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APPLICATION OF INDIRECT PLATE ELISA IN EARLY DIAGNOSIS OF PARAMPHISTOMOSIS USING PURIFIED POLYPEPTIDES OF SOMATIC ANTIGEN OF *PARAMPHISTOMUM EPICLATUM*

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ABSTRACT
An indirect Plate ELISA was standardized using purified polypeptides of somatic antigen of *Paramphistomum epiclitum,* sera of experimentally infected sheep (n=4) at different time intervals post infection and rabbit hyper immune sera. Plate ELISA was standardized using 2 µg/ml of antigen concentration with 1:200 and 1:1000 of sera and conjugate dilution, respectively. Whole somatic antigen of *P. epiclitum* was fractionated by gel filtration using seralose-6B gel filtration media for large molecular weight molecules, equilibrated with 50 mM Tris Hydrochloric acid (Hcl) - 20 mM and sodium chloride (NaCl) buffer. Out of total 19 peaks obtained as per the value of protein concentration in purified fractions of *P. epiclitum* antigen, 8 peaks were found to be broader. It was found that heterogeneous nature of whole somatic antigen of *P. epiclitum* contains large number of polypeptides of different molecular weights. These fractions were characterized for immunogenicity by indirect plate-ELISA using four experimentally *P. epiclitum* infected sheep sera. A pattern of antibody response assessed at weekly interval till the end of experiment (25th week). Antibody response showed peak values of responses at different time intervals (5th, 8th, 9th and 11th week post infection) in the four sheep.

KEY WORDS: Indirect Plate ELISA, Immunodiagnosis, *Paramphistomum epiclitum,* Purified polypeptides, Somatic antigen**.**

INTRODUCTION

Livestock health suffers at large from various gastro-intestinal helminthic infections, and paramphistomosis is such a trematodal infection imposing huge mortality and morbidity which account for significant loss in millions of rupees. India was considered as the most affected country of the world back in 1971 by Horak with *Paramphistomum epiclitum* and *Gastrothylax crumenifer* being the predominant species, with a prevalence rate 50-70% (Dutt 1980; Hafeez and Avastthi 1987; Matto and Bali 1991; Prasad and Varma 1999; Hassan *et al* 2005; Kaur *et al* 2008). Numerous outbreaks of acute paramphistomosis associated with high mortality among young sheep, goat, cattle and buffaloes have been recorded from time to time (Pande 1935; Bawa 1939; Katiyar and Varshney 1963; Panda and Misra 1980). In Punjab a high prevalence rate of paramphistomosis has been reported in large and small ruminants (Hassan *et al* 2005). Sensitivity to detect paramphistomosis with conventional parasitological analysis is paralyzed due to prolonged prepatency and non-availability of any parasitic stage in faecal materials. There is a need for diagnosis of the disease in its early stage when maximum pathogenicity is being caused by its immature stage. It can be fulfilled by using immunodiagnostic tests, among which, indirect plate ELISA holds good promise. In this study an attempt has been made to diagnose the disease serologically in its early stage by using purified polypeptides of somatic antigen of *Paramphistomum epiclitum* with indirect plate enzyme linked immunosorbent assay (ELISA) in experimentally infected sheep.

MATERIALS AND METHODS

Collection of adult flukes: *P. epiclitum* flukes were collected from rumen of sheep and goat from slaughter houses at Ludhiana, Bareilly and Delhi in live condition. After removing debris and mucous by thoroughly washing with several changes of normal saline and phosphate buffer saline (pH-7.4) these flukes were identified as per Soulsby, 1982 and stored at -20°C till further use.

