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Diagnosis of *Fasciola* infection by SDS–PAGE eluted excretory secretory (ES) protein fractions using dot-ELISA

M.A. Sabry [a,*], E.S. Taher [b], N. Farag Allah [b], A.M. Mahgoub [c]

[a] *Department of Zoonoses, Faculty of Veterinary Medicine, Egypt*
[b] *Department of Parasitology, Research Institute of Ophthalmology, Egypt*
[c] *Department of Parasitology, Faculty of Medicine, Cairo University, Egypt*

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Abstract Fascioliasis is now recognized as an emerging zoonotic disease in Egypt. Diagnosis in suspected patients still needs some degree of accuracy. In the present study, three *Fasciola gigantica* execratory secretory (ES) protein bands of molecular weight (MW) ranging from 14 to 20 kDa, 25 to 32 kDa and 45 to 65 kDa were eluted after fractionation of the parasite antigen using SDS–PAGE. The extracted kDa protein bands were concentrated and evaluated in diagnosis of *Fasciola* infection. Moreover the level of their cross reaction with other parasitic infections in infected and suspected patients of known parasite eggs/gram stool was evaluated using the dot-ELISA technique. Protein bands in the range of 14–20 kDa and that of 25–32 kDa were markedly specific and sensitive in diagnosis of different levels of anti-*Fasciola* antibodies (Ab) in sera of infected cases. These two groups of bands were able to exclude cross-reaction between anti-*Fasciola* Ab and other parasites recorded in stool of selected patients suffering from *Schistosoma mansoni*, *Ascaris*, and *Giardia*, either in single or mixed conditions with *Fasciola* eggs. While that of 45–65 kDa appeared less specific than the other previously mentioned bands. Protein bands in the range of 25–32 kDa appeared more sensitive than the other protein bands in detection of anti-*Fasciola* Ab at higher serum dilutions. The Dot-ELISA technique was proved to be more economic and easy in application. The dotted very small amount of antigens can be stored in a freezer and used at request in diagnosis of large numbers of samples.

1. Introduction

Several previous studies were done to induce early accurate diagnosis of *Fasciola* infection with special interest to exclusion of the cross reacted parasites using different serological techniques. In these studies, different *Fasciola* antigens (Ag) were

* Corresponding author.
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used including crude worm Ag [1], excretory secretory (ES) Ag [2] and egg antigens [3]. Each author clarified some advantages and disadvantages for his technique.

It was commonly understood that the ELISA technique is a sensitive and simple method for semi-quantitative determination of antibodies [4]. Specificity of ELISA in exclusion of cross reacted parasites depended mainly on the degree of specificity and purity of the used Ag as well as history of the tested sera [2].

More rapid, economic, direct and visually read, improved ELISA technique for the diagnosis of parasitic diseases as microenzyme-linked immunosorbent assay (dot-ELISA) was described by Rokni et al. [5]. This technique for diagnosis of bovine fascioliasis was used by Latchumikanthan et al. [6]. They cleared that nano-gram quantities of parasite antigen dotted onto a very small piece of nitrocellulose membranes were considered enough to obtain a marked direct and accurate diagnosis for the parasite directly.

Fractionated *Fasciola hepatica* ES Ag using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was done as described by Morphew et al. [7] and the selected protein bands of 16, 26–28, 35–36 and 56–58 kDa as specific bands were used for the diagnosis of *Fasciola* infection in sheep with different degrees of specificity. At the same time Kamel et al. [8] determined that the most specific protein band in *F. hepatica* ES Ag is that of 14–38 kDa band and the most specific one is that of 27 kDa.

In the present study, *Fasciola gigantica* ES Ag was fractionated using SDS–PAGE. Gel pieces containing the most common *Fasciola* protein bands were cut out; their antigenic contents were eluted and concentrated. The value of each fraction in diagnosis of *Fasciola* infection and exclusion of cross reaction with other infections was evaluated using the dot-ELISA technique versus sera collected from cases of known parasitic infection history as well as rabbit hyperimmune sera and negative sera as a control.

2. Materials and methods

2.1. Antigen preparation

2.1.1. F. gigantica excretory secretory antigen (E/S Ag)

F. gigantica ES Ag was prepared from living flukes collected from freshly condemned buffalo's livers according to Rivera Marrero et al. [9]. The clean active worms were incubated (40 worms per 100 ml) for 3 h at 37 °C in PBS (7.4 pH). The supernatant was separated after centrifugation at 5000 rpm at 4 °C for 1 h. Their protein content was increased by removal of excess PBS using poly ethylene glycol in molecular porous membrane tubing 6–8 MW cutoff (Spectrum Medical Inc., Los Angeles, CA 900060). After determination of their protein content by the method of Lowry et al. [10], the antigen was allocated into 1 ml vial and stored at −20 °C until use.

