Detection of anti–*Haemonchus contortus* antibodies in sheep by dot-ELISA with immunoaffinity purified fraction of ES antigen during prepatency

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Haemonchosis is a very common disease in small ruminants caused by *H. contortus*, a blood sucking parasite causing anaemia that may be fatal particularly to young animals. Therefore, detection of the infection during prepatent period is important for early treatment. Excretory-Secretory (ES) protein of *H. contortus* was purified through immunoaffinity chromatography. Dot-ELISA was performed with crude ES antigen as well as immunoaffinity purified fraction (F-1) with experimental and natural sera of sheep infected with *H. contortus*. Solid dot formation took place with 4 day, 1, 2 and 3 weeks post infection sera. Dot formation did not take place with negative control serum and uninfected control animal serum. When crude ES antigens was reacted to natural sheep sera having *H. contortus* infection, 60% sera samples showed solid dot formation whereas in F-1 fraction 75% of the sera samples showed solid dot indicating purified fraction was a more potent antigen. Crude ES and F-1 were also fractionated through SDS-PAGE. ES antigen revealed polypeptides in the range of 10 to 200 kDa of which 26, 32, 60 and 120 kDa were found more prominent. F-1 fraction on SDS-PAGE analysis revealed only four polypeptides of 26, 32, 60, and 120 of which 60 and 120 kDa were found to be most prominent. Results indicate that the purified fraction of ES antigen may be utilized for early diagnosis of haemonchosis. Further studies on cross antigenicity of this fraction with other nematode and trematode needs to be conducted.

**Keywords:** Dot ELISA, Excretory-secretory antigen, *Haemonchus contortus*, Immunoaffinity chromatography, SDS-PAGE

Haemonchosis is a predominant infection in small ruminants caused by *H. contortus* a blood sucking abomasal nematode causing severe anaemia which may be fatal particularly to young animals. Detection of infection during prepatent period has not been attempted although both 4th larval stage and the immature worm are blood sucking. Till the infection becomes patent and the eggs appear in the faeces, young animals suffer from anaemia resulting in sudden death. Gastro-intestinal nematodes occur as mixed infection in tropical countries but *H. contortus* is predominant and most pathogenic. Therefore, detection of prepatent infection is a prerequisite for effective control of infection in light of the problem of drug resistance in *H. contortus*. Early diagnosis of the infection is essential for timely treatment with suitable anthelmintics. Diagnosis of *H. contortus* infection during prepatency utilizing Excretory Secretory Antigen (ES) in ELISA has already been reported. In the present study, crude ES antigen and immunoaffinity purified ES antigen have been used in dot-ELISA a simple, easy to perform and less time consuming test for detecting early haemonchosis during prepatent period since Excretory-Secretory (ES) antigen is considered to be a potent antigen for diagnosis and immunoprophylaxis.

**Materials and Methods**

**Collection of parasites**—Adult *H. contortus* were collected from abomasa of sheep from local abattoir at Bareilly, Uttar Pradesh, India and carried to the laboratory in cold phosphate-buffered saline (PBS, pH 7.2). Identification of the worms was done as described.

**Preparation of excretory secretory (ES) antigen**—The worms were washed three times in PBS (pH 7.2, 0.85M) containing 1000 IU/ml penicillin and 1.0 mg/ml streptomycin. The adult worms were incubated at 37°C (500 worms were kept in 1 ml of the prepared medium) in an atmosphere of CO₂ in RPMI 1640 supplemented with 2% glucose, HEPES (25 mM) 4-(2-Hydroxyethyl)-1-piprazine-ethanesulfonic acid, L-glutamine (2 mM) and penicillin100 IU/ml/streptomycin (100 µg/ml). The medium was changed...
after 4 hr After overnight incubation worms were removed from the medium and the media were pooled and centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was collected and filtered through 0.2 µm filter membrane. PMSF (Phenyl methyl sulfonyl fluoride) was added to the filtrate and stored at -70°C.

