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Indirect ELISA and Western blotting as tools to diagnose fascioloidosis in a population of free-ranging red deer (*Cervus elaphus*)

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ABSTRACT

The aim of this research was to investigate the use of an indirect enzyme-linked immunosorbent assay (ELISA) and Western blotting test with native excretory/secretory *Fascioloides magna* antigen (ES-Ag) in free-ranging red deer (*Cervus elaphus*). The research was performed on 48 red deer shot during routine culls from two different areas, considering the occurrence of fascioloidosis. After coprological examination and gross pathology examination of the livers for *F. magna*, serum samples were divided into three groups as infected (n = 32), uninfected (n = 13) and previously infected deer (n = 3). Indirect ELISA results were significantly higher for serum samples from infected deer (percentage of positivity (PP) 65.1 ± 18.4) than uninfected (PP 11.6 ± 13.7) and previously infected deer (PP 20.3 ± 6.4). Samples from uninfected deer had fewer bands (30-33

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and 104 kDa) on Western blotting than samples from infected deer (6, 17, 22, 27, 30-33, 40, 45 and 104 kDa). The number of alive flukes positively correlated with the ELISA results and the number of *F. magna* eggs per gram of faeces (EPG). The results indicated that ELISA and Western blotting test could be useful in the early diagnosis of fascioloidosis in red deer and also in control in the population of free-ranging red deer.

Key words: *Fascioloides magna*, red deer, excretory/secretory antigens, indirect ELISA, Western blotting

Introduction

Fascioloidosis is a parasitic disease caused by the large American liver fluke *Fascioloides magna* (BASSI, 1875). Several wild and domestic ruminants are susceptible to this parasite. Red deer (*Cervus elaphus*), white tailed deer (*Odocoileus virginianus*) and fallow deer (*Dama dama*) are most common normal definitive hosts for *F. magna* in Europe (ERHARDOVÁ-KOTRLÁ, 1971). In Europe, fascioloidosis has been reported in Italy (BASSI, 1875), Germany (SALOMON, 1932), Poland (SLUSARSKI, 1955), Austria (PFEIFFER, 1983), Czech Republic (ERHARDOVÁ-KOTRLÁ, 1971), Slovakia (RAJSKY et al., 1994), Hungary (MAJOROS and SZTOJKOV, 1994), Croatia (MARINCULIĆ et al., 2002) and Serbia (TRAILOVIĆ et al., 2008).

Previous methods for epizotiological situation monitoring and preventive measures efficiency have been based on coprological and gross pathology examination of the livers collected from culled or dead animals (SEVERIN et al., 2007a). Dissection of livers with typical findings of capsules and adult flukes is pathognomical for *F. magna* infection (PYBUS, 2001). Thus, postmortal diagnosis is very simple. In coprological diagnosis, it can sometimes be a problem for inexperienced lab workers/young researchers to distinguish *F. magna* and *F. hepatica* eggs. Therefore there is a need for intravital diagnosis which enables early, sensitive and species specific diagnosis. This is the main reason for implementation of serology.

Knowledge of the humoral immune response of red deer to *F. magna* infection in natural conditions is the backbone of standardization and application of serological diagnostic methods in the diagnosis of fascioloidosis. Immunological tests such as enzyme-linked immunosorbent assay (ELISA) and various immunoblotting techniques are frequently used in the diagnostics of many parasitic diseases (NOVOBILSKY et al., 2007b). One of their main advantages is rapid confirmation of infection in comparison to the standard parasitological tests.

The aim of this research was to investigate the immunodiagnostic potential of excretory/secretory antigens (ES-Ag) of adult *F. magna* in a population of free-ranging red deer through an indirect ELISA test and Western blotting test. Coprological examination and gross pathology examination of the liver defined the degree of infection of individual animals. The obtained results were compared with the results of the immunodiagnostic tests.

