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Diagnosis of mixed gastrointestinal nematode infection in goat by an indirect-ELISA

Datta, S.¹; Dandapat, P.² and Jas, R.^{3*}

¹Veterinary Officer, Block Animal Health Centre, Barasat Block I, Barasat-700 128, Government of West Bengal; ²Eastern Regional Station, ICAR-Indian Veterinary Research Institute, Kolkata-700 037, West Bengal, India; ³Department of Veterinary Parasitology, Faculty of Veterinary & Animal Sciences, West Bengal University of Animal & Fishery Sciences, 37, Kshudiram Bose Sarani, Kolkata-700 037, India

*Correspondence: R. Jas, Department of Veterinary Parasitology, Faculty of Veterinary & Animal Sciences, West Bengal University of Animal & Fishery Sciences, 37, Kshudiram Bose Sarani, Kolkata-700 037, India. E-mail: rumajas@gmail.com

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Summary

An indirect-ELISA for the diagnosis of mixed gastrointestinal (GI) nematode infection comprising *Oesophagostomum*, *Haemonchus* and *Trichuris* species was standardized using crude somatic antigen of *Oesophagostomum columbianum* (CSAg-Oc) and sera of slaughtered goats with known parasitological status including *Oesophagostomum*, *Haemonchus*, and *Trichuris* (strong positive), *Haemonchus* and *Trichuris* (weak positive) and parasite free goats (negative). Two cut-off points, i.e. higher and lower cut-off were determined using the strong positive, weak positive and the negative control sera of goats. Thus the test sera having optical density (OD) values greater than the higher cut-off were considered positive for mixed infection with all the three nematode species, intermediate between the higher and the lower cut-off values were considered positive for mixed infection of *Haemonchus* and *Trichuris*, and less than the lower cut-off value were considered negative for any of these three nematode species. The sensitivity, specificity and accuracy of the ELISA for diagnosis of mixed GI nematodes were 81.25, 93.18% and 90.00%, respectively, while it was 92.86% sensitive, 75.00% specific and 91.67% accurate for the diagnosis of mixed infection with *Haemonchus* and *Trichuris*. The ELISA, so standardized, detected 27.78% sero-prevalence of *Oesophagostomum* plus *Haemonchus* and *Trichuris* infection and 38.89% percent of *Haemonchus* and *Trichuris* infection in the field goats. The standardized assay might be exploited as a diagnostic tool and also for sero-epidemiological study of two important GI nematodes of goats.

Key words: Diagnosis, Goat, *Haemonchus*, Indirect-ELISA, *Oesophagostomum*

Introduction

In India, including the state of West Bengal, parasitic gastroenteritis due to nematode infections is a major constraint to profitable goat rearing (Jas *et al.*, 2007; 2017a). The inherent habits and the husbandry practices followed for goat rearing in India make them highly prone to parasitic diseases, especially helminthoses due to infections with gastrointestinal (GI) nematodes. The GI helminth parasites recorded from different parts of India including West Bengal are *Haemonchus contortus*, *Oesophagostomum* spp., *Trichostrongylus* spp., *Gaigeria* sp., *Trichuris* sp., and *Strongyloides* sp. (Bhojane *et al.*, 2002; Brahma *et al.*, 2015; Jas *et al.*, 2017b). Helminth infections, with rare exceptions, are generally sub clinical and morbidity rather than mortality is the major impact. The major damage due to helminthoses is caused before the infection reaches its patency. But diagnosis of helminthic infections is still based on detection of eggs or larvae in the faeces by conventional parasitological methods. However, such methods fail to detect the pre-patent stages of the infections. Hence immunological methods are currently being exploited as an alternative to conventional parasitological examination for the diagnosis of parasitic infections (Johnson *et al.*, 2004; Jas *et al.*, 2010a, b). Under natural conditions, the infection with a single nematode species is very rare and mixed infection is more common. Therefore, an effective

diagnostic tool, which will be able to detect mixed GI nematode infections, will be very useful to adopt control strategy as well as for epidemiological studies. Although *Haemonchus contortus* is the predominant and pathogenic nematode species, a serious disease; “nodular enteritis” or “pimply gut” caused by *Oesophagostomum columbianum* also results in considerable morbidity and mortality in small ruminants. Although with minor clinical significance *Trichuris ovis* occurs as a common concurrent infection with *Haemonchus* and *Oesophagostomum* (Jas *et al.*, 2016). Major pathogenic effect of these nematodes is caused due to the migratory/immature stages of the parasite, which are not detectable by conventional parasitological methods. Hence there is a pressing need for developing a reliable serological assay like ELISA, which is known for its reliability in detecting subclinical infections.