**Protein estimation**—The protein in the ES antigen was estimated as Lowry et al.\textsuperscript{4}

**Collection of sera**

**Positive control sera**—Two rabbits (White New Zealand) were hyperimmunised with the ES antigen of *H. contortus* and immunoaffinity purified antigen of *H. contortus*. Initially 500 µg protein with FCA (Freund’s complete adjuvant) was injected through intramuscular route into the rabbit. After 15 days, 1\textsuperscript{st} booster dose was given with 750 µg of protein. Similarly 2\textsuperscript{nd}, 3\textsuperscript{rd} and 4\textsuperscript{th} booster dose was given with increasing dose of protein at the rate of 250 µg /booster. Finally, test bleed was performed 5 days after the last booster dose and then blood was collected from heart. Sera were separated and tested in DID (Double Immuno Diffusion).

**Negative control sera**—Negative control serum used during the study were 0 day lamb serum and uninfected control sera of sheep.

**Experimental sera**—Experimental sera against *H. contortus* were raised in 6 sheep about 1 year age and similar body weight infected *per os* with 10,000 L3 of *H. contortus*. 2 sheep of similar age and weight were kept as control. Sera were collected at weekly intervals and stored at -20°C. Animals were maintained in the Animal Shed of Division of Parasitology, Indian Veterinary Research Institute, Izatnagar, as per the approved guidelines of CPCSEA and were not allowed to graze outside the premises of shed.

**Natural sera**—Natural sera of sheep were collected from local abattoir. The serum samples (20) and abomasum from the same animal were collected. The abomasus were examined for the presence of *H. contortus*. All the animals examined were found to harbour *H. contortus* infection.

**Imunoaffinity chromatography with ES antigen of adult *H. contortus***—Imunoaffinity purification of the ES antigen of *H. contortus* was performed utilizing amino-link column containing amino-link as matrix which was also utilized for purification of several proteins and found better than cyanogen bromide activated sepharose 4B by several workers\textsuperscript{5}. The protocol for affinity purification of protein was modified as per the requirements of immunoaffinity chromatography. Anti-*H. contortus* antibodies were collected from sera of sheep infected with L3 of *H. contortus*. The infection was confirmed on autopsy and by obtaining larvae from the coproculture of faeces of infected animals. The antibody was dissolved in coupling buffer (5 mg antibody/ml gel). The Amino-link column was equilibrated to room temperature and washed with 5 ml of coupling buffer loaded with antibody and incubated at 4°C with gentle shaking. The supernatant was collected. The O.D. value of supernatant was read at 280 nm. The column was washed with PBS (pH 7.2) and the ES antigen was loaded to the column at the rate of 5 mg protein/ml gel. The column was allowed to embed the antigen into the gel bed completely keeping at 4°C with gentle shaking. After incubation the antigen was eluted. 30 ml of elution buffer (Glycine buffer 100 mM, pH 2.5-3.0) was added to the column and allowed to drip through slowly under gravity. Aliquots (20) of 1.5 ml each of unbound and bound fractions were collected. The collected fractions were neutralized by adding 100 µl of 1M Tris, (pH 7.5) and O.D. was taken at 280 nm. Each fraction was subjected to SDS-PAGE analysis and the bound fractions of interest were pooled and designated as F-1.

**Dot-ELISA**—Dot-ELISA was performed with crude ES antigen and immunoaffinity purified ES antigen which were reacted to experimental and natural sera of sheep infected with *H. contortus*. Positive sera (hyperimmune sera of rabbit) and negative sera (0 day lamb sera) and uninfected control sera were also utilized. 2-6 µl of antigens containing 2-4 µg protein was blotted on 0.2 µm nitrocellulose membrane strips and incubated at 37°C for 1 hr. Non specific sites were blocked with blocking solution. After washing with PBST the strips were incubated with the primary antibody (1:100) for 2 hr and washed with PBST. The strips were now incubated with donkey anti-sheep HRPO conjugate and anti rabbit HRPO conjugate. After washing, DAB substrate was added for development of colour.

