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Original Article

Detection of Coproantigens by Sandwich ELISA in Rabbits Experimentally Infected with *Fasciola gigantica*

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Abstract

Background: The study was targeted to report the appearance of coproantigens in feces and circulating antibodies in the serum of *Fasciola gigantica* experimentally infected rabbits.

***Methods*:** Copro Hyper Immune Serum (HIS) and Excretory-Secretory Hyper Immune Serum (ES HIS) antigens were used in a sandwich ELISA for the detection of *F. gigantica* antigens in feces of 12 rabbits experimentally infected with different doses of *F. gigantica* encysted metacercariae (EMC) (10, 25 and 30 EMC). The relation between time of appearance of coproantigens in feces and anti-*Fasciola* antibodies in serum was evaluated.

***Results*:** The earliest diagnostic coproantigen was recorded at 21st, 25th and 28th day post-infection (p.i.) in groups of rabbits infected with 30, 25 and 10 *F. gigantica* EMC respectively. Both HIS and ES HIS were able to detect coproantigens in feces of rabbits infected with 30 EMC at day 21 p.i. The appearance of *F. gigantica* coproantigens in feces of infected rabbits was concurrent to the appearance of anti-*Fasciola* antibodies in blood (3rd week p.i.). However, coproantigen has specific ability for direct assessment of active infection with minimal cross-reaction with other heterologous parasitic infections.

***Conclusion*:** The findings hold promise for a more accurate diagnostic technique in the near future for suspected *Fasciola* infection.

Introduction

Fasciola gigantica is an economically important parasite of domestic animals. This parasite constitutes a native health problem in Egypt because of its frequent occurrence and was therefore selected for the study. The development of suitable sustainable strategies for accurate early diagnosis is essential for controlling *F. gigantica* infection. Diagnosis is primitively based on identifying the parasite's eggs in the fecal samples. However, eggs can generally be detected after the maturation of the worms when they begin egg shedding (15-18 weeks post infection).

Rapid diagnostic methods would facilitate control of the parasite. Immunoassays as diagnostic tools can be employed either by antibody detection in the infected serum (1) or by antigen detection (2). The time required in the former 2 to 4 weeks p.i. whereas in the latter, 4 to 6 weeks p.i. Coproantigens are antigens prepared from fecal sample of infected animal and have shown to be effective for the diagnosis of a wide range of human and animal intestinal infections. In principle, antibodies raised against whole parasite extracts are coated on microtiter plates, and subsequently fecal antigen is captured and detected with the same or second parasite-specific antibody in a capture assay(3,4). A diagnostic antigen in the bile and feces from *F. hepatica* infected cattle was detected and characterized using Enzyme-Linked Immunotransfer Blot technique (EITB) (5). Coproantigen ELISA was developed for the detection of excretory secretory antigens in humans with fascioliasis (6). To monitor the efficacy of treatment, the detection of antigen rather than antibody is considered more reliable for diagnosing infection.

In the present study, we report the first time of appearance and dynamics of coproantigens in feces and circulating antibodies in sera in rabbits experimentally infected with different doses of *F. gigantica* EMC.

Material & Methods

Animal infection

Twelve parasite-free New Zealand white rabbits about 2 kg b.wt. were used in the experiment. Nine rabbits were orally infected with 10, 25 and 30 *F. gigantica* EMC (10 days old); (3 rabbits for each dose level) while the remaining 3 rabbits served as uninfected controls. Fecal and blood samples (for separation of serum) were collected immediately before infection and weekly for successively 12 weeks and stored at -20°C. Thereafter, the rabbits were sacrificed and flukes were collected after maceration of the liver using scalpel and needle in warm water. Rabbit hyper immune sera were raised separately against *Fasciola gigantica* coproantigens, ES antigen and egg antigen (7).

Excretory-Secretory (ES) antigen preparation

ES antigens were prepared from *Fasciola gigantica* (8) with slight modifications. Adult worms obtained from bovine livers were washed repeatedly (3-5 times) in M PBS, incubated for 3 h at 37 °C (one worm/5ml in 0.01 M PBS, pH 7.4). After incubation, the worms were removed and the supernatant fluid (PBS + ES) was collected and subjected to high speed centrifugation (12000 rpm) for 1 h at 4 °C. The supernatant was separated and designated as ES antigen. The protein content was measured (9). The antigen was aliquoted and stored at -70 °C until use.

