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Application of Western Blotting and Enzyme Linked Immunosorbent Assay (ELISA) for the Diagnosis of *Dicrocoelium dendriticum* in Sheep Using Excretory Secretory (E/S) Antigens

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Abstract: Protein bands of excretory/secretory (E/S) antigens of *Dicrocoelium dendriticum* were determined using SDS-PAGE and Western blotting. The E/S antigens were prepared from live adult *D. dendriticum*, which were obtained from slaughtered sheep at a local abattoir. In the SDS-PAGE analysis of *D. dendriticum* E/S antigen, 9 protein bands with molecular weights varying between 6 and 66 kDa with Coomassie staining and 14 bands between 6 and 205 kDa with silver staining were detected. Blood samples were obtained from sheep at the local abattoir. Following the examination of the organs for *D. dendriticum* and other helminths, serum samples were divided into 2 groups as positive (n = 23) and negative (n = 17) for *D. dendriticum* and the sera were tested using Western blotting. The bands obtained from the sera of positive and negative groups were compared and the molecular weight of specific protein band for *D. dendriticum* infection was determined as 205 kDa. On the other hand, the ELISA technique was used to study the response of IgG antibodies against E/S antigens of *D. dendriticum* in the sera of 40 sheep. The sensitivity and specificity of the test were 82.6% and 76.4%, respectively.

Key Words: *Dicrocoelium dendriticum*, E/S antigen, sheep, Western blotting, ELISA.

Koyunlarda Ekskresyon/Sekresyon (E/S) Antijeni Kullanılarak *Dicrocoelium dendriticum*'un Western Blotting ve Enzyme-Linked Immunosorbent Assay (ELISA) ile Tanısı

Özet: Bu çalışmada *Dicrocoelium dendriticum*'un ekskresyon/sekresyon (E/S) antijeninin protein bantları SDS-PAGE ve Western blotting yöntemleri kullanılarak tespit edilmiştir. Antijen, mezbanede kesimi yapılan koyunlardan elde edilen erişkin, canlı *D. dendriticum*'lardan hazırlanmıştır. *Dicrocoelium dendriticum* E/S antijeninin SDS-PAGE ile analizi sonucu yapılan Coomassie boyaması ile moleküler ağırlığı 6 ile 66 kDa arasında değişen 9 farklı protein bandı tespit edilmiş olup gümüş boyama neticesinde moleküler ağırlığı 6 ile 205 kDa arasında değişen 14 farklı band elde edilmiştir. Western blotting deneyi için mezbanede kesimi yapılan koyunlardan kan alınmış ve hem *D. dendriticum* hem de diğer helmintler bakımından yapılan muayeneye göre pozitif (n = 23) ve negatif (n = 17) olmak üzere iki grup oluşturulmuştur. Pozitif ve negatif örneklerin Western blotting ile analizi sonucunda elde edilen bantların karşılaştırılması neticesinde 205 kDa'luk bandın *D. dendriticum* için spesifik olduğu belirlenmiştir. Ayrıca, 40 koyunda *D. dendriticum*'un E/S antijenine karşı oluşmuş IgG antikorları indirekt-ELISA ile belirlenmiş ve bu testin sensitivitesinin % 82,6, spesifitesinin % 76,4 olduğu tespit edilmiştir.

Anahtar Sözcükler: *Dicrocoelium dendriticum*, E/S antijen, koyun, Western blotting, ELISA.

Introduction

The small liver fluke, *Dicrocoelium dendriticum* (Rudolphi, 1819) Looss, 1899, affects numerous mammalian species, mainly ruminants, in several countries in Europe, Asia, America, and North Africa (1). The biological cycle of *D. dendriticum* takes about 6 months, indirect species of land molluscs and ants are its primary and secondary intermediate hosts, respectively, and ruminants are its definitive hosts (2). The young

flukes migrate directly up the biliar duct system of the liver without penetrating the gut wall liver capsule, or liver parenchyma as seen in fasciolosis (3). Early reports (4) suggest that alteration in the ovine liver resulting from the invasion by the lancet fluke increases in severity and extent with duration of infection, leading ultimately to cirrhosis. The parasitic loads increase with continuous exposure to infection. As the worm burden increases, the pathological changes become more severe. It has been

postulated that the bile ducts are exposed to a continuous and long-standing irritation due to products of the parasite (4,5). This results in their inflammation and stimulation, and the deposition of connective tissue around the ducts. The connective tissue eventually spreads to obliterate most of the hepatic parenchyma (4).

