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Abstract

Antigens that were specific to *Fasciola gigantica* were obtained from the whole worm homogenate of the parasite by immunoaffinity chromatography in cyanogen bromide-activated sepharose 4B columns and used for the production of monoclonal antibodies. The *F. gigantica*-specific monoclonal antibody was labelled with horseradish peroxidase and used for the detection of circulating antigen by the direct ELISA method in the sera of cattle experimentally infected with the parasite. Circulating antigens were detectable in the sera of the animals as from the third week after infection while negative absorbance values were obtained 2 weeks after the termination of the infection by chemotherapy with oxyclozanide.

This immunodiagnostic method offers an attractive alternative as a supplement to the conventional coprological diagnosis of fasciolosis.

Keywords: Fasciola gigantica; Cattle–Trematoda; Monoclonal antibodies; ELISA; Immunoblotting

1. Introduction

Despite the fact that conventional coprological methods are the best options for the definitive diagnosis of *Fasciola gigantica* infection in ruminants, some advantages of immunodiagnostic techniques make them useful supplementary diagnostic methods. For instance, while the detection of eggs in faecal samples of cattle is only possible at about 10 weeks after infection, the immunodiagnostic detection of fasciolosis is possible in the prepatent stages before eggs begin to appear in faeces (Hillyer and Sanchez, 1985, Santiago and Hillyer, 1988, Hillyer and Galanes, 1988, Fagbemi, 1994). The value of immunodiagnostic assays for the detection of helminth infections is however diminished

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by a lack of specificity which emanates from the possession of common antigens by several unrelated helminths (Pelley and Hillyer, 1978, Kagan, 1974, 1979, Ambroise–Thomas, 1980, Fagbemi and Obarisiagbon, 1991). The first step therefore to enhance specificity in serodiagnosis of helminth infections is the production of specific antigens or antibodies.

Furthermore, most of the immunological tests in use over the past years for the diagnosis of fasciolosis focused on antibody detection which revealed both recent and current infections. On the other hand, the detection of parasite antigens in the serum of the host enables the diagnosis of current rather than past infections.

In this paper, we present the results of the detection of circulating *F. gigantica* antigens in the sera of experimentally infected cattle with a monoclonal antibody.

2. Materials and methods

2.1. Animal infection

Five trematode-free calves were infected orally with 500 *F. gigantica* metacercariae each. They were bled from the jugular vein every week commencing one week pre-infection to 20 weeks after infection. The calves were treated with 15 mg kg^{-1} oxyclozanide to terminate the infection in the 11th week after infection.

Faecal samples were collected from the rectum of the animals and examined for *Fasciola* eggs by the sedimentation method (Soulsby, 1965) from the 7th week after infection.

2.2. Preparation of whole worm antigens

Four trematode parasites, *F. gigantica, Paramphistomum microbothrium, Dicrocoelium hospes* and *Schistosoma bovis* were collected from animals at the local abattoir. Whole worm antigens of the worms were prepared by homogenizing them in 0.01 M phosphate buffered saline, pH 7.2 (PBS) in a Ten Broeck tissue blender followed by centrifugation at 4°C for 1 h at 100000 *g*. The PBS was supplemented with 10 mM leupeptin to inhibit proteases.

Excretory–secretory antigens were prepared from *F. gigantica* by incubating the worms (one worm per 5 ml) in leupeptin-supplemented PBS at 37°C for 3 h.

The protein concentrations of the antigens were determined by the dye-binding procedure described by Bradford (1976) which is based on the use of Coomassie Blue.

2.3. Immunoaffinity purification of Fasciola antigen

Affinity purification of the crude whole worm antigen of *F. gigantica* was done as described previously (Fagbemi et al., 1994). Briefly, hyperimmune sera were produced by immunising four groups of rabbits (three rabbits per group) with whole worm antigens of *F. gigantica, P. microbothrium, D. hospes* and *S. bovis*. Each group was immunised with one trematode antigen. IgG were extracted from these sera by ammonium sulphate precipitation followed by centrifugation at 50000 *g* for 30 min. Twelve mg IgG each from the four rabbit anti-sera were dissolved in 0.1 M $NaHCO_3$, 0.5 M NaCl and mixed with 6 ml CNBr–sepharose suspension overnight after which the

