

Detection of coproantigens by sandwich ELISA in sheep experimentally infected with *Fasciola gigantica*

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Abstract Two monoclonal antibodies to *Fasciola gigantica* excretory/secretory (ES) antigens were used in a sandwich ELISA for the detection of *Fasciola* antigens in faeces of 9 sheep experimentally infected with 300 metacercariae of *F. gigantica*. The detection of coproantigens was found in four of the seven sheep within 5 weeks of infection, and within 7 weeks of infection coproantigens were detected in all seven of the sheep. This technique was compared to an indirect ELISA for the detection of anti-*Fasciola* ES antigen specific antibodies in serum. The anti-*F. gigantica* antibodies were detected within 3 weeks of infection in all of the infected sheep, suggesting a greater sensitivity to detect early infections. However, following anthelmintic treatment and removal of parasites, the anti-*Fasciola* antibody levels still remained high for at least 6 weeks when the study was terminated. In contrast, the levels of coproantigens were no longer detected in the faeces within 2 weeks of anthelmintic treatment. This study demonstrates that our sandwich ELISA for the detection of *Fasciola* coproantigens is able to detect immature fluke infections and more importantly, was able to detect patent infection of fasciolosis.

Comment: how many weeks did you measure this for Endah?

INTRODUCTION

Diagnosis of fasciolosis caused by *Fasciola gigantica*, an economically important parasite of domestic animals is usually achieved by identifying fluke eggs in the faeces. However, the sedimentation technique for egg detection could not detect worms before they mature and begin egg shedding at approximately 15-18 weeks.

Two approaches of immunoassay have been investigated for diagnosing fasciolosis. One approach was antibody detection in the serum of infected animal. The antibody-based serologic test was used successfully to detect *F. hepatica* as early as 2-4 weeks after infection (Itagaki *et al.*,1989). The second approach was antigen detection. The circulating parasite antigen can be detected as early as 4 to 6 weeks after infection (Rodriguez-Perez & Hillyer, 1995). The detection of *F. hepatica* antigens in the faeces of infected animals have some advantages, one of them is only actively metabolizing flukes are detected. Diagnosis of fasciolosis by detection of coproantigen in human using an ELISA have been reported by Espino and Finlay (1994). The detection of antigen rather than antibodies is considered to be a more reliable method for evaluating the status of infection which could be used to monitor the efficacy of treatment.

In this study we reported our method in the assessment of coproantigens in the course of experimental *F. gigantica* infection in sheep.

MATERIALS AND METHODS

Animal infection

Nine *F. gigantica* free sheep were used in the experiment. Seven sheep were each orally infected with 100 *F. gigantica* metacercariae and two other sheep served as negative control (uninfected). Blood and faecal samples were collected weekly, one sample at the time of challenge and 25 samples weekly after challenge. At week 18 post infection all sheep were drenched with triclabendazole and serum and faecal samples collected for a further 7 weeks. Serum was collected for determination of anti-*Fasciola* antibody and faecal samples were processed for antigen detection and eggs examination.

Excretory/secretory (ES) antigen of *F. gigantica*

ES antigens were obtained from adult *F. gigantica* as described by Wijffels *et al* (1994) with slight modification. Briefly, adult flukes were removed from bile ducts of naturally infected cattle at local abattoir and about 50 flukes were put into 100 ml PBS at 37⁰ C for 15 minutes. Initial regurgitant, containing blood, bile and debris, was removed by washing parasites in RPMI culture media containing an antibiotic and then live flukes were removed and incubated in fresh RPMI medium for 4-6 hours at 37⁰ C (2 flukes/ml RPMI). After incubation, the culture media containing the ES antigen was centrifuged at 2,500 rpm at 4⁰ C for 10 minutes, and stored at -20⁰ C until required.

Monoclonal antibody

The monoclonal antibody (Mab) and the biotinylated Mab were bought from Walter ELISA Hall Institute, Melbourne, Australia. This Mab was raised against *Fasciola* excretory/secretory antigens.

Indirect ELISA

The method used for indirect ELISA was similar to that described by Wijffels *et al* (1994) with some modifications. Briefly, Polystyrene microtiter plates (Maxisorp;Nunc) were coated with 100 µl of the ES antigen solution per well (2 µg/ml) and incubated overnight at 4⁰ C. The plates were washed three times with PBST and blocked with 200 µl of 5% skim-milked in PBST. After incubation for 1 h at 37⁰ C, the plates were washed again 3 times with PBST. Then, 100 µl of diluted serum (1:100) in PBST were added into each well and incubated for 1 h at 37⁰ C. After washing 4 times with PBST, 100 µl of Affinity Purified Antibody Peroxidase Labeled Rabbit anti-Sheep IgG conjugate (1:1000) were added into each well and the plates were incubated for 1 h at 37⁰ C. The substrate was prepared by dissolving 1 tablet of Tetramethylbenzidine (TMB) to 1 ml of Dimethylsulfoxid (DMSO) and mixed with 9 ml of citrate phosphate buffer and 2 µl H₂O₂ (1 tablet of TMB for 1 plate). After washing again, 100 µl of substrate was added to each well, incubated for 10 minutes, then stopped with 25µl of H₂SO₄. The optical density (OD) was measured at 450 nm in an ELISA reader.

