

# Comparison between Different Immunological Techniques for Detection of Circulating Fasciola Antigen in sheep.

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**Abstract:** The detection of *Fasciola* antigen in serum or stool could be more valuable in diagnosis, hence early treatment before irreparable damage. In this study, fresh adult *Fasciola gigantica* worms were collected, then incubation in culture medium and collected medium was used to extract crude excretory-secretory (E/S) antigen. E/S was used to immunize rabbits to raise specific antibodies against *Fasciola spp.* Purified antibodies are further used as primary capture to coat ELISA plates. The secondary capture of antibodies was by conjugation with horse reddish peroxidase. Sandwich ELISA and DOT-ELISA were performed to detect *Fasciola* antigens in both serum and stool samples collected from a total of 152 sheep. 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* antigen detected in serum of sheep by ELISA showed 94.8 % sensitivity and 95 % specificity. Copro-antigen detected by ELISA showed 96.9% sensitivity and 96.7% specificity. The sensitivity and specificity of coproantigen by ELISA in stool sample were higher than that recorded by Sandwich ELISA for serum. Dot ELISA sensitivity was found to be 98.9% and specificity 98.3%. In conclusion the Dot ELISA gives better sensitivity and specificity than sandwich ELISA for serum and coproantigen in stool by ELISA. [New York Science Journal 2010;3(7):34-39]. (ISSN: 1554-0200).

**Key Words:** - Sandwich ELISA – Dot-ELISA – Fascioliasis - Coproantigen

## 1. Introduction

Fascioliasis is an infection caused by flukes of the class *Trematoda*, namely *Fasciola hepatica* and *Fasciola gigantica*. Human infection can result from the ingestion of encysted metacercariae attached to aquatic vegetation and plants as water-cress (Ragab and Farag, 1978; Hillyer, 1981; Marsden and Warren, 1984) or green vegetation as lettuce which is eaten raw (Stork *et al.*, 1973). The illness occurs world wide, particularly in regions with intensive sheep and cattle production (Abd- EL-Aziz *et al.*, 2001). The parasitological diagnosis of Fascioliasis is often unreliable because the parasite eggs are not found during the prepatent period (Nour Eldin *et al.*, 2004). Even when the worms have matured, the diagnosis may still be difficult since eggs are only intermittently released. Repeated examinations of stools are usually required to increase the accuracy of the diagnosis. Early diagnosis of Fascioliasis is necessary for institution of prompt treatment before irreparable damage of the liver occurs (Rokni *et al.*, 2004). For these reasons, serology is the most dependable diagnostic method. Attempts have been made to diagnose human Fascioliasis by detecting antibodies in the serum patient suspected of being infected with the flukes (Maleewong *et al.*, 1999). Advances in immuno-diagnosis have focused on detection of parasite antigens in host body fluid; these tests have an advantage over antibody detection because antigenemia implies recent and

active infection (Cornelissen *et al.*, 1999). The E/S antigens of *Fasciola spp* or their partially purified component are the commonest source of antigens used in protection trials and serodiagnosis (Gnen *et al.*, 2004). Immunodiagnosis of parasitic disease is mainly based on antibody detection (Fagbemi and Obarisiagbon, 1999) and revealed both recent and current infections with early diagnosis. To obtain reliable diagnostic method or to identify crude antigens, many authors prepared antigens from whole worm (Hillyer *et al.*, 1987 & Abdel-Rahman and Abdel-mageed, 2000) or from tegument (Charmy *et al.*, 1997) also Coproantigen (Allan *et al.*, 1996), egg antigen (Khalil *et al.*, 1989 and Abdel-Rahman *et al.*, 2000), and excretory secretory products (Espino and Finlay, 1994 and Ralston and Heath, 1995). The purpose of the present study is to isolate crude *Fasciola* antigens from E/S products, and preparation of anti-*Fasciola* antibodies, then evaluation of sandwich ELISA and Dot ELISA as an immunological assay for detection of *Fasciola* Copro-antigen and serum antigens in infected sheep, thus presenting an experimental trial that could be of value in providing a tool that may help in immunodiagnosis of human Fascioliasis.

## 2. Material and Methods

Preparation of crude E/S antigens

Adult *F. gigantica* worms were obtained from the livers of infected sheep at the local abattoir. The

worms were washed with sterile saline, and then incubated for 6 hr in RPMI 1640 culture medium (Sigma) according to the method of Maleewong and coworkers (1999). After incubation, the supernatant was collected and clarified by centrifugation at 10,000 rpm for 30 min at 4°C according to Hillyer (1979). The supernatant containing the E/S products was then concentrated by ultra filtration using Amicon 8400 membrane filter (Grace Co., Danvers, Ma), dialyzed against dist. H<sub>2</sub>O containing the proteinase inhibitors, and stored at -40°C before use.

