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APPLICATIONOF COMPETITIVE ELISA FOR DETECTION OF *FASCIOLA GIGANTICA* TEGUMENT-ASSOCIATED ANTIGEN IN HUMAN FASCIOLIASIS

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ABSTRACT

Tropical fascioliasis is considered to cause a significant economic loss in livestock industry in developing and underdeveloped countries. The aim of the current study was to evaluate the diagnostic capacity of *Fasciola gigantica (F. gigantica)* tegument-associated antigen to be compared with Kato-Katz method in diagnosis of human fascioliasis. The *F. gigantica* tegument-associated antigen was purified from the crude extracts by Superose-12 HR-10/300 column gel filtration chromatography. Polyclonal antibody (pAb) against purified *F. gigantica* tegument-associated antigen was prepared by immunization of rabbits with *F. gigantica* tegument-associated antigen. 83 blood and stool samples were collected from *Fasciola* patients, healthy control, and patients with other parasites. Detection of circulating *F. gigantica* tegument-

associated antigen in human serum and stool samples by competitive enzyme linked immunosorbant assay (ELISA) was done. The results revealed that the sensitivity and specificity of competitive ELISA for detection of *Fasciola* antigen in sera was 86.2% and 92.6 % in comparison to the sensitivity and specificity of competitive ELISA for detection of *Fasciola* antigen in stool samples which gave 96.3% and 96.3%, respectively. These results indicated the high sensitivity and specificity of competitive ELISA for immunodiagnosis of fascioliasis in both serum and stool samples using pAbs against *F. gigantica* tegument-associated antigen.

Key words: *Fasciola gigantic*a, tegument-associated antigen, serum, stool, indirect ELISA, Kato Katz method

INTRODUCTION

Fascioliasis is zoonotic disease that infects approximately 17 million people around the world. The disease is regarded as one of the parasitic diseases causing major economic and health problems (De Almeida *et al.,* 2007). *Fasciola gigantica*, causes a significant economic loss of 3 billion US dollars per year to the agriculture sector worldwide through meat and milk yield losses (Robinson and Dalton, 2009). Egypt is considered a hot spot for *Fasciola* infection especially the Nile Delta with estimated prevalence varying between 2 and 17% (Soliman, 2008).This fluke migrates in liver tissue after penetration of the liver capsule producing traumatic hemorrhage and necrosis, ending up in liver cirrhosis.

The most reliable means for diagnosis of human fascioliasis is the classical direct parasitological examination of stool using light microscopy. However, the technique is time-consuming, lacks sensitivity and sometimes unreliable as *Fasciola* eggs are not shedded in stool in the prepatent period; which lasts until approximately 3 to 4 months post infection (Noureldin *et al*., 2004). Moreover, eggs are frequently excreted at irregular intervals and repeated stool examinations of consecutive samples are required (Valero *et al*., 2009).

The most commonly used method for immunodiagnosis of fascioliasis is the ELISA. However, the antibody cross reacts with other trematode antigens giving false positive results (Hillyer 2005). Moreover, detection of *Fasciola* antibodies does not differentiate between previous and recent infections (Sabry and Taher, 2008). Thus, the use of specific antibodies to detect antigens secreted by the living flukes into their

host's body fluids may provide a better tool not only to diagnose active infection but also to assess the efficacy of treatment. Antibody-based antigen detection of *Fasciola* antigens may be also superior over microscopic examination in terms of sensitivity and facility of use, particularly for epidemiologic studies. Furthermore, because the release of *Fasciola* copro-antigens takes place before egg shedding, immunologic methods are also preferable to egg examination for diagnosis of acute infections (Ubeira *et al*., 2009).

