EVALUATION OF DIFFERENT TECHNIQUES FOR THE DIAGNOSIS OF BOVINE BABESIOSIS

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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 this thesis, entitled **"EVALUATION OF DIFFERENT TECHNIQUES FOR THE DIAGNOSIS OF BOVINE BABESIOSIS"** 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 **"EVALUATION OF DIFFERENT TECHNIQUES FOR THE DIAGNOSIS OF BOVINE BABESIOSIS"** is a record of research work done independently by **Dr. Rejitha.T.S.**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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We, the undersigned members of the Advisory Committee of **Dr. Rejitha.T.S.**, a candidate for the degree of Master of Veterinary Science in Veterinary Parasitology, agree that the thesis entitled **"EVALUATION OF DIFFERENT TECHNIQUES FOR THE DIAGNOSIS OF BOVINE BABESIOSIS"** may be submitted by Dr. Rejitha.T.S., in partial fulfilment of the requirement for the degree.

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REJITHA.T.S.

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Introduction

1. INTRODUCTION

The science of animal husbandry has been monitored by man in such a way that the interaction between animals and the environment in which they are maintained is optimised to his advantage. India is basically an agricultural country with livestock undertaking an important role in the national economy. The livestock population of India amounts to 222.3 million which includes 201.5 million cattle (Banerjee, 1999).

As livestock rearing in India is carried out amidst adverse climatic and environmental conditions, disease is an important constraint to the production of milk and meat for human consumption. Animals are constantly exposed to several infections due to various organisms. Nevertheless, those due to parasites often give vital blows to cattle production and population. The geographical and climatic conditions of Kerala are quite suitable for the proliferation and transmission of a large variety of parasites such as helminths, arthropods and protozoans and it remains difficult for any animal to be free from some of these parasites.

Haemoprotozoans assume a pivotal role in undermining the health of cattle. Babesiosis caused by protozoan parasites of the genus *Babesia* belonging to the order Piroplasmida of Phylum Apicomplexa is an important tick-borne haemoprotozoan disease of cattle occurring in the tropical and subtropical countries including India (Ristic, 1981). Indigenous animals in enzootic areas are generally more resistant than the exotic stock and their crossbred progeny. In the context of the import of exotic breeds and the introduction of crossbreeding programme on a large scale, the emergence of babesiosis has fetched considerable significance in developing countries (Gautam and Chhabra, 1983).

Traditional methods of diagnosis of bovine babesiosis rely on the detection of clinical signs like pyrexia (40-41.5°C), haemoglobinuria, anaemia,

anorexia, weakness etc., and also by the detection of organisms in the blood smears. The identification of organisms in thick or thin blood films is a true evidence of infection, however a negative result does not exclude the possibility for the presence of organisms. Moreover, the animals which recover from an acute infection become carriers of the haemoparasite in course of time. Diagnosis cannot be made by stained blood films using contemporary staining methods in such carrier animals and in those which have been treated therapeutically, as parasitaemia may be very low. Thus in order to identify these animals, detection of specific antibodies are found useful rather than the babesial parasites themselves (Todorovic, 1975). Little attention has been paid to this disease owing to their natural occurrence in the subclinical form and therefore they require detailed epidemiological understanding for evolving suitable control strategies.

Several serological tests have been developed and described by Bose *et al.* (1995) for the detection of babesial antibodies in animals. Indirect fluorescent antibody test (IFAT) being one of them has been widely used for the detection of antibodies to *Babesia* spp.

Serological tests are laboratory tests, tedious to perform, requiring considerable skill, expertise and equipment. Therefore, there is an obvious need for a rapid and simple serological test which can be applied under field conditions for the diagnosis of bovine babesiosis. The Slide-ELISA is a technique worthy of note, developed in recent years. In addition, the detection of parasitic stages in the vector is also essential, as this forms a component for assessing the infection rate in vectors and also the risk of babesiosis in an area.

Taking into consideration the aforementioned factors, the present study was undertaken to

1. identify the organisms of *Babesia* spp. involved in cattle, by blood smear examination.

2. demonstrate the presence of developmental stages of babesial organisms in ticks by dissection and staining.

3. detect the antibodies to *Babesia* spp. in the sera of cattle by indirect fluorescent antibody test (IFAT) and slide enzyme linked immunosorbent assay (Slide-ELISA) and to

4. suggest a suitable diagnostic measure for bovine babesiosis, by comparing the results of the above tests.

Review of Literature

2. REVIEW OF LITERATURE

2.1 PREVALENCE

2.1.1 Species of *Babesia* Identified

Dwivedi *et al.* (1976) identified *Babesia bigemina* by microscopic examination of blood in three cattle with clinical symptoms suggestive of babesial infection, in Hisar.

Roychoudhury *et al.* (1976) detected both intra and extra corpuscular babesial parasites in the blood smears of young calves with acute babesiosis. The organisms resembled *B. argentina* with a mean size of 2.47 x 1.56 microns, centrally located and an angle varying between 60° and 180° in between the pyriform pairs. As the dam of one of the calves had an attack of acute babesiosis six days prior to calving, the incidence of the disease could be attributed to the vertical transmission in addition to the presence of transmitting vector, *Boophilus microplus* in the farm premises.

Babesia bigemina was successfully induced experimentally by Pandey and Mishra (1978) in four spleenectomised heifers of 8 to 12 months of age. The same workers in 1979 demonstrated *B. bigemina* in Giemsa stained blood smears of cattle that suffered from acute infection.

Highlighting the advancements made in the study of babesiosis, Gautam and Chhabra (1983) reported that out of the four dominant species in bovines, only *B. bigemina* and *B. bovis* were known to occur in India.

Pandey and Mishra (1984) successfully conducted an experimental study on the morphological variations and intraerythrocytic multiplication of *B. bigemina* in four indigenous calves. They observed typical pyriforms with clearly visible chromatin at rounded ends, arranged at an acute angle either in the centre or occupying the whole space of the erythrocyte. The number of organisms

varied from one to four. Deviations from normal shape, size and arrangements to single pyriform, single or paired round forms, elongated and oval forms were also detected.

Bhikane *et al.* (2001) detected *B. bigemina* in forty six clinical cases of cattle recorded at the College of Veterinary and Animal Sciences, Latur by examination of Giemsa stained blood smears which revealed different morphological features of babesia inside the erythrocytes. The common forms observed were amoeboid, oval and vacuolar forms while annular ring forms with typical pyriforms forming angle at the pointed ends were less common. Small rounded or oval developmental stages could also be detected.

2.1.2 Age

Roychoudhury *et al.* (1976), Saha and Das (1978) and Kasaralikar *et al.* (1996) observed clinical cases of babesiosis in calves below 30 days of age, of which one was peracute in nature.

Latif *et al.* (1979) opined that the age of cattle was an important factor in prevalence of *B. bigemina* infection. They succeeded in inducing the infection in six month old and one year old cattle experimentally and observed typical signs of the disease in the latter case.

James *et al.* (1985) conducted a seroepidemiological study of bovine babesiosis in Venezuela and observed the maximum incidence of parasitaemia for the organism in calves between three and six months of age, with the presence of antibodies in those between 7 to 12 months of age.

In Tanzania, Woodford *et al.* (1990) detected antibodies to *B. bigemina* and *B. bovis* from calves, sampled five days after birth, and observed a fall in antibody level during the first month of life and then a rise by nine months. The changes in antibody levels were accounted to the presence and disappearance of maternal antibody and finally tick challenge respectively.

In a survey conducted by Guglielmone (1995) in Southern America, calves aged one to four months were found positive for babesial organisms. He was also able to detect positive cases in cattle above six months of age, but failed to notice any major incidence of the disease in older cattle.

Reports of Pandey and Mishra (1979), Hostis *et al.* (1995) and Bhikane *et al.* (2001) revealed that most cases of bovine babesiosis occurred in cattle of six months to five years, the maximum number being in between two and five years. Few reports were available with regard to older cattle.

2.1.3 Sex

As per the survey conducted by Bhikane *et al.* (2001) males were more susceptible than females to bovine babesiosis on account of the stress of heavy work load, adverse climatic condition and undernutrition.

2.1.4 Breed

Cattle with babesial organisms belonged to both pure and crossbreds which showed the cardinal signs of clinical babesiosis as per the case records maintained by Dwivedi *et al.* (1976).

However, James *et al.* (1985), did not notice any breed influence on the clinical incidence of *B. bigemina* in cattle.