In our previous study we have shown that there is sharing of antigen between *H. contortus* and *O. columbianum* and between *O. columbianum* and *T. ovis* and there are many cross-reactive antigens among these three nematode species (Jas *et al.*, 2016). Therefore, the present study was designed to develop a reliable serological assay for detection of mixed infection with *H. contortus*, *O. columbianum* and *T. ovis*. Furthermore, sero-epidemiological studies involving examination of a large group of animals will also benefit from a reliable ELISA.

Materials and Methods

Experimental animals

Two female Black Bengal goats, 5 months old were procured from the university farm and maintained in the departmental animal house after anthelmintic treatment. The goats were provided with concentrate feeds, chopped hay and tree leaves without any soil contamination and clean drinking water. The goats were maintained for three months as uninfected control. The serum of these goats was used as negative serum control in the assay. The entire experimental design was approved by the Institutional Animal Ethics Committee, West Bengal University of Animal and Fishery Sciences.

Preparation of antigen

For preparation of crude somatic antigen of *O. columbianum* (CSAg-Oc) caecum and colon of slaughtered goats were brought to the laboratory from the local abattoir at Kolkata. *Oesophagostomum* sp. were manually recovered from the dissected caecum and colon and collected in normal saline solution, then washed thrice in cold 0.15 M phosphate buffer saline (PBS), pH = 7.4. The CSAg-Oc was prepared following the method described by Jas *et al.* (2010b). The protein concentration of the antigen, as estimated by the method of Lowry *et al.* (1951), was 3 mg/ml. The antigen was preserved at -20°C till further use.

Collection of field goat sera samples

For standardization of ELISA and its evaluation for immunodiagnosis of mixed GI nematodes 120 sera samples of goats from local abattoir were collected following the standard technique. The parasitological status of the goats with respect to GI nematode infections was subsequently ascertained by screening their alimentary canal in the laboratory and was recorded separately for each serum sample. The sera samples of 108 goats with unknown parasitological status were collected randomly from a village in Bankura district of West Bengal. All the sera samples were preserved at -20°C without adding any preservative till further use.

Standardization and evaluation of indirect-ELISA

The indirect-ELISA using CSAg-Oc was standardized following the standard method (Voler *et al.*, 1976) for detection of antibodies against *Oesophagostomum* sp. as well as cross-reactive antibodies against *H. contortus* and *T. ovis* in goats. The optimal concentration of ELISA reagents including the concentration of the coating antigen, dilutions of positive and negative reference sera as well as anti-goat IgG-horse radish peroxidase (HRP) conjugate and optimal test conditions were determined by Checker Board dilution assay. Sera samples collected from the goats naturally infected with *Oesophagostomum*, *Haemonchus* and *Trichuris* species or *Oesophagostomum* with any of the two species were considered as strong positive and

that with *Haemonchus* and *Trichuris* species as weak positive, while the serum collected from the goats maintained in the departmental animal house under parasite free condition for a period of three months was considered as negative control.

Test procedure

Wells of ELISA plates (Nunc, Maxisorp) were coated with 100 µL (equivalent to 5 µg of antigenic protein) of CSAg-Oc as capture antigen diluted in 0.05 M carbonate-bicarbonate buffer, pH = 9.6 (coating buffer) and incubated overnight at 4°C. The plates were then thoroughly washed thrice with washing buffer (PBS containing 0.05% Tween 20). The uncoated sites of the wells were blocked with 200 µL of blocking buffer (0.05% PBST and 2% BSA) by incubating the plates at 37°C for 1 h with continuous shaking at a moderate speed. The plate was then washed as described above and then 100 µL test serum samples (1:50) were added to the test wells in duplicate keeping the appropriate controls (strong positive, weak positive, negative and conjugate) and the plates were incubated for 1 h at 37°C. After washing, 100 µL of anti-goat IgG-HRP conjugate (diluted 1:6000 in blocking buffer) was added to all the wells and the plates were incubated for 1 h at 37°C with constant shaking. Then 200 µL of freshly prepared substrate chromogen solution containing Orthophenylene diamine dihydrochloride (OPD tablets, Sigma, USA), citrate-phosphate buffer (pH = 5.6) and hydrogen peroxide (H₂O₂) was added to each well after washing and incubated at 37°C for 15 min. The reaction was then stopped by adding 50 µL of 2.5 M sulphuric acid (H₂SO₄) to all the wells. The optical density (OD) of the wells was measured at 492 nm in the ELISA reader (M/S Tecan Sunrise, Austria).

