

## Haemoprotozoa of cattle in Northern Kerala, India

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**Abstract.** A cross-sectional study was conducted using 150 blood samples collected from apparently normal / healthy crossbred cattle of Northern Kerala, South India, for detection of haemoprotozoan infections using staining techniques (Giemsa and Acridine Orange) and specific PCR. *Theileria* like piroplasms and *Babesia bigemina* were the only protozoan organisms detected in blood smears. Polymerase chain reaction using specific primers revealed amplification of products specific for *Trypanosoma evansi* (34.6%), *Theileria* sp. other than *T. annulata* (16%) and *B. bigemina* (0.6%). The higher prevalence rate of *Trypanosoma evansi* indicated that the subclinical parasitism can be due to higher prevalence of tabanid flies. The study also revealed the presence of a theilerial piroplasm other than *T. annulata* in North Kerala, which needs further investigation.

### INTRODUCTION

Indian cattle population of 185.18 million account for 14.13 per cent of world bovine population (Quinquennial Livestock Census, 2003) which contribute to the production of milk, making the country largest producer of milk in the whole world. But the average milk production per animal is below the world average with main reasons being absence of proper culling, poor nutrition and diseases (Birthal & Jha, 2005). Of the various diseases responsible for poor production, parasitic diseases are causing significant effects on growth and production. A recent estimate of US\$ 498.7 million per annum was calculated as the cost of controlling ticks and tick borne diseases in India (Minjauw & McLeod, 2003). A characteristic feature of many of these vector transmitted haemoparasitic

diseases is that, the animals which recover from acute infection, become carriers, creating a potential source of infection to healthy susceptible population (Callow, 1984). Hence diagnosis of these infections in carrier animals has great epidemiological significance, as they are the sources of infection for the vectors.

Kerala is a small state in the south-western corner of India spreading about 38,863 sq. km. Lying between north latitudes 8°18' and 12°48' and east longitudes 74°52' and 72°22', Kerala is well within the humid equatorial tropics. The seasons of Kerala can be broadly classified into hot and humid summer season from February to May, monsoon season from June to November and the fair season during December and January. The total annual rainfall in the state varies from 380 cm over the extreme northern districts to

about 180 cm to the south. These conditions favour the propagation of the vector ticks and flies. As per the recent compilation, a number of tick species have been reported from the animals of the state (Ghosh *et al.*, 2007). Previous reports of prevalence of haemoparasites from this state are scanty (Ravindran *et al.*, 2002, 2007b). Hence, the present communication focuses on a molecular survey of haemoprotozoan organisms prevalent in healthy crossbred cattle of five districts of Northern Kerala, South India using microscopic and PCR techniques.

## MATERIALS AND METHODS

### Animals and samples

A cross-sectional study (convenient sampling) was conducted using a total of 150 blood samples collected in heparinized eppendorf tubes from apparently normal / healthy crossbred cattle (crossbreds of Jersey, Holstein Fresian or Swiss Brown with the indigenous cattle) of Northern Kerala, 30 each from five districts *viz.*, Wayanad, Malappuram, Kozhikode, Palakkad and Kannur during June to December, 2007. The bovine populations (Quinquennial Livestock Census, 2003) in these districts are as follows; Wayanad (159858), Malappuram (134703), Kozhikode (163404) Palakkad (263763) and Kannur (103694). Six random samples from 5 different localities contributed 30 samples from each district (Figure1). Samples collected from animals were kept by individual households and not from farms. Two peripheral blood smears were also prepared from each animal and were fixed with methanol.

### Staining techniques

#### Giemsa staining

One smear from each animal was stained with Giemsa stain at 1 in 10 dilution for 30 minutes and examined under oil immersion objective of the microscope. A minimum of 5000 RBCs were screened before declaring

negative for presence of any blood protozoan.