Yet, the assessment of innate resistance of naïve *Bos taurus, Bos taurus x Bos indicus* and *Bos indicus* cattle to virulent *B. bigemina* and *B. bovis,* by Bock *et al.* (1997) revealed that *B. bigemina* infections were uniformly mild in all breeds of cattle while *B. bovis* was quite pathogenic in *Bos taurus* and the crossbreds.

Bhikane *et al.* (2001) reported a high prevalence of *B. bigemina* infection in indigenous animals compared to crossbreds and accounted it to the difference in managemental practices.

2.1.5 Season

Johnston (1968) reported that clinical babesiosis occurred mostly in winter in Northern Queensland and in autumn in Southern Queensland which was ascribed to seasonal incidence and increase in the number of ticks on cattle.

At the same time, James *et al.* (1985) recorded a peak incidence of *B. bigemina* parasitaemia in the dry season during the months of November, December and January in Venezuela.

In South America, Guglielmone (1995) observed an outbreak of bovine babesiosis during rainfall which was in concurrence with peak *B. microplus* infestation.

High incidence of bovine babesiosis due to *B. divergens* in France was observed during spring season (May to June), with a second peak in September and October, by Hostis *et al.* (1995).

Bhikane *et al.* (2001) recorded high incidence of *B. bigemina* infection in Monsoon (June to September), followed by summer (March to May) and in Winter (October to February), even though the disease was seen to occur throughout the year.

2.2 CLINICAL SIGNS

High rise of temperature and haemoglobinuria were recorded as the chief clinical signs of bovine babesiosis by Dwivedi *et al.* (1976), Roychoudhury *et al.* (1976), Pandey and Mishra (1978), Saha and Das (1978), Pandey and Mishra (1979), Knowles *et al.* (1982), Gupta and Sinha (1988), Kasaralikar *et al.* (1996) and Bhikane *et al.* (2001).

However, Pandey and Mishra (1979) concluded that there was no correlation between the rise in temperature and percentage of parasitaemia in subclinical infections. The reason for the subclinical form in indigenous cattle

enzootic to the area was attributed to the mild infection and the following resistance acquired in a relatively young age.

Jaundice was recorded as a clinical sign of babesiosis in bovines by Pandey and Mishra (1978), Knowles *et al.* (1982) and Kasaralikar *et al.* (1996).

Dwivedi *et al.* (1976), Pandey and Mishra (1978) and Bhikane *et al.* (2001) reported a reduction of milk yield in clinical babesiosis.

Bhikane *et al.* (2001) reported abortion in one pregnant cow due to babesiosis.

Pallor of mucosa was reported as one of the clinical signs by Saha and Das (1978), Pandey and Mishra (1978) and Bhikane *et al.* (2001).

Dwivedi *et al.* (1976), Pandey and Mishra (1979) Knowles *et al.* (1982) and Bhikane *et al.* (2001) found the animals anorectic when infected with *Babesia* spp..

Other clinical signs recorded in cases of bovine babesiosis were lacrimation and yellow dung (Roychoudhury *et al.*, 1976), salivation (Roychoudhury *et al.*, 1976; Saha and Das, 1978; Gupta and Sinha 1988), posterior weakness (Roychoudhury *et al.*, 1976; Pandey and Mishra, 1978), incoordination, fibrinous pneumonia and suspended rumination (Knowles *et al.*, 1982).

2.3 SPECIES OF TICKS IDENTIFIED

Roychoudhury *et al.* (1976) recovered *Boophilus microplus* from two exotic calves with high parasitaemia, maintained at the instructional livestock farm, College of Veterinary Science, Assam Agricultural University, Khanapara and from the shed premises, concluding that both the calves suffered from tick borne infection.

In a survey conducted by Rajamohanan (1980) on the common ticks of livestock in Kerala, *B. annulatus* was seen to be the most important vector of babesiosis.

According to Gautam and Chhabra (1983) the main vector of bovine babesiosis in India was *B. microplus*. *Hyalomma anatolicum anatolicum* and *H. marginatum isaaci* were also found to be involved in the dissemination of the disease.

James *et al.* (1985) observed that infection of cattle with *B. microplus* resulted in a higher prevalence of *B. bigemina* infection in the Centro-occidental region of Venezuela in the dry season during the months of November, December and January. On the contrary, influence of *B. microplus* on the prevalence of bovine babesiosis during rainy season due to favourable humidity and environmental temperature had been mentioned by Agarwal *et al.* (2003) in an epidemiological study conducted in Durg district of Chhattisgarh state.

Bhikane *et al.* (2001) observed mild to severe infestation with *B. microplus* in all the forty six clinical cases of bovine babesiosis reported at the College of Veterinary and Animal Sciences Clinic, Latur and neighbouring places.

2.4 DIAGNOSIS

2.4.1 Blood Smear Examination

Dwivedi *et al.* (1976) confirmed three clinical cases of babesiosis in exotic and crossbred cattle by microscopic blood smear examination which revealed *B. bigemina* organisms.

Similarly Roychoudhury *et al.* (1976) detected intra and extra corpuscular parasites in blood smears of two young calves with high fever, haemoglobinuria and posterior weakness and found the organisms morphologically similar to *B. argentina*.

Pandey and Mishra (1978) evaluated the percentage of parasitaemia by blood smear examination in experimental *B. bigemina* infection in calves and positively correlated it to the rise in body temperature. The parasitaemia varied from 9 to 22 per cent and the extent varied from animal to animal due to the variation in individual resistance.

Thompson (1978) opined that examination of thin blood smears was a crude method for determining the presence of *Babesia* in circulating blood because of the less number of erythrocytes that could be examined relative to the total number in the circulation.

Pandey and Mishra (1979) diagnosed acute cases of clinical babesiosis in cattle brought to Bihar Veterinary College and from the neighbouring areas by demonstrating *B. bigemina* in Giemsa stained blood smears. They observed deviation from the normal shape, size and arrangement of the piroplasms.

According to Gautam and Chhabra (1983) detection of parasites in stained blood and organ smears was helpful for ruling out babesial infection in cattle with suggestive clinical signs.

According to Akinboade and Dipeolu (1984), the failure to observe haemoparasites like *B. bigemina* 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.

But, Bose *et al.* (1995) claimed that the method of choice to detect babesial parasites in acute conditions was the examination of Giemsa stained thin blood films for, excellent demonstration of morphological details of the parasites and species identification were possible. They opined that thick films were necessary for detection of some strains of *B. bovis.*

Hostis *et al.* (1995) succeeded in directly examining stained thin blood smears for the confirmation of bovine babesiosis in a large scale survey in France and in identifying *B. divergens* in 357 and *B. major* in one out of 424 samples.

Agarwal *et al.* (2003) examined 2,293 Giemsa stained blood smears of cattle and assessed the prevalence of babesiosis as 12.87 per cent in Durg district of Chhattisgarh state.

2.4.2 Detection of Developmental Stages of *Babesia* in Vectors

Reik (1968) reviewed the work of many scientists on the developmental stages of *B. bigemina* in *B. annulatus* and *B. microplus*. He also studied the developmental forms in different stages of *B. microplus* in varying periods of their attachments.

Developing stages of *B. bigemina* similar to vermicules or spherical bodies were detected by Morzaria and Young (1977) in Giemsa stained haemolymph smears of replete *B. decoloratus* female engorged on infected animals. They also identified developing stages of *B. bigemina* from the haemolymph smears using IFA technique.

Achuthan *et al.* (1980) demonstrated the developmental stages of *B. bigemina* in *B. microplus* as bodies referred to the sporozoites in squash preparations of larva, nymph and adult, stained with Giemsa or Leishman stain and sections of nymph and adult stained with haematoxylin and eosin. The stages in the squash preparations were spherical in shape with little cytoplasm, either single or in multiples described as fission bodies. Sections revealed elongate bodies, pyriform in shape with peripheral chromatin and vacuolated cytoplasm. Similar bodies were also observed in the squash preparations of nymph and salivary glands of adult female *Rhipicephalus sanguineus* presumably the sporozoites of *B. canis*.

As literature on methyl green pyronine staining of tissues of *Boophilus* ticks to detect babesial developmental stages was scarce and not readily available, similar studies related to *Theileria* spp. in other Ixodid ticks have been reviewed.