**Preparation of hyper-immune anti-Fasciola serum**  
New-Zeland white rabbits were injected intramuscular. With 100 µg of crude E/S antigens and then 4 booster doses of antigens were given at weekly intervals. One week after the last booster dose, the rabbits were scarified and the sera were collected

#### Purification and labeling of polyclonal antibodies

The produced polyclonal antibodies were purified by ammonium sulfate treatment, then by ion exchange chromatography according to Goding (1986). The polyclonal antibodies were labeled by horse-radish peroxidase (Sigma) according to Tijssen and Kurstak (1984).

#### Identification and Characterization of polyclonal antibodies produced against ES antigen.

For identification and characterization of the chemical nature of polyclonal antibodies, The reactivity of polyclonal antibodies against the antigen before and after treatment with 20 mM sodium periodate and 4% trichloroacetic acid was tested by indirect ELISA according to Woodward *et al.* (1985).

#### Parasitological examination

Gross inspection of the liver of sheep after slaughter in abattoir to select Fasciola-infected animals and a number of non - infected sheep were taken as negative controls. Stool and serum samples were collected before slaughtering. Afterwards, samples collected from sheep harboring the fluke in their liver (as found by gross inspection), were considered Fasciola positive and these with normal healthy liver are Fasciola negative. Kato-Katz concentration technique (Martin and Beaver, 1968) and Formal-Ether sedimentation (Mansour-Ghanaei *et al.*, 2003) techniques were performed for all stool samples in order to identify Fasciola eggs or other helminthic ova.

Detection of circulating antigens in sheep sera and stool samples by sandwich ELISA.

After several optimization trials, the following sandwich ELISA originally described by Engvall and Perlmann (1971), was performed. Microtitration plates (Dynatech) were coated with 10 µg/ml of purified polyclonal antibodies in 0.1 M carbonate buffer, pH 9.6 dispensed as 100 µl/well and left overnight at room temperature. Plates were blocked by adding 200ul/well of 3% fetal calf serum/PBS/Tween for 1 hour at 37°C (3% FCS/PBS/T was used as diluting buffer and PBS/T as washing buffer). Undiluted sera or Undiluted Stool elutes were added (100 µl/well) and incubated for 2 hours at 37°C. Plates were washed with washing buffer. One hundred µl/well of 1:1000 dilution of peroxidase-conjugated polyclonal antibody (5µg/ml) were added and incubated for 2 hours at room temperature, and then plates were washed as before. The reaction was visualized by the addition of 100 µl/well of O-phenylene diamine (OPD) substrate solution for 30 minutes in the dark at room temperature. The reaction was stopped by adding 50 µl/well of 8 N H<sub>2</sub>SO<sub>4</sub> and plates were read at 492 nm using ELISA microplate reader (Bio Rad).

#### - Dot-ELISA (Antigen detection assay)

Dot ELISA was performed according to Boctor *et al.*, (1982) using Bio- Dot apparatus (BIO-RAD) for detection of circulating Fasciola antigen by double antibody sandwich procedures.

#### Statistical analysis

Data were expressed as mean ± standard deviation (SD) or number (%). Correlations between different parameters were performed using Spearman's rank correlation coefficient. SPSS computer program (version 13 windows) was used for data analysis. **3.**

### 3. Results

#### Parasitological Examination:-

Fasciola worms were detected in 97 sheep by gross inspection of the liver of sheep after slaughter in abattoir and used as our gold standard in succeeding experiments.

Parasitological examination of sheep stool by Kat-Katz technique for detection and count of Fasciola eggs was performed in total of 152 sheep. Fasciola eggs were detected in 78 sheep (80.62 %) with egg load ranging from 18 – 80 egg/gm stool. Other parasites including schistosomiasis, echinococcosis, anclystomiasis and ascariasis were detected in 30 sheep

#### Immunological Examination:

A pair of polyclonal antibodies (pAbs), 1F/6G and 2F/4G, were selected from a panel of anti-E/S antigens polyclonal antibodies for the present study. Isotypic analysis of polyclonal antibodies revealed that they were

of the IgG1 subclass with a K light chain. The specificity of the selected polyclonal antibodies against different parasite antigens (*S. mansoni* and *Echinococcus granulosus*) was determined by ELISA. Both polyclonal antibodies were strongly reactive with *Fasciola* antigen and non reactive with other parasite antigens (Table 1).