Interpretation of test results

In this study, two cut-off values i.e. higher and lower cut-off were determined. For calculation of cut-off points, OD values of four weak positive and four negative wells were considered. The higher and lower cut-off values were calculated as the mean OD_{492nm} plus three times the standard deviation of the OD_{492nm} of the sera of *Haemonchus* and *Trichuris* species infected goats and of parasite free goats (Lejon *et al.*, 2005) at 1:50 dilution, respectively. The test sera showing OD values greater than the higher cut-off values was considered positive for mixed infection with *Oesophagostomum* along with *Haemonchus* and *Trichuris* species. Whereas, the sera giving the OD values intermediate between the higher and lower cut-off values were considered positive for mixed infection with *Haemonchus* and *Trichuris* spp. and the sera samples showing OD values below the lower cut-off OD were considered free from any of the three nematode species as mentioned above.

Determination of sensitivity, specificity and accuracy of the indirect-ELISA

Sensitivity, specificity and accuracy of indirect-ELISA were calculated according to the standard

formulae (Thrusfield, 2003). The standardized ELISA was evaluated considering the ELISA OD values of 120 sera samples of slaughtered goats with known parasitological status as mentioned above.

Results

A total of 120 sera samples as well as GI tract (abomasum, caecum and colon) of goat were collected from the local slaughter house at Kolkata. Out of those, 32 GI tracts were found infected with *Oesophagostomum*, *Haemonchus* and *Trichuris* species and 80 goats were found positive for *Haemonchus* and *Trichuris*. The remaining eight goats were found negative for any of the three nematode species (Tables 1 and 2).

Serum samples of twenty-six goats out of 32 positive for *Oesophagostomum* along with the other two species showed OD values higher than the higher cut-off value. Six goats, although harbouring all the nematode species were not detected as positive for *Oesophagostomum* as they showed intermediate OD values between the higher and lower cut-off values, thereby resulting in false negative reaction. In contrast, out of the 88 goats negative for *Oesophagostomum*, six sera samples showed OD values higher than the higher cut-off value resulting in false positive reaction (Table 1). Therefore, six false positive reactions and six false negative reactions were observed in the standardized assay for detection of mixed infection with *Oesophagostomum*, *Haemonchus*, and *Trichuris* (Fig. 1). Thus the sensitivity, specificity and accuracy of the standardized assay were 81.25%, 93.18% and 90.00%, respectively (Table 1) for detection of mixed GI nematode infection.

Out of 112 goat sera positive for *Haemonchus* and *Trichuris* (32 samples positive for *Oesophagostomum*, *Haemonchus*, and *Trichuris* and 80 samples positive for *Haemonchus* and *Trichuris*) 104 serum samples showed

positive reaction and 8 samples showed false negative reaction by the standardized assay (Table 2). Out of 8 samples negative for any of the three nematode species two samples showed false positive reaction by the indirect-ELISA (Fig. 1). Thus the sensitivity, specificity and accuracy of the standardized assay were 92.86%, 75% and 91.67%, respectively for detection of mixed infection with *Haemonchus* and *Trichuris* (Table 2).

Out of 108 field goat sera samples with unknown parasitological status, 30 (27.78%) were positive for *Oesophagostomum* sp. plus *Haemonchus* and *Trichuris*, 42 (38.89%) for *Haemonchus* and *Trichuris* and the rest were negative for all the three nematode species infections (Fig. 2).