2.2. Production and separation of specific selected F. gigantica protein bands

2.2.1. Fractionation of F. gigantica E/S Ag using SDS–PAGE

F. gigantica ES Ag was fractionated using SDS–PAGE according to Laemmli et al. [11] in 10% polyacrylamide gel slabs in Tris–glycine buffer, pH 8.3 (Sigma Chemical Co.). Low and high MW standards were employed (Sigma SDS-100B). Gel strips containing the standard and part of *F. gigantica* fractionated Ag were cut out, fixed and stained with Coomassie-blue stain according to the method of Tsai et al. [12]. These strips were retained back to its original position for determination of the site of the selected protein bands.

2.2.2. Isolation of selected protein fraction from SDS–PAGE by electro elution

Continuous 10% gels (1.5 mm thickness) were isolated with 1 mg/ml of *F. gigantica* ES proteins. Individual slots in the same gel were used to electrophorese the molecular weight (MW) standards. Once the gel ran its full length, strips with the MW standards were cut and stained with Coomassie blue to determine the region where the antigens of interest would be according to the associated approximate MW. As shown in Fig. 1, three zones in the gel at the levels of 45–65 kDa (A), 25–32 kDa (B) and 14–20 kDa (C), were cut out horizontally across the whole gel. Each gel strip was transferred separately to elution tube membrane 6–8 MW cut off (Spectrum Medical Inc., Los Angeles, CA 900060). The tube was filled with PBS (pH 7.4) and kept in Bio-Rad elution unit at 10 V, 100 ml overnight at 4 °C. The gel material was removed and the volume was reduced using poly ethylene glycol in molecular porous membrane tubing 6–8 MW cut off according to Katrak et al. [13]. The protein content of the eluted concentrated materials was determined and kept in 1 ml vial at −70 °C till use.

2.3. Other antigens used for testing serum specificity

In order to test the specificity of the target antigens, the same sera were tested versus some selected antigens including hydatid cyst fluid antigen (HCFAg), *S. mansoni* crude Ag and *Toxocara canis* crude Ag. HCFAg was prepared from fertile HC extracted from freshly slaughtered camel lungs (slaughtered at Cairo abattoir) according to Osman et al. [14]. The fluid was clarified by centrifugation at 5000 rpm for 15 min at 4 °C, dialyzed against 5 mM Tris–HCl (pH 7.4) for 48 h at 4 °C, after determination of their protein content by the method of Lowry et al. [10]. The antigen was allocated into

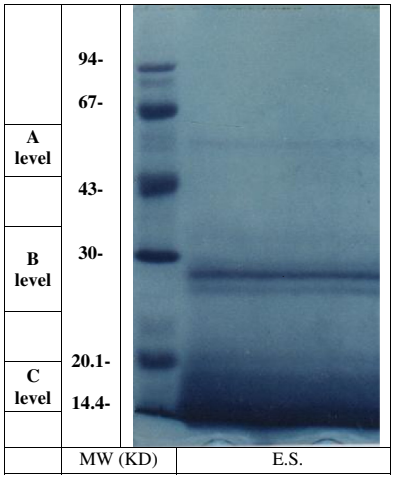

Figure 1 Gel electrophoresis showing the three selected protein bands before elution. A level at 45–65 kDa, B level at 25–32 kDa, C level at 14–20 kDa, MW. MW = Low M. W. protein standard; E.S. = fractionated *F. gigantica* excretory secretory antigen.

1 ml vial and stored at −20 °C until use. *T. canis* crude antigen was extracted from the anterior part of the worms extracted from the intestine of naturally infected dogs following the protocol briefly described by Sabry et al. [15]. *S. mansoni* whole worm crude antigen was prepared from lyophilized worms obtained from the Schistosoma Biological Materials Supply Program, Theodore Bilharz Research Institute, Giza, Egypt, according to Joseph et al. [16]. The protein content was evaluated and stored as before.