The first report on methyl green pyronine staining of salivary gland for the studies on the transmission of East Coast fever and tropical theileriasis to assess the infection rates of vector ticks rapidly and accurately with *T. parva* and *T. annulata* respectively was by Batungbacal (1974) followed by Amure (1975) and Martins (1977).

Walker *et al.* (1979) detected *T. parva* and *T. annulata* in whole salivary glands of *R. appendiculatus* and *Hyalomma anatolicum anatolicum* subspecies respectively by methyl green pyronine staining. The salivary acinar cell nuclei and the parasitic masses of *T. parva* and *T. annulata* stained blue or green in colour with methyl green with a clear cytoplasm. The immature parasitic mass stained red with pyronine. The nuclear material of the parasitic mass appeared diffuse, which diffused the overall staining effect and the parasitised cells had hyperplastic nuclei which stained intensely.

Irvin *et al.* (1981) described a rapid method for preparing and staining salivary glands of *R. appendiculatus* for the detection of *T. parva,* using a modified methyl green pyronine stain which minimised the loss of material and allowed the examination of salivary glands within minutes of preparation. Parasite masses in the salivary gland acini stained blue or purple and the acinar cell cytoplasm and nuclei stained pink and blue respectively. The bright red suffusion around the early parasite mass helped their easy detection.

Walker and McKellar (1983) were able to detect sporozoites of *T. annulata* in the methyl green pyronine stained whole salivary gland or Giemsa stained ground up salivary gland deposited on a microscopic slide. Infected acini were hypertrophied with pycnotic nuclei and many acini contained several parasite masses.

Methyl green pyronine staining of salivary glands of *H. a. anatolicum* was used as one of the methods by Walker *et al.* (1983) for assessing theilerial infection. The acinus with the parasite mass appeared hypertrophied with dense green stained nucleus. More than one cell per acinus was infected in heavy infections.

Sangwan *et al.* (1986) used methyl green pyronine staining of the salivary glands of Hyalomma ticks in Haryana for assessing theilerial infection. Cytoplasm of the infected acini stained pink with greenish blue colour to the acinar cell nucleus and chromatin particles of the theilerial mass. Infected acinus and the acinar cell nuclei were hypertrophied with a diffused theilerial mass.

Das and Sharma (1991 and 1994) assessed the prevalence of *T. annulata* in whole salivary gland of *H. a. anatolicum* by methyl green pyronine staining. The infected acini were denoted by a pink cytoplasm, blue nuclei and blue or purple parasitic mass. The earlier stages of parasites were dark blue, surrounded by an intense red area.

Sundar *et al.* (1993) determined the infection rate of salivary glands of *H. a. anatolicum* with *T. annulata* by methyl green pyronine staining. Both the parasite mass in the salivary gland and acinar cell nuclei stained blue with a pink colour for the cytoplasm. The parasitised acini were hypertrophied and the nucleus was pushed to the periphery by the parasitic mass.

2.4.3 Immunodiagnostic Tests

2.4.3.1 Antigen Preparation

Burridge *et al.* (1973) used parasitised erythrocytes of a spleenectomised calf experimentally infected with *B. bigemina* WRL 2035 strain for preparation of antigen coated slides. Erythrocytes were separated after centrifugation, washed and made as thin smears on glass slides for use as an antigen coated film for performing IFAT.

Antigens were prepared by Johnston *et al.* (1973) after passaging Lismorestrain of *B. argentina* through three spleenectomised calves. Erythrocytes were collected from the calf with 20 per cent parasitaemia, separated, washed and reconstituted in phosphate buffered saline. Thin smears were prepared and stored in liquid nitrogen (-100°C) till further use.

Kuttler *et al.* (1977) prepared *B. bigemina* antigen coated slides from the blood collected from an experimentally infected calf with 18 per cent parasitaemia. Thin blood films were made with washed erythrocytes to serve as antigen coated slides for IFAT and stored at –70°C after airdrying and fixing in acetone-methanol mixture till use.

Akinboade and Dipeolu (1984) served antigen slides from cultures of *B. bovis*. The erythrocytes were washed with phosphate buffered saline and then made into thin films on alcohol washed slides after adding bovine serum albumin. Air dried slides were stored at –70°C until use.

Kungu and Goodger (1990) made antigen coated slides from the whole blood, containing 10 to 15 per cent of infected erythrocytes. The blood collected with EDTA was washed with PBS, reconstituted to a 50 per cent suspension and thin smears were prepared after cytocentrifugation.

Araujo *et al.* (1998) described the preparation of *B. bovis* antigen slides for IFAT from a spleenectomised calf with 11.5 per cent parasitaemia. The slides were washed in PBS and stored at –20°C.

Mishra *et al.* (2001) performed IFAT using antigen coated films prepared from blood directly collected at the peak of parasitaemia and also with parasitised erythrocytes after removal of plasma and buffycoat. Washed and packed erythrocytes were used after diluting with PBS containing bovine serum albumin to prepare antigen films which were fixed in chilled acetone and stored at –20°C till use. They found that the removal of exoantigens and plasma improved the

quality of IFAT and facilitated the screening for unequivocal detection of *B. bigemina.*

Ravindran *et al.* (2001) prepared *B. bigemina* antigen coated slides using washed erythrocytes collected from an experimentally infected calf at the peak of parasitaemia. The slides were wrapped in aluminium foil and stored at –20°C.

2.4.3.2 Indirect Fluorescent Antibody Test

Madden and Holbrook (1968) described an indirect fluorescent antibody test for *B. caballi* in 20 carrier horses using horse blood with approximately four per cent parasitaemia as the antigen. They were able to obtain fluorescence of infected erythrocytes which they designated as 4+, 3+, 2+ and 1+ depending upon the brightness. The parasitised cells were found larger and rounder than the nonparasitised cells.

This test has been the most widely used test for diagnosis of all *Babesia* spp to date after Ross and Lohr (1968) used it for the detection of *B. bigemina.* They considered it highly specific and accurate and sufficiently sensitive to detect a positive response in animals infected two years previously.

Burridge *et al.* (1973) demonstrated the importance of dried blood sample as a source of antibody to *B. bigemina* in the IFA test in an experiment conducted in 12 bull calves in Kenya. Blood was collected and placed in a Whatman No.4 filter paper in a circular area of 1.5 cm diameter and allowed to dry. Antibodies were eluted from these dried blood samples using phosphate buffered saline and tested. They concluded that the use of dried blood samples in IFA test might facilitate epizootiological studies on *B. bigemina* since the titres of serum and dried blood samples collected simultaneously were in good correlation to each other.

Johnston *et al.* (1973) described the efficiency of IFAT to detect antibodies to *B. argentina* in both exposed and unexposed cattle of Queensland. This test was a reliable indicator of current infection, since out of 98 cattle of

exposed group, 97 gave a positive result with a titre of 64 and above with one false negative and of 290 unexposed cattle, 279 were negative and 11 were false positive.

Todorovic and Long (1976) found that IFA test was able to differentiate *B. bigemina* and *B. argentina* infections in Colombian cattle and suggested the test as useful in indicating the cause of mortality due to babesiosis.

Using the IFA test, Fujinaga and Minami (1980) differentiated a Japanese *Babesia* spp. from *B. bigemina, B. bovis* and *B. major,* in an experimental study conducted in five Holstein Friesian calves in Tokyo. They observed relatively low cross reaction between the four *Babesia* spp.

Akinboade and Dipeolu (1984) reported that the IFA test was accurate and sensitive in detecting *B. bigemina* and *B. bovis* infections and reliable for determining the endemicity of the disease in trade cattle of Nigeria. Out of the 200 sera tested, 93 per cent were positive for *B. bigemina* antibodies with high titres in 49 per cent.

Alonso *et al.* (1988) using IFA test, detected the presence of 29 and 35 per cent of *B. bigemina* and *B. bovis* antibodies respectively, in 158 blood samples from ten tick infested cattle herds in Cuba. At the same time, testing of 64 blood samples from two tick free herds, revealed four per cent samples as positive to *B. bigemina* and 1.5 per cent to *B. bovis.*

Linhares *et al.* (1992) tested 470 cattle in Goias region of Brazil for babesial infection using an IFA test and obtained 97.45 and 98.72 per cent positive for *B. bigemina* and *B. bovis* respectively, with no significant difference between the sexes, age groups or breeds.

Basalo *et al.* (1995) established an indirect fluorescent antibody test to diagnose bovine babesiosis at the diagnostic laboratory of Zulia University

Veterinary Clinic, Venezuela. The test on 74 samples indicated 100 per cent prevalence at 1:80 dilution and 71.6 per cent at 1:160 dilution.