The aim of the current study in immunodiagnosis of fascioliasis was to perform home-made diagnostic kit prepared at parasitology department, against purified *F. gigantica* tegument-associated antigen from adult liver fluke and evaluate its diagnostic capacity using competitive ELISA for detection of *Fasciola* copro-antigen and serum antigens in human fascioliasis

MATERIALS AND METHODS

Preparation of *F. gigantica* tegument-associated antigen

The tegument-associated antigen was isolated from adult *F. gigantica* flukes as previously described with some modifications (Gaudier *et al*., 2012). Briefly, *F. gigantica* adult flukes from freshly Livers of infected cattle were obtained from a local abattoir at Giza District, Egypt. *F. gigantica* adult flukes were washed several times with cold PBS. The flukes were subsequently incubated in RPMI 1640 medium, pH 7.4, supplemented with 100 U of penicillin and 100 µg of streptomycin per ml medium (Sigma Chemicals, St. Louis, USA) at 4°C for 24 h with gentle shaking for enrichment of the proteins around the surface (upper side of the basal membrane of the tegument) of the parasite. Following 24 h incubation the supernatant containing proteins from the surface of the parasite, was collected and centrifuged for 45 min at 27, 000 × g (4°C). The detergent was removed using an Extracti-GelD kit, and the preparation was concentrated by AMICON ultrafiltration using a YM-3 membrane (cutoff, >3 kDa). The protein content was determined by the Bio-Rad assay kit.

Purification of tegument-associated antigen by gel filtration chromatography

Five mg of partially purified tegument-associated antigen was applied onto a 50 ml Superose-12 HR-10/300 column (2.5 x 30 cm) (GE Healthcare

Biosciences, Pittsburgh, PA) equilibrated with 0.1 M Tris-HCl, pH 7.4. Samples were eluted with the same buffer at a flow rate of 0.5 ml/min. Elution was monitored by measuring the absorbance at 280 nm (A_{280}). Five ml fractions eluted from the column were collected, lyophilized, and resuspended in 3 ml distilled water (Morales and Espino, 2012).

Characterization of purified tegument-associated antigen

The purified tegument-associated antigen was characterized for determination of molecular weight range using discontinuous Sodium Dodecyl Sulphate-Polyacrylamid gel electrophoresis (SDS-PAGE) in 12.5% slab gels (1mm thick), under reducing conditions (+2- mercaptoethanol) and stained with Coomassie blue (Bio-Rad) as described by Laemmli (1970).

Preparation of Polyclonal Antibodies against purified tegument-associated 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 purified *F. gigantica* tegument-associated antigen mixed 1:1 in complete Freund adjuvant (CFA).Then two booster doses were given, at1 week intervals after the 1^{ry} injection each was 0.5 mg of Purified *F. gigantica* tegument-associated antigen emulsified in equal vol. of incomplete Freund adjuvant (IFA). One week after the last booster dose, the rabbit's sera were obtained and pAb fraction was purified by 50% ammonium sulphate precipitation method (Javois*et al*., 1999). More purification of pAb was performed by 7% caprilic acid method (McKinney and Parkinson, 1987) and finally with gel-filtration (Javois *et al*., 1999) . The produced IgG appeared in a very high degree of purity except for few serum protein contaminants (Fagbemi *et al*., 1995; Guobadia and Fagbemi, 1997).

Isotyping and labeling of polyclonal antibodies (pAb)

Classing and subclassing of pAb (isotyping) was done by indirect ELISA using a panel of anti- rabbit immunoglobulin peroxidase conjugates (goat anti- rabbit IgM, IgG, IgG_1, IgG_2, IgG_3 and IgA) (Sigma) as described by Mansour *et al.* (1998). The produced pAbs were used as capture pAb (IgG 1) and the other pAb (IgG A2) was labeled by horse-radish peroxidase (Sigma) according to Tijssen and

Kurstak (1984) and used as a conjugated antibody.