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IgG–CNBr–sepharose complexes were incubated with a mixture of 30 ml ethanolamine and lM Tris for 16 h at 4°C. The coupled IgG–CNBr–sepharose were packed in 20 × 1.5 cm columns and designated as *''Fasciola''*, *''Paramphistomum''*, *''Schistosoma''* or *''Dicrocoelium''* columns according to the IgG which they contained. Then 250 mg of *F. gigantica* whole worm antigen were passed serially through the three columns in the order; *''Schistosoma''*, *''Paramphistomum''* and *''Dicrocoelium''*. The run-through from the *''Dicrocoelium''* column was passed through the *''Fasciola''* column and the bound proteins were eluted thrice, first with PBS, pH 7.2 (eluate 1), then with Tris/HCl buffer, pH 8.5 (eluate 2) and finally with acetate buffer, pH 4.5 (eluate 3). The eluates were concentrated by vacuum dialysis using spectra/por 3 dialysis membrane tubing (mwco:3 500) (Spectrum Medical Industries, Inc., LA, USA) and stored at −20°C.

2.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

The proteins eluted from the *''Fasciola''* column were subjected to SDS-PAGE. The concentrated fractions were electrophoresed on 12.5% polyacrylamide gels 0.75 mm thick and transferred to nitrocellulose sheets in a mini-Transblot cell (BIORAD, Hercules, CA, USA) at 30 V overnight. The sheets were incubated with 3% gelatin overnight to block unreacted binding sites before they were cut into strips. The strips were incubated with the four anti-trematode rabbit anti-sera overnight before incubation in a 1:2000 dilution of goat anti-rabbit peroxidase-conjugated IgG for 1 h at room temperature. A concentration of 0.3 mg ml^{-1} of 3,3′, 5,5′-diaminobenzidine was used to develop the blots.

2.5. Production of monoclonal antibodies

The proteins in the PBS eluate (eluate 1) which consisted of the most *Fasciola*-specific fraction (Fagbemi et al., 1994) was used for the production of monoclonal antibodies.

Four BALB/c mice were immunised subcutaneously with the concentrated fraction of the eluate containing 40 μg protein and Freund's complete adjuvant. A second and third immunisation were done intraperitoneally with the same quantity of protein without adjuvant on days 14 and 21. Serum samples of the mice were assessed for antibody titre by ELISA on day 28. The mouse with the highest antibody titre was given a final intraperitoneal booster on day 35 and its spleen was obtained 4 days later.

The spleen cells were mixed with SP/o Ag-14 mouse myeloma cell line at a ratio of splenocytes:myeloma of 10:1 and fused in polyethylene glycol (mw 3350). The fused cells were grown in HAT medium (hypoxanthine/aminopterin/thymidine) in 24-well tissue culture plates at 37°C and 7% CO_2 micro-environment. Screening of the supernatant fluids for antibody production was done by ELISA.

Selection and cloning of the hybridomas were done as described by Solano et al. (1991, Fagbemi et al. (1994). Positive wells were selected and subcloned three times into 96-well microtitre plates by limiting dilution with normal BALB/c mouse peritoneal adherent cells serving as a feeder layer. Approximately 3×10^6 hybridoma cells in 0.5 ml of PBS were injected intraperitoneally into BALB/c mice primed 4 days earlier with 0.5 ml pristane (Aldrich Chemical Inc., Milwaukee, WI, USA). Ten days

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later, ascites fluid containing antibody was removed and stored at −20°C after centrifugation at 50 000 *g*. The isotypes of the antibodies in the supernatants from cloned hybrids were determined using the immunoglobulin subtype ELISA (BIORAD, Richmond, CA, USA).

2.6. Assessment of hybridomas

The hybridoma supernatants were assessed by ELISA. The optimum antigen concentration as determined by checkerboard titration was 25 μg ml^{-1}. Then 100 μl of eluate 1 in carbonate buffer (pH 9.6) per well was incubated overnight at 4°C. After three washes with PBS containing 0.05% Tween 20 and 1% skimmed milk (PBS-T-M), 100 μl of hybridoma or ascites fluid diluted in PBS-T-M was added per well and the plates were incubated for 1 h at 37°C. Horse radish peroxidase-conjugated anti-mouse IgG (Kirkegaard and Perry Laboratories, Gaithensburgh, MD, USA) at a dilution of 1:1000 in PBS-T-M) was added and incubated for 1 h at 37°C. O-phenylenediamine was used as the substrate and was incubated for 30 min at room temperature in the dark. A solution of 2.5 M H_2SO_4 was added (50 ml per well) to terminate the reaction. Absorbance values were read spectrophotometrically at 490 nm.

