Efficacy of Dot-ELISA using different antigens in detecting anti-schistosome antibodies among bovines in field conditions

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Abstract Schistosomosis has been recognised as one of the major parasitic diseases of livestock and human beings. *Schistosoma spindale* is the major cause of visceral schistosomosis among bovines of Kerala State. Besides pathology in animals, it has been long known that cercariae of *S. spindale* are a common cause of dermatitis in human beings in Asia. However, detection of this disease based on coprology has underestimated the prevalence of this economically important disease among cattle of the State. An efficient diagnostic tool providing unequivocal evidence of infection in living animals is perhaps, the key to formulate and deliver control measures to the target population. It is also crucial for an enhanced understanding of parasite epidemiology. The utility of excretory–secretory proteins as diagnostic and vaccine candidates for schistosomosis has been a focus of medical research since long. There exists a paucity of information with regard to analysis of ES proteins of *S. spindale* and their incorporation to develop sensitive and specific serodiagnostic tool. Hence a study

was designed to evaluate the efficacy of Dot-ELISA incorporating different antigens of *S. spindale* and to validate the test under field conditions.

Keywords *Schistosoma spindale* ·
Excretory–secretory antigens · Dot-ELISA

Introduction

Schistosomes are a group of digenean blood flukes which evoke a strong immunological response in the host which is potentially protective as well as incite an immunopathological reaction. The Indian species, *Schistosoma spindale*, located in portal and mesenteric veins of buffalo, ox, sheep, goat, rarely horse and donkey and rodents causes pseudotubercle formation in the liver and intestine along with thrombosis of periportal veins (Kalapesi and Purohit 1954). The disease is widely distributed in India, Sri Lanka, Thailand, Malaysia, Indonesia, Vietnam and Laos (Kumar 1999). It is noteworthy that abattoir surveys reveal a significant prevalence of *S. spindale* among slaughtered cattle of Kerala (Ravindran et al. 2007; Lakshmanan et al. 2011). As per the Animal Disease Surveillance Report (AHD 2008), which was based on routine coprological diagnosis, schistosomosis accounted for only 0.38 % of the total cases of parasitism in cattle of Kerala. This represents a true picture of underestimation of bovine schistosomosis in the State, which could be attributed to the fact that natural *S. spindale* infections characterised with diarrhoea and loss of production are seldom diagnosed by routine faecal sample examination (Agarwal 1999).

Serological assays have always played a crucial role as an adjunct to routine parasitological methods in diagnosis of parasitic diseases. Immunodiagnostic methods based on

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the detection of antibodies continued to be the most effective and practical method for diagnosis of imported schistosomiasis (Tsang and Wilkins 1997). Serodiagnosis of *S. haematobium* (Khalil et al. 1993), *S. japonicum* (Hwu et al. 1978) and *S. mansoni* (Bocor et al. 1987) have been attempted using various immunodiagnostic assays with antigens from adult worms and eggs. Enzyme linked immunosorbent assay (ELISA) is a simple, sensitive and rapid serodiagnostic technique which has been widely used for detection of the circulating antibodies and antigens related to many parasitic diseases (Voller et al. 1976). Different methods have been adopted of which enzyme linked immunosorbent assay is most widely developed for human schistosomiasis (Hamilton et al. 1998). Dot-ELISA, a modification of standard ELISA, is a widely employed practical tool for field studies. Only limited work has been attempted in diagnosis of *S. spindale* using whole worm antigens (Sumanth et al. 2003; Singh et al. 2004; Divya et al. 2013; Murthy et al. 2013). There has been no attempts to evaluate the diagnostic efficacy of excretory–secretory and egg antigens of *S. spindale*. The objective of the present study was to develop a simple field level Dot-ELISA for serodiagnosis of *S. spindale*, to compare the efficacy of different antigens for its suitability as diagnostic candidates as well as to compare the efficacy of Dot-ELISA with routine faecal sample examination in diagnosis of the disease among bovines, in field conditions, in Kerala, South India.

Materials and methods

Excretory–secretory antigens

Adult *S. spindale* worms were separated from the mesentery of cattle as per the method of Lakshmanan et al. (2011). Excretory–secretory antigens (ESA) of *S. spindale* were prepared based on the method of Liu et al. (2009) with some modifications. Eight hundred adult worms were soaked in 1X phosphate buffered saline (PBS, pH 7.4) for 2 h at room temperature followed by overnight incubation at 4 °C. The viability and tegumental integrity of the worms were checked under a stereo zoom microscope to ensure that all worms were intact and live. The worms were carefully removed under sterile conditions, the remaining fluid was centrifuged at 10,000×g for 30 min at 4 °C and the supernatant was used as ESA. The protein concentration of ESA was found to be 2.56 mg/ml using protein estimation kit by Lowry's method (Merck GeNei™, Bangalore) in a spectrophotometer (Lambda 750, Perkin Elmer) at 660 nm and was further aliquoted and stored at −20 °C.

