SURVEILLANCE OF HAEMOPROTOZOAN AND HAEMORICKETTSIAL DISEASES OF CATTLE OF NORTHERN KERALA

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Thesis submitted in partial fulfilment of the requirement for the degree of

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DECLARATION

I hereby declare that the thesis entitled **"SURVEILLANCE OF HAEMOPROTOZOAN AND HAEMORICKETTSIAL DISEASES OF CATTLE OF NORTHERN KERALA"** 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, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that the thesis entitled **"SURVEILLANCE OF HAEMOPROTOZOAN AND HAEMORICKETTSIAL DISEASES OF CATTLE OF NORTHERN KERALA"** is a record of research work done independently by **Dr.Archana. S. Nair.**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, associateship or fellowship to her.

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Introduction

1. INTRODUCTION

India is an agrarian country and animal husbandry is an important subsector of Indian agriculture. India has 185.18 million cattle, which account for 14.13 per cent of world bovine population (Quinquineal Census, 2003). The contribution of livestock sector to gross domestic product (GDP) is 4.36 per cent, which forms about 25 per cent of contribution from agriculture (Basic Animal Husbandry Statistics, 2006). Animal husbandry plays an important and vital role in supplementing family incomes and generating gainful employment in the rural sector, and it is considered as an important component in poverty alleviation programmes.

Diseases are one of the major hurdles in livestock development programmes. Livestock farmers are facing great economic loss due to loss of production or death of animals due to disease outbreaks. Birthal and Jha (2005) stated that diseases and feed scarcity are the major constraints of dairy production. According to the annual report of Project Directorate on Animal Disease Monitoring and Surveillance (2005- 06), the top nine economically important parasitic diseases affecting cattle include babesiosis, toxocarosis, schistosomosis, paramphistomosis, theileriosis, coccidiosis, fasciolosis, anaplasmosis and trypanosomosis and they cause huge economic loss in the form of death or reduction in milk, meat and wool production. The Animal Disease Surveillance Scheme, Kerala estimated the economic loss to cattle farming due to parasitic diseases including helminths, coccidiosis and haemoparasites as 139.32 crores for the year 2006.

The haemoparasitic diseases are mainly vector transmitted with the involvement of ticks or flies. A characteristic feature of many of these infections is that 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 (Das *et al.*, 2002).

It is assumed that about 80 percent of Indian herd is within areas endemic for *Babesia* and *Anaplasma* infections. The economic losses due to these diseases were estimated to be $57 million in India (Tick Cost Version 1.0, 1999). In India, *Theileria annulata* and *Babesia bigemina* constitute the predominant species responsible for widespread morbidity and mortality in cross-bred cattle (Bhatia *et al.*, 2006). Trypanosomosis is also a widely prevalent disease among different domestic and wild animals in India (Gupta *et al.*, 2003).

Annual report (2006) of Animal Disease Surveillance Scheme, Kerala depicts a total of 14578 cases of babesiosis, 1318 cases of theileriosis and 1008 cases of anaplasmosis. This report is based entirely on clinical symptoms. Confirmation by microscopy or other advanced techniques are rare. Proper identification of these infectious agents will help the veterinarians for accurate diagnosis in initiating timely treatment and control measures.

Microscopy remains the 'gold standard' for diagnosis of blood parasites and indeed it is simple, can give rapid results and doesn't require expensive equipment. Microscopic analysis is problematic for unskilled microscopists or when good quality microscopes are not available and parasitaemia is low. Diagnosis by microscopy is labour intensive when a large number of samples need to be screened in a relatively short time, such as during epidemiological studies. Serology can supplement microscopy, but the distinction between carriers and cured patients often remains problematic. Nowadays, Polymerase chain reaction based approaches have become powerful complementary tool to the taxonomists, systematists as well as epidemiologists as it allows the accurate identification of a parasite to the species and / or strain level (Gasser, 1999).

Authentic reports on the prevalence of haemoprotozoan and haemorickettsial organisms are scarce from the state of Kerala (Rejitha, 2003; Gopinath, 2004). For developing an effective disease control strategy, knowledge about the prevalence of disease is crucial. The present study was undertaken to bridge the gap of the information in this regard. Hence, the objective of this research project was to asses the intensity of haemoprotozoan and haemorickettsial organisms prevalent in healthy cross-bred cattle of five districts of Northern Kerala. Thus the study focuses on detection of carrier animals in Northern Kerala which have great significance in the vector borne transmission of diseases. In addition, the study will help in comparing the diagnostic efficacies of polymerase chain reaction and microscopic examination.

Review of Literature

2. REVIEW OF LITERATURE

Globally, parasitic and other endemic diseases continue to be a major constraint on profitable livestock production. They are rarely associated with high mortality and easily identifiable clinical signs and their effects are usually characterized by lower outputs of animal products, byproducts, manure and traction, all contributing to production and productivity losses (FAO, 2004).

2.1 EPIDEMIOLOGY

Of the total world cattle population, about three quarters are at risk to tick-borne diseases, the economic loss associated with tick-borne infections is almost too great to visualize (Purnell, 1979). Ticks and diseases they transmit are widely distributed in tropical and subtropical regions. It has been estimated that 80 per cent of world's cattle population is exposed to tick infestation (FAO, 1984).

2.1.1 **Haemoprotozoan Diseases**

2.1.1.1 *Babesia bigemina*

Babes (1888) first described *Babesia* in blood of African cattle showing haemoglobinuria, while Smith and Kilborne (1893) demonstrated that these parasites were tick transmitted and caused Texas fever.

Walker and Edward (1927) reported that *B. bigemina* was the predominant species in India and was reported from many states.

The vectors widely known in the transmission of bovine babesiosis in the country were *Boophilus microplus* and *Hyalomma anatolicum* (Achuthan *et al.*, 1980).

Rajamohanan (1982) reported *Boophilus annulatus* as the most prevalent cattle tick in Kerala.

Clinical manifestations of an acute babesiosis include fever, anorexia, dullness, weakness, ataxia, haemoglobinuria, icterus, anaemia and presence of intra-erythrocytic parasites (Callow, 1984).

Bovine babesiosis was responsible for considerable economic losses due to mortality, weight loss and reduction in milk yield, besides the costs of prophylactic measures and treatments. More than 500 million cattle world wide were at risk due to babesiosis alone (Ristic, 1988).

2.1.1.2 *Babesia bovis*

Babesia bovis is a small *Babesia* infecting erythrocytes and cause symptoms similar to *B. bigemina* such as high fever, anaemia and haemoglobinuria. But in addition, it cause cerebral babesiosis which results from the tendency of infected erythrocytes to clump, thus blocking blood capillaries of the cerebral cortex. The vectors of *B. bovis* are ticks of the genus *Boophilus* (Purnell, 1979).

Shastri and Kurundkar (1981) detected *B. bovis* piroplasms in impression smears from heart and kidney of a buffalo which died after showing symptoms of haemoglobinuria, fever and anorexia. They were not able to detect *Babesia* piroplasms from the peripheral blood smear when the animal was alive.

Infections caused by *B. bovis* were considered as the most important of all bovine babesial infections in the world, in economic point of view (Ristic, 1981).

2.1.1.3 *Trypanosoma evansi*

Gill (1965) stated that surra was an important disease of domestic animals in most countries of Asia.

Transmission of surra occurs mechanically by biting flies such as *Tabanus*, *Stomoxys* and *Lyperosia*. Trypanosomes did not survive for more than 10 to 15 min in the proboscis of the fly (Soulsby, 1982).

Trypanosoma evansi, the first trypanosome to be demonstrated as pathogenic to domestic livestock was recognized by Griffith Evans in 1880 from the blood of horses and camels in Pakisthan. Trypanosomosis in Indian cattle is underestimated because infection in them is usually sub-clinical. Stress of any kind like hard work, transportation, inclement weather, malnutrition, concurrent infection and vaccination may be responsible for peracute or acute form of the disease. This may lead to outbreak of the disease with 20- 90 per cent mortality in bovines (Gill, 1991).

The pathogenic effects of trypanosomosis were varied including anaemia, immunosuppression, retarded growth, weight loss and poor reproductive performance resulting in delay of oestrus, abortion and still birth (Luckins, 1992).

Saseendranath *et al.* (1993) observed facial oedema, swollen eye lids, conjunctivitis, purulent lacrymal discharge, anaemia, progressive loss of weight, swollen lymph nodes, orchitis and abortion to be the prominent symptoms in experimental *T. evansi* infection in sheep.

Kandavel and Nedunchelliyan (1994) reported that fever was not a constant sign in surra in bovines and parasitaemia was not accompanied by pyrexia.

Animals with subclinical infections of *T. evansi* contributed significantly to the overall morbidity and acted as reservoirs of infection and danger to uninfected, susceptible livestock (Luckins, 1998).

According to Kaur and Juyal (2003), cattle and buffalo acted as reservoir hosts for *T. evansi* due to subclinical nature of the disease.

Joshi *et al.* (2005) reported for the first time the human infection of *T. evansi* in a farmer with intermittent fever, from Maharashtra.

2.1.1.4 *Theileria annulata*

Theileria annulata, transmitted by *Hyalomma* ticks causes tropical theileriosis which is an important disease problem of cattle in tropical countries (Uilenburg, 1981).

Hyalomma anatolicum anatolicum was identified as the principal vector of *T. annulata* and the mode of transmission was trans-stadial (Bhattacharyalu *et al.*, 1990).

The projected annual economic loss due to theileriosis in adult cross-bred cows in India was estimated to be $ 210 million (Singh, 1991).

The carrier animals have an important role in transmission of theileriosis by ticks (d'Oliviera *et al.*, 1995).

Animals recovered from theileriosis show a long lasting carrier status in which low number of erythrocytes remain infected with *Theileria* piroplasms (Urquhart *et al.*, 1996).

Exotic cattle and their cross breds are highly susceptible to tropical theileriosis caused by *T. annulata* and hence it has been interfering with the successful implementation of cross breeding programmes, aimed at enhancing milk production in India (Beniwal *et al.*, 1997).

Though tropical theileriosis was recognized as early as 1905, the economic importance in India was realized only after 1970 when several cases of theileriosis in exotic and cross-bred cattle were recorded (Bhatia *et al.*, 2006).

2.1.1.5 *Theileria orientalis*

Theileria which are of low pathogenicity or avirulent in nature occur on all continents and cause benign theileriosis which are transmitted mainly by *Amblyomma*, *Rhipicephalus* and *Haemaphysalis* ticks (Uilenburg, 1981).

Erythrocytic piroplasms consisting predominantly of elongated rod and pear shaped forms which were considered to be *T. mutans* were recorded from India. But *Hyalomma anatolicum anatolicum* previously considered to be vectors of this *Theileria* species was not involved in its transmission (Shastri *et al.*, 1984).

Shastri *et al.* (1988) serologically confirmed the occurrence of *T. orientalis* from Maharashtra state in India and its transmission by *Haemaphysalis bispinosa*.

For decades, there have been difficulties in the classification of the *Theileria buffeli/ orientalis/ sergenti* group of parasites, which are the benign *Theileria* organisms responsible for oriental theileriosis, due to the unreliability of distinguishing these parasites by morphological criterion (Fujisaki *et al.*, 1994).

T. orientalis is responsible for oriental theileriosis or benign theileriosis, a mild disease characterized by moderate to severe anaemia in heavily parasitized cattle (Radostits *et al.*, 2000).

Theileria buffeli/ orientalis/ sergenti group of parasites though described as benign, are not harmless but the infection causes unthriftness, often with clinical symptoms and even death (Gill, 2006).

Ravindran *et al.* (2006a) reported anaemia as the major clinical symptom of *T. orientalis* infection and described the postmortem lesions as abomasal ulcers, spleenomegaly, massive pulmonary oedema and haemorrhage in heart and duodenum. *Haemaphysalis* species of ticks were identified from infected animals.

2.1.2 Haemorickettsial Diseases

2.1.2.1 *Anaplasma marginale*

Theiler in 1910 first described the intracellular inclusions which occurred in the erythrocytes of African cattle suffering from an acute infectious anaemia and called it *Anaplasma marginale* on the basis of its staining characters. Bovine anaplasmosis is a biologically and mechanically transmitted disease which is widespread throughout the tropical and sub-tropical regions of the world. Carrier cattle and wild deer have been shown to play an important role in epizootiology of anaplasmosis. Transmission of infection from deer to cattle also occurs.Carrier cattle usually do not reveal erythrocytic inclusions in their blood films (Ristic, 1981).

Anaplasmosis is an economically important disease affecting dairy and beef cattle in tropical and subtropical countries and many temperate countries (Drummond *et al.,* 1981).

Sharma (1988) suggested that latent infections of *A. marginale* in sheep constitute a source for spread of *Anaplasma* infection to highly susceptible cross-bred cattle.

Bovine anaplasmosis resulted in the development of mild to severe anemia and icterus without hemoglobinaemia and hemoglobinuria. Clinical symptoms may include fever, weight loss, abortion, lethargy, icterus and often death in animals over 2-year old (Kocan *et al.*, 2003).

Sivaseelan and Anna (2004) observed the gross pathological alterations in dairy cows died due to anaplasmosis like icteric discolouration and pallor of tissues and organs, splenomegaly, hepatomegaly, petechae under epicardium and catarrhal gastroenteritis.

Scoles *et al.* (2005) suggested that tick-borne biological transmission of *A. marginale* was at least two times efficient than direct fly borne mechanical transmission.

2.1.2.2 *Anaplasma bovis* (*Ehrlichia bovis*)

Karunamoorthy *et al.* (1992) observed fever, tremors, incoordination, convulsions, anorexia, pallor, lymphadenomegaly, salivation, lacrimation, grinding of teeth and frequent micturition in a bullock and blood tinged urine and diarrhoea in an aged non-descript buffalo, infected with *E. bovis*.

Symptoms of bovine ehrlichiosis include fever, enlargement of peripheral lymph nodes, apathy, anorexia and loss of weight, clinical signs being more evident in debilitated cattle. In the most characteristic acute form one ear, less often both, is held down over the parotid region and the head is tilted towards that side. The animal shakes its head frequently, as if to get rid of a foreign body, does not eat and is indifferent to its surroundings. Animals which have recovered from acute infections remain as carriers for variable periods and other immunodepressive factors may provoke a relapse (Woldehiwet and Ristic, 1993).

Devada *et al.* (1996) reported a case of ehrlichiosis with spherical intracytoplasmic inclusions in monocytes and lymphocytes of a cow showing clinical signs of fever, anaemia, emaciation and lymphadenomegaly.

The genus *Anaplasma* includes *Anaplasma bovis* which was formerly referred as *Ehrlichia bovis* (Dumler *et al.*, 2001).

Kolte *et al.* (2003) observed pyrexia, stiffness of neck, sudden drop in milk production, inappetance, nervous symptoms and arthritis in *E. bovis* affected buffaloes from Vidharbha, India.

2.1.3 Prevalence

2.1.3.1 Global

2.1.3.1.1 *Babesia bigemina*

Woodford *et al.* (1990) recorded 88 per cent of seroprevalence of *B. bigemina* in Pemba Island, Tanzania which indicates an enzootically stable situation.

Camus and Montenegro-James (1994) reported the prevalence of bovine babesiosis in the islands of Lesser Antilles and occurrence of both *B. bigemina* and *B. bovis*.

Chandrawathani *et al.* (1994) reported that infections with *B. bigemina* and *B. bovis* were widespread in cattle throughout Peninsular Malaysia.

Diagnostic activities done at parasitology laboratory of Veterinary Institute Research in Venezuela, during a period of 15 years (1986-2000) revealed a seroprevalence of 58.8 per cent *B. bigemina* infection (Guillen *et al.*, 2001).

Bell-sakyi *et al.* (2004) detected *B. bigemina* in two third of the blood smears examined from domestic cattle of Ghana.

B. bigemina is widespread throughout tropics and sub-tropical areas like central and South America, North and South Africa, Southern Europe, Australia and Middle east (Bhatia *et al.*, 2006).

2.1.3.1.2 *Babesia bovis*

Woodford *et al.* (1990) detected 96 per cent seroprevalence of *B. bovis* in Pemba Island, Tanzania which indicate an endemic situation.

Perez *et al.* (1994) reported 54 per cent seroprevalence of *B. bovis* and 55.4 per cent *B. bigemina* during the year 1991 in Costa Rica.

Rosignoli *et al.* (2000) reported a fatal outbreak of babesiosis due to *B. bovis* in a beef cattle unit in northern Italy.

Guillen *et al.* (2001) reported a seroprevalence of 47.6 per cent for *B. bovis* infection in Venezuela, during a period of 15 years from 1986 to 2000.

Babesia bovis occurs in cattle and some cervids in Europe, Russia, Africa, central and South America and Iran (Bhatia *et al.*, 2006).

2.1.3.1.3 *Trypanosoma evansi*

Payne *et al.* (1991) observed widespread prevalence of *T. evansi* infection in apparently normal cattle, buffaloes and horses in several areas of Indonesia.

Trypanosoma evansi is widely distributed in India, Pakisthan, Malaya, China, Indonesia, Philippines, Central America, South America, northern Africa, Asia minor, Russia, Egypt, Sudan, Somalia, Israel, Lebanon, Syria, Turkey, Iraq, Iran, Mauritius and Bulgaria (Bhatia *et al.*, 2006).

2.1.3.1.4 *Theileria annulata*

Flach and Ouhelli (1992) studied the epidemiology of tropical theileriosis in an endemic area of Morocco using Giemsa stained blood smears and reported that 47 out of 97 (48.5per cent) cattle carried *Theileria* piroplasms.

A prevalence of 76.5 per cent of *T. annulata* was recorded among cattle in the central area of Saudi Arabia (El-Metenawy, 2000).

Tropical theileriosis is a major constraint to livestock improvement programmes in many parts of the Middle East and Asia and about 200 million cattle are at risk (Radostits *et al.*, 2000).

Aktas *et al.* (2004) examined the *Hyalomma* ticks from cattle in the east of Turkey and reported that 12.6 per cent of the ticks were infected with *T. annulata.*

T. annulata occur commonly in cattle and water buffalo in North Africa, middle and Far East, Russia, Southern Europe, Asia Minor and India (Bhatia *et al.*, 2006).

2.1.3.1.5 *Theileria orientalis*

Benign *Theileria* parasites occur world wide and *Haemaphysalis* ticks act as vectors. European breeds are more susceptible than zebu breeds (Radostits *et al.*, 2000).

A survey of benign *Theileria* infection in cattle revealed a prevalence of 50.4 per cent in Combodia and 27.5 per cent in Vietnam (Inoue *et al.*, 2001).

The prevalence of benign *Theileria* infection in cattle and buffaloes of Thailand was reported by Sarataphan *et al.* (2003).

2.1.3.1.6 *Anaplasma marginale*

Kocan (1994) observed that anaplasmosis was widespread in Oklahoma as a result of increased movements of cattle and poor performance of the complement fixation test in detecting carriers.

Bovine anaplasmosis is common in tropical and subtropical regions and sporadic in temperate regions. Carrier animals are the source of infection. Disease transmitted biologically by ticks, mechanically by tabanid vectors, iatrogenically and transplacentally. Infection in cattle is endemic in areas that support large population of these vectors (Radostits *et al.*, 2000).

Prevalence of *A. marginale* among cattle at the central area of Saudi Arabia was reported as 0.98 per cent using microscopical examination of Giemsa stained blood smears (El-Metenawy, 2000).

Diagnostic activities done at parasitology laboratory of Veterinary Institute Research in Venezuela, during a period of 15 years (1986-2000) revealed a seroprevalence of 50.5 per cent of *A. marginale* infection (Guillen *et al.*, 2001).

Magona and Mayende (2001) reported 13.3 per cent prevalence of *A. marginale* in mixed farms in two districts of Uganda during the year 2000.

2.1.3.1.7 *Anaplasma bovis*

Radostits *et al.* (2000) stated that infection with *E. bovis* transmitted by *Rhipicephalus appendiculatus*, *Amblyomma variegatum* and *Hyalomma truncatum* occur in India and Africa.

Goethert and Telford (2003) reported the occurrence of *A. bovis* in cottontail rabbits of North America.

2.1.3.2 India

Jitendran (1997) reported that cross-bred animals exhibited higher infectivity to blood protista as compared to zebu cattle.

Roy *et al.* (2004) studied the prevalence of haemoprotozoan infections in four districts of Chattisgarh, by screening of blood smears from 1794 suspected animals. Overall prevalence of 22, 20.4 and 8.17 per cent were observed for trypanosomosis, babesiosis and theileriosis repectively. The prevalence was noted to be higher in stall-fed animals as compared to grazing animals. Highest prevalence of the disease was observed during the monsoon followed by the post-monsoon. Also the animals between 1-3 years of age showed maximum prevalence followed by animals between 3-6 years of age.

Gopinath (2004) observed that haemoparasites could be considered as an important etiological factor in development of anaemia in cattle. Majority of haemoparasite infections were observed in cattle above one year of age.

2.1.3.2.1 *Babesia bigemina*

Gautham and Chhabra (1983) reported that out of the four dominant species of *Babesia* in bovines, only *B. bigemina* and *B. bovis* were known to occur in India.

Banerjee *et al.* (1985) observed 28.6 per cent positivity using capillary tube agglutination test for *B. bigemina* infections in buffaloes in Hisar, India. They also observed that the buffaloes rarely suffer from clinical babesiosis and showed no significant change in body temperature and general health conditions.

Bhikane *et al.* (2001) reported that bovine babesiosis occur in cattle of six months to five years, the maximum affected were between two to five years of age.

Higher occurrence of babesiosis infection at the onset of monsoon was reported by Meshram and Kamble (2003), by examination of blood smears from equines, bovines, felines, caprines and canines in Nagpur, India.

Saud *et al.* (2005) observed *B. bigemina* piroplasms in seven out of 100 clinically suspected local hill cattle of Arunachal Pradesh.

Gupta *et al.* (2005) reported an outbreak of babesiosis in a herd of cattle in Jalandhar district of Punjab, after introduction of cross-bred dairy cattle having tick infestation into the herd.

Gupta *et al.* (2006) reported 74 outbreaks of bovine babesiosis in Haryana state during 2001-2003, in which the morbidity rate and cumulative mortality were maximum in rainy season followed by winter and summer.

2.1.3.2.2 *Babesia bovis*

The occurence of *B. bovis* or *B. bovis* like organisms in India is equivocal (Shastri and Kurundkar ,1981; Gautham and Chhabra, 1983; Muraleedharan *et al*,. 1984).

Shastri and Kurundkar (1981) identified *B. bovis* like piroplasms in impression smears of heart and kidney of a buffalo from Maharashtra.