2.7. Immunoblotting with monoclonal antibodies

The specificities of the monoclonals were ascertained by immunoblotting. Whole worm antigens of the four trematodes were electrophoresed on polyacrylamide gels and transferred to nitrocellulose sheets which were later cut into strips. Screening of the monoclonals was done by incubating them with strips. After incubation of the blots with the 1:2000 dilution of peroxidase-conjugated goat anti-mouse IgG for 1 h, colour development was done with 3,3′, 5,5′-diaminobenzidine.

2.8. ELISA with monoclonal antibodies

The specificities of the monoclonals were also ascertained by ELISA. One hundred μl of 4 mg ml^{-1} solution of whole worm antigens of the four trematode parasites were coated on ELISA plates in 0.06 M carbonate buffer (pH 9.6) overnight at 4°C. After three washes with 0.05% PBS–Tween 20, 0.5% skimmed milk in 0.5% PBS–Tween 20 was incubated for 1 h at 37°C to block the unreacted binding sites. Following the incubation, the plates were washed thrice with 0.05% PBS–Tween 20. Then 100 μl of monoclonal antibody diluted in 0.5% skimmed milk in 0.5% PBS–Tween were added per well followed by incubation at 37°C for 1 h. After three washes, 100 μl of horseradish peroxidase-conjugated goat anti-mouse IgG diluted 1:2000 in 0.5% skimmed milk in 0.5% PBS–Tween 20 was added and incubated for 1 h at 37°C. The substrate used was 3-ethylbenzthiazoline sulphonic acid (ABTS) at a concentration of 0.2 mM in 45 mM citrate buffer (pH 4.0). Incubation was done for 30 min at room temperature. Absorbance values were read spectrophotometrically at 410 nm.

2.9. Conjugation of monoclonal antibody to horseradish peroxidase

The monoclonal antibody which showed the highest specificity for *F. gigantica* was conjugated to horseradish peroxidase for the detection of circulating *F. gigantica* antigen by direct ELISA.

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The monoclonal antibody was purified from mice ascites on a column of Affi-gel Protein A (BIORAD) according to the manufacturer's protocol. Five mg horseradish peroxidase was dissolved in 0.1 ml of 1.25% glutaraldehyde in PBS and left overnight at room temperature. Then 0.5 ml of 0.1 M carbonate buffer (pH 7.2) was added before dialysis against carbonate buffer with two changes in 4 h. After this, 2.5 mg purified antibody in 0.25 M carbonate buffer was added and the mixture was incubated at room temperature overnight. One hundred μl of 0.2 M lysine was added before the addition of 0.7 ml glycerol and the preparation which was the peroxidase-labelled anti-*F. gigantica* antibody was kept at −20°C.

2.10. Detection of circulating antigen with monoclonal antibody by the direct ELISA method

One hundred μl of serum from each of the five infected calves were plated in each well of an ELISA plate overnight at 4°C. Washing was done thrice with 0.05% PBS–Tween 20. One hundred μl of peroxidase-conjugated anti-*F. gigantica* antibody diluted 1:2000 in 0.5% PBS–Tween 20 was added to each of the wells followed by incubation at 37°C for 1 h. After washing, the substrate ABTS was added and incubated

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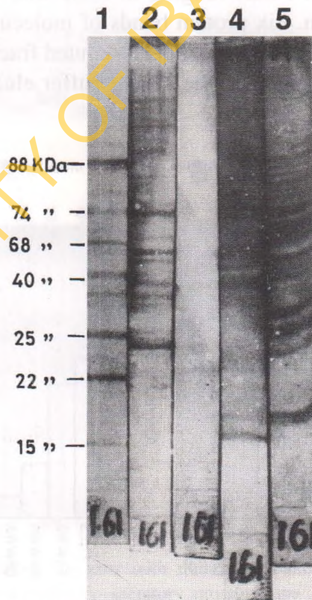

Fig. 1. Immunoblot of the eluted proteins obtained by the immunoaffinity chromatography of whole worm antigen of *F. gigantica* with rabbit anti-*F. gigantica* serum. Lane 1 – PBS eluate; Lane 2 – Tris/HCl eluate; Lane 3 – acetate buffer eluate; Lane 4 – run-through from "*Fasciola*" column; Lane 5 – whole worm antigen of *F. gigantica*.

for 30 min at room temperature. Absorbance values were read spectrophotometrically at 410 nm.

