29 and 17 kDa. But the band of 97 kDa was also revealed in the sera of negative animals. Therefore, we conclude that 97 kDa was not a specific protein band for *F. hepatica*. According to the results, the specific bands were 36, 29 and 17 kDa determined by Western blotting using the prepared excretory-secretory (E/S) antigen (Figure 2). These bands were revealed in the sera of all cattle infected with *F. hepatica*. These bands were not detected in any of the 5 negative sera. No bands observed in nitrocellulose membrane belong to other helminth infections in positive and negative groups for *F. hepatica*.

Discussion

In recent years, SDS-PAGE and the Western blotting techniques have been widely used in the diagnosis of parasitic diseases. The Western blotting test greatly

decreased the risk of cross-reactions in studies carried out in humans and animals with fasciolosis (15).

Ortiz et al. (17) used E/S, somatic (SO) and surface (SU) antigens of adult *F. hepatica* for antibody response determination in dairy cattle naturally infected with *F. hepatica*. They reported that antibody responses were developed against 60-66 kDa in E/S and SU antigens and 17 kDa in SO antigen. Qureshi et al. (18) used E/S antigens and reported that at approximately 15 kDa *F. hepatica* E/S antigens can be used for species specific diagnosis in cattle. Hillyer and Soler De Galanes (19), obtained sera from human patients, calves, sheep, and rabbits infected with *F. hepatica* and tested the enzyme-linked immunoelectrotransfer blotting (Western blotting) techniques with *F. hepatica* E/S antigens in order to evaluate their immunodiagnostic potential. Researchers reported that the serum samples from humans, rabbits, cattle, and sheep with fasciolosis recognized two antigenic polypeptides of 17 and 63 kDa in the form of sharp bands. Rivera Marrero et al. (20) used the Western blotting technique and reported that bands 25-30 kDa in E/S antigens were specific for acute and chronic fasciolosis in rabbits, cows and sheep. Three specific bands were detected in our study. These bands were 36, 29 and 17 kDa. Similarities can be seen when the studies of Ortiz et al. (17), Hillyer and Soler De Galanes (19), Rivera Marrero et al. (20) and ours are compared.

We conclude that specific protein bands were determined in E/S antigens obtained from *F. hepatica*. According to our study, using E/S antigens in serologic tests give reliable results. However, specific proteins should be purified by using modern equipment such as Prep-cell, Rotofor-Cell and Gel Eluter to prepare kits in the diagnosis of fasciolosis.

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

Groups	Helminth Species	The number of infected cattle (%)	Bands (kDa)			
			17	29	36	97
Positive	<i>F. hepatica</i>	1 (10)	+	+	+	+
	<i>F. hepatica</i> , <i>Trichostrongylidae</i> spp. <i>D. dendriticum</i>	2 (20)	+	+	+	+
	<i>F. hepatica</i> , <i>Trichostrongylidae</i> spp., <i>Cyst hydatid</i>	2 (20)	+	+	+	+
	<i>F. hepatica</i> , <i>Trichostrongylidae</i> spp.	3 (30)	+	+	+	+
	<i>F. hepatica</i> , <i>D. dendriticum</i>	1 (10)	+	+	+	+
	<i>F. hepatica</i> , <i>Paramphistomum</i> spp.	1 (10)	+	+	+	+
	<i>Trichostrongylidae</i> spp.	2 (40)	-	-	-	+
Negative	<i>Trichostrongylidae</i> spp., <i>D. dendriticum</i>	1 (20)	-	-	-	+
	<i>D. dendriticum</i> , <i>Paramphistomum</i> spp.	1 (20)	-	-	-	+
	<i>Cyst hydatid</i>	1 (20)	-	-	-	+

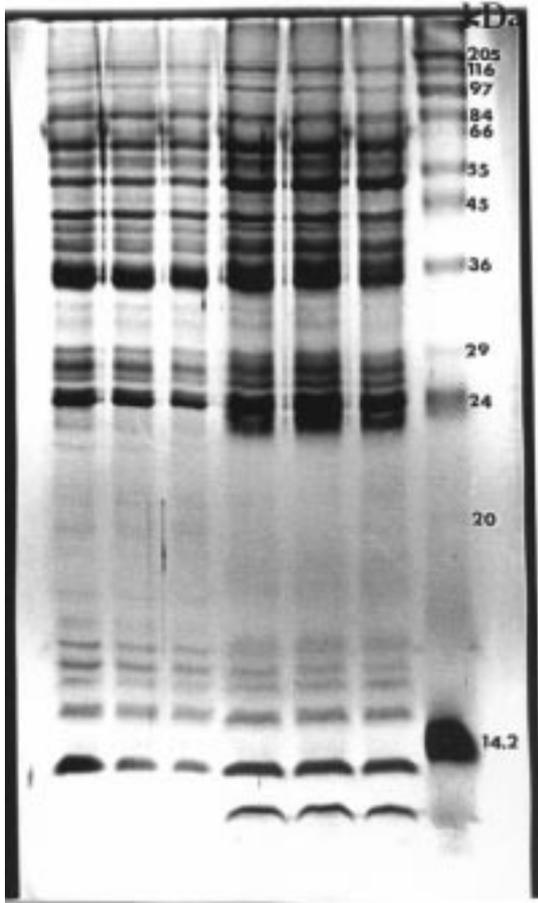


Figure 1. Detected protein bands in E/S antigens of *Fasciola hepatica* using SDS-PAGE.

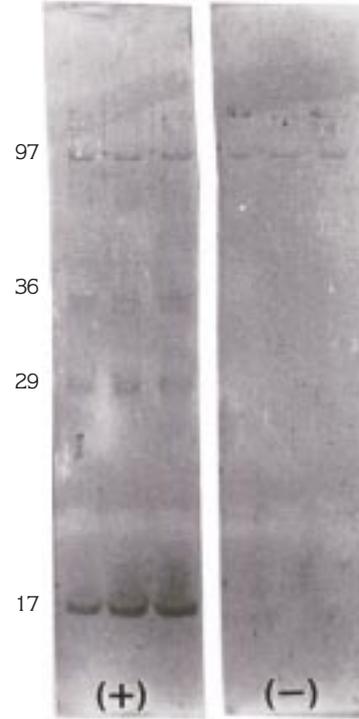


Figure 2. Detected bands in the sera of cattle positive and negative for *Fasciola hepatica* by Western blotting.

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