

Use of Somatic and Excretory-Secretory Antigens of *Fasciola hepatica* in Diagnosis of Sheep by ELISA

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Abstract: In the present study, ELISA kit was developed to diagnose infected sheep with *Fasciola hepatica*. The results were compared with eggs per gram of faeces samples. First, 30 blood and its corresponding faecal samples were collected from infected sheep (proven by inspection of fasciolosis in livers) slaughtered at Mashhad Abattoir as positive control. Ten blood samples were collected from 2 weeks old healthy lamb as negative control. Then, 100 random faeces and blood samples were collected from sheep slaughtered at Torghabe of Mashhad Abattoir. Serum samples were tested with indirect ELISA and faeces samples were also tested by Clayton Lane method. Result showed that from 30 serum samples of infected sheep, 27 samples were positive and from 10 healthy samples, 8 of them were negative. From 30 faecal samples, 3 samples were positive. The sensitivity and specificity of test were %90 and %80 respectively. From 100 random samples of sheep, 10 samples with somatic antigen and 8 samples with excretory/secretory antigen were positive by ELISA respectively. However only one faecal sample was positive by Clayton Lane method. It has been conducted that ELISA is an authentic test for diagnosis of infected sheep with *Fasciola hepatica*.

Key words: *Fasciola hepatica* • Fasciolosis • Antibody • ELISA • Sheep • Clayton Lane method

INTRODUCTION

Fasciola hepatica, a trematode parasite of ruminant and human, causes fasciolosis, a liver disease in most part of the world and particularly in the north of Iran. About 180 millions of human around the world are at risk of fasciolosis. Now, 2.4 million people are infected [1]. It is one of the most economically important parasitic diseases of livestock, causing disease in sheep and other domestic animals in Latin America, Africa, Europe and China. Of the 750 million people who live in endemic areas of China, over 40 millions are thought to be infected [2]. In 1998, 10000 people were infected with fasciolosis in north of Iran [3]. The detection of infections in ruminants relies on the microscopic observation of *Fasciola hepatica* eggs in the faeces of infected animals. However, early diagnosis by coprological examination is not possible because eggs are not found in the faeces until 10-12 weeks after infection, when flukes reach maturity and when hepatic injury has been produced [4]. To prevent this hepatic damage, several immunological methods have

been developed for the detection of early and specific antibodies to *Fasciola hepatica*, especially in cattle and sheep [5-7]. Methods such as immunoelectrophoresis and counterimmunoelectrophoresis, although are very specific, have limited sensitivity. Various immunoassays are available and ELISA tests are very sensitive and specific [8] and allows for early detection of fasciolosis in man and animals [9, 3]. The diagnosis was improved by the development of ELISA, using crude extracts [10], excretory-secretory products [11], purified or recombinant molecules such as cathepsin L-1 [12], by the detection of circulating antigens and coproantigens by sandwich ELISA [13]. The ELISA enables particularly clear discrimination between negative and positive populations, which would further safeguard against false positive or negative classification [14].

The purpose of this study was to test the sensitivity and specificity of the ELISA assay using somatic (S) and excretory-secretory (ES) antigens of *Fasciola hepatica* for diagnosis of sheep fasciolosis.

MATERIALS AND METHODS

Collecting Sera: Thirty blood and its corresponding faecal samples from naturally infected sheep were collected immediately post-slaughter from Mashhad abattoir as positive control. Fluke infection was confirmed, immediately, by the presence of adult *Fasciola hepatica* within the liver of sheep. Also, 10 blood samples were collected from the newborn lambs (uninfected 2 weeks old) with no history of fasciolosis as negative control. Then, 100 random blood and faecal samples were collected from sheep immediately post-slaughter from Torghabe of Mashhad abattoir. Blood samples were centrifuged at 3000 x g at 20°C for 15 minutes and sera were stored at -20°C until used. Faecal samples were stored in 10% of formalin at 1 to 3 ratios. Serum samples were tested with indirect ELISA and faecal samples were tested by Clayton Lane method [15].

Antigen Preparation

Somatic Antigen: Adult *Fasciola hepatica* were collected from livers of infected sheep from Mashhad abattoir. The worms were washed with sterile phosphate buffered saline (PBS). Somatic antigen was prepared by homogenizing fifteen adult worms in 10 ml PBS/pH 9.6 using electrical homogenizator for 3 minutes at 10000 rpm (Edmund Buhler Co.). Samples were sonicated in vicinity of ice (1 min, 0.5 amplitude) (Tommy Seiko model UP-200P, Tokyo) and then centrifuged at 10000g at 4°C for 30 minutes [16].

