IMMUNODIAGNOSIS OF BOVINE GASTROTHYLACOSIS USING COPROANTIGENS

By

A. MANDASAMY

THESIS SUBMITTED TO THE
KERALA AGRICULTURAL UNIVERSITY
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

Master of Veterinary Science

in

Veterinary Parasitology

COLLEGE OF VETERINARY AND ANIMAL SCIENCES
KERALA AGRICULTURAL UNIVERSITY
THRISSUR, KERALA

2000

DECLARATION

I hereby declare that this thesis entitled **"IMMUNODIAGNOSIS OF BOVINE GASTROTHYLACOSIS USING COPROANTIGENS"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

Mannuthy
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A. KANDASAMY

CERTIFICATE I

This is to certify that this thesis, entitled **"IMMUNODIAGNOSIS OF BOVINE GASTROTHYLACOSIS USING COPROANTIGENS"** submitted for the degree of M.V.Sc. in the subject of Veterinary Parasitology of the Kerala Agricultural University, is a bonafide research work carried out by **Shri.A. Kandasamy,** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

Dr.K. Devada
Major Advisor
Assistant Professor
Department of Veterinary Parasitology
College of Veterinary and Animal Sciences
Mannuthy

Mannuthy
14.8.2000

CERTIFICATE II

We, the undersigned members of the Advisory Committee of **Shri.A. Kandasamy,** a candidate for the degree of Master of Veterinary Science in Veterinary Parasitology, agree that the thesis entitled **"IMMUNODIAGNOSIS OF BOVINE GASTROTHYLACOSIS USING COPROANTIGENS"** may be submitted by Shri.A.Kandasamy, in partial fulfilment of the requirement for the degree.

14.8.2002

Dr.K. Devada
Assistant Professor
Department of Veterinary Parasitology
(Chairperson)

Dr.H.Subramanian
Associate Professor & Head
Department of Veterinary
Parasitology
(Member)

Dr.M.R.Saseendranath
Associate Professor & Head
Department of Veterinary
Epidemiology and
Preventive Medicine
(Member)

Dr.M.Mini
Assistant Professor
Department of Veterinary Microbiology
(Member)

EXTERNAL EXAMINER

ACKNOWLEDGEMENT

*My unreserved gratitude and indebtedness go to **Dr.K.Devada**, Assistant Professor, Department of Veterinary Parasitology and chairperson of the advisory committee. The unstinted support is something which words or deeds can not thank. Her motherly approach, personal attention, incessant encouragement and extreme patience throughout the course of my study were integral for the fulfillment of this work.*

*I would like to express my deep sense of gratitude to **Dr.H.Subramanian**, Associate Professor and Head, Department of Veterinary Parasitology for his meticulous guidance, criticism and support during the course of my study and research work.*

*I am grateful to the members of the advisory committee, **Dr.M.R.Saseendranath,** Associate Professor and Head, Department of Veterinary Epidemiology and Preventive Medicine and **Dr.M.Mini**, Assistant Professor, Department of Veterinary Microbiology, for their advice and help rendered.*

I am immensely thankful to Dr.C.George Varghese, Professor and Dr.Lucy Sabu, Assistant Professor, Department of Veterinary Parasitology for their generous help and encouragement.

I am grateful to Dr.Sulochana, Dean, College of Veterinary and Animal Sciences, Mannuthy for providing facilities needed for this work.

I am indebted to Dr.K.Madhavan Pillai, Professor (Retd.) and Dr.C.Pythal, Professor (Retd.), Department of Veterinary Parasitology for their valuable suggestions offered from time to time.

Special thanks are due to Dr.G.Krishnan Nair, Associate Professor, Department of Veterinary Microbiology for his technical advice and help rendered in preparing antigens and performing ELISA.

I sincerely thank all the staff members in the Department of Veterinary Parasitology for their help throughout my research work.

I offer my heartful thanks to my colleagues Dr.N.Baskar, Dr.K.Sreevidya Nambiar and Dr.R.Sangeetha for their co-operation, priceless help and friendliness which enabled a fairly strenuous task to remain pleasure throughout.

I sincerely thank Dr.Sree Ranjith Kumar, Research Associate, Department of Veterinary Microbiology for his help in reading ELISA results.

I am thankful to Dr.Arun Shaju, Dr.Shibu K. Jacob, Dr.Venkatesh Kumar, Dr.Lakshmi, Post graduate scholars for their timely help.

I express my thanks to my friends Dr.Mahendran, Dr.Ramakrishnan, Dr.Tresa Mery, Dr.Mohan, Dr.Kasi, Dr.Kanakasabai. Dr.Thirunavukkarasu, Dr.Vasudevan, Dr.Gopi, Dr.P.Senthilkumar, Dr.S.Senthilkumar, Dr.Balasubramanian and Dr.Rajalakshmi for their help at various phases of my work.

I am thankful to KAU for awarding me the fellowship for post graduate study.

I express my deep sense of indebtedness to my beloved mother, father, sister and brother for their constant encouragement and support in fulfilling this endeavour.

(A. KANDASAMY)

To

The Parasitologists of the world
living and dead
whose patient labours and personal sacrifices
have done so much to relieve human and
and animal suffering

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INTRODUCTION

INTRODUCTION

"Animals' health is Nation's wealth". This well known statement denotes the importance of maintaining health of livestock which plays a significant role in the Indian economy. In 1997-98 the livestock sector contributed nearly 9 per cent of the gross domestic product. Among them, bovines contributed more than 70 per cent, through milk, meat and dung (India 2000). Sustained growth and production of bovinesare therefore important for an overall economic improvement and poverty elimination.

However, parasitic diseases, apart from other diseases, often give vital blows to the bovine production. Among the gastrointestinal helminth parasites amphistomes form one of the most important group in undermining the health of bovines.

In India, for a long time, owing to their common occurrence in domesticated animals, amphistomes were regarded as harmless parasites. But investigations (Mukherjee and Deorani, 1962) have proved that the Indian livestock industry suffers much due to the fatal enteritis caused by flukes of various species.

In Kerala, the topography and climatic conditions are most favourable for the survival of amphistome parasites in domesticated animals.

Nath (1987) reported that *Gastrothylax crumenifer,* a species of amphistome, accounted for the highest prevalence (62.64 per cent) out of the 17 species belonging to eight different genera of family Paramphistomatidae in Kerala.

Gastrothylacosis, caused by *G. crumenifer*, is often a fatal disease of cattle (Radostits *et al.*, 1994). The adult fluke, located in rumen, is relatively harmless whereas the immature flukes cause serious disease characterized by enteritis and diarrhoea. Failure of timely diagnosis and treatment leads to death of the animal (Fischer and Say, 1989).

Conventional methods of diagnosis of gastrothylacosis rely on the detection of clinical signs such as general weakness, reduced feed consumption, oedema at the intermandibular region and diarrhoea with fluid foetid smelling dung mixed with blood or mucous, aided by quantitative and qualitative examination of amphistome eggs in faeces of suspected cases (Alwar, 1949). Yet, it is difficult to differentiate gastrothylacosis, by clinical signs, from nonspecific enteritis and other gastrointestinal helminth infections (Radostits *et al.*, 1994).

Microscopic detection of amphistome eggs in the faeces has many disadvantages; it lacks consistency; it is time consuming and labour intensive, requiring each sample to be examined individually by a parasitologist. It cannot be automated and on the contrary it cannot detect prèpatent infections (Johnson *et al.*, 1996) which should be considered because in gastrothylacosis, the disease occurs during the prepatent period when flukes are still immature and will not lay eggs.

Although the disease can be confirmed by necropsy (Urquhart *et al.*, 1988), it is time consuming.

An alternative approach to overcome such difficulties of diagnosis by parasitological means, is the detection of serum antibodies to specific antigens of *G. crumenifer* by various methods (Hafeez, 1975 and 1981). But serology also has its own limitations as the antibodies indicate post exposure to the parasite rather than the presence of active infection (Dumenigo *et al.*, 1996).

Several studies have shown that the detection of parasite antigens is more reliable than the detection of antibodies in the diagnosis of active parasitic infections (Kaliraj *et al.*, 1981). As a result, emphasis has been given on the detection of parasite antigens in biological samples especially if it does not involve collection of blood samples (Johnson *et al.*, 1996).

The detection of parasite antigens in the hosts' faeces (coproantigens) is a novel method showing good promise. The coproantigens have been proved to have good diagnostic characteristics. They are stable in the gastrointestinal tract and under various temperatures; they are shed by immature and mature flukes making early detection possible; they are present in detectable quantities and rapidly disappear after treatment.

Many immunodiagnostic tests have been developed to detect coproantigens for diagnosis of various parasitic infections. Among them, enzyme linked immunosorbent assay (ELISA) is found to be superior in terms of sensitivity and specificity. Thus ELISA using coproantigens has certain advantages over other diagnostic methods. The test avoids handling of blood

or serum products; indicates the presence of a current infection only and it is able to handle large number of samples (Fraser and Craig, 1997).

