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Use of Nickel Chloride in Dot-ELISA for Detection of Haemonchus sp. Infection in Sheep

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Abstract
Haemonchosis is a major cause of production losses in livestock, particularly sheep and goat. In this study, Nickel chloride is used in dot-ELISA for the detection of infection in sheep, experimentally-infected with 10,000 L 3 *Haemonchus contortus.* Serum samples from infected sheep were collected at weekly intervals. Adult somatic antigen was prepared from the worms collected from the local abattoir at Meerut (U.P.). Nitrocellulose membranes (0.2 µm) were dotted with 10 µl antigen that contained 8-10 µg protein, and were incubated at 37°C for 2 h. Non-specific binding sites were blocked with blocking agent casein in PBS(phosphate-buffered saline). Positive, negative and uninfected control sera from sheep, both experimentally- and naturally-infected, were used. Positive reaction with blue-black solid dot formation was obtained with the sera, as early as first week post-infection, with immunoaffinity- purified adult somatic antigen.
Keywords: dot-ELISA, *Haemonchus contortus*, immunoaffinity chromatography, Nickel chloride.

1. INTRODUCTION

Gastrointestinal parasitism represents a severe health problem in small ruminant production system and its consequences can be extensive ranging from reduced performance to mortality [1]. In India, parasitic gastro-enteritis due to nematode infection has been reported as a major constraint to profitable sheep production. *Haemonchus contortus*, one of the most pathogenic, blood suckling abomasal nematode which primarily infects sheep and is responsible for economic loss to sheep industry. Due to its blood feeding habit the nematode causes severe anaemia and sudden death [2] within a week [3]. During prepatency the clinical symptoms are not noticed. The present study dot- ELISA was utilized to detect the infection during prepatent period by the use of Nickel chloride ($NiCl_2$) by which we reduce the cost of diagnosis of *Haemonchus contortus* infection.

2. MATERIALS & METHODS

Experimental sera of sheep & Positive control sera was collected from another experiment going on , GIP, Division of Parasitology, IVRI, Izatnagar.

2.1 Coproculture of *H. contortus* larvae

Coproculture was performed in the laboratory as per the method described by Sahai, 1960 [4]. The source of faeces for culture were donor animals maintained in the sheds of Division of Parasitology, Indian Veterinary Research Institute which were not allowed to graze. The larvae (L_3) were stored in distilled water at 4^{O}C.

2.2 Collection of sera

Positive control sera: *Hyperimmunisation of rabbits,* Two rabbits were used for hyperimmunisation, with *H. contortus,* somatic antigen. Initially, 500 µg protein of antigen with FCA was injected by I/M route to the rabbits. After 15 days, first booster dose was given with 750 µg of antigen with IFA to immunized rabbits. Similarly, second, third and fourth booster was given with increasing doses of antigens at the rate of 250 µg/booster. Finally, test bleed was performed 5 day after the last booster dose and then blood was collected from heart. Sera was separated and tested for antibody titer by Double Immuno Diffusion (DID).
Negative control sera: Zero day lamb serum and uninfected control animal sera were used as a negative control.

Experimental sera of sheep: Eight sheep of same age group (one year) and weight were selected for the production of experimental sera. Six sheep free from helminthic infection were infected with 10,000 L_3 of *H. contortus* and two sheep were kept as control. Sera at weekly interval from experimental as well as control animals were collected. Sera samples were collected from 1^{st} week to 3rd week PI. The sera were kept at -20^{O}C and utilized for dot-ELISA.

Natural sera of sheep infected with *H. contortus*: Natural sera of sheep were collected from local abattoir at Bareilly (U.P., India). The sera samples as well as the abomasums of the same animal were collected. The abomasums were examined in the laboratory to check worm burden.

Uninfected natural sera: Natural sera not infected with *Haemonchus contortus* were collected from

sheep from abattoir at Bareilly.

2.3 Preparation of adult somatic antigen
Adult *H. contortus* were collected in PBS (pH 7.2) from the abomasum of sheep and washed with PBS three times. The worms were first homogenized in PBS (pH 7.2) and then sonicated and further centrifuged at 12,000 rpm at 4^{O}C. The supernatant was collected and filtered through 0.2 µm filter membrane. Filtrate was stored at -70^{O}C with PMSF for further use.

2.4 Protein estimation
The protein concentration of adult somatic antigen and immunoaffinity purified somatic antigen was estimated as per the method of Lowry *et al.* [5].

2.5 Immunoaffinity chromatography of somatic antigen of adult *H. contortus*
Immunoaffinity purification of the somatic antigen of *H. contortus* was performed utilizing prepacked column containing amino link as matrix (Pierce). The protocol for affinity purification of protein was modified as per the requirements of immuno affinity chromatography.