#### Acridine Orange staining

Acridine Orange staining was done as per the method of Lauer *et al.* (1981) with slight modifications (Ravindran *et al.*, 2007a). Briefly, blood smears were flooded with 0.01 percent Acridine Orange stain, allowed to act for two minutes and then washed slowly in tap water. The smears were mounted with coverslip and examined when moist, under the 100X magnification of fluorescent microscope (Olympus). A minimum of 50 peripheral fields were screened before declaring negative for presence of any blood protozoan.

#### Isolation of DNA

The blood samples were processed following the method described for detection of *Plasmodium* sp. from human blood (Tirasophon *et al.*, 1991) with slight modifications. Heparinized blood samples (150µl) were treated by 1300µl dehaemoglobinization buffer (0.015% saponin, 3.5mM NaCl and 1mM EDTA) and centrifuged at 12,000 rpm for 2 min. The supernatant was discarded. The pellet was washed once with 750µl of reaction mixture buffer (10mM Tris HCl, 50mM MgCl<sub>2</sub> and 0.01% gelatin) and centrifuged at 6000 rpm for one minute. Again the supernatant was discarded carefully. The final pellet was resuspended in 75µl of distilled water, boiled for 10 min and 10µl from this was used as template for PCR. The blood sample of a day old calf processed by the similar technique was used as negative control in all PCR reactions.

#### PCR reactions

The primer sequences, cycling conditions and the expected size of the amplified products are listed in Table 1. PCR protocols described previously for *Theileria* genus (d' Oliveira *et al.*, 1995), *Theileria annulata* (d' Oliveira *et al.*, 1995), *Trypanosoma evansi* (Chokesajjawattee,

Table 1. Details of PCR conditions and product size of the targeted genes of haemoprotozoan parasites

Parasite	Primers Sequence (5'-3')	Region amplified	Cycle Conditions	Product size (bp)	Reference
<i>Theileria sp.</i>	F-5'AGTTTCTGACCTATCAG 3' R-5'TTGCCCTTAAACTTCCTTG 3'	Small subunit ribosomal RNA gene	94°C-1 min; 60°C-1 min; 72°C- 1 min; 30 cycles	1098	d'Oliveira <i>et al.</i> (1995)
<i>T. annulata</i>	F-5'GTAACCTTTAAAAACGT3' R-5'GTTACGAACATGGGTTT3'	30kDa major <i>T. annulata</i> merozoite surface antigen gene	94°C-1 min; 55°C- 1 min; 72°C-1 min; 30 cycles	721	d'Oliveira <i>et al.</i> (1995)
<i>T. evansi</i>	F-5'TGCAGACGACCTGACGCTACT 3' F-5'CTCCTAGAAAGCTTCGGTG TCCT3'	Repetitive nuclear sequence	95°C- 2 min; 60°C- 2 min; 72°C-2.5 min; 30 cycles	227	Chokesajjawattee (1993), Wuyts <i>et al.</i> , (1994)
<i>B. bigemina</i>	F-5'CATCTAATTTCTCTCCATACCCTCC3' F-5'CCTCGGCTTCAACTCTGATGCCAAAG3'	<i>B. bigemina</i> SpeI-AvaI Restriction fragment	94°C-1 min; 65°C- 1 min; 73°C- 1.5 min; 35 cycles	278	Figueroa <i>et al.</i> (1992)

1993; Wuyts *et al.*, 1994) and *Babesia bigemina* (Figueroa *et al.*, 1992) were followed. For *T. evansi* and *B. bigemina*, genomic DNA of the respective parasites was used as the positive control. DNA isolated from the whole blood of a *T. annulata* infected cattle (from Namakkal, Tamilnadu) was used as positive control for *T. annulata* specific PCR and *Theileria* genus specific PCR.

The PCR reactions were carried out in a 25µl final reaction volume consisting of 0.2 mM dNTP mix, 1.25 U *Taq* DNA polymerase (Banglore Genei), PCR buffer containing 1.5 mM MgCl<sub>2</sub> and template DNA (10 µl). The reactions were performed in a thermal cycler with heated lid (Eppendorf, Germany).