Papadopoulos *et al.* (1996) demonstrated cross reaction against *B. bigemina* antigen with anti-*B. bovis* serum and a high correlation between antibody titres of these two species and *B. divergens* in sera collected from Macedonia, Greece, remarking that IFAT was not always satisfactory for diagnosing infections in regions where animals were infected with different piroplasms.

Reddy *et al.* (1997) standardised an indirect fluorescent antibody test to investigate the prevalence of *B. bigemina* in experimentally and naturally infected bovids. They obtained a sensitivity of 87.5 per cent seven days post infection and a seroreactivity of 9.4 per cent in 214 sera collected from *B. microplus* infested cattle.

Araujo *et al.* (1998) who evaluated the performance of IFAT for the detection of *B. bovis* in sera of cattle in Bahia State, Brazil, reported 440 out of 462 samples as positive. They considered the test as highly specific, accurate and sufficiently sensitive to detect antibodies for at least 276 days post-inoculation.

Quijada *et al.* (1998) examined 264 cross bred cattle of Lara State, Brazil for *B. bigemina* infection by IFAT and obtained 210 samples (79.6 per cent) as seropositive, thereby declaring the area as endemic for the disease.

Tewari *et al.* (2000) standardised an indirect fluorescent antibody test to investigate the prevalence of bubaline babesiosis caused by *B. bigemina* in 200 sera collected from a local abattoir at Izatnagar. They observed 12 samples as seropositive and also noted a serological cross reactivity of *B. bigemina* antigen to *B. bovis* reference serum and attributed it to the false positive reaction and apparent lack of specificity of the test.

Mishra *et al.* (2001) attempted an improved method of indirect fluorescent antibody test using antigen coated films devoid of babesial exoantigens for the detection of *B. bigemina* antibodies. Fifty out of seventy six samples collected from Wayanad district of Kerala were detected as seropositive.

Ravindran *et al.* (2002) conducted a seroprevalence study of *B. bigemina* infection in apparently healthy crossbred bovids of Wayanad district of Kerala using IFAT and obtained 67.6 per cent samples as positive out of 93 samples, which indicated the presence of subclinical infection in animals.

2.4.3.3 Slide Enzyme Linked Immunosorbent Assay

ELISA is a test system which holds the greatest promise for future in the diagnosis of babesiosis.

Kungu and Goodger (1990) described a modification of standard ELISA technique, the Slide-ELISA for diagnosing *B. bovis* infection and for screening of monoclonal antibodies in experimentally infected cattle maintained in Queensland. From 19 infected animals, 15 were positive and two were doubtful 12 months post infection, while 17 were positive three years post infection. They found Slide-ELISA as a sensitive, specific, economical and a very convenient test to perform and the test slides could be kept for at least one week at room temperature for later confirmation of results.

Bose *et al.* (1995) reported that ELISA was a very sensitive and efficient test. It had the advantage of objective reading of the result, computer evaluation of data and facility for handling large number of samples at a time.

Araujo *et al.* (1998) reported a high specificity for ELISA when used for detecting *B. bovis* antibodies in 462 sera collected from Bahia State, Brazil. They detected 449 samples as seropositive but, for performing ELISA, a well equipped laboratory was required.

Molloy *et al.* (1998) developed a competitive inhibition ELISA using monoclonal antibodies directed against a 58 KDa *B. bigemina* merozoite antigen. The sensitivity and specificity were 95.7 and 97 per cent respectively. They opined that the test was suitable for epidemiological studies where *B. bigemina* and *B. bovis* had overlapping distributions, as the specificity was not affected by antibodies to *B. bovis.*

Even though ELISA is easy and simple to perform under laboratory condition, its use as a field diagnostic test is limited.

Ravindran *et al.* (2001) first reported on the use of Slide-ELISA for the detection of *B. bigemina* by which they screened 48 cattle sera from an endemic area in Orissa and recorded 90 per cent samples as positive. They mentioned about the great potential of SELISA for use as a field diagnostic test.

2.5 COMPARISON OF DIFFERENT DIAGNOSTIC TESTS

Todorovic and Long (1976) compared indirect fluorescent antibody test and complement fixation test for the diagnosis of *Babesia* spp. infections in 372 sera collected from the Colombian cattle. IFA techniques detected *B. bigemina* and *B. argentina* antibodies 2.5 weeks and 4.6 weeks earlier respectively, than CF test. IFA had the advantages of simplicity, economy and speed of performance over the CF test.

Kuttler *et al.* (1977) made a comparison of IFA and CF test by evaluating 130 sera collected sequentially from five adult cattle artificially infected with *B. bigemina* and found IFA was most sensitive of the two. It detected antibodies after 90 days of infection whereas CF test needed a constant tick exposure to detect the antibodies.

Enzyme-linked immunosorbent assay (ELISA) was preferred to IFA and CF test by Bidwell *et al.* (1978) in a comparison study between the three tests for detecting antibodies against *B. divergens* and *B. major* in samples of artificially

infected cattle. Even though little was there to choose between IFA and ELISA tests, ELISA had advantages of more sensitivity and less operator bias or stress.

Fujinaga and Minami (1981) detected antibodies against *T. sergenti* and *B. ovata* in cattle in Japan by IFA and CF tests and found IFA more effective for diagnosing babesial infection than the other. Simultaneous testing of both the parasites were possible by a single IFA testing procedure.

Akinboade and Dipeolu (1984) compared blood smear and IFAT for detection of *B. bigemina* in trade cattle of Nigeria. It was confirmed by IFAT that 93 per cent of 200 cattle were positive for the infection while only 9 per cent out of 1200 cattle could be detected positive by smear examination. They concluded that serological diagnosis could monitor the infection better than by blood smear examination.

Alonso *et al.* (1988) were able to detect *B. bigemina* and *B. bovis* antibodies by IFAT on 35 and 29 per cent of 158 blood samples respectively, collected from ten tick infested cattle herds of Cuba, whereas blood smears were positive for the organisms only in two per cent and three per cent of the samples respectively.

Kungu and Goodger (1990) compared the sensitivity and specificity of Slide-ELISA with that of indirect haemagglutination assay and indirect fluorescent antibody test and found Slide-ELISA more sensitive as it detected antibodies to *B. bovis* 36 months after a single infection.

Indirect immunofluorescence (IIF) and ELISA were compared by Reddy *et al.* (1997) to investigate the prevalence of bovine babesiosis caused by *B. bigemina* in experimentally and naturally infected animals. Both IIF and ELISA were positive for the antibodies seven days after experimental infection, with 87.5 per cent and 100 per cent sensitivity respectively. Out of 214 sera collected from *B. microplus* infested cattle, seroreactivity was 33.6 per cent for ELISA and 9.4 per cent for IFAT.

The performance of an ELISA, IFAT and Rapid conglutination test (RCT) was compared and evaluated by Araujo *et al.* (1998) with 462 cattle sera from Bahia State, Brazil. The performance of these tests were similar with sensitivity of 97.2 per cent, 96.7 per cent and 92.4 per cent respectively for ELISA, IFAT and RCT.

Tewari *et al.* (2000) investigated the prevalence of bubaline babesiosis due to *B. bigemina* by IFAT and ELISA. Out of 200 sera tested, 12 (8 per cent) were positive by IFAT and 16 (6 per cent) by ELISA. It was seen that ELISA appeared marginally superior to IFAT.

Materials and Methods

3. MATERIALS AND METHODS

3.1 PREVALENCE

Cattle presented at the various Veterinary hospitals of Thrissur and Ernakulam districts and those belonging to the University Livestock Farm, Mannuthy and the Cattle Breeding Farm, Thumburmuzhi were screened for infections of *Babesia spp.* during this study conducted for a period of 14 months from July 2002 to August 2003.

Blood samples collected from animals showing suggestive clinical signs of babesiosis like fever, haemoglobinuria, anaemia, pale mucous membrane, general weakness, anorexia and with tick infestation formed the material for the present study. Samples were collected from a total of 71 cattle. A brief clinical history of the cases was also recorded, besides attending to factors such as age, sex, breed etc. of the suspected animals.

The age group of the animals were categorised as (a) one to six months (b) six months to one year (c) one to three years and (d) above three years.

Only two breeds were identified from among the cattle examined – indigenous and crossbreds.