Dicrocoeliosis often remains clinically undetected or undiagnosed, most likely because of its subclinical nature (6). Its diagnosis is mainly due to recovering adults from the liver at necropsy or detecting eggs at coprological examination (2). In general, the prepatent diagnosis for many parasitic infections could be carried out by detection of antibodies or circulating parasite antigens (7). Immunological methods for the detection of dicrocoeliosis have been developed in the recent years, as an alternative to coprological examinations and postmortem inspections of the liver (2). Many immuno-diagnostic techniques, as well as immuno-fluorescence precipitation (8), passive hemagglutination test, complement fixation, ELISA, and Western blotting (9-11) have been employed to detect anti-dicrocoelium antibodies in naturally and/or experimentally infected animals.

Immunological methods like the indirect-ELISA have an advantage over the standard parasitological (fecal) technique because *D. dendriticum* infection can be detected as early as 30 days after infection (11), although a positive ELISA result is an indication only of the exposure to the parasite. SDS-PAGE and Western blotting have been used as confirmation tests in the diagnosis of viral and bacterial infections at first, but lately these techniques have been utilized in the field of parasitology as well (12).

The current study aimed to characterize and isolate the specific protein bands for *D. dendriticum* antigens from the sera of sheep naturally infected with *D. dendriticum* using E/S antigen. On the other hand, another aim of this study was to evaluate the sensitivity and specificity of ELISA using the same antigen and sera. As a result, these 2 tests will be compared and a more sensitive test will be detected for the serodiagnosis of sheep dicrocoeliosis.

Materials and Methods

This study was carried out in sheep slaughtered at a local abattoir in Elazığ province (Turkey). Blood samples were obtained from Akkaraman sheep brought in for

slaughtering. After examining the organs of sheep for *D. dendriticum* and other helminthes carefully, especially the liver and bile ducts, serum samples were divided into 2 groups as positive (n = 23) and negative (n = 17) groups for *D. dendriticum*. Examination of all organs was done at the slaughterhouse.

Preparation of antigen

Excretory/secretory antigens of *D. dendriticum* adult flukes were prepared as previously described (11) with some modifications. Adult flukes were collected from the bile ducts of naturally infected sheep, and washed several times distilled water (approximately, 10 flukes per ml) and 0.01 M phosphate buffered saline (PBS) at 37 °C. The flukes were then left for 6 h in PBS at 37 °C to get rid of intestinal contents. Eggs were removed by sieving and centrifugation (30 min at +4 °C and 10,000 rpm) and the supernatant containing excretory/secretory (E/S) products filtered from 0.2 µm pore size filter. Then these products were dialyzed against distilled water for 24 h. After dialyzing, 5 mM EDTA and 5mM sodium azide were added to antigen for protection of protein. The samples were aliquoted and stored at -20 °C until used.

Polypeptide analysis

To determine specific protein bands of *D. dendriticum* E/S antigen, separating (12.5%) and stacking (5%) gel was subjected to SDS-PAGE (in reducing condition). Then 15, 20, 25 and 30 µl of the antigen were loaded onto this gel and 25 µl of antigen was found to be the optimum amount. One protein standard was used, and this was the Oncogene, pre-stained protein molecular weight markers (Oncogene Research Products, Cat No. MW03).

Antigenic analysis

After SDS-PAGE was applied, polypeptides were transferred onto nitrocellulose membrane using a transfer blot apparatus (EBU-102, C.B.C. Scientific Company. Inc.). Blotting was carried out for 2 h at 60 V, 0.22 A. Nitrocellulose containing-transferred samples were incubated overnight at +4 °C in 5% skimmed milk powder and rinsed twice by PBS (pH 7.4) containing 0.1% Tween-20. After washing, nitrocellulose membrane cut into strips and incubated with sera (diluted 1:50) for 2 h. Following 5 washes to remove unbound antibodies,

the strips were incubated with peroxidase labeled-antibodies against sheep IgG (Sigma A3415, St. Louis, MO, USA) (diluted 1:1000). Unbound conjugate was removed by 5 subsequent washes (PBS containing 0.1% Tween-20) before the addition of substrate solution containing 3,3- Diaminobenzidine (DAB) substrate tablets (Amresco Lot: 1571B66). Bands were visible within 15 min and the reaction was stopped by removing substrate with distilled water and air drying the strips. SDS-PAGE, staining and Western blotting were performed as described by Sambrook et al. (13).

