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Comparison of Fecal Egg Counts and Elisa for the Diagnosis of *Dicrocoelium dendriticum* Infection

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Abstract: From economical point of view, *Dicrocoelium dendriticum (D. dendriticum)* is considered to be a parasite which causes a lot of damages to the livestock industry annually. So, the rapid diagnosis of infection is very important. The rate of infection varies in some areas and it is reported to be 86%. The diagnosis of infection is based on egg per count of faeces (EPG) because diagnosis according to clinical symptoms is difficult. Since EPG is not accurate and sensivite, the serological methods become important for the diagnosis of this parasite. Serological tests are more accurate in comparison to EPG test and they are able to diagnose infection in a short time. Serological tests will also become important in Epidemiology studies.In this study, somatic and Excretory-secretory antigens (EsAg) were isolated. The ELISA test was set up according to positive and negative sera and the results which were obtained compared to the ones obtained by the EPG. The prevalence of infection in 550 samples by ELISA and EPG methods were 56% and 7% respectively, which shows the significant difference between these methods in examining the rate of infection. Based on the results, the specificity and sensitivity in ELISA test were 95% and 94% respectively. The results showed that the ELISA test is a more reliable test in comparison to EPG test for the rapid diagnosis of *D. dendriticum* infection.

Abbreviations: Elisa: enzyme-linked immunosorbent assay · SDS Page: Sodium dodecyl sulfate polyacrylamide gel electrophoresis · EPG: eggs per gram · EsAg: Excretory–secretory antigens · SoAg: Somatic antigens

Key words: *Dicrocoelium dendriticum* · ELISA · EPG · Somatic antigen and Excretory-secretory antigen

INTRODUCTION

Dicrocoelium dendriticum is one of the most prevalent liver trematodes in animals. This fluke lives inside the binary ducts of liver of cow, sheep, goat, pig and rarely in human [1-4]. Anemia, inflammation, Icterus, progressive liver cirrhosis and body wasting are among the symptoms of being infected by this parasite [5, 6]. The pathogenesis of disease does not usually cause death, but the chronic infection causes a lot of economic damages in livestock industry which includes a reduction in the production of meat, milk and wool. And also, a large number of infected livers are obliterated [7].

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The diagnosis of parasite is done by EPG method which is not an accurate test and the rate of the diagnosis of the infected animals is very low [8]. Since the number of eggs in faeces is relatively low, the diagnosis of infection with low intensity is difficult or impossible [9]. So an appropriate serology method is used to examine the rate of infection. One of the most common tests is the ELISA method. This method has many advantages which include: The presence of antibodies against the parasite can be examined in sheep blood four weeks after the infection [10], it is very simple and a large number of samples can be tested in a short time and this method is highly accurate and efficient. This method is used as the appropriate method for epidemiological diagnosis studies [11-14]. Rapid diagnosis and treatment of the infection reduces the economic damages.

In this study, EsAg and somatic antigens were isolated, the immunogenic antigens were identified by the use of SDS-page gel and western blot, then these antigens were purified and they were coated in ELISA microplates. The ELISA test was optimized by positive and negative samples and the sensitivity and specificity of the test was measured. Then ELISA was used for detection serum antibodies against *D. dendriticum* in 550 sheep randomly chosen in Mashhad (Iran). In the end, the serology results were compared with EPG method and the prevalence of infection was reported serologically for the first time in our country.

MATERIALS AND METHODS

Excretory–Secretory Antigen: EsAg antigens from *D. dendriticum* adult worms were obtained as previously described [10] with some modifications. The collected worms were washed in phosphate-buffered saline (PBS pH 7.4) with gentamycin (40 mg/l Sigma) at 37°C three times. Worm viability was checked on the basis of motility of the worms observed under astereomicroscope. After removing the parasites, medium containing EsAg products was collected and centrifuged at 10,000g for 15 min at 4°C. The supernatant was filtered through 0.22µm pore size filter units and then a cocktail of protease inhibitors (Sigma) was added. The fluid was finally concentrated by YM-50 membranes (50,000 molecular weight cut-off). Protein concentration was determined by the Bradford method [15]. Samples were aliquoted and stored at 80°C until used.

Somatic Antigen: Adults of *D. dendriticum* were washed several times in PBS pH 7.4. Approximately 200 washed flukes were placed in 8 ml PBS with the following protease inhibitors: EDTA (Merck) 1 mM, NEM (N-ethylmaleimide) (Merck) 1 mM, PMSF (phenyl methyl sulfonyl fluoride) (Merck) 1 mM and Pepstatin A (Merck) 0.1 lm. Then, they were sonicated by an ultrasound processor with pulse-type vibration for 1 min and 3 pulses of 30 sec each. Finally, the suspension was centrifuged at 10,000g for 30 min and the supernatant fraction collected [10]. After estimating the protein concentration by the method of Bradford, the antigen was stored in small aliquots at 80°C until used.

