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Efficacy of Sandwich and Dot-ELISA in Diagnosis of Fascioliasis using a Pair of Polyclonal Antibodies Against Cathepsin L Antigen in Naturally Infected Sheep

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ABSTRACT

The present study was designed to prepare a pair of polyclonal antibodies (PAb) (IgG₄ A7 and IgG₄ B12) against *Fasciola gigantica* Cathepsin L (CL) and used them in development of sandwich ELISA and dot-ELISA to detect Circulating *Fasciola* Antigens (CFA) in serum samples collected from a total of 157 sheep. The first PAb was used as antigen capturing Ab and the second as horse reddish peroxidase-conjugated antigen detecting Ab. After slaughtering, gross inspection of liver and parasitological stool examination, sheep were divided into *Fasciola* positive group (97 sheep), other helminthic infection group (30 sheep) and healthy control group (30 sheep). *Fasciola* antigens detected in serum of sheep by ELISA showed 94.8% sensitivity and 95% specificity. Dot-ELISA sensitivity was found to be 98.9% and specificity was 98.3%. In conclusion, Dot-ELISA gives better sensitivity and specificity than sandwich ELISA and easy to perform method for the rapid diagnosis of fascioliasis.

Key words: Fascioliasis, Cathepsin L., sandwich ELISA, dot-ELISA

INTRODUCTION

Fascioliasis, a chronic disease caused by helminthes, is a major cause of morbidity, mortality and decreased productivity in cattle, sheep and goats, resulting in serious economic losses in Egypt (Awad *et al.*, 2004; El-Shazly *et al.*, 2005). In addition, Nile Delta is considered one of the endemic areas in the world for human fasciolosis (Haseeb *et al.*, 2002; Safar *et al.*, 2005; Curtale *et al.*, 2007; Mas-Coma *et al.*, 2005, 2009).

Cathepsins are the main proteases present in the Excretory Secretory (ES) products of *Fasciola*. Cathepsin L (CL) is the major constituent (68%) of total cathepsins obtained from ES products (Dalton *et al.*, 2003). CL plays an important role in parasite survival, tissue digestion and escaping from immune evasion (Dalton *et al.*, 2003; O'Neill *et al.*, 2001). Therefore, it may be used in vaccination and diagnosis (Kuk *et al.*, 2005).

Coprological diagnosis of fasciolosis is useful in patent infections but it is not capable of detecting early infections. The pathology of immature fasciolosis is manifested as early as 3 weeks postinfection, whereas parasitological diagnosis is impossible until the 8th-12th week when eggs are detected in the faeces (Rokni *et al.*, 2003; Ghosh *et al.*, 2005; Kumar *et al.*, 2008). However, the serodiagnostic methods for the detection of antibodies are quite sensitive to detect the infection in the early stages but it did not discriminate previous exposure from current infection (Espino and Finlay, 1994; Dixit *et al.*, 2004; Mas-Coma *et al.*, 2005). Moreover, *Fasciola* shares antigens with other parasites such as *S. mansoni* increasing the possibility of cross-reactions when antibody

detection assay systems were used for diagnosis of infection (Espino and Finlay, 1994). Alternatively, antigen-detection assays has several advantages over other methods as it can identify animals with pre-patent infection and give a more accurate indication of current infection and the parasite load rather than past infection (Zheng *et al.*, 1990).

This study aimed to evaluate the diagnostic efficacy of a pair of polyclonal antibodies (PABs) raised against *Fasciola* CL antigen for detection of circulating *Fasciola* antigen using sandwich ELISA and dot-ELISA in serum samples of sheep naturally infected with *Fasciola*.

MATERIALS AND METHODS

Animal groups: This study was carried out in sheep (n = 157) slaughtered in a local abattoir located in Giza, Egypt. Blood and faecal samples were obtained from sheep brought in for slaughtering. Based on gross inspection, gold standard method, of sheep livers' after slaughter in abattoir, *Fasciola*-infected and -uninfected sheep were selected. Parasitological examination of stool samples using direct smear method, Kato-Katz concentration technique (Martin and Beaver, 1968) and formal-ether sedimentation (Mansour-Ghanaei *et al.*, 2003) was performed. Accordingly, sheep were classified into three groups, *Fasciola*-infected group (n = 97), other parasites infected group (n = 30) including schistosomiasis (n = 10), echinococcosis (n= 10), anclystomiasis (n = 10) and ascariasis and negative control group (n = 30). The collected blood from each sheep was centrifuged at 760 g at 4°C for 10 min and the obtained serum samples were stored at -20°C until analysis.