3. Results

3.1. Animal infection

All the five calves had *F. gigantica* eggs in their faeces 10 weeks after infection. The fluke eggs were not detectable in faecal samples one week after treatment with oxyclozanide.

3.2. Immunoblotting with affinity purified antigens and sera of rabbits hyperimmunised with whole worm antigens

The rabbit anti-*Fasciola* serum recognised proteins contained in the PBS-eluted affinity purified fraction while the other three rabbit anti-trematode sera did not. Further immunoblot analysis of the eluates (Fig. 1) revealed that the anti-*Fasciola* serum recognised about eight protein bands ranging in molecular weight from 15 kDa to 94 kDa in the PBS-eluted fraction. Six protein bands of molecular weight range of 19 kDa to 96 kDa were recognised in the Tris/HCl buffer-eluted fraction while the anti-*Fasciola* serum did not recognise any protein in the citrate buffer eluate.

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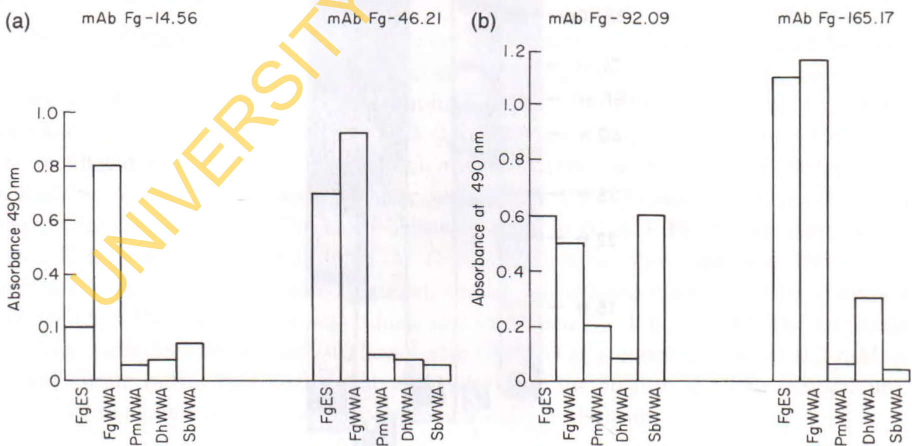

Fig. 2. (A) ELISA absorbance values for monoclonal antibodies MAb Fg-14.56 and MAb Fg-46.21 and various trematode antigens. FgES and FgWWA-*F. gigantica* excretory–secretory antigens and whole worm antigens: PmWWA-*P. microbothrium* whole worm antigen; DhWWA-*D. hospes* whole worm antigen; SbWWA-*S. bovis* whole worm antigen. Note the marked specificity of mAb Fg-46.21 for *F. gigantica* antigens. (B) ELISA absorbance values for monoclonal antibodies mAb Fg - 92.09 and mAb Fg-165.17.

3.3. Production of monoclonal antibodies

Forty-two hybridomas were produced. Eleven hybridomas produced antibodies of IgM isotype while 31 clones produced immunoglobulin isotypes IgG1, IgG2 and IgG3. Thirty eight out of the 42 monoclonals which were produced were non-specific for *F. gigantica* because they recognised a wide range of polypeptide antigens of *S. bovis*, *P. microbothrium* and *D. hospes* in the immunoblots. They also produced high ELISA absorbance values with these heterologous antigens. Four monoclonal antibodies revealed enhanced specificities for *F. gigantica* in ELISA and immunoblots (Figs. 2 and 3). Two of these four monoclonals, mAb Fg-14.56 and mAb Fg-46.21 were of IgG2 isotype while the other two monoclonals mAb Fg-92.09 and mAb Fg-165.17 were of