Fasciola gigantica Coproantigen preparation

Fecal supernatants were processed for coproantigen immune detection according to El-Bahy et al. (1992). Five grams of sample was mixed separately in an equal amount of distilled water and sonicated for 5 minutes under 150 watt interrupted pulse output at 50% power cycle using a sonifier cell disrupter . The fecal suspensions were centrifuged at 3000 rpm for 15 minutes .The supernatant was dialysed in 6 to 8 kDa dialysis tubes overnight at 4°C against

4 M urea . Fecal supernatants were then concentrated to fifth the original volume by absorption against 36 kDa polyvinyl pylorridone. The protein content was measured (9) and stored at -20°C until use.

Fasciola gigantica egg antigen preparation

Fasciola gigantica egg antigen was prepared (5). The eggs were collected from the bile of infected slaughtered animals by sedimentation method using several changes of tap water. The eggs were washed 3 times with distilled water containing penicillin G (500 IU / ml) and gentamycin (50 mg /ml). The eggs were collected after sedimentation for 30 minutes. Then the eggs were sonicated with 0.01 M PBS, pH 7.4 for 10 minutes under 150 watt interrupted pulse output at 50 % power cycle using a sonifier cell disrupter. Thereafter, the sonicated eggs were subjected to a high -speed centrifugation (10000 rpm) for one hour at 4°C. The supernatant was separated as egg antigen after measuring the protein content (9). The antigen was aliquoted and stored at -70 °C until use.

Preparation of rabbit hyper-immune sera

Rabbit hyper-immune sera were raised separately against *Fasciola gigantica* coproantigen, ES antigen and Egg antigen (7). Six other 2 month old white New Zealand rabbits were bled for negative control sera. Rabbits were injected with the previous antigens at a concentration of 1.2 mg protein for each antigen, mixed in an equal volume of Freud's complete adjuvant subcutaneously at different places in the rabbit's back. After 3 weeks, 3 consecutive injections of 0.4 mg protein antigen in equal volume of Freud's incomplete adjuvant were given intramuscularly at biweekly intervals. Rabbits were bled from the ear vein for serum collection 10 -14 days after the last injection. The collected sera were stored at -20°C until use.

Sandwich ELISA

A sandwich ELISA for detection of coproantigen was performed (10). In this test, antigen of the tested samples was captured between 2 antibodies. The first one is specific monoclonal antibody used in coating of ELISA plate, while the other one is laboratory prepared reference hyper immune sera of the other host. Antigen determination was performed in duplicate and the results were expressed as the mean absorbance for each determination. Antigen detection assay was evaluated in terms of its sensitivity (percentage of positive results among the total number of coprologically *Fasciola* positive animals) and specificity (percentage of negative results among total number of coprologically *Fasciola* negative animals).

Results

The first time appearance of *F. gigantica* coproantigen in feces of experimentally infected rabbits with different doses of *F. gigantica* encysted metacercariae (10, 25 and 30 EMC) at different intervals p.i. was determined. Fecal specimens at days 7, 15, 21, 25, 28 and 35 p.i. were tested for the presence of the target coproantigen. Pre-infection fecal supernatants from the same rabbits were used as non-infected controls. The results in Table 1 indicate that there is a positive correlation between the first time of diagnosis of coproantigen in feces and the dose of *F. gigantica* EMC used in induction of infection. The earliest diagnostic value of this antigen was recorded at 21th, 25th and 28th day p.i. in groups of rabbits infected with 30, 25 and 10 *F. gigantica* EMC respectively. With respect to sensitivity of the 3 different rabbit hyper immune sera (HIS) in early detection of *F. gigantica* coproantigen in feces, the same test was applied on fecal supernatant of one rabbit group only that was infected with 30 EMC using copro HIS, the other being heterologous rabbit hyper immune sera. In copro HIS, a detectable amount of *F. gigantica* coproantigen was present in all rabbit's feces at day 21 p.i. (Table 2).

Table 1: Appearance of *Fasciola gigantica* coproantigen in faeces of experimentally infected rabbits with different doses of *F. gigantica* encysted metacercariae using sandwich ELISA versus monoclonal antibody (F10)

Days post infection	Mean Sandwich ELISA OD Value Rabbit Group		
	Group I (30 EMC)	Group II (25 EMC)	Group III (10 EMC)
7th	0.28	0.27	0.25
15th	0.30	0.25	0.24
21st	0.42	0.29	0.28
25th	0.42	0.39	0.29
28th	0.42	0.36	0.33
35th	0.41	0.40	0.33
Positive control 10ug/ml	0.46	0.46	0.46
Negative control	0.28	0.28	0.28

Similar to copro HIS, Excretory-Secretory (ES) HIS was able to detect this antigen in rabbits feces at day 21p.i. with observation of slight increase in mean sandwich ELISA OD values rather than that with copro HIS where the mean sandwich ELISA OD values were 0.60, 0.65, 0.067 and 0.067 with ES HIS and 0.42, 0.42, 0.42 and 0.41 with copro HIS at 21st, 25th, 28th and 35th day p.i. respectively. However, egg HIS failed to detect *F. gigantica* coproantigen in the same rabbit's feces at different intervals p.i.