Indirect-ELISA

This technique was performed according to Sánchez-Andrade et al. (14) with little modifications. ELISA plates (Dynatech Laboratories, IA, USA) were coated with 100 µl of 4 µg/ml of E/S antigens in 0.01 M carbonate/bicarbonate buffer (pH 9.6) per well. Following overnight incubation the plates were washed twice with PBS containing 0.1% Tween 20 (PBS/Tween) and blocked with 130 µl per well of a solution containing 5% skimmed-milk powder in 0.1 M PBS (pH 7.4) for 1.5 h at 37 °C. After blocking, the plates were washed 3 times with PBS/Tween, and 100 µl sera diluted 1:20 in PBS containing 0.02% Tween 20 was added to the wells, followed by incubation for 1.5 h at 37 °C. The plate was again washed 5 times and 100 µl of a 1:1000 anti-sheep IgG conjugated to donkey peroxidase (Sigma Co. St. Louis, MO, USA Cat no: A3415) was added to the wells and the plate incubated at 37 °C for 2 h. Finally, after 5 washes, 100 µl of substrate containing O-phenylene diamine (OPD) and hydrogen peroxide (H₂O₂) in citrate/phosphate buffer was 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 sulfuric acid and the plate was read at 450 nm on an ELISA reader (Medispec ESR 200). The results are expressed as the mean of the optical density (OD). All samples were studied in duplicate with repetition when sera were up to 10% different. The cut-off value was calculated as the mean of the negative control sera absorbance values plus 3 standard deviations.

Results

In the macroscopic examination of the organs and carcasses of sheep, *D. dendriticum* was seen in 23 out of

40 sheep and hydatid cysts detected in 3 positive and 2 negative sheep. All liver bile ducts were examined, but *F. hepatica* was not encountered.

In this study, 9 protein bands with molecular weights varying between 6 and 66 kDa were detected in SDS-PAGE analysis and staining with Coomassie of *D. dendriticum* E/S antigen (Figure 1).

The same SDS-PAGE gel was stained by the silver staining method and 14 bands with molecular weights between 6 and 205 kDa were shown (Figure 2).

The bands revealed in the sera of positive animals (n = 23) for *D. dendriticum* were 205 and 98 kDa (Figure 3).

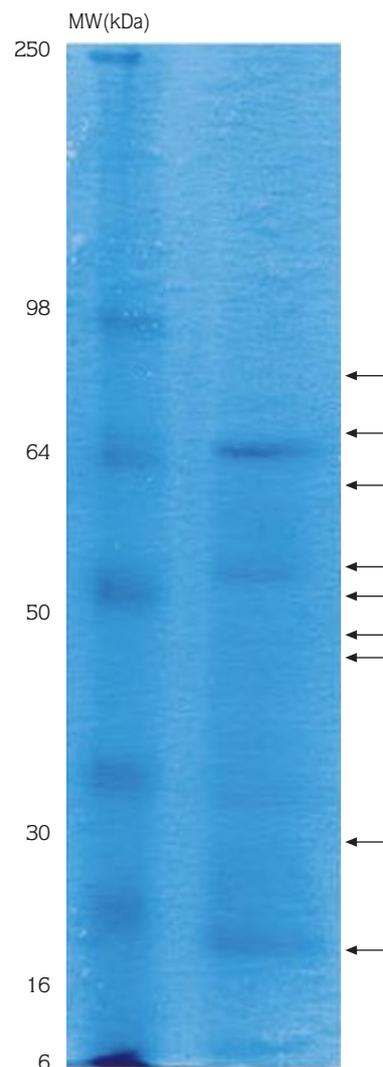


Figure 1. Detected protein bands in E/S antigens of *Dicrocoelium dendriticum* using SDS- PAGE and staining with coomassie blue.

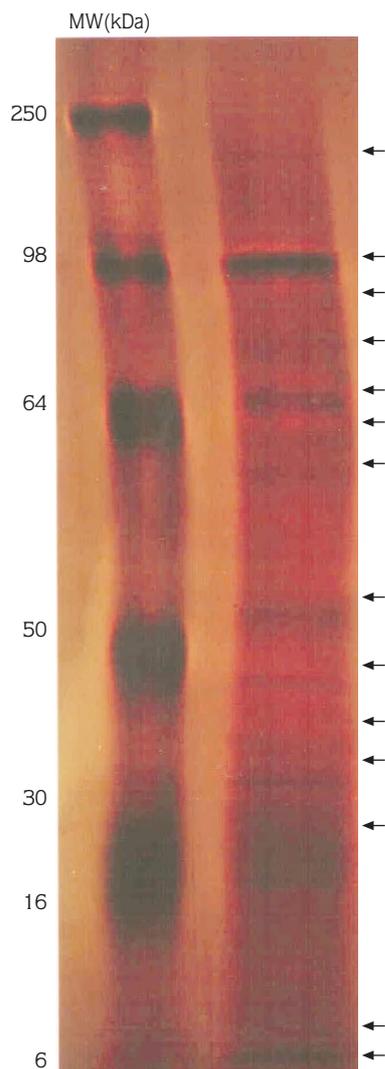


Figure 2. Detected protein bands in E/S antigens of *Dicrocoelium dendriticum* using SDS- PAGE and staining with the silver stain method.