Anti *H. contortus* antibodies collected from sera of sheep infected with L_3 of *H. contortus*. The infection was confirmed on autopsy as well as obtaining larvae from the coproculture of faeces of infected animal. The antibody was dissolved in coupling buffer (5 mg antibody/ml gel). The column was equilibrated to room temperature and washed with 5 ml of coupling buffer loaded with antibody and incubated at 4^{O}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 somatic 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 bed completely keeping at 4^{O}C with gentle-shaking. After incubation the antigen was eluted. Further, 20 ml elution buffer (Glycine buffer 100 mM, pH 2.5-3.0) was added to the column and allowed to drip through slowly under gravity. Twenty five aliquots of 1.5 ml each of unbound and bound fractions were collected. The collected fractions were neutralized by adding 100 ml 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.

2.6 Dot-ELISA
Dot-ELISA was performed with adult somatic antigen and immunoaffinity purified adult somatic antigen with experimental as well as natural sera of sheep infected with *H. contortus.* Positive sera (hyper immune sera raised in rabbit) and negative sera (0 day lamb sera) and control animal tested through DID were also utilized. 2-6 µl of antigens containing 2-4 µg *H. contortus* protein was blotted on 0.2 µm nitrocellulose membrane strips and incubated at 37^{O}C for 1 hour. Non specific sites were blocked with blocking solution. After washing with PBST, these strips were incubated with the primary antibody (1:100) for 2 hour and washed with PBST. The strips were then incubated with donkey anti sheep HRPO conjugate. After washing, finally put the solution which is prepared from 15 ml dilution buffer, pinch of Nickel Chloride, DAB solute, 10 µl Hydrogen Peroxide (H_2O_2) was added and blue-black dot formation was observed (Figure 1) .Stop the reaction by adding distilled water .

3. RESULTS

3.1 Coproculture of *H. contortus* larvae
L_3 larvae of *H. contortus* were obtained from coproculture and were identified by the presence of kinky tail and tubercles on the body surface as described by Levine [6]. All the larvae belonged to *H. contortus.*

3.2 Sera
Positive control: Hyper immune sera raised in rabbit were used as positive control in dot-ELISA previously tested in DID showing precipitin line with adult somatic antigen.

Experimental sera: Experimental sera were collected after infection with *H. contortus* larvae from 1st week to 3rd week PI. Carpological examination when conducted up to three week PI the faeces were found negative indicating prepatency.

3.3 Protein estimation
Protein concentrations as estimated by Lowry method in the adult somatic antigen was 6.3 mg/ml where as in the pooled immunoaffinity purified fraction of adult somatic antigen the protein concentration was 0.2 mg/ml.

3.4 Dot-ELISA

Dot-ELISA with adult somatic antigen and experimental sera of sheep infected with *H. contortus:*

Dot ELISA performed with adult somatic antigen and 1st, 2nd and 3rd week experimental sera as well as positive and negative sera as well as control animal sera. No dot formation was observed with 1st and 2nd week sera as well as negative control and control animal sera where as a solid dot formed with positive control sera. When 3rd week sera was reacted with the adult somatic antigen a solid dot was formed indicating recognition of antibodies at this stage

Dot ELISA with adult somatic antigen and naturally infected sera infected with *H. contortus:*

Dot ELISA performed with adult somatic antigen and naturally infected sera with *H. contortus* revealed solid dot formation with 5 sera samples out of ten sera samples at 1:100 sera dilution whereas solid dot formed with positive control and no dot with control animal sera.

4. DISCUSSION

Dot ELISA for the detection of *H. contortus* infection in sheep during prepatency preclinical stage has not been tried much although some workers [7] detected immune response to *H. contortus* during prepatent as well as patent period utilizing ELISA. Since detection of infection during prepatency is of more importance from control point of view the simplified field oriented test Dot ELISA was employed during the present study. Immunodiagnostic test based upon serodiagnosis has not been tried in haemonchosis on the ground that prepatent period of *H. contortus* is 18-21 days but Sahai [4] reported that the infection becomes patent by 27-28 days in sheep and when the L_4 and immature worms are present no eggs are present in faeces. L_4 causes sufficient damage and before the infection becomes patent the animal may die suddenly. Other G.I. nematode infections except oesophagostomosis have less intensity of infection and the degree of pathogenicity is of lower degree in comparison to *H. contortus [2]*.

