Evaluation of a polyclonal antibody based sandwich ELISA for the detection of faecal antigens in *Schistosoma spindale* infection in bovines

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Abstract Schistosomosis is a common parasitic infection in animals prevalent in cattle in Asia and Africa, where it is estimated that at least 165 million animals are infected. Out of the 10 species reported to naturally infect cattle only *Schistosoma nasale* and *Schistosoma spindale* have received particular attention, because of their recognized veterinary significance. Although animal schistosomes may, under rare conditions favouring intensive transmission, act as important pathogens in endemic areas occur at a subclinical level, causing significant losses due to long term effects on animal growth and productivity. The detection of *Schistosoma* antigens in serum or stool could be more valuable in diagnosis, hence early treatment before irreparable damage. In this study, fresh adult worms of *S. spindale* were collected from the mesenteric blood vessels, whole worm antigen was prepared. These were immunized to rabbit and guinea pig to raise antibodies against *S. spindale*. Polyclonal antibodies of rabbit are further used as primary capture antibodies to coat ELISA plates. The capture of antibodies of guinea pig was conjugation with horse reddish peroxidase was used as secondary antibodies. Sandwich ELISA was performed to detect *Schistosoma* antigens in faecal samples collected from a total of 86 infected cattle and buffaloes. The working dilutions of capture antibody, detecting antibody and conjugate were found to be 1:32, 1:20 and 1:5,000 respectively by checker board titration method. The dilution of faecal supernatant antigens of *S. spindale* antibodies was 1:80. Out of 86 faecal samples, 77 samples were positive by Sandwich

ELISA indicating 89.54 % infection. Where as in control samples none of the samples was positive. In mixed infection out of 20 samples positive for fasciola, amphistome and hydatid, Out of 20 samples 2 samples were positive indicating 10 % infection rate. The overall sensitivity of this test is 88.65 % and specificity was 90.90 %. It could be concluded that sandwich ELISA is a rapid, easy and sensitive assay for diagnosis of *S. spindale* infection in bovines.

Keywords Visceral schistosomes · Immunodiagnosis of schistosomosis · Sandwich ELISA of bovine schistosomes · Indian schistosomes

Introduction

Schistosomosis in bovines is widely reported as a disease entity in many parts of Asia and Africa. The infection due to schistosomes has not been paid attention. The main reason for the negligence of this disease could be the fact that this infection seldom assumed epizootic proportions. In the normal course, its effects are less dramatic and more insidious; however schistosomes may, in rare circumstances manifest high morbidity when intensive transmission is present. The pathogenicity has been described (Kulakarni et al. 1954; van Wyk et al. 1974 and Chen 1993) in endemic areas where it occurred at subclinical level. However, it has been established that high prevalence rate of subclinical infections cause significant losses due to long term effects on animal growth, productivity, reduced conception and pregnancy rates (McCauley et al. 1983, 1984; Dargie 1987) and increased susceptibility to other parasitic or bacterial diseases (Dargie 1980; Pitchford

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and Visser 1982; McCauley et al. 1983, 1984). Schistosomosis also causes loss due to liver condemnation in animal species (Kadam et al. 1977; Dargie 1987). Despite these observations, the schistosomes of the animals have received relatively little attention when compared to species affecting human beings.

Diagnosis of schistosomosis is usually achieved by identifying eggs in the faeces or nasal secretions. However, the concentration techniques for egg detection could not detect worms before they mature and begin egg shedding at approximately 8–11 weeks. Two approaches of immunoassay have been investigated for diagnosing schistosomosis. One approach was antibody detection in the serum of infected animal. The antibody based serological test was used successfully to detect infection as early as 2–4 weeks after infection. The second approach was antigen detection. In general, the circulating parasite antigens can be detected as early as 4–6 weeks after infection (Rodriguez-Perez and Hillyer 1995). 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.