However, the band of 98 kDa was also revealed in the sera of negative animals ($n = 17$) (Figure 4). Therefore, we conclude that 98 kDa was not a specific protein band for *D. dendriticum*. According to the results, the specific band was 205 kDa determined by Western blotting using the prepared E/S antigen. This band was revealed in the sera of all sheep ($n = 23$) infected with *D. dendriticum*. No bands specific for hydatidosis were observed in nitrocellulose membrane belonging to the hydatid cyst infections ($n = 5$) in the positive or negative groups.

In the analysis of the positive and negative sheep sera by ELISA, out of 23 positive sheep 19 were detected as positive and 4 as negative (Figure 5), whereas out of 17

negative sheep 13 were evaluated as negative and 4 as positive (Figure 6). As a result, the sensitivity of indirect-ELISA for dicrocoeliosis in sheep was found to be 82.6% with a specificity of 76.4%.

Discussion

There are few investigations on the ovine immune response against *D. dendriticum* (14). The somatic (SO) and excretory/secretory products (E/S) of *D. dendriticum* have been studied as antigens for ELISA and Western blotting, and their chemical characteristics have been examined in terms of both bile (9,10) and serum antibody response (10,11). The results showed that SO proteins and surface molecules of *Dicrocoelium* stimulate a more conspicuous antibody response (9), although problems of low specificity and sensitivity could occur when using entire worm extracts instead of E/S or purified antigens. In this study, we used E/S antigen for determining specific antibodies against *D. dendriticum* in sheep, because of high sensitivity and specificity as well as preparation being easier than for somatic antigen.

Our results on *D. dendriticum* antigenic composition detected by SDS-PAGE show a greater number of bands in E/S products. Wedrychowicz et al. (15), using the same technique, detected 8 to 9 polypeptides from 29 to 205 kDa in the parasite surface proteins and 17 bands in SDS-soluble or somatic proteins extracted with TBS. Also Revilla-Nuin et al. (7) found 36 polypeptides band in the somatic extracts of parasite as well as 18 bands in the E/S products. In the SDS-PAGE analysis of *D. dendriticum* E/S antigen, we detected 9 protein bands of molecular weight 6-66 kDa with Coomassie staining and 14 bands of 6-205 kDa with silver staining. Previously, Revilla-Nuin et al. (7) stressed that 205, 130, 62, 50, 44, 42 and 26 kDa proteins were not stained by Coomassie in the SDS-PAGE and the use of 10% polyacrylamide gels and silver stain is necessary to visualize the bands. Therefore, we stained the SDS-PAGE gel by both Coomassie and silver staining methods in order to show all bands.

In the Western blot analysis, we detected 2 polypeptides in E/S antigen with molecular weights of 98 and 205 kDa, using sera from sheep naturally infected with *D. dendriticum*. Our results partially coincide with those reported by Revilla-Nuin et al. (7), who used Western blot to analyze the somatic and E/S antigens of *D. dendriticum*, and found 8 main antigenic polypeptides ranging from 24 to 205 kDa in the somatic antigen and



Figure 3. Detected bands in the sera of sheep positive for *Dicrocoelium dendriticum* by Western blotting.

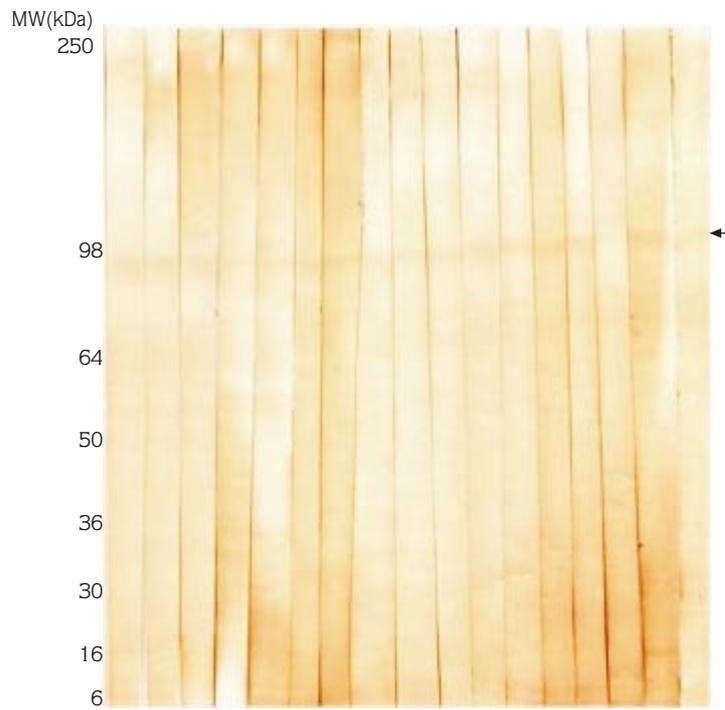


Figure 4. Detected band in the sera of sheep negative for *Dicrocoelium dendriticum* by Western blotting.