Materials and methods

Animals and sample collection. 48 red deer (*Cervus elaphus*), originating from four areas in Croatia, were used in the study. Thirty-eight red deer were from *F. magna* endemic areas (Podunavlje - Podravlje and Spačva hunting grounds) and ten were from *F. magna* free areas (Smrekova Draga and Bjelolasica hunting grounds) (Fig. 1). The whole liver, faecal samples and blood samples were collected from each deer. Faecal samples were evaluated by the sedimentation-flotation technique (ZAJAC, 1994) for detection and quantification of the *F. magna* egg count. Eggs were counted in Petri dishes under a microscope (magnification ×40) and expressed as eggs per gram of faeces (EPG). The livers were sliced perpendicularly into 1.0 - 2.0 cm thick slices, which were carefully examined for grossly visible flukes, migratory lesions, fibrous capsules, and accumulation of the haematin pigment. All recovered flukes were counted, measured and classified as immature (juvenile) or mature, according to BEHM and SANGSTER (1999). According to the morphological characteristics of migratory lesions and the fibrous capsule and its contents, *F. magna* infected deer were scored as FCMs (migratory lesions), FC1s (thin-walled fibrous capsules), FC2s (thick-walled fibrous capsules) and FC3s (fibrous capsules with necrotic and/or dry content). After the coprological examination and gross pathology examination of the livers for *F. magna*, sera samples were divided into three groups as infected, uninfected and previously infected deer. Blood samples were collected from the heart, centrifuged and sera were stored frozen at -20 °C until analysed.

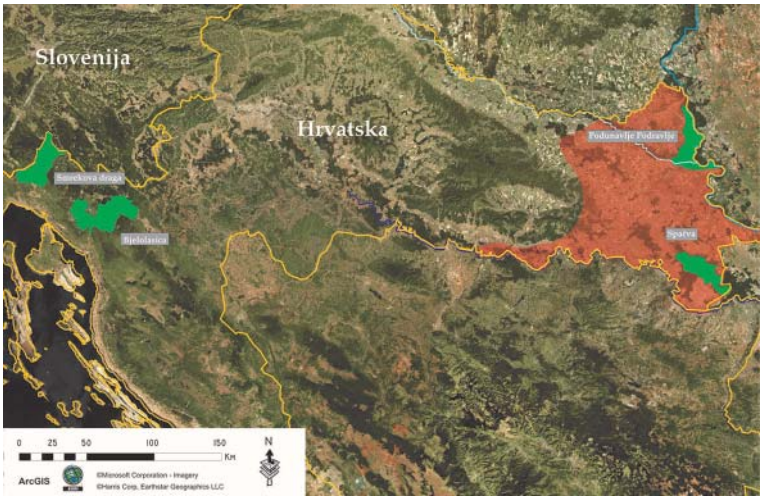

Fig. 1. Geographical distribution of sampling areas (green fields) and the *F. magna* endemic area (orange field).

Preparation of excretory/ secretory antigens. F. magna ES-Ag was prepared with modifications according to NOVOBILSKÝ et al. (2007b). Live adult flukes were collected from the livers of naturally infected red deer (hunted in the Podunavlje-Podravlje hunting ground) at a local slaughterhouse. Flukes were washed in 0.1 M phosphate buffer saline (PBS) and incubated in sterile RPMI 1640 medium, with 10000 UI of penicillin and 10 mg of streptomycin per millilitre of medium at 37 °C for 12 h. After incubation, the ES-Ag containing supernatant was collected and centrifuged at 2500*g* for 45 min, filtered through a syringe filter with 0.45 µm pore size, and concentrated using an AMICON Ultra-15 (Millipore, USA) with a PM10 membrane. Protein concentration was determined by bicinchoninic acid (BCA) assay (Sigma Aldrich, USA). The ES-Ag was stored at -20 °C until use.