During the study, care was taken that the experimental sheep do not graze outside the shed premises and before the experiment started all animals were treated with albendazole and coprological examination was conducted from 1st week to 4th week. The animals were treated as having monospecific infection with *H. contortus*. Dot ELISA was performed with monospecific sera since the animals were infected with L_3 stage of *H. contortus* raised through coproculture in the laboratory.

The test was performed with adult somatic antigen and immunoaffinity purified somatic antigen to detect antibodies in experimental sera during prepatency (1st week to 3rd week PI) as well as naturally infected sera from sheep having confirmed infection of *H. contortus*. In dot-ELISA with adult somatic antigen, antibodies could be detected only in 3rd week sera and positive control by formation of solid dot but not with 1st and 2nd week sera, negative and uninfected control. However, a solid dot was formed with the 3rd week sera and adult somatic antigen indicating presence of sufficient antibodies against *H. contortus* in the infected animal forming a solid dot. When dot-ELISA was performed with the same antigen and naturally infected sera solid dot was formed with 50% of the sera indicating presence of infection.

Dot-ELISA performed with immunoaffinity purified somatic antigen gave different results in terms of earlier detection of infection. Solid dot formation took place with 1st, 2nd and 3rd week sera as well as positive control and no dot in case of negative controls. It indicated that immunoaffinity purified antigen contained antigenic polypeptides reactive to anti *H. contortus* antibodies in sheep as early as first week PI. Similarly, the immunoaffinity purified antigen, when reacted to naturally infected sera, solid dot formed with 80% of the sera tested again indicating reactivity of antigenic polypeptides to anti *H. contortus* antibodies.

Kaur *et al. [8]* and Sood [9] utilized it for detection of antibodies in rabbit infected with *H. contortus* antigen. Sood *et al.* [10] reported competitive inhibition dot ELISA for detection of *H. contortus* antigen. However, ELISA has been reported by Schallig *et al.* [7] to detect antibodies in both experimentally as well as naturally infected sheep who could detect antibodies. Moreover, Dot ELISA is simpler to perform than ELISA.

Dot ELISA for diagnosis of parasitic infection has been reported by several workers including protozoan and helminthic parasites. Pappas *et al.* [11] reported dot-ELISA for rapid diagnosis of visceral leishmaniosis for human samples. Zimmerman *et al.* [12] reported dot ELISA for diagnosis of ovine fasciolosis in experimentally infected sheep infected with *Fasciola hepatica* ES antigen and the infection could be detected 4 weeks PI. Saxena *et al.* [13] reported dot ELISA for detection of *Fasciola sp.* infection in buffalo with crude antigen fractionated with gel exclusion chromatography and naturally infected serum.

The results of dot ELISA performed with immuno affinity purified antigen of *H. contortus* somatic antigen was comparable to ELISA and could detect the infection as early as first week PI during prepatency. However, unpurified somatic antigen could detect the infection as early as 3rd week PI which may be considered early patent period.

5. ACKNOWLEDGEMENTS

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Figure 1:

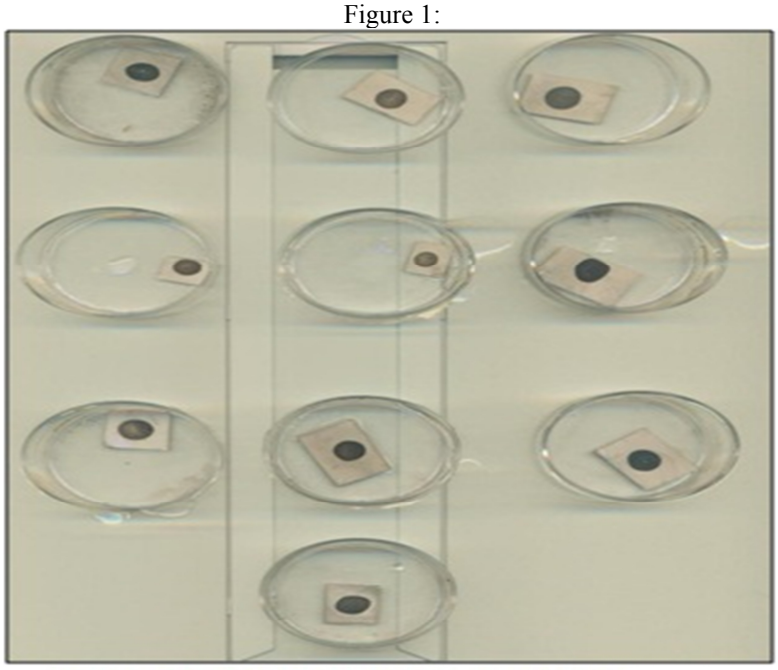

Figure 1- Blue-black Dot shows positive control

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