EMPLOYMENT OF INDIRECT DOT-ELISA FOR THE DETECTION OF ANTI-PARAMPHISTOMUM EPICLITUM ANTIBODIES IN Ruminants

Syed Shabih Hassan
Department of Veterinary Parasitology, College of Veterinary Science
Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana – 141004, Punjab, India

Keywords: Detection, Indirect Dot-ELISA, Paramphistomum epiclitum, Antibodies, Ruminants.

ABSTRACT

Paramphistomosis is one of the major parasitic diseases causing heavy economic losses to livestock production. Diagnosis of disease in early stage is very important for minimizing the losses through effective treatment. The conventional methods of diagnosis such as detection of eggs in faeces by sedimentation and floatation techniques have limitations while modern serology based tests like enzyme linked immune-sorbent assay requires well equipped laboratory. The immature and adult Paramphistomum epiclitum were collected from rumen of slaughtered goat and buffaloes in slaughterhouses from Bareilly, Delhi, Dehradun and Ludhiana. Collected parasites were thoroughly washed in normal saline and processed separately for antigen preparation. Parasites were homogenized in 0.1M PBS pH=7.4, sonicated in Soniprep-150 for 8 min (4 cycles of 2 min each) and centrifuged at 15000 rpm for 15 min at 4C. The supernatant obtained was filtered through 0.22m Millex–GV filter (Millipore, France) and stored in small aliquots of 0.5 ml each at -20C to be used as somatic antigen for ELISA test. Sera were collected at monthly interval from rabbits immunized by somatic P. epiclitum antigen for determining the titre variation with time and for Dot-ELISA tests. Indirect Dot-ELISA was standardized using antigen concentration ranging from 5μg/μl – 10ng/μl and goat anti-rabbit HRPO conjugate dilutions. P. epiclitum antigen was used for coating nitro cellulose membrane (NCM) pad on combs for Dot-ELISA and kept overnight at 4˚C. The combs were then incubated in 3% lactogen in PBS, pH=7.4, at 37˚C for 1 hr for blocking the non-specific antigen binding sites. Subsequently, the combs were incubated in rabbit anti-P. epiclitum sera in dilution range 1:50 – 1: 90000 at 37˚C for 1 hr followed by three washings in PBS of 2 min each. The combs were then incubated in goat anti-rabbit HRPO conjugate at 37˚C for 1 hr again followed by 4 washings in PBS of 2 min each. The combs were then incubated in substrate 3, 3'- Diamino-benzidine hydrochloride (5 mg/10 ml PBS + 10μl 0.06% H₂O₂) for 5– 15 min. Development of dark brown spot indicated positive reaction after using various control. The optimum antigen concentration was found to be 100ng/μl and optimum conjugate dilution was found to be 1:500. Anti-sera collected from rabbits at the interval of 30 and 60 days showed a maximum titre of 1:40,000.

The experimentally infected sheep sera were taken at weekly interval from four sheep, which were examined against adult somatic P. epiclitum antigen following concentrations 50-75 ng/μl and conjugate dilution 1:500 to 1:1000 and sera dilution at 1:600 and 1:800. Sheep sera showed reaction after 2 weeks post infection. A total of 200 sera samples were examined by indirect Dot-ELISA. Overall percent positive incidence rate was recorded to be 33 percent. The highest percent positivity (38.66%) was found in buffaloes followed by 31.7% in sheep, 26.66% in cattle and 25% in goats. In the present study, a rapid and simple test (Dot-ELISA) was developed for the diagnosis of paramphistomosis. The findings are helpful for detection of paramphistomum antibodies in naturally infected animals and can be used under field conditions.

Keywords: Detection, Indirect Dot-ELISA, Paramphistomum epiclitum, Antibodies, Ruminants.

