Diagnostic value of some *Fasciola gigantica* antigens

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Abstract The present study was aimed to select the specificity of antigens for *Fasciola gigantica* depending on its diagnostic utility and field applications. The tested antigens were coproantigen, excretory–secretory (ES) antigen and egg antigen. Coproantigen and Copro Hyper-immune serum were able to reflect the lowest level of cross-reaction with other tested *F. gigantica* antigens. By using SDS-PAGE, a structural homology was observed in *F. gigantica* ES and egg antigens. Intense cross reaction was observed between ES and egg antigens by ELISA technique even when there was no cross-reaction with coproantigen. The 27.6 kDa band proved to be the most specific in *F. gigantica* coproantigen and was different from the band at the same molecular weight by ES antigen. The results conclude that coproantigens show specific diagnostic ability for *Fasciola* and have low numbers of cross-reaction proteins reflecting its high specificity. Moreover, detection of coproantigen in faeces offers a new potential for diagnostics as compared to serum samples. This fact holds promise for a more accurate diagnostic

technique in the near future for suspected *Fasciola* infection.

Keywords Antigens · Cross reaction · Diagnosis · *Fasciola gigantica*

Introduction

Early and accurate diagnosis of *Fasciola* infection plays an essential role in designing a complete control plan for the disease. Diagnosis of *Fasciola* infection is usually achieved by identifying fluke eggs in faeces. However faecal sedimentation methods are time consuming and of low accuracy. In cattle, by the time eggs are produced, damage to the liver parenchyma may be severe.

In an effort to develop more sensitive diagnostic methods, two types of immunoassays have been investigated for the diagnosis of fascioliasis: detection of antibodies in serum as early as 2–4 weeks after infection; and detection by sedimentation technique after 10–14 weeks post-infection (p.i.). The second approach relies on detection of circulating parasitic antigens by ELISA; being positive after 4–6 weeks p.i.; but once flukes enter the bile ducts, the antigen products may no longer be circulating and this decreases the stability (Shalaby et al. 1998).

Concerning the detection of specific anti-*Fasciola* antibodies in serum, sensitivity and specificity of serological tests are affected by the antigens used; including *Fasciola* crude antigen, egg antigen and ES antigen (Espino et al. 1992). Despite their satisfactory sensitivity, lack of specificity was due to the presence of common antigens. This led to the discovery of specific coproantigen being extracted from faeces. The value of this specific technique

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can be increased with availability of specific purified hyperimmune serum (Dumenigno and Finlay 1998).

Cross-reaction in immunodiagnosis was studied by Hassan et al. (1989) using *Schistosoma* soluble egg antigen (SEA) and *Fasciola* adult worm antigen in patients infected with Fascioliasis, Heterophyiasis and Schistosomiasis by ELISA. False positive reactions occurred in 10 % of cases with Fascioliasis and Heterophyiasis when SEA was used. When crude antigen was used, false positive reaction occurred in 25 and 35 % of cases with Schistosomiasis and Heterophyiasis respectively. The specificity of SEA and *Fasciola* adult worm antigen were 87 and 70 % respectively.

Taking into account the previous facts, the present study aimed to test the antigenic relationship between the three selected *Fasciola gigantica* antigens (Coproantigen, excretory–secretory antigen and egg antigens) versus their related hyper-immune sera. Also, the relation between different protein bands in the three tested antigens using Sodium Dodecyl Sulphate Poly-Acrylamide Gel Electrophoresis (SDS-PAGE) and Enzyme-linked ImmunoTransfer Blot (EITB) versus reference sera was determined in order to search for the most specific antigen, being the best and easily applicable for the rapid diagnosis of Fascioliasis.

Materials and methods

Sample collection

This study was performed on 41 cattle and 68 buffaloes from private farms in Fayum Governorate (Egypt). Blood samples were collected, serum was separated and stored at -20°C till use. Fecal samples were examined directly for determination of parasitic status of each animal being used in the preparation of *F. gigantica* coproantigens.