Whole worm antigens

The recovered flukes were washed in several changes of chilled PBS (pH 7.2) and used for whole worm antigen (WWA) preparation as described by Sumanth et al. (2003) with some modifications. Around 200 adult male *S. spindale* flukes were homogenized at 4 °C in PBS, containing cocktail protease inhibitors (Sigma-Aldrich, Bangalore). The homogenate was subjected to sonication for 15 s each, for 2–3 min, interrupted by a five second pause at 80 kHz, on an ice bath. It was later centrifuged at 12,060×g for 30 min at 4 °C in a cooling centrifuge. The supernatant had a protein content of 12.6 mg/ml estimated as mentioned above and was stored at −20 °C.

Egg antigens

Eggs of *S. spindale* were concentrated by density gradient centrifugation with 20 % percoll in 0.25 M sucrose as per Baltz et al. (1982) and further processed for antigen as per Ashton et al. (2001). Egg antigen had a protein concentration of 264 µg/ml.

Serum

A total of 30 serum samples were collected from slaughtered animals in which the mesenteries harboured adult schistosomes. These formed the known positive sera. The serum collected from worm negative animals with a history of being reared in confined farms of non endemic areas served as known negative control (n = 30). Besides 100 serum samples were collected from cattle in field conditions reared in semi intensive system with access to water logged areas in Thrissur and Palakkad districts of Kerala State.

Raising hyper immune serum in rabbits

Hyper immune serum was raised against E/S, WWA and egg antigens of *S. spindale* in adult New Zealand White rabbits as per Murthy et al. (2013). Hyper immune serum raised against mixed amphistomes species WWA, available in the Department of Veterinary Parasitology, were also used as specificity controls.

Faecal samples

Rectal faecal samples collected from cattle in field conditions were processed using 10 % formalin and examined for the presence of typical napoleon hat shaped *S. spindale* ova.

Dot-ELISA

Three different dilutions of WWA and ESA each were prepared in carbonate bicarbonate buffer (pH 9.6) viz., 500, 250 and 100 ng/μl. The egg antigen was diluted to 250 and 100 ng/μl in the same buffer. One microlitre each of the diluted antigens was coated on the centre of nitrocellulose membrane (NCM) strips of approximately 1 cm² size. The air dried strips were incubated in blocking buffer at 37 °C for 1 h. Later the strips were incubated in serum samples (1:50 dilution of bovine serum/hyperimmune serum in blocking buffer) at room temperature for 1 h and then, washed six times with 0.05 % Tween-20 in 1X PBS (PSBT) for 10 min each. Further, the NCM strips were incubated with the conjugate (1:2,000 dilution of anti-species IgG-HRP conjugate was used as per the serum selected) at room temperature for 1 h. The strips were washed four times in PBST as described earlier and were immersed in chromogenic visualisation solution, containing diaminobenzidine, with mild rocking at room temperature, in dark, for 2 to 3 min. The reaction was terminated by washing the strips with distilled water. The NCM strips were air dried and photographed. Development of brown coloured spot indicated a positive reaction.

The three antigens were tested at different protein concentrations with serum from animals that were positive for schistosomes in the mesentery and the hyperimmune serum raised against schistosome antigens as the known positive controls. The different antigen combinations were also tested with serum of mesentery negative animals and foetal bovine serum which served as known schistosome negative controls. Sera from animals in which faecal samples revealed ova of amphistome, as well as the hyperimmune serum raised in rabbits against mixed amphistome species antigens, available in the department, were used to check the specificity of all the antigens in different dilutions. Field samples were also subjected to Dot-ELISA for testing seroprevalence of *S. spindale* in dairy cattle of endemic areas.

Results

The minimum antigen concentrations that yielded a positive Dot-ELISA signal were 100 ng/μl each for WWA and egg antigen and 250 ng/μl for ESA and hence were determined as optimum for further specificity studies. Serum dilution was fixed at 1:50 to rule out the possibility of cross reaction with heterologous serum. The test was performed with known schistosome positive and negative bovine serum, hyper immune serum raised against WWA of mixed amphistome species and against *S. spindale*, as well as with serum of cattle harbouring amphistome

infection. The test was repeated six times. The results are represented in the Fig. 1.

The ESA yielded positive Dot-ELISA signals with known positive bovine control serum and with hyperimmune serum raised against *S. spindale* in all replicates. It failed to react non specifically with either known negative bovine controls or amphistome positive controls. The WWA gave positive results with known schistosome positive bovine and hyperimmune sera but not with known schistosome negative bovine sera or foetal bovine sera, in all six replicates. However, WWA also gave a less intense positive signal with hyperimmune serum raised against amphistomes as well as with serum from amphistome infected cattle, in all replicates. Though egg antigen yielded positive signals with schistosome positive sera, weak positive signals were obtained using amphistome positive sera, as well in all the replicates. However, negative reaction was observed with known schistosome negative bovine serum and foetal bovine serum. Owing to the enhanced specificity, the ESA was selected as diagnostic antigen candidate for further validation of Dot-ELISA with field serum samples.