Excretory/Secretory Antigen: The Excretory/secretory (ES) antigen was prepared from a spent culture medium (RPMI 1640) containing 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM tosylamide 2-phenylethyl-chloromethyl ketone, 1 mM of L-trans-3-carboxyoxiran-2-carbonyl-L-leucylagmatine, 20mM hepes, 0.53 g/l N-acetyl-L-glutamine alanine, 7 ml/l Sodium bicarbonate 7.5% and 40 mg/ml gentamycine. The worms had been maintained in RPMI solution for 24 hours at 37°C with 5% CO₂ in air (3 flukes/ml). For checking aliveness of flukes, their motion was observed by Stereomicroscope every 12 hours. After incubation, the worms were removed and the collected spent medium was clarified by centrifugation at 10,000 rpm for 30 minutes at 4°C and centrifuged at 5000 g for 10 minutes [17]. The concentration of each antigen was measured using Bradford method [18] and then the samples were stored at -20°C until used.

ELISA: Somatic and excretory/secretory antigens were diluted 0.125, 0.25, 0.5 and 1 µl/ml with carbonate buffer (pH 9.6). Polystyrene micro titer plates were coated with 100µl of diluted antigens per well. After incubation for 1 hour at room temperature, plate was sensitized overnight at 4°C. Then, the wells were washed 5 times for 5 minutes, with 300 µl PBS-0.05% Tween 20. Two hundred µl of BSA 1% in PBS-T was added to each well as blocking and incubated for 1 hour at room temperature. Serum samples were diluted at 1:10, 1:20, 1:50, 1:100 and 1:500 with PBS-T. One hundred µl of diluted serum was added to each well and incubated at room temperature for 30 min. After a further washing, 100 µl anti-sheep IgG peroxidase conjugate diluted at 1:2000 and 1:5000 in PBS-T were added to each well. The plates were incubated at room temperature for 30 minutes and then washed as previously described. Then 100 µl of substrate solution containing TMB/H₂O₂ was added to each well and the plate was incubated for 15 min in darkness at room temperature and the reaction was stopped with 1M Sulfuric acid. The absorbance was measured at 492 nm using a micro plate ELISA reader. In the plate, 2 wells as the blank (without serum), 4 wells as the negative control and 4 wells as positive control. Each serum sample was tested in duplicate. The results were expressed as the mean of the obtained OD values. After optimizing the ELISA kit, one hundred random blood samples were tested by ELISA.

Faecal Egg Counts: For this purpose, Clayton Lane centrifuge and saturated salt solution was used as follows: Three grams of faeces sample were mixed with 42 ml water (in order to sediment fats, eggs and heavy scum of feces). The solution was filtered by Clayton Lane method and centrifuged at 2000 g for 2 minutes. 15 ml saturated sodium nitrate solution was added to the remained sediment (in order to the surface of liquid become convex). The lamella was set on the tube and centrifuged at 2000g for 2 minutes, the lamella was observed under microscope for checking the existence of fasciola eggs. Clayton lane method was performed on 100 faeces samples.

RESULTS

The best results were obtained by ELISA kit developing with 0.5 µl/ml of both somatic (Figure 1) and excretory/secretory (Figure 2) antigens. Moreover, 1:100 of antibody dilution and 1/5000 of anti-sheep IgG peroxidase conjugate had a good result. From 30 blood

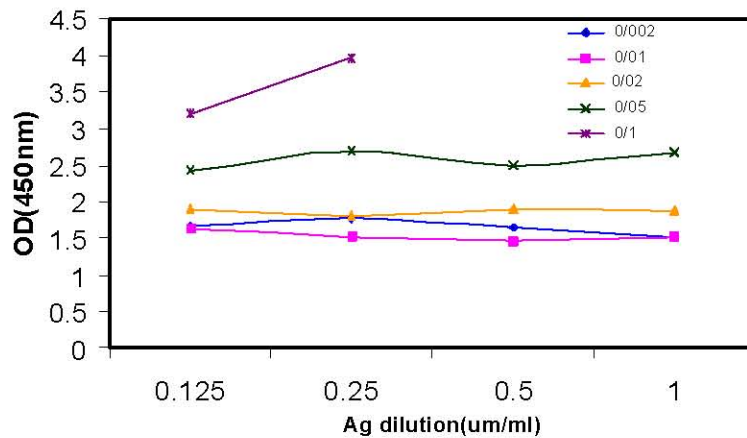


Fig. 1: Noise ratio of infected and uninfected sera of sheep with *Fasciola hepatica* using somatic antigen by Indirect ELISA. Noise ratio indicated (OD positive control / OD negative control).