As no references were available on the detection of coproantigens for the diagnosis of paramphistomatidosis, the related work done on other parasites was adopted in this study.

The present study was undertaken

1. to assess the feasibility of coproantigen detection by ELISA for the diagnosis of gastrothylacosis in cattle and
2. to compare the sensitivity of ELISA for detection of coproantigens and ELISA for detection of serum antibodies in the diagnosis of gastrothylacosis in cattle.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Paramphistomatidosis in ruminants is caused by trematodes belonging to the family Paramphistomatidae (Soulsby, 1982). The adult trematodes, located in rumen, are relatively non pathogenic whereas the immature trematodes in the small intestine cause severe disease characterized by severe enteritis (Fischer and Say, 1989 and Radostits *et al.*, 1994).

2.1 Prevalence of paramphistomatidosis

Although paramphistomatidosis is distributed world wide, it is of little veterinary significance in Europe and America (Urquhart *et al.*, 1988) but is a common infection throughout Africa and Asia (Fischer and Say, 1989).

Incidence of paramphistomatidosis is recorded in various parts of India from time to time (Pand e, 1935; Haji, 1935; Bawa, 1939; Mudaliar, 1945; Alwar, 1949; D'Souza, 1949; Ramakrishnan, 1951; Katiyar and Varshney, 1963; Balasubramaniam *et al.*, 1973; Ahluwalia *et al.*, 1976; Sharma and Asthana, 1978; Lalitha and Anandan, 1986; Varma *et al.*, 1989 and Manna *et al.*, 1994).

The prevalence of amphistome infection in different domestic ruminants in Kerala was studied by Nath (1987). The study indicated that the prevalence of infection was far more in cattle and buffaloes than in sheep and goats. A higher prevalence (38.1 per cent) was recorded during the rainy season particularly in June and July and low (10 per cent) in dry or summer

season particularly in March and April. It was seen that in cattle *Gastrothylax crumenifer*, a common amphistome species in India, accounted for the maximum prevalence (62.64 per cent), out of the 17 species belonging to eight different genera of family Paramphistomatidae.

2.2 Morphology of *Gastrothylax crumenifer*

Soulsby (1982) and Nath (1987) gave an account on morphology and identification characters of *G. crumenifer*. The gross morphology of the fluke is as follows: it is red when fresh, body nearly cylindrical, circular in transverse section, widened at the posterior end and having a slight constriction at the preacetabular region. The posterior sucker is large, terminal and has a raised border. The oral sucker is small. The uterus crosses from right to left at about the middle of the body, a characteristic morphological feature of *G. crumenifer*, which can be observed by pressing the fluke gently in between two microslides as reported by Nath (*loc.cit*).

2.3 Diagnosis of paramphistomatidosis

2.3.1 Traditional approach

Paramphistomatidosis in cattle is usually diagnosed by clinical signs involving young animals, history of the area, faecal examination for amphistome eggs or immature flukes and postmortem examination (Soulsby, 1982 and Urquhart *et al.*, 1988).

Apart from general weakness, characteristic clinical signs in cattle consist of oedema at intermandibular region – commonly known as bottle jaw

– which becomes prominent in evenings and reduces in mornings on account of grazing during day time with lowered head and rest at night respectively (Alwar, 1949), profuse fluid foetid diarrhoea (Soulsby, 1982; Fischer and Say, 1989 and Radostits *et al.*, 1994) where the dung may be darkish with mucous, with or without blood (Alwar, 1949) and foul smelling (Ramakrishnan, 1951) accompanied by intense thirst (Urquhart *et al.*, 1988).

In per acute cases, death is preceded by diarrhoea only and in acute conditions the animals may die in a day or two after the onset of diarrhoea (Alwar, 1949 and Ramakrishnan, 1951).

History of grazing is useful particularly in outbreaks of paramphistomatidosis in herds during a period of dry weather when animals graze around snail habitats (Urquhart *et al.*, 1988).

Faecal examination of amphistome eggs is of little value since the disease occurs during the prepatent period when the flukes are still immature and will not lay eggs (Urquhart *et al.*, 1988 and Fischer and Say, 1989).

Ramakrishnan (1951) reported that faecal samples from animals with diarrhoea were negative for eggs, during an outbreak of acute paramphistomatidosis among cattle in Nellore district, Andhra Pradesh.

In some circumstances the presence of large number of amphistome eggs in the faeces is also indicative of the disease since, although the pathogenic effects are caused by the immature forms, a large number of adult forms may also accompany the immature burden (Soulsby, 1982).

Detecting immature flukes in faeces is in fact a more common basis of diagnosis (Radostits *et al.*, 1994). Small pinkish-white seed like immature flukes can be found, when a sample of faeces is sifted under a stream of water (Fischer and Say, 1989).

Confirmation can be obtained by postmortem examination and recovery of small flukes from the duodenum (Urquhart *et al.*, 1988). The postmortem lesions include highly emaciated carcass, profound enteritis of small intestine especially duodenum with presence of innumerable number of immature amphistome flukes embedded in the mucous membrane (Ramakrishnan, 1951).

2.3.2 Serodiagnosis of paramphistomatidosis

Different serological and immunodiagnostic tests were evolved to diagnose intestinal paramphistomatidosis of sheep, goat and cattle (Hafeez, 1975 and 1981 and Reddy and Babu, 1987).

Hafeez and Avsatthi (1986) observed precipitates around live *Gigantocotyle explanatum* and *Paramphistomum epiclitum* flukes with homologous antisera raised in rabbits against whole worm extract antigens of these two parasites, in a simple *in vitro* precipitation test. They opined that this could be used for diagnosis of paramphistomatidosis in infected hosts. This opinion was upheld by their findings in 1987, when they tried to detect hepatic infection due to *G. explanatum* in naturally infected buffaloes by immunoelectrophoresis. All the serum samples from infected buffaloes gave

positive results. They concluded that immunoelectrophoresis could be useful as one of the diagnostic aids in hepatic gigantocotylosis.

Avsatthi *et al.* (1986) found that *P. epiclitum*, *G. crumenifer* and *G. explanatum* shared some common antigens which could possibly help in cross protection and immunodiagnosis.

Hafeez and Rao (1986) utilized gel diffusion, *in vitro* precipitation test and circumoval precipitation test to detect paramphistomosis due to *P. epiclitum* and studied the immune response in lambs and kids vaccinated with irradiated amphistome metacercariae. Gel diffusion and *in vitro* precipitation tests were found to be valuable in detecting the disease and the infected animals showed good antibody response.

This report was corroborated by Hafeez *et al.* (1987) in their attempt to diagnose paramphistomosis due to *P. epiclitum* in experimentally infected sheep by immunoelectrophoresis. They reported that antibodies could be detected as early as 15 days, well before the patent infection.

Reddy and Babu (1987) used counter immunoelectrophoresis (CIEP) technique in the diagnosis of paramphistomosis due to *P. epiclitum* in cattle and buffaloes. All the serum samples of cattle shown to have amphistome eggs in the faeces, gave positive reactions. Twenty per cent of cattle which showed no eggs in faeces were positive for serum antibodies, possibly due to the presence of immature parasites. They concluded that CIEP is a rapid and sensitive technique in the diagnosis of immature paramphistomosis.

Varma *et al.* (1991) obtained 91.17 per cent sensitivity in diagnosis of natural ruminal paramphistomosis caused by *P. cervi*, in sheep and goats by indirect haemagglutination assay (IHA) using adult fluke antigen.

Abidi and Nizami (1999) reported the occurrence of antigenic polymorphism as well as presence of common antigens among different amphistome species of buffalo and inferred that this could be significant for immunological diagnosis of paramphistomatidosis. In the same line of thought Saifullah *et al.* (2000) partially purified and characterized different immunostimulant somatic antigens of *G. crumenifer* for further studies in immunodiagnosis of the same.

2.4 Enzyme linked immunosorbent assay (ELISA)

Enzyme linked immunosorbent assay has been widely used and accepted as one of the best methods available for serodiagnosis of parasitic infections.

At present, no information is available on the use of ELISA in detection of serum antibodies for diagnosis of paramphistomatidosis in ruminants. So the use of ELISA in serodiagnosis of fasciolosis, also a fluke disease to paramphistomatidosis, is reviewed here.

Enzyme linked immunosorbent assay has been used for diagnosis of experimentally and naturally occurring fasciolosis in cattle (Farrel *et al.*, 1981; Welch *et al.*, 1987 and Anderson *et al.*, 1999), in buffaloes (Swarup *et al.*, 1987) and in sheep (Zimmerman *et al.*, 1982 and Santiago and Hillyer, 1988).

Farrel *et al.* (1981) concluded that ELISA offers the clinician the advantage of early diagnosis, several weeks earlier than the diagnosis accomplished by faecal examination and opportunity to treat while immature flukes migrated through liver. This conclusion was corroborated by Wescott *et al.* (1984) who tested a number of sera from cattle in a geographical area epizootic for *Fasciola hepatica* by ELISA.