3.2 SCREENING OF BLOOD SMEARS

Thin smears were prepared by collecting one drop of the peripheral blood from the eartip of the animal on a clean grease-free glass slide and stained using Giemsa's stain. Smears were examined under the oil immersion objective of a light microscope to detect the presence of babesial organisms.

3.3.2.2 Methodology

Ticks were cleaned with a smooth camel hair brush before dissection. They ticks were held in between the thumb and forefinger with the dorsal side up and dissected with a sharp blade from the posterior boarder proceeding anteriorly and the viscera exposed. Dissected ticks were immersed in PBS in a petri-dish and placed under a dissecting microscope (10 to 15 x). Paired salivary glands visible anteriorly on either side of the tracheae were removed carefully with a teasing needle and transferred with a drop of the medium (PBS) to a clean grease-free miscroscopic slide with the help of a camel hair brush. The slide was then brought under the dissecting microscope at the same magnification as before to separate the salivary glands, keeping the acinar cells intact. Gut that appeared as brownish strands in the central area and the ovaries visible as a bunch of grapes towards the posterior part were also separated and spread on different glass slides with a drop of the medium which was also done under dissecting microscope. Care was taken to complete the teasing and separation of the tick tissues on glass slides before the preparations dried up.

The preparations on the slides were then air dried and fixed for two to five min. in Carnoy's fixative. They were then rinsed for two min. in 70 per cent alcohol, followed by a rinse in distilled water for another two min. Then the slides were immersed in methyl green pyronine for seven to nine min. After staining, the slides were rinsed in distilled water, air dried and mounted under cover slip with DPX mountant.

Slides were scanned at 100 x magnification of a light microscope and then at 400 x magnification to detect the developmental stages of *Babesia* if any.

3.4 IMMUNODIAGNOSTIC TESTS

The indirect fluorescent antibody test (IFAT) and the slide enzyme linked immunosorbent assay (Slide-ELISA) were the two immunodiagnostic tests

employed in the present work to determine the presence of antibodies to babesial organisms in cattle suspected for the disease condition.

Borosil brand of glasswares, Tarson plastics and analytical or guaranteed reagent grade chemicals were used for the serology during the present study.

The materials were processed by standard methods and sterilised either in hot air oven or autoclaved, depending upon the material to be sterilised.

3.4.1 Collection of Blood

Whole blood in volumes of 10 ml approximately was collected from 71 cattle suspected for babesiosis for separation of sera. Sera were stored in polypropylene serum vials at –20°C for the detection of antibodies to babesial organisms.

3.4.2 Preparation of Antigen-coated Slides

Antigen coated slides for performing the immunodiagnostic tests, the IFAT and Slide-ELISA were prepared according to the method described by Mishra *et al.* (2001).

Whole blood procured from the cattle detected positive for babesial organisms by blood smear examination, was used for the preparation of antigen coated slides. Whole blood collected in EDTA vials was centrifuged at 3000 revolutions per min. for 10 min. After removing the plasma and buffy coat, the top most layer of packed erythrocytes was collected and washed five times in PBS (pH 7.2). The washed cells were then reconstituted to a 50 per cent suspension in PBS containing 0.5 per cent bovine serum albumin (BSA). Thin smears were prepared with one microlitre each of the suspension, on clean grease-free glass slides. They were air dried and fixed in chilled acetone for five min. Wrapped the slides in aluminium foil and stored at –20°C until use.

Babesia bigemina antigen coated slides procured from the Indian Veterinary Research Institute (IVRI), Izatnagar were also used for the immunodiagnostic tests.

3.4.3 Indirect Fluorescent Antibody Test (IFAT)

3.4.3.1 Buffers and Reagents

a. PBS stock (10 x) pH 7.4

Sodium chloride	-	80 g
Potassium chloride	-	2 g
Disodium hydrogen phosphate	-	15 g
Potassium dihydrogen phosphate	-	2 g
Distilled water	-	1000 ml

PBS working solution

PBS (10 x)	-	100 ml
Distilled water to make	-	1000 ml

Adjusted the pH to 7.4, autoclaved and used

b. Mounting medium

Glycerol	-	9 parts
PBS	-	1 part

c. Fluorescein isothiocyanate (FITC) conjugated rabbit - anti-bovine IgG procured from Genei, Bangalore.

d. Distilled water

e. Negative sera

Sera collected from healthy animals without any tick infestation and free from blood parasites were taken as negative control.

f. Positive sera

Serum from known positive cattle and the positive serum samples supplied from the IVRI, Izatnagar were used as positive control sera.

g. Test sera

3.4.3.2 Test Proper

A series of defined experiments to determine the operating conditions of the test were undertaken. The series included tests to determine incubation time, temperature and dilutions of the primary and secondary antisera. The final procedure adopted was in accordance to Mishra *et al.* (2001) with minor modifications.

1. All the sera including the positive and negative controls were thawed to room temperature and diluted to 1 in 40 with PBS pH 7.4.

2. The antigen coated slide was gradually brought to room temperature and incubated at 37°C for 10 minutes.

3. Circular areas of approximately 5 mm diameter were marked on a glass slide with an oil based marker pen.

4. Test sera of 10 µl aliquots were placed over each circle so as to form a bubble in a known sequence. Positive serum was charged to the bottom right hand circle and the negative control to the left of the positive control.

5. Incubated the slides for one hour in a moist chamber at room temperature.

6. The excess amount of sera was removed with a single and quick downward movement and then given a three, five minute rinse through PBS, followed by a final rinse through distilled water for five minutes.

7. Air dried the slides

8. Added 10 µl of appropriately diluted FITC conjugate (1 in 40 with PBS pH 7.4) to each circle.

9. Incubated the slides at room temperature for one hour in a moist chamber.

10. Repeated the washing procedure as described earlier and air dried the slides.

11. Applied a drop of mounting medium, covered with a coverslip and examined under the oil immersion objective of a fluorescent microscope (Olympus Bx 51).

3.4.3.3 Interpretation

A positive result was indicated by a specific fluorescence of the infected erythrocytes or piroplasms and a negative result by the absence of any specific fluorescence.

3.4.4 Slide Enzyme Linked Immunosorbent Assay (Slide ELISA)

Trials with different incubation temperature and time, dilution of the primary and secondary antisera and conjugate were undertaken for standardisation of the test. Eventually, the methodology standardised by Ravindran *et al.* (2001) was adopted with some modifications and employed in the present study.

3.4.4.1 Buffers and Reagents

a. PBS Stock (10 x) pH 7.4

Sodium chloride	-	80 g
Potassium chloride	-	2 g
Disodium hydrogen phosphate	-	15 g
Potassium dihydrogen phosphate	-	2 g
Distilled water	-	1000 ml

PBS working solution

PBS (10 x)	-	100 ml
Distilled water to make	-	1000 ml

pH was adjusted to 7.4, autoclaved and used

b. Tris-chloride buffer pH 7.6

Solution A - 0.2 M solution of tris (hydroxy methyl aminomethane)

Tris (hydroxymethyl aminomethane)	-	24.2 g
Distilled water	-	1000 ml

Solution B - 0.2 M HCl

Mixed 50 ml of solution A and 38.4 ml of solution B and made the volume to 200 ml with distilled water. Adjusted the pH to 7.6, autoclaved and used.

c. Horse-Radish Peroxidase (HRP) conjugated rabbit-anti-bovine IgG procured from Genei, Bangalore.

d. Substrate solution

Diamino benzidine (DAB)	-	5 mg
PBS (pH 7.4)	-	10 ml

Added 10 µl of hydrogen peroxide (30 per cent) to the freshly prepared substrate solution before charging on to the slides.

e. Mounting medium

Glycerol	-	9 parts
PBS	-	1 part

f. Distilled water

g. Negative and positive control sera

h. Test sera

3.4.4.2 Test Proper

1. The control and test sera were thawed to room temperature. The sera were diluted to 1 in 40 in PBS pH 7.4

2. Allowed the antigen coated slides to come to the room temperature.

3. Circular areas of about 5 mm diameter were marked on a slide with an oil based marker pen.

4. Placed 15 µl aliquots of diluted sera on each circle in a known sequence with the positive and negative control at the top and bottom on the right hand side respectively.

5. Incubated the slides at 37°C in a moist chamber for 90 min.

6. The slides were washed with PBS and rinsed in two changes of PBS for five min. each and air dried.

7. Fifteen microlitres of optimally diluted HRP rabbit-anti-bovine IgG (1 in 40) in PBS was added to each circle before incubating the slides at 37°C in a moist chamber for 30 min.