Purification of cathepsin L.: The standard protocol described by Coles and Rubano (1988) was followed with certain modifications to purify cathepsin L from *F. gigantica* regurgitant released *in vitro* (Dixit *et al.*, 2002). Briefly, viable flukes were incubated in 500 mL RPMI-1640 culture medium, pH 7.3 containing 2% (w/v) glucose, 30 mM HEPES and 25 mg L⁻¹ gentamycin. After 3-4 h incubation at 37°C, the culture medium (containing *in vitro* released regurgitate) was concentrated to a volume of 10 mL in an Amicon 8400 concentrator using an Amicon YM3 membrane (3 kDa molecular mass cut-off, Amicon) and applied to a Sephacryl S200HR gel filtration column (19×42 cm) equilibrated in 0.1 M Tris HCl, pH 7, at 4°C. The column was eluted with 0.1 M Tris HCl, pH 7 and 5 mL fractions were collected. Each fraction was assayed for Cathepsin L. activity using the fluorogenic substrate Tos-Gly-Pro-Arg-NHMec at a final concentration of 10 pM in 0.1 M glycine/HCl, pH 7. The Sephacryl S200 fractions containing Cathepsin L. activity were pooled and applied to a 50 mL QAE Sephadex column (2.5×10.0 cm) equilibrated in 0.1 M Tris/HCl, pH 7. The QAE Sephadex column was washed with 300 mL 0.1 M Tris/HCl, pH 7, then proteins eluted with 150 mL 75 mM NaCl in 0.1 M Tris/HCl, pH 7, followed by 250 mL 400 mM NaCl in 0.1 M Tris/HCl, pH 7. Five milliliter fractions (180 fractions in total) were collected and assayed for Cathepsin L. activity using the fluorogenic substrate Tos-Gly-Pro-Arg- NHMec. The Cathepsin L. activity was pooled then concentrated to 20 mL on an Amicon 8400 concentrator using a YM3 membrane. The concentrate was then diluted with distilled water to a volume of 100 mL and re-concentrated to a final volume of 10 mL to obtain a NaCl concentration of approximately 80 mM. The concentrated *Cathepsin* L. was stored as 1 mL aliquots at -80°C.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGE was performed according to Laemmli (1970) as follows; 10 ug/well of each isolated batch of Cathepsin L. antigen in 20 µL of sample buffer, were electrophoresed on 12% SDS-PAGE. The run

was continued until the sample reached the bottom of the gel. The protein bands in polyacrylamide gel were visualized by staining the gel with coomassie brilliant blue R-250 (Sigma).

Preparation of hyper-immune anti-*Fasciola* serum: Two kilogram New Zealand rabbits were immunized intramuscularly four times, 2-week intervals, with 1 mL of 1 mg of purified Cathepsin L. antigen diluted in 500 μ L PBS and 500 μ L of complete Freund's adjuvant in the 1st immunization and 500 μ g of purified Cathepsin L. antigen diluted in 500 μ L PBS and 500 μ L of incomplete Freund adjuvant in the subsequent immunizations. Three days after the last immunization dose, the rabbits were humanly sacrificed and the sera of each rabbit was collected separately and stored at -20°C until used (Espino *et al.*, 1987).

Purification and labeling of polyclonal antibodies (pAb): Classing and subclassing of pAb isotypes was done by indirect ELISA using a panel of anti-mouse immunoglobulin peroxidase conjugates (goat anti-mouse IgM, IgG, IgG₁, IgG_{2a}, IgG_{2b}, IgG₃, IgG₄ and IgA (Sigma). The produced subclasses of IgG pAbs were purified by ammonium sulfate treatment then by ion exchange chromatography (Goding, 1986). One used as capture pAb (IgG₄ A7; 1st rabbit) and the other pAb (IgG₄ B12; 2nd rabbit) was labeled by horse-radish peroxidase (Sigma) and used as a conjugated pAb (Tijssen and Kurstak, 1984).