Study Population

This study was conducted on patients admitted to Tropical Medicine Department, EL-Fayoum University Hospitals and outpatients of Theodor Bilharz Research Institute (TBRI), Giza. Those patients were complaining of abdominal pain, loss of body weight, dyspepsia, fever and diarrhea and were subjected to parasitological stool examination on three consecutive days using merthiolate-iodine-formaldehyde concentration method (Magambo *et al*., 1998). The number of eggs per gram stool was determined by the modified Kato thick smear technique (Martin and Beaver 1968; Kato-Hayashi, 2009). Three groups were used; *F.gigantica* infected group in whom the patients had the characteristic large operculated *Fasciola* eggs in their stool samples with no evidence of other parasitic infections (n = 29). Other parasites group (n = 34) included *S. mansoni* (n = 14), Hydatid (n = 10) and *Hymenolepis nana* (n = 10). Control group (n = 20) was age- and sex-matched parasite-free healthy individuals.

Stool Elute Preparation and Serum Samples Collection

Aqueous elutes of a portion of each stool specimen were prepared by adding approximately 3 parts of 0.01 M phosphate-buffered saline (PBS), pH 7.2, containing 0.05% Tween 20 (PBS/T) to 1 part of stool in a centrifuge tube (Demerdash *et al.,* 2011). The mixture was homogenized and then centrifuged at 900 × g for 5 min. The supernatant was aspirated and stored at -80°C until use. Whole Blood samples were collected from all cases and sera were separated by centrifugation at 760 × g at 4°C for 10 minutes, aliquoted and kept at -70°C until used.

Parasitological examination

Kato-Katz concentration technique (Martin and Beaver, 1968) and formal-ether sedimentation technique (Magambo *et al.,*1998) were performed for all patient's stool samples in order to identify *Fasciola* eggs or other helminthic ova.

Detection of Circulating *F. gigantica* tegument-associated antigen in Human Serum and stool samples by Competitive ELISA:

The microplates were coated with *F. gigantica* tegument-associated antigen in PBS 100 µl/well, and then placed in an incubator at 40-50 °C until the liquid completely evaporated. Next, 5 % glutaraldehyde solution in 0.1 M

sodium bicarbonate was added to fix the antigens and left at room temperature overnight. After blocking with 5 % skim milk at 37 °C for 1 h, 50 µl of the serum sample (1:20 diluted in blocking agents) was added to each well, followed immediately by the addition of 50 µl of IGg pAb (1:400 diluted in blocking agents). The plates were incubated at 37 °C for 1 h with shaking during the initial 3 min. Set up in duplicate wells. The controls included a strong positive, a weak positive, a negative control serum, and a buffer control. After washing with PBST, HRP-conjugated anti-human IgG 1:6000 diluted in PBST was added and incubated at 37 °C for 40 min. The plates were washed 5 times with washing buffer. Hundred µl per well of substrate solution one tablet of OPD (Sigma) dissolved in 25 ml of 0.05 M. Phosphate citrate buffer, pH 5 with added peroxidase H_2 O_2 (Sigma) and the plates were incubated in the dark at room temperature for 30 min. Fifty µl/well of 8 N H_2SO_4 was added to stop the enzyme substrate solution. The absorbance was measured at two wave length (450 nm and 630 nm) The OD value was determined using the formula: OD_{450}-OD_{630} (Mariani *et al*., l998).

Statistical analysis

The data were presented as mean (X) ± standard deviation (SD). The mean of the groups were compared by analysis of variance (Campbell, 1989) using either Student's t-test or ANOVA. The data were considered significant if $p \leq 0.05$. All statistical analyses were performed using the SPSS program.

RESULTS

Purification of *F. gigantica* tegument-associated antigen

The eluted fractions from *F. gigantica* worm following purification of whole *F. gigantica* worm homogenate bySuperose-12 HR-10/300 column gel filtration chromatography were monitored by measuring the absorbance at 280 nm (A280).The eluted antigens were represented by 2 peaks at faction no. 4 and 8 with a maximum OD value of 1.8. (Fig. 1).

