

## Evaluation of *Fasciola gigantica* Metacercarial Antigens for Early Diagnosis of Fascioliasis in Sheep

<sup>1</sup>Elsayed M. Bayoumy, <sup>2</sup>Gehan ElEnain,  
<sup>3</sup>Abeer Mahgoub, <sup>2</sup>Rabab Zalat and <sup>2</sup>Ibrahim Aly

<sup>1</sup>Department of Hydrobiology Researches, National Research Center, Giza, Egypt

<sup>2</sup>Department of Parasitology, Theodor Bilharz Research Institute (TBRI), Imbaba, Giza, Egypt

<sup>3</sup>Department of Parasitology, Faculty of Medicine, Cairo University, Giza, Egypt

**Abstract:** Immunological techniques have been developed over years using different *Fasciola* antigens for diagnosis of fascioliasis and as to replace the parasitological techniques which are time consuming and usually lack sensitivity and reproducibility. In this study, *Fasciola gigantica* purified metacercarial antigen was early detected in sera of infected sheep using both sandwich and Dot-ELISA in order to evaluate the efficacy of purified metacercarial antigen performance in diagnosing early fascioliasis. This work was conducted on 150 sheep blood samples which were classified according to their parasitological manifestation into 110 sera infected with fascioliasis, 20 infected with other different parasites and 20 healthy control sera. The sensitivity and specificity of sandwich ELISA compared to those of the Dot-ELISA were 92.7% and 93.7% versus 95.5% and 97.5%, respectively. On the other hand, the parasitological examination recorded 68.6% sensitivity and 100% specificity. The purified *F. gigantica* metacercarial antigens have shown a powerful antigenicity for early immune-diagnosis of fascioliasis in both ELISA techniques, however, Dot-ELISA was trouble-free, more sensitive, greater specific and affordable as well as feasible test; the features recommended for any technique to be used in detecting the light infection, particularly, at the early stages that precede the onset of parasite eggs.

**Key words:** *Lymnaea truncatula* · *Fasciola gigantica* · Metacercarial antigen · Polyclonal antibody · Sandwich ELISA.

### INTRODUCTION

*Fasciola gigantica* is a parasitic flatworm of the class Trematode, which causes tropical fascioliasis. It is regarded as one of the most important single plathyhelminth infections in Asia and Africa. Epidemiological studies demonstrated that the infection rates range between 80-100% in some countries [1]. There are so close characteristics between the two *Fasciola* species, *F. gigantica* and *F. hepatica*, that makes it difficult to distinguish between them. They are both alike in terms of genetics, behavior and morphological and anatomical structures [1, 2]. Thus, sophisticated molecular techniques are required to correctly identify and diagnose the infection [2]. *F. gigantica* causes outbreaks in tropical areas of

southern Asia, Southeast Asia and Africa. Infection is most prevalent in regions with intensive sheep and cattle production. In Egypt, *F. gigantica* exists in domestic animals since the era of Ancient Egypt [3, 4], 7000 years ago. Likely to other trematode, *Fasciola* develops in a Lymnaeidae snails which are well known for their importance as a freshwater intermediate host in the life cycle of *F. gigantica*. However, throughout the years an increasing number of other molluscan intermediate hosts of *F. gigantica* have been reported [3, 5]. Mammals including human and sheep get infected with *Fasciola* spp. when they accidentally eat raw vegetation contaminated with metacercariae shed from the snails. Once the metacercariae arrive to the small intestine, they penetrate its wall migrating to the peritoneal cavity and eventually reaching to the liver where adults mature in the

biliary ducts of the liver. Eggs are passed through the bile ducts into the intestine where they are then passed in the feces [3, 6]. As early diagnosis of fascioliasis is vital to avoid all the complications of that might accompany the disease, different techniques have been approached including direct parasitological methods, indirect clinical and biochemical assays and immunological techniques that measure the immune response to certain parasitic antigens and/or detecting circulating parasitic antigens. Direct parasitological methods usually lack sensitivity and reproducibility and are not reliable [4, 5&7-10].

The immunodiagnosis of fascioliasis through detecting the antibody levels and types in response to the specific worm antigen has several disadvantages such as the antibody cross-reactivity with other trematode antigens, including those of *Schistosoma spp.* Hillyer [11] and difficulty in deciding if the detected antibody levels are due to recent or previous infection. Therefore, the use of specific antibody to detect antigens secreted by the living flukes into their host's body fluids may be a better approach, not only in diagnosing active infection but also in assessing treatment efficacy and the effectiveness of future vaccines [3, 12]. The present study aimed to evaluate the diagnostic potential of purified metacercarial antigen in natural *F. gigantica*-infected sheep.