8. Repeated the washing procedure described earlier with tris-chloride buffer.

9. A volume of 15 µl of the substrate was charged to each circle and allowed to react in a moist chamber for 20 min. at room temperature.

10. Washed off the substrate with distilled water and air dried the slides.

11. Placed the coverslip after applying one drop of the mounting medium and observed under the oil immersion of a light microscope (1000 x).

3.4.4.3 Interpretation

Immunostained piroplasms and erythrocytes stained dark brown with positive serum while no colour development could be noted in the negative controls.

3.5 STATISTICAL ANALYSIS

Statistical analysis of the data on evaluation of different tests for the diagnosis of bovine babesiosis was done using the test for proportion as per the method described by Snedecor and Cochran (1994).

Results

4. RESULTS

4.1 PREVALENCE

A total of 71 blood samples collected from cattle suspected for babesiosis were screened for babesial infections. The four diagnostic methods used were, screening of blood smears, examination of ticks and serodiagnosis by IFAT and Slide-ELISA.

Both acute and subclinical forms of the disease were encountered during this study. Acute disease was observed in 11 (15.49 per cent) animals out of 71, while 60 samples (84.5 per cent) were collected from animals which were presumed to suffer from subclinical infections (Table 1).

Table 1. Status of babesial infections encountered

Status of infection	Number	Per cent
Acute	11	15.49
Subclinical	60	84.5
Total	71	

All the acutely infected cattle exhibited symptoms like haemoglobinuria, fever (40 to 41.6°C), pallor of mucosa and anorexia. Drastic reduction of milk yield was observed in five of them. Only seven animals were presented with ticks.

The signs shown by the subclinically infected animals were mild elevation of temperature (39.1 to 39.4°C) in five and a permanganate coloured urine In three. Another three of them showed pale, icteric mucous membrane, a slight colour change for urine, reduction in milk yield and severe anaemia. General weakness and pallor of mucosa were noted in 25 animals while three

animals were with general weakness, rough hair coat, anorexia and depression. Twenty animals were with tick infestation alone, while 23 showed presence of ticks along with other signs mentioned above.

During the present study, out of 71 cases, 16 animals were below six months of age, 25 animals were between six months and one year of age, 14 animals belonged to the age group of one to three years and 16 animals were above three years.

The 71 animals comprised of four males and the rest were females.

All of them were crossbreds except for two indigenous breeds.

4.2 SCREENING OF BLOOD SMEARS

On examination of Giemsa stained blood smears taken from 71 suspected animals, nine (12.68 per cent) revealed piroplasms similar to *B. bigemina*.

The organisms were arranged in pairs inside the erythrocytes in an acute angle, occupying almost the whole space of the erythrocytes. Single piroplasms were also detected both inside and outside the erythrocytes (Plate 1).

Out of the nine positive blood smears, eight (72.73 per cent) were from acutely infected animals and one (1.67 per cent) was from a subclinically infected calf (Table 2).

Table 2. Status-wise distribution of animals positive for babesiosis by blood smear examination

Status of the disease	Number examined	Number positive	Per cent positive
Acute	11	8	72.73
Subclinical	60	1	1.67
Total	71	9	12.68

The age-wise distribution of animals positive for babesiosis, by blood smear examination is presented in Table 3.

Among the 16 animals of one to six months of age group, one (6.25 per cent) was positive for babesial organisms. Although, 25 animals of six months to one year of age group were tested, none of them was found positive for the organisms in the blood smear. In one to three years of age group, three (21.43 per cent) were positive out of the 14 animals examined. On examination of blood smears collected from 16 animals above three years, five (31.25 per cent) were found positive for the causative agents.

Table 3. Age-wise distribution of animals positive for babesiosis by blood smear examination

Age group of animals	Number examined	Number positive	Per cent positive
1 – 6 months	16	1	6.25
6 months – 1 year	25	0	0
1 – 3 years	14	3	21.43
> 3 years	16	5	31.25
Total	71	9	12.68

In the present study, positive smears were obtained only from female animals.

The breed-wise distribution of animals positive for babesiosis by blood smear examination is furnished in Table 4.

Out of 69 crossbreds examined, nine (13.04 per cent) revealed babesial organisms in the blood smears, while organisms were not detected in the samples of the indigenous animals.

Table 4. Breed-wise distribution of animals positive for babesiosis by blood smear examination

Breed	Number examined	Number positive	Per cent positive
Crossbred	69	9	13.04
Indigenous	2	0	0
Total	71	9	12.68

4.3 EXAMINATION OF TICKS

4.3.1 Collection

The ticks collected from 50 suspected cattle were examined for the presence of the developmental stages of *Babesia* by methyl green pyronine staining of salivary glands, gut and ovaries.

Two hundred ticks were collected approximately and all of them were identified as *Boophilus annulatus*.

4.3.2 Dissection and staining

A tick was interpreted as positive if any one of the three tissues revealed the parasitic stages. Based on this concept, 27 (54 per cent) out of 50 animals with tick infestation were designated as positive for babesial organisms.

Infected salivary gland acini appeared hypertrophied with acinar cell cytoplasm pink, and the nucleus blue in colour. Deep blue coloured mass indicative of the parasite was also detected (Plate 2). In the gut, the presence of

the parasitic stages was indicated by the hypertrophy of the infected epithelial cells. The vacant spaces left by the developmental stages of the parasite in the cell cytoplasm were observed as vacuolations (Plate 3). The oocytes in the ovaries also revealed blue coloured spherical masses denoting the developmental stages of the parasite (Plate 4).

The distribution of the parasitic stages in the tick tissues is presented in Table 5.

The developmental stages of the protozoan could be detected in the salivary glands alone of the ticks collected from a single animal, while in another animal, salivary glands and the gut together revealed parasitic masses. The stages of the parasites were visible in the gut alone in the ticks collected from three out of 50 animals of the group; while in another group of three, both the salivary glands and the ovaries showed the developmental stages. In five animals, both the gut and the ovaries were shown to be positive for the parasitic stages. None of the ticks collected from the 50 animals maintained the parasitic stages in the ovaries alone.

Table 5. Distribution of infection among tissues of ticks collected from 50 animals

Tissue	Animals from which ticks have been collected	
	Number	Per cent
Negative	23	46
Positive	27	54
SG + G + o	14	28
SG	1	2
G	3	6
O	0	0
SG + G	1	2
SG + O	3	6
G + O	5	10

SG – Salivary glands ; G - Gut ; O - Ovaries

All the seven animals (100 per cent) from the above group of 50, that were exhibiting acute symptoms of babesiosis were found positive for the parasitic stages in the ticks. Only 20 animals (46.51 per cent) with a subclinical infection carried ticks that were maintaining the infection in them (Table 6).

Table 6. Status-wise distribution of animals infested with ticks positive for babesial developmental stages

Status of the disease	Number examined	Number positive	Per cent positive
Acute	7	7	100
Subclinical	43	20	46.51
Total	50	27	54

The age-wise distribution of animals with ticks positive for babesial developmental stages are presented in Table 7.

Three animals (37.5 per cent) were observed with positive ticks out of eight animals examined in an age group of one to six months. Out of 14 animals examined in six months to one year of age group, three animals (21.42 per cent) were found to have ticks positive for babesial developmental stages. Eleven animals (73.33 per cent) were found positive for infected ticks among 15 animals examined that belonged to an age group of one to three years. In 13 animals which were above three years of age, 10 (76.92 per cent) were detected with positive ticks.

Table 7. Age-wise distribution of animals infested with ticks positive for babesial developmental stages

Age	Number examined	Number positive	Per cent positive
1 – 6 months	8	3	37.5
6 months – 1 year	14	3	21.42
1 – 3 years	15	11	73.33
> 3 years	13	10	76.92
Total	50	27	54.0

None of the male animals were presented with ticks in the study conducted.

The breed-wise distribution of animals with ticks positive for the developmental stages of *Babesia* spp. is presented in Table 8.

Out of the 48 crossbred animals investigated, 25 (52.08 per cent) were found to be infested with ticks positive for babesial developmental stages. Ticks collected from the two indigenous cattle (100 per cent) were also found positive for the organisms.

