Short Paper

Immunodiagnosis of *Haemonchus contortus* infection in sheep by indirect enzyme linked immunosorbent assay (ELISA)

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Summary

Indirect plate enzyme-linked immunosorbent assay was standardized and evaluated for its effectiveness in immunodiagnosis of haemonchosis in experimental and clinical cases in sheep by using somatic whole adult antigen of *H. contortus*. Plate ELISA was standardized using 5 µg/well antigen concentration with 1:100 and 1:1000 of sera and conjugate dilution. Indirect plate ELISA was able to demonstrate the antibody titre at different weeks post infection in experimental sheep. A comparison of plate ELISA on suspected field sera and faecal sample examination by floatation method revealed that 74 samples were found to be positive by ELISA but only ten by faecal examination. Sensitivity of plate ELISA was found to be 80.0%, whereas specificity was 21.42% indicating that this test is quite sensitive for clinical cases; an early diagnosis, however, lacks specificity.

Key words: Indirect plate ELISA, Immunodiagnosis, *Haemonchus contortus*, Somatic antigens

Introduction

Production of sheep is an attractive feature for farmers in the Kashmir valley due to low capital input and the ability of sheep to thrive on native pastures. Almost every rural household has sheep, which serve the daily needs of milk, meat, wool, hides and valuable organic manure. However, the productivity of sheep is constrained by parasitic infections (Dhar *et al*., 1982; Tariq *et al*., 2006). Sheep are usually more prone to gastrointestinal tract (GIT) parasitism as they mostly graze on pastures contaminated with L_3 of parasitic nematodes. *Haemonchus contortus* is a major nematode infecting the sheep population (59.6%) of the Kashmir valley, which causes huge mortality and morbidity by affecting health, production, and reproduction of animals (Tariq *et al*., 2008).

Reliable detection of the active infection of *H. contortus* is usually based upon evaluation of clinical signs and faecal examination, which have their inherent limitations. Clinical signs usually become apparent only when the infection is heavy and the eggs are passed in the faeces after the prepatent period of approximately 41 days (Soulsby, 1983), when the infection is much advanced and the major damage is already done. In order to circumvent these limitations there is an acute need for developing a reliable serological assay like enzyme linked immunosorbent assay (ELISA) for early detection of the infection. Detection of serum antibodies against parasite by ELISA is a rapid and simple test with which a considerable number of samples could be processed at the same

time. Furthermore, seroepidemiological studies involving examination of a large group of animals might also benefit from a reliable ELISA. Usually such a test, in contrast to faecal examination, is less time consuming.

Therefore, an indirect ELISA based on crude somatic antigen of *H. contortus* was standardized and evaluated under field conditions as a diagnostic tool for detection of anti-*H. contortus* antibodies in sera of infected sheep.

Materials and Methods

Experimental animals

Eight healthy local sheep, 30–45-day-old, were maintained under intensive rearing conditions precluding accidental parasitic infections. At the age of five months, they were used for experimental infection with *H. contortus*.

Collection of *Haemonchus contortus* L_3 and experimental infection

The infective L_3 were obtained by culturing (Soulsby, 1983) the eggs separated from the adult female worms recovered from the abomasum of slaughtered sheep (Tariq *et al*., 2008). The L_3 at a dose rate of 600 per kg body wt. were orally administered in one of the 8 helminth free sheep, after overnight withdrawal of feed, to serve as donors for sufficient number of monospecific *H. contortus* eggs for artificial infection. After the patency of the infection, the faeces of the donor sheep were cultured (Soulsby, 1983) and the L_3 were harvested (Anon., 1971). These L_3 were used for artificial infection of four sheep as stated earlier, keeping the remaining three sheep as uninfected control. Serum samples of the infected, as well as the control sheep were collected on every third day post-infection (DPI) till 33 DPI, following the standard methods and they were preserved at -20°C for use in the assay.

Preparation of antigen

For preparation of crude somatic antigen (CSAg) *Haemonchus contortus* from the abomasum of freshly slaughtered sheep was collected (Johnson *et al*., 2004) in a Petri dish containing 0.15 M phosphate buffer saline (PBS), pH - 7.2. The worms were washed 3 times in the same buffer, and finally 200 worms were homogenized in 10 ml of cooled 0.15 M PBS (pH - 7.2) containing 25 mM phenylmethyl sulfonyl fluoride (PMSF) and 24 mM ethylene-diamine tetraaceticacid (EDTA) in a glass tissue homogenizer followed by sonication (Soniprep-150). The disintegrated parasite extract was centrifuged at 4°C at 10000 g for 15 min and the supernatant was collected as the CSAg with a protein concentration of 3.82 mg/ml specified according to Lowry *et al*. (1951). The antigen was stored at -20°C for use in the assay.