Antigen preparation: Somatic antigen of *P. epiclitum* was prepared by homogenizing whole flukes in 0.1M Phosphate buffer saline (PBS, pH 7.4) followed by sonication (Soniprep-150) at amplitude of 10 micron for 8 min in terms of 4 cycles. Each cycle was of 2 min with a rest for 1 min. The sonicated material was then centrifuged at 10000 rpm for 15 min at 4°C. The supernatant was filtered through 0.22µm Millex–GV filters (Millipore, France). The protein estimation was done as mentioned by Lowry *et al* (1951).This antigen was stored at –20°C after adding PMSF (Phenyl methane sulfonyl fluoride) @ 10µg/ml as an anti-proteolytic agent.

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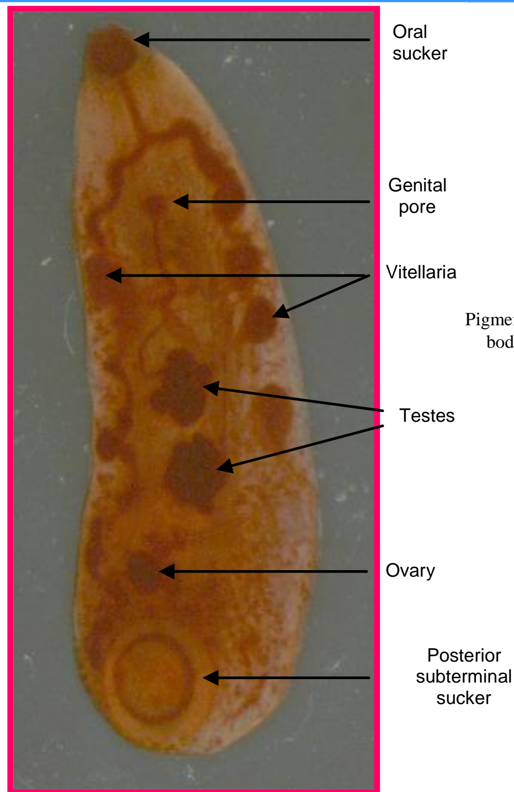

