

Detection of *Paramphistomum cervi* Antibodies in Rabbits using ELISA and DID

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ABSTRACT

Sheep (*Ovis aries*) is infected with a variety of gastrointestinal helminths, of which notably *Fasciola hepatica* and *Paramphistomum cervi* are prominent and pathogen causing a lot of morbidity and mortality (Mukherjee and Chauhan, 1965). Immature stages of *P. cervi* are highly pathogenic (Horak 1971) but the routine parasitological diagnosis is difficult, under the circumstances immunological test applied to the present study, is aimed at investigating the relative immunodiagnostic reliability and sensitivity of the Ouchterlony gel diffusion test and ELISA in paramphistomiasis.

Sheep blood was collected at the local abattoir naturally infected with *Paramphistomum cervi*. Using somatic antigen of whole worms derived from *P. cervi* was prepared by homogenisation, sonication and centrifugation at 10,000 rpm for 20 minutes at 4°C. Rabbits were immunized with the antigen mixed with Freund's complete adjuvant (1:1 ratio) for raising hyperimmune sera. Blood was collected at regular intervals by puncturing ear vein of the rabbit. The naturally infected sheep sera were also collected from the slaughter houses. The Ouchterlony test and ELISA were found to be positive as early as 2-4th weeks post-infection. By gel diffusion test, two precipitation bands were observed, and at 6th – 8th weeks of post infection by ELISA. An indirect ELISA standardized for detection of anti-*Paramphistomum* antibodies using antigen concentration of 2µg / ml was used on coating buffer. Indirect ELISA revealed antibody titre as high as 1: 12,800 in rabbit sera where as in sheep sera 1: 6400.

Key words: *P. cervi*, Indirect ELISA, Freund's Complete Adjuvant (FCA), Immunodiagnosis, Sheep and Rabbit.

INTRODUCTION

Paramphistomum is a member of the family paramphistomatidae which constitutes one of the most common and abundant groups of digenetic trematodes of domesticated livestock, especially in tropical and subtropical regions of the world. The disease paramphistomiasis caused massive infection of the small intestines with immature paramphistomes, characterized by sporadic epizootic outbreaks of acute parasitic gastroenteritis with high morbidity and mortality

rates, particularly in young stock (Horak, 1971). Diagnosis of paramphistomiasis during sub-clinical phase is of immense importance for early detection of disease so that the mortalities can be reduced by timely intervention and treatment. However early detection of the disease is difficult applying coprological techniques. There is an urgent need for the development of a reliable diagnostic test and an attempt is the present investigation for detection of anti-*paramphistomum cervi* antibodies by indirect enzyme linked immunosorbent assay (ELISA).

MATERIAL AND METHODS

Preparation of antigen

Adult *P. cervi* were collected from the rumen of infected sheep. The worms were washed with physiological saline and stored at -20°C until use. Somatic antigen of adult fluke was prepared by the following technique of Yadav and Gupta (1966). The paramphistome fluke were blotted over sterile filter paper and were freeze dried. 1 gram of fluke was immersed in 5 ml of PBS (pH 7.2) and then homogenized for 30 minutes, followed by sonication for 1 min at 4°C .

The emulsion was then centrifuged at 10000rpm for 30 minutes at 4°C . The supernatant was collected and stored at -20°C . The protein concentration was determined by the method of Doumas (1981). Finally 0.1% of thiomersol preservative was added to each antigen and stored in refrigerator.

Sera Samples

Blood samples were collected at necropsy from sheep, positive for *P. cervi* infection. The serum was separated and respectively labbled as positive and negative (control) sera.

Imune sera

Hyperimmune sera was raised in white New Zealand rabbits against respective antigen, by following standard protocol described by (Yadav and Gupta, 1995). The injection was given subcutaneously with equal quantity of Friunds Complete Adjuvant at 5 days intervals. Rabbits were bled after 7 days of last injection. The serum was separated and kept at -20°C for further use.