INTRODUCTION

Parasites are the ideal biological models for the study of speciation. The parasites can adopt various means and methods to evade the immune system. Now a days

*Corresponding author: fish_ab@rediffmail.com
transmission of parasitic diseases, its diagnosis and control have become a major concern throughout the world. Among the parasitic diseases, paramphistomosis is one of the most pathogenic disease in domesticated animals causing heavy economic losses to the livestock industry to the tune of several thousand crores of rupees annually. The disease is widely prevalent in India resulting in heavy losses in terms of mortality, morbidity, reduced wool, meat, milk production, reproductive disorders and expenditure on the purchase of antiparasitic drugs. In general, the parasitic diseases cause great economic losses. The total Livestock population is 535.78 million in the country (2019 census) showing an increase of 4.6% over Livestock Census2012. Total Bovine population (Cattle, Buffalo, Mithun and Yak) is 302.79 million in 2019 which shows an increase of 1.0% over the previous census. The total number of cattle and buffaloes in the country is 192.49 million and 109.85 million in 2019 showing an increase of 0.8% and 1.0% over previous Census respectively. The total sheep and goats in the country is 74.26 million and 148.88 million in 2019, increased by 14.1% and 10.1% over previous Census respectively. It has been estimated that more than 500 million cattle worldwide are at risk (Ristic, 1988). India accounts 51% of Asia and about 19% of world bovine population (Kadiravel, 2002). It is assumed that 80% of Indian herd is within areas endemic for infection.

Paramphistomosis is a group of disease caused by the various species of parasites; Paramphistomum epiclitum, P. cervi, and Gastrothylax crumenifer are found to be predominant in domestic ruminants. The other amphistome species viz. Cotylophoron bareilliensis and C. indicum found in sheep, C. bareilliensis in goats and P. dutti, Duttielacephaloporus, Oliveriobosi and O. indica found in buffaloes (Prasad and Verma, 1999). These parasites have complex life cycles and develop through various developmental stages thereby leading to the complexity of their antigenic moieties. These parasites share their antigenic epitopes with each other, which adds to the complexity of cross reactivity. The eggs of these parasites are detected during patent stages because parasites remain attached to their predilection sites of the host. The disease is a major concern in low-lying areas as the snail population viz. Indoplanorbis, Lymnaea and Gyraulus spp (intermediate host in the life cycle of paramphistome) increases mainly during monsoon and post monsoon season, which is characterized by sporadic epizootics of acute gastroenteritis with high morbidity and mortality in young domestic animals. Immature parasites are predominant in dorsal and ventral sacs of rumen of buffaloes, sheep and goats (Verma et al, 1989). Incidence of amphistomosis in cattle, buffaloes, sheep and goat has been reported in different states of India from time to time (Chhabra et al 1972; Chhabra and Gill, 1975; Gupta et al., 1978, 1980; Varma et al, 1989; Manna et al 1994; Saheb & Hafeez, 1995 and Magdy et al. 2009). The rate of paramphistomosis incidence was recorded to be highest in buffaloes 5.42% followed by cattle, sheep and goats in Punjab (Hassan et al, 2005). Dunn (1969) reported most of outbreaks in sheep and goats are due to transmission of infection through bovines. The mortality rate was recorded to be 10-20% in cattle, 38% in buffaloes and 80-83% in goats and sheep. The death rate due to immature paramphistomosis is very high and may go up to 80-90% in domestic ruminants. Besides chemotherapeutic control of the disease, of late the attention is being laid on the immunological based diagnostic techniques. The early detection of the disease and its diagnosis during sub-clinical phase is of utmost importance so that the mortality can be reduced by timely treatment of the disease. In fact, the use of conventional method to detect light infection during sub-clinical phase is difficult due to prolonged prepatency and non-availability of any parasitic stage during the period making conventional diagnosis difficult or impossible.