Identification and Characterization of anti-ES products pAbs: Identification and characterization of the chemical nature of pAbs was performed using indirect ELISA by testing the reactivity of pAbs against the antigen before and after treatment with 20 mM sodium periodate (Sigma) and 4% trichloroacetic acid (Sigma) (Woodward *et al.*, 1985).

Detection of circulating antigens in sheep sera by sandwich ELISA: The following sandwich-ELISA was adopted using a pair of pAbs against Cathepsin L., one as antigen capturing (IgG₄ A7) and the other as antigen detecting antibody (IgG₄ B12). The optimal dilutions of pAbs were determined by a checkerboard titration using a negative- and positive-control serum samples in each plate. For each step, 100 μ L well was added unless mentioned otherwise. Polystyrene microtiter plates (Thomas Scientific, USA) were sensitized overnight at room temperature with purified IgG₄ A7 pAb (10 μ g mL⁻¹ of 0.1 M carbonate buffer, pH 9.6). The plates were thoroughly washed with PBS/T (2 min/wash) and unbound sites were blocked with 200 μ l/well of 2.5% Fetal Calf Serum (FCS) (Sigma) diluted in PBS/T, pH 7.4. After 2 h incubation at 37°C, the plates were emptied by suction. Undiluted serum was added and the plates were incubated for 1 h at 37°C. After thorough washing as described above, peroxidase-conjugated IgG₄ B12 pAb (10 μ g mL⁻¹ of PBS/T) was added. The plates were incubated for 2 h at 37°C and washed with PBS/T. The substrate *O*-phenylenediamine dihydrochloride (Sigma) was added and the plates were incubated for 30 min in the dark at room temperature. The enzyme reaction was stopped with 50 μ L/well of 8 N H₂SO₄. The absorbance at 492 nm wavelength (A₄₉₂) of the plates was read using a microplate ELISA reader (Bio-Rad, Richmond CA, USA). 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 the cut off value were considered negative while those readings greater than the cut off value were considered positive.

Detection of circulating *Fasciola* antigens in serum by Dot-ELISA: Dot-ELISA was conducted as described earlier (Pappas, 1988; Rokni *et al.*, 2006). Briefly, 2 μ g/5 μ L of IgG₄ A7 was

dotted on nitrocellulose membrane discs and allowed to be dried thoroughly. The discs were placed into flat bottom micro plate wells. Non-specific binding sites were blocked by adding 100 μ L of 2.5% FCS tris buffer solution containing 0.5% Tween 20 (TBS/T) to each well. Blocking solution was then aspirated off and antigen disks were washed by shaking (three times, 10 min each) with PBS with 0.05% Tween 20 (Riedel de Haen, AG, Seelze, Hanover, Germany) in TBS (vol/vol). Five microliters of serum samples were added to each disk and incubated for 1 hr at room temperature. The serum samples were removed and washings were conducted as described above. The washing solution was removed and 2 μ g/5 μ L of horseradish peroxidase-labeled IgG₄ B12 conjugate diluted in PBS/T were added to each well and incubated for 1 hr at room temperature. The conjugate was removed and other washings were conducted as mentioned before. Ten mls of DAB substrate (diamino benzidine tetrahydrochloride) (Sigma) was added to the nitrocellulose membrane and incubated for 2 min at room temperature. The development of a deep brown colour dot on disks when compared with negative serum control was considered to be evidence of positivity. Colour development in controls was negligible or completely absent.

Statistical analysis: Data were expressed as mean \pm standard deviation. Standard diagnostic indices including sensitivity, specificity, positive and negative predictive values (PPV and NPV) were calculated as described by Galen (1980). Correlations between ova count and ELISA OD reading in serum samples were performed using Pearson correlation coefficient. SPSS computer program (version 12 windows) was used for data analysis.

RESULTS

Fasciola worms were detected in 97 sheep by gross inspection of the liver of sheep after slaughter in abattoir and used as a gold standard method. Parasitological examination of stool by Kato-Katz concentration technique for detection and counting of *Fasciola* eggs was performed for all sheep (n = 157). *Fasciola* eggs were detected in 78 out of 97 sheep (80.4%) with egg count ranging from 18-80 eggs g⁻¹ stool.