Indirect ELISA. Indirect ELISA was performed in 96 well microtiter plates (Sigma Aldrich, USA). To determine the optimal concentrations of the reagents for ELISA, criss-cross serial dilution analysis was performed, as described by CROWTHER (2009). The wells were coated with ES-Ag (6.5 µg/mL) diluted in PBS pH 7.2 and incubated overnight at 4 °C. After three washes for 5 min with PBS, containing 0.05 % Tween 20 (0.05 % PBS-T20), blocking agent (1 % PBS-T20) was added to each well and incubated for 30 min at 37 °C. Before adding deer sera diluted at 1:400 in 0.05 % PBS-T20, the washing step was repeated. Sera dilutions were done in duplicate. After incubation for 30 min at 37 °C, the washing step was repeated and peroxidase conjugated rabbit polyclonal anti-deer IgG (KPL Inc., USA) was added at a dilution of 1:500 in 0.05 % PBS-T20 for 30 min at 37 °C. Finally, after three further washes for 5 min with 0.05 % PBS-T20, substrate solution (azino-bis (3-ethylbenzthiazoline-6-sulfonic) acid (Sigma Aldrich, USA) at a concentration of 0.5 mg/mL in a sodium citrate solution, pH 4 containing 0.008 % hydrogen peroxide) was added and incubated for 15 min at room temperature. The plates were read at 405 nm in a MULTISCAN Spectrophotometer (Labsystems, Finland). Optical densities (OD) were obtained from duplicate samples and were expressed as a percentage of positivity (PP) using the following calculation (WRIGHT et al., 1993): PP = [(sample mean OD)-(negative pool mean OD)]/[(positive pool mean OD)-(negative pool mean OD)]*100. Sera pools from five naturally infected and five uninfected deer were used as positive and negative controls, respectively. These standards were repeated on each plate.

The cut-off value was calculated as the mean OD reading of negative controls (uninfected animals and previously infected) ± 2 standard deviation, and OD levels equal to or less than the cut-off value were considered negative. The diagnostic sensitivity, specificity, and accuracy were calculated as described by GALEN (1980). These values were calculated and expressed as follows: sensitivity = [no. of true positives/(no. of true positives + no. of false negatives) × 100]; specificity = [no. of true negatives/(no. of false

positives + no. of true negatives) × 100]; and accuracy = [no. of true positives + no. of true negatives/(no. of true positives + no. of false negatives + no. of true negatives + no. of false positives) × 100].

Western blotting. The *F. magna* ES-Ag samples were mixed with Laemmli sample buffer and heated at 95 °C/3.5 min (LAEMMLI, 1970). Electrophoresis of the denatured samples was carried out using NuPage 12 % Bis Tris gel ready precast gel (Invitrogen) at 200 V for 60 minutes at room temperature in running buffer (25 mM Tris, 192 mM glycine) (pH 8.3), containing 1 % SDS. Proteins were then electro-transferred onto a nitrocellulose membrane (NCM) (0.45μm) (Sigma Aldrich, USA) at 100 V for 75 minutes in transfer buffer (25 mM TrisHCl, 192 mM glycine, 20 % methanol) (pH 8.1- 8.4). After transfer, the membranes were stained with Ponceau S solution (Sigma-Aldrich, USA) to visualize the protein bands, cut into individual strips and distained with 0.05 % PBS-T20. The strips were blocked with 1 % PBS-T20 for 60 minutes at 37 °C. After blocking, the strips were incubated with deer sera, diluted 1:200 in 0.05 % PBS-T20 overnight at room temperature using a bench rocker (ROCKER25, LABNET International, USA) with a constant speed of 30 rpm. Strips were washed three times and incubated for 30 min at 37 ºC, with peroxidase conjugated rabbit polyclonal anti-deer IgG (KPL Inc., USA), diluted at 1:2000 in 0.05 % PBS-T20. The washing step was repeated and strips were stained with substrate solution containing 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma Aldrich, USA) and 3 % hydrogen peroxide. Bands were visible within a few minutes, after which the stop reaction was performed by removing the substrate with distilled water and air drying the strips.

Statistical analysis. The significance of differences in antibody levels between two groups of data (infected and uninfected) was determined by the Student *t*-test, at the level of 0.5.

The correlation between the number of live flukes taken from the livers and EPG count in the faeces of infected animals, the PP when using ES-Ag and the number of live flukes removed from the livers of animals, was calculated using Spearman's coefficient correlation. Statistical analysis of all these indicators was performed using the MedCalc® program, version 10.2.0.0 (MedCalc Software bvba, Mariakerke, Belgium).