Materials and methods

Preparation of faecal supernatant

The protocol was followed as per Dumenigo et al. (1996). Two grams of faecal sample was mixed (1:2) with phosphate buffered saline (PBS) containing 0.3 % Tween 20 (PBS-T). It was vigorously mixed in a 15 ml centrifuge tube until slurry was formed. The faecal slurry was centrifuged at 2,000 rpm for 20 min at room temperature. The supernatant was collected in a 2 ml screw capped tube and was labeled with the reference number and stored at -20°C until used for coproantigen detection. The sediment was screened for ova under the microscope.

Collection of intestines and mesenteric plexuses and recovery of adult schistosomes

Immediately after slaughter, 216 intestinal tracts of cattle and buffaloes were collected and these were brought to the laboratory and washed thrice with water. The fascia was separated from the mesenteric plexus and surface of the lumen. The mesenteric blood vessels were held towards sunlight and viewed for the presence of schistosomes. The intestines were cut into small pieces and soaked in PBS for about 6 h. The pieces of intestines were removed and contents were sieved. On the top of the sieve the worms were recovered. A perfusion technique was followed, by injecting small quantity of PBS in the mesenteric vessels

and small puncture was made on the blood vessel. By squeezing the contents of blood vessels from the opposite direction, the worms were recovered.

Preparation of the adult worm homogenates

The adult worms of *Schistosoma indicum* and *Schistosoma spindale* were collected from mesenteric veins of slaughtered animals in sterile PBS. After three washings in sterile PBS, the parasites were suspended in cold absolute alcohol and stored at -20°C until processing for antigen extraction. Since most cattle had mixed infections of *S. indicum* and *S. spindale*, the parasites were collected and segregated species wise on the basis of morphological features.

Preparation of whole worm antigen (WWA)

About 500 worms of *S. spindale* were taken into the petri dish and washed thrice with PBS. Then these worms were triturated with 5 ml of PBS by using glass tissue homogenizer (Potter–Elvehjem glass Teflon) at moderate speed for a total time of about 10 min at 4°C . The contents were sonicated at 16 kHz for 10 cycles for 60 s each with a gap of one minute each. Then the contents were passed through petroleum ether and supernatant was discarded. The sediment was centrifuged at $9,500\times g$ for 15 min at 4°C in a high speed centrifuge (C30 Remi, India). The supernatant was used as antigen and to this antigenic solution one drop of 1 % sodium azide and 1 mM of Phenyl methyl sulfonyl fluoride (PMSF 170 $\mu\text{g/ml}$) was added and aliquoted at -20°C .

Estimation of protein concentration in antigens

The protein concentration of antigens was estimated as per the method of Bradford (1976) using protein estimation kit obtained from Bangalore Genei.

Raising of hyper immune serum (HIS) in rabbit and guinea pig

One rabbit and one guinea pig were used for raising HIS against WWA of *S. spindale* (permission was accorded by ethical committee bearing the No 493/01/9/CPSEA/veterinary college/Bangalore dated 31-10-2010). About 0.5 ml (80 mcg/ml) of crude antigen was mixed with equal volume of Freund's complete adjuvant (FCA) and the mixture was injected subcutaneously to the rabbit and guinea pig. After seven days, one more booster containing antigen injection with Freund's incomplete adjuvant (FIA) was given and three more injections given at weekly intervals with the same concentration of antigen. Both the animals were bled by puncture of marginal ear vein and serum was

separated. Then serum was checked for the presence of the antibodies using agarose gel precipitation test (AGPT). Then, 10 days after the final injection, blood was collected by jugular puncture and cardiac puncture. Serum was separated under sterile conditions, aliquot and stored at -20°C till use. Then it was tested by CIEP with corresponding antigen for the presence of antibodies.