Characterization of *F. gigantica* tegument-associated antigen

The SDS-PAGE analysis and Coomassie brilliant blue staining of the crude and purified antigens of *F. gigantica* are shown in Figure 2. The eluted protein fractions resulted from gel filtration chromatography column purification method were analyzed by 12.5% SDS-PAGE under reducing condition and showed 3 major bands at

115, 95, and 70 kDa and two minor bands at 27 kDa and 12 kDa. (Figure 2).

Estimation of total protein content:

The total protein content of *F. gigantica* tegument-associated antigen was measured crude antigen, partially purified and after purification steps including gel filtration chromatography column method. It was8.5 mg/ml, 5.9 mg/ml and dropped to 3.1 mg/ml, respectively.

Parasitological examination

Parasitological examination of human stool samples was done by Kat-Katz technique for detection and count of *Fasciola* eggs in total of 83 human samples. *Fasciola* eggs were detected in 29 patients (34.9 %) with egg load ranging from 4.1 – 24.7 egg/gm stool (Table 1).Other parasites included *S. mansoni* (n = 14), Hydatid (n = 10) and *Hymenolepis nana* (n = 10) were detected.

***Production of pAbs against F. gigantica* tegument-associated antigen**

Blood samples were withdrawn from New Zealand white rabbits before the injection of each immunizing dose. They were tested for the presence of specific anti-*F. gigantica* antibodies by indirect ELISA. An increasing antibody level started one week after the first booster dose. A high titer of specific anti- *F. gigantica* antibodies was observed at thirty-five days from the start of immunization dose with OD of 2.1 at 1/250 dilution (Table 2). The total protein content of specific anti-*F. gigantica* antibodies was 2.1 mg/ml.

Isotypic Analysis of Abs

Classing and subclassing of anti-*F. gigantica* Ab was done by indirect ELISA using a panel of anti- rabbit immunoglobulin peroxidase conjugates (goat anti- rabbit IgM, IgG, IgG_1, IgG_2, IgG_3 and IgA. Strong reaction against *F. gigantica* tegument-associated antigen with mean OD reading equal to 1.903± 0.152 using total IgG and high reaction with mean OD equal to 1.352±0.201 using IgG1which revealed that the produced pAb was IgG1 subclass (Table 3).

Detection of Circulating *F. gigantica* tegument-associated antigen in Human Serum

The circulating *F. gigantica* antigen level was measured at two wave length (450 nm and 630 nm). The OD value was determined using the formula: OD_{450}-OD_{630} Cut off values were calculated as mean OD reading of negative controls +2 standard deviation (SD) of the mean, tested samples showing OD values > cut off value

were considered positive for *F. gigantica*. The cut off value was 0.25. When detecting *F. gigantica a*ntigen in human sera, the results were positive in 25 cases (86.2%) of fascioliasis group while 4 cases were negative (13.8%) **(Table 4).**

In group infected with other helminthic parasites, 4 cases were detected as positive (2 with *S. mansoni*, 1 with Hydatid and 1 with *H. nana* infection) while the other 30 cases were negative. All healthy control patients were negative. There was a statistical significant (*P<0.001* and *P<0.01*) difference comparing the positivity in *F. gigantica* infected group and the other two tested (**Table 4)**.

Detection of coproantigen of *F. gigantica* tegument-associated antigen in human

Table 5 showed the results of OD value of coproantigen for human stool samples in different studied groups. The cut off values for positivity was 0.224.The OD values of *F. gigantica* infected group (1.68±0.201) were significantly higher than both the negative control group (0.195±0.09) and the other parasites groups (0.147±0.02) for *S. mansoni*, 0.123±0.07 for Hydatid and 0.142±0.05 for *H. nana*. In group infected with other helminthic parasites, 2 cases were detected as positive (1 with *S. mansoni*, 1 with Hydatid) while the other 32 cases were negative (Table 5). All healthy control patients were negative. There was a statistical significant (P<0.001 and P<0.01) difference comparing the positivity in *F. gigantica* infected group and the other two tested (**Table 5**).