## MATERIALS AND METHODS

**Collection of Sheep Blood Samples:** Sera and feces samples were collected randomly from groups of sheep before being slaughtered to local abattoir at the Giza slaughter-house. Medical examination and liver biopsy were carried out to detect the presence of *Fasciola* worms in sheep livers.. Blood samples were collected during slaughtering. Sera were prepared in 0.2 ml/aliquot, heat-inactivated and stored at -20°C until used.

**F. Gigantica Miracidiae:** Eggs were collected from the gall bladder of sheep slaughtered at slaughter house. Recovered eggs from *F. gigantica* flukes were transferred to petri dishes containing distilled water and incubated at 26°C for 15 days for the development of miracidia that needed lights to stimulate its shedding from the eggs.

**Snails:** *Lymnaea truncatula* were collected (n=350) from different water bodies from different villages around Kafr-EL-Sheikh Governorate, Egypt. They were collected in large plastic containers with care to avoid shell damage and transported to laboratory and screened for *F. gigantica* infection in vitro.

The collected snails were divided into two groups and placed in feet glass aquaria. Each aquarium was provided approximately 10 lts of de-chlorinated water with aeration. The aquaria were maintained on wooden shelves with natural light exposure. The water temperature and pH were monitored day after day. Snails were fed with lettuce leaves. Uncolored plastic sheets were set into the aquarium to collect eggs. The laid egg mass seen in the plastic sheets or sides and bottom of the tanks were transparent and jelly. The faeces were removed regularly by siphon method and the water was changed for once in two days [12].

**Infection of the Snails with the Miracidiae:** Three to four snails were placed in individual Petri-dishes and exposed to infection with 10-12 miracidia for 3-6 hours to ensure the full contact. After exposure, all snails were transferred to individual test tubes. Four weeks later, the infection of these snails was demonstrated by checking the shedding of metacercariae after their exposure of the sunlight. [13]

**Preparation of Metacercarial Antigen (MAG):** The collected metacercariae were homogenized in the presence of cocktail of protease inhibitors (1m M each of ethylene diamine tetracetic acid, ethylene glycol bis-4N-tetracetic acid, N-ethylmaleimide and phenylmethyl sulphonyl fluoride) for 20 min under ice bath using a Teflon glass homogenizer. The homogenate was, then, subjected to ultrasonication for 5 min to disrupt remaining intact metacercariae using an ultrasonicator (Misonix, USA). After sonication, the preparation was centrifuged at 14,000 g for 45 min at 4°C. The protein content of the supernatant containing metacercarial antigenic material was determined by Bradford [14] and stored at -20°C until use.

**Preparation of Polyclonal Antibodies (pAb) Against Metacercarial Antigen:** Just before immunization, rabbit's sera were assayed by ELISA for *Fasciola* antibodies and cross-reactivity with other parasites. Rabbits were injected intramuscularly (i.m.), with 1 mg of metacercarial antigen mixed 1:1 in complete Freund adjuvant (CFA) [5]. Then two booster doses were given, at 1 week intervals after the 1<sup>st</sup> injection each was 0.5 mg antigen emulsified in equal vol. of incomplete Freund adjuvant (IFA) [15]. One week after the last booster dose, the rabbit's sera were obtained and pAb fraction was purified by 50% ammonium sulphate precipitation method [18] More purification of pAb was performed by 7% caprylic acid method [19] and finally with gel-filtration [16]. The

produced IgG appeared in a very high degree of purity except for few serum protein contaminants. Partially purified pAb was further adsorbed with fetal calf serum (FCS) to eliminate any non-specific binding with bovine antigen.

**Characterization of Metacercarial Antigen:** The purified metacercarial antigen was characterized for determination of molecular weight range using discontinuous SDS-PAGE in 12% slab gels (1 mm thick), under reducing conditions (+2-mercaptoethanol) and stained with Coomassie blue, followed by enzyme-linked immunoelectrotransfer blot technique (EITB). Apparatus and chemicals were purchased from Bio-Rad and both assemblies of apparatus and gel preparations were performed according to the operation manual [17].