Determination of optimal faecal antigen dilution

100 μl of different dilutions (1:10, 1:20 and 1:40, 1:80, 1:160, 1:320, and 1:640 per ml of carbonate buffer) of faecal antigen of *S. spindale* was added to 24 wells of a 96 well ELISA plate. The plate was incubated overnight at 4°C and washed thrice (3×5 min) with washing buffer. The blocking buffer was added to block the non specific sites and incubated at 37°C for one hr, 1:80 dilutions were found to be optimum, beyond this dilution the OD values are starts declining.

Determination of optimal serum dilution (capture antibodies and detecting antibodies)

100 μl of faecal antigens of *S. spindale* in carbonate buffer was added to 24 wells of a 96 well ELISA plate. The plate was incubated overnight at 4°C and washed thrice (3×5 min) with washing buffer. The blocking buffer was added to block the non specific sites and incubated at 37°C for 1 h wash the plates. Add 100 μl of positive serum (rabbit origin or capture Abs) dilutions (1:2, 1:4, 1:16, 1:32, 1:64 & 1:128) was added in duplicate and incubated at 37°C for 1 h. After washing the plates, 100 μl of positive serum (Guinea pig detection antibodies) dilutions of 1:10, 1:20, 1:40 and 1:80 was added in duplicate and incubated at 37°C for 1 h.

Determination of working strength of anti bovine IgG conjugate

To determine the working dilutions of anti bovine conjugate, 100 μl of normal bovine serum (1:100) was coated on to 96 well flat bottom polystyrene ELISA plate (Maxisorp, Nunc) by diluting with coating buffer and incubated at 37°C for 1 h. The ELISA plate was washed with washing buffer thrice. The blocking buffer was added to block the non specific reactive sites and incubated at 37°C for 1 h. The plate was washed with washing buffer thrice. The different anti bovine IgG test conjugate dilutions of 1:1000, 1:2000, 1:3000, 1:4000, 1:5000 and 1:10000 were prepared in blocking buffer and 100 μl of each dilution was added to the wells in duplicate and incubated at 37°C for 1 h.

Sandwich ELISA

The sandwich ELISA for detection of coproantigen was as followed as per the Dumenigo et al. (1996). Polystyrene microtiter plates (Maxisorp, Nunc) were coated with 100 μl capture polyclonal antibody (1:50 dilution) i.e. hyper immune sera raised rabbits against *S. spindale* in bicarbonate buffer. The plate was covered with an adhesive plastic and incubated at 4°C overnight. The coating solution was removed and washed twice by filling the wells with 200 μl PBS. The plate was flicked over a sink. The remaining drops were removed by patting the plate on a paper towel. The remaining protein binding sites in the coated wells were blocked by adding 200 μl of 5 % non fat dry milk blocking buffer in PBS.

Again the plates were covered with an adhesive plastic and incubated for 2 h at room temperature and the milk was discarded and the plate patted on a paper towel. 100 μl of diluted faecal samples (in PBS-T) were added to each well in duplicate and the last well was kept as a blank, these plates were then incubated for 90 min at 37°C . The samples were removed and plates were washed thrice with PBS-T by filling the wells with 200 μl PBS. A total of 100 μl HIS of guinea pig (1:20 dilution) against *S. spindale* (detection antibody) was added in bicarbonate buffer, the plates were covered with plastic adhesive and incubated for 2 h at 37°C , then the plate was washed three times with PBS-T.

Then 100 μl of goat anti guinea pig IgG HRP conjugate (1:5,000 dilution), diluted in PBST was added and incubated for 2 h at 37°C . Plates were washed three times with PBST. Then 100 μl of substrate chromogen (TMB) working solution was added and incubated for 15 min, and then the color reaction was monitored. The reaction was stopped by adding 100 μl of 2 M H_2SO_4 .

The absorbance values were read in a Multiscan plus P (Lab systems) ELISA reader at 450 nm. All positive and negative controls were incubated in the assay in duplicate. The cut off point was obtained by the 6 mean plus three times the standard deviation (SD) of the mean absorbance value obtained from known negative cattle.

