



STANDARDISATION OF COPROANTIGEN DOT ELISA FOR DIAGNOSIS OF BOVINE AMPHISTOMOSIS

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ABSTRACT

Amphistomosis is a snail borne trematode disease in ruminants causing high morbidity and mortality in tropical and subtropical areas causing great economic losses. The disease is caused mainly by immature flukes and seasonally egg producing trematodes which makes conventional ova detection inefficient. Early diagnosis using coproantigens is a novel method showing good promise. The present study was carried out to standardise a rapid field level diagnostic test for early detection of coproantigens in amphistomosis. Hyper immune polyclonal antisera raised in rabbits against excretory secretory antigens of amphistomes were used in the study for coproantigen detection. Out of the fifty known amphistome ova positive samples, 38 gave positive brown dots indicating 76 per cent infection. Twelve positive samples turned negative in Dot ELISA which may be due to the low fluke burden in these animals thus excreting undetectable coproantigens in faeces. Immunodot did not give any false positive reaction and had 100 percent specificity, 76 per cent sensitivity, 100 per cent positive predictive value (PPV) and 62.5 per cent negative predictive value (NPV) with a accuracy of 83 per cent. Use of monoclonal antibodies and more purified antigens can increase the sensitivity of the present test and can be developed to a commercial rapid dipstick ELISA for early diagnosis of amphistomosis.

Key words: Amphistomosis, Dot ELISA, standardisation, coproantigen, diagnosis

I. INTRODUCTION

Amphistomosis is a parasitic disease of livestock animals, more commonly of cattle, sheep and goats. The species responsible for acute outbreak of disease in cattle are *Paramphistomum cervi*, *Cotylophoron cotylophorum*, *Calicophoron calicophorum*, *Gastrothylax crumenifer*, *Fischoederius cobboldi*, *Orthocoelium scoliocoelium* and *Olveria indica*. The disease causes high morbidity and mortality resulting in great economic loss through lowered production. Several outbreaks of acute amphistomosis have been recorded worldwide [1, 2]. Immature paramphistomes cause sporadic outbreaks of acute parasitic gastroenteritis associated with high mortality in young sheep, goats, cattle and buffaloes. Diagnosis during this acute sub clinical phase is of utmost importance for early treatment since conventional ova detection fails in early diagnosis. Eventhough several serodiagnostic tests are developed, antibody detection tests are not conclusive. Coproantigen detection appears to be a promising alternative in early detection of infection with high specificity and sensitivity. The present study was designed to standardize a coproantigen dot-ELISA for early diagnosis of amphistomosis.

II. MATERIALS AND METHODS

Mature rumen flukes were collected in phosphate buffered saline (PBS), from cattle slaughtered at Municipal Corporation slaughter house, Thrissur were washed several times with phosphate buffered saline premainained at 37°C and were used to collect excretory secretory antigen (ESA) as per the method of [3]. A total number of 30 adult active flukes comprising of *G. crumenifer*, *F. cobboldi*, *F. elongatus*, *Carmyerius* spp. and *Paramphistomum* spp. were incubated in sterilized phosphate buffered saline, pH 7.4 for five hours at 37°C. After incubation, flukes were removed and the suspension containing ES products were centrifuged at 10,000 g for 30 min at 4°C. The supernatant obtained was dialysed against double distilled water for 24 h and then lyophilized. The freeze dried material stored at 4°C served as ESA to be used for raising hyper immune serum.

Protein estimation of ESA was done by Lowry's method (Merck Genei™, Bangalore) in a spectrophotometer (Lambda 750, Perkin Elmer) at 660nm.

A. Preparation of hyperimmune sera

Hyper immune sera was raised in two Newzealand White rabbits by immunizing subcutaneously with sensitising inoculum prepared by mixing 0.5 ml of diluted antigen containing 500 µg excretory secretory protein mixed with equal quantity of Freund's complete adjuvant (Merck GeNei™, Bangalore). This was followed by three booster doses of antigen mixed with Freund's incomplete adjuvant (Merck GeNei™, Bangalore) at one week intervals. The rabbits were bled a week after the final booster dose, and the sera separated was stored at -20°C.

B. Preparation of Coproantigens

Fresh dung samples were collected from rectum of dairy cattle and brought to the laboratory and processed for coproantigens as per [4]. About one gram of dung was mixed with 2 ml of carbonate-bicarbonate buffer (1:2) and shaken vigorously in a vortex until slurry was formed. The faecal slurry was then centrifuged at 2000 g for 30 min at 4°C and the supernatant obtained was stored at -20°C to be used for coproantigen detection.

C. Standardisation of Dot ELISA

Standardisation was done to determine the optimum dilution of rabbit hyperimmune sera and conjugate needed to detect coproantigen in faecal sample. Rabbit hyperimmune serum was used at a dilution of 1:25, 1:50 and 1:100 in dilution buffer (0.05% skim milk in PBS-T). Goat antirabbit IgG HRP conjugate was checked at 1:1000, 1:2500 and 1:5000 dilutions. The optimum dilution of hyper immune sera and conjugate which gave brown coloured spots were considered positive. Positive and negative controls were maintained. Faecal eluents prepared from one month old calf served as negative control while amphistome ova positive faecal samples served as positive control.

