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Serodiagnosis of visceral schistosomosis by using *Schistosoma spindale* whole worm antigen in bovines by enzyme linked immuno sorbent assay in Bengaluru, Karnataka*

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ABSTRACT

In the present study indirect ELISA was aimed to detect specific antibodies of *Schistosoma spindale* using whole worm antigen. Out of 250 serum samples screened in cattle, 46 were positive indicating 18.40% infection. Out of 250 serum samples screened in buffaloes, 40 were positive with 16.0% rate of infection. The overall sensitivity of this test was 77.27% and specificity 75%. Out of 12 serum samples collected from animals positive for fasciolosis, amphistomosis and hydatidosis to check for specificity in ELISA, 8 serum samples showed cross reaction with *Fasciola* and Amphistome infection.

Key words: ELISA, Indian Schistosomes, *Schistosoma spindale,* Visceral schistosomes

Schistosomosis is a common parasitic infection in animals, mainly in cattle in Asia and Africa, and estimatedly at least 165 million animals are infected (De Bont and Vercruysse 1995). Among 10 reported species that naturally infect the cattle, *Schistosoma nasale* and *Schistosoma spindale* have received attention because of their veterinary importance. They act as an important pathogen in endemic areas as occur at a subclinical level. However, these subclinical infections can still cause significant loss due to long term effects on animal growth and productivity, as well as an increased susceptibility to other diseases (De Bont and Vercruysse 1998). Natural *S. spindale* infection is not diagnosed frequently by faecal examination since direct smear and floatation methods have poor sensitivity and eggs are often missed, hence immuno diagnosis is considered essential for correct means of confirmatory diagnosis (Agrawal 2004). The enzyme linked immunosorbent assay was used for detection of antigens (Fu *et al*. 1990, Hayunga *et al*. 1986) and antibodies (Hancock *et al*. 1986) in schistosomosis, though cross reaction was also reported (Janitschke *et al*. 1981). Serodiagnosis of bovine visceral schtosomosis has not been tried to elucidate the prevalence and prepatency of the disease in Karnataka state. Hence the study was undertaken to know the status of seroprevalence of schistososmosis using ELISA around Bengaluru, Karnataka.

MATERIALS AND METHODS

Collection of serum samples: Serum samples were collected from cattle (250) and buffaloes (250) and screened for the antibody titers. Ten serum samples of known negative for helminth infections and 12 samples from other mixed parasitic infections of *Fasciola*, *Amphistome* and *Hydatid* disease were collected.

Collection of mesenteric plexuses and recovery of schistosomes: Immediately after the 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 to sunlight and viewed for presence of schistosomes. A perfusion technique was followed, by injecting small quantity of PBS in the mesenteric blood vessels and small puncture was made on the blood vessel. By squeezing the contents of blood vessels from the opposite direction, the live worms were recovered.

Preparation of whole worm antigen (WWA): The adult worms of *S.spindale* and *S.indicum* and were collected from mesenteric blood vessels and kept in sterile PBS. After 3 washings in sterile PBS, the parasites were suspended in cold absolute alcohol and stored at –20°C until processing for

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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.

About 500 worms of *S.spindale* were taken into the petri-dish and washed thrice with PBS. These worms were triturated with 5 ml of PBS by using glass tissue homogenizer at moderate speed for 10 min at 4 °C. The contents were sonicated at 16 kHz for 10 cycles for 60 sec each with a gap of 1 min each. The contents were passed through petroleum ether and supernatant was discarded. The sediment was centrifuged at 9,500 ×g for 15 min at 4 °C in a high speed centrifuge. The supernatant was collected and used as antigen. For this antigen one drop of 1% sodium azide and 1mM of phenyl methyl sulfonyl fluride (PMSF 170µg/ml) was added and aliquoted at –20°C. The protein concentration of antigens was estimated as per Bradford *et al.* (1976) using protein estimation kit.

Raising of hyper immune serum in rabbits: Rabbits were used for raising HIS against whole worm antigen of *S. spindale.* About 0.5 ml (80 mcg/ml) of crude antigen was mixed with equal volume of freund's complete adjuvant and the mixture was injected subcutaneously to the rabbit. After 7 days, one more booster containing antigen injection with Freund's incomplete adjuvant was given and 3 more injections given at weekly intervals with the same concentration of antigen. Both the animals were bled by puncture of marginal ear vein and checked for antibody response by agar gel precipitation test. Ten days after the final injection, blood was collected by jugular and cardiac punctures. Serum was separated under sterile conditions, aliquot and stored at –20°C till the use. It was tested by CIEP with *S.spindale* antigen for the presence of antibodies by means of antigen antibody arcs.

Enzyme linked immuno sorbent assay (indirect ELISA): Indirect ELISA was conducted as per Law *et al*. (1996). The absorbance values were read in a ELISA reader at 450nm. Positive control and negative control were included in the assay in duplicate.