Enzyme linked immunosorbent assay gave the best results when Hillyer *et al.* (1985) compared it with gel diffusion and counter immunoelectrophoresis in the diagnosis of *F. hepatica* in bovines and this finding was supported by Swarup *et al.* (1987) who compared the efficiency of agar gel precipitation test (AGPT), CIEP and ELISA in the diagnosis of *F. gigantica* infection in buffaloes.

Wedrychowicz *et al.* (1995) used ELISA for measuring the bile antibody response to somatic antigens of adult *Dicrocoelium dendriticum* in naturally infected cattle and reported that ELISA was highly sensitive and specific.

Anderson *et al.* (1999) in comparing faecal egg count and antibody measurement by ELISA in detecting bovine fasciolosis due to *F. hepatica* and *F. gigantica*, observed that ELISA had sensitivity and specificity of 86.1 per cent and 70 per cent as against 66.7 per cent and 100 per cent of faecal egg count.

2.5 Coproantigen detection

Gottstein and Deplazes (1989) reviewed the indirect methods for determining parasitic infections of animals like serum antibody detection and detection of antigens circulating in serum or excreted in faeces (coproantigens in intestinal parasitosis).

According to Fraser and Craig (1997), coproantigen detection has certain advantages over microscopical examination of eggs in faeces and serum antibody or antigen detection. It avoids handling of blood or serum products; indicates the presence of current infection. Coproantigens rapidly disappear from faeces after treatment and it has good specificity and sensitivity.

2.5.1 Trematodes

Enzyme linked immunosorbent assay (ELISA) using coproantigens has been developed for the detection of several parasitic trematodes with varying successes.

Studies in cats with natural opisthorchosis showed a close relationship between level of antigens in faeces and number of flukes/eggs in the faeces and between antigen titres in faeces and bile (Teplukhin *et al.*, 1988).

Mallewong *et al.* (1997) developed a capture ELISA for detection of *Paragonimus heterotremus* infection in experimentally infected cats by detecting the excretory-secretory (E/S) antigens in the faeces. They found that the sensitivity of ELISA was 73.7 per cent and specificity was 100 per cent.

Capture ELISA was found to be positive for coproantigens during the early stage of infection when worms were still immature and when eggs have not been released into the faeces.

The sensitivity of ELISA was found to be 83.3 per cent by Moustafa *et al.* (1998) who developed the test for detection of *F. hepatica* antigens in the faecal sample of experimentally infected animals (mice, rats and rabbits) using polyclonal antibodies raised against somatic antigens of the parasite. He concluded that ELISA was an easier, more rapid and sensitive test for diagnosis of *F. hepatica* coproantigens than the routine stool examination for *Fasciola* eggs.

Detection of *F. hepatica* coproantigens by monoclonal antibody based capture ELISA was successfully used in cattle (Dumenigo *et al.*, 1996 and Rahman *et al.*, 1998 and 1999) and in sheep (Dumenigo and Mezo, 1999 and Dumenigo *et al.*, 1999). It was found that coproantigen capture ELISA had a higher sensitivity (83.7 to 100 per cent) and specificity (100 per cent). It was more sensitive and easier to perform than microscopic examination for eggs. Moreover prepatent infections, before the flukes matured, were detectable. The assay correlated well with the number of flukes in the animals, suggesting that it was possible to estimate the fluke burden also.

2.5.2 Cestodes

Coproantigen ELISA has been described for a number of different cestode infections based on the use of parasite specific capture antibodies. The simplest method involves polyclonal rabbit hyperimmune sera directed

against a crude somatic extract of immature and adult proglottids (Fraser and Craig, 1997). Despite the polyclonal nature of the antibodies, the assay proved to be remarkably genus specific and sensitive.

A capture ELISA using rabbit hyperimmune sera raised against somatic antigens was used for detection of coproantigens of *Hymenolepis diminuta* in rats (Allan and Craig, 1989 and 1994), *Taenia pisiformis* (Allan *et al.*, 1992) and *Echinococcus multilocularis* (Deplazes and Eckert, 1996; Sakai *et al.*, 1998 and Deplazes *et al.*, 1999) and *E. granulosus* (Allan *et al.*, *loc.cit*; Craig *et al.*, 1995 and Hoida *et al.*, 1998) in dogs and *Taenia solium* in humans (Allan *et al.*, *loc.cit*). The coproantigen detection was considered more sensitive than microscopical examination for eggs in faeces as the antigens were present in faeces before patency and independent of egg output. Antigens could be detected in faecal samples from infected host stored in 5 per cent formalin for 6 months (Allan *et al.*, *loc.cit*). Deplazes and Eckert (*loc.cit*) opined that the application of coproantigen ELISA has the potential for replacing parasite detection at necropsy.

Ahmad and Nizami (1998) evaluated three immunodiagnostic tests *viz.*, indirect ELISA, immunodot and counter current immunoelectrophoresis (CCIEP) for detection of *E. granulosus* specific coproantigens in experimentally infected dogs. They reported that coproantigens were detected in the first week post infection by ELISA as against the fourth week by immunodot and CCIEP. It was evident that ELISA was more sensitive and detected the coproantigens earlier than immunodot and CCIEP.

There are some evidences to suggest that improved specificity may occur with the use of capture antibodies directed against adult excretory/secretory (E/S) antigens.

Studies using anti E/S capture antibodies for the detection of coproantigens of *T. solium* in humans (Maass *et al.*, 1992), *T. hydatigena* (Deplazes *et al.*, 1990) and *E. granulosus* (Deplazes *et al.*, 1992; Walters and Craig, 1992 and Malgor *et al.*, 1997) in dogs and *E. multilocularis* in foxes (Morishima *et al.*, 1998) showed that the assay had a high degree of sensitivity and specificity.

Walters and Craig (*loc.cit*) found that coproantigen detection was two and half times more sensitive than serum antibody detection.

Monoclonal antibodies offered a higher degree of species specificity for cestode coproantigen assays.

A sandwich ELISA using monoclonal antibody against the parasite of interest was used in detection of coproantigens of *E. multilocularis* in dogs (Kohno, 1991; Kohno *et al.*, 1995; Sakashita *et al.*, 1995 and Furusawa, 1997) and in foxes (Nonaka *et al.*, 1996 and 1998). It was shown that the coproantigen ELISA was more sensitive and specific than the egg detection assay. Detection of coproantigen using monoclonal antibodies was a more effective diagnostic method than detection of antibody because of its capacity to diagnose infection during the prepatent period (Sakashita *et al.*, *loc.cit*).

2.5.3 Nematodes

For gastrointestinal nematode infections there are only a few specific examples of coproantigen immunodiagnostic tests.

Ellis *et al.* (1993) developed a capture ELISA that could identify *Haemonchus contortus* antigens in the faeces of sheep naturally infected with the parasite. The assay could detect all infected sheep and was specific for *H. contortus.*

The possibility of coproantigen detection in the diagnosis of strongyloidosis was investigated in rats experimentally infected with *Strongyloides ratti* using a capture ELISA. The result showed that the test was specific showing only a low cross reactivity (Nageswaran *et al.*, 1994).

Johnson *et al.* (1996) described a sandwich ELISA assay for the quantitative detection of E/S antigens liberated by intestinal nematode *Heligmosomoides polygyrus* and voided in the faeces of mice. The assay was proved to be highly sensitive and specific. It detected the parasite antigens in the faeces stored for eight weeks at -20°C, 4°C or 20°C and could detect prepatent infections.

2.5.4 Protozoa

Detection of coproantigens for immunodiagnosis of human protozoan infections like entamoebosis and giardiosis has been developed.

Jain *et al.* (1990) developed a simple, sensitive and stable ELISA for the detection of coproantigens in the faeces of individuals with intestinal entamoebosis caused by *Entamoeba histolytica*. In the test, all trophozoite

positive patients and 40 per cent of cyst passers showed the presence of coproantigens while all the healthy individuals were negative for coproantigens.

Shewy *et al.* (1999) detected a 18 to 20 kDa *Cryptosporidium parvum* coproantigens in the stool of infected humans and calves which could be used for specific diagnosis of cryptosporidiosis in human and calves.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Prevalence of paramphistomatidosis

Specimen registers of the University Veterinary Hospitals at Mannuthy and Kokkalai and that maintained at the Department of Veterinary Parasitology, College of Veterinary and Animal Sciences, Mannuthy were referred. The number of bovine faecal samples and the number of faecal samples positive for amphistome eggs were noted for the period from June 1999 to May 2000. The period of minimum and maximum prevalence of the infection was also observed.

3.2 Collection of flukes

Live flukes from the rumen were collected in chilled saline from infected cattle sacrificed at the local slaughter house, Thrissur. *Gastrothylax crumenifer* flukes were identified based on gross morphology and pressing the flukes gently in between two microslides as described by Nath (1987) and separated from other flukes in the laboratory.