Indirect ELISA: Antigenic bands purified by electro elution and plates were coated with pure antigen (Es or So) in NaHCO3/Na2CO3 (Merck) buffer pH 9.6 at 100 µl/well and then incubated overnight at 4°C. After removal of the antigen, the plates were washed three times with PBS containing 0.05% Tween 20 (PBST) (Merck) for 5 min each. The unbound sites in each well were blocked by incubation in a humidity chamber for 1 h at 37 °C with 100 µl 5% skimmed milk diluted in PBST. Then, 100 µl sera from infected sheep were added and incubated for 1 h at 37 °C. After incubation for 2 hr in the sera, the plates were washed three times with PBST. All sera were tested in duplicate. Then 100 µl anti-sheep IgG peroxidase conjugate (Jackson) (diluted to 1:5,000 in PBST) was added to each well. The plates were incubated for 1 h at 37 °C and then washed three times as previously described. After a new wash, 100 µl of a solution of 3,3',5,5' - tetramethylbenzidine (TMB) (Sigma) was added to each well and the plates were incubated for 15 min in darkness at room temperature [11]. The color reaction was stopped by the addition of 100 µl 1N sulfuric acid (Merck) and optical density values were read at 450 nm using an Automated Microplate Reader Elx800 (Bio-Tek Instruments, USA). Each serum sample was tested in duplicate and the results were expressed as the mean of the OD values obtained. For antibody detection we considered as positive those samples in which mean OD values exceeded the average of negative controls plus three standard deviations of that mean (the cut-off point). Twenty sheep were used as negative controls. The data were analyzed by ANOVA and Tukey's multiple comparison test.

SDS-PAGE: 30 µg of somatic and EsAg were electrophoresed using 12.5% SDS-PAGE under reducing condition. Gels were stained with Coomassie blue (Fermentas) [21].

Western Blot: immunoblotting was used to reveal which antigen is immunized. Small aliquots (10 ìl) of extracts were reserved for protein determination using Bradford assay reagent from Fermentas (Fermentas, USA). Proteins were separated by SDS-poly acrylamide electrophoresis in slab gels of 12.5% poly acrylamide, made in duplicate and transferred to nitrocellulose membranes (Problat MN 0.45mm, Takara, Otsu, Japan). Afterwards, the transferred proteins were blocked with blocking solution (tris-buffered saline, TBS, containing 5% skimmed milk) at room temperature for 30–60 min. Membranes were washed (2-5 min) in TBST (TBS with 0.05% Tween-20) and incubated for 1-2 h with anti-*D. dendriticum* sheep sera. The membranes were washed again (2-5 min) in TBS and developed with rabbit anti-sheep IgG peroxidase (Jackson) conjugate for 1 h in the dark at room temperature. Another 2-3 washes were carried out. After evaluating different quantities of proteins, 40 µg of antigen protein by lane for SDS-PAGE; 1/50 dilution of the anti-*D. dendriticum* or other parasite sheep sera and 1/500 dilution of the rabbit anti-sheep IgG for Western blot analysis. Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibody rabbit anti-sheep IgG (H + L) peroxidase conjugated (Pierce Biotechnology Inc., USA) for 1 h in the dark at room temperature. The ECL Plus immunoblotting detection system (GE Healthcare Biosciences, Buckinghamshire, UK) was used to detect HRP activity on a chemiluminescence detector system (G Box, Syngene, Cambridge, England) [21].

Faecal Egg Counts: Faecal egg counts (egg per gram of faeces: EPG) were determined by the McMaster technique. In each case to assess the level of infection, 4 gram of faeces was mixed in 56 ml of tap water and the sample was mixed with a stirring device. The faecal suspension was left for 30 min at room temperature. The suspension was poured through a tea strainer into a clean new container and a 10-ml tube was filled to capacity with the filtered suspension. The tube was centrifuged for 5 min at 1,200 RPM and the supernatant was removed. Shortly before commencing the egg count, 4 ml of flotation fluid was added to a centrifuge tube. The sediment was then carefully resuspended and both sides of the McMaster counting chamber were filled. The filled McMaster chamber was left for 3–5 min to rest before counting. The EPG was obtained by multiplying the total number of eggs counted in the two squares of the counting chambers of the McMaster slide. Helminth eggs present were identified using standard parasitological criteria [16, 17].