Muraleedharan *et al.* (1984) recorded two clinical cases of *B. bovis* infection in buffaloes from Karanataka through detection of piroplasms in peripheral blood smears.

Muraleedharan *et al.* (2005) recorded the incidence of *B. bovis* infection in four indigenous and two Jersey cross-bred cattle from Karnataka based on Giemsa stained smear examination. Higher incidence was noted during north east monsoon.

2.1.3.2.3 *Trypanosoma evansi*

The livestock all over India particularly in Rajasthan, Haryana, Punjab, Madhya Pradesh, Uttar Pradesh, Maharashtra, Tamil Nadu, Kerala and Andhra Pradesh have been reported to suffer from *T. evansi* infection (Gill, 1991).

Prasad *et al.* (1997) undertook a survey in and around Gudivada of Krishna delta in Andhra Pradesh for the detection of surra in buffaloes. A total of 299 (4.05per cent) were found positive by wet film examination out of 7377 animals screened. The seasonal prevalence of infection recorded was 5.08, 2.78 and 2.20 per cent in rainy, summer and winter season respectively.

Malik *et al.* (2000) opined that poor nutrition aggravates the harmful effects of *T. evansi* infection in buffalo calves.

An outbreak of trypanosomosis which resulted in death of 22 animals was reported from horses of Kampli, Karnataka (Harish *et al.*, 2004). In a herd of 60 horses, 52 exhibited acute form of the disease showing fever, anorexia, edema of dependent parts and staggering gait. The occurrence of disease was correlated to high population of blood sucking tabanid flies in the affected area.

In India, *T. evansi* infection was most common in areas where environment for the breeding of insect vectors like tabanid flies was most suitable. The incidence coincided with considerable rains, floods and inundations (Bhatia *et al.*, 2006).

2.1.3.2.4 *Theileria annulata*

Occurrence of theileriosis among calves and adult cattle at Chennai has been reported by Anandan *et al.* (1983). Calves below three months of age were found suffering from the disease more commonly.

Sinha and Verma (1986) examined blood smears from a total of 442 cows with fever from Ranchi during a period of 1982-1986 and found out 27.8 per cent prevalence of *T. annulata.*

Bansal *et al.* (1987) reported a high prevalence of sub-clinical theileriosis (47-90 per cent) through IFAT in four military dairy farms at Bareilly, Lucknow, Panagarh and Banglore.

Tropical theileriosis caused by *T. annulata* was an important disease of exotic cattle and their cross-bred progeny leading to morbidity and mortality especially in calves causing considerable economic loss (Chandra *et al.*, 2000).

Soundararajan *et al.* (2000) reported seroprevalence of 66.4 per cent in cattle and 41.9 per cent in buffaloes for *T.annulata*, using ELISA in Tamil Nadu.

During the period of 2001 to 2003, 162 outbreaks of theileriosis were recorded in Haryana state (Gupta *et al.*, 2006). Of these, the maximum outbreaks occurred in winter followed by rainy season and the least in summer. But the percent morbidity and cumulative mortality were more in rainy season than winter.

Aulokh and Singla (2006) reported a prevalence of 6.94 per cent of *T. annulata* infection in suspected cattle of Ludhiana.

Soundararajan and Rajavelu (2006) reported an incidence of 28.2 per cent for *T. annulata* in cattle in and around Chennai by blood smear examination and found that the disease occurs more in adult cattle than in calves.

2.1.3.2.5 *Theileria orientalis*

Benign *Theileria* parasite of bovine, *T. orientalis* was reported from Maharshtra, India by blood smear examination and serological test (Shastri *et al.*, 1984, 1988).

Harikrishnan *et al.* (2001) reported the occurence of *T. orientalis* in a calf in Tamil Nadu based on the morphology and failure of transmission through *Hyalomma* ticks.

Screening of blood samples from 375 cattle of ten farms in Karnataka by wet mount technique and Giemsa staining technique revealed 15.2 per cent prevalence of T. *orientalis*. Adults showed higher infection than calves (Ramesh *et al.*, 2003).

2.1.3.2.6 *Anaplasma marginale*

A. marginale infection was found more in cross-bred cattle compared to the indigenous cattle (Yadav *et al.*, 1985; Mallick *et al.*, 1987).

Magesh and Dhanapalan (2001) reported an unusual case of anaplasmosis with occasional clonic convulsions along with fever and anaemia in a cross-bred cow.

Garg *et al.*, (2004) reported 33 per cent prevalence of sub-clinical anaplasmosis in an organized farm out of 182 cross-bred cattle in Haryana through blood smear examination.

Higher incidence of anaplasmosis was noticed in cattle of 4-8 years of age, and that too more in cross-bred cattle (Muraleedharan *et al.*, 2005).

Soundararajan and Rajavelu (2006) reported an incidence of 7.78 per cent of *A. marginale* in cattle in and around Chennai through examination of stained blood smears.

Harish *et al.* (2006) reported an overall prevalence of 6.6 per cent for anaplasmosis out of 11,755 blood samples from cattle, sheep, goats, buffalo, dogs, horses and wild animals from various parts of Karnataka.

2.1.3.2.7 *Anaplasma bovis*

Karunamoorthy *et al.* (1992) reported ehrlichiosis in a bullock and she buffalo from Tamil Nadu.

Sreekumar *et al.* (2000) diagnosed bovine ehrlichiosis in Tamil Nadu by isolation of *E. bovis* in peripheral blood monocyte cultures, IFAT and blood smear examination.

Vijayalakshmi and Sreekrishnan (2005) reported bovine ehrlichiosis in Pondichery

Soundararajan and Rajavelu (2006) reported a prevalence of 0.53 per cent for *E. bovis* out of 150 blood smears of animals examined in and around Chennai.

2.1.3.3 Kerala

2.1.3.3.1 Babesiosis

Carrier status of bovine babesiosis from Wayanad district of Kerala was demonstrated as 67.6 per cent using IFAT (Ravindran *et al.,* 2002) and 70.9 per cent using slide ELISA (SELISA) (Ravindran *et al.*, 2007a).

Rejitha (2003) studied 71 cows brought to veterinary hospitals in Thrissur and Ernakulam districts, Kerala and found *B. bigemina* piroplasms in 12.5 per cent animals while *B. bigemina* antibodies were detected in 54.9 per cent animals by SELISA.

Gopinath (2004) diagnosed babesiosis in 15 out of 50 cows with anaemia through blood smear examination.

Animal Disease Surveillance Scheme, Kerala (2006) recorded 14578 cases of bovine babesiosis based on the clinical symptoms.

2.1.3.3.2 Trypanosomosis

Balakrishnan *et al.* (1994) recorded three clinical cases of canine trypanosomosis from Thrissur district, during the period from 1988 to 1993. All the cases were diagnosed by wet film and blood smear examinations.

Tresamol *et al.* (2003) reported a case of trypanosomosis in a cross-bred cow from a dairy farm in Thrissur district.

Ajithkumar *et al.* (2004) reported a case of trypanosomosis in a Murrah cross-bred she-buffalo with profuse nasal bleeding for four days.

2.1.3.3.3 Theileriosis

Gopinath (2004) reported that four out of 50 anaemic cows examined suffered from theileriosis, based on blood smear and lymph node smear examination.

Epidemiology Report of Animal Husbandry Department, Kerala, 2006 recorded 1318 cases of bovine theileriosis based on the clinical symptoms.

Ravindran *et al.* (2006a) reported mortality among cross-bred cattle of Wayanad due to *T. orientalis* infection.

2.1.3.3.4 Anaplasmosis (*A. marginale*)

Anaplasmosis was diagnosed in 12 out of 50 cows with anaemia through blood smear examination by Gopinath (2004).

Animal Disease Surveillance Scheme, Kerala (2006) recorded 1008 cases of bovine anaplasmosis based on the clinical symptoms.

2.1.3.3.5 Ehrlichiosis (*A. bovis*)

Devada *et al.* (1996) reported a case of ehrlichiosis in a cow by blood smear examination.

Gopinath (2004) diagnosed ehrlichiosis in one cattle out of 50 animals with anaemia.

2.1.3.4 **Mixed infections**

Kandavel *et al.* (1990) reported a fatal case of mixed infection due to *Theileria*, *Babesia* and *Anaplasma* in a 20 day old cross-bred calf from Chennai.

Nalinikumari *et al.* (2000) reported mixed infection of theileriosis and trypanosomosis in a cross-bred cow by examination of stained blood smear.

Harikrishnan (2003) noticed that cross-bred calves that were exposed to *T. annulata* infected *Hyalomma* ticks, developed a mixed infection of *T. annulata* and *B. bigemina*. The development of concurrent infection was assumed to be due to the activation of the latent *B. bigemina* caused by stress associated with the development of theileriosis. Piroplasms of *B. bigemina* were detected only after the appearance of *T. annulata* in the peripheral blood.

Concurrent subclinical babesiosis and anaplasmosis were identified in 4.9 per cent of cross-bred cattle of an organized farm through examination of blood smears (Garg *et al.*, 2004).

Bell-Sakyi *et al.* (2004) reported the increased severity of anaemia in mixed infections of *Anaplasma* and *Babesia* as well as *Anaplasma* and *Theileria* infections in ruminants compared to single infections.

Hofman-Lehman *et al.* (2004) investigated the cause for a fatal infectious disease outbreak in a large cattle herd and found concurrent infections with vector-borne pathogens like *A. marginale*, *B. bigemina* and *T. orientalis*. They

could find that *A. marginale* was the major cause of the haemolytic anaemia while coinfections with other agents exacerbated the disease.

Ramesh *et al.* (2008) detected *B. bigemina* and *E. bovis* in peripheral blood smear of a cow from Tamil nadu.

2.1.4 Molecular epidemiology

Parasite identification procedures using the tools of molecular biology are highly sensitive and specific, with parasite DNA being identified amongst overwhelming quantities of host DNA and these tools are becoming increasingly important in epidemiological studies. As DNA is very stable, provided protein enzymes which may degrade it are inactivated by protein denaturation or freezing, parasite DNA detection techniques becomes more reliable (Prichard, 1997).

The integration of molecular biology into traditional epidemiological research is referred as molecular epidemiology. It provides new tools for studying disease occurrence at the molecular level (Smith, 2006).

2.1.4.1 *Babesia bigemina*

Smeenk *et al.* (2000) reported 35 per cent prevalence for *B. bigemina* infection in bovines using molecular techniques in Zimbabwe.

Almeria *et al.* (2001) reported six per cent prevalence for *B. bigemina* infections in cattle in Minorca, Spain using PCR.

Torina *et al.* (2007) assessed the prevalence of tick-borne pathogens of domestic animals in sicily, Italy during 2003-2005 using serological tests (ELISA and IFAT) and DNA tests (PCR) and found that the most prevalent tick-borne pathogens were *Anaplasma* and *Babesia* species.

2.1.4.2 *Babesia bovis*

Smeenk *et al.* (2000) detected 47 per cent prevalence for *B. bovis* infection in bovines of Zimbabwe using PCR.

Fornelio *et al.* (2003) reported the presence of *B. bovis* in suspected cows of Portugal using PCR.

2.1.4.2 *Trypanosoma evansi*

Solano *et al.* (1999) used PCR as a tool for the survey of bovine trypanosomosis in the agro-pastoral zone of Sideradougou, Burkina Faso and identified an overall prevalence of 11.5 per cent while the prevalence according to the parasitological examination was only 5.3 per cent.

In a survey conducted on apparently healthy water buffaloes of North Vietnam, PCR detected 5 per cent of animals positive for *T. evansi* infection while the seroprevalence was 16 per cent (Holland *et al.*, 2004).

Singh *et al.* (2004) detected 17.05, 9.67, 4.6 and 4.14 percent of prevalence of *T. evansi* infection in camels using PCR, Ag-ELISA, blood smear and wet film examinations respectively.

2.1.4.3 *Theileria annulata*

d'Oliveira *et al.* (1995) detected 75 per cent prevalence of *T. annulata* out of 92 samples of cattle of Spain using PCR.

Almeria *et al.* (2001) found that more than 94 per cent of the examined animals were positive for *Theileria* sp., of which 41.3 per cent were positive for *Theileria annulata* in Minorca, Spain, with the use of PCR.

2.1.4.4 *Theileria orientalis*

Tanaka *et al.* (1993) reported higher sensitivity of PCR for detection of *T. sergenti* than the conventional methods.

Benign *Theileria* organisms were detected in 50.4 per cent cattle in Combodia and 27.5 per cent in Vietnam using allele-specific PCR (Inoue *et al.*, 2001).

The prevalence detected for benign *Theileria* infection in cattle and buffaloes of Thailand was higher (82.6 per cent) by allele-specific PCR than by microscopical examination (27.5 per cent) of Giemsa stained blood smears (Sarataphan *et al.*, 2003).

Kim *et al.* (2004) conducted molecular epidemiological and phylogenetic studies of benign *Theileria* species in Japan by PCR, amplifying major piroplasm surface protein (MPSP) gene.

2.1.4.5 *Anaplasma marginale*

Hofmann-Lehmann *et al.* (2004) conducted a molecular investigation of a fatal haemolytic anaemia in a herd in Switzerland and identified *A. marginale* as the major cause.

Molecular epidemiological investigation of human and bovine anaplasmosis in Southern Europe suggested that *A. marginale* infections are maintained in cattle and deer, and *A. phagocytophilum* infections may occur in humans and are maintained in cattle, donkeys, deer and birds (Naranjo *et al.*, 2006).

Torina *et al.* (2007) assessed the prevalence of tick-borne pathogens of domestic animals in Sicily, Italy during 2003-2005 using serological tests (ELISA and IFAT) and DNA tests (PCR). The most prevalent tick-borne pathogens were *Anaplasma* and *Babesia* species.

2.1.4.6 *Anaplasma bovis*

Kawahara *et al.* (2006) reported the prevalence of 26 per cent for *A. bovis* in the blood samples of wild deer in Japan, through PCR and DNA sequence analysis.

2.2 DIAGNOSIS

Microscopy remains the 'gold standard' for diagnosis of parasites and indeed it is simple, can be rapid and does not involve expensive equipment. But diagnosis by microscopy is labour intensive, especially when a large number of samples need to be screened as in epidemiological studies. Moreover the sensitivity of detection is low and there is difficulty in discrimination up to the species level of the parasites. In order to overcome some of these difficulties, serological diagnostic techniques have been developed. But, these techniques could not differentiate between current and previous infections. In addition, the cost of producing specific purified antigens is very high and consequently crude antigen preparations are often used, resulting in reduced specificity and sensitivity. Molecular methods have been developed for parasite detection to address some of the problems encountered using conventional diagnostic methods (Singh, 1997).

2.2.1 Staining Techniques

2.2.1.1 Giemsa staining

According to Akinboade and Dipeolu (1984), the failure to observe haemoparasites by blood smear examination in cattle of endemic area could not be considered as absence of infection. They also concluded that blood smears could not monitor the level of infection in animals maintaining a state of premunity, due to the low parasitaemia.

2.2.1.1.1 *Babesia bigemina*

Pandey and Mishra (1984) conducted an experimental study on morphological variations and erythrocytic multiplication of *B. bigemina* in indigenous calves. Morphological variations in shape, size and arrangements included single pyriform, single or paired round forms, elongated and oval forms. Intra-erythrocytic multiplication of *B. bigemina* was found to be by budding and binary fission. A positive correlation was evident between rise in body temperature, increase in parastaemia and appearance of atypical forms of *B. bigemina.*

Babesia in blood films, often appear in pairs arranged at a characteristic angle with narrow ends opposed. Typically they are pyriform but they may be round, elongated or cigar shaped. With Romanowsky dyes, the cytoplasm appears blue and nucleus red (Urquhart *et al.*, 1996).

The piroplasms of *Babesia* detected in carrier cases showed different morphology, varying from ovoid or vacuolar forms to annular ring forms. The typically paired pyriforms of *Babesia* were comparatively infrequent (Garg *et al.*, 2004).

Bell-sakyi *et al.* (2004) detected *B. bigemina* as large pleomorphic piroplasms in Giemsa stained smears.

2.2.1.1.2 *Babesia bovis*

B. bovis piroplasms were mostly pear shaped but ovoid and round forms were also fairly present. When more than one ovoid form was present, the members of the pair were oriented side by side or at divergent angles. The pear shaped forms were present either in the centre of erythrocytes, or a little to the periphery and formed divergent angles with each other (Shastri and Kurundkar, 1981).

B. bovis were identified as small intraerythrocytic piroplasms, vacuolated signet ring forms are particularly common, consisting of a centrally placed vacuole with a nuclear mass at one pole (Soulsby, 1982).

Muraleedharan *et al.* (1984) detected *B. bovis* piroplasms in Giemsa stained blood smears as paired organisms either in divergent angle or side by side, or single pyriform or vacuolated form, situated in the centre or near to the periphery in the erythrocytes.

2.2.1.1.3 *Trypanosoma evansi*

The typical form of *T. evansi* is slender fusiform with subterminal kinetoplast, well developed undulating membrane and substantial free flagellum (Soulsby, 1982).

According to Boid *et al.* (1985), the diagnosis of *T. evansi* infection by microscopical examination of Giemsa stained blood smears was insensitive and led to underestimation of the prevalence of infection.

Comparison of six parasitological methods in the diagnosis of *T. evansi* infection by Monzon *et al.* (1990) revealed the sensitivity of Giemsa stained blood smears to be 45.6 per cent.

Based on the studies conducted on sheep experimentally infected with *Trypanosoma evansi*, Saseendranath (1993) reported that parasitaemia could not be detected by wet film and blood smear examination consistently. In two replications of experimental infection, blood smear examination could detect parasitaemia in 61.18± 13.6 and 58.54± 7.66 percent of the examinations.

Saseendranath and Ramkrishna (1995) compared four different parasitological techniques *viz.*, wet film examination, Giemsa stained blood smear, microhaematocrit and mouse inoculation for detection of surra in sheep and found that parasitological diagnosis of trypanosomosis is satisfactory in animals with acute infection or high intensity of parasitaemia, but is not so reliable in chronic or latent disease when parasitaemia may be very low. The combination of microhaematocrit and mouse inoculation technique was the most effective among the parasitological methods.

Arunachalam et al. (2008) detected the presence of intercellular polymorphic forms *viz.*, short and stumpy, slender, akinetoplastic parasites from Giemsa stained blood smears of a buffalo infected with *Trypanosoma evansi*.

2.2.1.1.4 *Theileria annulata*

The shape of *Theileria* piroplasms varied from round and oval to elongate, rod and comma shaped, but the percentage of these different forms were to some extent variable and could not be considered as a reliable criterion for the distinction of species (Uilenburg, 1981).

In gross morphology, the erythrocytic forms of *Theileria* resemble small Babesiids. It is not always easy to distinguish them in blood film from some of the smaller *Babesia* (Kreier and Baker, 1987).

With Giemsa stain, cytoplasm of the piroplasms appeared blue with a red chromatin dot at one end. The piroplasms appeared rod shaped or round, oval and ring shaped with more than one parasite in each erythrocyte. In lymphnode smears, cytoplasm of lymphocytes revealed Koch's blue bodies which were the schizonts (Urquhart *et al.*, 1996).

2.2.1.1.5 *Theileria orientalis*

The erythrocytic piroplasms of *T. orientalis* were predominantly elongated, pear shaped, bacillary or rod shaped and show 'veil' or 'bar' structures or both in most cases (Shastri *et al.*, 1988).

A 'bar' like structure was associated with *T. orientalis* piroplasms which were not clearly apparent in Giemsa staining, but it could be easily detected in wet mount technique. In Giemsa stained blood smears, while the organisms were seen with cytoplasm and nucleus taking deep stain, the organelle, *i.e.*, the bar structures were not clearly seen in most of the infected erythrocytes. In a small percentage of infected erythrocytes barely negligible outline of a rectangular bar-like structure or 'dot' like forms could be seen. These structures were difficult to be appreciated in over-stained smears or smears with stain particles (Goud and Setty, 1994).

Ramesh *et al.* (2003) studied the prevalence of *T. orientalis* in cattle of Karnataka state by screening of blood samples by wet mount technique and Giemsa staining.

Rod shaped, round, oval or ring shaped piroplasms of *T. orientalis* were observed by Ravindran *et al.* (2006a) in Giemsa stained blood smears of cows showing anaemia.

2.2.1.1.6 *Anaplasma marginale*

In Giemsa stained blood films, the organisms of *Anaplasma marginale* were seen as small, round, dark red 'inclusion bodies' within the red cell. Often there was only one organism in a red cell and characteristically that occured at the outer margin, however these two features were not constant (Urquhart *et al.*, 1996).

In Giemsa stained smears, *A. marginale* appear as dense, round and homogenously stained blue-purple intraerythrocytic bodies, most of which were located near the margin of the erythrocyte (Kahn and Line, 2005).

2.2.1.1.7 *Anaplasma bovis*

Devada *et al.* (1996) observed *E. bovis* as spherical intracytoplasmic inclusions in monocytes and lymphocytes of a cow with fever, anaemia, emaciation and lymphadenomegaly.

Sreekumar *et al.* (1996) studied the morphology and staining characters of *E. bovis*. It could be identified as elementary bodies, initial bodies and morula in the host cell cytoplasm and the inclusions were solid and compact.

2.2.1.2 Acridine Orange staining

Acridine Orange stain is a fluorochrome stain, which produced a red fluorescence of ribonucleic acid (RNA) and a green fluorescence of deoxyribonucleic acid (DNA) (Hansen *et al.*, 1970).

Ristic (1981) recommended Acridine Orange staining as an alternative for Giemsa staining technique in detection of anaplasmosis.

Acridine Orange fluorescent microscopy of capillary centrifuged blood offer an alternative technique over Giemsa stained smears in detection of low density parasitaemia in malarial infections (Wongsrichanalai *et al.*, 1991).

Bose *et al.* (1995) observed a higher sensitivity of Acridine Orange staining compared to Giemsa staining in the detection of *Babesia* sp.

Damodar (1996) stated that Acridine Orange staining of buffy coat smear was sensitive, rapid, simple and superior over Giemsa staining in detection of malarial parasite.

Ravindran *et al.* (2007b) reported the detection of *B. bigemina* and *E. canis* through Acridine Orange staining of thin blood smears.

2.2.3 Molecular diagnostics

Molecular diagnostics rely on techniques that enable the detection of minute quantities of nucleic acids. DNA hybridization or DNA probe methods were the earliest molecular biological methods that were developed for diagnosis but the sensitivity of DNA probes were just comparable to conventional microscopy. In polymerase chain reaction, amplification of the target DNA is achieved using a thermostable enzyme *Taq* polymerase and specific primers. PCR-RFLP is a method which detects minor variations in a gene. After amplification of a region of a gene by PCR, the amplicon is subjected to digestion by one or more restriction endonucleases (Singh, 1997).