2.4. Dot-ELISA technique

The technique described by Shaheen et al. [17], one microliter (1 μl) of the antigen (1.0 mg protein/ml) in PBS-T (pH 7.4) was dotted on 6 mm diameter nitrocellulose membrane disks, after drying at 56 °C for 10–15 min, it placed into flat bottom of polystyrene 96 wells ELISA plate. Non-specific binding sites were blocked using 3% BSA-PBS-T. After 3 time wash using PBS-T, disks were used immediately or stored at −20 °C. For testing, 20 μl of diluted sera was applied on top of the dotted antigens in the wells in duplicates. The plates were incubated at room temperature for 1 h. After 3 time wash, 20 μl of horseradish peroxidase conjugated goat anti-human IgG (Sigma) and goat anti-rabbit IgG (Sigma) diluted at 1:1000, was added and incubated for 30 min at room temperature. After 3–5 time washing, 50 μl of freshly prepared 4-chloro 1-naphthol 340 μg/ml substrate buffer with 0.03% hydrogen peroxide solution was added. After color development, (within 15 min) the disks were washed and air-dried. The intensity of the blue-purple color was judged by the naked eye and evaluated in comparison with the reference control and tested sera at 1:100 serum dilutions. Sensitivity was calculated as the number of true positive/(True positive + False negative), while specificity calculated as the number of true negative/(False positive + True negative) [18].

2.5. Selected serum samples

A total of 63 selected serum samples from patients of known parasitic infection history and 4 experimentally infected *F. gigantica* rabbits, as well as 10 healthy people and 4 control rabbits' sera were selected for evaluation of the eluted antigens.

The examined sera were assigned into 8 groups including; G-1 as 10 serum samples from patients harboring *Fasciola* eggs only in their stool and still positive for 3 successive examinations (1–3 egg/gram), G-2 as 20 *S. mansoni* infected patients. G-3 as 20 samples from virus hepatitis infected patients, G-4 as 3 serum samples from x-ray proved hydatid cyst infected patients, G-5 as 10 samples from diarrheic patients harboring *Giardia* trophozoites and cysts in their stool, G-6 as 4 serum samples from experimentally infected rabbits by *F. gigantica* 70 days post infection proved to have immature fluke in their liver (by personal communication from Sabry et al. [19]). G-7 as 10 serum samples collected from healthy non-infected people as well as 4 healthy non-infected rabbits' sera (G-8) as a control.

3. Results

Checkerboard titration before the dot-ELISA technique determined the optimum concentration for the test to be 20–30 μg of tested antigen per dot which was enough to detect specific antibodies of the target antigen, at 1:100 serum dilutions for 1 h and 1:1000 conjugate for 30 min at room temperature. These were found to be optimum for marked differentiation between positive and negative serum samples by the naked eye.

In the present work, testing 3 protein band groups extracted from fractionated *F. gigantica* ES antigens at molecular weight (MW) ranges of 45–65 kDa, 25–32 kDa and 14–20 kDa (Fig 1) cleared marked specificity (100%) toward detection of anti *Fasciola* antibodies (Ab) in all tested sera. At the same time they did not react falsely with Ab in other selected sera of patients infected with hydatid cysts and *Giardia*. The fractions at the level of 14–20 kDa reacted with 2 patients infected with hepatitis C virus (HCV). The fractions at the level of 45–65 kDa reacted also versus 2 *S. mansoni* infected sera as well as 3 HCV infected sera using the dot-ELISA technique (Table 1).

Treatment of the same serum samples versus their original parasite antigens revealed positive reaction for 6 and 2 HCV infected sera versus *S. mansoni* and HFAgs reducing the specificity to 70% and 90% respectively. Moreover 2 *Giardia* infected sera were positive reacting versus *S. mansoni* Ag also (Table 1). Moreover; none of all the tested antigens produced any type of reaction when tested versus the control non-infected people and rabbit sera (Table 1).

The obtained results evidenced marked superiority in specificity of the two *F. gigantica* ES fractionated eluted protein band groups of MW 14–20 kDa and 25–32 kDa than that of 45–65 kDa in detection of anti-*Fasciola* Ab and that did not cross react with any of the tested serum samples from patients infected with other parasites.

The sensitivity of these two protein band groups (14–20 kDa, and 25–32 kDa) in detection of low level of anti-*Fasciola* Ab by serial dilutions of the known *Fasciola* infected patients' and rabbits' sera is demonstrated in Table 2. Moreover; inspecting the conditions of HCV infected cases that cross reacted with these fractions at low serum dilution using dot-ELISA technique.

The results emphasized that both MW protein band groups (14–20 kDa, and 25–32 kDa) were successful in detection of anti-*Fasciola* Ab in different tested sera till 1:250 dilution.

By increasing the serum dilution to 1:500; the selected protein bands of MW 25–32 kDa appeared to be more sensitive (100%) than the other (14–20) kDa MW groups (80% sensitivity) in sera of *Fasciola* infected patients. The same phenomenon was still true using dot-ELISA test by increasing the serum dilutions to 1:1000. The eluted Ag of 25–30 kDa MW still proved high sensitivity (100%). The sensitivity of both band groups decreased to 60% and 90% for the dots carrying 14–20 kDa and 25–32 kDa respectively after treatment by the same *Fasciola* infected sera (Table 2).