Agar Gel Diffusion test

Immunodiffusion test was carried out as per method described by Ouchterlony 1958. The test was carried out using five ml of 1% agar solution (Defco) prepared in 100 ml of baritone buffer (pH 8.6). The agar solution was poured onto slides @ 5 ml/slide. Then slides were kept at room temperature so that gel solidifies. At the centre of gel plate, wells were made at a distance of 3 mm between the central and peripheral wells. The wells were charged with antigen and antibody, the diffusion allowed in moist chamber at 37°C for 48hr, two

precipitin lines were noted after staining the gel with comassive brilliant blue R – 250 stain.

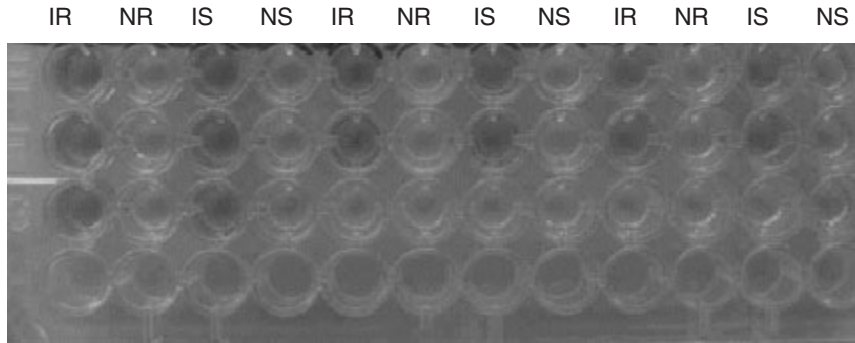
ELISA

Microtitration plate (Nunc) were coated with antigen diluted (2 $\mu\text{g}/\text{ml}$) in carbonate buffer (pH 9.6) and incubated at 37°C for 1hr before keeping overnight at 4°C , coated plates were blocked with 3% skimmed milk in PBS for 2 hrs at 37°C , after washing the plate with phosphate buffer (3 times). Test sera was added (in appropriate dilution 1:100, 1:200, 1:400 etc) and normal sera into each well @ 100 μl / well. Serum was diluted in PBS-1% skimmed milk and then incubated at 37°C for 2 hr. Then plates were again washed and 100 μl of antiovine and antirabbit IgG- peroxidase conjugate (1:5000 dilution, sigma USA) was added and kept at 37°C for 2 hr. After washing five times, in substrate (orthophenylene diamine – OPD Sigma USA.) Plates were kept in dark for 7 minutes (Ghosh and Gupta, 2005). The reaction was stopped by adding 50 μl of 3N HCl per well and the optical density was measured at 492 nm using ELISA reader (labsystem multiscan).

RESULTS

The crude somatic antigens of *P. cervi* injected into the rabbits found to be highly antigenic. The ELISA was carried out by the following method of Ghosh and Gupta, (2005) with slight modification. The checker board titration was performed to find out optimum concentration of antibody.

For the ELISA, ovine sera and rabbit sera were tested using a 2 – fold dilution from 1: 100 to 1: 12800 dilution (Fig 1a), in sheep sera from 1:100 to 6400 dilution (Fig 1b). Before ELISA, antibody titre was detected by Ouchterlony test, during 2 – 3 weeks of post infection, only one band was found and after 3rd – 4th weeks of post infection, two bands were found, the antibody titre obtained in DID was 1: 16, with the increasing serum dilution the antibody absorbance values were found to be decreased sharply and could not be detected by double immunodiffusion test. At 6th week of post infection, antibody was detected by ELISA. The sera were tested using 2 fold dilution from 1:100 dilution, the sera had increased antibody absorbance values at 6th – 8th week of post infection, then due to



IR – Infected Rabbit sera NR – Normal Rabbit sera
 IS – Infected Sheep sera NS – Normal Sheep sera

