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Diagnostic potential of *Fasciola gigantica*-derived 14.5 kDa fatty acid binding protein in the immunodiagnosis of bubaline fascioliasis

G. Allam[1,2]*, I.R. Bauomy[3], Z.M. Hemyeda[1], T.M. Diab[3] and T.F. Sakran[1]

[1]Department of Zoology, Faculty of Science, Beni-Suef University, Beni-Suef, Egypt: [2]Department of Microbiology, College of Medicine, Taif University, Taif, Saudi Arabia: [3]Department of Parasitology, Theodor Bilharz Research Institute, Giza, Egypt

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Abstract

The 14.5 kDa fatty acid binding protein (FABP) was isolated from the crude extract of adult *Fasciola gigantica* worms. Polyclonal anti-FABP IgG was generated in rabbits immunized with prepared FABP antigen. Sandwich enzyme-linked immunosorbent assay (ELISA) was applied to detect coproantigen in stools and circulating *Fasciola* antigen (CA) in sera of 126 water buffaloes by using purified and horseradish peroxidase (HRP)-conjugated anti-FABP IgG. Sandwich ELISA sensitivity was 96.97% and 94.95%; while specificity was 94.12% and 82.35% for coproantigen and CA detection, respectively. However, sensitivity and specificity of the Kato–Katz technique was 73.74% and 100%, respectively. The diagnostic efficacy of sandwich ELISA was 96.55% and 93.1% for coproantigen and CA detection, respectively. In contrast, the diagnostic efficacy of the Kato–Katz technique was 77.59%. In conclusion, these results demonstrate that the purified 14.5 kDa FABP provides a more suitable antigen for immunodiagnosis of early and current bubaline fascioliasis by using sandwich ELISA.

Introduction

Fascioliasis is an infection of herbivores caused primarily by the parasitic trematodes *Fasciola hepatica* and *F. gigantica*. The former has a worldwide distribution mainly in temperate climates; whereas the latter is primarily of tropical climates in Africa and Asia (Hillyer, 2005). Heavy economic losses are inflicted on the livestock industry in tropical countries due to *F. gigantica* infection (Raina *et al.*, 2006). Early diagnosis of fascioliasis is vital to avoid all the complications of this disease. There are three different known approaches for diagnosis of parasitic infections: direct parasitological methods, indirect methods relying on clinical and biochemical assays, and immunological methods measuring the immune response to certain parasitic antigens and/or detecting circulating parasitic antigens (Feldmeier & Poggensee, 1993). Direct parasitological methods usually lack sensitivity and reproducibility and are not reliable (Hillyer, 1988; Shehab *et al.*, 1999; Carnevale *et al.*, 2001). On the other hand, immunodiagnosis of fascioliasis by detecting antibody to the antigen produced from *Fasciola* worms has several disadvantages. First, the antibody cross-reacts with other trematode antigens, including those of *Schistosoma* spp., giving a false-positive result (Hillyer, 2005). Second, detection of *Fasciola*-specific antibodies does not discriminate between previous and current infection. Therefore, the use of specific antibody to detect antigens secreted by the living flukes into their host's body fluids may be a better approach, not only in diagnosing active infection but also in assessing treatment efficacy and the

*Fax: + 966 2 7250528
E-mails: g.allam@tu.edu.sa; gm_allam@yahoo.com

effectiveness of future vaccines (Fagbemi *et al.*, 1997; Guobadia & Fagbemi, 1997).

Antigen detection assays are considered of prime importance for immunodiagnosis, as the detection of circulating *Fasciola* antigens and coproantigens can indicate an active infection (Espino *et al.*, 1990, 1992; Abdel-Rahman *et al.*, 1999). Several antigens are needed for efficient diagnostic methods. *Fasciola* antigens are mostly released from rapid turnover of the external covering, the tegument. In *Fasciola*, fatty acid binding proteins (FABPs) are the carrier proteins that help in the uptake of fatty acids from the hosts' fluids (Ockner, 1990). Several attempts have been made to utilize both native and recombinant FABPs with different molecular weights for protection and vaccine development against both fascioliasis and schistosomiasis (Tendler *et al.*, 1996; Estuningsih *et al.*, 1997; Ramajo *et al.*, 2001; Sirisriro *et al.*, 2002; Hillyer, 2005; Nambi *et al.*, 2005). On the other hand, Rabia *et al.* (2007) reported that FABPs could be used as a diagnostic antigen for human fascioliasis. In our earlier study on human fascioliasis, the 14.5 kDa *F. gigantica* FABP showed high diagnostic efficacy in the detection of this infection in human patients (Allam *et al.*, 2011). The present study was aimed at evaluating the diagnostic potential of this antigen in natural *F. gigantica*-infected buffaloes.