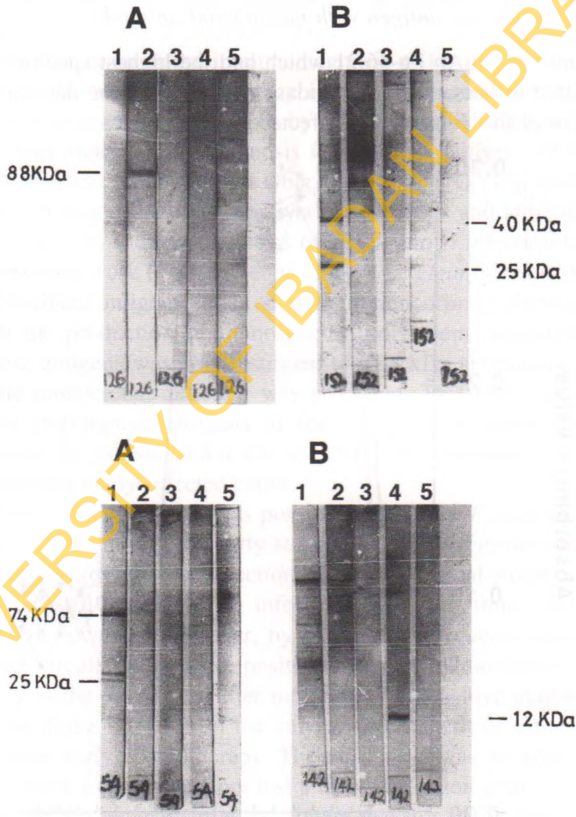

Fig. 3. (A) Immunoblot of trematode antigens with monoclonal antibodies mAb Fg-14.56 (a) and mAb Fg-46.21 (b). Lane 1 – *F. gigantica* excretory–secretory antigen; Lane 2 – *F. gigantica* whole worm antigen; Lane 3 – *P. microbothrium* whole worm antigen; Lane 4 – *D. hospes* whole worm antigen; Lane 5 – *S. bovis* whole worm antigen. Note the specificity of mAb Fg-46.21 for the antigens of *F. gigantica*. (B) Immunoblot of trematode antigens with monoclonal antibodies mAb Fg-92.09 (a) and mAb Fg-165.17 (b). Monoclonal mAb Fg-92.09 has a higher specificity for *F. gigantica* antigen in the immunoblot than in ELISA tests.

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IgG1 isotype. Monoclonal mAb Fg-14.56 showed very high ELISA reactivity with *F. gigantica* whole worm antigen (Fig. 2(A)) and it prominently recognised a 74 kDa polypeptide of this antigen in the immunoblot (Fig. 3(A)). Monoclonal mAb Fg-46.21 exhibited the highest specificity for *F. gigantica*. It showed very high ELISA reactivity for the excretory–secretory and whole worm antigens of the fluke but very low absorbance values with the antigens of the other trematodes (Fig. 2(A)). Besides it had distinct recognition for only the 25 kDa and 40 kDa polypeptides of the *F. gigantica* excretory–secretory antigens and 41 kDa and 88 kDa polypeptides of the whole worm antigens of this fluke (Fig. 3(A)). Monoclonals mAb Fg-92.09 and mAb Fg-165.17 revealed more cross-reactivities with the other trematode parasites than mAb Fg-14.56 and mAb-46.21.

3.4. Detection of circulating antigen with monoclonal antibody

Monoclonal antibody mAb Fg-46.21 which had the highest specificity for *F. gigantica* was conjugated to horseradish peroxidase and used for the detection of circulating antigen in the sera of the *F. gigantica*-infected calves.