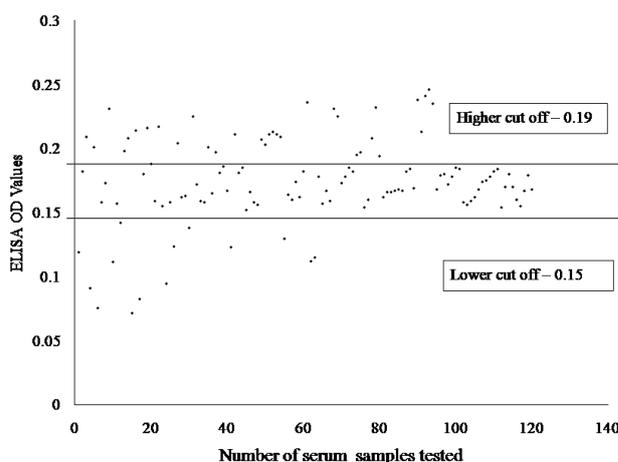


Fig. 1: Serum antibody response (OD values) in field goats (n=120) with known parasitological status to the standardized indirect-ELISA. Serum samples showing ELISA OD values > higher cut-off values considered positive for all three nematode species, serum samples showing intermediate ELISA OD values between the higher and lower cut-off values considered positive for *Haemonchus* and *Trichuris* only, and serum samples showing ELISA OD values < lower cut-off value considered negative for any of the three nematode species

Table 1: Performance results of the indirect-ELISA for detection of *Oesophagostomum*, *Haemonchus*, and *Trichuris* infection on field sera of goat with known parasitological status on necropsy

Observable Parameters	No. of slaughtered goats whose serum samples as well as g.i. tract were examined	No. of sera of slaughtered goats whose GI tract, positive for all three nematodes, showed ELISA OD > higher cut-off	No. of sera of slaughtered goats whose GI tract, negative for any nematodes, showed ELISA OD > higher cut-off	No. of sera of slaughtered goats whose GI tract, positive for all three nematodes, showed ELISA OD < higher cut-off	No. of sera of slaughtered goats whose GI tract, negative for any nematodes, showed ELISA OD < higher cut-off
Observation results	120	26	6	6	82

Table 2: Performance results of the indirect-ELISA for detection of *Haemonchus* and *Trichuris* infection on field sera of goat with known parasitological status on necropsy

Observable Parameters	No. of slaughtered goats whose serum samples as well as GI tract were examined	No. of sera of slaughtered goats whose GI tract, positive for the two nematodes, showed ELISA OD > lower cut-off	No. of sera of slaughtered goats whose GI tract, negative for any nematodes, showed ELISA OD > lower cut-off	No. of sera of slaughtered goats whose GI tract, positive for the two nematodes, showed ELISA OD < lower cut-off	No. of sera of slaughtered goats whose GI tract, negative for any nematodes, showed ELISA OD < lower cut-off
Observation results	120	104	2	8	6

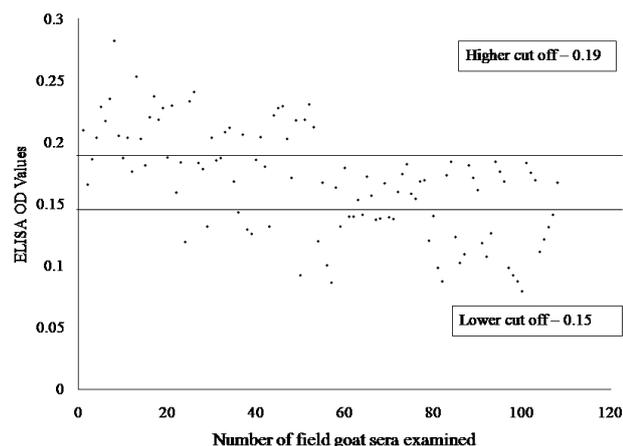


Fig. 2: Serum antibody response (OD values) in field goats (n=108) with unknown parasitological status to the standardized indirect-ELISA. Serum samples showing ELISA OD values > higher cut-off values considered positive for all three nematode species, serum samples showing ELISA OD values intermediate between the higher and lower cut-off values considered positive for *Haemonchus* and *Trichuris* only, and serum samples showing ELISA OD values < lower cut-off value considered negative for any of the three nematode species