A pair of pAbs against CL antigen was used in the present study. Isotypic analysis of pAbs revealed that they were of the IgG₄ subclass with a κ light chain. The specificity of the selected pAbs against different parasite antigens (*S. mansoni*, *Echinococcus granulosus*, *Ancylostoma duodenale* and *Ascaris lumbricoides*) was determined by ELISA. Both pAbs were strongly reactive against *Fasciola* antigen and non reactive with other parasite antigens. The enzyme-linked immunoelectrotransfer blott (EITB) technique revealed that the 2 selected PABs recognized 31 and 50 kDa bands of electrophorsed ES antigen. Binding of the two pAbs, IgG₄ A7 and IgG₄ B12, to CL antigens coated plates were strongly inhibited by treatment with 4% trichloroacetic acid (54.5 and 62%, respectively) and slightly inhibited by 20 mM sodium periodate (9.0 and 12%, respectively) denoting that they are glycoprotein in nature.

From the standard curve, the lower detection limit of ELISA assay was 4 ng/mL. In serum, the cutoff was 0.401 and CL antigen was detected by ELISA in 92 out of 97 *Fasciola*-infected sheep leading to overall sensitivity 94.8%. The specificity of the assay was determined as the sum of results of negative control group and other parasites group, 27 out of the 30 other parasites sheep group and all negative controls had undetectable circulating *Fasciola* antigen leading to overall specificity 95% (57/60). The positive and predictive values of ELISA assay were 96.8% (92/95) and 84.4% (27/32), respectively (Fig. 1, Table 1).

The optimal criteria for conducting Dot-ELISA were detected as 1 μ g of antigen per dot, *Fasciola* antigen was detected in 96 out of the 97 *Fasciola* infected sheep sera leading to

Table 1: Sensitivity and specificity of the two techniques for detection of circulating *Fasciola* antigen in naturally *Fasciola* infected sheep

Parameter	Sandwich ELISA	Dot-ELISA
Sensitivity	92/97 (94.8%)	96/97 (98.9%)
Specificity	57/60 (95%)	59/60 (98.3%)
PPV	92/95 (96.8%)	96/97 (98.9%)
NPV	27/32 (84.4%)	96/97 (98.9%)

PPV: positive predictive value; NPV: Negative predictive value.

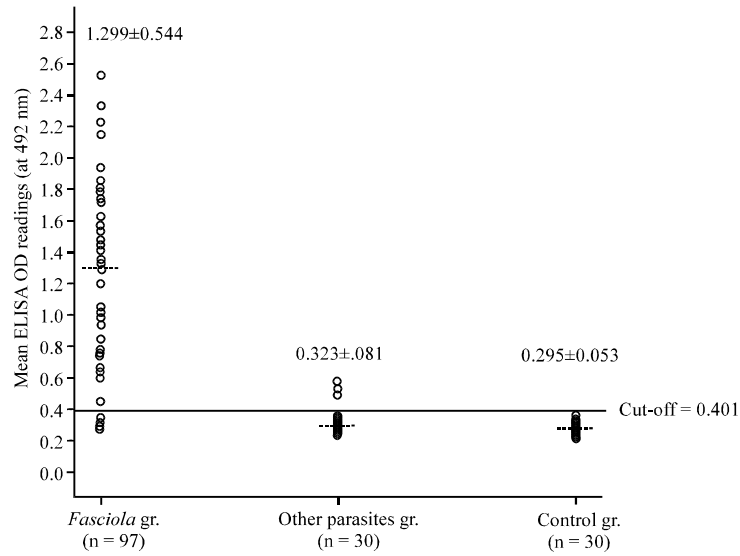


Fig. 1: Level of *Fasciola* CL antigen detected in serum samples of different studied groups measured by sandwich ELISA (OD reading at 492 nm). The horizontal line represents the cut-off value of the assay (the lower detection limit). Dotted lines represent the mean OD value