Preparation of faecal supernatants

Faecal supernatants were prepared for antigen detection according to Espino *et al* (1990) with some modifications. Briefly, a sample of faeces from an individual animal was taken and homogenized using a mortar and pestle. One gram of homogenized faeces was then suspended in 2 ml of 0.1% PBST, and mixed by vortexing, then centrifuged at high speed for 10 minutes. The supernatant was collected and stored at -20° C until used.

Sandwich ELISA

A sandwich ELISA for detection of coproantigen was as follows: Polystyrene microtiter plates (Maxisorp;Nunc) were coated with 100 μ l of non-biotinylated monoclonal (2.5 μ g/ml) and incubated at 4° C overnight. The plates were washed (4x, PBST), blocked with 5% skim-milked in PBST and incubated for 1 hr at 37° C. After washing (4x, PBST), the undiluted faecal supernatant was added (100 μ l/well) in duplicate wells, and incubated for 1 hr at 37° C. Plates were washed (6x, PBST) and 100 μ l/well of biotinylated monoclonal antibody (0.63 μ g/ml) was added and incubated for 1 hr at 37° C. Following incubation, the plates were washed again (6x, PBST) and 100 μ l/well of extravidin (peroxidase conjugate) diluted 1:1000 was added to the wells and incubated for 45 minutes at 37° C. Plates were washed for a final time (8x, PBST) and 100 μ l/well of substrate solution was added to the well and incubated at RT for 10 minutes. The reaction was stopped by addition of 25 μ l/well of 1 M H_2SO_4 . Absorbance values were measured at 450 nm in an ELISA reader. For negative controls, faecal supernatant from non-infected sheep were used. For positive controls, 25 μ g/ml of *F. gigantica* ES antigen was added to the corresponding *Fasciola*-naïve faecal samples. The cut-off point was obtained by the

mean plus three times the standard deviation (SD) of the mean absorbance value obtained from uninfected sheep.

RESULTS

The results of the sandwich ELISA to detect coproantigens are shown in Figure 1. The antigens in faeces were detected within 5 weeks of infection in four of the seven infected sheep (57%) and within 9 weeks of infection in five sheep (71%) and within 11 weeks of infection, coproantigens were detected in all of seven infected sheep (100%). The OD values of the positive coproantigen in the week 5-11 post infection ranged between 0.21 and 0.59 with a mean of 0.30 (SD \pm 0.09). The mean of uninfected (control) animal was 0.17 (SD \pm 0.02). Sandwich ELISA OD values for the detection of antigen in the faeces considered positive when they were \geq 0.23 (0.17+3(0.02)). Following anthelmintic treatment and removal of parasites, the level of coproantigens were no longer detected in the faeces within 2 weeks after treatment. The mean OD values in the week 3-7 after treatment was 0.19 (SD \pm 0.01).

The anti-*F. gigantica* antibody in the serum was detected within 3 weeks of infection in all of the infected sheep (100%) and the antibody absorbance increased smoothly the following week and reached a peak within 9-14 weeks after infection. Two weeks after treatment the antibody level decreased but the absorbance still remained high (Figure 2). The mean OD values obtained by indirect ELISA was 1.66 (SD \pm 0.69) with a range of 0.44 to 2.69 during the course of infection. Furthermore, the mean OD values in the week 3-7 after treatment was 1.14 (SD \pm 0.41). The *F. gigantica* eggs were detected in

two of seven infected sheep (29%) within 15 weeks of infection and six of seven infected sheep (86%) within 17 weeks of infection (Figure 3). One week after treatment the eggs could not be detected in the faeces.

DISCUSSION

Two monoclonal antibodies to *F. gigantica* ES antigens were used for immunodiagnosis of fasciolosis in infected sheep by the detection of coproantigen in a sandwich ELISA. The detection of infection by examination of faecal samples for the presence of *Fasciola* eggs is often difficult because they are not found during prepatent period. In some cases it is also difficult during the patent period because eggs excretion is intermittent (Santiago & Hillyer, 1988; Chauvin *et al.*, 1995). Various serological methods including ELISA to measure antibodies of *Fasciola* using ES antigen have been developed (Hillyer *et al.*, 1996; Anderson *et al.*, 1999). However, this method has limitations because the presence antibodies indicate previous exposure to the parasites rather than the existence of a current infection. The sandwich ELISA using Mab ES 78 demonstrated that it is a good and specific method to detect *F. hepatica* ES antigen in serum and faeces from animal and human fasciolosis (Espino *et al.*, 1990; Castro *et al.*, 1994; Dumenigo *et al.*, 1996). In this study, it was found that between 57-71% of infected sheep had detectable *F. gigantica* antigen in the faeces within 5-9 weeks of infection and all infected sheep had detectable *F. gigantica* antigen in the faeces at weeks 11 until the time of treatment using the detection of coproantigen by sandwich ELISA. Following anthelmintic treatment the level of coproantigens were no longer detected within 2 weeks of treatment. This suggested that

this method would be suitable for detection of active ovine fasciolosis at least four weeks earlier than the time of egg appearance in the faeces as reported by Dumenigo *et al.*(2000).