Table 1: Reactivity of anti- E/S antigens polyclonal with *Fasciola gigantica*, *S. mansoni* SEA, and *Echinococcus* antigens compared to normal rabbit serum

Coating antigen	ELISA OD at 492 nm		
	Normal rabbit serum	pAb 1F/6G	pAb 2F/4G
<i>Fasciola gigantica</i> antigen	0.049	1.98	2.13
<i>S. mansoni</i> SEA	0.051	0.26	0.24
<i>Echinococcus</i> antigen	0.038	0.17	0.18

Characterization of target antigen recognized by monoclonal antibody treatment with 4% trichloroacetic acid (54.5%) and slightly inhibited (9.0%) by 20 mM sodium periodate with denoting that target antigen for MAb 12D/10F binding of pAb 1F/6G to E/S antigens coated microtitration plates was strongly inhibited by was a glycoprotein (Table 2).

Table 2:- Identification and characterization of target monospecific antibodies produced against E/S antigens.

Target antibodies	XOD±SE	XOD±SE	% Reduction
	Before treatment	After treatment	
Periodate oxidation	1.20 ±0.01	1.08 ±0.06	12 %
Trichloroacetic acid treatment	1.38 ±0.01	0.642 ±0.06	54.5 %

Cut off point = 0.207      OD at 492 nm      SE = standard error

Sensitivity of the selected polyclonal antibodies by capture ELISA

Polyclonal antibodies against E/S antigens 1F/6G and 2F/4G were employed, one for coating and the other one as peroxidase conjugated antibody

in a sandwich ELISA to establish the sensitivity and linearity of the assay, a standard curve using serial dilutions of E/S antigens (0.0025–10µg/ml) in PBS/T buffer was set up. Optical density (OD) readings were plotted against the concentration of the antigen preparations. A lower detection limit of 3 ng E/S antigens /ml was reached (Fig . 1).

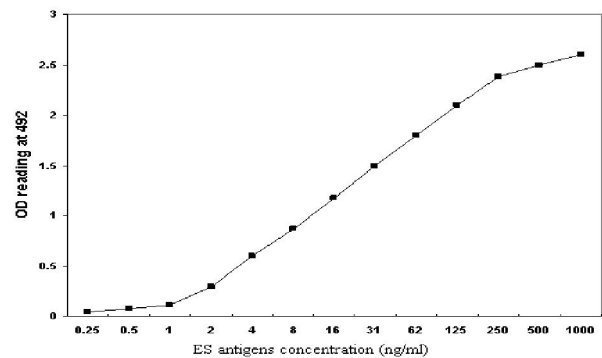


Fig. (1): Standard curve to test E/S antigens concentrations (ng/ml) in sandwich ELISA with pAbs 1F/6G and 2F/4G.

#### Detection of coproantigens in sheep stool by sandwich ELISA

All stool elutes from negative control sheep had antigen levels below cut-off value. Furthermore, 28 out of 30 stool elutes belonging to group of sheep

harboring other parasites had undetectable fasciola antigen. The overall specificity of the assay is 96.7% (58/60). Fasciola antigen was detectable in 94 of 97 stool elutes from infected sheep. The overall sensitivity of the assay is 96.9%. (Table 3).

Table 3:- Detection of Coproantigens in sheep stool by sandwich ELISA.

Groups	Positive cases			Negative cases		
	X (OD) $\pm$ SE	No.	% positivity	X (OD) $\pm$ SE	No.	% Negativity
normalcontrol (N = 30)	-	-	-	0.350 $\pm$ 0.24	25	100%
Infected sheep (N=97)	0.450 $\pm$ 0.37	94	96.9%	0.280 $\pm$ 0.29	18	19%
Other parasites(N=30)	0.391 $\pm$ 0.32	2	3.3 %	0.300 $\pm$ 0.40	28	96.7%

Cut off point = 0.207      OD at 492 nm      SE = standard error      N : number

#### Detection of circulating antigens in sheep sera by sandwich ELISA

As depicted in table (4), it was evident that the negative control sera were 100 % negative for circulating fasciola antigens. On the other hand, 27 out of 30 sera belonging to group of

sheep harboring other parasites had undetectable fasciola antigen. The overall specificity of the assay is 95 % (57/60). Fasciola antigen was detectable in 92 of 97 sera from infected sheep. The overall sensitivity of the assay is 94.8%.

Table 4 :-Detection of circulating antigens in sheep sera by sandwich ELISA.

Groups	Positive cases			Negative cases		
	X (OD) $\pm$ SE	No.	% positivity	X (OD) $\pm$ SE	N0	% Negativity
Healthycontrol (N=30)	-	-	-	0.361 $\pm$ 0.39	30	100%
Infected sheep (N=97)	0.876 $\pm$ 0.31	92	94.8%	0.307 $\pm$ 0.29	5	5.2%
Other parasites(N=30)	0.411 $\pm$ 0.22	3	5 %	0.294 $\pm$ 0.37	24	95 %

Cut off point = 0.207      OD at 492 nm      SE = standard error      N : number

#### Detection of circulating antigens in sheep sera by Dot- ELISA

Using Dot-ELISA the sera from negative control sheep were completely negative. On the other hand, 29 out of 30 sera belonging to group of sheep harboring other parasites had undetectable fasciola antigen. The overall specificity of the assay was 98.3

% (59/60). Fasciola antigen was detectable in 96 of 97 sera from infected sheep. The overall sensitivity of the assay is 98.9%. (Table 5)

Table 5 :- Sensitivity and specificity of different techniques for detection of circulating antigen.