**Reactivity of Anti- Metacercarial Antibody by Indirect ELISA:** The reactivity of anti- metacercarial antibodies against *Fasciola* antigens was assessed using indirect ELISA [5]. The anti- metacercarial antigen IgG was purified on sequential use of 50% ammonium sulfate precipitation method [19], caprylic acid treatment [20] and ion exchange chromatography method. Tijssen and Kurstak [15] Anti- metacercarial antigen IgG antibodies were conjugated with horseradish peroxidase using sodium periodate oxidation method [20] and the antibody concentration was measured.

**Parasitological Examination:** Kato-Katz concentration technique (Martin and Beaver, 1968) and formal-ether sedimentation technique [21] were performed for all stool samples in order to identify *Fasciola* eggs or other helminthic ova.

**Sandwich ELISA:** Sandwich-ELISA based on the original [5] and the modified methods [22] Plates were coated with 100 µl/well of purified anti- metacercarial antigen IgG pAb (20 µg/ml for in carbonate buffer (0.06M, pH 9.6) and incubated overnight at room temperature. Plates were washed thrice with 0.1 M PBS/Tween-20, then, wells were blocked by 200 µl of 2.5% FCS (Sigma)/PBS/T-20 and incubated at 37°C for 2 hr after washing, negative, positive and other parasitic samples were put into wells and incubated for 1 hr at room temperature and rewash. 100 µl of peroxidase-conjugated anti- metacercarial antigen IgG pAb diluted 1/250 was added and incubated for 1 hr at room temperature. 100 µl of freshly prepared substrate solution (Phosphoric/citrate buffer as 1.02 g citric acid,

3.68 g Na<sub>2</sub>HPO<sub>4</sub>.12H<sub>2</sub>O in 100 ml dist. H<sub>2</sub>O) were dispensed in each well for 5 min. 50 µl from stopping buffer (4N H<sub>2</sub>SO<sub>4</sub>) were added to stop the enzyme-substrate over reaction. The absorbance was measured at 492 nm using ELISA reader (Bio-Rad microplate reader, Richmond, Ca). Cut off value was calculated [23] as the mean O.D reading of negative control+3 Standard deviation of the mean (mean + 3SD).

**Sandwich Dot ELISA:** To create a simple and rapid assay, the described [24] S-D-ELISA was modified to detect the circulating metacercarial *-Fasciola* Ags, using the purified anti- metacercarial IgG pAb. S-D-ELISA depends on the binding of anti-*Fasciola* pAb to a sensitive surface of nitrocellulose membrane (NC) as a capture matrix using Bio-Dot apparatus (BIO-RAD). The pre-wetted NC membrane was transferred to Bio-Dot apparatus and washed once with coating buffer (0.1 M carbonate buffer (pH 9.6). NC membrane was coated with 10-15 µg/well of anti- metacercarial pAb. Membrane was washed 3 times with 100 µl/well of PBS/T then blocked with 10-15 µl/well of 5% skimmed milk for 45 min at room temperature. Negative, positive and other parasitic serum samples were applied, diluted (1:1-1:32) in diluent's blocking buffer and incubated for 15-45 min. NC membrane was washed thrice with 100 µl/well of PBS/T and 10-15 µg /well of anti-metacercarial IgG pAb HRP conjugate was added and the membrane was incubated for 45 min. The membrane was washed 5 times with 100 µl/well of PBS/T, then 2 times with PBS only and immersed in freshly prepared 2% DAB substrate solution (diaminobenzidine tetra hydrochloride). The reaction was stopped after 30 min with cold dist. H<sub>2</sub>O. The color intensity was measured visually.

**Statistics:** All statistical analyses were performed using student's *t*-test. The data were considered significant if *P* values were equal to or less than 0.05. According to Galen [25], standard formulas were used for calculation of sensitivity, specificity, percentage of positive and negative predictive values, also, false positive and negative rates.

## RESULTS

The average number of metacercariae liberated from the snails is directly related to the infection and the size of the snails, the largest average number of metacercariae was produced by large snails, while the lowest average number was produced by small snails.