The PCR products were electrophoresed on a 1.5 per cent agarose gel containing ethidium bromide (0.5 µg/ml) using 70V power supply for 90 min and the amplified fragments were visualized by gel documentation system (Alpha Innotech, USA) and photographed.

Results were statistically analysed using Chi square test.

## RESULTS

### Microscopical examination of blood smears

#### Giemsa staining

Examination of Giemsa stained smears revealed *Theileria* like piroplasms in 61 samples and *B. bigemina* piroplasms in 4 samples. Various morphological appearances of theilerial piroplasms were observed. They were thin and thick rod shaped or annular with light staining trailing cytoplasm. All these forms could be detected in almost all the positive smears with annular forms being found less frequently while the thin rod forms the maximum.

#### Acridine Orange staining

Acridine Orange stained smears revealed *Theileria* like piroplasms in 71 samples and *B. bigemina* piroplasms in only four. The nucleus of the protozoa fluoresced green while the cytoplasm with light orange colour. Various morphological appearances of *Theileria* like piroplasms were observed (Fig. 2).

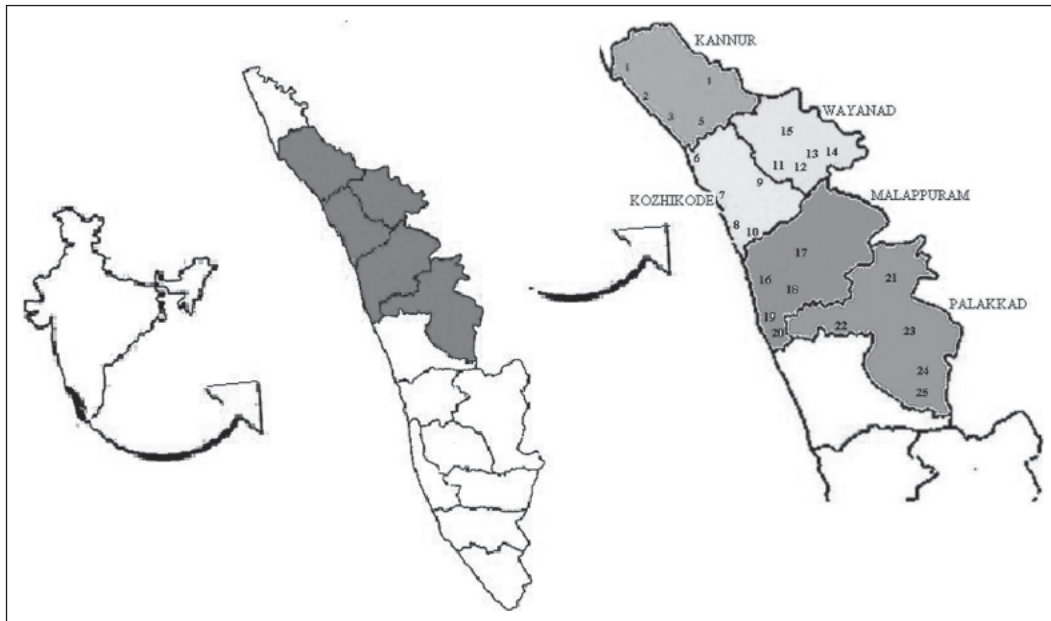


Figure 1. Map of India and Kerala showing the area of collection of blood sample

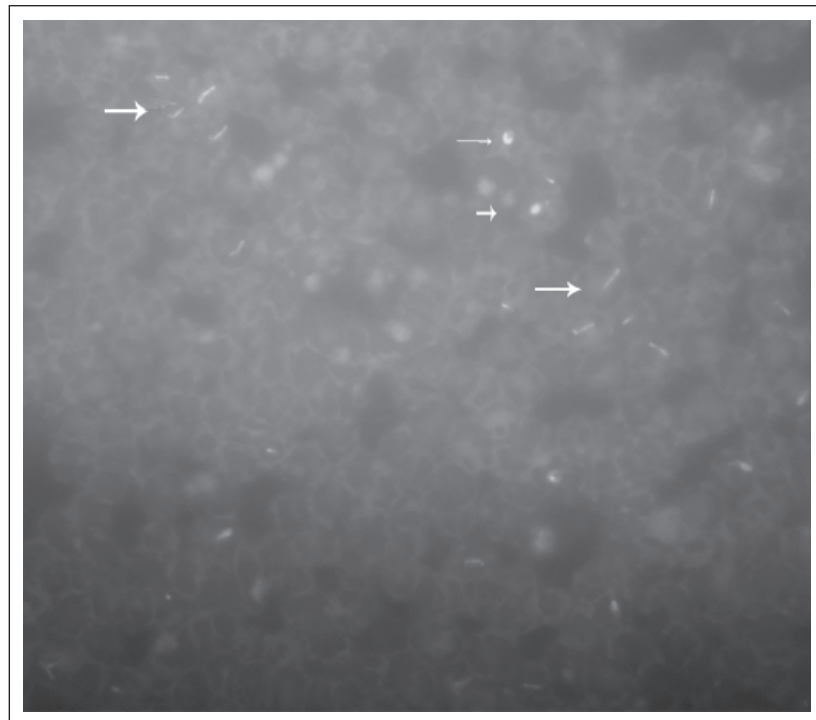


Figure 2. Acridine orange stained blood smear showing *Theileria* like piroplasms  
Rod shaped piroplasms (Bold arrows)  
Annular piroplasms (Thin arrow)

### **Polymerase Chain Reaction**

The specific primers for *Theileria* genus, *T. annulata*, *T. evansi* and *B. bigemina* amplified the PCR products of expected size when the respective positive control DNA was used as template while no amplification was seen with negative control.