On screening the sensitivity of these 2 MW (14–20 kDa, and 25–30 kDa) eluted protein bands versus rabbit hyperimmune sera, both groups still reacted sensitively with the 4 tested rabbits' sera till dilution of 1:500. By increasing the dilution to 1:1000 the protein bands in the range of 25–32 kDa appeared to be more sensitive (100%) than the other one (14–20 kDa) which gave lower sensitivity (only 50%) as shown in Table 2.

Two serum samples of HCV infected patients showed positive reaction versus the dots containing 14–20 kDa fractions only till 1:250 serum dilutions. This cross reaction disappeared by increasing the serum dilution to 1:500 or more. At the same time none of the tested band groups reacted versus the tested non-infected control samples (Table 2).

Table 1 Specificity of *F. gigantica* ES antigen in diagnosis of *Fasciola* infection using the dot-ELISA technique at (1:100) serum dilution.

Antigens used in dot-ELISA		Number and history of tested patients serum samples					
		(G-1) *Fasciola* infected patients ($n = 10$)	(G-2) *S. mansoni* infected patients ($n = 20$)	(G-3) Hepatitis C infected patients ($n = 20$)	(G-4) Hydatid infected patients ($n = 3$)	(G-5) *Giardia* infected patients ($n = 10$)	(G-6) *Fasciola* infected rabbits ($n = 4$)
F. gigantica 14–20 kDa	No. of +Ve*	10	0.0	2	0.0	0.0	4
	Specificity %	100	100	90	100	100	100
F. gigantica 25–32 kDa	No. of +Ve*	10	0.0	0.0	0.0	0.0	4
	Specificity %	100	100	100	100	100	100
F. gigantica 45–65 kDa	No. of +Ve*	10	2	3	0.0	0.0	4
	Specificity %	100	90	85	100	100	100
S. mansoni crude Ag	No. of +Ve*	0.0	20	6	0.0	2	0.0
	Specificity %	100	100	70	100	80	100
Hydatid fluid Ag	No. of +Ve*	0.0	0.0	2	3	0.0	0.0
	Specificity %	100	100	90	100	100	100
T. canis crude Ag	No. of +Ve*	0.0	0.0	0.0	0.0	0.0	0.0
	Specificity %	100	100	100	100	100	100

(G-7) Sera of healthy people ($n = 10$) and (G-8) sera of 4 non-infected rabbits. Both groups did not show any reaction versus the tested antigens

* No. of +Ve = number of positive samples.

Table 2 Sensitivity of *F. gigantica* ES antigen fractions in diagnosis of *Fasciola* infection using the dot-ELISA technique.

Dilution of the tested sera	Antigenic fractions used in dot-ELISA		*Fasciola* infected patients ($n = 10$)	*Fasciola* infected rabbits ($n = 4$)	Hepatitis C infected ($n = 20$)	Control non infected	
						People ($n = 10$)	Rabbits ($n = 4$)
1:100 serum dilution	*F. gigantica* 14–20 kDa	No. +Ve*	10	4	2	0	0
		%	100	100	90	100	100
	F. gigantica 25–32 kDa	No. +Ve*	10	4	0	0	0
		%	100	100	100	100	100
1:250 serum dilution	*F. gigantica* 14–20 kDa	No. +Ve*	10	4	2	0	0
		%	100	100	90	100	100
	F. gigantica 25–32 kDa	No. +Ve*	10	4	0	0	0
		%	100	100	100	100	100
1:500 serum dilution	*F. gigantica* 14–20 kDa	No. +Ve*	8	4	0	0	0
		%	80	100	100	100	100
	F. gigantica 25–32 kDa	No. +Ve*	10	4	0	0	0
		%	100	100	100	100	100
1:1000 serum dilution	*F. gigantica* 14–20 kDa	No. +Ve*	6	2	0	0	0
		%	60	50	100	100	100
	F. gigantica 25–32 kDa	No. +Ve*	9	4	0	0	0
		%	90	100	100	100	100

* No. of +Ve = number of positive samples.

For conclusion, both selected purified *F. gigantica* E/S fractionated eluted protein band (*s*) groups of MW range of 25–32 kDa and 14–20 kDa succeeded in accurate diagnosis of fascioliasis and exclusion of cross reacted antibodies of other parasites. The protein band (*s*) groups of MW range of 25–30 kDa appeared more sensitive than the other ones.