Results

F. magna eggs were found in 26 of 38 (68.4 %) animals originating from the *F. magna* endemic area. The average EPG count was 44 ± 87 per sample. In 35 out of 38 examined livers (92.1 %) one or more changes typical for *F. magna* infection were noticed, whose intensity and quantity are numerically presented in Fig. 2. FC2s were found in the highest number of samples, in 23 livers, with a total prevalence of 69.7 %, followed by FC1s, which were detected in 18 livers, with a total prevalence of 54.5 %, whilst FC3s and FCMs

were found in 15 livers, with a total prevalence of 45.5 %. In two livers connective tissue fibres were found in the form of wide or narrow traces and accumulation of the haematin pigment without the presence of FC3s, and they were considered as previously infected. In general, cases of several changes per examined liver were recorded in the highest number (four types of change in 4 livers, three types of change in 8 livers and two types of change in 11 livers), whereas the presence of a single change was noticed in only 9 livers. Furthermore, three livers were without typical *F. magna* changes and were considered as negative. Parasitological examination of 32 livers identified live flukes (juvenile and mature) in an average quantity of 15.5 ± 14.15 flukes per liver. Statistically significant and positive correlations were observed between the number of live flukes removed from the livers and the EPG count in the faeces of infected animals ($r = 0.690$; $P = 0.0001$). The results of coprological findings in terms of EPG are shown in Fig. 2 in correlation to the numerical values of the established indicators of necropsy and parasitology findings. No changes typical for *F. magna* infection and trematode eggs were found in animals from *F. magna* free areas.

A concentration of antigen at 6.5 μg/mL, dilution of the test sera at 1:400 and dilution of the peroxidase conjugated rabbit polyclonal anti-deer IgG at 1:500 was the optimal combination of reagents. Here, the value of OD in the negative control samples was 0.34 ± 0.17, whilst the value in the positive control samples was 1 ± 0.23. From the negative control results, the cut-off value was 0.67 for *F. magna* ES-Ag. The sensitivity, specificity and diagnostic efficacy of ELISA was 100 % (32/32), 92.3 % (15/16) and 97.8 % (47/48), respectively. The per cent of positivity in ten of the thirteen uninfected deer samples was ≤9.9 and in the other three 26.9, 29.2 and 45.7, respectively. In all infected deer samples the PP was ≥41.3. Fig. 3 shows the mean values and standard deviations of PP in the three different groups of samples. Statistically significant and positive correlations were observed between the mean values obtained for PP and the total number of live flukes (juvenile, mature) ($r = 0.468$; $P = 0.0092$).

The Western-blotting test was used to examine a total of 22 samples of sera: 11 samples were from coprologically positive animals with live *F. magna* in their liver (P+), 4 from coprologically negative (P-) with gross lesions typical for *F. magna* infection, 3 from previously infected animals with gross lesions typical for *F. magna* infection, but without live flukes in the liver (PK), and 4 from uninfected animals from the *F. magna* free area (N). Sera P+ and P- reacted with a total of 8 (6, 17, 22, 27, 30-33, 40, 45 and 104 kDa), PK with three (6, 30-33 and 104 kDa) and N with two (30-33 and 104 kDa) specific ES-Ag proteins. The recognition percentages of individual specific proteins are shown in Fig. 4.