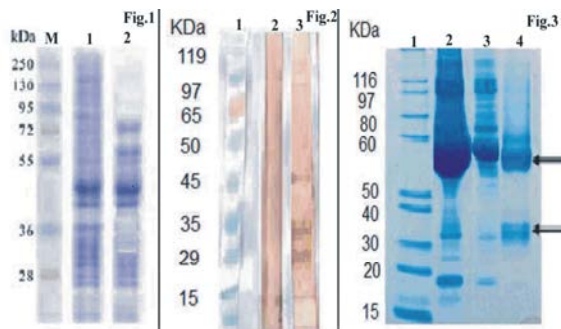


Fig. 1: SDS-PAGE M: Molecular standard weight (1). Crude Metacercarial Antigen (2). Purified Metacercarial Antigen.

Fig. 2: Immunoblot (1). Molecular standard weight (2). Negative sera (3). Positive sera .

Fig. 3: Purification of pAb (1). Molecular standard weight (2). Crude rabbit Sera (3). Purified Ab by Amm. Sulfate 4- Purified Ab by caprylic acid

Table1: Reactivity of purified *F. gigantica* metacercarial by indirect ELISA

Table Serum Samples	OD readings at 492 nm (m ± SD)
Fascioliasis (=110)	2.44±0.143
Echinococcosis (10)	0.21±0.094
Ancylostomiasis (10)	0.18±0.082
Ascariasis (10)	0.20±0.105

**Reactivity of *F. Gigantica* Metacercarial Antigen by Indirect ELISA:** As shown in Table (1), the antigenicity of the purified *F. gigantica* metacercarial antigen was tested by indirect ELISA technique and serum samples from naturally *F. gigantica* infected sheep gave strong reaction against *F. gigantica* metacercarial and no cross reactions were recorded with sera of sheep infected with other parasites e.g. hydatid and Hookworm.

**SDS-PAGE and Immunoblot Analysis:** The SDS-PAGE analysis and Coomassie brilliant blue staining of *F. gigantica* metacercarial Ags are shown in Figure 1. The fractionated *F. gigantica* metacercarial Ags containing several polypeptide bands ranged from 95 to 12 KDa. Three bands, 45, 31 and 14 KDa were recognized when pooled sera from naturally *F. gigantica* infected sheep were probed with fractionated metacercarial antigen in immunoblot analysis (Fig. 2).

**Purification of Rabbit Anti-Fasciola Metacercarial pAb:** It was done via removing most of albumin by 50% ammonium sulphate-precipitated protein. Several bands of precipitated proteins were detected. Further purification of whole Igs by 7% caprylic acid precipitation removed the

remaining non-IgG proteins. Purified IgG was represented by H- and L-chain bands at 31 and 53 kDa, respectively (Fig. 3).

**Stool Analysis for Fasciola Infection (Kato):** By naked eye examination during slaughtering, 110 sheep were found to harbor *Fasciola* worms in the liver and the bile ducts. However, according to stool analysis by Kato-Katz quantitative technique, only 70 animals were true positives and the rest of the animals (40) gave false-negative results (68.6% sensitivity). Three slides were counted for each sheep and the mean number of eggs/g (epg) in faeces was calculated. The intensity of infection was estimated and animals were classified into three subgroups: low, moderate and high infection. The mean number of epg ± SD was 14.81 ± 1.59, 31.2 ± 1.32 and 85.7 ± 1.06, respectively. Presence of *Fasciola* worms in liver was used as our gold standard in succeeding experiments. Other parasites, including echinococcosis, ancylostomiasis and ascariasis were detected in 20 sheep. The other 20 sheep were used as normal control.

**Reactivity of Rabbit anti-Fasciola Metacercarial Ags pAb:** Sandwich (S)-ELISA was performed to determine the reactivity of pAb which gave a strong positivity to *Fasciola* metacercarial till 1:2500 dilutions. The optimization of various reagents was assayed by S-ELISA. The minimum metacercarial concentration used was 3 ng/ml; the optimum concentration of purified IgG pAb was 20 mg/dl, whereas conjugated IgG pAb was found to be 10 mg/dl.

**Detection of Circulating Fasciola Metacercarial Ags in Sheep Sera by S-ELISA:** The cut-off value was calculated as the mean OD reading of negative controls + 2 standard deviation of the mean. The OD readings equal to or less than cut-off value were considered negative while those readings greater than the cut off value were considered positive. The specificity of the assay was determined as the sum of results of negative control group and other parasites group. The cut-off value was 0.263 and circulating *Fasciola* metacercarial Ag was detected in 102 out of 110 *Fasciola*-infected sheep and the sensitivity of the assay was 92.7% all the 20 negative controls had metacercarial Ag levels below the cut-off value in serum samples. Furthermore, 19 out of 20 serum samples belonging to sheep harboring other parasites had undetectable metacercarial Ag of *Fasciola* leading to overall specificity 95%. The diagnostic efficacy of the assay was 93.9% (Fig. 4 & Table 3).