Discussion

Two cut-off values i.e. higher and lower cut-off values were determined in the present assay. Higher cut-off and lower cut-off values were determined considering the OD values of weak positive and negative control sera, respectively. Under field conditions goats are naturally infected with different types of nematode parasites and infection with single type of nematode is very rare. The conventional method of diagnosis primarily relies on the detection of eggs in the faecal sample but the eggs are detected in faeces only after the patency. In most of the cases of GI nematodoses the major damage is caused by the larval stages or immature stages which could not be detected by conventional faecal examination technique. It is thus imperative to detect mixed nematode infection rather than single infection at the prepatent stage to reduce the associated economic losses in goat farming. Indirect-ELISA standardized using easily available crude somatic antigen in the present study yielded promising results for detection of mixed GI nematode infection in goats. The standardized assay was evaluated using field goat serum with known parasitological status proved to have sensitivity, specificity and accuracy of about 81.25%, 93.18% and 90.00%, respectively for detection of mixed infection with *Oesophagostomum*, *Haemonchus* and *Trichuris*. In contrast the assay for detection of mixed infection with *Haemonchus* and *Trichuris* showed high sensitivity (92.86%), specificity (75.00%) and accuracy (91.67%).

Therefore, the present study with two cut-off (higher and lower) holds considerable promise for detection of mixed infection and also for its exploitation in sero-epidemiological survey for these economically important helminth infections. Under field conditions of India as

well as in West Bengal mixed infection of *Haemonchus* along with *Oesophagostomum* and *Trichuris* are of common occurrence (Jas and Ghosh, 2007; Brahma *et al.*, 2015, 2017; Jas *et al.*, 2017b).

Cross reactivity among helminth parasites is a common problem in the development of a reliable tool for immunodiagnosis as well as for immunoprophylaxis (Cuquerella *et al.*, 1994; Molina *et al.*, 1999; Jas *et al.*, 2016). Sharing of immunopeptide (56 kDa) between *Haemonchus* and *Oesophagostomum* and between *Oesophagostomum* and *Trichuris* (47 kDa) has been reported by Jas *et al.* (2016) and the authors also observed cross reactivity among these nematode species. Indirect-ELISA for diagnosis of *Haemonchus* (Schallig *et al.*, 1995) and *Oesophagostomum* species (Jas *et al.*, 2010b) was standardized earlier. In the present study our aim was to develop an assay which could detect both the economically important parasites due to presence of shared as well as cross reactive antigens by a single test.

Introduction of higher cut-off value was to detect mixed infection of both *Oesophagostomum* and *Haemonchus* and lower cut-off value to detect infection of *Haemonchus* which could never be ignored in goat husbandry. In the present assay six animals positive for *Oesophagostomum* and 8 samples positive for *Haemonchus* showed false negative reaction and this might be due to low worm burden (Jas *et al.*, 2010a) 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 level.

False positive reaction with postmortem negative samples recorded in the assay might be due to the persistence of serum antibodies to the recent past infection with any of the three parasites, which might have been treated with anthelmintics (Jas *et al.*, 2010b) or due to the prepatent infection of *Oesophagostomum* or *H. contortus*. False positive reaction must also be due to the cross-reactivity of crude somatic antigen of *Oesophagostomum* with helminth parasites other than *Haemonchus* and *Trichuris* (Jas *et al.*, 2010b). Further, the assay was employed for detection of mixed infection of *Oesophagostomum*, *Haemonchus* and *Trichuris* in goats and about 66.67% of goats were found positive for either mixed infection of *Oesophagostomum*, *Haemonchus* and *Trichuris* or with *Haemonchus* and *Trichuris*. The sero-prevalence of GI nematodes in goats as observed in the present study was in agreement with the earlier reports on prevalence of GI nematodes as determined by faecal sample examination (Brahma *et al.*, 2015; Jas *et al.*, 2017b).

The performance of the assay in terms of sensitivity and specificity is quite satisfactory and it holds potential for its development as immunodiagnostic tool for detection of mixed GI nematodes comprising *Haemonchus*, *Oesophagostomum* and *Trichuris* infection in goats. The indirect-ELISA standardized in the present study might be exploited for sero-epidemiological study

as it is able to detect two most important nematodes of goats.

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Conflict of interest

We have no conflict of interest with any people or organization.

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