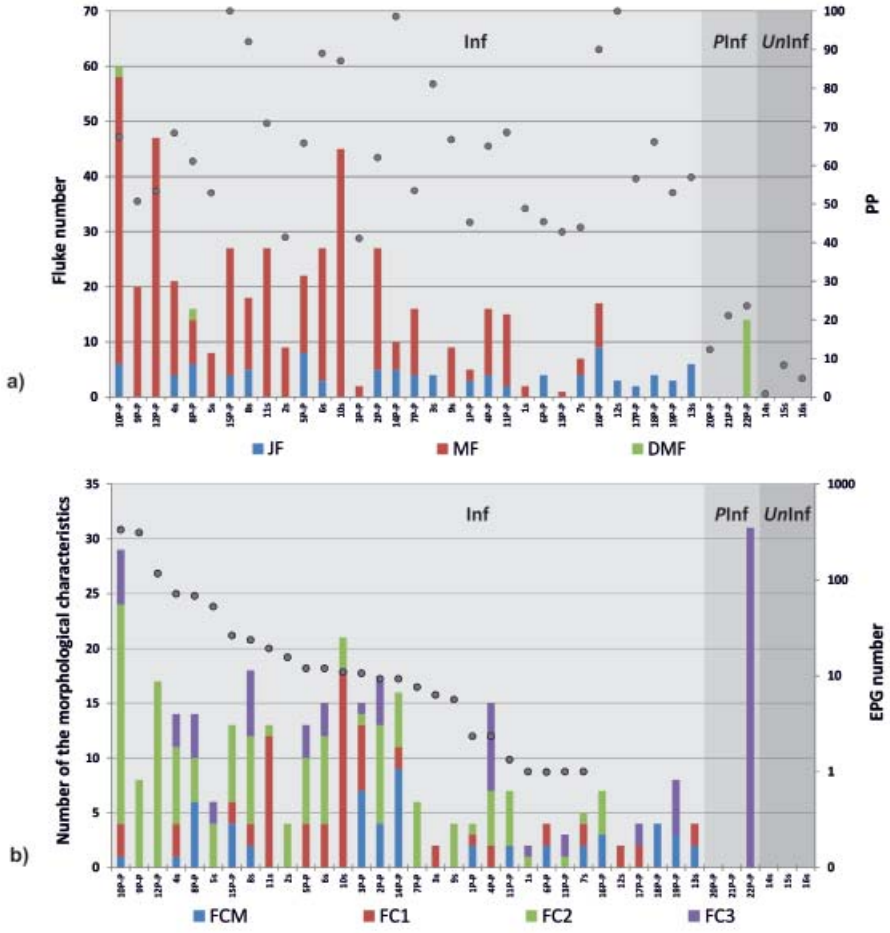

Fig. 2. The numerical values of the indicators of a) parasitology with the percentage of positivity (PP) and b) necropsy findings of examined livers compared with eggs per gram (EPG) counts in faeces of deer from *F. magna* endemic area.

Necropsy findings indicators: FCM (migratory lesions), FC1 (thin-walled fibrous capsules), FC2 (thick-walled fibrous capsules) and FC3 (fibrous capsules with necrotic and/or dry content). Parasitology findings indicators: JF (juvenile flukes), MF (mature flukes) and (death mature flukes). Inf - infected, *P*Inf - previously infected and *Un*Inf - uninfected deer.

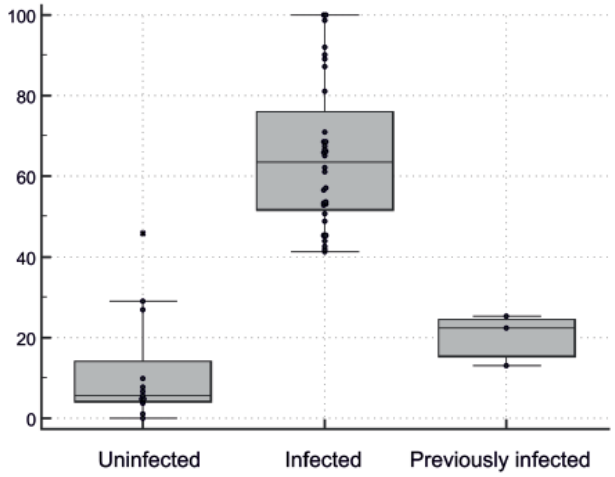

Fig. 3. The bars represent the PP of uninfected (column 1), infected (column 2), and previously infected deer (animals with gross lesions typical for *F. magna* infection, but without live flukes in the liver) (column 3). The horizontal line inside each box represents the arithmetic mean of PP in each group; whiskers are the 95 % confidence interval

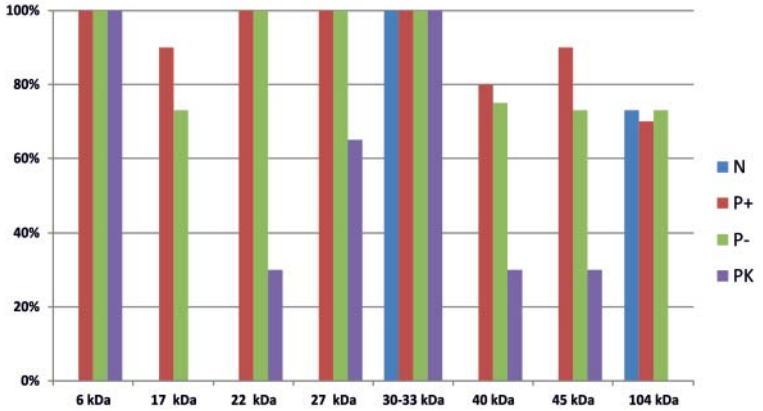

Fig. 4. Recognition percentage of the specific *F. magna* ES-Ag proteins recognised by deer sera of uninfected deer from *F. magna* free area (N), coprologically positive (P+), coprologically negative (P-) and previously infected deer (animals with gross lesions typical for *F. magna* infection, but without live flukes in the liver) (PK)