In contrast to the PCR amplification from a particular region of the genome, RAPD or arbitrary primed PCR is based on the amplification of random fragments of genomic DNA with single primer of arbitrary sequence. It enables screening of entire genome using a simple approach without prior DNA sequence information (Gasser, 1999).

Modern molecular technologies have substantial applications in parasitology. In particular, PCR coupled approaches have found broad applicability because their sensitivity permits the enzymatic amplification of gene fragments from minute quantities of nucleic acids from tiny amounts of parasite material (Gasser, 2006).

Loop mediated isothermal amplification (LAMP) is a new method that can amplify a few copies of DNA to million fold in less than an hour under isothermal conditions with high specificity and sensitivity. Positive LAMP reaction was visualized by naked eye. The technique was simple, rapid and did not require expensive heating devices. Inoue *et al.* (2007) developed a LAMP for the specific detection of human African trypanosomosis. Thekisoe *et al.* (2007) reported the development of LAMP assay for detection of trypanosomosis and theileriosis in cattle.

2.2.3.1 Polymerase chain reaction

By using *Taq* DNA polymerase, a thermostable enzyme isolated from the thermophilic bacterium *Thermus aquaticus*, it was possible to amplify original target DNA a million-fold with the method called polymerase chain reaction (Saiki *et al.*, 1988).

The advent of PCR (Saiki *et al.*, 1985; Mullis *et al.*, 1986) has revolutionized biological research and is extensively used today in molecular biology research, diagnostics, population genetics, forensic analysis *etc.* The

technique is so sensitive that even a single DNA molecule can be amplified from a complex mixture of genomic sequences. Specific application of molecular biology in veterinary parasitology include the classification of parasites, diagnosis of infection, understanding parasite pathophysiology, the development of antiparasite vaccines and drugs, the selection of hosts with genetic resistance and perfecting systems of biological control of parasites (Prichard,1997).

Khaminsou *et al.* (2007) studied the comparative efficacy of Giemsa staining, Acridine Orange staining and semi-nested PCR for diagnosis of human malarial infections and suggested that PCR is suitable for detecting low level parasitaemia than staining techniques.

2.2.3.1.1 *Babesia bigemina*

Figueroa *et al.* (1992) developed a PCR assay in which the primers derived from a sequence contained in P^{16} insert were used to amplify a 278 bp fragment from the genome of *B. bigemina.*

Gayo *et al.* (2003) used *Babesia* species specific PCR to confirm the transovarian transmission of *B. bigemina* in ticks.

Ravindran *et al.* (2007c) developed a PCR-RFLP method for the simultaneous detection of *B. bigemina* and *T. annulata* infections in cattle which showed a good potential for epidemiological screening.

Singh *et al.* (2007) used PCR for detection of *B. bigemina* in carrier animals. Specific amplification for *B. bigemina* was detected in nine out of 20 suspected samples examined while Giemsa stained blood films detected none of the samples as positive.

2.2.3.1.2 *Babesia bovis*

Polymerase chain reaction amplification of *B. bovis* DNA has been used to demonstrate the presence of this protozoan directly from bovine blood (Wagner *et al.*, 1992).

Polymerase chain reaction amplification of the apocytochrome b gene of *Babesia bovis* DNA had been used to demonstrate the presence of the organism in bovine blood (Fahrimal *et al.*, 1992).

Figueroa *et al.* (1993) developed a highly sensitive multiplex PCR assay for simultaneous detection of *B. bigemina*, *B. bovis* and *A. marginale* DNA in bovine blood.

Calder *et al.* (1996) used a PCR-based diagnostic test which targets the small-subunit rRNA gene of *B. bovis* with sensitivity much higher than CFT.

Lew *et al.* (1997) demonstrated much genetic diversity among the Australian isolates of *B. bovis* using PCR based techniques.

Patarapadungkit *et al.* (2004) described a new primer set for specific detection of *B. bovis*, which showed a higher sensitivity than the primers described by Fahrimal *et al.* in 1992.

2.2.3.1.3 *Trypanosoma evansi*

Polymerase chain reaction technique had been used for diagnosis of African trypanosomosis by Moser *et al.* (1989) on blood samples of experimentally infected animals and confirmed its higher sensitivity and specificity when compared to the parasitological methods.

A PCR-based detection technique for *T. evansi* was developed which had a sensitivity of 0.5 pg of parasite or one parasite in ten microliter of blood samples. The primers used generated 227 bp fragment from *T. evansi* DNA (Wuyts *et al.*, 1994).

Basagoudanavar *et al.* (1998) reported the detection of cameline surra from endemic area of Rajasthan, India using crude blood samples by PCR.

A study on comparison of diagnostic sensitivity of five parasitological methods and PCR for of *Trypanosoma evansi* in water buffaloes by Holland *et al.* (2001) showed that the sensitivity of PCR was the highest (78.2 per cent), closely followed by mouse inoculation (74 per cent), microhaematocrit centrifugation technique (69.6 per cent) and mini-anion exchange centrifugation technique (62.4 per cent). The sensitivity of buffy coat technique was much lower.

Gaysen *et al.* (2003) described a single PCR-RFLP assay to identify all important bovine trypanosomes. Delespaux *et al.* (2003) used the same method and confirmed that the technique was a useful tool in the field for the diagnosis of natural mixed infections with trypanosomes.

Comparative evaluation of parasitological, serological and DNA amplification methods for diagnosis of trypanosomosis in camels of Rajasthan, India was done by Singh *et al.* (2004). The best diagnostic efficacy was found with PCR followed by antigen ELISA and the least with blood film examination.

Shailaja *et al.* (2005) standardized PCR for identification of trypanosomosis in captive wild animals. Scraped blood smear was used as the source of DNA. They identified 14 out of 44 samples as positive for trypanosomosis using PCR while Giemsa staining identified 11 samples as positive.

PCR using a set of DNA amplimers based on structural 18S and 5.8S ribosomal DNA sequences specific for kinetoplastida taxon was employed for detection of *T. evansi* in Indian camels in Bikaner, India. Out of ten blood samples from apparently healthy camels, six samples were detected as positive by showing amplification specific to *T. evansi* (Shahardar *et al.*, 2007).

2.2.3.1.4 *Theileria annulata*

A primer set amplifying a 721 bp fragment was used for detection of *Theileria annulata* by d'Oliviera *et al.* (1995) on bovine blood and Jongejan *et al.* (1998) on lysates of experimentally infected *Hyalomma* ticks.

Martin-Sanchez *et al.* (1999) reported the higher sensitivity of PCR in detection of *T. annulata* from cattle blood samples than IFAT and Giemsa staining.

Roy *et al.* (2000) used PCR for detection of *T. annulata* in carrier animals using primer sets for amplification of small subunit ribosomal RNA genes. The detection limit of the PCR was an erythrocytic parasitaemia of 0.00008 per cent.

Parasitaemia at different stages of the disease was studied by Das *et al.* (2002), using PCR on blood samples from calves experimentally infected with *T. annulata.* They reported significant correlation between intensity of PCR bands and parasitaemia. Infection was detected in majority of the recovered calves in which parasites were difficult to find in stained blood smears.

Fornelio *et al.* (2003) used PCR amplification of 18S rRNA gene for phylogenetic analysis of *T. annulata* in Portugal.

2.2.3.1.5 *Theileria orientalis*

Polymerase chain reaction was used to identify benign *Theileria* DNA in salivary gland lysates of *Haemaphysalis* ticks (Kawazu *et al.*, 1995). Primers were used to amplify an 870 bp fragment of the genes encoding the 33- and 34- kDa major piroplasm antigens.

Kuboto *et al.* (1995) developed allele-specific PCR for the major piroplasm surface protein gene to differentiate parasite populations of benign *Theileria.*

Chansiri *et al.* (1998) used RAPD-PCR to obtain genomic finger printing for inter-species differentiation of benign theilerial organisms.

Jeong *et al.* (2003) reported more than two fold increase in sensitivity of PCR compared with microscopic examination in detection of *T. sergenti* infection.

2.2.3.1.6 *Anaplasma marginale*

Barbet (1995) reviewed the recent developments in molecular biology of anaplasmosis and suggested that merozoite surface protein genes (msp) 1, 2 and 4 have potential for the development of vaccines and msp 3 and 5 for improved diagnostic assays.

A highly sensitive and specific PCR based assay for the detection of the minute levels of *A. marginale* present in the blood of the long term carrier cattle was developed by Gale *et al.* (1996). The sensitivity limit of the PCR-ELISA was 0.00015per cent parasitaemia (24 infected erythrocytes per microlitre of blood). No cross-reactivity or false positive results were obtained.

Lew *et al.* (2002) developed a PCR assay to amplify the major surface protein 1 alpha gene of *A. marginale* both as an inter and intra-specific test.

Shimada *et al.* (2004) used PCR-RFLP for specific detection of *Anaplasma marginale* and reported that it was a very sensitive and specific diagnostic tool.

Carelli *et al.* (2007) developed a real time PCR assay for the diagnosis of *A. marginale* infection in cattle which allowed the simultaneous detection and quantification of the *A. marginale* DNA in bovine blood, to assess the carrier status of the animals and to evaluate the efficacy of vaccines and drugs.

2.2.3.1.7 *Anaplasma bovis*

A PCR-based assay was developed for detecting DNA of granulocytic ehrlichiae in blood samples from dogs, horses and cattle (Engwall *et al.*, 1996).

Monocytes generally comprise less than one percent of all leukocytes in circulating blood and so *A. bovis* sequences in the extracted DNA would be rare. Therefore nested PCR is preferred for identification of *A. bovis* (Goethert and Telford, 2003).

Parola *et al.* (2003) detected *A. bovis* from *Haemaphysalis lagrangei* ticks collected from a bear in Thailand through PCR amplification of 16S rRNA gene and sequence analysis.

Kawahara *et al.* (2006) conducted a study to determine the species of *Ehrlichia* and *Anaplasma* that naturally infect deer in Japan and detected the presence of *A. centrale*, *A. bovis*, *A. phagocytophilum* and a novel *Ehrlichia* spp. using PCR and sequence analysis.

Materials and methods

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Animals

A cross-sectional study was conducted with randomly selected 150 cross bred cattle from Northern Kerala. Blood samples were collected from 30 apparently normal healthy animals each from five districts: *viz.*, Wayanad, Malappuram, Kozhikode, Palakkad and Kannur. Six random samples were collected from 5 different localities of each district. Figure 1 shows the districts of sample collection. Following were the different localities of collection from each district (Table 1, Fig 2).

Table 1. Different localities of sample collection from five districts of Northern Kerala

District	Locality
Wayanad	Panamaram, Meenangadi, Sulthan Bathery, Pookot, Trikkaipetta
Malappuram	Marakkara, Manjery, Thirunavaya, Ariyalloor, Tavannur
Kozhikode	Kozhikode, Tamarassery, Ramanattukara, Chemenchery, Vadakara
Palakkad	Muthalamada, Kollenkode, Kodumbu, Shornur, Mannarkad, Olavakkodu
Kannur	Payyannur, Kannur, Edakkad, Iritty , Paral

3.1.2 Staining

3.1.2.1 Liquid Giemsa stain (Qualigens)

3.1.2.2 Microscope (Carl Zeiss)

3.1.2.3 Acridine Orange stain (AO stain) 0.01 per cent

Acridine Orange powder (Himedia) 20 mg

Sodium acetate buffer 190 ml

Stock solution of the buffer was prepared by adding 13.6 g of sodium acetate to 100 ml of distilled water and 90 ml of 1 N HCl. The final pH was adjusted to 3.5 by adding 1N HCl.

3.1.2.4 Fluorescent microscope (Olympus)

3.1.3 Polymerase Chain Reaction

3.1.3.1 Extraction of DNA

3.1.3.1.1 Lysis Buffer / Dehaemoglobinization Buffer

Saponin	0.015 per cent
NaCl	3.5 mM
EDTA	1 mM

3.1.3.1.2 Reaction Mixture Buffer

Tris HCl	10 mM
$MgCl_2$	50 mM
Gelatin	0.01 per cent

3.1.3.2 DNA amplification

3.1.3.2.1 *Taq* DNA polymerase (Banglore Genei)

Taq Buffer (10X)	Tris HCl	20 mM
	KCl	50 mM
	$MgCl_2$	1.5 mM

3.1.3.2.2 Deoxy nucleotide triphosphate (dNTP) mix containing 200 µM of each of the following:

Deoxy adenosine triphosphate

Deoxy guanosine triphosphate

Deoxy cytosine triphosphate

Deoxy thymidine triphosphate

The working solution was prepared by mixing 2 µl of 10 mM stock (Bioenzyme, USA) of each dNTP in 92 µl distilled water.

3.1.3.2.3 Primers for PCR of each species of haemoparasites were custom synthesized from IDT, USA (through Imperial, Chandigarh).

3.1.3.2.3.1 Primer sequences for *Anaplasma* genus

Initial amplification (Wilson *et al.*, 1990; Chen *et al.*, 1994)

Forward 5' TAC CTT GTT ACG ACT T 3'

Reverse 5' AGA GTT TGA TCM TGG 3'

Nested PCR for *Anaplasma bovis* (Kawahara *et al.*, 2006)

Forward 5'CTC GTA GCT TGC TAT GAG AAC-3'

Reverse 5'TCT CCC GGA CTC CAG TCT G-3'

Nested PCR for *Anaplasma phagocytophilum* (Kawahara *et al.*, 2006)

Forward 5'-GCT GAA TGT GGG GAT AAT TTA T-3'

Reverse 5'-ATG GCT GCT TCC TTT CGG TTA-3'

3.1.3.2.3.2 Primer sequences for *Anaplama marginale* (Shimada *et al.*, 2004)

Forward 5'- GCA TAG CCT CGC CGT CTT TC -3'

Reverse 5'- TCC TCG CCT TGG CCC TCA GA -3'

3.1.3.2.3.3 Primer sequences for *Trypanosoma evansi* (Chokesajjawattee, 1993)

Forward 5'- TGC AGA CGA CCT GAC GCT ACT -3'

Reverse 5'- CTC CTA GAA GCT TCG GTG TCC T -3'

3.1.3.2.3.4 Primer sequences for *Theileria* genus (d'Oliveira *et al.*, 1995)

Forward 5'AGT TTC TGA CCT ATC AG- 3'

Reverse 5'- TTG CCT TAA ACT TCC TTG -3'

3.1.3.2.3.5 Primer sequences for *Theileria annulata* (d'Oliveira *et al.*, 1995)
Forward 5'-GTA ACC TTT AAA AAC GT-3'
Reverse 5'- GTT ACG AAC ATG GGT TT-3'

3.1.3.2.3.6 Primer for sequences *Theileria orientalis* (Tanaka *et al.*, 1993)
Forward 5'- CAC GCT ATG TTG TCC AAG AG-3'
Reverse 5'- TGT GAG ACT CAA TGC GCC TA-3'

3.1.3.2.3.7 Primer sequences for *Babesia bigemina* (Figueroa *et al.*, 1992)
Forward 5'- CAT CTA ATT TCT CTC CAT ACC CCT CC-3'
Reverse 5'- CCT CGG CTT CAA CTC TGA TGC CAA AG-3'

3.1.3.2.3.8 Primer sequences for *Babesia bovis* (Fahrimal *et al.*, 1992)
Forward 5'- GGG TTT ATA GTC GGT TTT GT-3'
Reverse 5'- ACC ATT CTG GTA CTA TAT GC-3'

3.1.3.2.4 Negative control

Leukocytic DNA isolated from blood of a one day old calf was used as negative control in all PCR reactions.

3.1.3.2.5 Positive controls

The positive control for each reaction was the respective DNA isolated from the parasite or from parasite infected bovine whole blood. For *Trypanosoma evansi* and *Babesia bigemina,* genomic DNA was used. DNA isolated from the whole blood of a *Theileria annulata* infected cattle (obtained from Veterinary college and Research Institute, Namakkal) was used as positive control for *T. annulata* specific PCR and *Theileria* genus specific PCR. DNA isolated from the whole blood of cattle infected with *Anaplasma marginale* (from Madras Veterinary College, Chennai) and *Anaplasma bovis* (from a clinically infected

animal revealing morulae of *A. bovis* in blood smear) were used as positive controls. Specific primers for *T. orientalis*, *B. bovis* and *A. phagocytophilum* were used for PCR without positive control.

3.1.3.2.6 Thermal cycler (Eppendorf, Germany)

3.1.3.3 Analysis of PCR product- Submarine Agarose Gel Electrophoresis

3.1.3.3.1 Tris Borate EDTA (TBE) buffer (5X)

Tris	27 g
Boric acid	13.75 g
EDTA (0.5M)	10 ml
Aqua dest ad	500 ml

pH was adjusted to 8.3. Working solution of 0.5X TBE was made by mixing 50 ml of 5X TBE with 450 ml of distilled water.

3.1.3.3.2 Agarose Molecular Biology grade (Biogene,USA)

3.1.3.3.3 Ethidium bromide (Himedia)

3.1.3.3.4 Gel loading buffer (6X)

Bromophenol blue	0.09 per cent
Xylene cyanol	0.05 per cent
Glycerol	60 per cent
EDTA (0.5M, pH 8)	60 mM

3.1.3.3.5 100 bp DNA ladder (Banglore Genei)

Reconstituted with triple distilled water, 6X gel loading dye and 100bp ladder respectively in the ratio 4:1:1.

3.1.3.3.6 Submarine gel electrophoresis apparatus (Banglore Genei)

3.1.3.3.7 Gel documentation system (Alpha Innotech, USA)

3.2 METHODS

3.2.1 Staining

3.2.1.1 Blood smears

Two thin blood smears were prepared using peripheral blood collected from ear vein of each cattle. Blood smears were labeled and fixed using methanol. One smear was used for Giemsa staining and the other for Acridine Orange staining.

3.2.1.2 Giemsa staining

One smear from each sample 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 50 peripheral fields were screened for detection of any blood parasites.

3.2.1.3 Acridine Orange staining

The blood smears were fixed with methanol and were used for staining with Acridine Orange (Lauer *et al.,* 1981). The methanol fixed 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.

3.2.2 Polymerase chain reaction

3.2.2.1 Collection of whole blood

One milliliter of blood from cattle was collected in heparinized eppendorf tubes (1.5 ml) from jugular or ear vein.

3.2.2.2 Isolation of DNA

The blood samples were processed following the method described for detection of *Plasmodium* spp. from human blood (Tirasophon *et al.*, 1991). Heparinized blood samples (150 µl) were treated by 1300 µl dehaemoglobinization buffer and centrifuged at 12,000 rpm for 2 min. The supernatant was discarded. The pellet was washed once with 750 µl of reaction mixture buffer and centrifuged at 6000 rpm for one minute. Again the supernatant was discarded carefully. The final pellet was resuspended in 75 µl of distilled water, boiled for 10 min and 10 µl from this was used as template for PCR.

3.2.2.3 Amplification of DNA

Polymerase chain reaction was set up in a total volume of 25 µl.

Reaction mix:

10X PCR buffer	2.5 µl
dNTP mix	0.2 mM
Taq DNA polymerase	1.25 U
Primers – (concentration for each species described elsewhere)	
Template DNA	10 µl

The volume was made upto 25 µl with autoclaved triple distilled water.

3.2.2.3.1 *Babesia bigemina*

Primers for the PCR amplification of a 278 bp *B. bigemina* specific fragment (Figueroa *et al.*, 1992) were used in a concentration of 15 pmol. The cycling conditions were as follows: after an initial denaturation at 95^{0}C for five min, 35 cycles of denaturation (95^{0}C for 60s), annealing (65^{0}C for 60s), and extension (73^{0}C for 90s) were conducted. For the first two cycles, two minutes denaturation were allowed. The final extension was at 73^{0}C for 15 min.

3.2.2.3.2 *Babesia bovis* (Fahrimal *et al.*, 1992)

Primers for the PCR amplification of a 711 bp *B. bovis* specific fragment were used in a concentration of 25 pmol. The cycling conditions were as follows: after an initial denaturation at 94^{0}C for five min, 30 cycles of denaturation (94^{0}C for one min), annealing (55^{0}C for two min) and extension (72^{0}C for three min) were followed. For the first cycle, five minutes denaturation was given. The final extension was at 72^{0}C for seven min.

3.2.2.3.3 *Anaplama marginale*

Polymerase chain reaction was performed as per the protocol described by Shimada *et al.* (2004) using primers (20 pmol each) for the partial amplification of msp 5 gene with a product size of 457 bp. The cycling conditions were: initial denaturation of five min at 95^{0}C, 35 cycles each consisting of denaturation at 95^{0}C for one min, annealing at 65^{0}C for one min and extension 72^{0}C for two min. The final extension was at 72^{0}C for 10 minutes.

Polymerase chain reaction - Restriction fragment length polymorphism

To determine specificity of amplicon, Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) was performed as per the protocol described by Shimada *et al.*, (2004). After the amplification reaction, the PCR product (457 bp) was digested with *Eco* RI and *Hind* III restriction endonucleases (Bangalore GeNei, India) according to the manufacturer's instructions.

3.2.2.3.4 *Trypanosoma evansi*

The primers developed from a repetitive sequence probe pMUTec6.258 (Chokesajjawattee, 1993) amplifying 227 bp product were used at a concentration of 10 pmol and the reaction was performed as per the method of Basagoudanvar *et*

al. (1998). A hot start was performed for seven min at 90 ^{0}C to completely denature the DNA followed by 30 cycles each of 30 s at 90 ^{0}C (to denature), 30 s at 60 ^{0}C (to anneal) and 30 s at 72 ^{0}C (to extend), and then with one extensive polymerization at 72 ^{0}C for seven min.

3.2.2.3.5 *Theileria* genus specific PCR

Polymerase chain reaction was performed according to the protocol described by d'Oliveira, *et al.* (1995). *Theileria* genus specific PCR primers which amplify a 1098 bp product were used at a concentration of 10 pmol each. Cycling conditions were as follows: Initial denaturation of 94^0 C for five min was followed by 30 cycles, each cycle consisting of a denaturation step of one min at 94^0 C, an annealing step of one min at 60^0 C and an extension step of one min at 72^0 C. Final extension was provided at 72^0 C for five min.

3.2.2.3.6 *Theileria annulata*

Polymerase chain reaction was performed according to the protocol described by d'Oliveira *et al.* (1995). Primers amplifying 721 bp product were used at the rate of 40 pmol for 25 µl reaction.

Initial denaturation of 94^0 C for 5 min was followed by 30 cycles with each cycle consisting of a denaturation for one min at 94^0 C, an annealing for one min at 55^0 C and an extension for one min at 72^0 C. Final extension at 72^0 C for five min was also provided at the end of 30 cycles.