Fig 1a. ***Paramphistomum epiclitum***

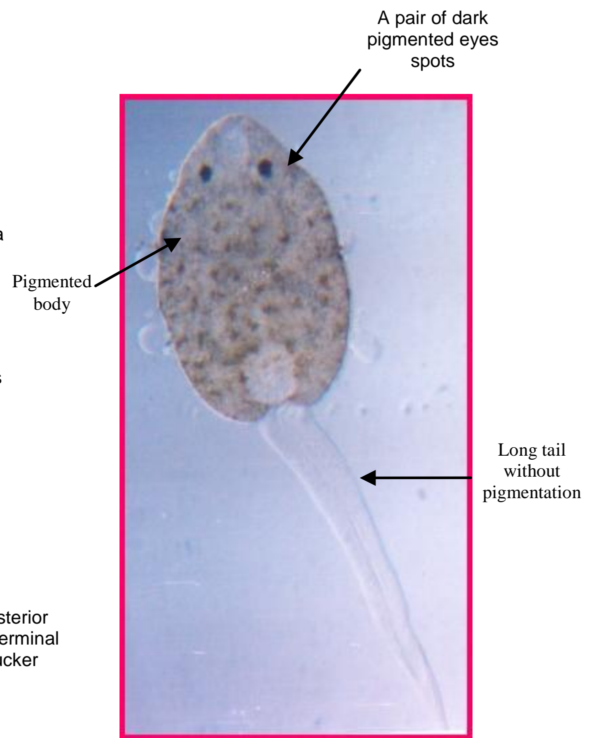

Fig 1b. ***Cercariae pigmentata***

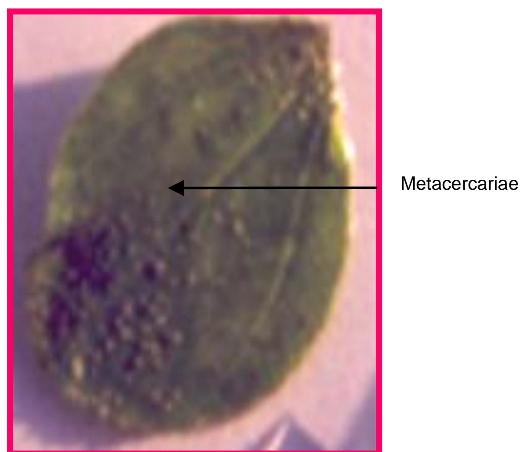

Fig1c. Metacercariae on leaf

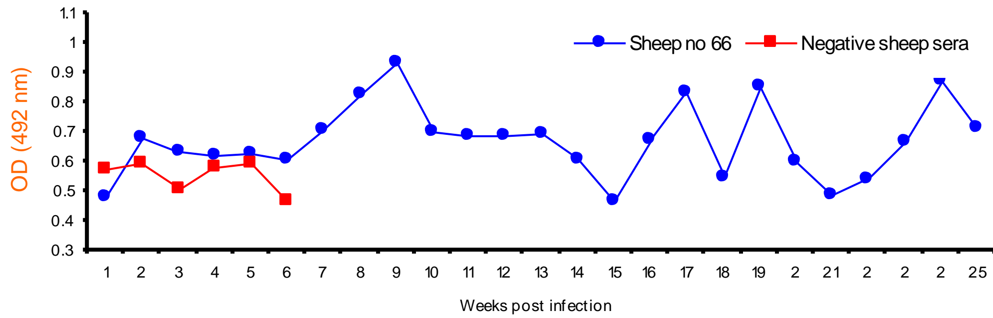

Fig. 2(a)

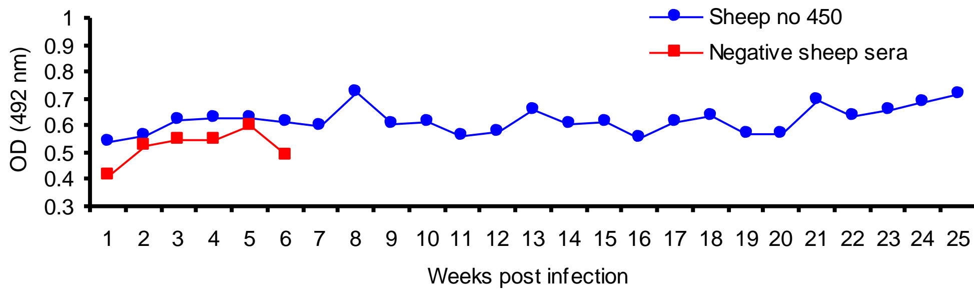

Fig. 2(b)

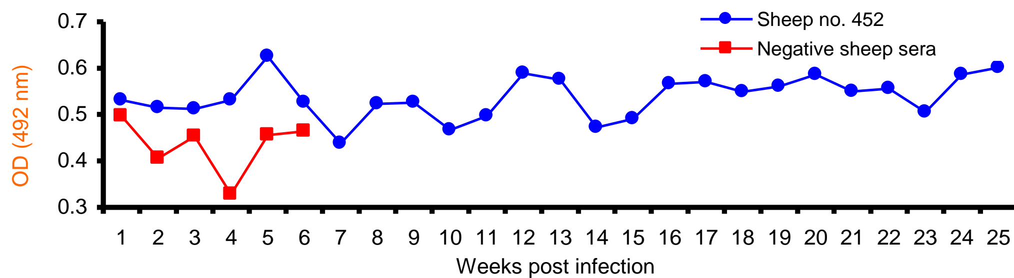

Fig. 2(c)