Table 8. Breed-wise distribution of animals with ticks positive for the babesial development stages

Breed	Number examined	Number positive	Per cent positive
Crossbred	48	25	52.08
Indigenous	2	2	100
Total	50	27	54

4.4 IMMUNODIAGNOSTIC TESTS

4.4.1 Collection of Blood

Sera separated from the whole blood collected from 71 animals suspected for babesiosis were screened by both IFAT and Slide-ELISA.

4.4.2 Preparation of Antigen-coated Slides

A specific positive or negative reaction could not be obtained with either the positive or negative control sera when tested with the antigen-coated slides prepared in the laboratory. Differentiation between positive and negative reactions was not defined. Hence, the antigen-coated slides procured from the IVRI, Izatnagar were used for the serological examination for *B. bigemina* antibodies by IFAT and Slide-ELISA, in the present study.

4.4.3 Indirect Fluorescent Antibody Test (IFAT)

Sera collected from 71 animals suspected for bovine babesiosis were screened by IFAT to detect the presence of babesial antibodies.

Thirty seven sera out of 71 (52.11 per cent) were detected positive by IFAT. A positive reaction in the test sera was indicated by a specific fluorescence of either the infected erythrocytes or piroplasms (Plate 5). No specific fluorescence could be observed for the erythrocytes or the piroplasms when negative sera were applied.

All the sera obtained from the 11 acutely infected cattle (100 per cent) were detected positive for *B. bigemina* antibodies by IFAT. Out of 60 animals that showed subclinical symptoms, 26 (43.33 per cent) were seropositive (Table 9).

Table 9. Status-wise distribution of animals positive for *B. bigemina* antibodies by IFAT

Status of the disease	Number examined	Number positive	Per cent positive
Acute	11	11	100
Subclinical	60	26	43.33
Total	71	37	52.11

The age-wise distribution of animals positive for *B. bigemina* antibodies by IFAT is presented in table 10.

Sixteen animals belonging to the age group of one to six months were tested and six (37.5 per cent) were denoted as seropositive by IFAT. Twenty five sera from animals of six months to one year of age were tested, with 11 (44.0 per cent) being seropositive. Ten (71.43 per cent) were seropositive among 14 animals which were of one to three years of age. Out of 16 sera tested from animals above three years of age, 10 (62.5 per cent) gave a positive fluorescence.

Table 10. Age-wise distribution of animals positive for *B. bigemina* antibodies by IFAT

Age group	Number examined	Number positive	Per cent positive
1 – 6 months	16	6	37.5
6 months – 1 year	25	11	44.0
1 – 3 years	14	10	71.43
> 3 years	16	10	62.5
Total	71	37	52.11

The sex-wise distribution of animals positive for *B. bigemina* antibodies by IFAT is presented in Table 11.

Among the four males examined, only one (25.0 per cent) was found positive by IFAT. Sera of 67 females collected, revealed *B. bigemina* antibodies in 36 samples (53.73 per cent).

Table 11. Sex-wise distribution of animals positive for *B. bigemina* antibodies by IFAT

Sex	Number examined	Number positive	Per cent positive
Male	4	1	25.0
Female	67	36	53.73
Total	71	37	52.11

The breed-wise distribution of animals positive for *B. bigemina* antibodies by IFAT is presented in Table 12.

Out of 69 crossbreds tested by IFAT, 36 animals (52.17 per cent) revealed *B. bigemina* antibodies. Only one (50 per cent) of the two indigenous animals tested, showed a positive fluorescence.

Table 12. Breed-wise distribution of animals positive for *B. bigemina* antibodies by IFAT

Breed	Number examined	Number positive	Per cent positive
Crossbred	69	36	52.17
Indigenous	2	1	50.0
Total	71	37	52.11

4.4.4 Slide-Enzyme Linked Immunosorbent Assay (Slide-ELISA)

Slide-ELISA was performed to screen 71 sera of cattle against *B. bigemina* antibodies. Thirty nine animals (54.93 per cent) were diagnosed as positive by the test.

A positive reaction was indicated by a dark brown staining of the *B. bigemina* infected erythrocytes or the piroplasms or both (Plate 6). In negative reactions, no colour developed for the erythrocytes or for the organisms in them.

All the 11 sera collected from the acutely infected animals showed a positive reaction (100 per cent). Slide-ELISA revealed a positive reaction in 28 (46.67 per cent) out of 60 samples collected from those animals with a subclinical infection (Table 13).

Table 13. Status-wise distribution of animals positive for *B. bigemina* antibodies by Slide - ELISA

Status of the disease	Number examined	Number positive	Per cent positive
Acute	11	11	100
Subclinical	60	28	46.67
Total	71	39	54.93

The age-wise distribution of animals positive for *B. bigemina* antibodies by Slide-ELISA is presented in Table 14.

Screening of sixteen animals of one to six months of age revealed seven of them (43.75 per cent) with a positive reaction. Thirteen animals (52.0 per cent) were positive, out of 25 tested in six months to one year of age group. Among the 14 animals belonging to an age group of one to three years, 10 (71.43 per cent) were seropositive. In 16 animals of above three years that were

screened, nine (56.25 per cent) had antibodies to *B. bigemina* as revealed by the test.

Table 14. Age-wise distribution of animals positive for *B. bigemina* antibodies by Slide - ELISA

Age	Number examined	Number positive	Per cent positive
1 – 6 months	16	7	43.75
6 months – 1 year	25	13	52.00
1 – 3 years	14	10	71.43
> 3 years	16	9	56.25
Total	71	39	54.93

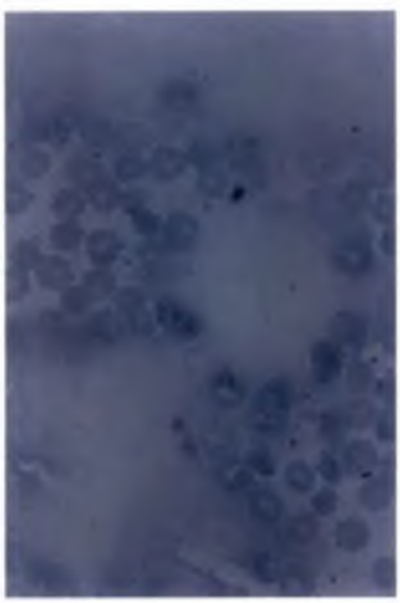
On testing the sera of four male animals, none could be detected positive for antibodies to *B. bigemina* while antibodies were detected in all the female animals.

The breed-wise distribution of animals positive for *B. bigemina* antibodies by Slide-ELISA is presented in Table 15.

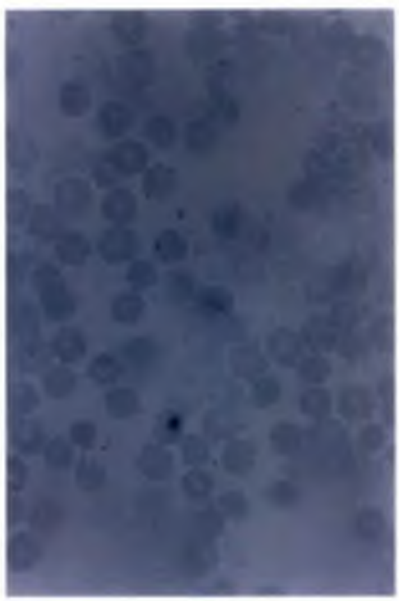
The screening of sera from 69 crossbreds revealed 38 samples (55.07 per cent) as seropositive, while only one sample from the two indigenous cattle tested was seropositive to *B. bigemina* antibodies by Slide-ELISA.