	Sandwich ELISA in sera	Sandwich ELISA in stool	DOT-ELISA
% Sensitivity	94.8	96.7	98.9 %
% Specificity	95%	96.6	98.3 %

#### 4. Discussions

Several studies have reported the purification of the immunodominant antigens from the E/S products of liver flukes for immunodiagnosis of Fascioliasis. A recent study Rokni *et al.* (2004) compared the diagnostic potential of crude somatic antigen and E/S

antigen using enzyme linked immunotransfer blot (EITB) technique. The sensitivity and specificity values for somatic antigens were 91.0% and 96.2% respectively, as for E/S antigen this parameter were 95.2% and 98.0% respectively. On the other hand,

Shaker *et al.* (1994) found that ELISA was 100 % sensitive and 93 % specific for detecting circulating *Fasciola* antibodies using purified somatic antigen. But, Rodriguez-Perez and Hiller (1995) determined that ES antigens were more specific than somatic and surface antigens for serodiagnosis of Fascioliasis in sheep. Another study Yamasaki *et al.* (1989), demonstrated high sensitivity and specificity in an ELISA for immunoglobulin detection using E/S antigens of *Fasciola*, showing only a single case of cross-reactivity with sera from *Schistosomiasis japonicum* infected subjects Maleewong *et al.* (1999), showed that 25.9 % of sera from patients with cholangiocarcinoma showed cross-reactivity with *Fasciola* antigen, they added that this could be due to subclinical *Fasciola* infection, since in Thailand people habitually consume raw aquatic plants that harbor metacercariae. In this work, crude E/S antigen was used to prepare specific antibody, the later was used in sandwich ELISA to detect circulating *Fasciola* antigens. It is thought that these tests have an advantage over antibody detection in that antigenemia implies recent and active with more sensitive criteria (Deplazes *et al.*, 1991). In this study, there was cross reactivity showing with sera of tow sheep infected with Schistosomiasis, one with hydrated cyst, one with Trichostrongyloides and one with hook worm infection. Our results showed 82.47 % sensitivity and 90.9 % specificity in sandwich ELISA for Fascioliasis in naturally infected sheep. The somewhat low sensitivity may be due to the preparation of crude E/S antigen. Further purification of E/S antigen is needed to produce specific antigen; the continuous-elution SDS-PAGE would be recommended (Maleewong *et al.*, 1999). In addition the antigen should be purified from cross reacting antigenic part by immunoaffinity purification method using cyanogen bromide activated sephorose 4B coupled to rabbit antisera raised against other helminthic antigens particularly Schistosomiasis (Gottstein, 1983). Antigen detection assays may facilitate earlier diagnosis than antibody tests; hence the detection of Copro-antigen has the advantage of diagnosing Fascioliasis in stool even before egg detection in stool samples (Youssef *et al.*, 1991). Also production of detectable levels of Immunoglobulin needs time. An immunodiagnostic sandwich ELISA assay was performed for the detection of *F. hepatica* antigens in stool samples of experimentally infected animals. Using affinity-purified polyclonal antibodies rose against crude somatic antigen. The test showed 83.3% sensitivity and 80 % specificity, and antigens were detected as early as 4 weeks post infection (Moustafa *et al.*, 1998). In our work sandwich ELISA showed 81.44 % sensitivity and 91.78 % specificity, in stool of

naturally infected sheep. Specificity in our study was higher than that of the above mentioned work (Moustafa *et al.*, 1998), probably because the antigen prepared from E/S products of adult flukes yield more immunodominant antigen than somatic antigen (Rodringuez-Perez and Hillyer, 1995). Higher sensitivity (100%) and specificity (95%) of ELISA were observed by Darwish and EL-Gammal (1995), for detection of *Fasciola* Copro-antigen in human cases in comparison to results obtained in this study. This may be due to variability of parasite species collected from the abattoir for this work, and the fact that human beings had many factors which may cause Cross-reactions. It could be concluded that sandwich ELISA rapid, easy and sensitive for diagnosis of Fascioliasis. It would be more valuable to apply this test in screening for Fascioliasis, being a non- invasive procedure, also detecting Copro-antigen by ELISA suggests recent and active *Fasciola* infection but still more purification is needed to overcome cross-reactivity and improvement of diagnosis.

## 5. References

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