Out of the 150 animals screened, 45.3% were identified as carriers for at least one of the haemoprotozoa. Also, among the 150 animals screened, 52 (34.6%) were positive for *T. evansi*, 24 (16%) for *Theileria* genus specific PCR, and one (0.6%) for *B. bigemina*. Ten samples revealed concurrent infection of *T. evansi* and *Theileria* sp. No samples showed amplification specific for *T. annulata*.

### **Comparison of Giemsa, Acridine Orange and PCR techniques**

PCR showed the maximum sensitivity in detecting these organisms. Acridine Orange staining was more sensitive compared to Giemsa staining in detecting haemoparasites. Statistical analysis using Chi square test for comparison of results of microscopical examination (Acridine Orange staining) and PCR for *Theileria* and *T. evansi* revealed significant difference ( $p > 0.05$ ), but no significant difference ( $p < 0.05$ ) was observed when same tests were compared for detection of *B. bigemina*. When the two staining techniques were compared statistically for detection of *Theileria*, non-significant difference ( $p < 0.05$ ) was observed.

Thus, the most important haemoprotozoan organisms affecting cattle of the study area were *T. evansi* and a *Theileria* like organism other than *T. annulata*. Trypanosomosis was the predominant infection followed by theileriosis in all the districts except Malappuram, where reverse pattern was seen.

## **DISCUSSION**

Vector-borne parasitic protozoa are detected and identified by staining and microscopic examination of specimens

from infected host animals and arthropod vectors, by propagating parasites in tissue culture and experimental animals and by characterizing the organisms using isoenzymes and serological reagents (Bose *et al.*, 1995). Each of these methods had drawbacks, making parasite detection problematic and often unreliable. These traditional methods are now supplemented and in many instances replaced by new methods employing molecular techniques (Gasser, 2006). The present study forms the most comprehensive study on epidemiology of blood protozoan infections of dairy cattle of South India employing staining and molecular methods.