Materials and methods

Collection of flukes

Adult live *F. gigantica* flukes were collected from the liver and the bile ducts of naturally infected bubaline host (water buffaloes, *Bubalus bubalis*) at a local abattoir (Moneeb, Giza, Egypt). Worms were extensively washed with chilled physiological saline and phosphate-buffered saline (PBS, pH 7.2).

Maintenance of rabbits

Three parasite-free, 8-month-old female New Zealand white rabbits (about 2 kg in weight) were used for monospecific anti-FABP antibody production. Rabbits were kept under standard laboratory conditions at 21°C, 45–55% humidity, filtered drinking water and standard diet (24% protein and 4% fat).

Preparation and purification of F. gigantica *FABP*

Adult live *F. gigantica* flukes were homogenized in 20 mM Tris-HCl buffer (BDH Chemicals, England) containing 5 mM phenylmethylsulphonyl fluoride (PMSF) as a protease inhibitor (Sigma-Aldrich, St. Louis, Missouri, USA). The parasite homogenate was centrifuged at 10,000 *g* for 15 min at 4°C. The supernatant was collected and the protein content was determined according to Bradford (1976). The crude extract was subjected to the ammonium sulphate precipitation method according to Nowotny (1979). FABP was purified from the crude extract by a combination of ion-exchange chromatography on DEAE-Sephadex A-50 at pH 7.0 and gel filtration using Sephacryl HR-100. At pH 7.0 FABP binds strongly to the column and is selectively eluted with a salt gradient (van Nieuwenhoven *et al.*, 1991). Absorbance of each fraction was measured at 280 nm and the purity of the produced protein was assayed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, according to Laemmli (1970).

Production, purification and labelling of anti-FABP polyclonal antibodies

One milligram of *F. gigantica* FABP was mixed with an equal volume of complete Freund's adjuvant and injected intramuscularly into each rabbit, according to Guobadia & Fagbemi (1997). Booster doses (0.5 mg mixed with an equal volume of incomplete Freund's adjuvant) were administered at week 2 and 3 after the initial dose, according to Fagbemi *et al.* (1995). Sera were collected from an ear vein 4 days after the last injection, to detect the titre of antibodies produced. When the titre was high, the animal was scarified and blood samples were collected and antisera were stored at −80°C until used.

Anti-FABP IgG was purified by the ammonium sulphate precipitation method (Nowotny, 1979), followed by the caprylic acid purification method (McKinney & Parkinson, 1987), and finally DEAE-Sephadex A-50 ion-exchange chromatography (Sheehan & FitzGerald, 1996). The protein content was estimated by a Bio-Rad protein assay (Bradford, 1976) and the purity of the produced IgG was identified by SDS-PAGE according to Laemmli (1970). Anti-FABP IgG was conjugated with horseradish peroxidase (HRP) according to the periodate method of Tijssen & Kurstak (1984).

Collection and examination of samples from naturally infected buffaloes

A total of 126 samples (faeces and blood) were collected from buffaloes during several visits to a local abattoir (Moneeb area, Giza, Egypt), out of which 99 samples were collected from buffaloes infected with *F. gigantica*, 10 samples infected with *Ascaris*, 7 samples infected with *Schistosoma mansoni*, and 10 samples from healthy buffaloes used as negative control samples. Blood was collected during slaughtering. Sera were prepared and stored at −80°C until used. The liver, gall bladder and the general viscera of each animal were checked for adult flukes and for other parasites. Samples of faeces were collected from the rectum in clean, wide-mouthed containers with tight-fitting covers. After repeated sieving of these samples, coprological examination was carried out microscopically by the Kato–Katz technique according to Engels *et al.* (1997). Buffaloes with mixed infections were excluded from this study.