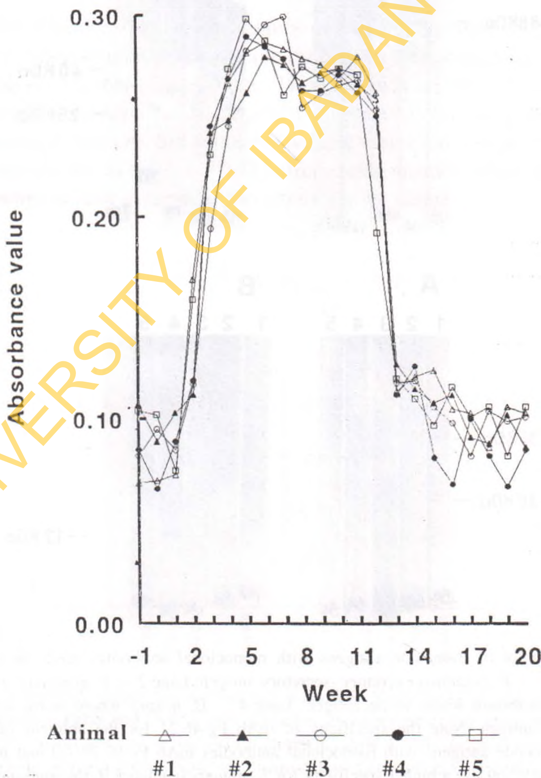

Fig. 4. Detection of *F. gigantica* antigen with monoclonal antibody in cattle.

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All the five calves had detectable levels of circulating *F. gigantica* antigens as from the third week after infection, 7 weeks before eggs were detected in their faeces (Fig. 4). Peak levels of circulating antigen were obtained by the 6th and 7th weeks after infection. Negative absorbance values for circulating antigen were recorded as from 2 weeks after treatment with oxyclozanide.

4. Discussion

In this paper, a monoclonal antibody which was produced by immunising BALB/C mice with immunoaffinity purified antigenic extract of *F. gigantica* was labelled with peroxidase and used for the immunodiagnosis of fasciolosis by the detection of circulating antigen in the sera of infected calves.

The most important issue in the immunodiagnosis of fasciolosis is the specificity of the assay. It is well known that the efficacy of immunodiagnostic tests is hampered by the possession of similar antigenic epitopes by several helminth parasites which lead to a general lack of specificity in serodiagnosis (Pelley and Hillyer, 1978, Kagan, 1979, Ambroise–Thomas, 1980, Fagbemi and Obarisiagbon, 1991). The first question in the course of this investigation therefore was whether antigens and antibodies which could enhance the specificity of immunodiagnosis of *F. gigantica* infection in cattle could be produced. In a previous work (Fagbemi et al., 1994), *F. gigantica*-specific antigens were found in the PBS-eluted antigenic fraction after immunoaffinity chromatography. However, success in the production of monospecific polyvalent rabbit antisera that were reactive with these antigens was only restricted to a 88 kDa antigen. In this work, an *F. gigantica*-specific monoclonal antibody was produced. This antibody distinctly recognised about four polypeptide antigens in the excretory–secretory and whole worm antigenic extracts of the parasite and it was useful for the detection of circulating antigen in the sera of experimentally-infected cattle.

The second issue was whether it was possible to have an *F. gigantica*-specific assay that could detect an infection in the early stages. In the conventional diagnostic method which is based on coprology, the detection of eggs in faecal samples of cattle is not possible until at least 10 weeks after infection, by which time considerable hepatic damage would have occurred. However, by using an *F. gigantica*-specific monoclonal antibody to detect circulating antigen, positive diagnosis of fasciolosis was possible in this study as early as the third week after infection. This positive diagnosis persisted for the duration of the fluke infection in the calves. One benefit of this early diagnosis is that it can encourage early chemotherapy. The third issue was whether an immunodiagnostic test could detect a current and not just a recent infection with *F. gigantica*. Again, the approach used in this investigation has shown that this is the case. Negative ELISA absorbance values were obtained from just 2 weeks after the termination of the infection by chemotherapy. Generally, antigen detection tests have an advantage over antibody detection methods in that antigenemia indicates current rather than past infections and this principle has been applied to the diagnosis of other helminth infections such as *Schistosoma, Dirofilaria, Echinococcus* and *Trichinella* infections (Craig and Nelson, 1984, Craig, 1986, Santoro et al., 1978, Weil, 1987).

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Although this immunodiagnostic test has been observed to have a high specificity, its sensitivity remains to be ascertained. The proportion of cattle harbouring a natural infection with *F. gigantica* in a herd that can be positively detected as infected will have to be determined. However, this immunodiagnositic method offers a promising alternative as a supplement to the conventional method of faecal egg detection in the diagnosis of fasciolosis in cattle.

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