D. Immunodot Analysis of Faecal Supernatants

The undiluted faecal eluent (2 µl) was coated onto the centre of the nitrocellulose strips (1.5 x 1.5 cm). The strips were allowed to dry at room temperature. The unbound sites of the NCM strips were incubated with blocking buffer (5% skim milk in PBS) at 37°C for one hour. Hyper immune serum raised in rabbits against ESA of amphistomes was used as the primary antibody. The strips were incubated in the hyper immune serum samples (1:25 dilution in blocking buffer) at room temperature for one hour and washed with PBS-T for five to 10 min with five to six changes of wash buffer. Further, the

NCM strips were incubated with horse radish peroxidase conjugated goat anti-rabbit immunoglobulin G at a dilution of 1:2500 in blocking buffer for one hour at room temperature. The strips were washed four times in PBS-T and were put into the chromogenic visualisation solution at room temperature, with mild rocking for two to three min. The reaction was terminated by washing NCM strips with distilled water. The NCM strips were air dried and observed for the development of a brownish spot in positive cases. The sensitivity and specificity was calculated by taking ova positive samples as the standard.

III. RESULTS AND DISCUSSION

Indirect Dot ELISA was standardised to detect coproantigens of amphistomes using rabbit hyperimmune sera at a dilution of 1:20 and antirabbit IgG HRP conjugate at 1:2500 dilution maintaining positive and negative controls (Fig. 1).

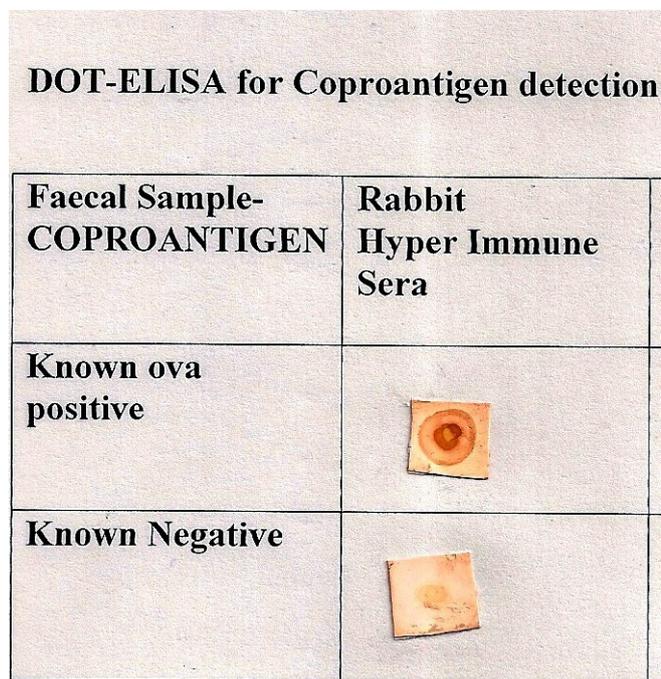


Figure 1. Dot ELISA for coproantigen detection

Fifty faecal samples positive for amphistome ova were subjected to the standardised Dot ELISA. Dot ELISA gave only 76 per cent positive with twelve known positives yielding false negative results, though all known ova negative samples were Dot ELISA negative (Table. 1).

Table 1. Comparison of diagnostic tests

Total Number of samples	Diagnostic Test	Number positive	Number negative	Per cent positive
50	Microscopy	50	0	100
	Dot ELISA	38	12	76

The levels of coproantigens of amphistomes vary between individual infected animals. Low coproantigen concentration might be the reason for a negative result in Dot ELISA in the present study. This false negative result may be attributed to the low fluke burden with faecal excretion of presumably non-detectable quantity of coproantigens. The concentration of helminth excretory secretory antigen in the faeces of the infected host directly correlates with the number of parasites in the intestine [5].

Statistical analysis using Cochran Q test revealed that the results of Dot ELISA was significantly different from ($p < 0.05$) microscopy. Sensitivity and specificity was ascertained for Dot ELISA with a sensitivity of 76 per cent, specificity of 100 per cent, positive predictive value (PPV) of 100 per cent and negative predictive value (NPV) of 62.5 per cent and with a accuracy of 83 per cent.

Eventhough immunodot has the advantage of being rapid, cheaper and more suitable for field conditions, in the present study it revealed a lower sensitivity of 76 per cent in detecting amphistome faecal antigens but showed 100 per cent specificity. Immunodot in the present study did not give false positive reactions. Low sensitivity may be due to the non-detectable quantity of coproantigen due to low fluke burden. Affinity purification of both antigen and immunoglobulins, also the use of monoclonal antibodies can increase the sensitivity of the test thus ruling out the limitations in the present study. Conventional ova detection often underestimates the true prevalence of the disease and ova detection is possible only 6-8 weeks post infection. Sensitivity of ELISA procedure is very high and can detect the infection at an early stage from seventh day onwards whereas immunodot detected the faecal antigens four weeks post infection as reported by [6].

Dot ELISA is a simple inexpensive rapid test having potential for wider application in coprological diagnosis even in poorly equipped laboratories. It is concluded that faecal antigens can be detected at a very early stage of infection by a simpler, cost-effective dot ELISA technique and can be developed to a commercial dipstick test for the early diagnosis of amphistomosis in bovines.

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