3.3 Preparation of somatic antigens from flukes

The preparation of somatic antigens from *G. crumenifer* was done as per Jithendran *et al.* (1996).

1. Washed the collected flukes in several changes of chilled phosphate buffered saline (PBS) pH 7.2 to remove the debris.
2. Ground them over the ice using sterile pestle and mortar.
3. Sonicated the material (60S, 40W, 80% pulse) (Deplazes *et al.* (1990)

4. Centrifuged the material at 10000xg in a REMI C 24 cooling centrifuge at 4°C for 30 min.
5. Collected the supernatant fluid containing somatic antigens and stored in separate aliquot s at -20°C until use. The sediment at the bottom was discarded.
6. The protein content of the somatic antigen was determined by Biuret method (Weichselbaum, 1946) by using photometer 5010 (Boehringer Mannheim).

3.4 Raising hyperimmune sera against somatic antigens in rabbits

The production of rabbit hyperimmune sera was carried out as described by Johnson *et al.* (1996).

3.4.1 Animals

Two six-month-old male Newzealand White rabbits were used for raising hyperimmune sera.

Rabbits were maintained in a rabbit cage and fed with compound feed obtained from Small Animal Breeding Station, KAU, Mannuthy and green grass. They were maintained for two months.

3.4.2 Preparation of inoculum

The antigen suspension was emulsified with equal volumes of Freund's complete adjuvant for the first injection and Freund's incomplete adjuvant for the successive booster doses.

The sensitizing inoculum contained 2.0 mg protein and subsequent inoculum contained 1.0 mg protein.

3.4.3 Immunization of rabbits

Both rabbits were inoculated subcutaneously with 1 ml of inoculum over the shoulder, followed by three booster doses at two weekly intervals. The rabbits were bled two weeks after the final booster dose and the serum was separated from the blood and stored at -20°C in sterile storage vials. The sera were tested for antibodies by agar gel precipitation test (AGPT).

3.4.4 Agar gel precipitation test (AGPT)

AGPT was done as per the method described by Kagan and Norman (1976).

3.4.4.1 Reagents

Agarose	- 0.8 gm
Sodium chloride	- 0.8 gm
Phenol	- one drop
Distilled water	- 100 ml

Staining solutions

Amido black 10 B	- 1 gm
Sodium chloride	- 8.5 gm
Distilled water	- 1000 ml

Decolouriser-I

Methanol	- 40 parts
Acetic acid	- 10 parts
Distilled water	- 10 parts

Decolouriser-II

Absolute alcohol	- 35 parts
Acetic acid	- 5 parts
Distilled water	- 10 parts

3.4.4.2 Test proper

The melted agarose about 2 to 3 ml was poured onto glass slides and allowed to solidify. Wells were cut in a three well pattern. The central well was loaded with the prepared somatic antigen of *G. crumenifer* and the peripheral wells were loaded with anti-somatic antigen of *G. crumenifer* hyperimmune serum raised in rabbits and normal serum of rabbits. After charging, the slides were kept in a humid chamber at room temperature for 48 h and examined against light for the development of any precipitin line.

3.4.4.3 Staining

The slides were washed after soaking in two changes of normal saline for 24 h each and then in distilled water for another 24 h to remove unreacted proteins. The slides were dried by clamping them between moist filter paper over night at 37°C. After drying, they were stained with amidoblack

for 15 min and decolourised in solution I and II for 20 min each. The slides were then dried at 37°C for 1 h and mounted in DPX.

3.5 Collection of sera

Sera were collected from 100 cattle with gastrothylacosis sacrified at the slaughter house, Thrissur as described by Ahmad and Nizami (1998).

1. Venous blood was collected from jugular vein of infected animals at the time of slaughtering and placed in a slanting position for 2 h at room temperature.
2. The blood samples were then stored at 4°C overnight.
3. After centrifugation at 2000xg for 10 min, the sera were collected in sterile vials and stored at -20°C until further use.

3.6 Collection of faecal samples and preparation of coproantigen

Faecal samples of the same 100 cattle infected with gastrothylacosis, from which the sera were obtained, were collected just before slaughter in small plastic vials. The coproantigens were prepared by the method described by Ahmad and Nizami (1998).

1. Faecal materials were mixed with carbonate-bicarbonate buffer pH 9.6 (coating buffer) at a ratio of 1:10 and shaken vigorously until a slurry was formed.
2. This was centrifuged at 2000xg for 30 min in order to get a clear supernatent which was retained and stored at -20°C.

The faecal supernatent was used as the source of antigens. The protein concentration was determined by Biuret method (Weichselbaum, 1946) by using Photometer 5010 (Boehringer Mannheim).

3.7 Immunodiagnostic tests

The diagnosis of gastrothylacosis in cattle was carried out by Enzyme linked immunosorbent assay (ELISA) using coproantigens and serum antibodies.

3.7.1 Enzyme linked immunosorbent assay using coproantigens

3.7.1.1 Buffers and reagents

During the course of study Borosil brand of glasswares, laxbroplastics and analytical or guaranteed reagent grade of chemicals were only used.

The materials were processed using standard methods (Hoskins, 1967) and sterilized either in hot air oven or in autoclave depending upon the material to be sterilized.

1. Phosphate buffered saline (PBS) 10x concentrated solution

Sodium chloride	- 80 gm
Potassium chloride	- 2 gm
Disodium hydrogen phosphate	- 11.3 gm
Potassium dihydrogen phosphate	- 2 gm
Distilled water	- 1 litre

2. Phosphate buffered saline - working solution, pH 7.2

PBS (10x)	- 100 ml
Distilled water to make	- 1 litre

3. PBST - wash buffer, pH 7.2

PBS (10x)	- 100 ml
Tween 20	- 500 µl
Distilled water to make	- 1 litre

4. Carbonate-bicarbonate buffer, pH 9.6

Sodium carbonate (Na_2CO_3)	- 1.59 gm
Sodium bicarbonate ($NaHCO_3$)	- 2.93 gm
Distilled water	- 1 litre

5. Bovine Serum Albumin (BSA) - 2 per cent

BSA (Hi-media)	- 2 gm
Distilled water	- 100 ml

6. Substrate solution

3,3', 5,5' - Tetramethyl benzidine (TMB/H_2O_2 Genei, Pvt., Ltd.) was used as substrate and diluted 20 folds just before use.

7. IN sulphuric acid - stopping reagent

3.7.1.2 Standardisation

The optimum concentrations of the coproantigen prepared, anti-somatic antigen hyperimmune serum raised in rabbits and Goat anti-rabbit IgG-HRP conjugate (Genei, Pvt., Ltd.) were standardised by the checker board titration. Optimum dilution of the reagents which gave the highest titre

with the positive sample and lowest titre with negative sample was selected as the working dilution for the present study. The optimum concentration of coproantigen prepared, anti-somatic hyperimmune serum and Goat anti-rabbit IgG-HRP conjugate were found to be 1:64, 1:100 and 1:1000 respectively.

3.7.1.3 Test proper

Enzyme linked immunosorbent assay was performed essentially as described by Ahmad and Nizami (1998) with minor modifications.

1. The ELISA plates (96 wells flat bottom micro titre plate - Tarsons) were coated with 50 µl of coproantigen suspected faecal supernatants prepared, diluted to 1 in 64 in carbonate-bicarbonate buffer and incubated at 4°C overnight.
2. The plates were then washed by emptying, filling with PBST and leaving for three minutes. This process was repeated three times.
3. Then, 100 µl of two per cent BSA was added to each well to block the unreacted sites in the wells and incubated for one hour at 37°C.
4. After incubation, the plates were washed with PBST as above and dried by tapping gently against filter paper (Whatman No.1).
5. Then, 50 µl, of 1:100 diluted anti-somatic antigens hyperimmune serum raised in rabbits was added to each well and incubated at 37°C for one hour.
6. The plates were washed again with PBST as above.

7. Then, the wells were charged with 50 µl of 1:1000 diluted Goat anti-rabbit IgG-HRP conjugate (Genei, Pvt., Ltd.) and incubated at 37°C for one hour.
8. The wells were washed once with PBST.
9. Then, 100 µl of 20 fold diluted TMB/H_2O_2 (Genei, Pvt., Ltd.) was added to each well and incubated at room temperature in dark for 20 min.
10. Hundred microlitres of 1N sulphuric acid was added to each well to stop the reaction and plates were read in a multiskan MS ELISA reader at 450 nm. HRP control well was blanked to read the samples.

3.7.1.4 Interpretation of results

The samples having an optical density (OD) value above the sum of mean of negative controls and three times the standard deviation were taken as positive.