Concerning appearance of anti-*Fasciola* antibodies in sera of experimentally infected rabbits with 30 EMC throughout 12 weeks p.i., the mean absorbance values diagnosed fascioliasis at 3 -12 weeks p.i. with a range of 0.38 to 0.57 as shown in Table 3. The mean antibody levels, that were expressed as mean absorbance values (OD 490) reached the highest level at week 4 p.i. (0.57) then decreased at week 5 p.i. (0.40) and rose again thereafter. This was repeated consistently in two tests while, the OD 490 value in controls was significantly different ($P<0.01$). *F. gigantica* coproantigens were not detected in feces during 1-2 weeks p.i in the same experimentally infected rabbits. However, by the 3rd week p.i., this antigen was detected in all infected rabbits and the mean absorbance values (OD 490) diagnosed fascioliasis at 3-12 weeks p.i. with a range of 0.40 to 0.53 (Table 3).

Table 2: Sensitivity of different rabbit hyperimmune sera in early detection of *Fasciola gigantica* coproantigen in faeces of experimentally infected rabbits with 30 EMC using monoclonal antibody (F10) sandwich ELISA

Days post infection	Mean Sandwich ELISA OD Value Using Rabbit hyper-immune sera		
	Copro HIS	Egg HIS	ES HIS
7th	0.28	0.35	0.50
15th	0.30	0.33	0.50
21st	0.42	0.32	0.60
25th	0.42	0.36	0.65
28th	0.42	0.36	0.67
35th	0.41	0.37	0.67
Positive control 10ug/ml	0.46	0.38	0.77
Negative control	0.28	0.35	0.50

Table 3: Appearance of *Fasciola gigantica* antibodies (sera) and coproantigens (faeces) in experimentally infected rabbits at different intervals post infection

Weeks post infection	Mean ELISA OD values* (antibodies in sera)	Mean sandwich ELISA OD values (coproantigens in faeces)
0	0.25	0.27
1st	0.24	0.27
2nd	0.27	0.29
3rd	0.38	0.40
4th	0.57	0.43
5th	0.40	0.43
6th	0.47	0.43
7th	0.48	0.47
8th	0.46	0.49
9th	0.46	0.53
10th	0.50	0.52
11th	0.52	0.50
12th	0.50	0.50
	0.30 (normal rabbit sera)	0.28 (negative control)

*At 1: 200 serum dilution

The mean coproantigen levels, that were expressed as mean absorbance values (OD490), increase by 3rd week p.i. peaking (0.53) between 8th and 10th week p.i. and became stable (0.50) afterwards while the OD 490 value in controls was significantly different ($P<0.01$) (Fig. 1).

It is worthy to mention that in the given experiment, anti-*Fasciola* antibodies as well as *F. gigantica* coproantigen in feces appeared at the same week post-infection (3rd week p.i.)(Fig. 2).