Serum samples of 24 animals selected from the previously examined cases were used depending on their parasitic status. This included 10 animals shedding *Fasciola* species eggs, 10 helminthes eggs other than *Fasciola* spp. and 4 animals which did not shed eggs. These sera were used for identification of specific protein bands of tested *F. gigantica* antigens by EITB technique.

Antigen preparation

Faecal supernatant was used for coproantigen immunodetection according to El-Bahy et al. (1992). The protein content was measured by the method of Lowry et al. (1951). The antigen was aliquoted and stored at -20°C until used.

Egg antigen was prepared according to El-Bahy et al. (1992). The protein content was measured by the method of

Lowry et al. (1951). The antigen was aliquoted and stored at -70°C until used.

ES antigen was prepared according to River Marrero et al. (1988). Protein content was measured according to Lowry et al. (1951). The antigen was aliquoted and stored at -70°C until used.

Hyperimmune rabbit sera (HIS) related to the three antigens was prepared according to El-Bahy et al. (1992).

Antigen detection

Two ELISA techniques were used; Indirect ELISA for current determination of circulating antibodies and Monoclonal –Antibody Sandwich ELISA for capturing of antigens in tested samples. This helped in evaluation of *F. gigantica* coproantigen as a tool for diagnosis of fascioliasis at field level.

Fractionation of different *F. gigantica* antigens was done using SDS-PAGE. Electrophoretic transfer of proteins from SDS-PAGE to nitrocellulose sheet was done according to Towlin et al. (1979). Immunodetection of antigenic bands on nitrocellulose strips was performed by Enzyme –Linked Immunotransfer Blot (EITB).

Results

Testing the ability of 3 selected *F. gigantica* antigens (Coproantigen, egg antigen and ES antigen) in binding with specific antibodies of related hyper immune rabbit sera (HIS) revealed that the rate of cross-reaction between the three selected antigens and sera decreased with increase in serum dilution from 1:50 to 1:1,600.

Copro HIS reacted specifically with coproantigen at different levels of serum dilution with decreasing mean ELISA OD value from 0.4 to 0.08 on increasing serum dilution from 1 :50 to 1:1,600 respectively. At the same time, coproantigen was not involved in cross reaction with other tested HIS (egg HIS and ES HIS). On the contrary, ES HIS cross- reacted with egg antigen at all tested dilutions and egg HIS was able to specifically detect its homologous antigen at different levels of dilution but it cross reacted with ES antigen at 1:50, 1:200 and 1:400 serum dilution. The data in Table 1 characterizes coproantigen as well as copro HIS as antigen and antibodies which were able to reflect the lowest level of cross-reaction with other selected *F. gigantica* antigens.

In order to clarify the main cause of cross-reaction recorded by ELISA technique using the three tested *F. gigantica* antigens versus their specific hyperimmune rabbit sera, this part of the study aimed to throw light on the relation between different protein bands in the 3 tested *F.*

Table 1 Antigenic relationship between 3 selected *F.gigantica* antigens versus specific hyper-immune rabbit sera using ELISA technique

HIS dilution	Rabbit hyper-immune sera (HIS)	Mean ELISA O.D. value		
		Coproantigen	Egg antigen	ES antigen
1:50	Copro HIS	0.40	0.30	0.14
	Egg HIS	0.10	0.75	0.54
	ES HIS	0.21	0.70	0.57
	Normal serum	0.23	0.16	0.10
1:100	Copro HIS	0.30	0.23	0.07
	Egg HIS	0.11	0.71	0.43
	ES HIS	0.14	0.70	0.61
	Normal serum	0.22	0.09	0.08
1:200	Copro HIS	0.22	0.17	0.03
	Egg HIS	0.09	0.75	0.52
	ES HIS	0.08	0.63	0.58
	Normal serum	0.16	0.08	0.04
1:400	Copro HIS	0.14	0.04	0.05
	Egg HIS	0.06	0.61	0.32
	ES HIS	0.05	0.44	0.39
	Normal serum	0.13	0.05	0.03
1:800	Copro HIS	0.11	0.04	0.03
	Egg HIS	0.04	0.66	0.14
	ES HIS	0.03	0.40	0.41
	Normal serum	0.08	0.02	0.02
1:1600	Copro HIS	0.08	0.03	0.02
	Egg HIS	0.04	0.52	0.07
	ES HIS	0.03	0.34	0.34
	Normal serum	0.05	0.00	0.01

gigantica antigens using SDS-PAGE and EITB versus reference sera.