Detection of circulating antigen and coproantigen by sandwich ELISA

This method was performed according to Allam *et al.* (2011). Briefly, for detection of circulating *Fasciola* antigen in sera, the microtitre plates (Dynatech, Chantilly, Virginia, USA) were coated with 100 µl/well of purified anti-FABP IgG (10 µg/ml in 0.06 M carbonate buffer, pH 9.6) and incubated overnight at room temperature. Plates were washed three times with washing buffer (0.1 M PBS/T20, pH 7.4), then blocked with 200 µl/well of 1% bovine serum albumin (BSA) in 0.1 M PBS for 2 h at 37°C. The plates were washed three times with washing buffer, then 100 µl of

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serum samples were added into the wells in triplicate and incubated for 2 h at 37°C. The plates were washed three times as before. Then 100 µl of peroxidase-conjugated anti-FABP IgG of dilution 1/250 was added and the plates were incubated for 1 h at room temperature. After washing the plates five times, 100 µl of ortho-phenylenediamine (OPD) substrate solution was added to each well and the plates were placed in the dark at room temperature for 30 min. To stop the enzyme–substrate reaction 50 µl/well of 2 N H_2SO_4 was added to each well. The absorbance (OD) was measured at 492 nm using an ELISA reader (Bio-Rad, Hercules, California, USA) .

For detection of coproantigen, stool was diluted in a ratio of 1:3 with saline and the exact method used for serum ELISA was applied to detect the presence of coproantigen.

Key features in reliability of test results

The cut-off point for positivity was measured as mean OD reading of negative controls +3 standard deviations (SD) of the mean. The tested samples showing OD values greater than cut-off value were considered positive. Test sensitivity, specificity and efficiency were calculated according to the following formulae (Zane, 2001):

$$\text{Sensitivity}\,(\%) = (\text{no. of true positive results} \times 100)/(\text{no. of positive results} + \text{no. of false negative results}).$$

$$\text{Specificity}\,(\%) = (\text{no. of true negative results} \times 100)/(\text{no. of negative results} + \text{no. of false positive results}).$$

$$\text{Efficiency}\,(\%) = (\text{no. of true positive results} + \text{no. of true negative results}) \times 100/(\text{no. of true positives} + \text{no. of false positives} + \text{no. of false negatives} + \text{no. of true negatives}).$$

Statistical analysis

The data were presented as mean (x) ± SD. The means of the groups were compared by analysis of variance (Snedecor & Cochran, 1981) using either Student's t-test or ANOVA. The correlation analysis between circulating antigen (CA) and ova count was performed by correlation coefficient (r). The data were considered significant if $P \leq 0.05$. All statistical analyses were performed using the SPSS program (SPSS Inc., Chicago, Illinois, USA).

Results

Purification of FABP from crude extracts of F. gigantica *adult worms*

The total protein of crude extracts that were obtained from adult *F. gigantica* worms was 20 mg/ml as measured by Bio-Rad protein assay. Crude extracts of *F. gigantica* were subjected to 50% ammonium sulphate saturation. The protein content of the post-saturation supernatant was 5 mg/ml. This protein was purified by DEAE-Sephadex A-50 ion-exchange column chromatography at pH 7.0 and the protein content after this step was 2.5 mg/ml. The two-step procedure showed a high degree of reproducibility. The yield of FABP as a protein content after gel filtration chromatography was 1.5 mg/ml.

Production and purification of anti-FABP polyclonal IgG

Rabbits were immunized three times with 1 mg of FABP at 1-week intervals. The antibody level reached the highest titre (2.75 OD reading at 492 nm) after the second booster dose. The total protein content of crude rabbit serum containing anti-*Fasciola* antibody was 10.2 mg/ml. The yield of purified anti-FABP IgG antibody following each purification step was determined by the assessment of protein content. Using the 50% ammonium sulphate precipitation method, the protein content was 4.7 mg/ml. However, the content dropped to 2.3 mg/ml after the 7% caprylic acid precipitation method. Finally, the protein content of highly purified anti-FABP IgG antibody after ion-exchange chromatography was 1.1 mg/ml.