**SDS-PAGE of ES and immunoaffinity purified ES antigen of *H. contortus***—Excretory secretory antigen was fractionated by SDS-PAGE as described\textsuperscript{6}. Mini vertical slab gel electrophoresis system utilizing tris-glycine gel under non-reducing condition was employed at 100 V. After complete run the gel was stained with coomassie brilliant blue G-250 and destained. Molecular weight of the fractionated polypeptides was determined by using appropriate molecular weight marker.
Results

Positive control sera—Sera of the hyperimmunised rabbits were found positive in DID against ES antigen and F-1 fraction.

Protein estimation—Protein in the ES antigen was 1.2mg/ml and in the pooled immunoaffinity purified fraction 0.15 mg/ml.

Dot-ELISA with crude ES antigen and experimental sera of sheep—Dot-ELISA with ES antigen and experimental sera of sheep (4 day, 1, 2 and 3 weeks P.I.) was performed. Solid dot formation was observed with 1, 2 and 3 weeks experimental sera and with the positive sera. Dot formation did not take place with 4 day experimental sera, negative and uninfected control animal sera. The results indicated recognition of antibodies against *H. contortus* as early as 1 week P.I (Fig. 1).

Dot-ELISA with crude ES antigen and naturally infected sera of sheep—Dot-ELISA with the ES antigen and naturally infected sera of sheep (confirmed for *H. contortus* infection) was performed. Solid dot formation with uniform boundary was considered positive where as those dots having diffused material without uniform boundary were considered negative. Solid dot formation was observed with 12 sera samples (1, 2, 4, 5, 8-10, 11-13, 17 and 19) out of 20 and with the positive control sera. Dot formation did not take place with the negative control serum and uninfected control animal serum. The results indicated presence of antibodies against *H. contortus* in the naturally infected sheep sera (Fig. 2).

Dot-ELISA with immunoaffinity purified ES antigen (F-1) and experimental sera of sheep—Dot-ELISA with immunoaffinity purified ES antigen (F-1) and experimental sera of sheep (from 4 day P.I to 3rd week P.I.) was performed. Solid dot formation was observed with 4 day, 1, 2 and 3 week P.I. experimental sera as well as positive control sera where as no dot formation with the negative sera and control animal sera. The results indicated recognition of antibodies against *H. contortus* as early as 4 day P.I. (Fig. 3).

![Fig. 1—Dot-ELISA with 4 day, 1, 2 and 3 week PI sera and ES antigen of *H. contortus*.](image1)

![Fig. 2—Dot-ELISA with natural sera of sheep infected with *H. contortus* and ES antigen of *H. contortus*.](image2)

![Fig. 3—Dot-ELISA with 4 day, 1, 2 and 3 week PI sera and immunoaffinity purified ES antigen of *H. contortus*.](image3)
Dot-ELISA with immunoaffinity purified ES antigen (F-1) and naturally infected sera of sheep—Dot-ELISA with the immunoaffinity purified ES antigen and naturally infected sera of sheep having confirmed infection for *H. contortus* revealed solid dot formation with 15 sera samples (1-8, 12-14, 16-19) out of 20, as well as with the positive sera. Dot formation did not take place with the negative control and uninfected control animal sera. The results indicated recognition of antibodies against *H. contortus* in the naturally infected sheep sera (Fig. 4).

**SDS-PAGE**

**SDS-PAGE of ES antigen**—ES antigen of *H. contortus* revealed polypeptides ranging between 10-200kDa. Prominent polypeptides were 15, 26, 60 and 120 kDa. In addition some faint bands between 30-40 kDa and two bands above 120 kDa were also observed (Fig. 5).

**SDS-PAGE of immunoaffinity purified ES antigen (F-1)**—SDS-PAGE analysis of immunoaffinity purified ES antigen (F-1) revealed 4 polypeptides of 26, 32, 60 and 120 kDa in the fraction. Out of these polypeptides 60 and 120 kDa were found more prominent as compared to 26 and 32 kDa polypeptides (Fig. 6).