The effective control of the disease is possible with integrated programmes that utilize appropriate diagnostic technologies, sound management practice, selective use of drug and effective vaccines. Microscopy although remains the gold standard for detection of parasite but it lacks sensitivity. However many sero-diagnostic tests such as the widely used test i.e., indirect fluorescent antibody test (IFAT), and latest technique ELISA (Enzyme linked Immunosorbent Assay) hold promise. Dot-ELISA is a modified version of the microplate ELISA suitable for field application. ELISA is being practiced as the most effective diagnostic technique for detection of anti-parasitic antibodies. The early detection of the disease and its diagnosis during sub-clinical phase is of utmost importance so that the mortality can be reduced by timely treatment of the disease. An attempt has been made in the present work to study the prevalence of paramphistomosis in Punjab by Dot-ELISA technique. Dot-ELISA is a simple, fast, and sensitive test
recommended for field diagnosis of parasitic diseases like fascioliasis, paragonimosis and haemonchosis (Ibarra et al. 1998 and Zhang et al. 2000).

MATERIALS AND METHODS
Live flukes were collected from rumen of slaughtered goats, sheep and buffaloes from slaughter houses in Bareilly, Delhi, Dehradun and Ludhiana. The collected parasites were washed thoroughly in normal saline and then stored at –20°C till further use.

Preparation of Antigen
The collected parasites were washed thoroughly in normal saline and homogenized in 0.1 M PBS (pH 7.4) and sonicated in Soniprep 150 at 10 (amplitude) for 8 min (4 cycles of 2 minute each). The homogenate was then centrifuged at 15000 rpm for 15 min at 4°C. The supernatant obtained was filtered through 0.22 m Millex GV filter and stored in small aliquots of 0.5 ml each at –20 °C, after protein estimation by Lowry et al (1951). The collected filtrate was used as antigen for immunodiagnostic tests.

Production of Antibodies in rabbits
The New Zealand white rabbits were acclimatized in the laboratory for 15 days before starting the experiment. They were immunized subcutaneous with 400 g of _P. epiclitum_ somatic antigen mixed with Freund's Complete Adjuvant (FCA). This was followed by booster dose of Ag with FIA (Freund's Incomplete Adjuvant) after an interval of 7days and then immunized the rabbit with 400 g of the antigen alone after an interval of 7 days till desired Ab titre obtained. The animals were bled before starting the experiment for collection of pre-immune sera to be used as control and also before each booster immunization and the serum was refrigerated at –20 °C till use.

Production of Antibodies in sheep
For raising experimentally infected sheep sera, six sheep below one year old were procured. Para Amphistome metacercariae were collected for experimental production of disease in sheep after exposing the snails against artificial light and sunlight. The metacercariae were collected and counted. Of which, 4000 metacercariae were given to four sheep individually for raising paramphistome infection in experimental condition. The animal was starved for 12 hours before giving the infection. Metacercariae was kept in small quantity of water in a glass tube then transferred in glass syringe and poured into the oral cavity. Faecal samples examined microscopically and found positive for paramphistome eggs after 135 days post infection. Serological samples were collected twice in a week for Dot-ELISA immunodiagnostic tests with various kind of paramphistome antigen.

Detection of Antibodies by Dot-Enzyme linked Immunosorbent Assay
Indirect Dot-ELISA was used for the detection of anti-_P. epiclitum_ antibodies. The Nitrocellulose membrane (NCM) pad fixed over the combs was used for _P. epiclitum_ antigen coating for Dot-ELISA and kept overnight at 4°C. The combs were incubated in 3% lactogen in PBS, pH=7.4, at 37°C for 1 h for blocking the non-specific antigen binding sites and then in rabbit anti–_P. epiclitum_sera in dilution range 1:50 – 1:90000 at 37°C for 1 h followed by three washings in PBS of 2 min each. The combs were then incubated in goat anti-rabbit HRPO and bovine anti-rabbit HRPO conjugate at 37°C for 1 h again followed by 4 washings in PBS of 2 min each and then in substrate 3,3'-Diamino-benzidine hydrochloride (5 mg/10 ml PBS + 10μl 0.06% H₂O₂) for 5- 15 min. Development of dark brown spot indicated positive reaction. Various controls (positive and negative) were also included.