Discussion

The majority of the research published so far about fascioloidosis aims in three different directions. These are: to establish the degree of infected population (NOVOBILSKÝ et al., 2007a), monitoring prevention and therapy efficiency in an affected area (JANICKI et al., 2005; URSPRUNG et al., 2006; JANICKI et al., 2012) and monitoring the efficiency of measures implemented to prevent the spreading of fascioloidosis into non-affected areas (NOVOBILSKÝ et al., 2007a). The implementation of these measures requires knowledge of different diagnostic methods to confirm fascioloidosis in a certain population. It is understandable that diagnostic precision will affect the implementation efficiency of these measures. So far, diagnostic methods for monitoring the occurrence of fascioloidosis have been based on live animal faeces examination and the necropsy of culled or otherwise dead animals (NOVOBILSKÝ et al., 2007b). Recently, new diagnostic methods have appeared in several studies (SEVERIN et al., 2007b, 2012) as a result of coprological method inefficiency (sedimentation/flotation) in discovery of the existence of infection during the first three to seven months after infection, i.e. the prepatent period (ERHARDOVÁ-KOTRLÁ, 1971; FOREYT and TODD, 1976). Also, in 25 out of 38 animals culled in the infected population, independently from infection confirmation based on liver examination and the finding of live flukes, the absence of eggs was shown in a significant number of faecal samples. The presence of *F. magna* eggs was not determined in the faecal samples of six animals despite the finding of live flukes in the liver. It should be noted here that mature flukes were found in only one liver, while juvenile flukes were seen in the other five.

In fact, in some cases, despite the fact that the infected animal is in the patent period, due to the non-continuous release of eggs by the flukes, coprological examination may be negative. On the other hand, necropsy and parasitological examination of the liver are reliable diagnostic methods in culled or dead red deer. These diagnostic methods are widely used in enzootic areas, primarily to monitor the infection level in the population and to control the prevention measures applied (URSPRUNG et al., 2006).

It is necessary to point out that the possibility of cross-reactions between other parasites and *F. magna* also exists. For instance, it has been shown that cross reactivity exists between *F. hepatica* and *Fasciola gigantica* excretory-secretory products (EL RIDI et al., 2007), *Dicrocoelium dendriticum* and *F. hepatica* somatic proteins (WEDRYCHOWICZ et al., 1995), while *Paramphistomum cervi* whole worm antigen reacted with animal sera infected with *F. gigantica* but not strongylids (ANURACPREEDA et al., 2008). Therefore, one of the priorities in future immunodiagnostic research should certainly be to determine cross-reactivity with other parasites, primarily those that are present in the same area and in the same population. Since it is not possible to use the ELISA test for detection of specific antigen components of the ES-Ag responsible for binding with

specific antibodies, or whether it is only specific for that parasite, KURIEN and SCOFIELD (2009) suggested the use of the Western-blotting method. In our research, the antibodies of naturally infected animals reacted with *F. magna* ES-Ag proteins of the following molecular mass: 6, 17, 22, 27, 30-33, 40, 45 and 104 kDa. In negative animals a reaction was proven with 30-33 and 104 kDa proteins, and therefore it may be concluded that these proteins are non-specific and hence diagnostically meaningless. It is important to point out that *F. hepatica* and *D. dendriticum* infection was not proven in any faecal sample or examined liver. Since the research did not cover serological testing of red deer for other types of parasites, especially *F. hepatica* or *D. dendriticum* ES-Ag, it was not possible to determine the specificity/cross-reactivity of each individual protein. For comparison, QUERESHI et al. (1995) showed that the proteins from 17 and 22 kDa are species specific in the sera of experimentally infected white tailed deer, and propose their use in differential diagnosis. They also showed that the 27 kDa protein cross-reacted with the sera of cattle experimentally infected with *F. hepatica* metacercariae. Recently, there has been increasing use of individual parts of ES-Ag, obtained by various methods of purification and recombination, for which high immunogenicity and specificity have been detected. For example, in research aimed at finding species specific protein components in *F. hepatica* ES-Ag, a high level of specificity has been shown for 8, 17, 26 and 28 kDa proteins in revealing fasciolosis in cattle and sheep (ABDEL-RAHMAN et al., 1998; HILLYER and GALANES, 1988; KIM et al., 2003). One of the future tasks is thorough research of *F. magna* ES-Ag and comparison with other trematode ES-Ag (*F. hepatica, D. dendriticum*).