3.2.2.3.7 *Theileria orientalis*

A pair of oligonucleotide primer sequences which are localized within the coding region in a 32-kDa surface protein gene of *T. orientalis* were used for PCR amplification as described by Tanaka *et al.* (1993), at a concentration of 25 pmol.

The reactions were carried out in 30 cycles, each consisting of a two min denaturation at 95^0 C (three min for the first cycle), two min of annealing at 60^0 C, and two and half min of extension at 72^0 C, with an additional seven min elongation at 72^0 C after the last cycle.

3.2.2.3.8 *Anaplasma* whole gene

Universal eubacterial Primers (PC5 and PO mod) which recognize conserved sequences of the 3' end and 5' end of the 16S rDNA (Chen *et al.*, 1994), and amplify nearly the entire gene (1500 bp) were used at the rate of 25 pmol for 25 µl reaction.

Cycling conditions consisted of initial denaturation for three min at 95 ^{0}C then 25 cycles, each of 30 s at 94 ^{0}C, one min at 52 ^{0}C, and two min at 72 ^{0}C. Final extension at 72 ^{0}C for five min was also provided.

Anaplsma bovis (Ehrlichia bovis)

Nested PCR was performed as per the protocol described by Kawahara *et al.* (2006). One microliter of the product from the first amplification (*Anaplasma* genus specific PCR) was used as the template DNA amplifying a 551bp product with *A. bovis* 16S rRNA gene specific primers. Both primers were used at the rate of 25 pmol for 25 µl reaction. The amplification consisted of initial denaturation for three min at 95 ^{0}C followed by 40 cycles each of one min at 94^0 C, one min at 55^0 C and one min at 72^0 C and a final extension of 72^0 C for five min.

Anaplasma phagocytophilum

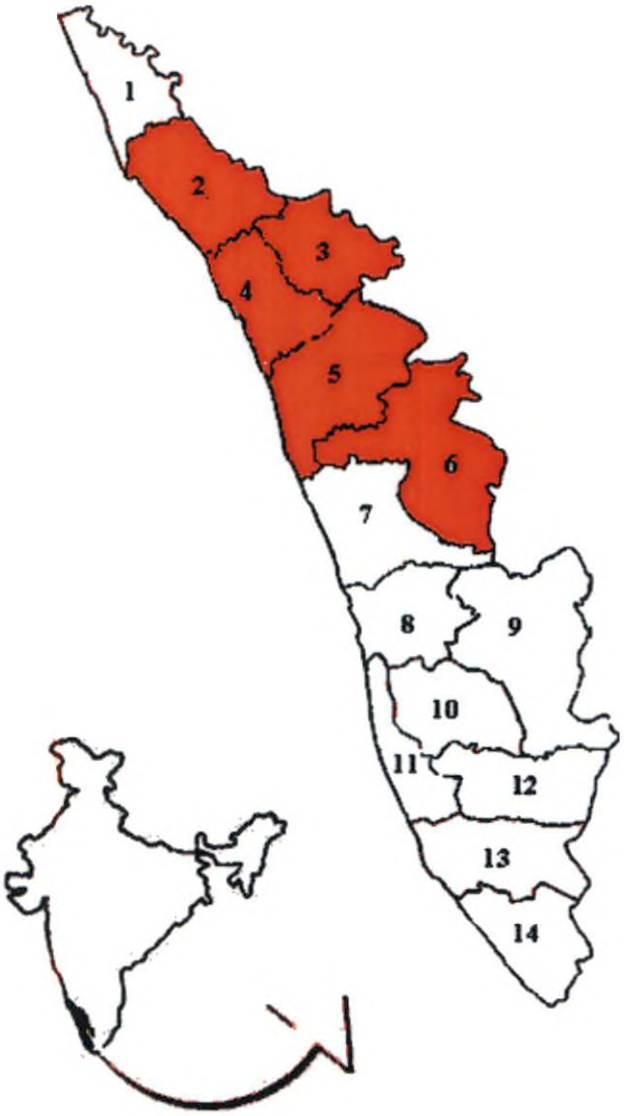
Nested PCR was performed as per the protocol described by Kawahara *et al.* (2006). One microliter of the product from the first amplification (*Anaplasma* genus specific PCR) was used as template DNA amplifying a 641bp product with

A. phagocytophilum 16S rRNA gene specific primers. Both primers were used at the rate of 25 pmol for 25 µl reaction. The amplification consisted of initial denaturation for three min at 95 ^{0}C followed by 40 cycles each of one min at 94^0 C, one min at 55^0 C and one min at 72^0 C and a final extension of 72^0 C for five min.

3.2.2.4 Agarose electrophoresis and visualization

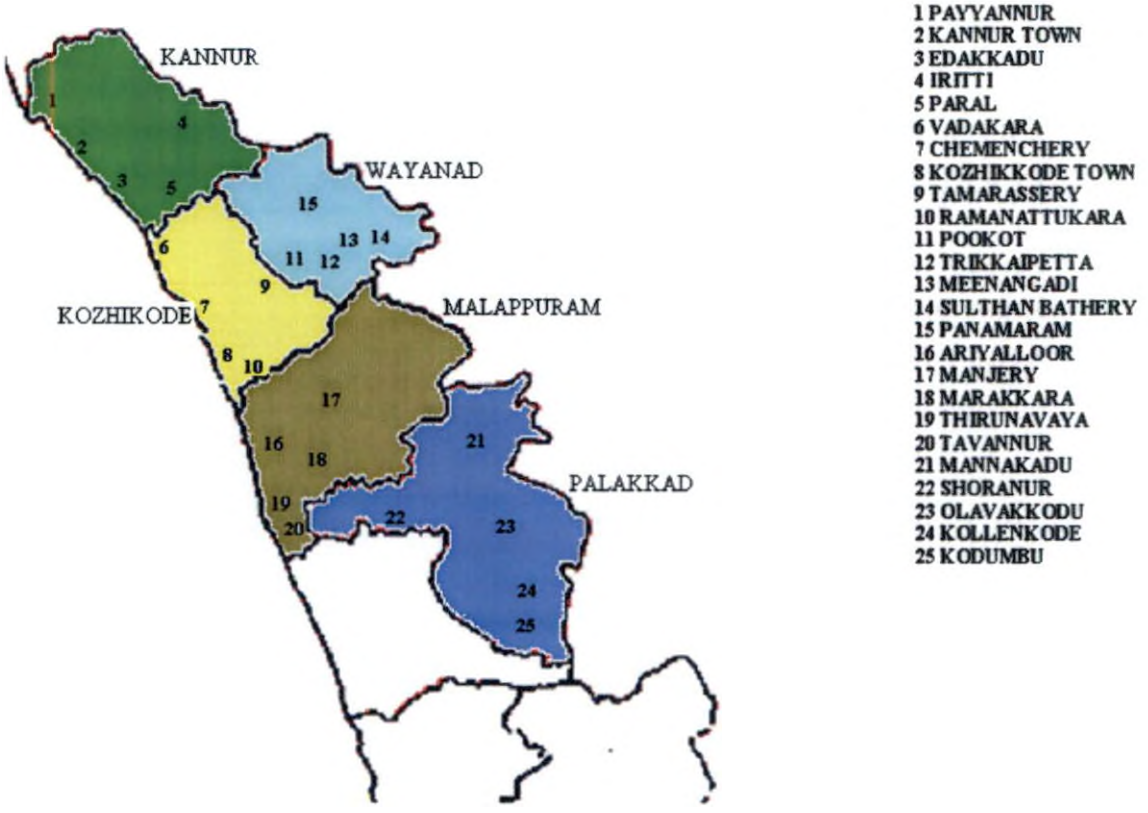
The products of PCR or PCR-RFLP were checked for the amplification and lack of spurious products by electrophoresis on a 1.5 per cent agarose gel using 70 V power supply for 90 min. The products were visualized by gel documentation system and photographed.

Fig 1. Districts of sample collection



1. Kasargod
2. Kannur
3. Wayanad
4. Kozhikode
5. Malappuram
6. Palakkad
7. Trissur
8. Eranakulam
9. Idukki
10. Kottayam
11. Alappuzha
12. Pathanamthitta
13. Kollam
14. Thiruvananthapuram

Fig 2. Different Localities of Sample Collection from Northern Kerala



Results

4. RESULTS

4.1 Screening of Animals

In the present study, blood samples from 150 apparently normal adult crossbred cattle from five districts of Northern Kerala *viz.*, Wayanad, Malappuram, Kozhikode, Palakkad and Kannur were screened for the presence of haemoprotozoan and haemorickettsial organisms. The diseases under investigation included trypanosomosis, theileriosis, babesiosis and anaplasmosis. The methods used for detection of these organisms were Giemsa staining, Acridine Orange staining and polymerase chain reaction.

4.1.1 Microscopical Examination

4.1.1.1 Giemsa staining

Peripheral blood smears stained using Giemsa stain were examined for the presence of haemoparasites. *Babesia bigemina* (Plate 1) and *Theileria* like piroplasms were the major protozoan organisms detected. Various morphological appearances of the *Theileria* piroplasms were observed. The piroplasms were thin or thick rod shaped or annular with light staining trailing cytoplasm (Plate 3-6). All the forms could be detected in almost all the positive smears with annular forms being found less frequently while the thick and the thin rods the maximum. *Anaplasma marginale* inclusions (Plate 11) were detected as the major haemorickettsial organism. *Theileria* like piroplasms were observed in 61 samples, *B. bigemina* piroplasms in four samples and *Anaplasma* inclusions in two samples, out of the 150 samples examined. District-wise data is shown in table 2.

4.1.1.2 Acridine Orange (AO) staining

Peripheral blood smears stained using AO stain were examined for the presence of haemoparasites. *B. bigemina* (Plate 2) and *Theileria* like piroplasms (Plate 7-10) were detected as the major protozoan organisms. *A. marginale* inclusions (Plate 12) were detected as the major

haemorickettsial organism. *Theileria* like piroplasms were observed in 71 samples, *B. bigemina* piroplasms in four samples and *A. marginale* inclusions in three samples, out of the 150 samples examined. District-wise data is shown in table 2.

Table 2. Results of blood smear examination

District	Staining technique	TLP		*B.big*		*A.mar*	
		No:	per cent	No:	per cent	No:	per cent
WAYANAD	Giemsa	21	14	4	2.6	1	0.6
	AO	22	14.7	4	2.6	1	0.6
MALAPPURAM	Giemsa	13	8.7	0	0	0	0
	AO	14	9.2	0	0	1	0.6
KOZHIKODE	Giemsa	11	7.3	0	0	0	0
	AO	14	9.2	0	0	0	0
PALAKKAD	Giemsa	9	6	0	0	1	0.6
	AO	9	6	0	0	1	0.6
KANNUR	Giemsa	8	5.2	0	0	0	0
	AO	11	7.3	0	0	0	0
TOTAL	Giemsa	61	41.2	4	2.6	2	1.3
	AO	71	46.4	4	2.6	3	2

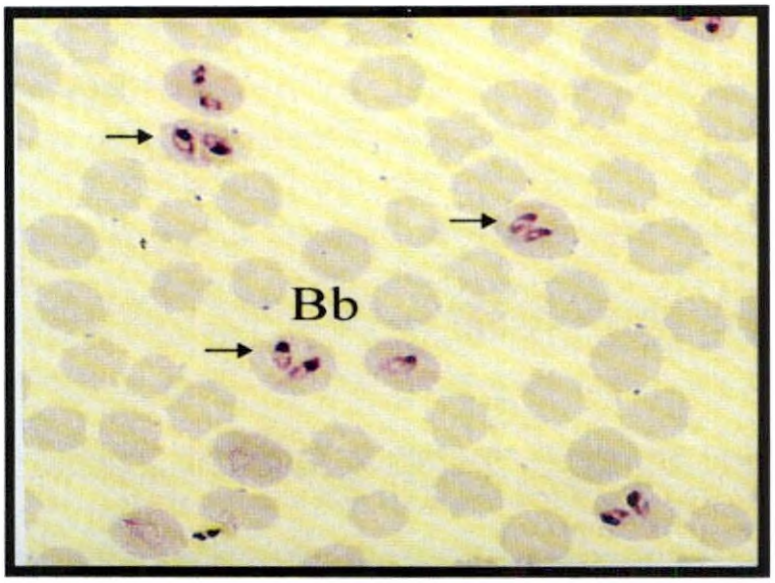
No: -Number of smears positive

TLP- *Theileria* like piroplasms

B.big- B. bigemina

A.mar- A. marginale

Plate 1. Giemsa stained blood smear showing *Babesia bigemina* piroplasms



Bb : *Babesia bigemina* piroplasms

Plate 2.Acridine Orange stained blood smear showing *Babesia bigemina* piroplasms

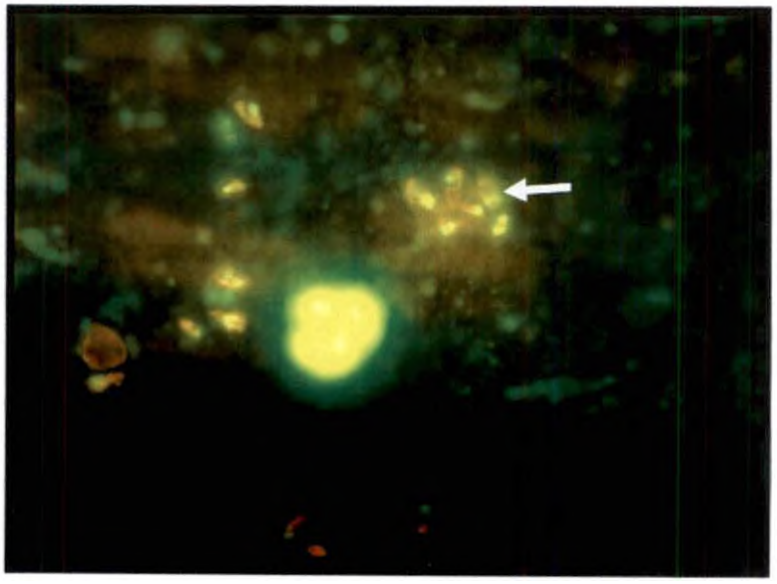


Plate 3. Giemsa stained blood smear showing *Theileria* like piroplasms (Annular form)

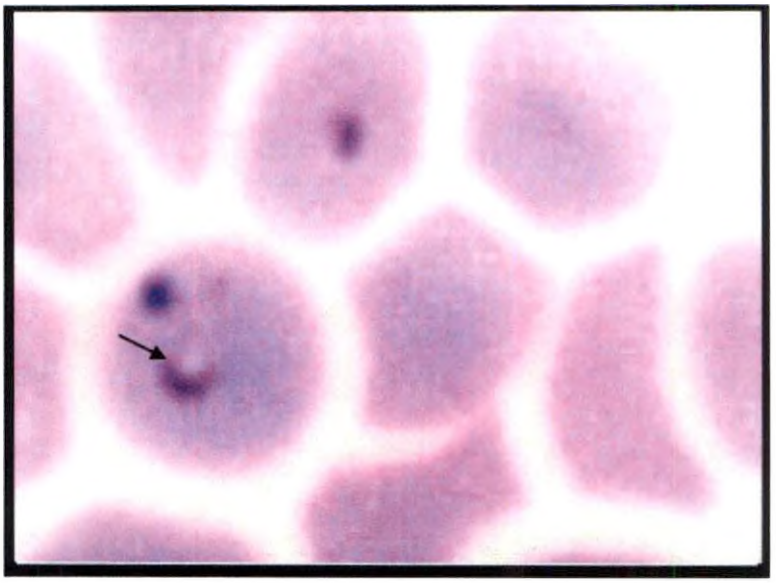


Plate 4.Giemsa stained blood smear showing *Theileria* like piroplasms (Rod shaped form)

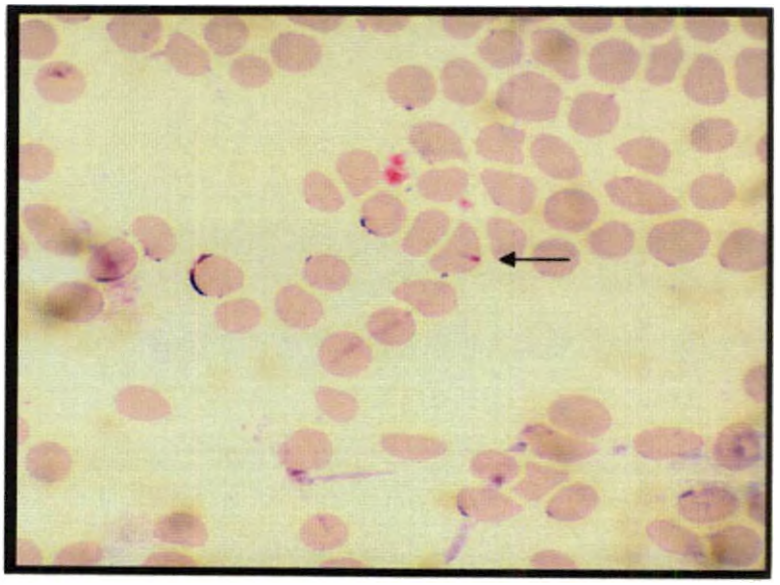


Plate 5. Giemsa stained blood smear showing *Theileria* like piroplasms (Rod shaped with trailing cytoplasm)

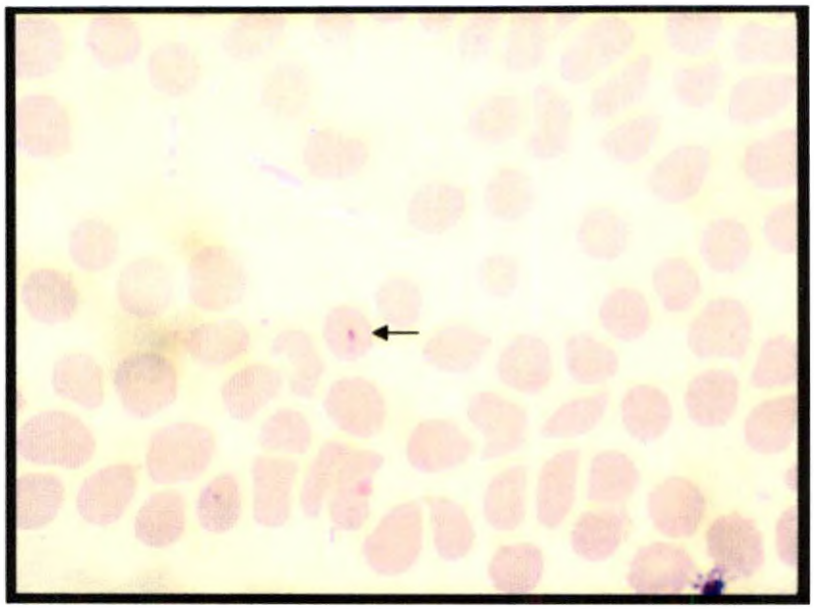


Plate 6. Giemsa stained blood smear showing *Theileria* like piroplasms (Thin rod, comma shaped)

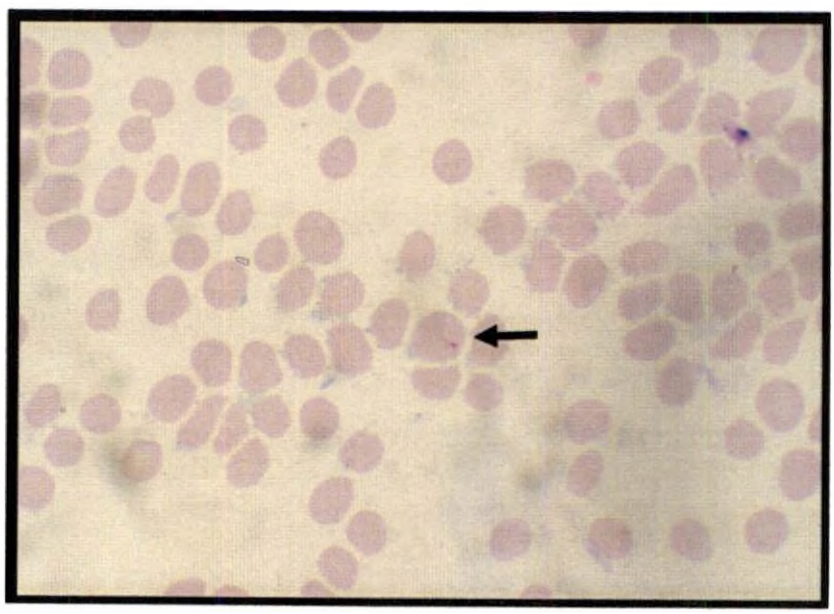


Plate 7. AO stained blood smear showing *Theileria* like piroplasms (short rod forms)

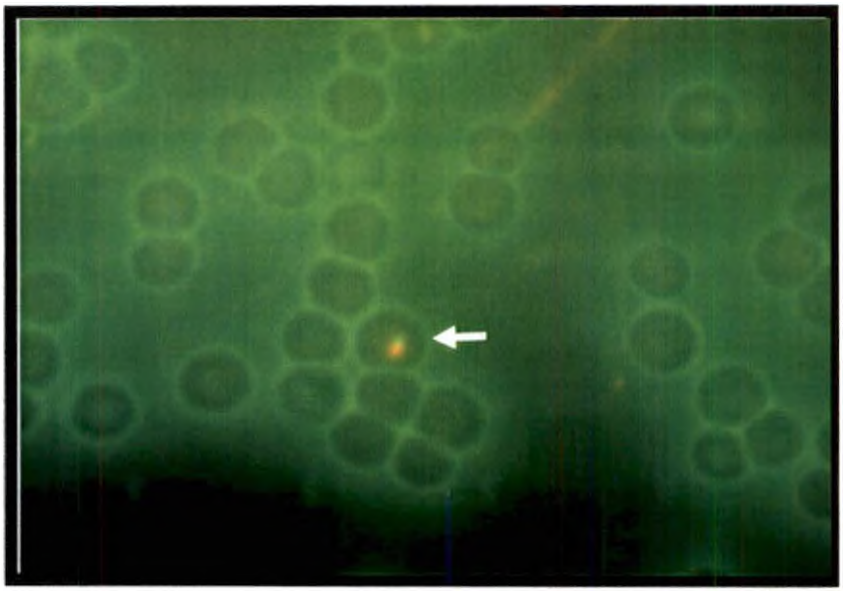


Plate 8. AO stained blood smear showing *Theileria* like piroplasms (cap form with trailing cytoplasm)