Parasitological examination of stools using Kato–Katz technique

By naked eye examination during slaughtering, 99 buffaloes were found to harbour *Fasciola* worms in the liver and the bile ducts. However, according to stool analysis by Kato–Katz quantitative technique, only 73 animals were true positives and the rest of the animals (26) gave false-negative results (73.74% sensitivity). Three slides were counted for each buffalo and the mean number of eggs/g (epg) in faeces was calculated. The intensity of infection was estimated and animals were classified into three subgroups: low, moderate and high infection. The mean number of epg ± SD was 11.81 ± 6.59, 34.6 ± 5.63 and 75.4 ± 16.06, respectively.

Detection of coproantigen in faeces of naturally infected buffaloes by sandwich ELISA

Sandwich ELISA was applied using purified and HRP-conjugated anti-FABP polyclonal IgG to detect FABP antigen in stools of naturally infected buffaloes. The antigen level was measured as OD reading at wavelength 492 nm. As depicted in table 1, mean OD value of the *Fasciola*-infected group was significantly ($P < 0.01$) higher than both the healthy control group and the other-parasites-infected group. Three out of 99 *Fasciola*-infected animals showed false-negative results and the sensitivity of the assay was 96.97%. Negative controls were below the cut-off value (0.361), while 1 of 17 other-parasite-infected groups was at the borderline of the cut-off value, giving 94.12% specificity.

In the fascioliasis group, the highest OD readings were observed in the highly infected cases, giving 100% positivity, followed by the moderately infected cases (97.14% positivity), and cases with low infection showed 92% positivity. However, schistosomiasis mansoni cases

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Table 1. Detection of coproantigen in faeces, and circulating antigen in sera, of naturally infected buffaloes.

Groups	Coproantigen						Circulating antigen					
	Positive cases			Negative cases			Positive cases			Negative cases		
	$x \pm$ SD	n	Positivity (%)	$x \pm$ SD	n	Negativity (%)	$x \pm$ SD	n	Positivity (%)	$x \pm$ SD	n	Negativity (%)
Healthy control ($n = 10$)	–	–	–	0.259 ± 0.034	10	100	–	–	–	0.276 ± 0.24	10	100
Fasciolaisis ($n = 99$)	2.216 ± 1.009	96	96.97	0.249 ± 0.04	3	3.03	2.065 ± 0.912	94	94.95	0.306 ± 0.057	5	5.05
Low infection ($n = 25$)	0.771 ± 0.1	23	92	0.222 ± 0.014	2	8	1.127 ± 0.17	22	88	0.253 ± 0.052	3	12
Moderate infection ($n = 35$)	2.045 ± 0.298	34	97.14	–	1	2.86	1.834 ± 0.259	33	94.29	0.346 ± 0.005	2	5.71
High infection ($n = 39$)	3.203 ± 0.443	39	100	–	–	–	3.02 ± 0.426	39	100	–	–	–
Other parasites ($n = 17$)	0.362	1	5.88	0.159 ± 0.044	16	94.12	0.377 ± 0.047	3	7.65	0.174 ± 0.031	14	82.35
Schistosomiasis ($n = 7$)	0.362	1	14.29	0.201 ± 0.028	6	85.71	0.409 ± 0.02	2	28.57	0.203 ± 0.02	5	71.43
Ascariasis ($n = 10$)	–	0	0	0.133 ± 0.029	10	100	0.349	1	10	0.158 ± 0.024	9	90

Data are expressed as mean (x) optical density (OD) ± standard deviation (SD) at 492 nm. Cut-off value was equal to 0.361 and 0.348 for coproantigen and circulating antigen detection, respectively. n, Number of animals.

showed only 14.29% positivity and ascariasis cases gave 0% positivity (table 1).

Detection of FABP in serum of naturally infected buffaloes by sandwich ELISA

As shown in table 1, the mean OD value of *Fasciola*-infected buffaloes was significantly ($P < 0.01$) higher than those of the negative control group and the other-parasites-infected group. Out of 99 cases of *Fasciola*-infected animals, 94 cases were detected as positive samples. The sensitivity of the assay was 94.95%. However, buffaloes infected with other parasites showed 82.35% specificity. All the 10 negative controls were below the cut-off value (0.348).

In the fascioliasis group, the highest OD readings were observed in those cases with high infection, giving 100% positivity, followed by moderately infected animals, giving 94.29% positivity, while the lowest readings were observed in the group with low infection, giving 88% positivity. However, schistosomiasis and ascariasis cases showed 28.57 and 10% positivity, respectively (table 1).