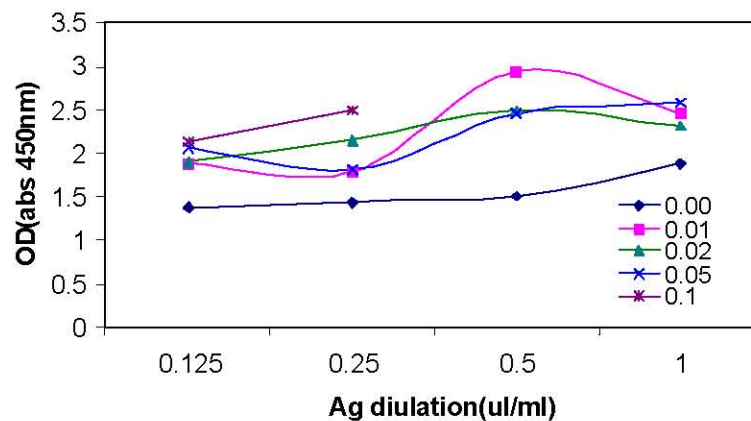


Fig. 2: Noise ratio of infected and uninfected sera of sheep with *Fasciola hepatica* using excretory- secretory antigen by Indirect ELISA.

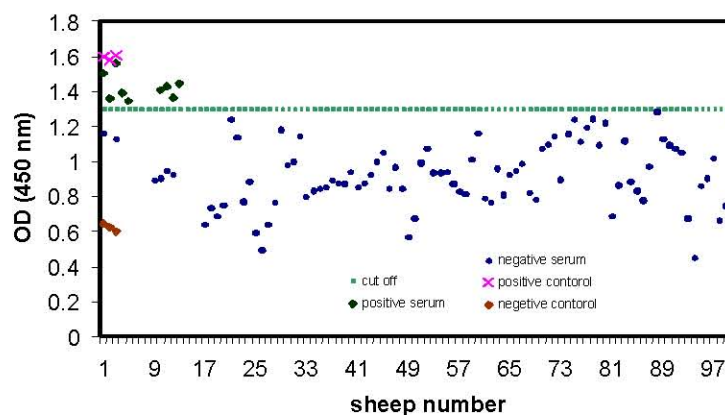


Fig. 3: Immune response in 100 random blood samples with somatic antigen by ELISA.

samples of naturally infected sheep, 27 samples were positive by ELISA and also from 30 faecal samples of these sheep, 3 samples were positive by Clayton Lane method. The sensitivity and specificity of the test were

%90 and %80 respectively. From one hundred random blood samples of sheep, 10 samples with somatic (Figure. 3) and 8 samples with excretory/secretory antigens (Figure. 4) were positive by ELISA, respectively.

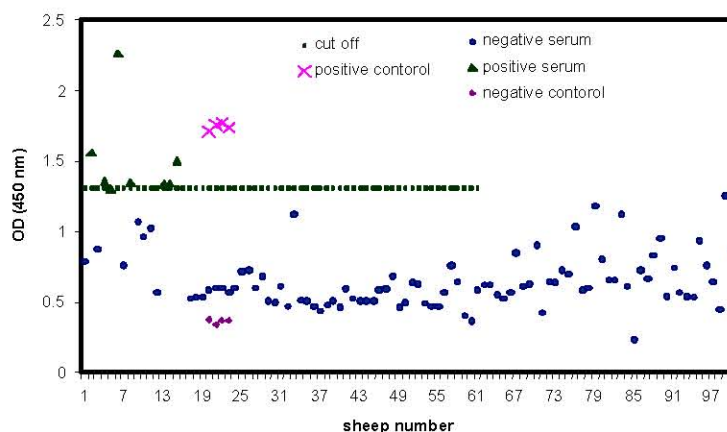


Fig. 4: Immune response in 100 random blood samples of infected sheep with Excretory/Secretory antigen by ELISA.

Also from 100 faecal random samples of these sheep, only one sample was positive by Clayton Lane method. According to these results, ELISA is more sensitive than faecal test for detection of fasciolosis.

DISCUSSION

Serological studies are now the main diagnostic method. It allows diagnosis of fasciolosis, even in the acute stage and before the parasite eggs can be identified in feces. Any ELISA used for the immunodiagnosis of *Fasciola hepatica* infections must have high sensitivity and specificity and be able to detect infection early, before liver damage has occurred [6].