Anti-schistosome antibodies could be detected in 32 out of the 100 bovine sera collected from field, by Dot-ELISA using *S. spindale* ESA at a concentration of 250 ng/μl. Schistosomosis could be diagnosed only in 12 animals (12 %) by routine coprology. There was statistically significant difference between the tests [Cal $\chi^2 >$ Table χ^2 (5 %)] and coprology was found to have a low detection level. The results of Dot-ELISA with known negative and positive sera, was also compared with mesentery sample examination. It revealed that Dot-ELISA could detect anti-schistosome antibodies with 100 % sensitivity and specificity when mesentery sample examination was fixed as the test standard (Table 1).

	WWA 100ng	ESA 250ng	Egg Antigen 100 ng
HIS SS (<i>S. spindale</i>)
HIS Amphistome
Known SS positive bovine serum
Known SS negative bovine serum
Known Amphistome positive bovine serum
Foetal bovine serum

Fig. 1 Dot-ELISA with different antigens of *S. spindale*

Table 1 Comparative evaluation of Dot-ELISA using ESA to detect anti-schistosome antibodies

	Abattoir samples		Field samples	
	Mesentery examination	Dot-ELISA	Coprology	Dot-ELISA
No. positive	30	30	12	32 ^a
No. negative	30	30	88	68
Total	60	60	100	100

^a Significant [Cal $\chi^2 >$ Table χ^2 (5 %)]

Discussion

Excretory–secretory antigens were found to be more specific for detection of anti-schistosome antibodies in cattle. The alternate methodology adopted for ESA preparation in the present study was also relatively simple when compared to the traditional in vitro culture technique in supplemented RPMI-1640 medium described for schistosome ESA preparation. Moreover according to Morphew et al. (2007) the excretion and secretion behaviour of the parasites could be altered due to removal from natural host tissues and in vitro maintenance in chemical mixture.

Though crude antigens were reported to possess a high sensitivity and specificity equivalent to those of defined or characterised antigens, cross reactions of *S. japonicum* WWA and egg antigens with *Ascaris* sp. and *Paragonimus* sp. infected human sera have been documented (Mott et al. 1987). But Boctor et al. (1987) could not observe any cross reaction between *S. mansoni* WWA and *Fasciola* sp. infected human serum in Dot-ELISA. Soluble WWA preparations of *S. mansoni* at a concentration of 250 ng/dot were also reported to yield false positive results in malaria and filaria positive human subjects (Montenegro et al. 1999). Noya et al. (2002) had also observed false positive reactions in western blot and ELISA with crude WWA and egg antigens of *S. mansoni* particularly in individuals infected with nematodes, though these antigens possessed high sensitivity for the diagnosis of infection. In the present study *S. spindale* ESA had not exhibited cross reaction with amphistome positive sera in Dot-ELISA. The high specificity of ESA in serodiagnosis has been observed by several workers for detecting other trematode infections as well viz, *Clonorchis sinensis* (Choi et al. 2003), *Paragonimus* sp. (Narain et al. 2005), *F. hepatica* (Awad et al. 2009), *Opisthorchis felinus* (Gomez-Morales et al. 2013) and *Paramphistomum cervi* (Anuracpreeda et al. 2013). On perusal of available literature, it is understood that ESA of *S. spindale* have not yet been utilised for Dot-ELISA. The absence of cross reaction with amphistomes, one of the most prevalent flukes infecting cattle of Kerala, highlights the utility of ESA as a potential diagnostic candidate for detecting intestinal schistosomosis serologically. However

future studies are to be conducted towards purifying the antigens and to rule out the possibility of cross reaction with other helminths.

Detection of anti-schistosome antibodies does not preclude the possibility of existence of past infection or persistence of antigens after specific treatment. However such conclusions do not seem to influence the positive results of Dot-ELISA in the present study. This is because the only available anti-schistosome drug, praziquantel, is not being used to treat bovine schistosomosis in Kerala. The current practice of using antimony compounds leads to energy depletion, reduced fecundity and “hepatic shift” (Kumar 1999) instead of destroying the adult worms. Hence ELISA positive animals in the study could be considered as those harbouring schistosomes.

In the present study, schistosomosis could be detected among 32 % of bovines in Kerala especially in water-logged panchayats of the State, using Dot-ELISA. The wide disparity between immunodiagnosis and egg detection, revealed in this study points out to the possibility that *S. spindale* infection among bovines of the State was under diagnosed. To strengthen our view, abattoir surveys have revealed a significant prevalence of *S. spindale* in various parts of Kerala. The significant prevalence of infection among live animals demands urgent attention as the disease is characterised with morbidity, mortality, reduced productivity and poor subsequent reproductive performance (Mc Cauley et al. 1984) and increased susceptibility to other diseases (Dargie 1980). Dot-ELISA is simple, cheap and easy to perform under field conditions and offer best possibility for timely and effective diagnosis of the disease, identification of endemic areas and thus in planning prevention and control strategies.

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