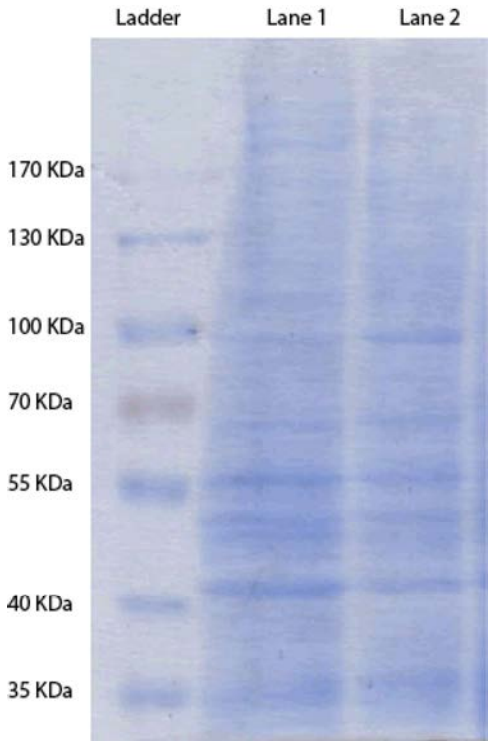

Fig. 1: Detected protein bands in somatic antigen (lane 1) andEsAg (lane 2)of *Dicrocoeliumdendriticum* using SDS- PAGE and staining with coomassie blue.

RESULTS

Detection of Antigenic Polypeptides: The concentration of the Somatic and EsAg antigens was 2 milligrams and 0/4 milligrams, respectively. In order to reach the concentration of the EsAg to 1 milligram, the samples were concentrated. 40 milligrams of each antigen was run on 12/5% gel and was stained by coomassie blue (Fig. 1). In order to find immunogenic antigens, antigens were transferred to paper and western blot was done. Two antigenic bands with the size of 67 and 130 kDa were identified (Fig. 2). In order to examine the cross reaction with *Fasciola hepatica* and verify the specification of related bands to *Dicrocoelium*, the anti fasciola antibodies were used and it was shown that the two identified bands are specific to *Dicrocoelium* (Fig. 2).

Evaluation by Indirect ELISA: In ELISA method, the higher the purity of the coated antigens, the higher the sensitivity of the test and a more accurate diagnosis will become possible. So in this study, the EsAg and somatic antigens were first run by SDS-page gel and immunogenic antigens were identified by western blot. These antigens

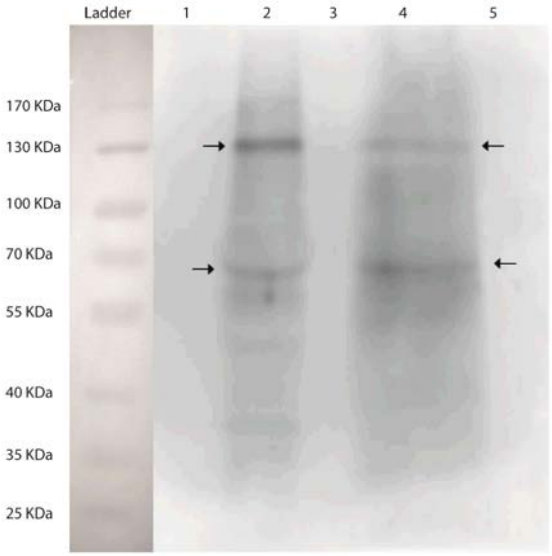

Fig. 2: Detected bands in the sera of sheep positive for *D. dendriticum*by Western blotting. Lane 1. Used of anti-*fasciloa hepatica* serum with somatic antigen. Lane 2, somatic antigen with anti- *D. dendriticum*serum. Lane 3, Used of anti-*fasciloa hepatica* serum with exceratory antigen. Lane 4, exceratory antigen with anti- *D. dendriticum*serum. Lane 5, antigen without use anti-sera.

Fig. 3: Optical Density (OD) values obtained by indirect ELISA technique using positive sheep sera (Cut-off: 0.2008).

Fig. 4: Optical Density (OD) values obtained by indirect ELISA technique using negative sheep sera (Cut-off: 0.2008).

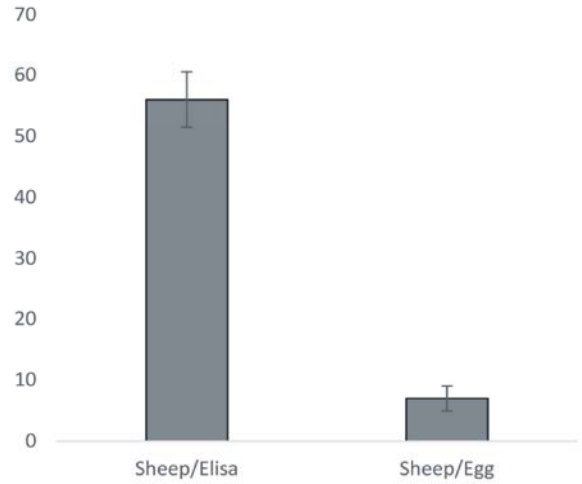

Fig. 5: Prevalences (%) of D. dendriticum egg-output and serum antibodies in 550 sheep. (mean±standard deviation) (P<0.05).