Table 6 summarizes the sensitivity, specificity, PPV and NPV of competitive ELISA which was used for detection of *F. gigantica* in human serum and stool samples. It was found that the sensitivity, specificity, PPV and NPV of serum samples were 86.2%, 92.6%, 87.8% and93.1% respectively. While the sensitivity, specificity, PPV and NPV of stool samples were 93.1%**,** 96.3%**,** 93.5% and 96.4% respectively**.**

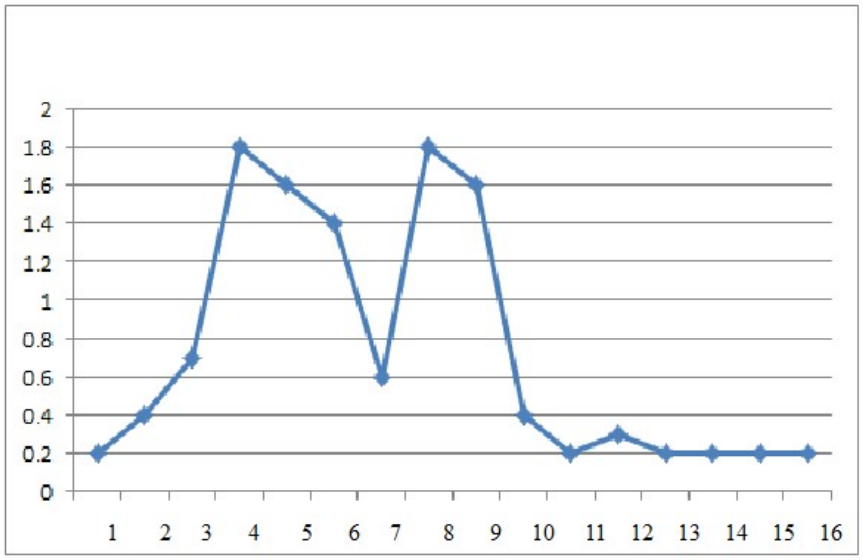

Figure (1): Fractions eluted from gel filtration chromatography columns monitored by measuring the absorbance at 280 nm (A280)

Table (1): Intensity of *F.gigantica* infection in relation to egg count by Kato-katz method

Groups		Positive cases		Negative cases	
		(n)	(OD_{450}-OD_{630} ± SD)	(n)	(OD_{450}-OD_{630} ± SD)
I	*Fasciola*(n=29)	27	(1.68±0.201)	2	(0.182±0.03)
II	Other parasites				
	Schistosomamansoni(n=14)	1	(0.721±0.21)	13	(0.147±0.02)
	Hydatid(n=10)	1	(0.801±0.321)	9	(0.123±0.07)
	Hymenolepis nana(n=10)	0	-	10	(0.142±0.05)
III	Healthy control(n=20)	-	-	20	(0.195±0.09)

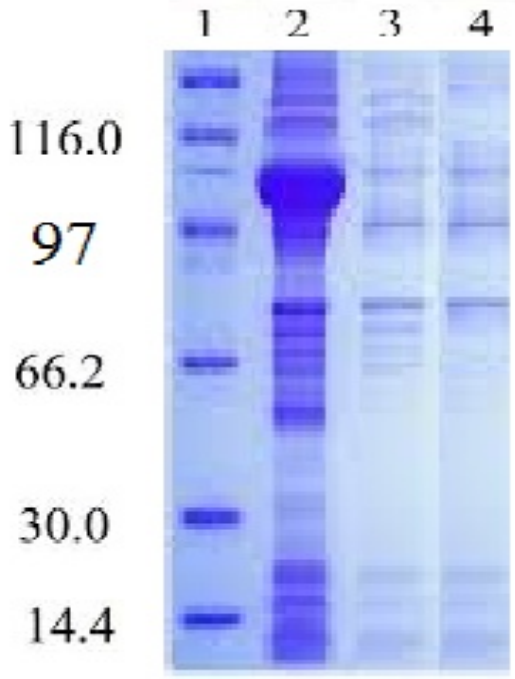

Figure (2): SDS-PAGE of target antigens eluted from affinity chromatography columns
Lane 1: Low molecular weight standard; Lane 2: Crude Antigen; Lane 3:Partially purified antigen; Lane 4: Purified tegument-associated antigen