In our study, the sensitivity and specificity of indirect ELISA were %90, %80 respectively. From 100 random blood samples, 10 samples with somatic and 8 samples with excretory/secretory antigens were evaluated as positive by ELISA, whereas only 1 faecal sample were diagnosed positive by Clayton lane method. The results indicated that ELISA is more sensitive than faecal test. Indirect ES-ELISA has been reported to be a rapid and sensitive test (sensitivity %92, specificity %94.4) [19]. ELISA has been evaluated for diagnosing sheep fasciolosis and they compared the results of this method with indirect haemagglutination (IHA). The sensitivity and specificity of ELISA were estimated %100 [20]. However, the serological methods, especially the ELISA test, are highly sensitive and specific when compared to *F. hepatica* diagnosing by coprological means. ELISA, Dot ELISA and indirect ELISA have been used for diagnosing cattle fasciolosis. The sensitivity and specificity of these methods were (%97.5, %80), (%93.1, %95.4), (%96.5, %98.8) respectively [21]. It has been

reported that the sensitivity, estimated using the sera from sheep with confirmed infections, was %96.9 and the specificity of ELISA, estimated using sera from sheep in uninfected flocks, was %99.4. Of a total of 249 sheep in the infected flocks, %60.2 was positive by ELISA and %52.2 was positive by the faecal egg count method [22]. Fas2-ELISA has been used and exhibited %95 sensitivity and %100 specificity in 38 individuals infected with *Fasciola hepatica* diagnosed by finding eggs in stools and 46 serum samples from healthy volunteers [23]. It has been concluded that the ELISA is a %100 sensitive and also a %100 specific test for the early serodiagnosis of sheep fasciolosis [24]. They indicated that this method may also be useful for the determination of anti-*Fasciola hepatica* antibodies in serum and milk of other ruminants. By applying the ELISA to herds in England, the prevalence of *Fasciola hepatica* infection in England was estimated to be %48 [25]. An ELISA with a diagnostic sensitivity of %98 and specificity of %96 was evaluated as a means of assessing the intensity of *Fasciola hepatica* infection in cattle [26]. It has been stated that excretory/secretory fasciola antigen can be used for specific diagnosis of cattle fasciolosis [27]. In their study, the excretory/secretory antigen in comparison with somatic antigen had better response. In fact the average of negative control OD value with excretory/secretory antigen was less than average of negative control OD value with somatic antigen. Considering the obtained cut off point of excretory/secretory antigen was more than somatic antigen, therefore ELISA with excretory/secretory antigen had more specificity and it evaluated fewer samples as positive. The ELISA is clearly more sensitive than the faecal egg count method, partly because antibodies are present approximately 8 weeks before the

infection matures and eggs are shed in the faeces [28] and partly because many animals with mature infections do not shed detectable numbers of eggs. The ability to diagnose and treat infections early is a big advantage of the ELISA because it minimizes tissue damage in the infected animal caused by immature flukes as they migrate through the liver. More importantly, early treatment prevents shedding of eggs in the faeces, thus contributing to effective management by reducing the rate of infection. The only real disadvantage of antibody tests is that they do not discriminate between active and past infections [22]. There was not any information about the accurate time of sheep infection, the false negative results may be obtained because of low antibody titer in sampling phase. For solving this problem, it has been suggested that young adult sheep should be medicated by anti-parasitic drug for 2 weeks, after that they should be infected with metacercaria experimentally and the antibodies should be divided in certain distances. In fact by this method, the cross-reaction with *Dicrocoelium dendriticum* would be reduced and high antibody titer would be formed against *Fasciola hepatica* [29]. Furthermore, using polyclonal secondary antibody instead of monoclonal antibody increases the test sensitivity [6]. Sera obtained from human patients infected with *Fasciola hepatica* were tested by the Enzyme linked immunotransfer blot (EITB) technique with the parasite's somatic and excretory-secretory (ES) antigens. The sensitivity, specificity, positive and negative predictive values for somatic antigen were %91.0, %96.2, %95.2 and %92.7 respectively, while these parameters as for ES antigen were %95.2, %98.0, %97.5 and %96.2, correspondingly [16]. In another experiment, using excretory-secretory antigens of *Fasciola hepatica* by Dot-ELISA, the sensitivity, specificity, positive and negative predictive values were %96.8, %96.1, %96.8 and %96.1, respectively [30].

In conclusion, the ELISA is useful as a screening test to examine anti-*Fasciola hepatica* antibodies for the diagnosis of fasciolosis in sheep. The assay will be a valuable tool for managing liver fluke on infected properties, particularly if it can be used to estimate the prevalence of infection using pooled serum samples.

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