Determination of working strength of anti bovine IgG conjugate: To determine the working dilutions of anti bovine conjugate, 100µl of normal bovine serum (1:100) was coated on to 96 well flat bottom polystyrene ELISA plate by diluting with coating buffer and incubated at 37°C for 1 h. The ELISA plate was washed with washing buffer thrice (3×5 min). The blocking buffer was added to block the non specific reactive sites and incubated at 37°C for 1h. The plate was washed with washing buffer thrice (3×5 min). Test conjugate dilutions (1:1,500) were prepared in blocking buffer and 100µl of each dilution was added to the wells in duplicate and incubated at 37°C for 1h.

Determination of optimal test serum dilution: 100µl of antigen 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. After washing the plates, 100µl of positive serum dilutions was added in triplicate and further steps were as described for ELISA.

Determination of cut off value: Ten known negative serum samples were obtained from a confined cattle dairy farm with regular deworming schedules. The cut off value was calculated by taking mean absorbance values of known negative sera plus 3 standard deviation. Any serum with OD values above the cut off value was regarded as positive.

RESULTS AND DISCUSSION.

Indirect enzyme linked immunosorbent assay: In the present study the whole worm antigen was prepared and utilized for the standardization of ELISA. The protein concentration of the whole worm antigen was 80 µg/ ml of antigen. The working solutions of antigen, positive serum and conjugate were standardized by checker board titration method and is 110 ng/ml/well (10 µg protein / ml of antigen), 1:100 and 1:1,500 respectively. Results of the preliminary assay conducted on 10 sera from cattle reared in a confined farm negative for helminth ova and with no history or symptoms of schistosomosis yielded a background mean absorbance OD values of 0.275 with standard deviation of 0.084 for whole worm antigen. The cut off value for whole worm antigen was determined as 0.480 (mean±3 SD).

Out of 250 serum samples screened in cattle, 46 were positive indicating 18.40% infection (Table 1). Out of 250 serum samples screened in buffaloes, 40 were positive (16.0%). The overall sensitivity of this test was 77.27% and specificity was 75%.

Table 1. Detection of *Schistosoma spindale* infection by indirect ELISA using whole worm antigen

Host species	Positive cases			Negative cases	
	Number of serum samples	Positives	% positives	Number of serum samples	% negatives
Control	10	0	100	10	0
Cattle	250	46	18.40	204	81.60
Buffaloes	250	40	16.00	210	84.00
Other parasitic infection	12	8	66.66	4	33.34

In this study the positive samples further confirmed by ELISA using the whole worm antigens because of its potential for discriminating false positives from true positive samples. In a limited study, to address the question of antigenic cross reaction among *Fasciola*, *Amphistome* and *Hydatid* samples. Serum samples (12) collected from animals positive for *Fasciola*, *Amphistome* and *Hydatid* were used to check the specificity of the infection. In ELISA, 8 serum samples showed cross-reaction to *Fasciola* and *Amphistome* serum samples. Using homologous and heterologous antigens to block schistosome antibodies in ELISA, the extent of antigen cross reactivity was examined. Percentage decrease in A450nm at rabbit hyperimmune serum dilutions was maximum by homologous antigen i.e. *Schistosoma* whole worm antigen followed by heterologous amphistome and *Fasciola* whole worm antigens. Amphistome exhibited more cross reaction than the *Fasciola* and no cross reactions were observed in the hydatid serum samples because it fitted in the different clade/group even though it affects liver.

In the present study the sensitivity was 77.27% and specificity was 75% with whole worm antigen and the results were slightly lower compared to others (Pardo *et al.* 2004) who reported sensitivity of 94% and specificity of 97% with *S. bovis* adult worm antigen. Janitschke *et al.* (1987) utilized the nitrocellulose dot-ELISA for serodiagnosis of schistosomosis with sensitivity of 100% for *S.mansoni* and 93.6% for *S. haematobium*. The specificity was also 100% and it was concluded that it is one of the large scale diagnostic tests utilized under field conditions. Ganayni and Youssef (1992) concluded that ELISA is less sensitive using both cercarial and adult antigens than IHAT test.

Raouf *et al*. (2007) developed ELISA using a purified immunogenic fraction from *S.mansoni* adult worm (Sm 31/32 KDa), serum from active and inactive schistosomosis. ELISA IgG reactivity to Sm 31/32 KDa fraction by ELISA was significantly higher in sera of schistosomosis (active and inactive), compared to normal controls, while no significant difference was detected between active (OD=0.79 +/- 0.23) and inactive (OD=0.87 +/- 0.37) samples. No cross reaction was detected using *Faciola* or *Hydatid* sera, the overall level of specificity and sensitivity attained was 90% and 93%, respectively, and 31/32 KDa peptide might be of high value in serodiagnosis of active and inactive intestinal *S.mansoni* infection in human patients and also it had a similar role in animal visceral schistosomosis. The only disadvantage of antibody test is they do not discriminate between active and past infections.

In is concluded that, the *Schistosoma spindale* worm antigens were useful for serodiagnosis of visceral schistosomosis in bovines by enzyme linked immunosorbent assay. This tool could form the basis for evolving of a uniform system of seromonitoring of *Schistosoma spindale* in local bovine population in turn enabling implementation of a suitable disease control strategy in the country.

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