Percentage positivity for coproantigen in stool and circulating FABP antigen in serum of naturally infected animals

As shown in table 1, the data of sandwich ELISA with coproantigen showed that 39 cases out of 39 of the high infection group were positive (100%), and 34 cases out of 35 of the moderate infection group were positive (97.14%), while 23 cases out of 25 of low infection group were positive (92%).

Similarly, the data of sandwich ELISA with circulating FABP antigen in buffalo sera showed that 39 cases out of 39 of the high infection group were positive (100%), 33 cases out of 35 of the moderate infection group were positive (94.29%), while 22 cases out of 25 of the low infection group were positive (88%).

To clarify the cross-reactivity with other helminths, sandwich ELISA was applied to detect the FABP in the stool and the serum of buffaloes infected with *Schistosoma mansoni* and *Ascaris*. The positivity of sandwich ELISA with coproantigen in the stool was 14.29 and 0% for schistosomiasis and ascariasis, respectively. On the other hand, the positivity of sandwich ELISA with circulating antigen in the serum was 28.57 and 10% for schistosomiasis and ascariasis, respectively (table 1).

Sensitivity, specificity and diagnostic efficacy of sandwich ELISA for detection of coproantigens and circulating FABP antigens in buffaloes with low and moderate Fasciola *infection*

The sensitivity of sandwich ELISA for detection of both coproantigen and circulating FABP antigens in groups with low and moderate infection was 95 and 91.67%, respectively; while the specificities were 94.12 and 82.35%, respectively; and the diagnostic efficacies were 94.81 and 89.61%, respectively. The present data demonstrated that sandwich ELISA is more sensitive and more specific in coproantigen detection than circulating antigen detection in cases of low and moderate infection.

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Sensitivity, specificity and diagnostic efficacy of parasitological analysis and sandwich ELISA for diagnosis of bubaline fascioliasis

In *Fasciola*-infected buffaloes, the sensitivity, specificity and the diagnostic efficacy of parasitological analysis were 73.74, 100 and 77.59%, respectively. However, the sensitivity, specificity and the diagnostic efficacy of sandwich ELISA for coproantigen detection were 96.97, 94.12 and 96.55%, respectively. Similarly, the sensitivity, specificity and the diagnostic efficacy of sandwich ELISA for circulating FABP antigen detection were 94.95, 82.35 and 93.10%, respectively (table 2).

The present data clearly showed a higher diagnostic efficacy of sandwich ELISA in diagnosis of bubaline fascioliasis than parasitological analysis. Moreover, the use of purified and HRP-conjugated anti-FABP IgG in sandwich ELISA is more sensitive and more specific for detection of *Fasciola* coproantigen than detection of circulating antigen in *Fasciola*-infected animals.

Discussion

The present study was conducted to evaluate the diagnostic capacity of one of the *F. gigantica* antigens, 14.5 kDa FABP, in diagnosis of bubaline fascioliasis. The FABP antigen was purified from the crude adult worm extracts by DEAE-Sephadex A50 ion-exchange chromatography and Sephacryl HR-100 gel filtration methods. FABP appeared as a single band at 14.5 kDa by reducing SDS-PAGE. In our previous study, the reactivity of the purified anti-FABP IgG was tested by indirect ELISA against FABP antigen. It was found that anti-FABP IgG was highly sensitive, highly specific and reliable for the detection of circulating FABP antigen until 1 ng/ml (Allam *et al.*, 2011).

The data of the present study showed that the sensitivity, specificity and the diagnostic efficacy of coprological analysis were 73.74, 100 and 77.59%, respectively. These data are parallel to those reported by Anderson *et al.* (1999), who indicated that the sensitivity of the egg counting method was 66.7% and specificity was 100%, whereas the overall accuracy was 73.9%. On the other hand, the sensitivity, specificity and diagnostic efficacy of sandwich ELISA for detection of coproantigens were 96.97, 94.12 and 96.55%, respectively. Such a finding coincides with that obtained by Hassan *et al.* (2008) who found that use of monoclonal antibody against some 26–28 kDa antigens of *F. gigantica* in sandwich ELISA, for detection of coproantigen in stool of naturally *F. gigantica*-infected animals, revealed 81.8% sensitivity and 90.9% specificity. In a field study, a survey screening for human fascioliasis using ELISA and coprology in endemic locations had 95.5% sensitivity and 86.6% specificity (Espinoza *et al.*, 2005). In addition, Moustafa *et al.* (1998) concluded that ELISA proved to be a rapid, easy and sensitive test for diagnosing fascioliasis, by detection of *F. hepatica* coproantigens earlier than routine stool examination. *Fasciola* coproantigens were detected in the stools of both infected animals and human patients several weeks before eggs were detectable in the stool (Youssef *et al.*, 1991). Therefore, detection of coproantigens in stools is useful for early diagnosis of fascioliasis to avoid clinical complications of the disease.