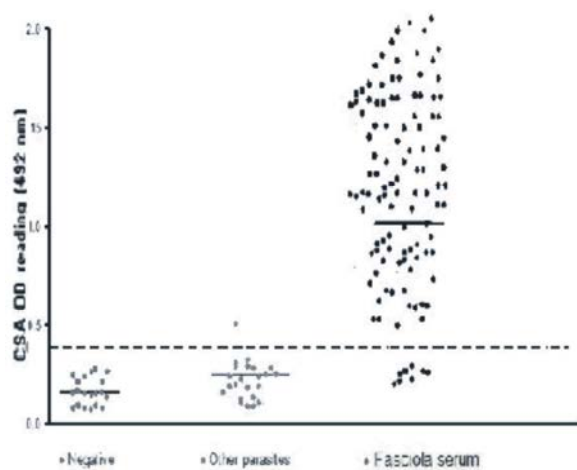


Fig. 4: Detection of circulating Fasciola metacercarial Ags in sheep sera by S-ELISA

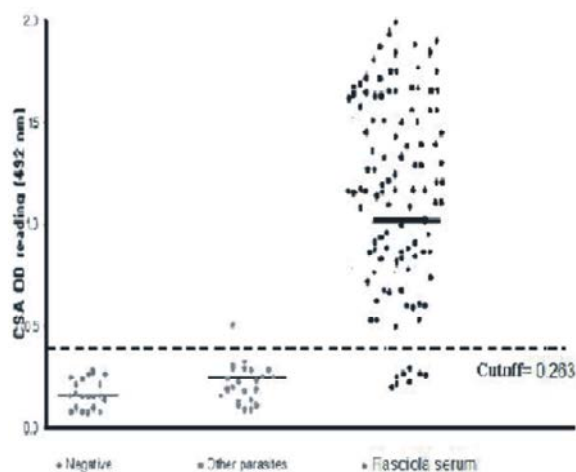


Fig. 5: Detection of circulating Fasciola metacercarial Ags in sheep sera by Dot-ELISA

Table 2: Stool examination of sheep for *Fasciola* egg count

Sheep groups	Ova Count (mean ± SD)
Healthy control (n = 20)	-
Light infection (n = 20)	14.81 ± 1.81
Moderate infection (n = 30)	31.2 ± 1.32
Heavy infection (n = 20)	85.7 ± 1.06
Other parasites (n = 20)	-

Table 3: Sensitivity, specificity and diagnostic efficacy of sandwich ELISA and Dot-ELISA for detection of *Fasciola* antigens in serum samples.

Item	S-ELISA	Dot-ELISA
Sensitivity	92.7%	95.5%
Specificity	95 %	97.5%
Diagnostic efficacy	93.9%	94.2%

A positive correlation was found between ova count in stools of *Fasciola*-infected sheep and the OD readings of ELISA serum samples ( $r = 0.730$ ,  $p < 0.001$ ).

**Detection of Circulating Fasciola Metacercarial Ags in Sheep Sera by DOT-ELISA:** The optimal criteria for conducting Dot-ELISA were detected as 1 ig of antigen per dot; *Fasciola* antigen was detected in 105 out of the 110 *Fasciola* infected sheep sera leading to a sensitivity of 95.5%. All negative control sheep and 19 out of the 20 other parasites group sheep had undetectable *Fasciola* antigen and the overall specificity of the assay was 97.5% (39/40). Only one case of *Schistosoma* showed false positive reactions. The diagnostic efficacy of the assay was 94.2% (Fig. 5 & Table 3). In *Fasciola*-infected sheep, a positive correlation was recorded between ova count/gm stool and the intensity of colour reading in serum samples ( $r = 0.58$ ,  $p < 0.01$ ).

## DISCUSSION

Fascioliasis is an infectious parasitic disease caused by *F. hepatica* or *F. gigantica* affects millions of people world-wide. Up to 17 million people are infected and around 91.1 million are at risk of infection [26]. Today, fascioliasis is recognized as an emerging and re-emerging vector-borne disease with the widest latitudinal, longitudinal and altitudinal distribution known for any zoonotic disease. Hence, the World Health Organization has classified fascioliasis as an important human parasitic disease that merits international attention [5, 27].