4. Discussion

Cross-reaction between *Fasciola* species and other parasites is still a questionable point creating some difficulties in the accurate evaluation of the infection status of suspected cases using some serological techniques, especially at the level of field collected polyclonal sera Rokni et al. [5].

Accuracy of these techniques was affected markedly by the degree of purity and specificity of the used antigens. More accurate results can be obtained using Enzyme Linked Immune Transfer Blot (EITB) but this technique is usually non-practical for current field application in comparison with the ELISA technique [20].

According to Valero et al. [4], ELISA is a sensitive serological test able to analyze many samples simultaneously but it needs some sophisticated equipment. The new modified dot-ELISA was more economic, more suitable for accurate diagnosis using very few amounts of reagents especially in case of small amounts of valuable purified antigen, and very convenient for field study where the results can be read visually [5].

The present study evaluated the efficacy of 3 selected groups of *F. gigantica* SDS–PAGE fractionated ES antigen

corresponding to MW ranges of 45–65 kDa, 25–30 kDa and 14–20 kDa in diagnosis of *Fasciola* infection after concentration of the eluted fractions using the dot-ELISA technique. Selection of these antigens depended on previously published work on *F. hepatica* [21] and [22] and *F. gigantica* [23] and [6] all over the world.

At the same time, crude antigens of some parasites such as *S. mansoni*, hydatid cysts and *T. canis* as well as sera of *F. gigantica* experimentally infected rabbits were used simultaneously in the technique as references for detection of the cross reacted antibodies in tested sera.

The three tested fractions proved high specificity in detection of anti-*Fasciola* Ab in the examined sera at 1:100 serum dilution, while fraction of (14–20 kDa) showed 90% specificity versus HCV infected patients. Moreover; the fraction at MW of 45–65 showed 90% and 85% specificity versus *S. mansoni* and HCV infected patients. These data proved superiority of fractions at the MW of 25–32 kDa as they did not cross react with any of the previously mentioned cases, followed by that of 14–20 kDa. Specificity of these two purified *Fasciola* band antigens appeared in the same MW range previously described by Intapan et al. [23] and by Escalante et al. [24] using the EITB technique.

The decreased specificity of the band groups at 14–20 versus HCV infected sera in comparison with that of 25–32 kDa may reflect a degree of cross reactivity or may be as a result of the presence of true infection in the incubation period. For these reasons the second part of the present study focused on more characterization to the diagnostic value of the two specific *F. gigantica* band groups (25–30 kDa and 14–20 kDa MW) from the aspect of sensitivity toward the present antibodies in the cross reacted cases.

The protein MW bands of 25–32 kDa appeared more sensitive in detecting low levels of anti-*Fasciola* Ab than that of 14–20 kDa. This sensitivity still even (100%) by increasing the serum dilution till 1:500 in sera of *Fasciola* infected patients decreased to 90% sensitivity at 1:1000 dilutions. At the same time sensitivity of the other fractions (14–20 kDa) decreased to 80% and 60% at increasing the serum dilution to 1:500 and 1:1000 respectively, high sensitivity versus natural infected rabbit sera in-comparison with that collected from other patients. This may be attributed to the nature of infection in the tested patients in relation to the level of anti-*Fasciola* Ab in their sera.

Failure of the selected Ag in detection of *Fasciola* infection in some cases may be related to the presence of low antibody titer which is usually associated with chronic infection. Superiority of *Fasciola* Ag fractions in the range of (25–32) kDa in accurate diagnosis was proved previously by several authors as Farghaly et al. [1] for (23–28 kDa) [23] and [8] for 27 kDa using the EITB technique.

In the authors' opinion, the increase in the sensitivity of the tested fractions versus HCV infected sera in the present study proved that the previous falsely reacted cases reflected a degree of cross reactions than the presence of true unapparent *Fasciola* or mixed infection.

At the same time some authors clarified the role of other kDa in diagnosis of fascioliasis as 17 kDa, 16 kDa [21] and 14.4 kDa [25]. All of these fractions were in the range of the second selected group of bands (14–20 kDa) of the present study.

For conclusion, *F. gigantica* antigens (25–32 kDa and 14–20 kDa) proved their ability to induce accurate diagnosis of infected and suspected cases as well as they are able to exclude cross reaction with cross reacted parasites in the tested serum samples.

It is worthy to mention that the new approach adopted in the present study facilitates commercial use of purified specific antigens in induction of accurate diagnostic test (dot-ELISA) more easily in comparison with EITB especially as it can be read visually.

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