Table 2. Sensitivity, specificity and diagnostic efficacy of parasitological analysis and sandwich ELISA used for detection of *Fasciola* antigens in stool and serum of *Fasciola*-infected buffaloes.

		Fasciola antigen detection by sandwich ELISA	
	Parasitological analysis	Stool	Serum
Sensitivity (%)	73.74	96.97	94.95
Specificity (%)	100	94.12	82.35
Diagnostic efficacy (%)	77.59	96.55	93.1

In the current study, anti-FABP IgG was tested by sandwich ELISA to detect *Fasciola* antigen in sera collected from naturally infected buffaloes. The sensitivity, specificity and diagnostic efficacy of the test were 94.95, 82.35 and 93.1%, respectively. These data are parallel to those reported by Rabia *et al.* (2007) who recorded 91% sensitivity and 86.7% specificity when they used rabbit anti-FABP as antigen capture for detection of this *Fasciola* antigen by indirect ELISA in sera of infected patients. The sensitivity and specificity of serodiagnosis were slightly different, depending on the antigen and techniques that were used in many different studies; 91% sensitivity and 92% specificity have been achieved by using anti-49.5 kDa *Fasciola*-specific excretory-secretory (ES) fraction and sandwich ELISA for detection of circulating antigens in fascioliasis patients (Espino & Finlay, 1994; Espino *et al.*, 1998).

The results presented here indicate that the sandwich ELISA for detection of coproantigen is more sensitive and specific than circulating antigen for immunodiagnosis of fascioliasis in cases of low and moderate infection as well as high infection. This result could be attributed to immune complex formation with host antibodies that tend to decrease the potential rate of circulating antigens. Therefore, levels of coproantigens are less affected by immune complex formation than circulating antigens (Mezo *et al.*, 2007). Moreover, the use of serum samples has several disadvantages; for example, antigenaemia develops mainly during the acute phase of infection (prepatent phase), whereas after this period, antigens decrease and become undetectable over the course of infection (Espino *et al.*, 1998). Accordingly, coproantigen assay could be the most feasible procedure for diagnosing active acute and chronic infections, while serodiagnosis of fascioliasis is recommended only for the early stages of infection.

Cross-reactivity with other parasites is a major problem in specificity of the immunodiagnosis of fascioliasis, especially in countries where schistosomiasis and fascioliasis are endemic (Carnevale *et al.*, 2001; Rabia *et al.*, 2007). Interestingly, the present study revealed minimal cross-reactivity between 14.5 kDa *F. gigantica* FABP antigen and either circulating antigens or coproantigens of both *Schistosoma mansoni* and *Ascaris*. This finding indicates that 14.5 kDa FABP is a more specific

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antigen for diagnosis of fascioliasis than other tested antigens (O'Neill *et al.*, 1998; Rabia *et al.*, 2007; Hassan *et al.*, 2008). Such slight cross-reactivity may be due to the presence of shared antigenic epitopes between 14.5 kDa *F. gigantica* FABP antigen and one of *Schistosoma mansoni* and *Ascaris* antigens.

In conclusion, the present study clearly showed that the purified 14.5 kDa FABP obtained from crude extracts of *F. gigantica* worms could be introduced as a suitable candidate antigen for immunodiagnosis of bubaline fascioliasis by using sandwich ELISA. The sensitivity and specificity of sandwich ELISA for the detection of *Fasciola* coproantigen in stools are higher than those obtained by sandwich ELISA for the detection of circulating *Fasciola* antigen in serum. In addition, coproantigen detection was shown to be a good correlate to intensity of infection and hence could be used as an alternative to counting ova.

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