Results

The working dilutions of capture antibody, detecting antibody and conjugate were found to be 1:32, 1:20 and 1:5,000 respectively by checker board titration method.

The dilution of faecal supernatant antigens of *S. spindale* antibodies was 1:80. Out of 86 faecal samples which were positive by indirect ELISA, 77 samples were positive indicating 89.54 % positives. Whereas in negative controls, none of the samples was positive. Evaluation of 20 faecal

Table 1 Detection of schistosomal antigens in faecal sample by sandwich ELISA

Positive cases					Negative cases		
Sl. no	Species	Total number of faecal samples	Positive (OD $\pm$ SE)	Percent positive	No. of faecal samples	Negative (OD $\pm$ SE)	Percent negative
1	Control	20	0	0	20	0.350 $\pm$ 0.24	100
2	Cattle and Buffaloes	86	77 0.450 $\pm$ 0.37	89.54	11	0.280 $\pm$ 0.29	11
3	Other infection	20	2 0.391 $\pm$ 0.32	10.0	18	0.300 $\pm$ 0.40	90

samples collected from animals positive for fasciola, amphistome and hydatidosis by Sandwich ELISA, two faecal samples were (fasciola) positive in the test. Out of 20 samples 2 samples were positive indicating 10 % positives. The overall sensitivity of this test was 88.65 % and specificity was 90.90 % (Table 1).

Discussion

Sandwich ELISA was performed for the detection of schistosomal antigens in faecal material of infected animals. Antigen detection assays facilitated earlier diagnosis than antibody tests (Li et al. 1994; Qiu et al. 2000) hence the detection of coproantigen in fasciolosis was possible (Youssef et al. 1991; Endah et al. 2004). In the present study, sandwich ELISA with faecal supernatant antigens (1:80 dilution) of *S. spindale* reacted with hyper immune sera of rabbit sera (raised against *S. spindale* which were used as capture antibodies). Out of 86 faecal samples, ELISA revealed 77 samples to be positive indicating 89.54 % infection. Where as in negative control samples, none of the samples was found positive. In 20 mixed infection samples, two samples of fasciola species were positive in this test giving 10 % positive. The overall sensitivity of this test is 88.65 % with specificity of 90.90 %. Specificity in the present study was higher probably due to the antigen prepared from WWA of adult flukes that yielded more immune dominant antigenic portions (Rodriguez-Perez and Hillyer 1995). Higher sensitivity (100 %) and specificity (95 %) of ELISA were observed by Darwish and Elgammal (1995). Qiu (2000) reported sensitivity of 84.7 % and no false positive reaction in healthy control and two samples showed cross reaction with paragonimosis. Similar reports were recorded by the various authors on trematode parasite circulating antigens. Endah et al. (2004) and Ibrahim et al. (2010) reported the study with two monoclonal antibodies of *Fasciola gigantica* ES antigens in the immuno diagnosis 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 the

prepatent period. In some cases it is difficult during the patent period because egg excretion pattern is intermittent (Santiago and Hillyer 1988; Chauvin et al. 1995). Various serological methods including ELISA was developed by using *Fasciola* ES antigen to detect antibodies (Hillyer et al. 1996; Anderson et al. 1999). However, this method had limitations because the presence of antibodies could 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 good and specific method to detect *Fasciola hepatica* ES antigen in serum and faeces from animal and human fasciolosis (Espino et al. 1990; Castro et al. 1994; Dumenigo et al. 2000).

It could be concluded that sandwich ELISA is a rapid, easy and sensitive assay for diagnosis of schistosomosis. It would be more valuable to apply this test in screening for schistosomosis, being a non invasive procedure could also detect coproantigen by ELISA Indicating recent and active schistosome infection. However more purification may be necessary to overcome cross-reactivity and improvement of diagnosis. This happened to be the first attempt of Sandwich ELISA in the coproantigen detection of *S. spindale* infection.

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