3.7.2 Enzyme linked immunosorbent assay using serum antibodies

3.7.2.1 Buffers and reagents used were the same as used for the previous test

3.7.2.1.1 Standardisation

Optimum concentration of the somatic antigen prepared, test serum samples and the Rabbit anti-bovine IgG-HRP conjugate (Genei, Pvt., Ltd.) were standardised by the checker board titration. Optimum dilution of the reagents which gave the highest titre with positive serum and lowest titre with negative serum was selected as the working dilution for further use. The

optimum dilution of somatic antigens prepared, test serum samples, Rabbit anti-bovine IgG-HRP conjugate, were found to be 1:200, 1:100 and 1:1000 respectively.

3.7.2.3 Test proper

An indirect ELISA was performed as per the method described by Craig *et al.* (1995) and Anderson *et al.* (1999).

1. The ELISA plates (96 wells flat bottom microtitre plate - Tarsons) were coated with 50 µl of somatic antigen prepared, diluted to 1 in 200 in carbonate-bicarbonate buffer and incubated at 4°C overnight.
2. The plates were then washed by emptying, filling with PBST and leaving for 10 min. This process was repeated three times.
3. Then, 100 µl of two per cent BSA was added to each well to block the unreacted sites in the wells and incubated for one hour at 37°C.
4. After incubation the plates were washed with PBST as above and dried by tapping gently against the filter paper (Whatman No.1).
5. Then, 50 µl of 1:100 diluted bovine serum samples were added to each well and incubated at 37°C for one hour.
6. The wells were washed again with PBST as above.
7. The wells were charged with 50 µl of 1:1000 diluted Rabbit anti-bovine IgG-HRP conjugate (Genei, Pvt., Ltd.) and incubated at 37°C for one hour.
8. The wells were washed three times with PBST and three times with distilled water.

9. Hundred microlitres of 20 fold diluted TMB/H_2O_2 (Genei, Pvt., Ltd.) was added to each well and incubated at room temperature in dark for 20 min.
10. The reaction was stopped by adding 100 µl of 1N sulphuric acid.

The plates were read in a multiskan MS ELISA reader at 450 nm. HRP control well was blanked to read the samples.

3.7.2.4 Interpretation of results

The samples having OD value above the sum of mean of negative controls and three times the standard deviation were taken as positive.

RESULTS

4. RESULTS

4.1 Prevalence of paramphistomatidosis

The prevalence of amphistome infection in bovines determined by referring to specimen registers revealed that faecal samples of a total of 1534 bovines that suffered from nonspecific enteritis, anorexia, stunted growth and parasitism were examined at the hospitals. Out of these, 253 (16.5 per cent) samples were found to be positive for amphistome eggs.

The maximum prevalence (23 per cent) was observed during the months of June and July 1999 and the minimum prevalence (10 per cent) was observed during the months of January 2000 (Table 1).

4.2 Collection of flukes

The flukes were collected from infected cattle, sacrificed at local slaughter house, Thrissur. *G. crumenifer* were identified based on morphology as described by Soulsby (1982) and Nath (1987). They were red in colour when collected. Body was cylindrical to round with a small oral sucker and a large ventral sucker which had a raised border. By pressing the flukes gently in between two microslides, crossing of the uterus from one side to the other was clearly visible.

Table 1.

Prevalence of paramphistomatidosis in the University Hospitals of KAU from June 1999 to May 2000

Months	Faecal samples		Percent positive
	Number examined	Number positive	
June 1999	128	29	23*
July 1999	98	23	23*
August 1999	144	29	20
September 1999	126	24	19
October 1999	114	16	14
November 1999	119	19	16
December 1999	94	16	17
January 2000	128	13	10**
February 2000	144	27	19
March 2000	154	20	13
April 2000	144	17	12
May 2000	141	20	14
Total	1534	253	16.5

* Period of maximum prevalence

** Period of minimum prevalence

4.3 Somatic antigens of *G. crumenifer*

The protein content of the somatic antigens that were prepared from *G. crumenifer* was found to be 4 mg/ml.

4.4 Raising hyperimmune serum in rabbits

The immunized rabbits were bled on the 56th day after the first injection and the sera were subjected to AGPT.

4.4.1 Agar gel precipitation test (AGPT)

After 48 h a clear single precipitin line was observed between the wells charged with somatic antigens and anti-somatic antigen hyperimmune serum raised in rabbits. No precipitin line was observed between wells charged with somatic antigens and control serum (Plate I, Fig.1).

4.5 Collection of sera

Sera were collected from 100 cattle known to be infected with *G. crumenifer* sacrificed at the local slaughter house, Thrissur.

4.6 Collection of faecal samples and preparation of coproantigens

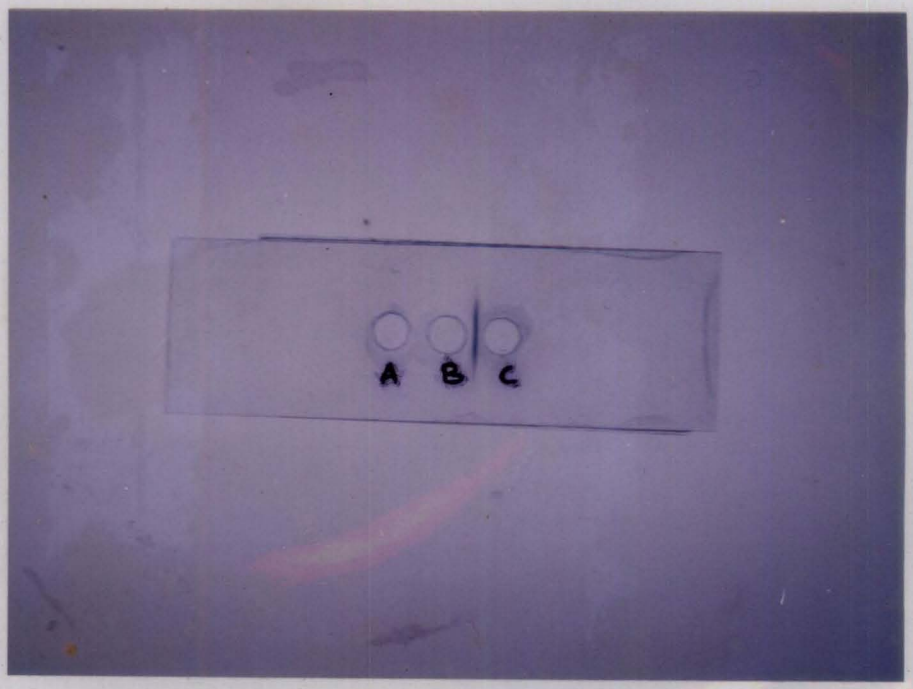
Faecal samples were collected from the same 100 known *G. crumenifer* infected cattle, from which sera were obtained.

The protein content of the faecal supernatant containing the coproantigens was found to be 1 mg/ml.

Fig. 1. Agar gel precipitation test

A – Control serum
B – Somatic antigens of *G. crumenifer*
C – Hyperimmune serum against somatic antigens of *G. crumenifer*

PLATE I



4.6.1 Storage of faecal supernatant

Coproantigens were detected in faecal supernatants stored up to 8 weeks at -20°C.

4.7 Immunodiagnostic tests

4.7.1 Enzyme linked immunosorbent assay using coproantigens

An indirect ELISA was used to detect coproantigens in the faecal supernatant of 100 known *G. crumenifer* infected cattle. The cut-off point was determined by the sum of the mean optical density (OD) value of the negative controls and three times the standard deviation. The sample was considered positive for coproantigens if their OD value was higher than the cut-off point. Using this criterion, 74 faecal samples were found to be positive for coproantigens as tested by ELISA. The sensitivity of ELISA was 74 per cent (Plate II, Fig.1) (Table 2).

4.7.2 Enzyme linked immunosorbent assay using serum antibodies

An indirect ELISA was performed to detect serum antibodies in 100 known *G. crumenifer* infected cattle. A positive serum sample was defined as having OD value greater than the sum of the mean negative OD value and three times the standard deviation. Using this criterion, 51 serum samples were found positive for serum antibodies to *G. crumenifer*. The sensitivity of ELISA was 51 per cent (Plate II, Fig.2) (Table 3).