Fractionation of the three tested *F. gigantea* antigens revealed several bands located in the three main band groups between the protein molecular weight standard at 94–31, 31–25 and 25–14 kDa (Fig. 1a). Fractionation of dialyzed (6–8 kDa) *F. gigantea* coproantigen using SDS-PAGE revealed 9, 3 and 5 bands located at 94–31, 31–25 and 25–14 kDa band ranges, respectively. On the other hand, fractionated *F. gigantea* ES antigen showed 5, 3 and 8 bands at the previous band ranges, respectively. With respect to *F. gigantea* egg antigen, there were 7, 2 and 5 bands at the previous band ranges, respectively (Table 2).

Protein intensity of each band of fractionated *F. gigantea* of the three antigens of known molecular weight was measured using Imaging Densitometer (Table 2). The results displayed in Table 3 reveal that the 27.6 kDa band of all the tested *F. gigantea* antigens was the one most enriched in protein whereas, the least protein content was

recorded at 77.8, 21.3 and 18 kDa in *F. gigantea* coproantigen, ES and egg antigens respectively.

Fasciola gigantea coproantigen was analysed by SDS-PAGE then transferred onto nitrocellulose (NC) sheet for EITB technique. The fractionated and transferred coproantigen was allowed to react with rabbit hyperimmune sera raised against *F. gigantea* coproantigen, ES antigen, egg antigen as well as normal rabbit sera (Fig. 1b). EITB against coproantigen transferred to NC sheet revealed reaction by copro HIS to 7 bands; 21.5, 27.6, 30.5, 61.7, 72.1, 77.8 and 83.4 kDa (Table 4). The 27.6 kDa band was one of the most prominent bands detected whereas, there was a weak reaction with bands of 30.5, 77.8 and 83.4 kDa. On reaction of similar NC strips versus ES HIS and egg HIS, 2 bands of 77.8 and 83.4 kDa showed positive results besides the 21.6 kDa band with egg HIS while normal rabbit sera reacted non-specifically with the three bands of 61.7, 77 and 83.4 kDa.

From the above results it is clear that the 3 bands at 27.6, 30.5 and 72.1 kDa can be considered to be specific bands of *F. gigantea* coproantigen where they had no cross-reaction with ES HIS, egg HIS and normal rabbit sera. The fractionated and transferred ES antigen was allowed to react with ES HIS, copro HIS as well as normal rabbit sera (Fig. 1c). It was found that the ES HIS detected numerous immune-reactive bands. Bands of 18.1, 20.4, 24.4 27.7, 30.9, 35.5, 39.8, 44.2, 50.4 and 57.9 kDa were prominent (Table 5).

On reaction of similar NC strips blotted with fractionated *F. gigantea* ES antigen versus the other heterologous sera, few bands showed non-specific reaction. One band only at 30.9 kDa showed non-specific reaction with copro HIS whereas egg HIS reacted non-specifically with 4 bands at 18.1, 20.4, 27.7 and 30.9 kDa. There was a non-specific reaction in the form of a fine band at 30.9 kDa by using normal rabbit sera. These data recognized the bands of 24.4, 35.5, 39.8, 44.2, 50.4 and 57.9 kDa as specific bands of *F. gigantea* ES antigen since they had no cross-reaction with copro HIS, egg HIS and normal rabbit sera.

Fasciola gigantea egg antigen was analyzed by SDS-PAGE and then probed using rabbit hyperimmune sera raised against the previous 3 tested *F. gigantea* antigens and normal rabbit sera using EITB technique (Fig. 1d). The illustrated densitogram in Fig. 1d shows the patterns of different protein bands and their density indicating that there was a similar pattern between ES and egg antigens; however, they were not identical. It was found that egg HIS identified 8 immunoreactive bands of 18.03, 20.4, 27.6, 33.1, 42.7, 52.9, 65.2 and 71.3 kDa (Table 6).