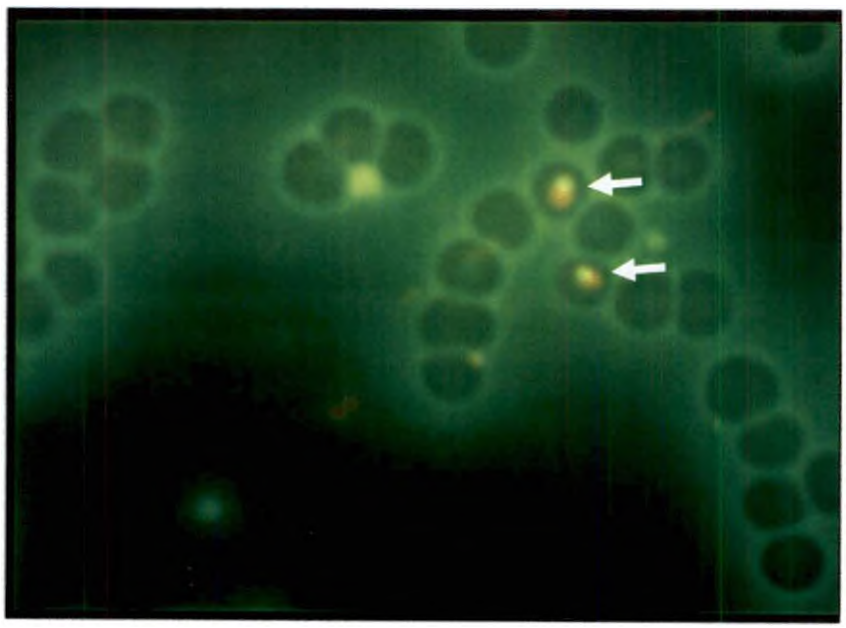


Plate 9. AO stained blood smear showing *Theileria* like piroplasms (Thin rods)

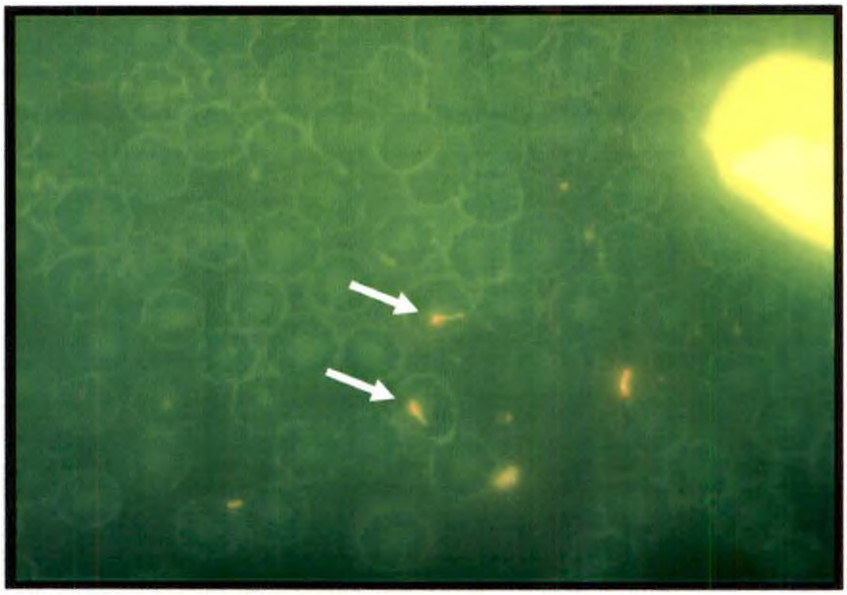


Plate 10. AO stained blood smear showing *Theileria* like piroplasms (thin rods)

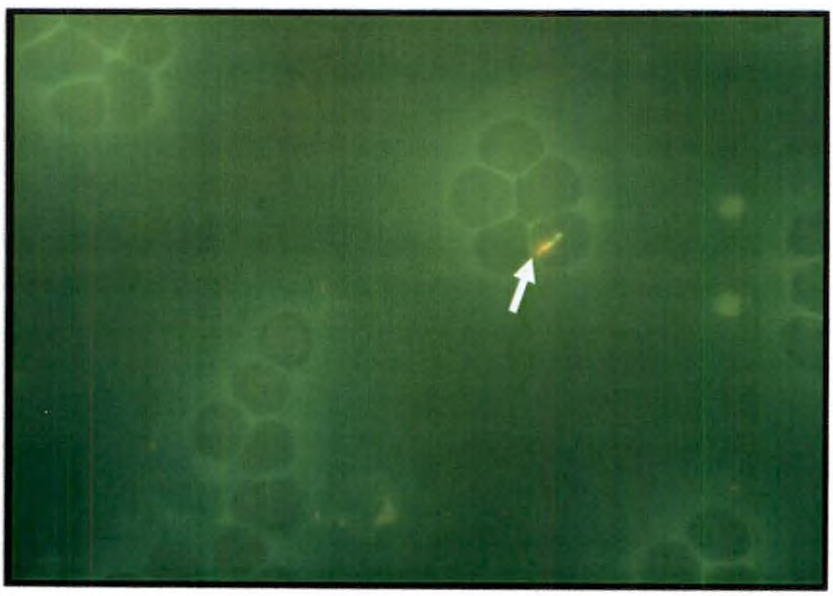


Plate 11. Giemsa stained blood smear showing *Anaplasma marginale* inclusions

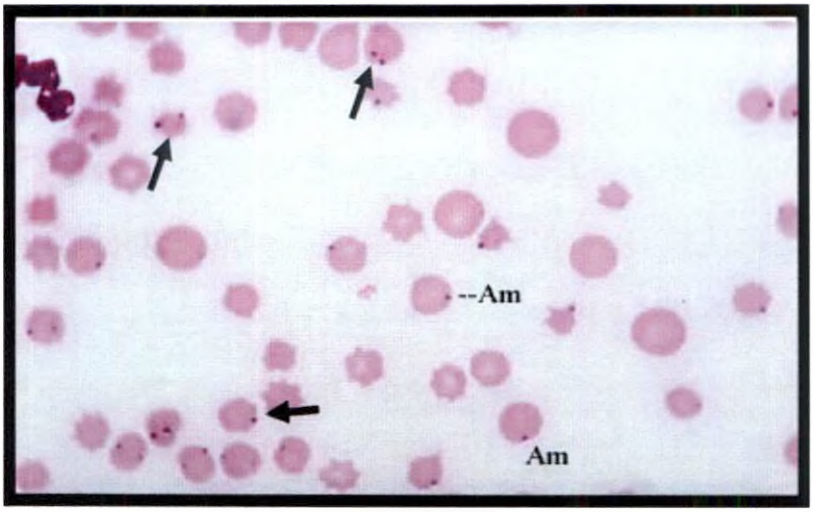
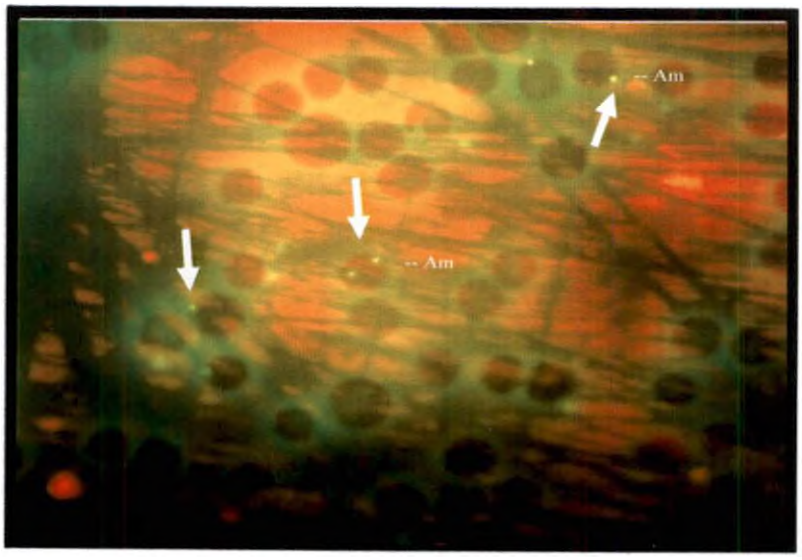


Plate 12. AO stained blood smear showing *Anaplasma marginale* inclusions



Am: *Anaplasma marginale*

4.1.2 Polymerase Chain Reaction

Heparinized blood samples from 150 animals were collected and processed for PCR. Out of the cattle screened, 85 (56.7 per cent) were found to be carriers for at least one of the haemoprotozoan or haemorickettsial organism and 65 (43.3 per cent) were free from any of these organisms. Among these, 52 samples were found positive for *Trypanosoma evansi*, 24 samples were positive for *Theileria* genus specific PCR, two samples were positive for *Theileria orientalis* and one sample was positive for *B. bigemina*. *A. marginale* specific product was seen in 25 samples and *A. bovis* specific product in five samples. Consolidated district-wise data is shown in table 3.

4.1.2.1 Trypanosomosis

The *T. evansi* specific primers amplified 227 bp fragment without any spurious products when genomic DNA was used as the template (Plate 13). The bovine leucocyte DNA which was used as the negative control did not amplify any product. Out of the 150 samples screened, 52 were positive for the presence of *T. evansi* DNA.

4.1.2.2 Theileriosis

The *Theileria* genus specific primers amplified a 1098 bp fragment from genomic DNA without any spurious products (Plate 14).

The primers specific for *T. annulata* amplified 721 bp product from positive control (DNA isolated from clinically positive animal) without any spurious amplicons (Plate 15).

An amplicon of size 857 bp without any spurious products confirmed the presence of *T. orientalis* when its specific primers were used (Plate 16).

The primers did not amplify any product when bovine leucocyte DNA was used as template. Of the total 150 samples, 24 showed the presence of *Theileria* genus specific product, only two samples showed the presence of *T. orientalis* specific product. None of the samples amplified *T. annulata* specific product.

4.1.2.3 Babesiosis

B. bigemina and *B. bovis* specific PCR were performed for all the samples. The *B. bigemina* specific primers amplified a 278 bp product without any spurious products from genomic DNA (Plate 17). One of the samples gave positive result for *B. bigemina*. An amplicon of 711 bp was expected for *B. bovis* but none of the samples gave positive result. The primers did not amplify any product when bovine leucocyte DNA was used as template.

4.1.2.4 Anaplasmosis

The *A. marginale* specific primers amplified a 457 bp product without any spurious products from DNA isolated from blood of infected cattle from Madras which was used as the positive control (Plate 18). The primers did not amplify any product when bovine leucocyte DNA was used as the template.

A. marginale specific PCR-RFLP was performed on samples which yielded the desired 457 bp product. On digestion with *Eco* RI, products of size 265bp and 192 bp were observed. On digestion with *Hind* III, products of size 227 bp and 230 bp were revealed (Plate 19). Out of 150 samples 25 were positive for *A. marginale*.

Nested PCR was performed for detection of *A. bovis* (*Ehrlichia bovis*) and *A. phagocytophilum*. A product size of 1500 bp fragment was expected after initial

amplification with primers specific for *Anaplasma* genus (Plate 20). An amplicon of size 551 bp confirmed the presence of *A. bovis* (Plate 21) while 641 bp product confirmed the presence of *A. phagocytophilum.* Seven samples revealed the presence of Anaplsma genus specific amplification. Five samples revealed the presence of *A. bovis* while none was positive for *A. phagocytophilum.*

Table 3: Results of PCR amplification for the detection of haemoprotozoan and haemorickettsial diseases.

District	No: of samples	Tryps		*Theil*		*T.an*		*T. or*		*B.b*		*Ana.*		*A.b*		*A. m*	
		No:	per cent	No:	per cent	No:	per cent	No:	per cent	No:	per cent	No:	per cent	No:	per cent	No:	per cent
Wayanad	30	3	10	3	10	0	0	0	0	1	3.3	1	3.3	1	3.3	5	16.6
Malappuram	30	4	13.3	10	33.3	0	0	0	0	0	0	2	6.6	2	6.6	4	13.3
Kozhikode	30	6	20	1	3.3	0	0	1	3.3	0	0	0	0	1	3.3	5	16.6
Palakkad	30	18	60	6	20	0	0	0	0	0	0	4	13.3	0	0	9	30
Kannur	30	21	70	4	13.3	0	0	1	3.3	0	0	0	0	1	3.3	2	6.6
Total	**150**	52	**34.6**	24	**16**	0	0	2	**1.3**	1	**0.6**	7	**4.7**	5	**3.3**	25	**16.7**

Tryps-*T. evansi*

Theil- Theileria genus

T.an- T. annulata

T.or- T. orientalis

B.b-B. bigemina

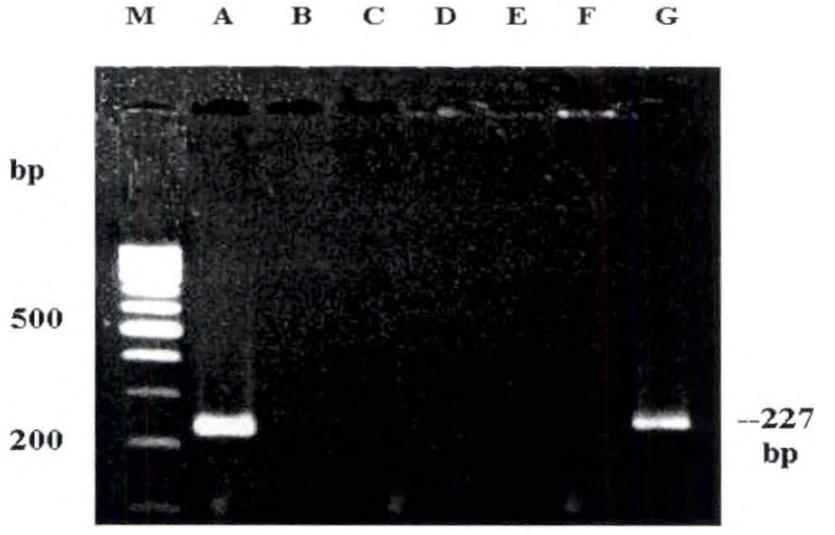
Ana.-Anaplasma genus

A.b- A. bovis

A.m-A. marginale

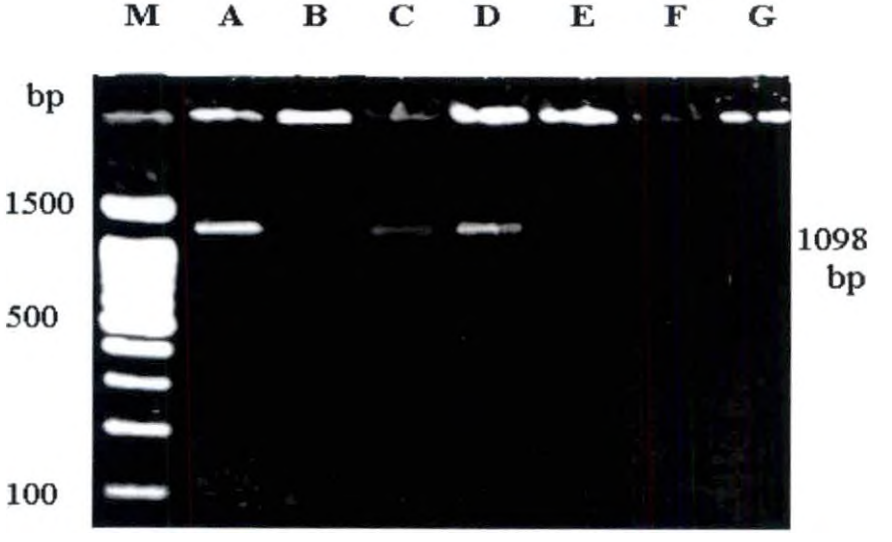
No: - number of samples positive

Plate 13. Gel documentation of PCR products after *Trypanosoma evansi* specific PCR



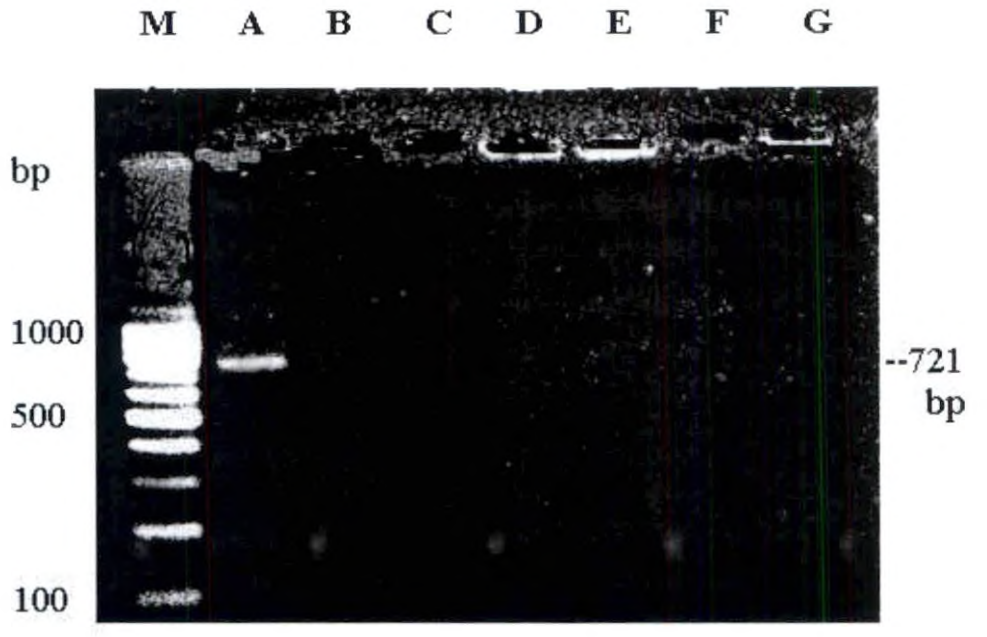
M- Standard molecular marker A- Known positive control
B- Negative control C-G – Field samples
Samples loaded on well G shows positive band

Plate 14. Gel documentation of PCR products after *Theileria* genus specific PCR



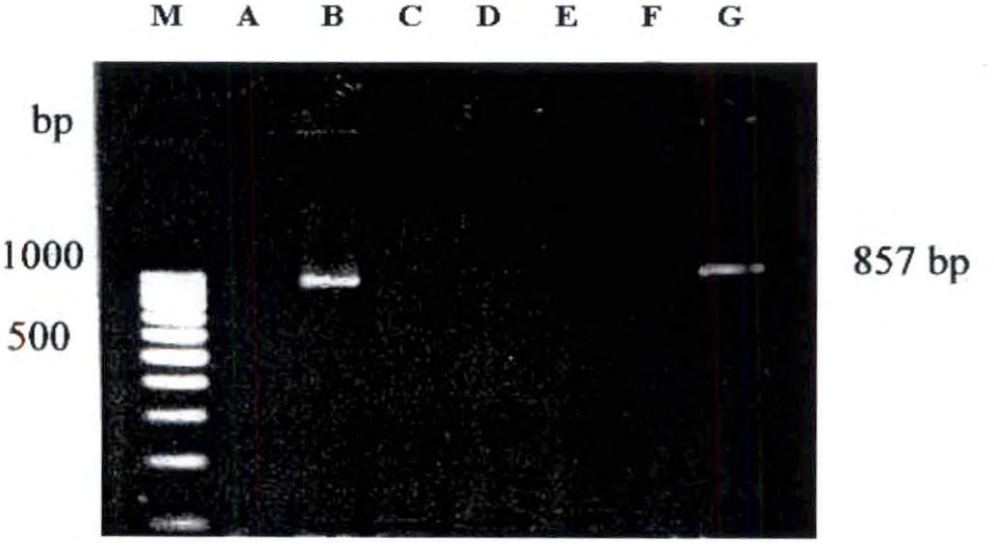
M- Standard molecular marker A- Known positive control
B- Negative control C-G – Field samples
Samples in wells C and D are positive

Plate 15. Gel documentation of PCR products after *Theileria annulata* specific PCR



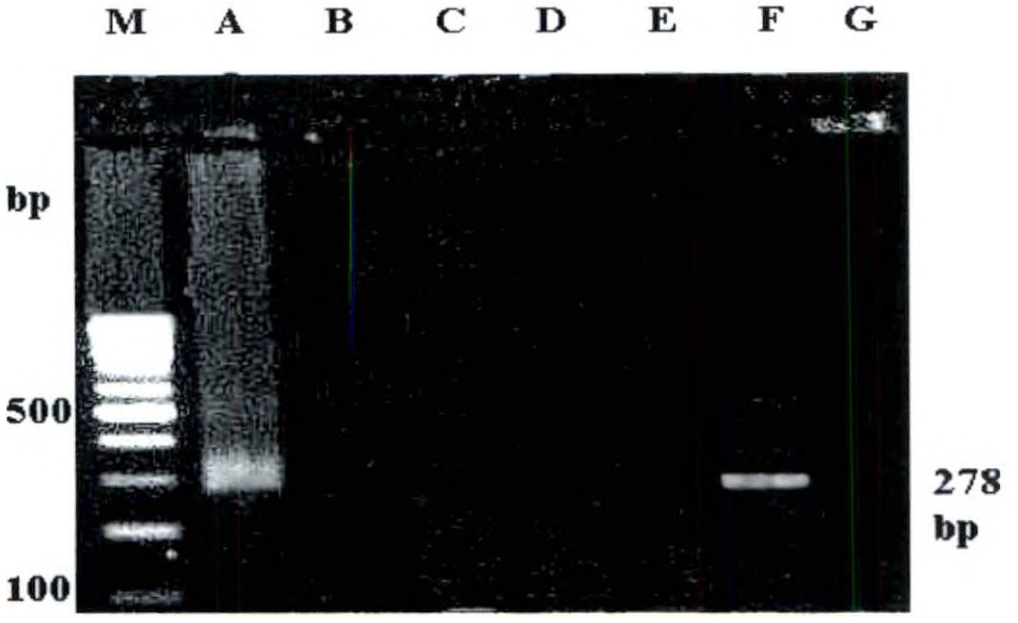
M- Standard molecular marker
B- Negative control
A- Known positive control
C-G – Field samples

Plate 16. Gel documentation of PCR products after *Theileria orientalis* specific PCR



M- Standard molecular marker
B-G Field samples
A- Negative control
Sample in wells B and G are positive