Table 2: Antibody titer of immunized rabbit anti-*F.gigantica* IgGAb against *F. gigantica* tegument-associated antigen by indirect ELISA

	OD Value at 492 nm				
	0 Day	15 Days	21 days	28 Days	35 Days
Normal Rabbit	0.2	0.3	0.3	0.4	0.3
Immunized Rabbit	0.2	1.2	1.4	1.8	2.1

Table 3: Isotypic Analysis of Abs

Type of Immunoglobulin	OD readings at 492 nm
IgG	1.903± 0.152
IgG1	1.352± 0.201
IgG2	0.979± 0.112
IgG3	0.263± 0.224
IgM	0.142±0.102
IgA	0.903± 0.152

Table 4:-Detection of circulating *F. gigantica* tegument-associated antigen in human Serum

Egg count (no. of eggs/10ml)	Mean Ova	Males	Females	Total	Frequency (%)
Light (1-9 eggs)	4.1± 0.052	5	8	13	44.8%
Moderate (10-20 eggs)	14.4± 0.122	10	2	12	41.4%
Heavy (>20 eggs)	24.7± 0.158	3	1	4	13.8%
Total		18	11	29	100%

Table 5:- Detection of circulating *F. gigantica* tegument-associated antigen in human stool summarizes the reliability of results

		(n)	(OD_{450}-OD_{630} ± SD)	(n)	(OD_{450}-OD_{630} ± SD)
I	*Fasciola*(n=29)	25	(0.818±0.11)	4	(0.19±0.04)
II	Other parasites				
	Schistosomamansoni(n=14)	2	(0.321±0.09)	12	(0.141±0.021)
	Hydatid(n=10)	1	(0.469±0.07)	9	(0.128±0.031)
	Hymenolepis nana(n=10)	1	(0.529±0.05)	9	(0.151±0.02)
III	Healthy control(n=20)			20	(0.169±0.047)

Table 6: Summarizes the sensitivity, specificity, PPV and NPV of Competitive ELISA that used for detection of *F. gigantica* antigen in human serum and stool samples

*F. gigantic a*ntigen detected in :	Sensitivity	Specificity	PPV	NPV
Serum	86.2%	92.6%	87.8%	93.1%
Stool	93.1%	96.3%	93.5%	96.4%

DISCUSSION

Tropical fascioliasis caused by *Fasciola gigantica* infection is one of the major diseases infecting ruminants in the tropical regions of Asia and Africa, and causes a significant economic loss in the livestock industry in developing and underdeveloped countries for more than 3.2 billion US dollars per year (Anuracpreeda *et al*., 2016).The most widely method used for immunodiagnosis of fascioliasis is the ELISA. However, the antibody cross reacts with other trematode antigens, including *Schistosoma spp*., giving false positive results (Hillyer, 2005). Moreover, detection of *Fasciola*-specific antibodies does not