Testing of fractionated *F. gigantea* egg antigen blotted at 2 bands only (20.4 and 71.3 kDa). On the other hand, there was a non-specific reaction at 5 bands of 18.03, 20.4, 27.6, 65.2 and 71.3 kDa by using ES HIS. The normal rabbit sera identified non-specifically 2 bands of 65.2 and

Fig. 1 **a** SDS-PAGE of *F. gigantica* coproantigen (**a**), egg antigen (**b**) and ES antigen (**c**). **b** Immunoblot reaction demonstrating specific protein bands of *F. gigantica* coproantigen using rabbit hyperimmune sera and normal rabbit sera. **c** Immunoblot reaction demonstrating specific protein bands of *F. gigantica* ES antigen using rabbit hyperimmune sera and normal rabbit sera. **d** Immunoblot reaction demonstrating specific protein bands of *F. gigantica* egg antigen using rabbit hyperimmune sera and normal rabbit sera. *St* low molecular weight standard

71.3 kDa. These data recognized the bands of 33.1, 42.7 and 52.9 kDa as specific bands of *F. gigantica* egg antigen since they had no cross-reaction with copro HIS, ES HIS and normal rabbit sera.

The above results of fractionation and identification of the 3 tested *F. gigantica* antigens indicate that in spite of using specific hyper-immune sera, 3 bands at 18, 20.4 and 27.6 kDa region were common between ES and egg antigens showing the ability to react with both types of their homologous hyperimmune sera and at the same time, did not show any reaction towards normal rabbit sera. These bands

were considered to be the main cause of cross reaction that was recorded previously by ELISA technique. On the other hand, the band at 27.6 kDa regions of *F. gigantica* coproantigen appears to be more accurate for diagnosis using EITB technique.

Discussion

Fascioliasis is one of the most important limiting parasites all over the world. The presence of eggs in stools was the

Table 2 Different protein bands recorded by Densitometer scan of SDS-PAGE gel of *F. gigantica* coproantigen, ES and Egg antigens

Lanes bands	Molecular weight (kDa)		
	Coproantigen	ES antigen	Egg antigen
1	83.358	57.926	71.278
2	77.787	50.430	65.168
3	72.061	44.209	52.936
4	61.654	39.734	42.679
5	57.526	35.515	39.647
6	47.053	30.914	36.323
7	42.201	27.624	33.072
8	38.425	25.572	27.559
9	34.336	24.406	25.198
10	30.453	22.952	23.580
11	27.596	22.121	22.505
12	26.080	21.269	20.398
13	24.707	21.009	18.029
14	21.531	20.348	15.427
15	19.668	18.094	
16	17.84	15.996	
17	15.351		

standard method for diagnosis of Fascioliasis. However, it lacked sensitivity since parasitic eggs did not appear during acute fascioliasis. As antibody titers persisted after curing, serological tests were also of limited value (Espino et al. 1992). A lack of specificity was observed to cross-reactions between *Fasciola* and other parasites (Hillier and Galanes 1988). Therefore, diagnosis of fascioliasis is nowadays directed towards the immunological detection of *Fasciola* antigens either in serum or faeces. Antigen detection assays have several advantages over other diagnostic methods. They can diagnose in the prepatent period or occult infection, give more accurate indication of active infection than many serodiagnostic tests (El-Bahy et al. 1992).

A variety of antigens were excreted and secreted by *F. gigantica* worms in blood and faeces of infected hosts. These antigens had potential for use in immunodiagnosis. Thereafter, although several diagnostic assays for fascioliasis were described by detection of coproantigen in faeces, little is known about the immunological characterization of this antigen. In the present study, the antigenicity and diagnostic value of *F. gigantica* coproantigen in comparison with that of ES and egg antigens were evaluated.