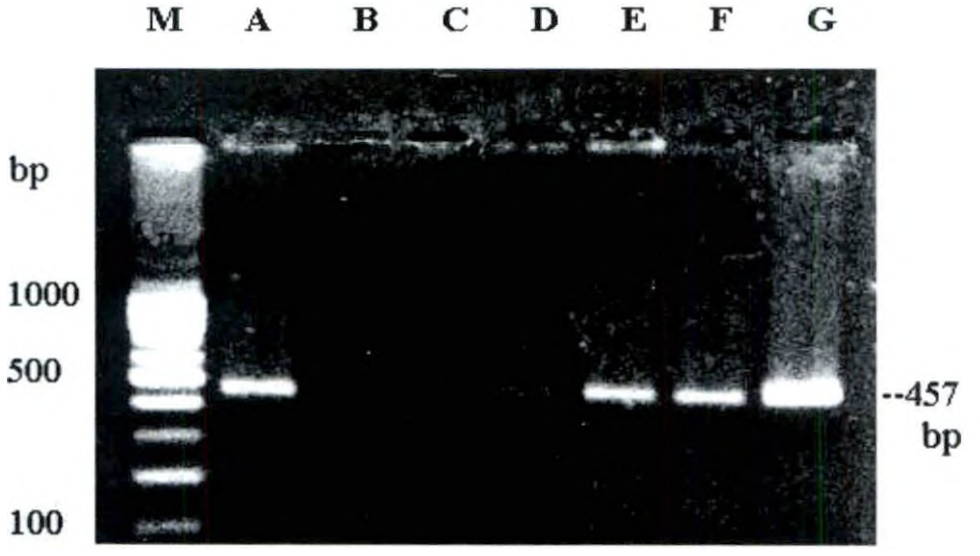
Plate 17. Gel documentation of PCR products after *Babesia bigemina* specific PCR



M- Standard molecular marker
A- Known positive control
B- Negative control
C-G – Field samples

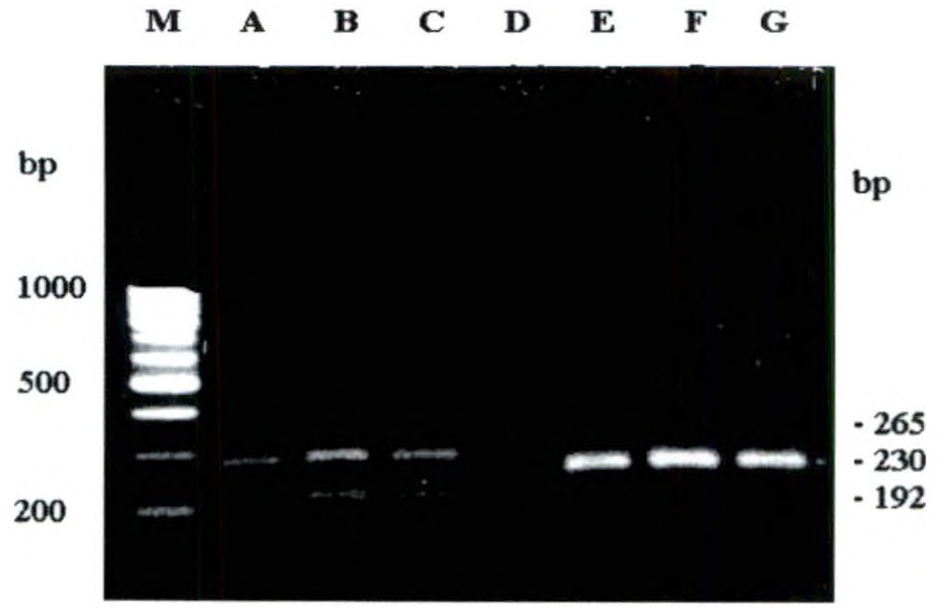
Sample in well F is positive

Plate 18. Gel documentation of PCR products after *Anaplasma marginale* specific PCR



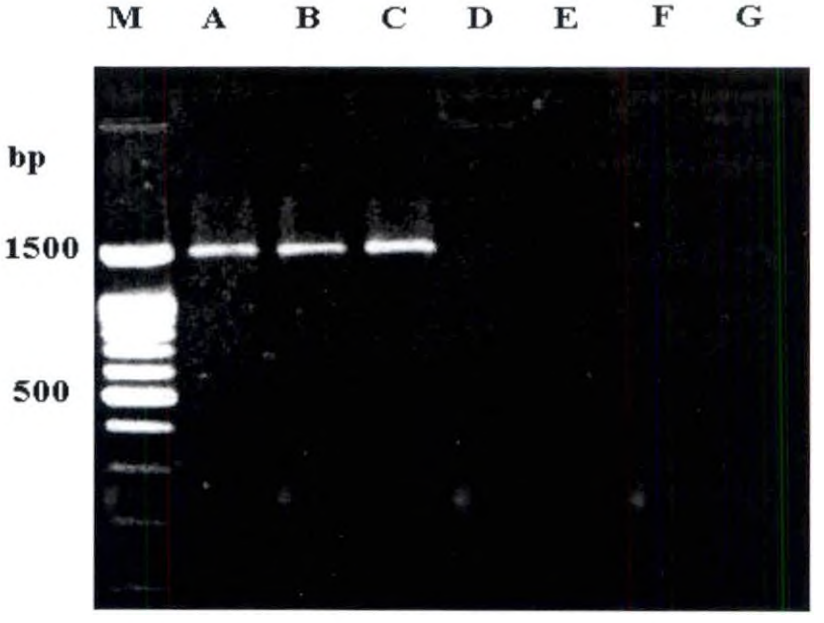
M- Standard molecular marker
A- Known positive control
B- Negative control
C-G – Field samples
Samples in well E, F and G are positive

Plate 19. Gel documentation after PCR-RFLP for *Anaplasma marginale*



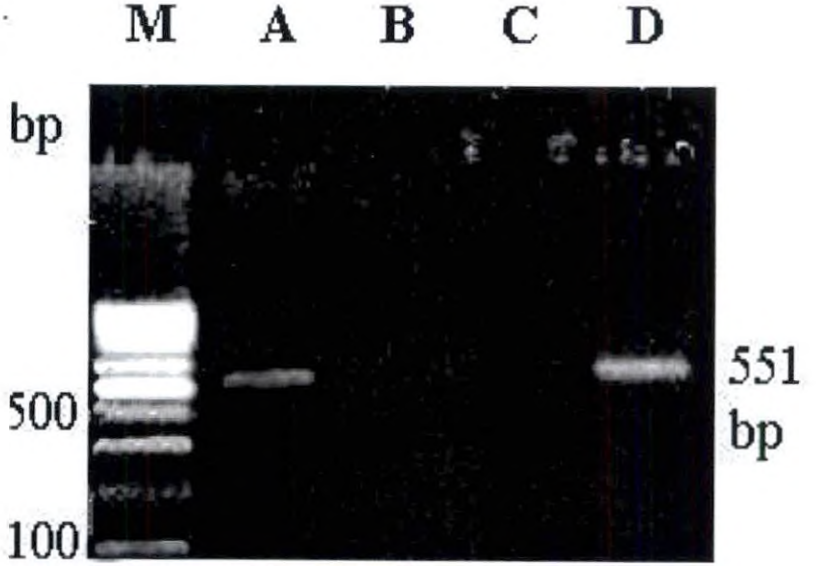
M- Standard molecular marker
A, B, C – product after digestion with *Eco* RI
D – Negative control
E, F, G – product after digestion with *Hind* III

Plate 20. Gel documentation of PCR products after *Anaplasma genus* specific PCR



M- Standard molecular marker A- Positive control
B-F –Field samples G- Negative control
Samples on well B and C are positive

Plate 21. Gel documentation of PCR products after *Anaplasma bovis* specific nested PCR



M- Standard molecular marker A- Known positive control
B- Negative control C-D – Field samples
Sample D is positive

4.2 District-wise prevalence of haemoprotozoan and haemorickettsial diseases

4.2.1 Wayanad

Six samples were collected each from five different localities *viz.*, Meenangadi, Pookot, Sulthan Bathery, Panamaram and Trikkaipetta. Thirty samples were subjected to microscopical examination and PCR. Thin blood smears stained with Giemsa stain, revealed *Theileria* like piroplasms in 21 samples, *B. bigemina* in four samples and *A. marginale* inclusions in only one out of the 30 samples examined. Acridine Orange technique detected *Theileria* like piroplasms in 22, *B. bigemina* in four and *A. marginale* inclusions in one of the sample. PCR revealed *T. evansi* and *Theileria* genus specific products in three samples each and for *B. bigemina* specific product in one sample out of the total 30 samples. Five samples gave positive PCR product for *A. marginale* and one sample for *A. bovis*. The results obtained were tabulated in table 4.

4.2.2 Malappuram

A total of 30 samples were collected from five different localities *viz.*, Thirunavaya, Marakkara, Ariyalloor, Manjery and Tavannur. Out of the 30 samples screened, examination of blood smears stained with Giemsa stain, 13 revealed the presence of *Theileria* like piroplasms. Acridine Orange stained smears revealed *Theileria* like piroplasms in 14 samples and *A. marginale* in only one sample. PCR revealed product specific to *T. evansi* in four, *Theileria* genus in 10, *A. bovis* in two and *A. marginale* in four samples. The results obtained were tabulated in table 5.

4.2.3 Kozhikode

Thirty samples were collected from five different localities of the district *viz.*, Kozhikode town, Tamarassery, Ramanattukara, Chemenchery and Vadakara. Out of the 30 samples tested, smears stained with Giemsa revealed *Theileria* like piroplasms in 11 samples. *Theileria* like piroplasms were observed in 14 samples through microscopical examination of Acridine Orange stained blood smears. Out of 30, six samples were showing positive PCR product specific for *T.*

evansi and five samples for *A. marginale*. One sample showed PCR product specific for *T. orientalis* and another for *A. bovis*. The results obtained were tabulated in table 6.

4.2.4 Palakkad

Six samples were collected each from Kodumbu, Olavakkode, Shornur, Kollenkode and Mannarkad, making a total of 30 samples from the district. Of the 30 blood smears stained by Giemsa technique, *Theileria* like piroplasms were detected in nine samples and *A. marginale* inclusions in one sample. Acridine Orange technique also gave the same result. Out of 30 samples, 18 showed PCR product specific for *T. evansi*, six samples for *Theileria* genus and nine samples for *A. marginale*. The results obtained were tabulated in table 7.

4.2.5 Kannur

Six samples were collected each from five different localities of the district *viz.*, Iritty, Edakkad, Paral, Kannur and Payyannur. Out of the 30 samples tested, microscopical examination revealed the presence of *Theileria* like piroplasms in eight blood smears after Giemsa staining and 11 blood smears after Acridine Orange staining. Polymerase chain reaction revealed *Theileria* genus specific fragment in four, *T. orientalis* in one, *A. marginale* in two and *A. bovis* in only one sample out of the 30 samples tested. The results obtained were tabulated in table 8.

Table 4. Result of microscopical examination and PCR for detection of carrier animals of Wayanad District.

Place	No. of samples	Giemsa	AO	PCR							
				Tryp	*Theil*	*T.an*	*T.or*	*B.b*	*Ana*	*A.b*	*A.m*
Meenagadi	6	TLP-4	TLP-5	1	2	0	0	0	1	1	1
Pookot	6	TLP-5	TLP-6	0	0	0	0	0	0	0	1
S.bathery	6	TLP-6	TLP-6	0	0	0	0	0	0	0	2
Panamaram	6	TLP-4	TLP-3	0	0	0	0	0	0	0	0
Trikkaipetta	6	TLP-2 *B.b*-4 *A.m*-1	TLP-2 *B.b*-4 *A.m*-1	2	1	0	0	1	0	0	1
Total	**30**	**TLP-21** ***B.b*-4** ***A.m*-1**	**TLP-22** ***B.b*-4** ***A.m*-1**	**3**	**3**	**0**	**0**	**1**	**1**	**1**	**5**

TLP-*Theileria* like piroplasms

Tryp-T. evansi

Theil- Theileria genus

T.an- T. annulata

T.or- T. orientalis

B.b-B.bigemina

Ana -Anaplasma genus

A.b-A. bovis

A.m -A.marginale

Table 5. Result of microscopical examination and PCR for detection of carrier animals of Malappuram District

Place	No. of samples	Giemsa	AO	PCR							
				Tryp	*Theil*	*T.an*	*T.or*	*B.b*	*Ana*	*A.b*	*A.m*
Thirunavaya	6	TLP-3	TLP-3 A.m-1	2	0	0	0	0	1	0	1
Marakkara	6	TLP-4	TLP-4	0	4	0	0	0	0	0	0
Ariyalloor	6	TLP-3	TLP-3	0	2	0	0	0	0	2	1
Manjery	6	TLP-3	TLP-2	0	1	0	0	0	1	0	2
Tavannur	6	-	TLP-2	2	3	0	0	0	0	0	0
Total	**30**	**TLP-13**	**TLP-14 A.m-1**	**4**	**10**	**0**	**0**	**0**	**2**	**2**	**4**

Table 6. Result of microscopical examination and PCR for detection of carrier animals of Kozhikode District

Place	No. of samples	Giemsa	AO	PCR							
				Tryp	*Theil*	*T.an*	*T.or*	*B.b*	*Ana*	*A.b*	*A.m*
Town	6	TLP-4	TLP-5	0	0	0	0	0	0	0	0
Tamarassery	6	TLP-6	TLP-6	1	0	0	0	0	0	1	2
Ramanatukara	6	-	TLP-1	0	0	0	0	0	0	0	0
Chemenchery	6	-	TLP-1	1	0	0	1	0	0	0	2
Vadakara	6	TLP-1	TLP-1	4	1	0	0	0	0	0	1
Total	**30**	**TLP-11**	**TLP-14**	**6**	**1**	**0**	**1**	**0**	**0**	**1**	**5**

TLP-*Theileria* like piroplasms

Tryp-*T. evansi*

Theil- Theileria genus

T.an- T. annulata

T.or- T. orientalis

B.b-B. bigemina

Ana-Anaplasma genus

A.b-A. bovis

A.m-A. marginale

Table 7. Result of microscopical examination and PCR for detection of carrier animals of Palakkad District

Place	No. of samples	Giemsa	AO	PCR							
				Tryp	*Theil*	*T.an*	*T.or*	*B.b*	*Ana*	*A.b*	*A.m*
Kodumbu	6	TLP-4 A.m-1	TLP-4 A.m-1	3	1	0	0	0	3	0	5
Olavakkodu	5	TLP-1	TLP-1	4	0	0	0	0	1	0	2
Shornur	6	TLP-1	TLP-1	3	0	0	0	0	0	0	1
Kollenkode	6	-	-	6	3	0	0	0	0	0	1
Mannarkad	6	TLP-3	TLP-3	2	2	0	0	0	0	0	0
Total	**30**	**TLP-9 A.m-1**	**TLP-9 A.m-1**	**18**	**6**	**0**	**0**	**0**	**4**	**0**	**9**

Table 8. Result of microscopical examination and PCR for detection of carrier animals of Kannur District

Place	No. of samples	Giemsa	AO	PCR							
				Tryp	*Theil*	*T.an*	*T.or*	*B.b*	*Ana*	*A.b*	*A.m*
Iritty	6	TLP-1	TLP-4	1	2	0	0	0	0	0	1
Edakkad	6	-	-	5	0	0	0	0	0	0	0
Paral	6	TLP-4	TLP-4	4	0	0	0	0	0	0	0
Kannur	6	-	-	4	0	0	0	0	0	1	0
Payyannur	6	TLP-3	TLP-3	6	2	0	1	0	0	0	1
Total	**30**	**TLP-8**	**TLP-11**	**20**	**4**	**0**	**1**	**0**	**0**	**1**	**2**

TLP-*Theileria* like piroplasms

Tryp-T. evansi

Theil- Theileria genus

T.an- T. annulata

A.m-A. marginale

T.or- T. orientalis

B.b-B. bigemina

Ana-Anaplasma genus

A.b-A. bovis

4.3 Comparison of staining techniques and polymerase chain reaction

The entire 150 field samples were subjected to three methods of detection of haemoparasites *viz.*, Giemsa staining, Acridine Orange staining and PCR. Polymerase chain reaction showed the maximum sensitivity in detecting the organisms in the sample. Results obtained from the two different staining techniques were almost comparable. Consolidated data on the comparison of diagnostic techniques is given in table 9 and figure 3.

Table 9. Consolidated data on results of staining techniques and PCR on samples collected from North Kerala

Diseases	Total No. of samples	Giemsa		Acridine Orange		PCR	
		No. positive	Percentage positive	No. positive	Percentage positive	No. positive	Percentage positive
B. bigemina	150	4	2.6	4	2.6	1	0.6
T. evansi	150	0	0	0	0	52	34.6
Theileria genus	150	61	40.6	71	47.2	24	16
T. orientalis	150					2	1.3
A. marginale	150	2	1.3	3	2	25	16.7
A. bovis (*E. bovis*)	150	0	0	0	0	5	3.3

4.4 Prevalence of mixed infections of haemoparasites under study

Out of the 150 samples tested, microscopical examination revealed the presence of both *A. marginale* and *Theileria* in one sample and another sample for both *B. bigemina* and *Theileria* (Plate 22).Ten samples have shown specific product for *Theileria* and *T. evansi,* seven for *T. evansi* and *A. marginale*, four for *Theileria* and *A. marginale,* one each for *T. evansi* and *A. bovis, Theileria* and *A. bovis, A. bovis* and *A. marginale* simultaneously when PCR was used. *Theileria*, *T. evansi* and *A. marginale* were detected concurrently in one sample. The data is tabulated in Table 10.

Table 10. Data of mixed infections (based on result of PCR)

Concurrent infections	No: of samples
Theileria & *A. marginale*	4
Theileria & *T. evansi*	10
T. evansi & *A. marginale*	7
T. evansi & *A. bovis*	1
Theileria & *A. bovis*	1
A. bovis & *A. marginale*	1
Theileria, *T. evansi* & *A. marginale*	1
Total samples with mixed infections	**25**

4.5 Prevalence of haemoprotozoan and haemorickettsial diseases of Northern Kerala (based on PCR results)

The major haemoprotozoan and haemorickettsial disease affecting cattle of each district are ranked based on the per cent prevalence in corresponding district and the data is shown in table 11.

Table 11. Important haemoprotozoan and haemorickettsial disease of five districts of Northern Kerala

Rank	Wayanad	Malappuram	Kozhikode	Palakkad	Kannur
1	Anaplasmosis (*A.marginale*)	Theileriosis	Trypanosomosis	Trypanosomosis	Trypanosomosis
2	Trypanosomosis	Anaplsmosis (*A.marginale*)	Anaplsmosis (*A.marginale*)	Anaplsmosis (*A.marginale*)	Theileriosis
3	Theileriosis	Trypanosomosis	Theileriosis	Theileriosis	Anaplsmosis
4	Babesiosis	Ehrlichiosis (*A.bovis*)	Ehrlichiosis (*A.bovis*)	----------	Ehrlichiosis (*A.bovis*)

The most important haemoparasite affecting cattle of Northern Kerala is *T. evansi* with a prevalence of 34.6 per cent, followed by *A. marginale* (16.7 per cent) and an uncharacterized *Theileria* organism (16 per cent). Prevalence of important haemoparasitic infections in the five districts of Northern Kerala is shown in figure 4.

The relative importance of different haemoprotozoan and haemorickettsial diseases in Northern Kerala is depicted based on their prevalence in figure 5.

Fig 3. Consolidated result of staining techniques and PCR on samples collected from North Kerala