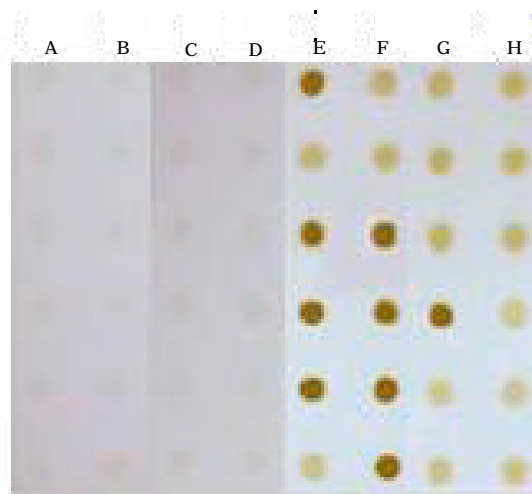


Fig. 2: Application of Dot-E LISA for detection of circulating *Fasciola* antigen in different studied groups. Lane A-B: Negative control group. Lane C-D: Other parasites group. Lane E-H: *Fasciola*-infected group

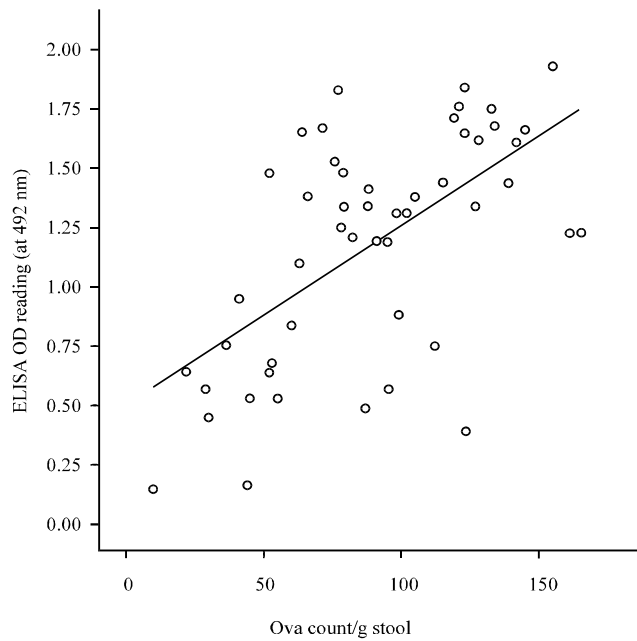


Fig. 3: Correlation between *Fasciola* egg count/g stool and OD reading of ELISA ($r = 0.58$; $p < 0.01$) in serum samples of *Fasciola gigantica* infected sheep

sensitivity of 98.9%. All negative control sheep and 29 out of the 30 other parasites group sheep had undetectable *Fasciola* antigen and the overall specificity of the assay was 98.3% (59/60). Only one case of *Schistosoma* showed false positive reactions in this serum titer. The positive and negative predictive values of the assay were 98.9% (96/97) and 98.3% (29/30), respectively (Table 1, Fig. 2).

In *Fasciola*-infected sheep, a positive correlation was recorded between ova count/gm stool and ELISA OD reading in serum samples ($r = 0.58$, $p < 0.01$) (Fig. 3).

DISCUSSION

Herein, purification of *F. gigantica* CL antigen by alcohol precipitation method followed by ion-exchange chromatography led to a removal of host components and an increase in antigen purity and hence an increase in both the sensitivity and specificity of ELISA and dot-ELISA assays. Electrophoretic separation on 12% gel resolved CL antigen into 1 protein band with the molecular weight of 27 kDa. Affinity chromatography has been shown to be a very effective tool for isolation of candidate diagnostic and vaccine molecule (Fagbemi *et al.*, 1995, 1997; Yadav *et al.*, 2005; Kumar *et al.*, 2008).