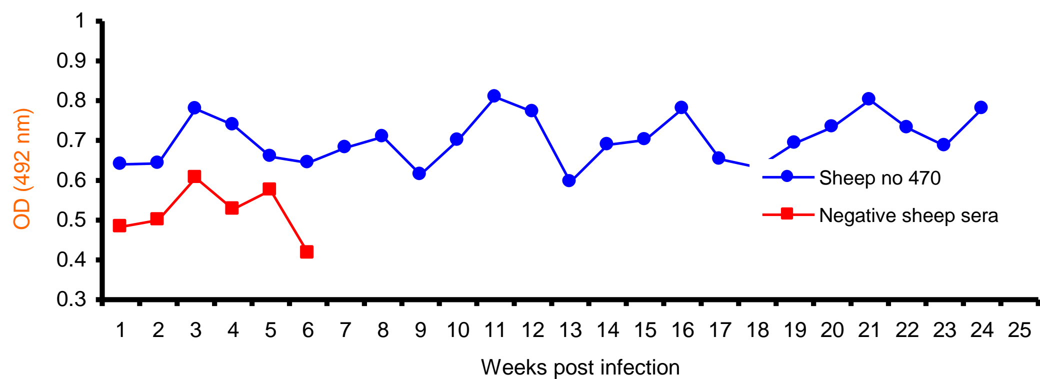

Fig. 2(d)

Figure 2: Antibody response of four experimental sheep against purified fractions of somatic *P. epiclitum* antigen using indirect plate ELISA

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Table 1. Antibody response of four experimental sheep against purified fractions of somatic *Paramphistomum epiclitum* antigen using indirect plate ELISA

S. No.	Sheep No.			
	66	450	452	470
1	0.482	0.543	0.515	0.640
2	0.678	0.564	0.531	0.642
3	0.634	0.622	0.512	0.779
4	0.619	0.629	0.531	0.739
5	0.628	0.634	0.625	0.659
6	0.604	0.614	0.526	0.644
7	0.709	0.604	0.438	0.682
8	0.826	0.729	0.523	0.709
9	0.935	0.613	0.526	0.614
10	0.702	0.617	0.467	0.700
11	0.685	0.563	0.497	0.809
12	0.685	0.579	0.589	0.772
13	0.693	0.660	0.575	0.596
14	0.608	0.606	0.472	0.689
15	0.467	0.619	0.491	0.701
16	0.670	0.560	0.566	0.780
17	0.831	0.620	0.570	0.653
18	0.548	0.636	0.549	0.633
19	0.850	0.574	0.561	0.693
20	0.601	0.576	0.586	0.733
21	0.488	0.700	0.550	0.802
22	0.538	0.637	0.556	0.732
23	0.665	0.658	0.505	0.687
24	0.873	0.692	0.586	0.780
25	0.713	0.720	0.601	0.806

Purification of polypeptides in the somatic antigen of *P. epiclitum* by seralose-6B gel-filtration chromatographic technique: Whole fluke somatic antigen of *P. epiclitum* was fractionated by gel filtration using seralose-6B gel filtration media (40-190 microns; SISCO Research Laboratories, India) for large molecular weight molecules, equilibrated with 50 mM Tris Hydrochloric acid (HCl) -20 mM Sodium Chloride (NaCl) buffer, pH - 8.0.**.** Adult whole fluke somatic antigen of 1.5 ml volume having protein concentration 17.7 mg/ml was filtered through 0.22 µm Millex - GV filter (Millipore, France) for removing unwanted materials. The filtered products were applied on column bed, and eluted with 50 mM Tris Hcl - 20 mM NaCl buffer, pH - 8.0 (Flow rate, 0.6 ml/min). First four fractions were collected of 5.0 ml each. Afterwards, 75 fractions of 1.0 ml each were collected in eppendorf tube and kept at -20°C. The protein concentration of each fractions of purified *P. epiclitum* antigen was done with Lowry`s method. The antigens were tested in duplicate and optical density was read at 520 nm and taken in the form of mean of the OD values. The protein content of purified fractions of *P. epiclitum* antigen obtained from seralose-6B gel filtration chromatographic techniques was estimated. A total of 19 peaks were obtained as per the value of protein concentration in purified fractions of *P. epiclitum* antigen. Out of total, 8 peaks were found to be broader. Gel-filtration findings revealed the heterogeneous nature of whole fluke somatic antigen of *P. epiclitum* containing large number of polypeptides of different molecular weights. The fractions were characterized for immunogenicity by indirect plate-ELISA using four experimentally *P. epiclitum* infected sheep sera.