Table 1: Dicrocoelium. dendriticum egg-output and seropositivity in 550 sheep.

Faecal-output result	Elisa	Number of sheep
–	–	242
+	+	39
–	+	269

Table 2: Egg-output and seropositivity in the 50 sheep positive for Dicrocoelium dendriticum

Faecal-output result	Elisa	Number of sheep
-	-	3
+	+	4
-	+	43

coated in ELISA microplates. 50 positive control samples and 20 negative control samples were used to set up the indirectly ELISA test. Positive samples were chosen from infected livers of sheep in abattoir, which were only infected by *D. dendriticum*. The negative samples were chosen from uninfected lambs. From 50 positive samples, 47 samples were positive with ELISA method (Fig. 3) and from 20 negative samples, 19 ones were negative (Fig. 4). The sensitivity and specificity of the test was 94% and 95% respectively,which is considered to be high in comparison to other studies.

Compare ELISA and EPG Techniques: The results obtained from ELISA method was compared to the results obtained from EPG (Table 1). Among 550 samples which were chosen randomly from suburbs of Mashhad, there were 308 positive and 242 negative samples with ELISA method, while there were 39 positive and 511 negative samples with EPG method (Table 1). For positive samples only in four samples were observed eggs of *D. dendriticum* in stool (Table 2). This shows the 56% infection prevalence with ELISA method and 7% infection prevalence with EPG method (Fig. 5). In order to calculate the cutoff point, positive values were the mean optical density (OD) of negative sera plus three S.D.

DISCUSSION

The present study was put into practice with the purpose of using indirect ELISA method with the help of purified antigens in diagnosing *D. Dicrocoelium* in sheep, detecting the prevalence of infection and comparing this method with EPG. Definitive diagnosis of *Dicrocoelium*, because of imprecise symptoms and shared symptoms with other parasities, needs para clinic experiments [18]. Different methods such as EPG and serology methods have been used for diagnosis so far [19-22]. The EPG method is a routine procedure which is not able to show the precise amount of infection and it is difficult to work with, also it very time consuming. On the other hand, many factors affect egg excretion of the parasites and the number of eggs in 1 gram of faeces which include: 1- The amount of egg excretion changes with the season change. In winter this amount is less in comparison to spring. Even the amount of egg excretion changes in different months or even during a single day. In the case of *Dicrocoelium*, the excretion of eggs is more in the afternoon than in the morning [23]. 2- The diet and the use of anti-parasite medicine affect egg excretion. 3- Depending on the number of parasite, the amount of egg excretion will be different. The more the number of parasites, the greater the amount of egg excretion [24]. 4- According to the parasite period, it takes a long time for the egg to appear in faeces. In a study done by Campo [25], the greatest amount of egg excretion was 150 to 180 days after the infection. All of these factors lead to the fact that the EPG method is not a convenient and reliable method and we need to use serology methods. Serological methods are of significant importance due to their simplicity, cheapness and accuracy. So far, different serological methods have been used for diagnosis of Dicrocoeliosis.

Among them, the ELISA method has been more studied because we can study a great number of samples simultaneously in a short time. Also it is highly sensitive in rapid diagnosis of the disease. According to a study by Bode and Geye [26] in which six different serological methods were used to diagnose *Dicrocoelium* in hamsters with were infected experimentally, the compliment fixation

and ELISA experiments showed the highest sensitivity in rapid diagnosis of antibodies against *Dicrocoelium*. In a study by Savitskii [27] which different serological methods were used for diagnosis of *Dicrocoelium*, the passive hemagglutination and ELISA tests had the highest specificity. In study done on sheep infected experimentally [10], the antibodies against *Dicrocoelium* could be identified after 30 days using indirect ELISA method. This immunity refers to the migration of premature parasites to the liver and the highest amount of antibodies is 60 days after infection which persist up to 180 days.

In a study by Sek *et al* [22], the sensitivity of the test was calculated to be 86%, but the amount of specificity was low. In the present study, the sensitivity and specificity of the ELISA test was 94% and 95% respectively with was high in amount and it may be due to the fact that we used purified antigens for the ELISA test. We identified two, 67 and 130 kDa immunogenic bands and after purification, these antigens were used to set up the ELISA test; while, in a study by Revilla *et al* [21], only one immunogenic antigen band was identified.

In most of the Epidemiologic studies in Iran the EPG test has been used and this is the first study which uses the indirect ELISA test for examining the level of infection to this parasite. The level of infection prevalence was 56% which shows that these areas are highly infected by *Dicrocoelium* parasite.

Consequently, the ELISA test is a precise and reliable method for examining the prevalence of infection. With ELISA test, infection can be diagnosed quickly and with treatment, economic damages can be prevented. In contrary, EPG test has a higher percent error and it is not suitable for epidemiologic studies.

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