Standardization of the assay

Plate ELISA was performed in 96 wells polystyrene microtitre plates with all incubation times previously determined by checkerboard titration following Hudson and Hay (1989). The antibody detection was performed according to the method of Voller *et al*. (1976) with some modifications. The optimal concentration of ELISA reagents including the concentration of the coating antigen (5 µg/well), dilution of the positive and negative reference sera (1:100) as well as rabbit anti-goat IgG-horseradish peroxidase (HRP) conjugate (1:1000) and the optimal test conditions, respectively were determined by checkerboard dilution assay using flat-bottom 96-well micro-ELISA plate. The absorbance (optical density; OD) of the wells was measured at 492 nm by an ELISA reader (Biorad II). The mean OD plus three times the standard deviation of the negative control sera was taken as the cut-off value for considering a sample as test positive (Lejon *et al*., 2005).

Performance of the assay

The sensitivity, specificity and accuracy of the ELISA were determined (Thrusfield, 1997) using 96 serum samples of field sheep. The parasitological status of animals with regard to nematodes trematode and cestodes was carefully examined and recorded.

Results

The cut-off OD value for the

standardized ELISA as determined was 0.268. *H. contortus* antibodies in all experimentally infected sheep were detected as early as 18 to 27 DPI. The seroconversion was relatively long before the patency of the infection, which in this study was on 42 DPI. Specific antibodies were consistently present during the subsequent observation period i.e. till 33 DPI.

A total of 96 sheep samples suspected for Haemonchosis were examined by indirect plate ELISA. Various negative and experimental positive control sheep were raised and sera from both positive and negative sheep were taken as reference sera for ELISA. A cut off value of means of negative controls ±3SD was taken into account for the detection of each positive case. Out of the total 96 examined sera samples, 74 were found to be positive with plate ELISA having an incidence rate of 77.08%.

The sensitivity, specificity, positive predictive value, and negative predictive value were calculated from the two way analysis table (Table 1) using the following formula:

Sensitivity of plate ELISA was found to be 80.0%, whereas specificity was 21.42%, indicating that this test is quite sensitive for clinical cases: an early diagnosis, however, lacks specificity. The plate ELISA positive and negative predictive values were found to be 10.81% and 90.0%, respectively.

A marked difference was observed between the proportion of ELISA positive and faecal examination (floatation) negative samples. Seventy four samples were found to be positive by ELISA but only ten by faecal examination, indicating that ELISA is a significantly more sensitive method.

Discussion

Diagnosis of gastrointestinal nematode infections has conventionally relied upon detection of the clinical signs, aided by qualitative detection of the eggs in the faeces of suspected animals. Feacal examination methods are effective, since clinical signs are apparent only in heavy infection and the faecal eggs are detected in *Haemochus* infection only after its patency on approximately day 41 (Soulsby, 1983) when the major damage is already done by the parasites. It is thus imperative to detect the infection at this stage in order to minimize the associated economic losses, especially in weaned kids and lambs.

The standardized ELISA using easily available crude somatic antigen yielded promising results for detection of prepatent and patent haemochosis in sheep. In experimentally infected sheep, the infection was detected between 18-27 days after the infection and it was obviously much earlier than the time required for the infection to reach the patent period (42 days in the present study). ELISA is known for its potential to detect the antibodies at quite an early stage of the infection. Nevertheless, it could detect the artificially induced haemonchosis only after 17 days of the infection. The indirect ELISA was evaluated on field sera and the results were compared with the post-mortem findings with regard to the actual parasitological status. The assay proved high sensitivity (80.20%), specificity (21.42 %) and holds considerable promise for its exploitation in seroepidemiological studies of this economically significant helminthosis. Sequestration of antibodies and the formation of circulating immune complexes (Gasser *et al*., 1993) and immune evasion mechanisms of the parasite (Spinelli *et al*., 1996) might be responsible for wide variations in the antibody level in the necropsy positive samples as shown by indirect ELISA. Two false negative results in the present assay might be due to low

Table 1: Two way analysis table for sensitivity and specificity of parasitological method and ELISA plate

		ELISA test		
		No. of positive animals	No. of negative animals	Total
Faecal examination (Flotation)	No. of positive animals	8	2	10
	No. of negative animals	66	18	84
	Total	74	20	94

worm burden or poor immune response of the host (Gasser *et al*., 1994). Besides, host nutritional status (Jenkins *et al*., 1991; Gasser *et al*., 1992) and physiological and environmental factors like reinfection or coinfection with other parasites (Carmena *et al*., 2005) might also have an impact on the antibody levels. False positive result of the assay with postmortem negative samples might be due to the persistence of antibodies to the past infection, which might have been eliminated by anthelmintic medication. False positive result is probably due to cross reactivity of *H. contortus* with other helminths. Cross reactivity amongst different helminths is a common and limiting factor in the development of serological tests against helminth infection (Philipp and Rumjaneck, 1984; Cuquerella *et al*., 1994; Molina *et al*., 1999).

Copro-ELISA assay could be the best possible tool for detection of current infection of gastrointestinal parasites and attempts in this regard have already been made (Johnson *et al*., 2004; Nayebzadeh *et al*., 2008; Jas *et al*., 2010). The standardized assay offers the potential for its development as one of the best diagnostic tools for haemonshosis. The use of purified antigen may minimize cross reactivity with other worms for ELISA. In conclusion, the use of ELISA is the best serodiagnostic technique for *H. contortus* compared to coprological examination, because ELISA is more sensitive than any other tests.

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