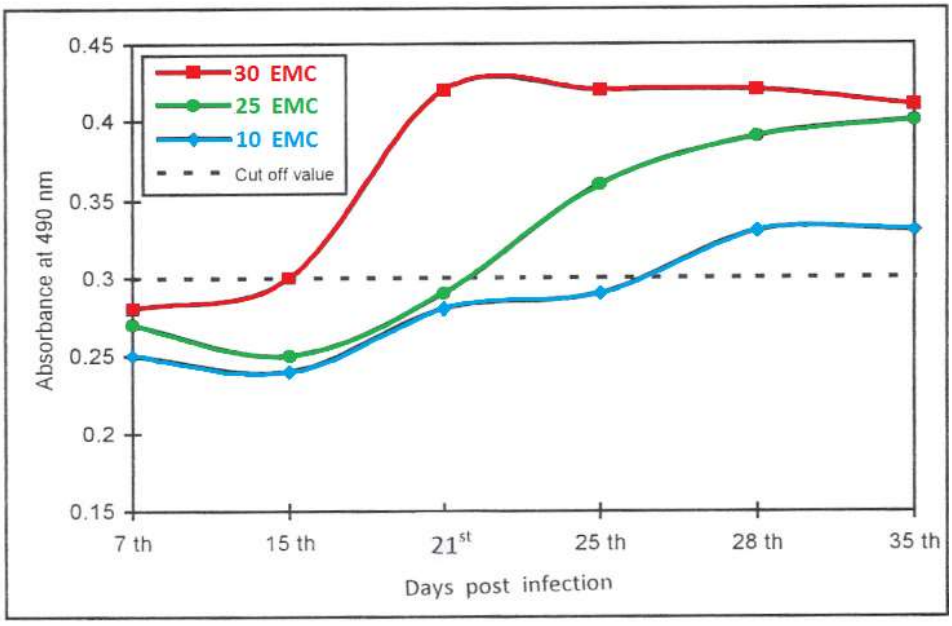

Fig. 1: Optical densities of sandwich ELISA for faecal specimens from experimentally infected rabbits with different doses of *Fasciola gigantica* encysted metacercariae (EMC)

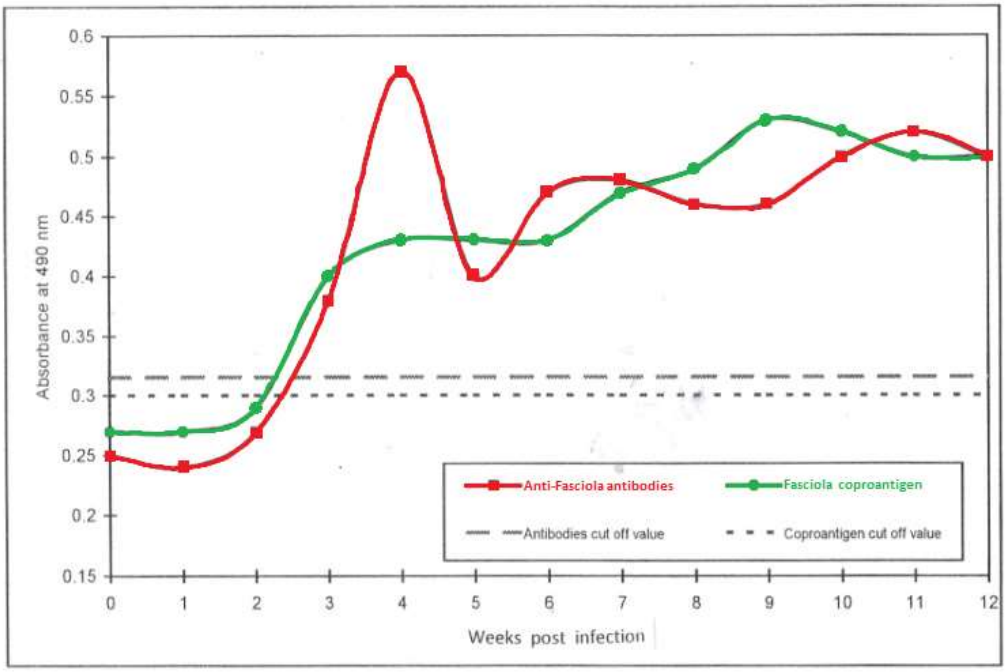

Fig. 2 : Appearance of anti- *Fasciola* antibodies in sera and *Fasciola gigantica* coproantigen in faeces of experimentally infected rabbits at different intervals post infection

Discussion

The significance of *F. gigantica* coproantigen as a powerful tool for specific diagnosis of fascioliasis encouraged interest to determine the first time appearance of this antigen in feces of experimentally infected animals. This was done in rabbits experimentally infected with 3 doses of *F. gigantica* EMC (10, 25 and 30 EMC). Weekly examination of the collected fecal specimens showed a positive correlation between the first time detection of coproantigen in feces and the dose of *F. gigantica* EMC used in induction of infection. The obtained results concurred with those reported earlier (11, 12) who detected *F. gigantica* coproantigen in feces of experimentally infected rats 4th week p.i. On the contrary, *F. hepatica* coproantigen in feces of experimentally infected rabbits were detected later than 7th week p.i. using ELISA test (13). This delay might be due to the sensitivity of monoclonal antibody used in this study that was not used in the other one.

Low mean worm burden was collected from each infected rabbit group as 1, 3 and 3.3 in those infected by 10, 25 and 30 *F. gigantica* EMC respectively. These collected worms were considered as a confirmation of successful infection, irrespective of the infection dose of EMC while rabbit was considered as an unsuitable host for development of *F. gigantica* (14, 15).