**Discussion**

Detection of *H. contortus* infection in sheep during prepatent period of 15 days and early patent period of 27-28 days is essential for effective control of haemonchosis on farms and field since the infection is most pathogenic among gastro-intestinal nematodes. During the present study dot-ELISA was utilized to detect anti *H. contortus* antibodies in the sera of both experimentally and naturally infected sheep utilizing crude and immunoaffinity purified fraction of ES antigen. Results indicated that the antibodies were present in the infected sera as early as 1st week P.I. when tested with ES antigen in dot-ELISA where as earlier detection of the infection at 4 day P.I was possible with immunoaffinity purified fraction. These
antigens could also detect antibodies in the naturally infected sera. With crude ES antigen, antibodies could be detected in 60% of the naturally infected sera whereas immunoaffinity purified fraction could detect antibodies in 75% of the sera.

Dot-ELISA has been utilized for the early detection of some helminthic and protozoan infections. Dot-ELISA with ES antigen in experimental *F. hepatica* infection detect antibodies as early as 4 week P.I. Dot-ELISA was utilized for diagnosis of leishmaniasis. Importance of dot-ELISA has been reviewed for rapid diagnosis of parasitic infection. Importance of membrane disc in ELISA for diagnosis of bancroftian filariasis has already been reported. Cellulose acetate membrane required only 60 picogram ES antigen protein to detect antibody and found better than plate ELISA. The test has also been utilized for serodiagnosis of *F. gigantica* infection in buffalo. *F. gigantica* infection in buffalo was detected by dot-ELISA utilizing crude somatic antigen fractionated by gel exclusion chromatography. In *H. contortus* infection very few attempts have been made. Some workers have attempted dot-ELISA for serodiagnosis of *H. contortus*. During the present study dot-ELISA could detect the antibodies against *H. contortus* as early as 1st week P.I with crude ES antigen. However, with the immunoaffinity purified fraction the antibodies could be detected as early as 4 day P.I. Early detection of antibodies in haemonchosis has already been reported in ELISA. Production of antibodies against larval ES antigen in rabbit and sheep has also been reported. Anti-*H. contortus* antibodies raised in rabbit react with 96 hour ES product in culture medium maintaining L3 larvae developing to L4 larvae. During the present study recognition of ES proteins by 4 day P.I. sera of sheep is supported by the fact that when L3 molts to L4, ES proteins are secreted in the blood circulation and become immunogenic stimulating production of antibodies. It takes about 48-72 hr for development of L4 from L3, therefore, antibodies are generated from 4 day onward. The study therefore, strongly supports that Dot-ELISA may be a good field oriented test to detect early haemonchosis. The results also indicate that the adult ES product shares some polypeptides with the larval ES product since it has already been described that in culture medium L3 larvae developing to L4, larvae ES product are secreted. The ES product of larvae stimulates production of antibodies as described.

In order to have an idea about the polypeptide profile of the crude ES antigen and immunoaffinity purified ES antigen, SDS-PAGE was performed. In the crude ES antigen the polypeptides present ranged between 10-200 kDa. More prominent polypeptides were of 26, 32, 60 and 120 kDa. In addition some polypeptide bands between 30-40 kDa and two bands above 120 kDa were also observed. Polypeptides observed in the immunoaffinity purified antigen were 26, 32, 60 and 120 kDa. Polypeptides of 24 and 30 kDa have already been observed in SDS-PAGE. 60 kDa and 110 kDa polypeptide have been reported in ES product. 60 kDa polypeptide has been determined to be glutamate dehydrogenase and 110 kDa to be amino peptidase. During the present study polypeptides of 26 and 32 kDa have been reported in ES antigen purified through immuno-affinity chromatography. Protein of low molecular weight has been described to be of diagnostic value. Similarly 32 kDa polypeptide may be cysteine protease which has been found in ES product. Enzymes present in the ES product have already been established to induce antibody production in natural host. Thus these polypeptides fractionated by immunoaffinity purification of the ES antigen have diagnostic polypeptide reactive in dot-ELISA. The enzymatic status of these polypeptides in Indian strains of *H. contortus* needs to be investigated further for their diagnostic or immunoprophylactic potential.

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**References**