Polyclonal antibodies were tested and found positive by Dot-ELISA using _P. epiclitum_somatic antigen. The test antigen was diluted to concentration between 10 g/l to 10 ng/l. The dilution range of sera was used between 1:100 to 1:80000.

RESULTS AND DISCUSSION
Dot-Enzyme Linked Immunosorbant Assay (Dot-ELISA)
Indirect Dot-ELISA against adult somatic _P. epiclitum_ antigen was performed using 12 leg nitrocellulose membrane pad coated comb ELISA strips with the help of 96 well ELISA microtitre plate (Greiner). Dot-ELISA for paramphistomosis was standardized using the concentration of adult somatic antigen of _P. epiclitum_ ranging from 10 ng/µl to 2µg/ml and goat anti rabbit HRPO conjugate dilution ranging from 1:100 – 1: 5000 and sera dilution ranging from 1:200 – 1:32000.

The combs were incubated in 3% lactogen in 0.1M PBS (pH = 7.4) at 37 °C for 1 hr for blocking the non-specific binding sites. Subsequently the combs were incubated in rabbit anti-_P. epiclitum_ hyper immune sera in dilution range 1:50 – 1:12800 at 37 °C for 1 hr followed by three washings in PBS of two minutes
each. The combs were then incubated in goat anti-rabbit HRPO conjugate at 37°C for 1 hr again followed by 3 washings in PBS of two minutes each. The combs were then incubated with Diaminobenzidine dihydrochloride (DAB) 25mg/10ml PBS + 10µl 0.06% of H₂O₂ for 5 – 15 minutes. The reaction was stopped with distilled water and dark brown coloured spot developed on the nitrocellulose membrane in the region where antigen was coated (Fig.1). It indicated positive reaction between antigen and antibody. The optimum antigen concentration was found between 50-100 ng/µl, anti-rabbit HRPO conjugate concentration at 1:500 and 1:1000 and sera dilution at 1:400 to 1:800. Antibody titre of rabbit hyperimmune sera of different time interval was also checked and found positive following serial dilutions ranging from 1:200 to 1:12,800. Anti-P. epiclitum sera dilution from rabbit No. 1 and 2 at the interval of 30 days post immunization showed a maximum titre of 1:10,000.

Indirect Dot-ELISA with Clinical/Field Sera
A total of 200 sera samples (82 sheep, 75 buffaloes, 28 goats and 15 cattle) were examined by indirect Dot-ELISA. Various negative and positive controls were included. Out of the total examined sera 66 samples (29 buffaloes, 26 sheep, 7 goats and 4 cattle) were found to be positive with the incidence rate of 33%. The highest percent positivity (38.66%) was found in buffaloes followed by 31.7% in sheep, 26.66% in cattle and 25% in goats. The observation for the detection of paramphistome infection in 200 clinical/field sera samples by indirect Dot-ELISA is depicted in Table -1.

Table 1: Paramphistome antibodies detection by Dot-ELISA in field cases.

<table>
<thead>
<tr>
<th>Animal Sera</th>
<th>No. Examined</th>
<th>No. Positive</th>
<th>Percent (%) positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffaloes</td>
<td>75</td>
<td>29</td>
<td>38.66</td>
</tr>
<tr>
<td>Cattle</td>
<td>15</td>
<td>4</td>
<td>26.66</td>
</tr>
<tr>
<td>Sheep</td>
<td>82</td>
<td>26</td>
<td>31.7</td>
</tr>
<tr>
<td>Goats</td>
<td>28</td>
<td>7</td>
<td>25.00</td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
<td>66</td>
<td>33.0</td>
</tr>
</tbody>
</table>