In this study the sensitivity and specificity of sandwich ELISA was 94.8 and 95%, respectively. Cathpsin L was found to be a promising antigen used in the diagnosis of *F. Hepatica* and *F. gigantica* infections during early prepatency in sheep, cattle and buffaloes (Dixit *et al.*, 2002, 2004) and has given a satisfactory level of sensitivity, though several workers have reported varying degrees of cross-reactivity of CP antigen with other related helminths. Cross-reactivity of *F. gigantica* 28 kDa CP with *Schistosoma bovis*, *Paramphistomum microbothrium* and *Dicrocoelium hospes* in cattle has been reported by Fagbemi and Goubadia (1995). Cross-reactivity

of native *F. hepatica* Cathepsin L. with sera from cattle infected with *Dictyocaulus viviparus* was observed by Cornelissen *et al.* (1999). Cornelissen *et al.* (2001) reported a specificity of 75.3% in naturally infected cattle using *F. hepatica* recombinant Cathepsin L. protease as antigen. However, O'Neill *et al.* (1998) observed no cross-reactivity of Cathepsin L. antigen with sera from human patients with *S. mansoni*, hydatidosis, cysticercosis and Chagas disease by using IgG₄ ELISA. Carnevale *et al.* (2001) found that recombinant pro-Cathepsin L. was 100% specific in the detection of human *F. hepatica* infection. A commercially available ELISA kit based on the fas-2 antigen, CL1 (Levieux and Levieux, 1994) for the serodiagnosis of *F. hepatica*, by Institute Pourquier, Montpellier, France, has been found 98.2 and 98.3% sensitive and specific, respectively, in cattle and 96.9 and 99.4% sensitive and specific, respectively, in sheep (Molloy *et al.*, 2005). Yadav *et al.* (2005) used purified Cathepsin L. CP for detection of anti-*F. gigantica* antibody in experimentally infected sheep and buffaloes using ELISA, Western blotting and dipstick ELISA and reported that it detected infection as early as 4 weeks post-infection.

Dot-ELISA has been considered as one of the valuable methods in diagnosis of different parasitological diseases including fasciolosis (Hassan *et al.*, 2002; Intapan *et al.*, 2003), toxoplasmosis (Elsaid *et al.*, 1995), schistosomiasis (Schaefer *et al.*, 1995; Mansour *et al.*, 2009), hydatidosis (Hadighi *et al.*, 2003) and cysticercosis (Liu *et al.*, 1996). In this study, the sensitivity and specificity of dot-ELISA were 98.9 and 98.3%, respectively. In fact, all 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.* (2003) 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.* (2004) 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.* (2006) 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 (Dixit *et al.*, 2004; Mas-Coma *et al.*, 2005) and decreasing the possibility of cross reaction with other parasites due to sharing the same epitopes (Espino and Finlay, 1994). The improved specificity in the present study could be attributed to the use of a pair of pAbs of IgG₄ isotype as antigen capturing and binding antibodies, in this manner, Hassan *et al.* (2002) conducted dot-ELISA used for measurement of different anti-*Fasciola* IgG isotypes and reported that IgG₂ and IgG₄ demonstrated the highest specificity (>99%), followed by IgG₁ (90%) and the least specific test was obtained with detection of IgG (85%). Levieux and Levieux (1994) concluded that detection of anti-*Fasciola* isotypes especially IgG₄ was very specific for accurate diagnosis of fasciolosis. Also, Wongkham *et al.* (2005) developed ELISA using recombinant *F. gigantica* CL1 antigen to detect specific immunoglobulin G (IgG) subclass antibodies (IgG₁, IgG₂, IgG₃ and IgG₄) in human fasciolosis and reported that IgG₄ enhanced both specificity and accuracy of ELISA test.

Generally, the difference in specificities could also be attributed to the different target antigens used and the different protocols used in preparation and purification of these antigens and finally the type of antibody used (Cornelissen *et al.*, 1992; Dumenigo *et al.*, 2000; Neyra *et al.*, 2002). Also, polyparasitosis was considered an important factor that may decrease the specificity especially when an animal harbour two or three parasitic infections at the same time raising the possibility of cross-reaction.

Dot-ELISA has several advantages over sandwich ELISA, i.e., nitrocellulose papers spotted with antigen are stable for at least three months at -20°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 (Intapan *et al.*, 2003; Wongkham *et al.*, 2005; Rokni *et al.*, 2006).

It is concluded that, dot ELISA is an easier, more rapid, sensitive and specific test for diagnosing fascioliasis and cheaper than sandwich ELISA, especially in case of light infection, denoting that it could detect the infection at earlier stages. Besides, it is a more feasible test in the field.

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