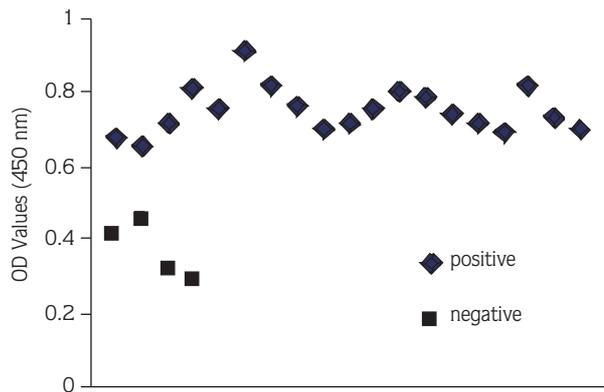


Figure 5. Optical Density (OD) values obtained by indirect ELISA technique using positive sheep sera (Cut-off: 0.629).

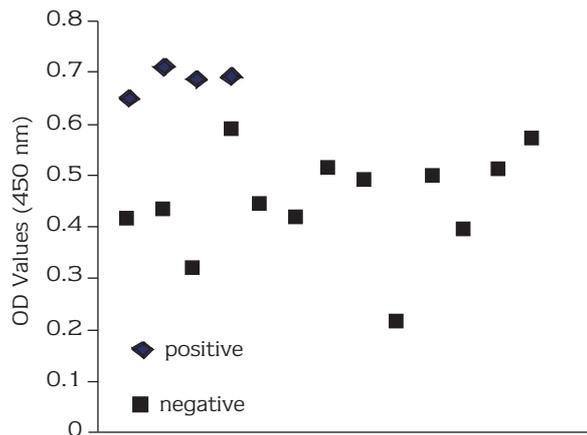


Figure 6. Optical Density (OD) values obtained by indirect ELISA technique using negative sheep sera (Cut-off: 0.629).

7 polypeptides (from 26 to 205 kDa) in the E/S products. However, other researchers (7), according to the Western blot and indirect-ELISA results, reported that the 130 kDa protein is suitable for the immunodiagnosis of dicrocoeliosis. Savitskii and Benediktov (16) obtained 6 protein fractions, although only 3 of them, ranging from 31 to 40 kDa, showed serological activity.

In our study, in order to eliminate cross reactions all sheep were carefully inspected especially for *F. hepatica* infection, and it was not found. In the Western blot analysis of sheep sera *F. hepatica* specific protein bands reported from Gönenç et al. (17) were not detected. Actually, Gönenç et al. (17) indicated that 42, 39.5 and 33 kDa bands were specific for *F. hepatica* E/S antigens for sheep.

Wedrychowicz et al. (10) clearly demonstrated that antibodies specific for certain *D. dendriticum* antigens occur in the sera and bile of naturally infected sheep. Wedrychowicz et al. (10) stressed that IgG levels against E/S antigen were approximately 4 times higher than surface protein antigens in the same sheep. We detected a specific antibody response against E/S antigen by Western blotting in sheep.

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Sánchez-Andrade et al. (14) detected the sensitivity and specificity of indirect-ELISA in sheep as 86% and 93%, respectively. In our study, while the sensitivity rate was similar to that former (14), the specificity rate was lower. Although both the antigen and the number of sheep were the same, the reason for the low specificity could not be explained. Many researchers (11,14,18,19) have reported that ELISA is effective for an early diagnosis of dicrocoeliosis. According to the present findings, indirect-ELISA is a moderate diagnostic test for dicrocoeliosis in sheep, whereas Western blotting is a good confirmation test and more sensitive and specific than indirect-ELISA.

In conclusion, only a very limited number of papers have been published on the detection of *D. dendriticum* antibodies in contrast to those on the other liver fluke such as *Fasciola* spp. in livestock. Therefore, the results of the present study demonstrate that the Western blotting test is a valid technique for the determination of *D. dendriticum* infections in sheep.

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