Fig.1. Enzyme linked immunosorbent assay using coproantigens

Row A . 1 – Substrate control
2, 3 – Conjugate control
4 to 6 – Negative controls
7 to 12 – Positive controls

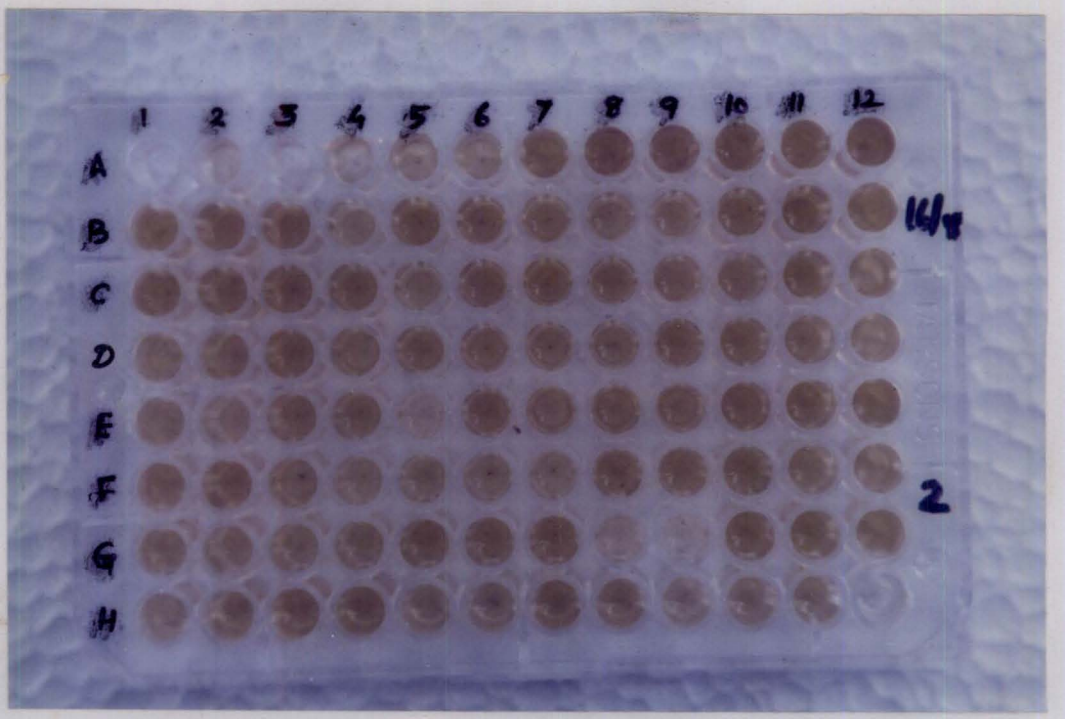
Row B to H, 1 to 12 – Faecal supernatants containing coproantigens (1:64 dilution)

Fig. 2. Enzyme linked immunosorbent assay using serum antibodies

Row A . 1 – Substrate control
2, 3 – Conjugate control
4 to 6 – Negative controls
7 to 12 – Positive controls

Row B to H, 1 to 12 – Serum samples (1:100 dilution)

PLATE II



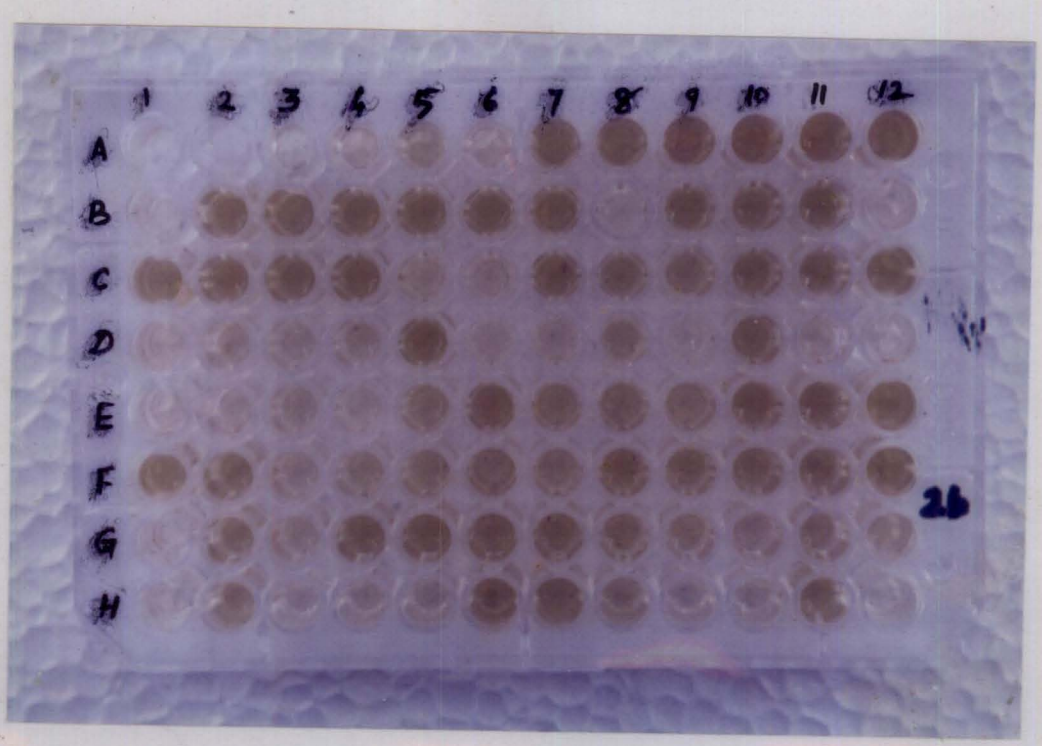


Table 2.

Result of enzyme linked immunosorbent assay using coproantigens in cattle with gastrothylacosis

Sample No.	OD value [1]	Result[2]	Sample No.	OD value [1]	Results[2]
1	0.257	+	26	0.266	+
2	0.397	+	27	0.257	+
3	0.349	+	28	0.274	+
4	0.179	-	29	0.296	+
5	0.253	+	30	0.266	+
6	0.357	+	31	0.235	+
7	0.374	+	32	0.247	+
8	0.272	+	33	0.381	+
9	0.423	+	34	0.441	+
10	0.282	+	35	0.243	+
11	0.415	+	36	0.270	+
12	0.209	-	37	0.275	+
13	0.316	+	38	0.183	-
14	0.353	+	39	0.288	+
15	0.377	+	40	0.276	+
16	0.290	+	41	0.200	-
17	0.197	-	42	0.426	+
18	0.256	+	43	0.262	+
19	0.318	+	44	0.284	+
20	0.241	+	45	0.270	+
21	0.233	+	46	0.313	+
22	0.240	+	47	0.370	+
23	0.380	+	48	0.304	+
24	0.302	+	49	0.335	+
25	0.282	+	50	0.412	+

Contd.

Table 2.

Continued

Sample No.	OD value [1]	Result[2]	Sample No.	OD value [1]	Results[2]
51	0.263	+	76	0.236	+
52	0.192	-	77	0.180	-
53	0.149	-	78	0.217	-
54	0.261	+	79	0.243	+
55	0.265	+	80	0.233	+
56	0.361	+	81	0.202	-
57	0.426	+	82	0.171	-
58	0.412	+	83	0.177	-
59	0.256	+	84	0.180	-
60	0.207	-	85	0.081	-
61	0.272	+	86	0.292	+
62	0.384	+	87	0.237	+
63	0.196	-	88	0.195	-
64	0.332	+	89	0.325	+
65	0.427	+	90	0.342	+
66	0.374	+	91	0.195	-
67	0.442	+	92	0.365	+
68	0.199	-	93	0.189	-
69	0.204	-	94	0.289	+
70	0.351	+	95	0.315	+
71	0.444	+	96	0.080	-
72	0.363	+	97	0.321	+
73	0.193	-	98	0.190	-
74	0.268	+	99	0.200	-
75	0.43	+	100	0.198	-

1 - cut-off point = 0.225
2 - + positive; - negative

No. of positive = 74
No. of negative = 26

Table 3.

Result of Enzyme linked immunosorbent assay using serum antibodies in cattle with gastrothylacosis

Sample No.	OD value [1]	Result[2]	Sample No.	OD value [1]	Results[2]
1	0.220	-	26	0.201	-
2	0.367	+	27	0.218	-
3	0.309	+	28	0.213	-
4	0.321	+	29	0.330	+
5	0.331	+	30	0.194	-
6	0.403	+	31	0.206	-
7	0.325	+	32	0.196	-
8	0.160	-	33	0.096	-
9	0.309	+	34	0.387	+
10	0.334	+	35	0.216	-
11	0.348	+	36	0.481	+
12	0.332	+	37	0.141	-
13	0.337	+	38	0.207	-
14	0.389	+	39	0.212	-
15	0.385	+	40	0.212	-
16	0.451	+	41	0.194	-
17	0.418	+	42	0.341	+
18	0.179	-	43	0.201	-
19	0.420	+	44	0.187	-
20	0.210	-	45	0.210	-
21	0.131	-	46	0.346	+
22	0.438	+	47	0.299	+
23	0.375	+	48	0.403	+
24	0.403	+	49	0.299	+
25	0.207	-	50	0.349	+

Contd.

Table 3.