Table 3 Protein intensity of each band of fractionated *F. gigantica* coproantigen, ES and Egg antigens using Imaging Densitometer

Lanes bands	Low mol. wt. standard (kDa)	Coproantigen protein amount (%)	Low mol. wt. standard (kDa)	ES antigen protein amount (%)	Low mol. wt. standard (kDa)	Egg antigen protein amount (%)
	94		67		94	
1		3.8		5.7	67	8.0
2		1.3		4.0		5.0
3	67	4.5	43	6.4	43	5.2
4		6.5		12.7		5.0
5		5.6		9.8		4.2
6	43	1.4	30	9.3		4.4
7		3.4		17.3	30	12.0
8		4.6		3.6		17.3
9		5.0		6.1		8.6
10	30	6.7		5.7		4.4
11		19.2		3.6		10.0
12		4.9		0.9	20.10	9.5
13		13.6		1.2		2.6
14	20.10	4.8	20.10	4.0		3.5
15		4.4		5.4		
16		4.5		4.1		
17		5.5				
	14.40		14.40		14.40	
Sum		99.7		99.8		99.5
In lane		100.0		100.0		100

Table 4 Detection of *F.gigantica* coproantigen specific bands using EITB technique

Lanes bands sera	Mol. wt. (kDa)	Rabbit hyper-immune sera (HIS)			Normal rabbit
		Copro HIS	ES HIS	Egg HIS	
1	83.358	±	±	±	±
2	77.787	±	±	±	+
3	72.061	+			
4	61.654	+			+
5	57.526				
6	47.053				
7	42.201				
8	38.425				
9	34.336				
10	30.453	±			
11	27.596	+			
12	26.080				
13	24.707				
14	21.531	+		+	
15	19.668				
16	17.834				
17	15.351				

+ positive, ± weak reaction

Table 5 Detection of *F.gigantica* ES antigen specific bands using EITB technique

Lanes Bands	Mol. wt. (kDa)	Rabbit hyper-immune sera (HIS)			Normal rabbit sera
		ES HIS	Copro HIS	Egg HIS	
1	57.926	±			
2	50.430	±			
3	44.209	+			
4	39.744	+			
5	35.515	+			
6	30.914	+	+	+	±
7	27.664	+		+	
8	25.572				
9	24.406	±			
10	22.952				
11	22.121				
12	21.269				
13	21.009				
14	20.348	+		+	
15	18.094	+		+	
16	15.996				

+ positive, ± weak reaction

Table 6 Detection of *F.gigantica* egg antigen specific bands using EITB technique

Lanes Bands	Mol. wt. (kDa)	Rabbit hyper-immune sera (HIS)			Normal rabbit sera
		Egg HIS	Copro HIS	ES HIS	
1	71.278	+	+	+	+
2	65.168	±		±	±
3	52.936	+			
4	42.679	±			
5	39.447				
6	36.323				
7	33.072	+			
8	27.559	+		+	
9	25.198				
10	23.580				
11	22.505				
12	20.398	+	±	+	
13	18.029	+		+	
14	15.427				

+ positive, ±weak reaction

The present study concluded that according to the level of cross-reaction among the 3 tested *F. gigantica* antigens, it could be characterized by coproantigen as well as copro HIS as antigen and antibodies which were able to reflect the lowest level of cross-reaction with other tested *F. gigantica* antigens. Hassan et al. (2008) showed that copro- and excretory–secretory hyperimmune sera detected *Fasciola* copro-antigen in faeces of animals while egg antigen failed to do so. However, in the present study, coproantigen and copro-hyperimmune sera were able to reflect the best level of cross reaction with other *F. gigantica* antigens.

The absence of coproantigens from any cross-reaction proteins may be due to fact that this antigen is excreted by flukes in the bile duct, then moves through the digestive tract; where it is subjected to different digestive enzymes; including proteases. These digestive enzymes free copro-antigens from somatic cells creating a character of high specificity of this antigen. Consequently, at low sera dilutions, *F. gigantica* coproantigen was able to detect specifically antibodies of copro HIS and had no positive ELISA values with both egg and ES antigens and gave positive ELISA values with other heterologous rabbit hyperimmune sera at the same level of sera dilutions. However, ELISA O.D. values obtained in homologous assays were higher than those in heterologous ones (Abdel-Rahman and Abde-Megeed 2000).