Various immunodiagnostic techniques are being employed for titre determination like agar gel precipitation test (AGPT), Indirect haemagglutination test (IHA), Conglutinating complement absorption test (CCAT) and ELISA. Among such tests enzyme linked immunesorbent assay (ELISA) has been extensively used (Burden Hammet, 1978; Farrel, 1981; Zimmerman et al., 1982). ELISA is being practiced as the most effective diagnostic technique for detection of anti-parasitic antibodies. Zimmerman et al. (1985) observed antibodies against crude formulations of excretory-secretory preparations derived from adult Fasciola hepatica at 4 weeks post-infection in sheep using Dot-ELISA. ELISA especially Dot-ELISA has been observed as a specific and sensitive serodiagnostic method for Paragonimosis sp. (Zhang et al. 2000). It is a fast, simple and inexpensive test and also found suitable for field diagnosis of fascioliosis in cattle. The test showed a sensitivity of 82%, specificity of 90% with 95% confidence level (CL), good repeatability and a significant association with reference to ELISA (Maisonnave, 1999). Furthermore in comparison to diffusion in gel (DIG) ELISA and indirect ELISA tests, Dot-ELISA (with sensitivity of 93.1% and specificity 95.4%) has been found to be highly effective and may be recommended for use in sero-epidemiological surveys of F. hepatica (Ibarra et al.1998). ELISA has also been observed to be
suitable for widespread use in the diagnosis of cryptosporidiosis (El–Shazly-AM et al. 2002). In Haemonchus contortus also a high titre of 1:40000 were observed with rabbit hyperimmune sera raised against somatic antigen of H. contortus (Kaur et al. 2002a, b). In the present study, prevalence of paramphistomosis by the application of Dot-ELISA technique was recorded to be 31 percent. The highest percent positivity (36.84%) was found in buffaloes followed by 30% in sheep, 28.57% in cattle and 20% in goats. It may be due to cross reactivity with other metazoan parasites as well as other reason for increased prevalence rates recorded by serological tests (Chan and Ko, 1990). It has been found that false positive and false negative results in other diagnostic assays can often be resolved by immunoblotting (Molina et al. 1993). Percentage prevalence by Dot-ELISA in different animals revealed that the early diagnosis is very important for curtailing the loss due to disease by appropriate treatment. Hence the high titre observed in the present study may prove to be of great significance in diagnosis of paramphistomosis in field cases for the immunological control of the disease. The application of Dot-ELISA diagnostics against paramphistomosis in domestic ruminants is very sensitive and specific.

CONCLUSION
Paramphistomosis is a pathogenic disease in domesticated animals causing morbidity and mortality in livestock industry. The control of disease will benefit the farmers in terms of economic status. The sera of anti-P. epiclitum and experimental sheep can be used as reference sera with paramphistome antigens for

Figure 1: Standardization and Development of Dot-ELISA for the detection of anti-P. epiclitum Antibodies in Ruminants.
immunodiagnosis of paramphistomosis. Immature flukes causing paramphistomosis was identified through the dynamics of serodiagnostic tests in domestic animals. Immunodiagnostic tests such as Dot-ELISA are very sensitive tests and useful for the detection of paramphistome antibodies in clinical field cases. These observations are very important for the diagnosis of paramphistomiasis and immunological control of the disease occurring in livestock industry. The mortality of animal due to paramphistomosis may be significantly reduced by detecting infection at the earliest.

ACKNOWLEDGEMENTS
Author is thankful to Science & Engineering Research Council, Department of Science & Technology, Ministry of Science & Technology, Govt. of India New Delhi for financial support in the form of DST-SERC-FAST-Track project and Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, for facilities provided.

REFERENCES
17. Maisonnave, J. (1999) Standardization of a dot immunoperoxidase assay for field diagnosis of
Fasciola hepatica infected cattle. *Veterinary Parasitology,* 85 (4) 259-268.