Continued

Sample No.	OD value [1]	Result[2]	Sample No.	OD value [1]	Results[2]
51	0.161	-	76	0.189	-
52	0.174	-	77	0.170	-
53	0.176	-	78	0.355	+
54	0.161	-	79	0.150	-
55	0.177	-	80	0.125	-
56	0.384	+	81	0.173	-
57	0.327	+	82	0.181	-
58	0.316	+	83	0.325	+
59	0.359	+	84	0.192	-
60	0.348	+	85	0.310	+
61	0.142	-	86	0.146	-
62	0.379	+	87	0.196	-
63	0.180	-	88	0.210	-
64	0.430	+	89	0.380	+
65	0.307	+	90	0.457	+
66	0.319	+	91	0.210	-
67	0.358	+	92	0.328	+
68	0.149	-	93	0.184	-
69	0.399	+	94	0.431	+
70	0.423	+	95	0.200	-
71	0.428	+	96	0.198	-
72	0.406	+	97	0.340	+
73	0.180	-	98	0.160	-
74	0.446	+	99	0.201	-
75	0.160	-	100	0.240	-

1 - cut-off point = 0.300
2 - + positive; - negative

No. of positive = 51
No. of negative = 49

4.8 Comparison of results of enzyme linked immunosorbent assays using coproantigens and serum antibodies

The comparison of results of ELISA using coproantigens and ELISA using serum antibodies are presented in Table 4.

Out of 100 animals with known *G. crumenifer* infection, 74 animals were positive for coproantigens and 51 animals were positive for serum antibodies. Forty three animals revealed both coproantigens and serum antibodies while 18 animals did not show either of them. Thirty one animals which were negative for serum antibodies were found positive for coproantigens. Eight animals which were negative for coproantigens were found positive for serum antibodies.

Table 4.

Comparison of results of enzyme linked immunosorbent assays using coproantigens and serum antibodies

		ELISA using coproantigens		Total
		Positive	Negative	
ELISA using serum antibodies	Positive	43	8	51
	Negative	31	18	49
	Total	74	26	100

DISCUSSION

5. DISCUSSION

5.1 Prevalence of paramphistomatidosis

During the present study out of 1534 faecal samples from bovines, 16.5 per cent animals were found positive for amphistome eggs. Generally the infection was prevalent throughout the year with a slight increase (23 per cent) during the months of June and July.

This observation is in agreement with Moghoe (1945) who reported that amphistome infection in bovines existed throughout the year, in a survey conducted in Central Province, Berar and Central India.

But the observations of the present study differ from that of Nath (1987) who detected amphistome infection throughout the year with peak infections during the South West monsoon (July to August) (38.1 per cent) and North East monsoon (September to November) (34. 5 per cent) in Kerala, observing a definite relationship between the infection and season.

The slight increase in the prevalence of paramphistomatidosis during the months of June and July noted in the present study does not appear significant for the following reasons. The records may not be a true indicator of infection in the area. There are chances of many cases of paramphistomatidosis going unnoticed or unrecorded. Faecal examination is not a reliable tool for the detection of immature amphistomosis when flukes are still immature and will not lay eggs. Moreover, no postmortem examination was done in these cases.

But this observation truly reflects on the extent of infection, nature of carrier animals and chances of susceptibility in young animals in and around Thrissur, Kerala.

5.2 Morphology of *Gastrothylax crumenifer*

The gross morphology of the *G. crumenifer* flukes collected was similar to that described by Soulsby (1982) and Nath (1987). Nath (*loc.cit*) studied about the various species of amphistome flukes infecting domestic ruminants in Kerala and reported that *G.crumenifer* was the only species of genus *Gastrothylax* encountered in Kerala, having the highest prevalence.

5.3 Enzyme linked immunosorbent assay using coproantigens

An indirect ELISA was performed to detect coproantigens in 100 known *G. crumenifer* infected cattle. Seventy four animals were found to have detectable levels of coproantigens. The sensitivity of ELISA was 74 per cent.

The results obtained were in accordance with Maleewong *et al.* (1997) who reported that the sensitivity of ELISA was 73.7 per cent in detecting *Paragonimus heterotremus* coproantigens in infected cattle, Moustafa *et al.* (1998) who reported a sensitivity of 75 per cent against *F. hepatica* coproantigens in rats by ELISA and Craig *et al.* (1995) who reported a sensitivity of 76.9 per cent against *E. granulosus* coproantigens in dogs by ELISA.

The present observations are slightly less when compared to the reports of Moustafa *et al.* (*loc.cit*) who detected 83.3 per cent sensitivity against

F. hepatica in mice, Deplazes and Eckert (1996) who recorded 80 per cent sensitivity against *E. multilocularis* in foxes and Deplazes *et al.* (1999) who observed 83.6 per cent sensitivity against *E. granulosus* in dogs, in a coproELISA.

In the detection of coproantigens of *F. hepatica* by Dumenigo *et al.* (1996), Rahman *et al.* (1998 and 1999) and Dumenigo *et al.* (1999), *H. diminuta* by Allan and Craig (1989 and 1994), *T. pisiformis* and *T. solium* by Allan *et al.* (1992), *E. granulosus* by Malgor *et al.* (1997) and Ahmad and Nizami (1998), *E. multilocularis* by Sakashita *et al.* (1995) and Kohno (1995), *S. ratti* by Nageswaran *et al.* (1994) and *E. histolytica* by Jain *et al.* (1990), the sensitivity of ELISA was 100 per cent.

The difference in sensitivity of coproantigen ELISA may be related to the difference in the nature of antigens *viz.*, somatic and excretory/secretory (E/S) antigens used to produce antiparasite antibodies in the test as opined by Allan *et al.* (*loc.cit*). The present study utilized somatic antigens of *G. crumenifer* to raise antiparasite antibodies in rabbits. This might be a reason for the difference in sensitivity when compared to tests utilizing E/S antigens of parasites. There are some evidences as suggested by Fraser and Craig (1997) that improved sensitivity may occur with the use of capture antibodies directed against adult E/S antigens.

The sensitivity of coproantigen ELISA could also be affected by the nature of antiparasite antibodies used in the test like the polyclonal and monoclonal antibodies. Although many scientists like Sakashita *et al.* (*loc.cit*)

and Kohno *et al.* (1995) opined that the lowered sensitivity of ELISA could be due to the use of polyclonal antibodies, Jain *et al.* (1990) reported that polyclonal antibodies could be successfully used for detection of *E. histolytica* coproantigens in human.

Reduced antigen secretion due to the ageing of the parasite could also be one of the factors affecting sensitivity of coproantigen assay as remarked by Allan *et al.* (1992).

Maleewong *et al.* (1997) attributed the difference in sensitivity of ELISA to the fact that antigen products of parasites being exposed to many digestive enzymes in gastrointestinal tract before passage in the faeces. But Rahman *et al.* (1998) objected to this stating that coproantigens were stable in host gastrointestinal tract and that they could achieve 100 per cent sensitivity in coproantigen detection of *F. hepatica* in cattle by ELISA.

Another factor that could affect the sensitivity of coproantigen ELISA is the storage of coproantigens for different periods, at varying temperatures. In the present study the faecal supernatants prepared were stored for eight weeks at -20°C according to Jain *et al.* (1990) who recommended storage of stool extracts at -20°C for delayed screening of *E. histolytica* coproantigens in human.

It was noteworthy that in the present study coproantigens could be detected in faecal supernatants stored up to eight weeks at -20°C concurring with the results of Deplazes *et al.* (1990) and Craig *et al.* (1995) who reported

the stability of coproantigen for weeks under various storing conditions i.e. 4°C, room temperature or deep freezing.

Allan *et al.* (1992) claimed that coproantigen assay for detection of *Taenia* and *Echinococcus* spp. were found to be reliable even after storage of faecal samples for six months, although five per cent formalin was used to preserve the materials. Further analysis to elucidate fully, the use of formalin fixed samples in coproantigen assays has also been putforth. However, Johnson *et al.* (1996) reported that faecal samples could be stored for a period up to eight weeks at -20°C, 4°C and 20°C without addition of formalin in detecting coproantigens of *H. polygyrus* in mice.

Another factor which could affect the sensitivity of coproantigen assay is the sampling procedure i.e. the portion of faeces taken for assay. According to Allan *et al.* (*loc.cit*) the non-uniform distribution of antigens in the faeces is said to interfere with the interpretation of results since the concentration of antigens depend on the portion of faeces taken. He minimised this variation in the distribution of antigens, in his study to detect *T. pisiformis* coproantigens in dogs, by mixing thoroughly the whole faeces excreted during the previous 24 h before assessment of coproantigens.

Yet another factor affecting the sensitivity of coproantigen ELISA is the consistency of faeces. Jain *et al.* (1990) reported that coproantigen assay gave specific results in detection of *E. histolytica* in human, even if the diarrhoeic stool specimens contain blood which is common in amoebic dysentery, while Dumenigo *et al.* (1996) who studied the consistency of faeces

and level of antigen in faeces in detecting *F. hepatica* coproantigens in cattle observed no relationship between them.

5.4 Enzyme linked immunosorbent assay using serum antibodies

An indirect ELISA using adult fluke somatic antigens was performed to detect serum antibodies for diagnosis of gastrothylacosis caused by *G. crumenifer* in cattle. The sensitivity of ELISA was found to be 51 per cent in the present study.