Plate 1: Microtitration plate

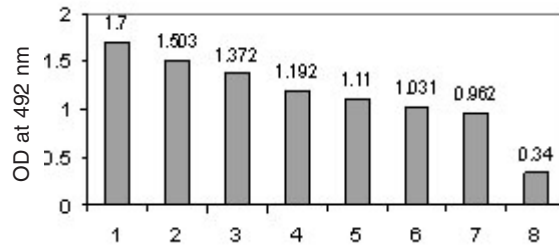


Fig. 1(a): Infected Rabbit serum dilution

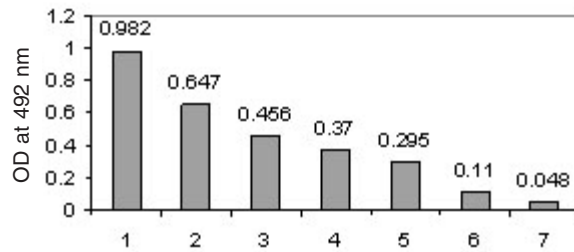
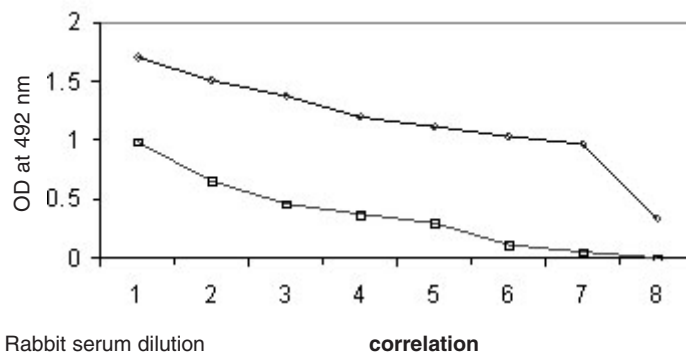


Fig. 1(b): Infected Sheep serum dilution



○ Infected Rabbit serum dilution
 □ Infected Sheep serum dilution

Fig. 2:

increasing dilution, the antibody absorbance values was found to be decreased, ultimately from 1:128, 00 dilution no reaction was positive. At 9th week of infection antibody was not found in the dilution (Plate a). No reaction was observed in the control sera. A strong absorbance values was noted in the sera of rabbit experimentally immunized with *P. cervi* antigen when tested with homologous antigen, at 6th week of post infection and no positive absorbance were detected in the sera collected on 9th week of post infection. The absorbance value of hyperimmune sera in 1:100 was 1.710, in the dilution of 1: 12800 was 0.34, where as in case of ovine sera highest absorbance values was 0.892, and at last dilution 16400, it was 0.048 (Fig. II). The present study reveals that ELISA is the most effective diagnostic technique for the detection of antibodies. ELISA has been reported specific and sensitive serodiagnostic tool for the paragonimosis (Zhang *et al.*, 2000).

DISCUSSION

ELISA test has been reported to be good from the point of sensitivity and specificity (Maisonave, 1999). In the present study in comparison to diffusion in gel, the ELISA test has been found to be highly effective and may be employed for the wide use in seroepidemiological survey (Ibarra *et al.*, 1998) of paramphistomiasis. In *Haemonchus contortus* infection also a highest titre of 1:40, 000 observed with rabbit hyperimmune sera raised by somatic antigen of *H. contortus* (Kaur *et al.*, 2002). The high titre observed in the present

study may be the significance in diagnosis of paramphistomiasis in field. The serodiagnosis of the disease is the only alternate coproscopic detection of the fluke eggs (Hammond, 1973, El – Hareth, 1980; Gupta and Yadav, 1992).

The immunodiffusion test and ELISA have been found suitable and test of choice for serodiagnosis of fascioliasis Hillyer, 1975; cechini and Kasalin 1989; Gomez, *et al.*, 1984 and Gorman *et al.*, 1993) due to their high specificity and satisfactory sensitivity.

The present study aimed to identify partially antigenic polypeptides of *P. cervi* may help in the Immunodiagnosis of paramphistomiasis. There is no information on the antigenic polypeptides of this parasite, although (Maji *et al.*, 1997) investigated antigenic cross reactivity among three species of paramphistomes.

The preliminary investigation indicated that IgG antibodies can be detected even upto a dilution of 1:12800 by the ELISA. It is thus concluded that soluble extracts of *P. cervi* are highly antigenic.

CONCLUSION

The present study reveals that ELISA is the most effective diagnostic technique for the detection of antibodies against *paramphistomum cervi*. It has been detected that ELISA is more specific, sensitive and reliable serodiagnostic technique than DID for paramphistomiasis.

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