Acridine orange staining is an easy and reliable technique, which enables rapid detection of haemoparasites. Eventhough this technique requires costlier equipment; it is more sensitive compared to Giemsa staining technique (Ristic, 1981; Wongsrichanalai *et al.*, 1991; Bose *et al.*, 1995; Damodar *et al.*, 1996). The higher sensitivity of PCR over microscopical methods is well established (Almeria *et al.*, 2001; Khaminsou *et al.*, 2007).

Out of the 150 blood samples screened, 45.3% were found to be carriers for at least one of the haemoprotozoan organism. It was interesting to observe that 34.6% of the studied animals were found to be carriers for *T. evansi*. But, blood smear examination failed to detect *T. evansi* in any of these samples. This indicates the higher sensitivity of PCR for detection of occult trypanosomosis (Wuyts *et al.*, 1994). Cattle are usually sub-clinical carriers of trypanosomosis (Gill 1991). Parasitological diagnosis of chronic or latent trypanosomosis is difficult due to the intermittent or low parasitaemia (Luckins, 1998). The higher prevalence rate observed in this study may be due to the hot humid environmental conditions of the northern districts of Kerala, which favour the survival of tabanid flies.

Microscopy detected *Theileria* organisms in 61 (Giemsa staining) and 71 (AO staining) samples, out of the 150 samples tested. But, *Theileria* genus specific PCR could detect only 24 samples

(16 per cent) as positive. This can be due to the low sensitivity of this PCR because of the large size of amplicon (Wilson *et al.*, 1990). *Theileria annulata* specific PCR product could not be amplified in any of these samples. Hence, the presence of a theilerial piroplasm other than *T. annulata* in North Kerala is very much evident from these findings. *Theileria orientalis*, a benign *Theileria* of cattle was already reported from the state of Maharashtra (Shastri *et al.*, 1988). However, in gross morphology, it is not easy to distinguish the erythrocytic forms of *Theileria* and small *Babesia* (Kreier & Baker, 1987; Irvin 1987). Hence, these uncharacterized piroplasms need further investigation and characterization.

There were no previously confirmed reports of bovine tropical theileriosis from this state, even though the disease is widely prevalent in Northern and Southern India, including neighbouring states like Tamilnadu and Karnataka. This may be due to the absence or low abundance of the principal tick vector, *Hyalomma anatolicum anatolicum* in Kerala (Ghosh *et al.*, 2007). It is suspected that the rod shaped *Theileria* like piroplasms identified in this study, is maintained through domestic-sylvatic herbivore interaction through the widely prevalent *Haemaphysalis* sp. of vector ticks.

The present study revealed a very low carrier status of *B. bigemina* in bovines of Northern Kerala in contrast to the previous reports *viz.*, 67.6% using IFAT (Ravindran *et al.*, 2002) and 70.9% using SELISA (Ravindran *et al.*, 2007b). This may be due to the inherent shortcomings of serological tests like, cross reactions and inability to indicate an existing infection (Montenegro-James & James, 1998). Previous reports of *Babesia bovis* in the country were equivocal (Shastri & Kurundkar, 1981; Gautham & Chhabra, 1983).

Kerala has a cattle population of 2.12 millions and more than 80 per cent of it is constituted by crossbreds (Quinquennial Livestock Census, 2003). The economic loss to cattle farming in Kerala, due to parasitic diseases including helminths,

coccidiosis and haemoparasites was US\$ 27.86 million as per the 2006 estimate (Animal Disease Surveillance Scheme Annual Report, 2006). The high prevalence of haemoprotozoan infections observed in the healthy crossbred cattle, is a matter of concern for states, like Kerala, which had laid emphasis on genetic upgradation of their germplasm for higher production. Obviously, the future of improved management of these disease lies in the understanding of vector, pathogen and host relationship through a pragmatic approach of designing accurate epidemiological tools and diagnostics and introducing proper control measures against such diseases.

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