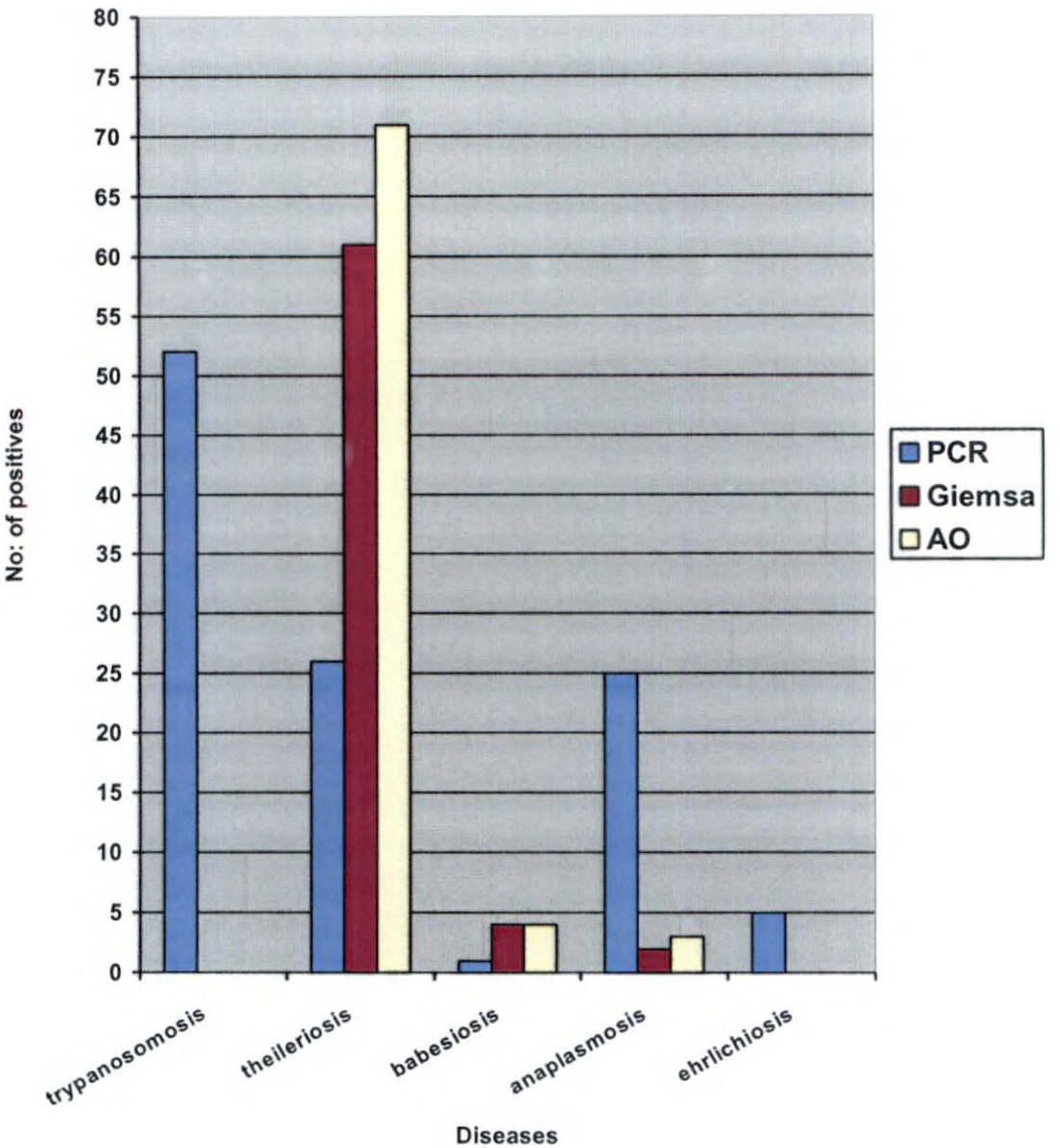
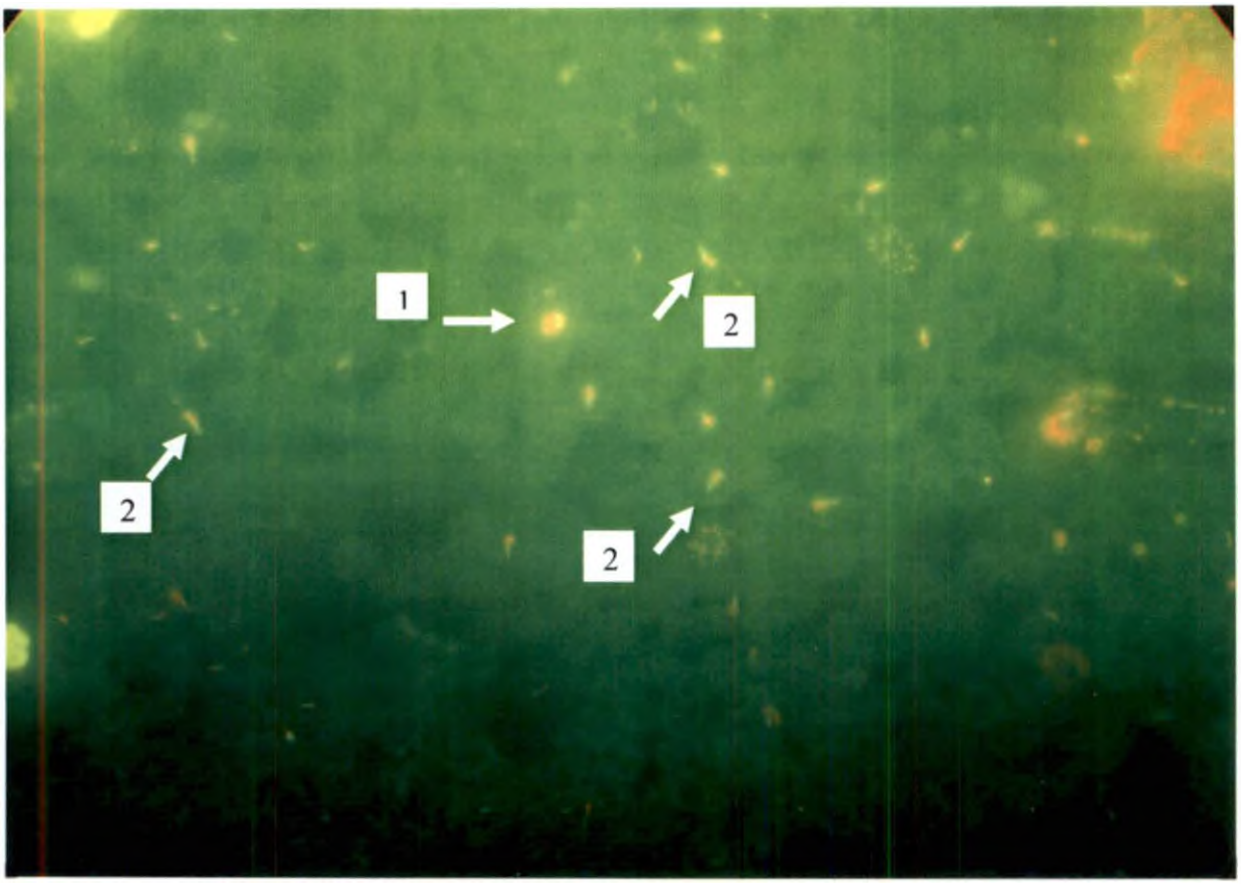


Plate 22. AO stained blood smear showing concurrent presence of *Babesia* and *Theileria* piroplasms



1- *Babesia* piroplasms

2 - *Theileria* like piroplasm

Fig 4. Percentage Prevalence of different haemoparasitic diseases in five districts of Northern Kerala

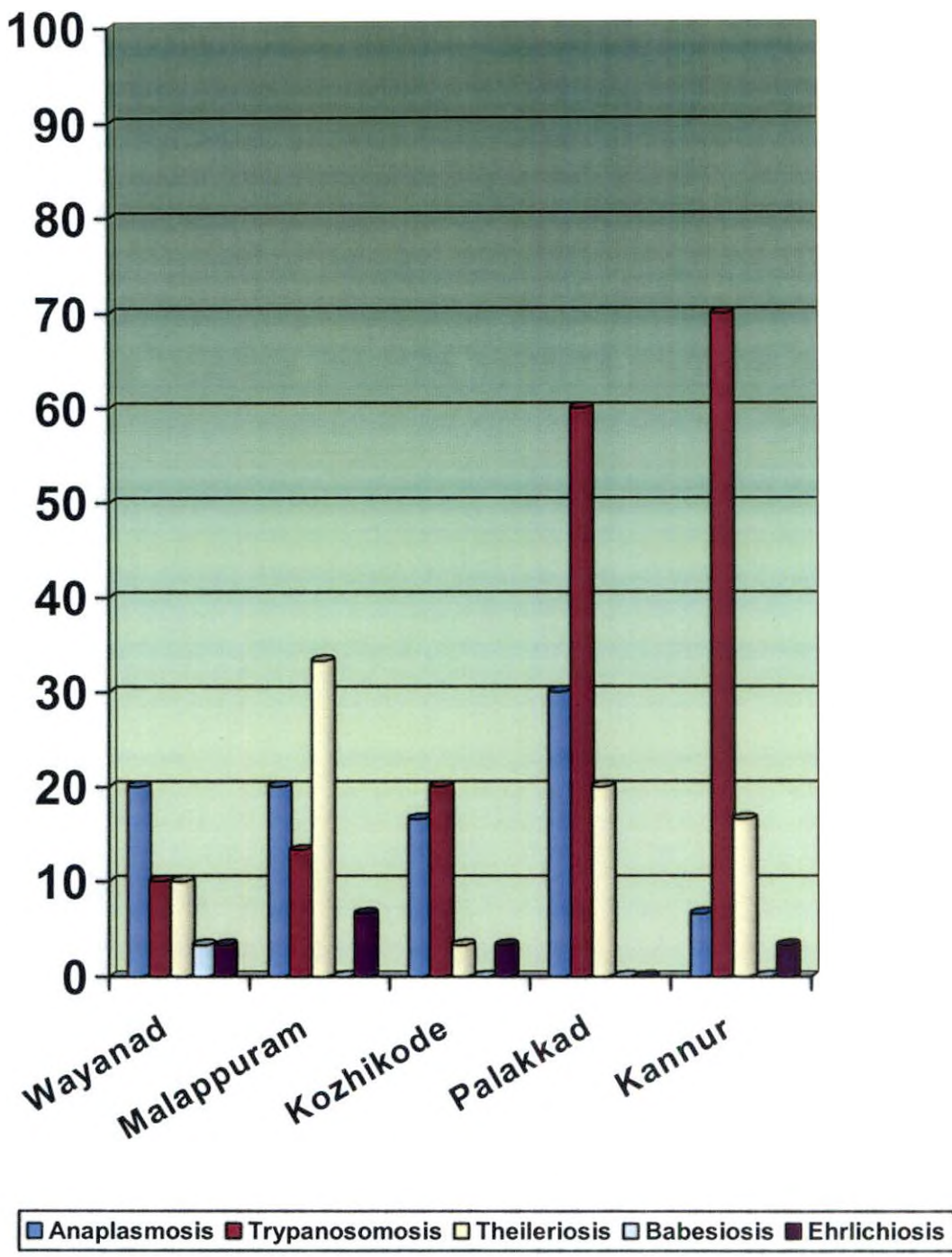
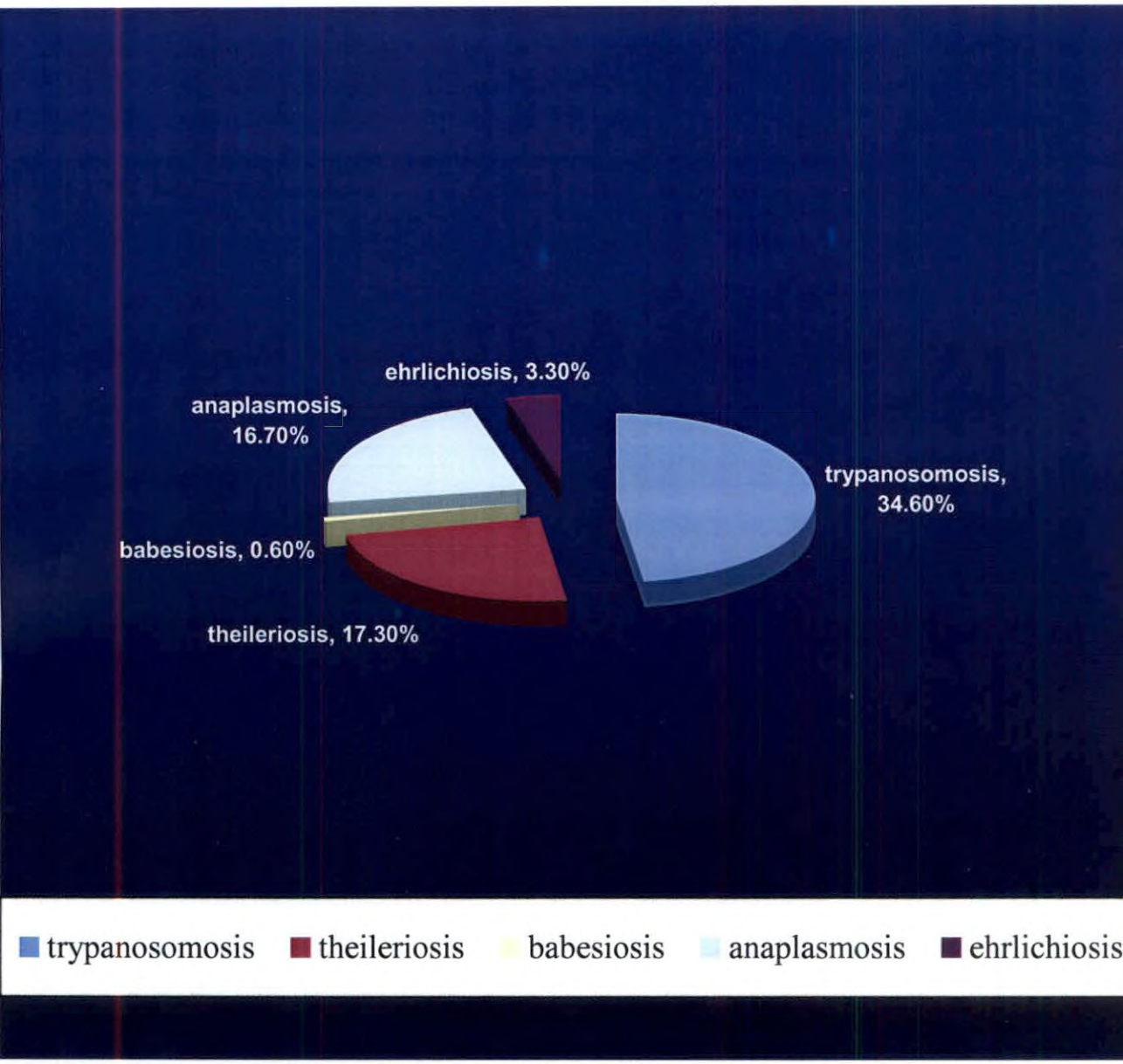


Fig 5. Haemoprotozoan and haemorickettsial diseases in Northern Kerala



Discussion

5. DISCUSSION

5.1 Screening of animals

Haemoprotozoan and haemorickettsial infections of cattle from five districts of Northern Kerala *viz.*, Wayanad, Malappuram, Kozhikode, Palakkad and Kannur were studied. Blood samples and smears were collected from 150 apparently normal cross-bred cattle. Six samples were collected from five different localities of these districts, forming a total of 30 samples from each district. Microscopical examination of Giemsa stained and Acridine Orange stained blood smears and PCR were used to screen these samples.

5.1.1 Microscopical examination

Blood smears of each animal were examined by Giemsa as well as Acridine Orange staining. The protozoan organisms that could be detected were piroplasms of *Babesia bigemina* and *Theileria*. *Anaplasma marginale* was the rickettsial organism detected by the blood smear examination. Microscopical examination of blood smears is a cheap and rapid technique still used widely in India for the detection of bovine haemoparasites. Several epidemiological studies have been conducted previously based on blood smear examination (Flasch and Ouchelli, 1992; El-Metenawy, 2000; Bell-Sakyi *et al.*, 2004).

Blood smears stained with Giemsa stain detected *B. bigemina* piroplasms in four samples (2.6 per cent). Rejitha (2003) found *B. bigemina* piroplasms in Giemsa stained smears of 12.5 per cent animals among 71 cows brought to veterinary hospitals in Thrissur and Ernakulam districts. Gopinath (2004) diagnosed babesiosis in 47 per cent of anaemic cases of cattle by blood smear examination. The lower prevalence rate observed in this study may be due to the fact that the animals screened were apparently healthy animals.

Two samples (1.3 per cent) revealed the presence *of A. marginale* inclusions. But, Gopinath (2004) reported anaplasmosis as the cause of anaemia in 37.5 per cent of cases by blood smear examination.

Microscopical examination of blood smears after Giemsa staining detected the presence of *Theileria* like piroplasms in 61 samples. Various morphological appearances of these piroplasms were observed. The piroplasms were thin or thick rod shaped or annular with light staining trailing cytoplasm (Plate 3-6). All the forms could be detected in almost all the positive smears with annular forms being found less frequently while the thick and the thin rods the maximum. Shastri *et al.* (1988) described different types of *T. orientalis* piroplasms as elongated, pear shaped, bacillary or rod shaped and show 'veil' or 'bar' structures or both in most cases. Rod shaped, round, oval or ring shaped piroplasms of *Theileria* were observed by Ravindran *et al.* (2006a) in Giemsa stained blood smears of cows showing anaemia.

Giemsa staining technique revealed *Theileria* like piroplasms in 61 blood smears, *Babesia* piroplasms in four and *Anaplasma* inclusions in two smears. Acridine Orange staining detected *Theileria* like piroplasms in 10 more samples than Giemsa stained smears (71 samples) and *A. marginale* inclusions in one more sample (Table 2). Hence, Acridine Orange staining was more sensitive compared to Giemsa staining in detecting haemoparasites as previously reported (Ristic, 1981; Wongsrichanalai *et al.*, 1991; Bose *et al.*, 1995; Damodar *et al.*, 1996). Acridine Orange staining is very easy and reliable technique which enables rapid detection of haemoparasites. But it requires costlier equipment compared to Giemsa staining technique. Ravindran *et al.* (2007b) also reported the ease and higher sensitivity of Acridine Orange over Giemsa staining in detection of haemoparasites.

T. evansi and *A. bovis* were not detected in any of the blood smears examined after Giemsa or Acridine Orange staining. Cattle are usually sub-

clinical carriers of trypanosomosis and parasitological diagnosis is difficult with chronic or latent infections in which parasitaemia is intermittent or low (Luckins, 1998). Saseendranath and Ramkrishna (1995) also reported the low sensitivity of parasitological techniques in detecting latent infections of *T. evansi.*

Microscopy revealed *A. marginale* in two samples after Giemsa staining and three after Acridine Orange staining. In the present study microscopy could detect very low prevalence of anaplasmosis due to *A. marginale* from Northern Kerala. Previously high prevalence of anaplasmosis was reported from Tamil Nadu and Haryana using the same technique (Garg *et al.*, 2004; Soundararajan and Rajavelu, 2006). The low prevalence observed in the present study may be due to the fact that carrier cattle usually do not reveal inclusions in their blood films (Ristic, 1981).

Microscopy could not detect a single case of anaplasmosis due to *A.bovis* in the present study. Earlier Soundararajan and Rajavelu, 2006 reported a prevalence of 0.53 per cent out of 150 blood samples of cattle collected from in and around Chennai. Sreekumar *et al.* (2000) could not detect *A. bovis* (*E. bovis*) in blood smear of a buffalo with ehrlichiosis and identified the etiology by culturing the organism in blood mononuclear cells.

5.1.2 Polymerase Chain Reaction

Out of the 150 healthy cattle screened, 85 (56.7 per cent) were found to be carriers for at least one of the haemoprotozoan or haemorickettsial organism and 65 (43.3 per cent) were free from any of these organisms (Table 3). The higher prevalence rate of haemoprotozoan and haemorickettsial diseases in northern districts of Kerala indicate the abundance of biological or mechanical vectors of these agents. The districts like Kozhikode, Malappuram and Wayanad have ample forest cover and many localities from where blood collection was conducted were really high range. The abundant tick fauna of these regions help in vector

transmission. A recent study revealed 18 species of ticks *viz., Haemaphysalis bispinosa, H. intermedia, H. turturis, H. aculeata, H. cuspidata, H. spinigera, H. knobigera, Rhipicephalus haemaphysalioides, R. sanguineus, R (Boophilus). annulatus, R(Boophilus). microplus, R(B). decoloratus, Hyalomma marginatum issaci, H. anatolicum anatolicum, H. hussaini, Nosomma monstrosum, N. keralensis* and *Amblyomma integrum* from various domestic animals from Kerala especially from northern districts (Prakasan and Ramani, 2007). Palakkad and Kannur are hot and humid districts ideally suited for breeding of tabanid flies especially soon after a monsoon. The close proximity of these northern districts to the neighbouring states of Karnataka and Tamil Nadu may act as another factor. There were so many reports of blood parasitic infection of bovines from these states (Ramesh *et al.*, 2003; Soundararajan and Rajavelu, 2006; Harish *et al.*, 2006). Increased animal movement through these border districts may pave way for transmission of these disease agents to native cattle of Kerala. Srinivasan and Samuel (1999) observed that haemoprotozoan infection was an important contributing factor for the development of bovine anaemia in India. Samanta *et al.* (1995) opined that anaemia results in low productivity, depressed reproduction and suppressed resistance thus affecting the viability of livestock industry.

Trypanosomosis was the major haemoprotozoan affecting cattle of Northern Kerala followed by theileriosis caused by a previously unreported and uncharacterized theilerial organism as detected by PCR (Table 3). The rickettsia *A. marginale* was also widely prevalent in Northern Kerala and may contribute to jaundice and death in the affected cattle.

The polymerase chain reaction detected 34.6 per cent cattle examined as carriers of *T. evansi*. It was the major haemoparasite detected by PCR. Smitha (2005) reported a sero-prevalence of 2.35 per cent of *T. evansi* using Dot-ELISA among 510 cattle from various farms of Kerala Agriculture University and households around Thrissur district. The higher prevalence rate obtained in this study may be due to the higher sensitivity of molecular techniques and also to the

hot humid environmental conditions of the northern districts of Kerala which favour the survival of tabanid flies.

Polymerase chain reaction revealed the presence *T. orientalis* in 1.3 per cent of animals examined. But the PCR products could not be reamplified and hence could not be further confirmed. Ravindran *et al.* (2006a) reported *T. orientalis* infection in cattle causing anaemia, from Wayanad, Kerala based on the morphological appearance of the organism in Giemsa stained blood smears. Twelve deaths were recorded during a period of 2005-2006 from Wayanad.

Sixteen per cent of the samples examined in the present study revealed amplification with *Theileria* genus specific PCR but failed to give any amplification with *T. annulata* and *T. orientalis* specific PCRs. It suggested the presence of a theilerial organism other than *T. annulata* and *T. orientalis* in cattle of Northern Kerala.

T. annulata could not be detected in any of these samples using microscopy or PCR. Prakasan and Ramani (2007) reported *Hyalomma anatolicum anatolicum,* the vector for *T. annulata,* from buffalo and cow brought for slaughter at Chelari, Malappuram. But, majority of animals slaughtered in Kerala, especially buffaloes, are brought from neighboring states and hence we presume that this study do not reflect the real tick fauna of domestic animals of Kerala. Hence, the absence of *T. annulata* in cattle may be due to the absence or low prevalence of the tick vector, *Hyalomma anatolicum anatolicum* in Kerala. But, Gopinath (2004) reported *T. annulata* infection in four animals of Thrissur district with signs of anaemia, but this finding was entirely based on the blood smear examination and clinical symptoms.

B. bigemina could be detected only in one sample (0.6 per cent) out of the total 150 samples using PCR. Even though a high prevalence of carriers of bovine babesiosis was reported, 67.6 per cent using IFAT (Ravindran *et al.,* 2002) and

70.9 per cent using SELISA (Ravindran *et al.*, 2007a) from Wayanad district of Kerala, the present study revealed a very low carrier status of *B. bigemina* in bovines of Northern Kerala. However, serological tests have inherent shortcomings like the cross reactions and inability to indicate an existing infection (Nantulya, 1990). Most enzyme linked immunosorbent assays (ELISAs) used in diagnosis of haemoparasites employ relatively crude antigens, derived from infected erythrocyte preparations. These preparations are therefore, heavily contaminated with host erythrocyte components, leading to false positive results and over-estimation of the existing disease status (Montenegro-James and James, 1998).

The molecular detection of rickettsial organisms revealed 16.7 per cent samples positive for *A. marginale* and 3.3 per cent for *A. bovis*. There were many reports of anaplasmosis from India (Sreekumar *et al.*, 2000; Garg *et al.*, 2004; Harish *et al.*, 2006). But the present study confirmed the occurrence of *A. marginale* and *A. bovis* through molecular techniques for the first time in India.

Field veterinarians and researchers in developing countries are in need for improved methods for accurate detection of parasitic agents in animals. Vector-borne parasitic protozoa and rickettsiae were 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 diagnosis and parasite detection problematic and often unreliable. These traditional methods may now be supplemented and in many instances replaced by new methods employing molecular techniques (Gasser, 2006).

5.2 Prevalence of haemoparasitic diseases in Nortern Kerala

The most important haemoparasite affecting cattle of Northern Kerala was *T. evansi* with a prevalence of 34.6 per cent of the sampled animals, followed by *A. marginale* (16.7 per cent) and then an organism belonging to *Theileria* genus (16 per cent) other than *T. annulata* and *T. orientalis* (Table 9). The higher prevalence of *T. evansi* and *A. marginale* may be attributed to the abundance of tabanid flies in the Northern Kerala, due to the hot humid climate prevailing in the region conducive for fly breeding.