Results obtained in the present study appear to be lower when compared to Hafeez and Avsatthi (1987) who found 100 per cent sensitivity in detecting serum antibodies to *Gigantocotyle explanatum* in naturally infected cattle by immunoelectrophoresis using whole worm extract. A sensitivity of 90 per cent was obtained by Hafeez *et al.* (1987) in detecting serum antibodies against *P. epiclitum* in experimentally infected sheep by immunoelectrophoresis using adult fluke somatic antigens. Varma *et al.* (1991) supported them by reporting a sensitivity of 91.17 per cent in investigating immune response to *P. cervi* by IHA using adult fluke somatic antigens.

The sensitivity of ELISA could also be affected by the nature of antigens like somatic and E/S antigens. According to Farrel *et al.* (1981) an improved sensitivity of ELISA could be achieved by using partially purified somatic antigens of *F. hepatica*. Welch *et al.* (1987) and Anderson *et al.* (1999) reported an increase in sensitivity of ELISA using E/S antigens in the diagnosis of *F. hepatica* in cattle.

Though the reasons of low sensitivity of serum antibody ELISA in the present study are not clear, the formation of circulating immune complexes, parasite induced immunosuppression, nutritional status or possible antigenic strain variation might be the factors involved in lowering antibody levels, corroborating the findings of Craig *et al.* (1995).

5.5 Comparison of results of Enzyme linked immunosorbent assays using coproantigen and serum antibodies

At present no report is available on the comparison of coproantigen ELISA and serum antibody ELISA in the diagnosis of gastrothylacosis in cattle.

In the present study coproantigen ELISA had a higher sensitivity (74 per cent) than serum antibody ELISA (51 per cent).

From the results, it is observed that 43 cattle were positive for both coproantigens and serum antibodies which reflects the possibility of patent infections. Eighteen cattle were negative for both coproantigens and serum antibodies which possibly reflects the limitations of the assays discussed as above.

Thirty one animals which were negative for serum antibodies were found positive for coproantigens. This indicates the advantages of coproantigens that they are devoid of immune complex formation and parasite induced immunosuppression which might have interfered with the detection of serum antibodies.

Eight animals which were negative for coproantigens were found positive for serum antibodies. This observation suggests the possibility of a previous exposure as remarked by Craig *et al.* (1995).

So the present study upholds the feasibility of coproantigen detection in the diagnosis of gastrothylacosis caused by *G. crumenifer* in cattle. Though much work remains to be done in the assessment of such technique as a diagnostic tool, the results indicate the need to develop coproantigen assay for use in detection of paramphistomatidosis of domestic animals.

Diagnosis during the prepatent period is one of the advantages of coproantigen assays and it has been widely discussed for many species of parasites. The findings of the present study encourage research in detection of coproantigens for diagnosis of immature amphistomosis when faecal examination does not reveal any egg.

Research is to be carried out in clarifying the nature of antigens of amphistome species and coproantigens present in the faeces. Avsatthi *et al.* (1986) reported that there were common antigens shared by many amphistome species, which may interfere in the diagnostic tests of the disease. But in veterinary medicine it is more important to identify animals carrying heavy infections with amphistomes irrespective of their species. The exact identification of the species is not generally necessary as broad spectrum anthelmintics alone can reduce parasite burdens.

The present study indicates that coproantigen assay is relatively easy to handle and further simplification for field use appears to be feasible. However, several questions are to be clarified such as those regarding the lowest quantitative level of infection to be detected and those regarding sensitivity and specificity of the test under field conditions.

SUMMARY

6. SUMMARY

Conventional methods of diagnosis of gastrothylacosis, caused by *Gastrothylax crumenifer*, in bovines like observation of clinical signs, aided by faecal examination for amphistome eggs and serological methods have many limitations. Detection of antigens is a more reliable and suitable method especially if it does not involve collection of blood samples. The present study was undertaken to note the prevalence of paramphistomatidosis in bovines in Thrissur from June 1999 to May 2000, assess the feasibility of coproantigen detection and to compare the sensitivity of enzyme linked immunosorbent assay (ELISA) using coproantigens and ELISA using serum antibodies in the diagnosis of gastrothylacosis in cattle.

It was noted from the registers maintained at the University Veterinary Hospitals, Kokkalai and Mannuthy and that maintained at Department of Veterinary Parasitology, College of Veterinary and Animal Sciences, Mannuthy, that, out of a total number of 1534 faecal samples examined from bovines, 253 (16.5 per cent) animals were found to be positive for amphistome eggs. Generally the infection was prevalent throughout the year though a slight increase in incidence (23 per cent) was noted in the months of June and July.

Hundred cattle known to be infected with *G. crumenifer* brought for slaughter at the local slaughter house, Thrissur were selected at random for the study. *Gastrothylax crumenifer* flukes were identified based on morphology

and separated from the flukes of other species for the preparation of somatic antigens.

The antigens were prepared by grinding and centrifuging at 10000xg at 4°C for 30 min for immunization of rabbits. The protein content of the somatic antigens was estimated and found to be 4 mg/ml. It was stored until use at -20°C.

Hyperimmune serum was raised in rabbits against the somatic antigens prepared. The immune response elicited in rabbits was observed by agar gel precipitation test (AGPT).

The immunodiagnosis of gastrothylacosis in cattle was carried out by an indirect ELISA detecting coproantigens in faecal samples and antibodies in serum.

Faecal samples were collected from the 100 cattle known to be infected with *G. crumenifer*, brought to the slaughter house, Thrissur. They were processed for the preparation of coproantigens. The faecal supernatants containing the coproantigens were stored at -20°C. Similarly blood samples collected from the same cattle were processed and the sera separated and stored at -20°C for the immunodiagnostic tests.

An indirect ELISA was performed to detect coproantigens in faecal supernatents using rabbit hyperimmune serum against somatic antigens of *G. crumenifer*. Seventy four faecal samples were found to contain detectable levels of coproantigens indicating a sensitivity of 74 per cent.

Serum samples collected were tested for antibodies to *G. crumenifer* by an indirect ELISA using somatic antigens. The sensitivity of the assay was 51 per cent.

From the results, it was observed that 43 cattle were positive for both coproantigens and serum antibodies which denotes the possibility of patent infections. Eighteen cattle were negative for both coproantigens and serum antibodies which possibly reflects the limitations of the assay caused by various factors. Thirty one cattle which were negative for serum antibodies were found positive for coproantigens. This indicates the advantages of coproantigens over serum antibodies that they are devoid of immune complex formation and parasite induced immuno suppression. Eight animals which were negative for coproantigens were found positive for serum antibodies. This observation suggests the possibility of a previous exposure.

The results indicate the feasibility of coproantigen detection by ELISA, which appears to have a higher sensitivity than detection of serum antibodies by ELISA, for diagnosis of gastrothylacosis in cattle. However, questions regarding specificity of the test and application of the test in the field are to be clarified.

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IMMUNODIAGNOSIS OF BOVINE GASTROTHYLACOSIS USING COPROANTIGENS

By

A. Kandasamy

Abstract of a Thesis submitted to the
Kerala Agricultural University
in partial fulfil ment of the requirements for the degree of

MASTER OF VETERINARY SCIENCE

in

VETERINARY PARASITOLOGY

College of Veterinary and Animal Sciences
Kerala Agricultural University
Thrissur, Kerala

2000

ABSTRACT

A study was conducted on the prevalence of paramphistomatidosis in Thrissur from June 1999 to May 2000, feasibility of coproantigen detection by ELISA and comparison of sensitivity of ELISA using coproantigens and ELISA using serum antibodies in diagnosis of gastrothylacosis, caused by *Gastrothylax crumenifer*, in cattle.

It was noted from the registers maintained at the University Veterinary Hospitals at Kokkalai and Mannuthy and that at the Department of Veterinary Parasitology, College of Veterinary and Animal Sciences, Mannuthy, that out of a total number of 1534 faecal samples from bovines examined, 253 (16.5 per cent) animals were found to be positive for amphistome eggs with the maximum prevalence (23 per cent) in June and July. Generally the infection was prevalent throughout the year.

An indirect ELISA using rabbit hyperimmune serum against somatic antigens of *G. crumenifer* was performed to detect coproantigens in faecal samples collected from 100 known *G. crumenifer* infected cattle. Seventy four samples were found to contain detectable levels of coproantigen indicating a sensitivity of 74 per cent.

Serum samples collected from the same infected cattle were tested for antibodies to *G. crumenifer* by an indirect ELISA using somatic antigens. Fifty one samples were found positive for antibodies indicating a sensitivity of 51 per cent.

It was seen that when 43 cattle were positive for both coproantigens and serum antibodies, 18 cattle were negative for both of them. Although 31 cattle which were negative for serum antibodies were found positive for coproantigens, eight cattle negative for coproantigens were found positive for serum antibodies.

The results showed that coproantigen detection, which revealed a higher sensitivity than the detection of serum antibodies by ELISA, is feasible for the diagnosis of gastrothylacosis in bovines.