Experimental sheep: Five healthy Corridale sheep were procured from Mattewara Sheep Breeding Farm, Ludhiana for raising experimental infection. The animals were divided into two groups. Under Group 1 four sheep were kept for giving infection. Group 2 included one sheep as control. Deworming of all sheep was done, first with Fenbendazole (Panacur) @7.5mg/Kg body weight PO and then after 21 days with Ivermectin (Hitek) @ 25ml/100Kg body weight orally to clear them from any parasitic infection. To make sure that these sheep were free of any endoparasitic infection faecal samples of these animals were periodically checked microscopically for endoparasitic infection with

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sedimentation and floatation techniques. The animals of Group 1 were subjected to experimental infection by feeding metacercariae of *P. epiclitum* orally. Sera from experimental sheep were collected from day zero and then at an interval of 7 days post infection. Sera were stored at –20°C after adding Thiomersal (conc.10mg/ml) @ 5µl/ml till further use.

Raising Antiserum: After 15 days of acclimatization, two Soviet Chinchilla rabbits (3-4 months old) were immunized using adult somatic antigen of *P. epiclitum*. Primary immunization was done with 500 µg of somatic antigen intramuscularly along with Freund's complete adjuvant. Three booster doses (250 µg) were given in Freund's Incomplete Adjuvant intramuscularly at an interval of one week each. Preimmunized sera collected before immunization was used as control sera. Sera were also collected before each booster immunization and were stored at –20°C after adding thiomersal (10mg/ml) @ 5µl/ml in 1.5 ml aliquots.

Plate enzyme linked immunosorbent assay: The ELISA was performed following the method of (Hudson and Hay 1989). The ELISA was performed on 96 wells microtitre plates (Greiner). The optimum antigen, antibody and conjugate concentrations with all incubation times were previously determined by checkerboard titration. The wells of polystyrene microtitre plates were coated with 100µl of 2µg/ml concentration of antigen (diluted in 0.1M carbonate-bicarbonate buffer, pH-9.6) and kept at 4°C overnight. Next day, the plate was washed thrice with 0.1% PBS Tween-20 for 15 m each washing for 5 m. The plate was blocked with 3% lactogen (300µl/well) and incubated at 37°C for 2 hrs. The plate was given three washings with 0.1% PBS Tween -20 for 15 m each washing of 5 m. 50µl of serially diluted experimental sera was added in triplicate wells and plates was kept at 37°C for 1 hr. The plate was given three washings with 0.1% PBS Tween -20 for 15 m each washing of 5 m. 50µl of 1:1000 rabbit raised anti goat HRPO conjugate was added in wells and plate was incubated at 37°C for 1 h. The plate was given three washings with 0.1% PBS Tween-20 for 15 m each of 5 m. 50µl of substrate ortho-phenylenediamidine (OPD) (Sigma, USA) containing H_2O_2 was added in wells and plate was incubated in dark place for 10-15 m. The reaction was stopped by addition of 2M H_2SO_4. The OD of microtitre plate was read at 492nm by ELISA reader (Multiskan). Sera of naturally paramphistomosis infected sheep and anti *P. epiclitum* rabbit hyperimmmune sera were used as positive control and serum of control sheep was kept as negative control.

RESULTS AND DISCUSSION

Identification of parasites: The mature flukes for preparation of antigens were identified as per the morphological characters given by Soulsby (1982) by pressing them between the slides and further confirmation was done by their permanent staining with borax carmine stain (Fig 1a.) *P. epiclitum* flukes were thick and fleshy with elongated, comma shaped body. Anterior oral and posterior sub terminal sucker were present. Simple intestinal caeca extending upto the level of acetabulum and two lobed testes were present tandem and anterior to ovary. Seminal vesicles were long, strongly coiled and thin walled. Well-developed vitellaria had been seen on the lateral sides of the fluke (Fig 1a).