With respect to sensitivity of the 3 different rabbit hyper immune sera (copro HIS, ES HIS and Egg HIS) for early detection of *F. gigantica* coproantigen in feces of experimentally infected rabbits, monoclonal antibody sandwich ELISA was applied to fecal supernatants of rabbits in group infected with 30 EMC. It was found that both copro HIS and ES HIS were able to detect *F. gigantica* coproantigen in all rabbits feces at day 21p.i. with slight increase in mean sandwich ELISA OD values with ES HIS rather than that with copro HIS. This confirmed the previously mentioned fact that coproantigen was less antigenic than ES antigen. On the other hand, egg HIS failed to

detect *F. gigantica* coproantigen in the same rabbit's feces at different intervals post-infection.

Concerning appearance of anti-*Fasciola* antibodies in sera of experimentally infected rabbits with 30 EMC throughout 12 weeks p.i., the mean absorbance values made a diagnosis of fascioliasis at 3-12 weeks p.i. The mean antibody levels reached the highest level at week 4 p.i. then decreased at week 5 p.i. and rose again thereafter. Decreased antibody levels at week 3 p.i. and rise again thereafter in sera of *F. hepatic*a experimentally infected mice were recorded earlier (7) and the present findings fall on the same lines.

Regarding appearance of *F. gigantica* coproantigen in feces of the same experimentally infected rabbits, this antigen was not detected during the period 1-2 weeks p.i. The mean coproantigen levels increased by 3rd week p.i. peaking between 8th and 10th week p.i. and became stable afterwards. These results concurred with those obtained in *F. hepatica* experimentally infected rats (11). The higher detection observed between 8th and 10th week p.i. might be attributed to the rapid growth of the flukes associated with a higher metabolic rate and increased shedding of antigens (10). It is noteworthy that in the given experiment, anti-*Fasciola* antibodies as well as *F. gigantica* coproantigen in feces appeared at the same week post-infection (3rd week p.i.).

Antigen assays detected recent infections rather than clear infections when the animal still had elevated antibody levels specific for *Fasciola* (5). The authors suggested that a band detected by EITB using a densitometer in the area corresponding to 26-kDa reacted with rabbit anti-fresh fluke antigen and infected cattle sera but not with fluke negative rabbit sera, rabbit anti-*Fasciola hepatica* egg sera, *Fascioloides magna* positive or negative cattle sera. This band was not detected by Coomassie blue Sodium Dodecyl sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) gels or by Ponceau S stained nitrocellulose strips. They added that diagnosis of *F. hepatica* through detection of specific, stable antigen in feces of infected animals offered potential advantages over serum-based tests in having better sample accessibility, discrimination between previous and current infections and possible semi-quantitation of fluke burdens. The antigens of *F. hepatica* that were present in the feces of patients with chronic fascioliasis and in the feces of rats infected experimentally with *F. hepatica* metacercariae were defined (16). The authors used affinity chromatography to purify the antigens present in feces of rats that had been infected for 6 to 12 weeks using ES 78 monoclonal antibody bound to activated sepharose 4 B. Through this approach, they identified 6 polypeptides in feces of rats that had been infected for 10 -12 weeks and suggested that these polypeptides could be antigens common to both parasitic stages. This was particularly true for the polypeptides 14, 24 and 51 kDa because they reacted with the immune sera, human sera and ES 78 monoclonal antibody and could thus be important markers for the detection of acute and chronic fascioliasis.

It is obvious that the detection of coproantigen in feces with monospecific antibody offers an attractive alternative as a supplement to conventional antibody-based serological techniques in the diagnosis of fascioliasis. Furthermore, there is a possibility of semi-quantitatively measuring worm numbers by color intensity reaction related to the amount of antigen present in feces. Besides, feces are more readily accessible than serum samples that require animal handling and physical and clinical restraint.

Conclusion

It can be concluded that there is a marked relationship between number of migrating flukes and level of *F. gigantica* coproantigen in feces. The coproantigen can be diagnosed in feces of infected rabbits at the same time as its antibody is raised in the blood. But the spe-

cific ability of coproantigens to directly assess active infection, in having minimal cross-reaction with the other heterogeneous parasitic infections especially with availability of specific monoclonal antibody and the ease of accessibility of the sample holds promise for a better diagnostic technique for suspected *Fasciola* infection utilizing sandwich ELISA technique for coproantigen detection.

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