Cross reaction elucidated in the present study between *F. gigantica* and ES antigens were observed at low sera dilutions. But clear distinction between specific and cross

reactive binding activities was clearly observed with highly diluted sera. This is in agreement with Shalaby (1998).

The present work demonstrated that *F. gigantica* coproantigen was least antigenic among the tested antigens. This was proved by the lowest ELISA O.D. values for copro HIS versus its homologous antigen rather than that of both egg HIS and ES HIS versus their homologous antigens of lowest antigenicity coproantigen is due to its 70 % carbohydrate content and due to its exposure to digestion during its transit from bile ducts to the intestines.

A structural homology was observed in *F. gigantica* ES and egg antigens by the use of SDS-PAGE. This homology resided in components of similar molecular weights between both antigens as 18, 20.4, 27.6 and 39.7 kDa. These components might be responsible for a possible cross-reaction between both antigens. Electrophoretic analysis has for long been used to better clarify the homogeneity and heterogeneity of protein composition of helminthes as mentioned by Siles-Lucas and Bandera (1996).

EITB reaction of fractionated *F. gigantica* coproantigen versus rabbit hyperimmune sera revealed 3 bands at 27.6, 30.5 and 72.1 kDa as specific bands of coproantigen, with no cross-reaction with either ES HIS, egg HIS or normal rabbit sera. This appeared to be in tune with the findings of Espino et al. (2000) considering them to be markers for the diagnosis of acute and chronic fascioliasis. *F. gigantica* coproantigen bands of 27.6 and 72.1 kDa were specific for diagnosis of animal fascioliasis, but the 72.1 kDa was less sensitive than the 27.6 kDa. In our study, the 27.6 kDa band proved to be the most specific band in *F. gigantica* coproantigen and was different from the band at same molecular weight in ES antigen. Similarly, Hassan et al. (2008) reported that the 26–28 kDa coproantigen was sensitive (81.8 %) and specific (90.9 %) for the diagnosis of fascioliasis. Regarding EITB of fractionated *F. gigantica* ES antigen versus ES HIS, only 6 from 10 bands at molecular weight of 24.4, 35.5, 39.8, 44.2, 50.4 and 59.9 kDa were considered to be specific bands of *F. gigantica* ES antigen; since they had no cross-reaction with copro HIS, egg HIS and normal rabbit sera. These data are in agreement with Gorman et al. (1994). EITB of fractionated *F. gigantica* egg antigen versus egg HIS, 8 immunoreactive bands reacted specifically. From these bands, only 3 bands at molecular weights of 33.1, 42.7 and 52.9 kDa were considered to be specific bands of *F. gigantica* egg antigen; since they had no cross-reaction with copro HIS ES HIS and normal rabbit sera. These results are in agreement with Abdel-Rahman et al. (2000).

It may be worth mentioning that the intense cross-reaction observed between ES and egg antigens by ELISA technique may be attributed to the presence of common bands at 18, 20.4 and 27.6 kDa between them. These bands

showed the ability to react with both ES and egg hyper-immune sera.

The 27.6 kDa coproantigen band identified by copro HIS might not be related to that band at the same molecular weight in both ES and egg antigens. This can be justified by failure of ES HIS and egg HIS to identify this band. In our opinion, this band might result from shifts in the band size of ES and/or tegument-related worm antigen due to proteolytic cleavage that occurred in the small intestine.

It can be safely concluded that 27.6 kDa band was the most specific band in *F. gigantica* coproantigen. Moreover, this band is different from that of the same molecular weight in ES antigen. This band is protein enriched as evaluated using Imaging Densitometer. Coproantigen showed specific diagnostic ability for *Fasciola* and had low number of cross-reaction proteins that reflecting its high specificity. Thus based on the present studies, detection of coproantigen in faeces offers a new and simple method as compared to collection of serum samples. This fact holds promise for a more accurate diagnostic technique in the near future for suspected *Fasciola* infection.

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