Cercariae of *P. epiclitum* were dark brown in color having pigmented body with an anterior and posterior sucker and identified as *Cercariae pigmentata.* They had numerous cells in it when viewed microscopically. The tail was long and had no pigmentation. A pair of dark pigmented eyespots was present in the upper half of body of cercariae (Fig 1b) and metacercariae (Fig 1c) were fed to sheep to establish experimental infection.

Protein concentration of antigen: The protein content of somatic antigen of *P. epiclitum* was estimated to be 17.7 mg/ml. The sera raised against this somatic antigen in two rabbits were tested after fourth booster of immunization. Double immunodiffusion showed white precipitin lines against adult somatic antigen of *P. epiclitum* suggesting the development of antibody titre in rabbit sera.

Standardization of indirect plate ELISA for anti *P. epiclitum* antibodies: Indirect plate ELISA was standardized using purified somatic antigen of *P. epiclitum* ranging from 1µg/ml to 10µg/ml, goat anti rabbit HRPO conjugate and rabbit anti goat HRPO conjugate dilutions ranging from 1:1000 to 1:8000 and the range of sera dilution from 1:50 to 1:16,000. The optimum concentration of purified somatic *P. epiclitum* antigen was observed to be 2 µg/ml, conjugate dilution 1:1000 and sera dilution at 1: 200.

Signs after experimental infection: Group 1 experimental sheep (n=4), after experimental infection were observed regularly for disease development. The signs observed post infection in the animals were more severe in early phase of infection. The experimental sheep were dull, lethargic and anorexic and foetid diarrhea was observed. Signs were in agreement with earlier workers (Panda and Misra 1980, Gupta *et al* 1978, Saheb and Hafeez 1995). Juyal *et al* (2002) observed that clinical stage was developed in experimentally infected animals 2-4 weeks post infection with more

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severe disease in lambs than in kids. The disease is characterized by foetid diarrhoea associated with gastroenteritis, rapid emaciation and high mortality as recorded by Dutt (1980) and Chowdhury (1994) in their reviews. The group 2 healthy control sheep (n=1) was found normal without showing any clinical signs throughout the experimental period.

Indirect plate ELISA for experimental sheep using purified fraction of *P. epiclitum* antigen: Highest peak OD value of sheep no. 66 (0.935) in the sera was observed after 9th wpi. After 9th week the antibody level decreased and OD fluctuated between 0.702-0.873 (Fig 2 a). Similarly in sheep no. 450 highest peak OD value (0.729) in the sera was observed after 8th wpi. After 8th week the antibody level decreased and OD fluctuated between 0.613-0.692 (Fig 2 b). Highest peak OD value in sheep no. 452 (0.625) in the sera was observed after 5th wpi. After 5th week the antibody level decreased and OD fluctuated between 0.526-0.586 (Fig 2 c). Highest peak OD value (0.809) in the sera of sheep no. 470 was observed after 11th wpi. After 11th week the antibody level decreased and OD fluctuated between 0.772-0.78 (Fig 2 d). The response in the form of OD values is tabulated in (Table 1).

The study showed possibility of multiplicity of antigenic epitopes which were recognized at different weeks post infection by immune system of individual sheep. Immune response seen as soon as 5th 8th and 9th weeks post infection, justify the objective of early diagnosis of disease as the prepatent period of paramphistomosis in sheep is 71 days (Soulsby 1982). Usefulness of ELISA for detecting *P. cervi* infection in sheep 15 days post infection has been reported by Keller (1983). Similarly positive ELISA titre were seen as early as 8-24 days post infection in sheep by Boch *et al* (1983). They observed rise in titres 24-50 days post infection. Similar results were observed in immunoperoxidase ELISA in experimental sheep infection by Alabay (1981). Variability of results regarding highest antibody titres experimentally infected sheep were observed using whole somatic antigen of *P. epiclitum* (Kaur *et al* 2009).

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