About one third of the animals screened were carriers for *T. evansi*. But the reports of clinical trypanosomosis reported by field veterinarians were very less (Animal Disease Surveillance Report of AHD, Kerala, 2006). This underestimation may be due to the non-characteristic clinical symptoms and low sensitivity of parasitological diagnostic methods and cryptic nature of the organism. Gill (1991) stated that trypanosomosis in Indian cattle is underestimated because infection in them is usually sub-clinical. Saseendranath and Ramkrishna (1995) reported that parasitological diagnosis of trypanosomosis is satisfactory in animals with acute infection or high intensity of parasitaemia, but is inconsistent in chronic or latent disease when parasitaemia may be very low. Dwivedi (2000) stated that *T. evansi* inhabits in the deep blood vessels in case of low parasitaemia. So he recommended that blood for diagnosis should be obtained from both the peripheral and deep blood vessels.

Anaplasmosis, one of the major haemorickettsial diseases of cattle also showed a higher prevalence in cattle of Northern Kerala. Animal Disease Surveillance Report of AHD, Kerala (2006) revealed 1008 cases of Anaplasmosis (0.05 per cent of the total cattle population) in Kerala. But here also, clinical anaplasmosis often go unreported by the field veterinarians because of lack of facilities for confirmatory diagnosis and non-characteristic clinical symptoms.

Bovines of Northern Kerala revealed the presence of an uncharacterized *Theileria* and another piroplasms which is *Theileria* like. In gross morphology, it is not easy to distinguish the erythrocytic forms of *Theileria* and small *Babesia* (Kreier and Baker, 1987). Further investigations are needed for characterization of these organisms.

A high prevalence of haemoprotozoan and haemorickettsial infections was observed in the animals of Northern Kerala. All the animals sampled were apparently normal which indicate sub-clinical infection or carrier status of these vector borne diseases. Though the carrier animals didn't exhibit any symptoms, they remain patent to the vectors and remain as a silent source of infection to other susceptible animals (Kieser *et al.*, 1990; d'Oliviera *et al.*, 1995; Luckins, 1998). Also when they encounter a stress condition, the sub-clinical infection may flare up to clinical disease. In this way the carrier animals contribute to heavy economic loss to cattle production by increasing the morbidity of the haemoparasitic diseases. Hence for development of effective disease control measures, identification of carrier status of the apparently healthy animals is very important.

The epidemiology of parasitic diseases is changing, especially because of changes in the animal management conditions and also climatic conditions. These modifications can provoke an increase in the stock of vectors, an increase in contact rate between cattle and vectors, and an increase in the contact rate between cattle and wild fauna. This results in likely modifications of the endemic situation, with a higher risk of clinical diseases (L'Hostis and Seegers, 2002). With the changing geo-climatic conditions, gradual replacement of multi-host tick, *H. anatolicum* by one host tick, *B. microplus* is increasingly reported (Khan, 1990,1994; Sangwan *et al.*, 2000).

5.2.1 Wayanad

Out of the 30 samples collected from cattle of Wayanad, microscopical examination revealed *Theileria* like piroplasms in blood smears of 22 samples, *B. bigemina* in four samples and *A. marginale* inclusions in one of the sample. PCR detected three samples (10 per cent) as positive for *T. evansi* and *Theileria* genus each. *B. bigemina* was detected in only one sample (3.3 per cent). Five samples (16.6 per cent) were detected as positive for *A. marginale* and one sample (3.3 per cent) for *A. bovis* (Table 4).

Wayanad is a small hilly district neighbouring Tamil Nadu and Karnataka and has a considerable forest cover. The geographic and climatic features are favourable for the proliferation of ticks. It is suspected that the *Theileria* like piroplasms, widely prevalent in cattle of Wayanad, is maintained through domestic-sylvatic herbivore interaction through vector ticks. This protozoan needs urgent attention and further investigation. The morphology of these piroplasms suggested that they were similar to *Theileria* genus. PCR could detect an organism of *Theileria* genus other than *T. annulata* and *T. orientalis* in three samples from this district. Ravindran *et al.* (2006a) detected mortality among cross-bred cattle of Wayanad due to *T. orientalis* infection. They observed rod shaped, round, oval or ring shaped piroplasms suggestive of *T. orientalis*. This report was based solely on the morphological characters of the intraerythrocytic piroplasms.

Earlier reports on sero-prevalence of bovine babesiosis were with the use of IFAT (67.6 per cent) (Ravindran *et al.*, 2002) and SELISA (70.9 per cent) from Wayanad district of Kerala (Ravindran *et al.*, 2007a). But in present study only one (0.6 per cent) of the 30 animals was positive for *B. bigemina* specific PCR. The higher prevalence rate obtained in serological tests may be due to cross reactivity with other protozoa or due to previous outbreaks. Animal Disease Surveillance Report of AHD, Kerala documented 179 cases (0.17 per cent of

cattle population of Wayanad) of anaplasmosis, 643 cases (0.62 per cent) of babesiosis and 79 (0.07 per cent) of theileriosis during the year 2006.

5.2.2 Malappuram

Microscopiçal examination of the 30 blood smears detected *Theileria* like piroplasms in 14 and *A. marginale* in one sample. PCR revealed the specific product of *T. evansi* in four (13.3 per cent), *Theileria* genus in 10 (33.3 per cent), *A. bovis* in two (6.6 per cent) and *A. marginale* in four samples (13.3 per cent) out of the 30 samples (Table 5).

Theileria like piroplasms were observed in 14 blood smears and 10 samples were positive for *Theileria* genus specific PCR. This indicates the presence of a theilerial organism other than *T. annulata* and *T. orientalis* in addition to an unknown piroplasm. Malappuram is also a hilly district favouring the abundance of tick population. Maximum number of samples amplifying PCR product specific to *Theileria* genus was obtained from this district. While the number of *T. evansi* and *A. marginale* specific PCR positive cases were comparatively less. Animal Disease Surveillance Report of AHD, 2006 reported 58 clinical cases (0.04 per cent of district population of cattle) of analasmosis, 942 babesiosis (0.69 per cent) and 124 theilerioisis (0.09 per cent) reported from this district. But this report was based on the clinical symptoms and not confirmatory. The present study couldn't identify any carriers of babesiosis from Malappuram, eventhough field veterinarians reported large number of clinical babesiosis based on the major clinical symptom haemoglobinuria. This may be due to the low sample size in the study.

5.2.3 Kozhikode

Out of the 30 samples, *Theileria* like piroplasms were observed in 14 samples through microscopical examination. Using PCR, six samples (20 per

cent) were positive for *T. evansi* and five (16.6 per cent) for *A. marginale*. One sample (3.3 per cent) showed specific PCR product for *T. orientalis* and *A. bovis* each, when PCR was used for detection (Table 6).

Kozhikode district consist of a considerable area of high range adjacent to Wayanad district. Here also *Theileria* like piroplasms were detected in almost half of the samples but the parasitaemia was not as high as in samples from Wayanad. Animal Disease Surveillance Report of AHD, Kerala, 2006 have recorded 330 cases (0.2 per cent) of anaplasmosis, 1223 cases (0.74 per cent) of babesiosis and 306 cases (0.18 per cent) of theileriosis from Malappuram.

5.2.4 Palakkad

A total of 30 blood smears were examined after staining with Giemsa, in which nine samples showed the presence of *Theileria* like piroplasms and one for *A. marginale* inclusions. Using PCR, 18 samples (60 per cent) were positive for *T. evansi*, six samples (20 per cent) for *Theileria* genus and nine samples (30 per cent) positive for *A. marginale* (Table 7).

A very high prevalence of *T. evansi* and *A. marginale* was observed. Trypanosomosis and anaplasmosis are transmitted mechanically by biting flies. The high prevalence of carrier animals may be due to the increased fly breeding activity in this district. Sub-clinical trypanosomosis can be a reason for debility in bovines in this district (Gill, 1991; Luckins, 1992). Anaplasmosis may contribute to jaundice and death in affected cattle due to 'gall sickness' (Kocan *et al.*, 2003). There were 303 clinical cases (0.29 per cent) of anaplasmosis, 1802 (0.68 per cent) babesiosis and 296 (0.11 per cent) theilerioisis reported from the field (Animal Disease Surveillance Report of AHD, Kerala, 2006) based on clinical symptoms.

5.2.5 Kannur

Out of the 30 blood smears stained with Giemsa, 11 showed the presence of *Theileria* like piroplasms. Using PCR, 21 samples (70 per cent) were positive for *T. evansi*, four (13.3 per cent) were positive for *Theileria* genus, one (3.3 per cent) each for *T. orientalis* and *A. bovis*. Two samples (6.6 per cent) were positive for *A. marginale* (Table 8).

The maximum numbers of carrier animals with *T. evansi* were observed from Kannur out of the five districts screened. But, no evidence of *T. evansi* was seen in microscopical examination which suggests the subclinical occurence of the organism in cattle. Here unlike in Palakkad, the number of *A. marginale* positive cases was very less. *B. bigemina* was not observed in any of the samples. This suggests the importance of trypanosomosis in cattle of Kannur district. Animal Disease Surveillance Report of AHD, Kerala, 2006 reported 472 (0.29 per cent) anaplasmosis, 1435 (0.89 per cent) babesiosis and 275 (0.17 per cent) theileriosis cases based on the clinical signs.

5.3 Comparison of staining techniques and PCR

The number of positive cases identified by PCR was much higher compared to both staining techniques (Table 9). Eventhough trypanosomes were detected as the major protozoan using PCR, this organism could not be demonstrated by microscopy in any of the samples. There were many reports of low sensitivity of parasitological techniques in detection of trypanosomosis owing to low circulating parasitaemia (Boid *et al.*, 1985; Monzon *et al.*, 1990, Saseendranath and Ramkrishna, 1995). Cattle are usually sub-clinical carriers of trypanosomosis and parasitological diagnosis is difficult with chronic or latent infections in which parasitaemia is intermittent or low (Luckins, 1998). *A. marginale* was detected in only three out of 150 blood smears while PCR detected

25 samples as positive. *A. bovis* was not at all detected by any of staining techniques while PCR revealed five positive samples.

Thus, PCR based detection was highly sensitive and specific compared to the staining techniques. Microscopical detection of parasites with low level parasitaemia is very difficult. The failure to observe haemoparasites by blood smear examination in cattle of endemic area could not be considered as absence of infection (Akinboade and Dipeolu, 1984). Such carrier animals are important contributors to the transmission of diseases to susceptible animals by ticks and other vectors. Thus, the detection of infection in carrier animals is an important epidemiological parameter (d'Oliveira., 1995). There are several reports of the increased reliability of molecular tools for diagnosis of parasites of veterinary importance (McManus and Bowles, 1996; Gasser, 1999; Sparagano, 1999).

Species-wise discrimination of parasites which was difficult with microscopical examination was possible with PCR. *Theileria* like piroplasms observed by microscopical examination in many of the blood smears were detected as not *T. annulata* or *T. orientalis* only with the help of PCR. Using microscopy, 71 samples revealed *Theileria* like piroplasms, but 59 of these samples didn't give amplification when *Theileria* specific PCR was used. They did not amplify *T. annulata* or *T. orientalis* specific PCR product. Hence, an intraerythrocytic piroplasm not related to *Theileria* genus in bovines of Northern Kerala was established. In gross morphology, it is not easy to distinguish the erythrocytic forms of *Theileria* and small *Babesia* (Kreier and Baker, 1987). So the new piroplasm detected can be a small *Babesia* spp. Also 12 samples which were amplified using *Theileria* genus specific primers, indicated the occurrence of some unknown *Theileria* in Northern Kerala. Almeria *et al.* (2001) detected *Theileria* spp. in more than 94 per cent of the studied animals of which only 41.3 per cent were positive for *Theileria annulata* in Minorca, Spain, with the use of PCR. Cattle can be infected by at least seven species of *Theileria*, yet more unidentified species, mostly non-pathogenic, exist world wide (Uilenberg, 1981).

Almeria *et al.* (2001) reported the higher efficacy of PCR compared to microscopy of blood smears in detection of *Theileria* spp. and *Babesia* spp. and discrimination between pathogenic and non-pathogenic *Theilerias*. Khaminsou *et al.* (2007) reported the higher sensitivity PCR over Giemsa staining and Acridine Orange staining for diagnosis of human malarial infections.

In the present study microscopical examination of stained blood smears revealed *B. bigemina* piroplasms in four samples where as PCR revealed *B. bigemina* specific amplification in only one sample. This suggests the lower sensitivity of the primer set which was used for detection of *B. bigemina*. Figueroa *et al.* (1992) reported the sensitivity limit of this primer set as 10 pg while working with purified DNA samples from Mexican isolate of *B. bigemina*. But Ravindran *et al.* (2006b) could not get the reported sensitivity using these primers for detection of Indian isolates of *B. bigemina* under the recommended assay conditions. And they suggested that variation in the sequence of the target DNA in comparison with Mexican isolate may be responsible for the lower sensitivity. Hence primer with a higher sensitivity has to be used for epidemiological surveys in future.

Microscopy although, remains the gold standard for detection of parasites, it lacks sensitivity (10^{-5} to 10^{-6} *i.e.* one parasite per 10^5 to 10^6 erythrocytes), while thick blood films stained with Acridine Orange (sensitivity approximately 10^{-7}) and quantitative buffy coat analysis system (10^{-7} to 10^{-8}) are useful diagnostic adjuncts with some limitations (Bose *et al.*, 1995). For identification of carrier animals, this sensitivity (approximately 10^{-5} to 10^{-6}) is generally not adequate. And further, direct microscopy for the diagnosis of haemoparasitic infections requires experienced microscopists for accurate diagnosis and is labour intensive. In order to replace such conventional methods for routine diagnosis, a method has to be simple, rapid, specific, sensitive and inexpensive. The DNA based methods involve multiple steps and the cost of setting up the laboratory equipment and

purchase of assay reagents for the PCR based detection methods are high. However these methods are extremely valuable research tools for epidemiological studies and have been used to provide accurate epidemiological data for parasites from many different geographical locations (Singh, 1997).

5.4 Mixed Infections

Microscopical examination, revealed only two samples with mixed infection. One sample showed presence of both *A. marginale* and *Theileria*, and another sample revealed both *B. bigemina* and *Theileria*. Out of the 150 samples, 25 samples (16.7 per cent) showed the presence of more than one haemoparasite when PCR was used (Table 10). Ten samples have shown the simultaneous presence of *Theileria* and *T. evansi*. Seven samples have shown the presence of both *T. evansi* and *A. marginale*. Four samples have shown the presence of *Theileria* and *A. marginale*.

Mixed infections of *T. annulata* with *A. marginale* and with *A. bovis* were detected in 4.3 per cent and one per cent of cases respectively, from Tamil Nadu (Soundararajan and Rajavelu, 2006). Ramesh *et al.* (2008) reported the concurrent infection of *B. bigemina* and *A. bovis* in a cow from Coimbatore, Tamil nadu using Giemsa stained blood smear examination. Kandavel *et al.* (1990) reported a fatal case of mixed infection due to *Theileria*, *Babesia* and *Anaplasma* in a 20 day old cross-bred calf from Chennai. Garg *et al.* (2004) reported the mixed infection of *A. marginale* and *B. bigemina* in 9 out of the 182 blood smears screened, from crossbred cattle of Uttaranchal.

Tick vectors are responsible for transmission of a huge diversity of microorganisms and there is chance for interaction between these pathogenic species. Severity of symptoms is often associated with co-infection by different pathogens. Moreover mixed infections may affect therapeutic strategies (Dib *et al.*, 2008). Dib *et al.* (2008) also suggested that immunocompromising effect

produced by one agent open the gate to infection by other parasites as in the case of anaplasmosis and babesiosis. Bell-Sakyi *et al.* (2004) reported the increased severity of anaemia in mixed infections of *Anaplasma* and *Babesia* as well as *Anaplasma* and *Theileria* infections in ruminants compared to single infections. According to Magona and Mayende (2001), occurrence of mixed parasitic infections exacerbate the pathogenic conditions of the affected cattle, either through incremental anaemia or through synergistic effects due to interaction among various parasitic infections.

In the present study, all major bovine haemoprotozoa and haemorickettsiae were detected from the same samples using conventional and molecular techniques, which were not attempted so far in the whole country. The higher prevalence rate for haemoprotozoans and haemorickettsiales were observed in Northern Kerala. Trypanosomosis due to *T. evansi* was the major protozoan disease detected by PCR in carrier cattle even though none of the blood smears from these animals revealed this organism. The study conclusively proved higher prevalence of anaplasmosis due to *A. marginale* and *A. bovis*. The unidentified piroplasms (*Babesia*?) and uncharacterized *Theileria*, reported in the study, need further investigations.

Summary

6. SUMMARY

Haemoprotozoan and haemorickettsial diseases like trypanosomosis, babesiosis, theileriosis, anaplasmosis and ehrlichiosis cause huge economic losses to dairy farming in the form of death or reduction in milk yield due to fever, anaemia and emaciation. In the present study, prevalence of haemoprotozoan and haemorickettsial diseases of cattle of Northern Kerala was assessed. Specific diagnosis was conducted using polymerase chain reaction and microscopical examination of blood smears by Giemsa and Acridine Orange staining.

Thin peripheral blood smears and heparinized blood samples were collected from a total of 150 apparently healthy adult cross-bred cattle from Wayanad, Malappuram, Kozhikode, Palakkad and Kannur. Thirty samples were collected randomly from five different localities of each district.

The blood smears were stained by Giemsa and Acridine Orange techniques, and screened for the presence or absence of haemoparasites. Whole blood samples were processed and used for specific PCRs for different haemoprotozoan and haemorickettsial diseases of cattle. Separate primer pairs for specific detection of *T. evansi*, *T. annulata*, *T. orientalis*, *Theileria* genus, *B. bigemina*, *B. bovis*, *A. marginale*, *A. bovis* and *A. phagocytophila* were used in PCRs.

Giemsa staining technique revealed *Theileria* like piroplasms in 61 samples, *Babesia* piroplasms in four samples and *Anaplasma* inclusions in two samples. Acridine Orange stained smears revealed *Theileria* like piroplasms in 71 samples, *Babesia* piroplasms in four samples and *Anaplasma* inclusions in three samples. Microscopical examination by both the staining techniques detected mixed infection of *A. marginale* and *Theileria* and also *B. bigemina* and *Theileria* in one sample each.

Polymerase chain reaction detected 52 samples (34.6 per cent) as positive for *T. evansi*, 24 samples (16 per cent) for an uncharacterized *Theileria* organism, two samples (1.3 per cent) for *T. orientalis* and one sample (0.6 per cent) for *B. bigemina*. *Anaplasma marginale* was amplified from 25 samples (16.7 per cent) and *A. bovis* from five samples (3.3 per cent). Polymerase chain reaction specific to *T. annulata*, *B. bovis* and *A. phagocytophila* didn't give any amplification in any of the samples. Mixed infections were detected in 16.6 per cent of the samples. Ten samples showed the concurrent presence of *Theileria* and *T. evansi*, seven samples for the presence of both *T. evansi* and *A .marginale* and four samples for the presence of *Theileria* and *A. marginale* when specific PCRs were conducted.

High prevalence of haemoprotozoa and haemorickettsia was detected in the bovines of Northern Kerala indicating a higher carrier status of the animals. *T. evansi* was the major haemoparasite detected in bovines of Northern Kerala followed by *A. marginale* and an uncharacterized *Theileria* organism. The high prevalence of haemoprotozoan and haemorickettsial diseases suggests the abundance of ticks and flies in Northern Kerala which acts as vectors for these parasites.

Microscopical examination of blood smears of cattle from the hilly districts of Wayanad, Malappuram and Kozhikode revealed the presence of a *Theileria* like piroplasm which were not amplified using PCRs specific for *Theileria* genus, *T. annulata* or *T. orientalis* needs further investigation. But PCR clearly revealed that high prevalence of carriers of trypanosomosis exist in Kannur and Palakkad districts. More over, anaplasmosis is also widely prevalent in Palakkad. Both these diseases are transmitted mechanically by tabanid flies.

Polymerase chain reaction showed very high sensitivity than staining techniques for detection of haemoprotozoan and haemorickettsial diseases. Carrier animals exhibited very low parasitaemia which was difficult to be detected by staining techniques. Polymerase chain reaction was highly sensitive giving

amplification from a very minute quantity of parasite DNA. The detection of carrier animals of haemoprotozoan and haemorickettsial diseases is very important in epidemiology so that effective control measures are devised. The present study revealed that polymerase chain reaction can be used as a reliable diagnostic technique in epidemiological surveys.

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SURVEILLANCE OF HAEMOPROTOZOAN AND HAEMORICKETTSIAL DISEASES OF CATTLE OF NORTHERN KERALA

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ABSTRACT

A cross-sectional study was conducted to assess the carrier status of haemoprotozoan and haemorickettsial diseases of cattle of Northern Kerala. The diagnostic methods employed were microscopical examination of blood smears stained with Giemsa and Acridine Orange and polymerase chain reaction. A total of 150 blood samples were collected from the five districts of Northern Kerala (30 samples from each district). Microscopical examination of Giemsa stained smears revealed *Theileria* like piroplasms (41.2 per cent), *Babesia* piroplasms (2.6 per cent) and *Anaplasma* inclusions (1.3 per cent) while Acridine Orange stained smears detected *Theileria* like piroplasms in 47.2 per cent, *Babesia* piroplasms in 2.6 per cent and *Anaplasma* inclusions in 2 per cent of blood smears examined. Acridine Orange staining is more easy, time saving and reliable in detecting of haemoparasites than Giemsa staining technique. Polymerase chain reaction detected haemoprotozoan like *T. evansi* (34.6 per cent), *B. bigemina* (0.6 per cent), an uncharacterized *Theileria* organism (16 per cent) and *T. orientalis* (1.3 per cent). *A. marginale* (16.7 per cent) and *A. bovis* (3.3 per cent) were the haemorickettsial organisms detected by PCR. Mixed infections were detected in 16.6 per cent of the samples. *T.annulata*, *B. bovis* and *A. phagocytophila* were not detected in any of the samples. Polymerase chain reaction showed much higher sensitivity compared to examination of stained smears in detecting carrier animals. Trypanosomosis is the most prevalent protozoan disease among carrier animals of Northern Kerala which were detected only with the help of PCR while microscopy totally failed to detect such carriers. The high prevalence of haemoprotozoan and haemorickettsial diseases suggests the abundance of ticks and flies in Northern Kerala which acts as vectors for these parasites. Kannur and Palakkad districts revealed maximum prevalence of *T. evansi*. In addition, anaplasmosis was also widely prevalent in Palakkad district. Microscopical examination of blood smears from cattle of the hilly districts of Wayanad, Malappuram and Kozhikode revealed the presence of a *Theileria* like piroplasm

which were not amplified with PCRs specific for *Theileria* genus, *T. annulata* or *T. orientalis* and this uncharacterized piroplasm needs urgent attention.