This study was done to evaluate the sensitivity and specificity metacercarial antigens as early diagnostic agents of fascioliasis. Exploring an effective early diagnosis might be the keystone of the prevention and eradication of the disease. Our goal was achieved through detection of specific circulating *Fasciola* antigen in naturally infected sheep sera using the sandwich and dot-ELISA techniques.

The preparation of pAb against *Fasciola* metacercarial antigen was carried out by immunization of rabbits with purified metacercarial antigen, pAb was purified by ammonium sulfate precipitation and caprylic acid treatment and was identified as a double band at 31 kDa and 53 kDa by reducing SDS-PAGE. These yields were valuable as purified Ig following similar purification procedures by Perosa *et al.* [28] to purify the human pAb. The activity of purified pAb was detected by indirect ELISA and showed that the pAb was highly sensitive, specific and reliable for the detection of circulating *Fasciola* antigen.

In the present study, 150 blood samples were collected from naturally infected sheep and classified to fascioliasis ( $n = 110$ ), healthy control ( $n = 20$ ) and other parasites ( $n = 20$ ). According to parasitological investigations (egg count/g), fascioliasis group was classified into light infection ( $n = 20$ ) (14.8 epg), moderate infection ( $n = 30$ ) (31.2 epg) and heavy infection ( $n = 20$ ) (85.7 epg), the sensitivity of parasitological method using Kato-katz technique was 68.6% while the specificity was 100 %. The results obtained in this study are in agreement with those recorded from other studies [29, 30].

In this study, the sensitivity and specificity of sandwich ELISA for detection of circulating metacercarial antigen in sera was 92.7% and 95%, in comparison to the parasitological examination which gave 68.6% and 100%, respectively. These results indicating highly sensitivity and specificity of sandwich ELISA for immunodiagnosis of fascioliasis using purified pAb against metacercarial antigen. That is clinically vital to diagnose fascioliasis as early as possible in serum to avoid all the complications of such a disease. Previously Kumar *et al.* [31], evaluation of *F. gigantica* somatic antigen 27 kDa for potential detection of *F. gigantica* and *F. hepatica* infection in buffaloes by indirect ELISA showed 81% sensitivity and 97.98% specificity, which supports the potential usage of these antigens in early diagnosis.

Dot-ELISA has been considered as one of the valuable methods in diagnosis of different parasitological diseases including fasciolosis [32, 33], toxoplasmosis [33], schistosomiasis [35, 36], hydatidosis [37] and cysticercosis [38]. In this study, the sensitivity and specificity of dot-ELISA were 95.5% and 97.5%, respectively. Previous studies used dot-ELISA for screening anti-*Fasciola* antibodies in both ruminant and human but not in detecting *Fasciola* antigen as in this study. In this manner, Intapan *et al.* [33] used *F. gigantica* 27 kDa (FG 27) as a target antigen in dot-ELISA and found that the sensitivity, specificity and accuracy were 100%, 97.4% and 98.2%, respectively. Dalimi *et al.* [39] used *F. gigantica* partially purified antigen in *Fasciola* infected patients in dot-ELISA test and reported 94.2% sensitivity and 99.4% specificity. Also, Rokni *et al.* [40] used ESP as a target antigen in *F. hepatica* infected patients in dot-ELISA and found that the sensitivity and specificity were 96.8% and 96.1%, respectively. Antigen detection assays have an apparent advantage over antibody detection assays in that antigenemia implies

active infection [5,41,42] and decreasing the possibility of cross reaction with other parasites due to sharing the same epitopes [43].

Dot-ELISA has several advantages over sandwich ELISA, i.e. nitrocellulose papers spotted with antigen are stable for at least three months at  $-20^{\circ}\text{C}$ , all incubation steps are performed at room temperature and the results can be read with the naked eye, thus an expensive spectrophotometer is not required. The test is applicable to diagnose in the field setting as well as in laboratories that are not well equipped. The dot-ELISA is simpler and allows testing of multiple samples at the same time [33, 40, 44, 45].

To sum up, not only dot- ELISA was proved to be more rapid, sensitive, specific and feasible technique used with the candidate antigen in diagnosing fascioliasis but metacercarial antigens are efficient indicators for the early stages of the disease, as well. These promising results provide affordable technique to be used in the field and to detect the light infection.

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