discriminate between the previous and the recent infections. Thus, the use of specific antibodies to detect antigens secreted by the living flukes into their host's body fluids may be a better approach not only to diagnose active infection but also to assess treatment efficacy and determine the effectiveness of future vaccines (Guobadia and Fagbemi, 1997). Therefore, the present study was conducted to evaluate the diagnostic capacity of *F. gigantica* tegument-associated antigen, in diagnosis of human fascioliasis. Antibody-based antigen detection of *Fasciola* antigens may be advantageous over microscopic examination in terms of sensitivity and facility of use, particularly for epidemiologic studies in endemic areas. Moreover, because the release of *Fasciola* coproantigens takes place before egg shedding (Ubeira *et al*., 2009), immunologic methods are also preferable to egg examination for detection of acute infections. However, because microscopic examination is currently the only method that is 100% specific for diagnostic of human fascioliasis, we used the Kato-Katz method as a "gold standard" for comparisons in the present study. The *F. gigantica* tegument-associated antigen was purified from the crude extracts by Superose-12 HR-10/300 column gel filtration chromatography. The purified *F. gigantica* tegument-associated antigen appeared as 3 major bands at 115, 95, and 70 kDa and two minor bands at 27 kDa and 12 kDa. Polyclonal antibody (pAb) against purified *F. gigantica* tegument-associated antigen was prepared and used for the detection of coproantigen in stool samples as well as the circulating *Fasciola* antigen in serum of infected human. Anti-*F. gigantica* antibody was prepared by immunization of rabbits with *F. gigantica* tegument-associated antigen. The purification procedures followed in this study were satisfactory for polyclonal IgG. Two purification steps were undertaken: ammonium sulfate precipitation and caprylic acid treatment. The yield of anti-*F. gigantica* antibody as a protein content by these methods was 2.1 mg/ml. This yield was reasonable in comparison with the yield of purified immunoglobulin from any biological fluid following similar purification procedures (Bride *et al*. 1995; Yang and Harrison,1996). The reactivity of the anti-*F. gigantica* pAb was tested by indirect ELISA against *F. gigantica* tegument-associated antigen. It was found that anti-*F.gigantica* pAb was

highly sensitive, specific, and reliable for the detection of the purified *F. gigantica* tegument-associated antigen.

From a panel of pAbs raised against *F. gigantica* tegument-associated antigen, a pair selected due to their high reactivity to *F. gigantica* antigen by indirect ELISA. Both pAbs showed no crossreactions with *S. mansoni*, Hydatid or *H. nana* antigens. Isotypic analysis of the produced pAbs revealed that they were of IgG1 and IgG2 subclasses.

In this study, we optimized competitive ELISA method for detection of *Fasciola* copro antigens in human stools as well as in serum samples. As regards the specificity of the competitive ELISA using anti-*Fasciola* pAb, there were no problems related to cross-reactivity when common helminthes were present as presented in our results, the specificity of competitive ELISA using anti-*Fasciola* pAb in serum samples was 92.6 % and was 96.3 for detection of *Fasciola* copro antigens in human stools. This is not surprising because the specificity of the test mainly depends on the recognition of specific antigen. Detection of coproantigen of *Fasciola gigantica*, already proved to be highly specific in previous studies with fecal samples of bovine and ovine origin (Mezo *et al*., 2004 and 2008).

In the present study, 83 blood and stool samples were collected from patients who complained of abdominal pain, loss of body weight, dyspepsia, fever and diarrhea and classified to fascioliasis (n = 29), healthy control (n = 20), and other parasites (n = 34) (14*S. mansoni*, 10 Hydatid and 10 *H.nana*).

In this study, the sensitivity and specificity of competitive ELISA for detection of *Fasciola* antigen in sera was 86.2% and 92.6 % in comparison to the sensitivity and specificity of competitive ELISA for detection of *Fasciola* antigen in stool samples which gave 96.3% and 96.3%, respectively. These results indicating highly sensitivity and specificity of competitive ELISA for immunodiagnosis of fascioliasis in both serum and stool samples using pAbs against *F. gigantica* tegument-associated antigen. The importance of such fact is that clinically it is vital to diagnose fascioliasis as early as possible in serum and stool samples to avoid all the complications of such a disease. This is in agreement with EL Ridi *et al*. (2007) who reported the most prominent nine bands of *F. gigantica* E/S (62-60,40, 30, and 28 kDa). The

data indicated that E/S-based indirect ELISA reached 100% sensitivity and specificity inimmuno-diagnosis of sheep fascioliasis. Also, Kumar *et al*. (2008) evaluated *F. gigantica* somatic antigen 27 kDa for potential detection of *F. gigantica* and *F. hepatica* infection.

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