In comparison with an indirect ELISA, the anti-*F. gigantica* antibodies were detected within 3 weeks of infection suggesting a greater sensitivity to detect earlier infections. However, following anthelmintic treatment the anti-*F. gigantica* antibody levels still remained high at least 6 weeks when the study was terminated. Generally, antigen detection tests have an advantage over antibody detection methods in that antigenemia indicates current rather than past infections. Furthermore, serological assay require blood tubes and needles for collecting sera thereby increasing cost, while faecal collection is simpler and easier to obtain. This study demonstrates that our sandwich ELISA for the detection of *Fasciola* coproantigens is able to detect early infection (5-9 weeks) and more importantly was able to detect patent infections of fasciolosis.

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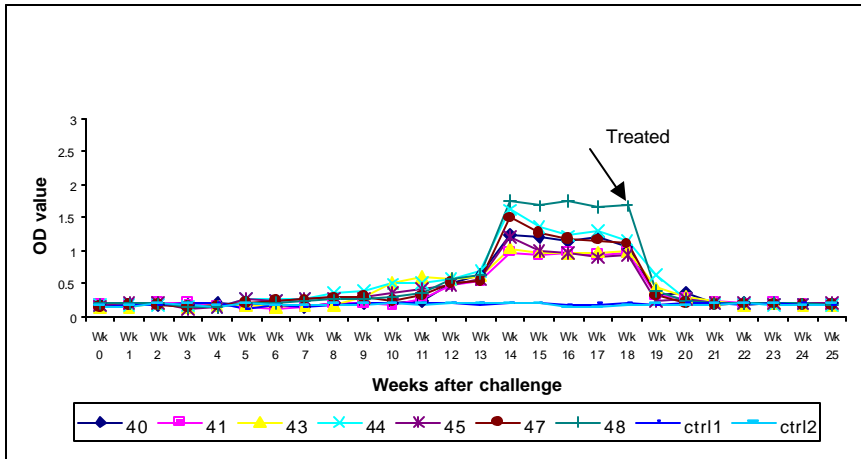


Figure 1. Antigen responses from sheep challenged with *Fasciola gigantica* metacercariae

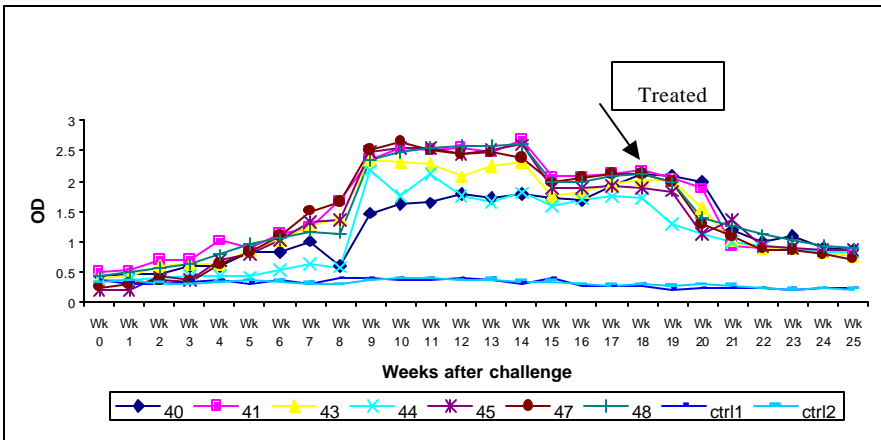


Figure 2. Antibody responses from sheep challenge with *Fasciola gigantica* metacercariae

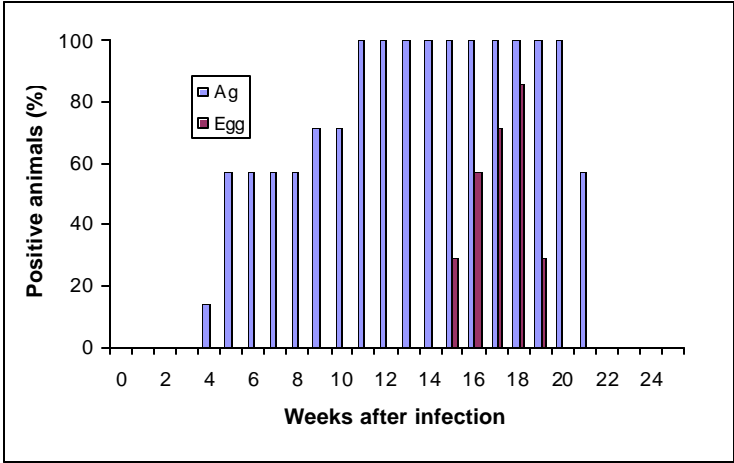


Figure 3. Percentage of experimentally infected sheep positive by sandwich ELISA and eggs examination