ANDERSON et al. (2007) point out that there are certain difficulties in collecting blood samples from game animals immediately after culling. Problems most often arise due to the conditions at the time of in-field work (evisceration of culled animals at the site of culling and the later transport to the place where the game animals are processed, which means that there is an extended time period from the moment of death until the sample is taken, strong variations in ambient temperature, contamination of samples by dirt, the storage conditions of samples etc.), which means that the samples could be of poor quality. In such samples there is often degradation of sera proteins or haemolysis, which may lead to errors in diagnostics using the ELISA test. GARDNER and GREINER (2006) point out that if it is not possible to collect good quality samples, in our case blood samples, it is necessary to use methods which will be more reliable in the given circumstances. For example, DUBEY et al. (2009) mentioned haemolysed sera samples from negative animals, which were positive by the ELISA test. In contrast to the ELISA test, a special feature of the Western-blotting test is direct visualization of specific molecular mass protein bands, linked to specific antibodies (ANDERSON et al., 2007). Using the Western-blotting test, due to the reliability of the results, the requirements are met for the precise diagnostic evaluation of poor quality samples. In our research, in three sera samples collected from

F. magna free area, a high PP was noticed (26.9, 29.2 and 45.7). All samples were very haemolytic, but following the Western-blotting test no reaction was detected with the specific fractions of ES-Ag.

The results indicated that ELISA and Western blotting test could be useful in the early diagnosis of fascioloidosis in red deer, and also for control in a population of free-ranging red deer. However, the number of tested animal sera was relatively small, and further evaluation of both methods on a larger number of sera samples, compared with different trematode ES-Ag (*F. magna, F. hepatica, D. dendriticum*) is needed.

Acknowledgements

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SAŽETAK

Cilj ovog rada bio je istražiti imunodijagnostički potencijal ekskretorno/sekretornog antigena (ES-Ag) dobivenog od odraslih *Fascioloides magna* u populaciji slobodno živućih jelena običnih primjenom neizravnoga imunoenzimnog i Western blotting testa. Istraživanje je provedeno na 48 jelena običnih odstrijeljenih u okviru planiranog odstrjela na dva različita područja s obzirom na pojavu fascioloidoze. Nakon koprološke pretrage i razudbe jetara na prisutnost *F. magna*, uzorci seruma bili su podijeljeni u tri skupine: invadirani (n = 32), neinvadirani (n = 13) i prethodno invadirani jeleni (n = 3). Rezultati neizravnog ELISA testa bili su značajno viši u uzorcima seruma od invadiranih jelena (postotak pozitivnosti (PP) 65,08 ± 18,40) nego neinvadiranih (PP 11,6 ± 13,7) i prethodno invadiranih jelena (PP 20,31 ± 6,43). Uzorci od invadiranih imali su nekoliko linija (30-33 i 104 kDa) primjenom Western blottinga za razliku od uzoraka od invadiranih jelena (6, 17, 22, 27, 30-33, 40, 45 i 104 kDa). Broj živih metilja u pozitivnoj je korelaciji s rezultatima ELISA testa i broja *F. magna* jajašca po gramu izmeta (EPG). Rezultati ukazuju da neizravna ELISA i Western blotting test mogu biti korisni s ciljem ranog otkrivanja fascioloidoze u jelena običnog te isto tako u njenoj kontroli u populaciji slobodno živućih jelena običnih.

Ključne riječi: *Fascioloides magna*, jelen obični, ekskretorno/sekretorni antigeni, neizravna ELISA, Western blotting