Table 15. Breed-wise distribution of animals positive for *B. bigemina* antibodies by Slide - ELISA

Breed	Number examined	Number positive	Per cent positive
Crossbred	69	38	55.07
Indigenous	2	1	50.0
Total	71	39	



A

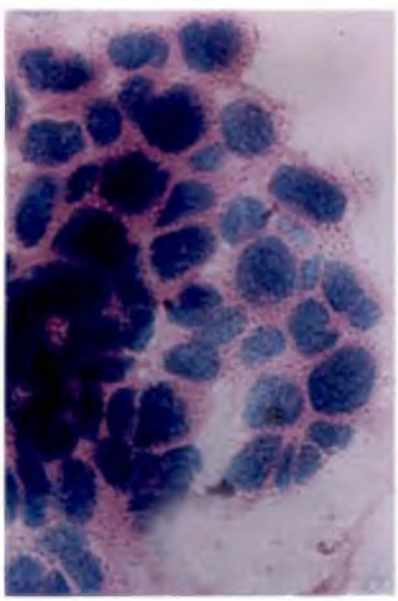


B

Plate 1. Blood smear - Giemsa staining (x1000)

A. Paired piroplasms inside the erythrocytes

B. Single piroplasms inside and outside the erythrocytes



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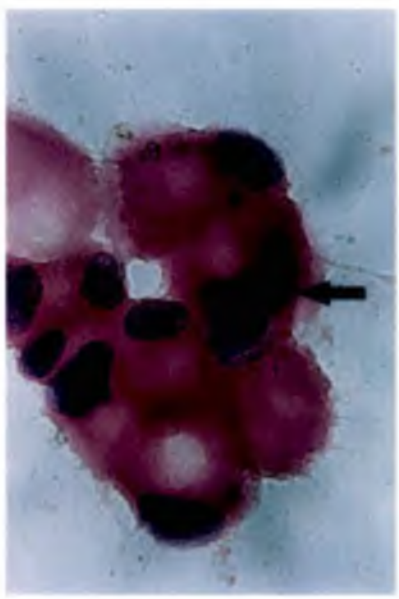
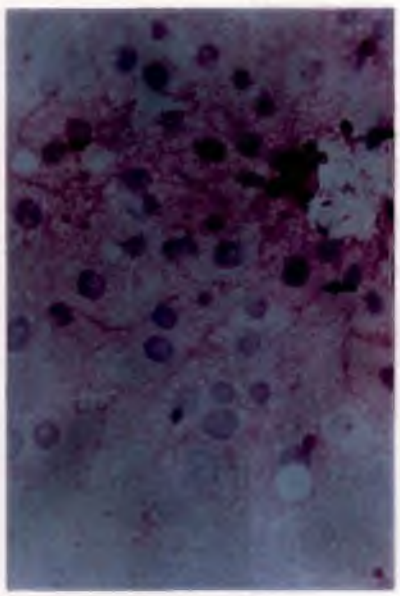


B

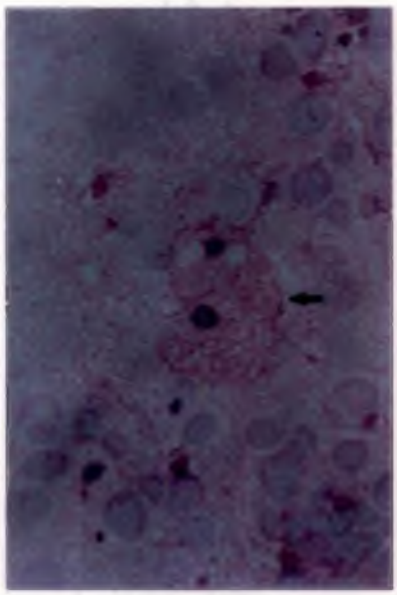
Plate 2. Tick salivary gland -Methyl green pyronine staining (x400)

A. Normal acini

B. Hypertrophied acini with parasitic mass



A

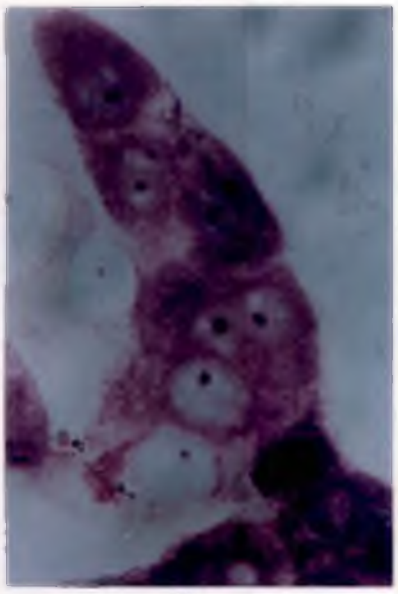


B

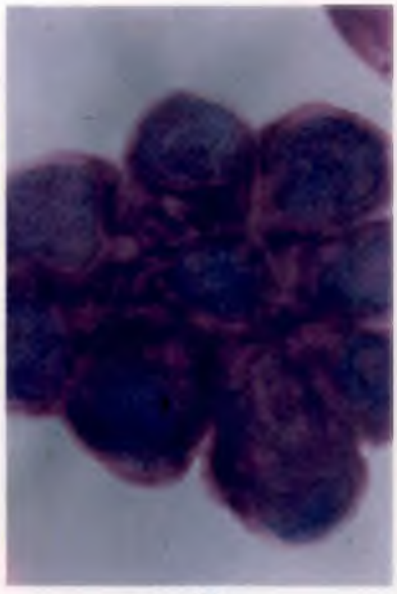
Plate 3. Tick gut - Methyl green pyronine staining (x400)

A. Normal epithelial cells

B. Infected epithelial cells with hypertrophy and vacuolation of cytoplasm



A



B

Plate 4. Tick ovary - Methyl green pyronine staining (x400)

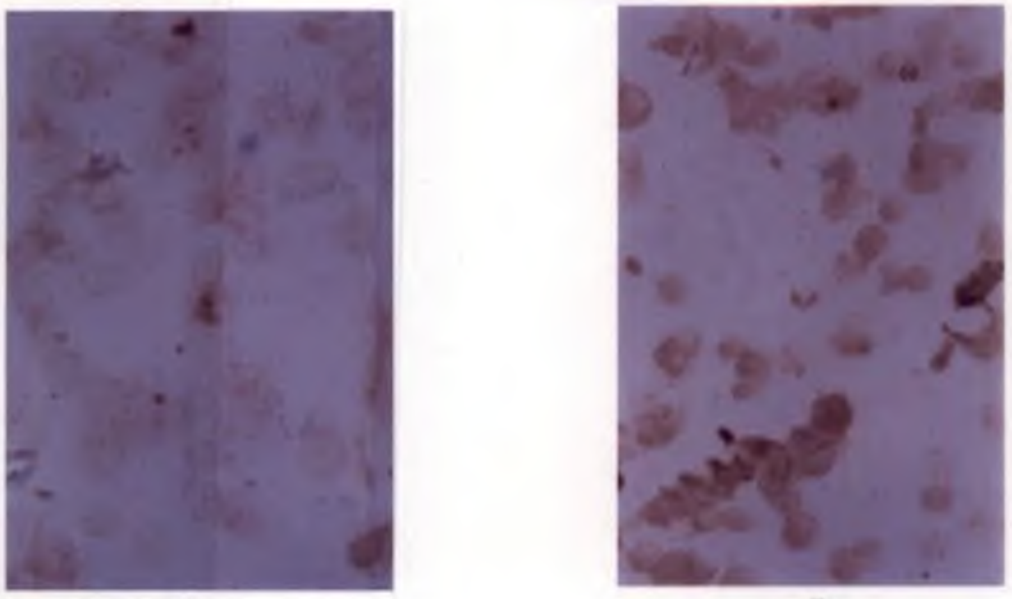
A. Normal oocytes

B. Infected oocytes with parasitic mass

A B

Plate 5. Indirect Fluorescent Antibody Test

A. Negative B. Fluorescence of piroplasms



A B

Plate 6. Slide-Enzymelinked Immunosorbent Assay

A. Dark brown staining of Piroplasms

B. Dark brown staining of piroplasms and erythrocytes

4.5 COMPARISON OF DIFFERENT DIAGNOSTIC TESTS

The performance of different diagnostic tests namely blood smear examination, examination of ticks, IFAT and Slide-ELISA to detect *B. bigemina* infections in cattle is presented in Table 16 and Fig.1.

A total of 71 animals were tested and the blood smears were found positive in nine animals (12.68 per cent). The application of IFAT revealed 37 samples (52.11 per cent) as seropositive, while Slide-ELISA showed a positive reaction in 39 samples (54.93 per cent). Examination of ticks collected from 50 animals denoted 27 (54 per cent) as positive based on the detection of parasitic stages in the tick tissues.

On comparison of these tests by test for proportion, an overall significant variation was observed in their performance.

On further analysis it was found that the IFAT, Slide-ELISA and tick examination did not vary considerably from one another with regard to its performance but all the three tests differed significantly when compared with the results of blood smear examination.

Table 16. Performance of the various diagnostic tests to detect *B. bigemina* infections in cattle

Diagnostic test	Number tested	Number positive	Per cent positive
Blood smear	71	9	12.68
IFAT	71	37	*52.11
Slide-ELISA	71	39	*54.93
Examination of ticks	50	27	*54

* Significant ($P<0.05$)

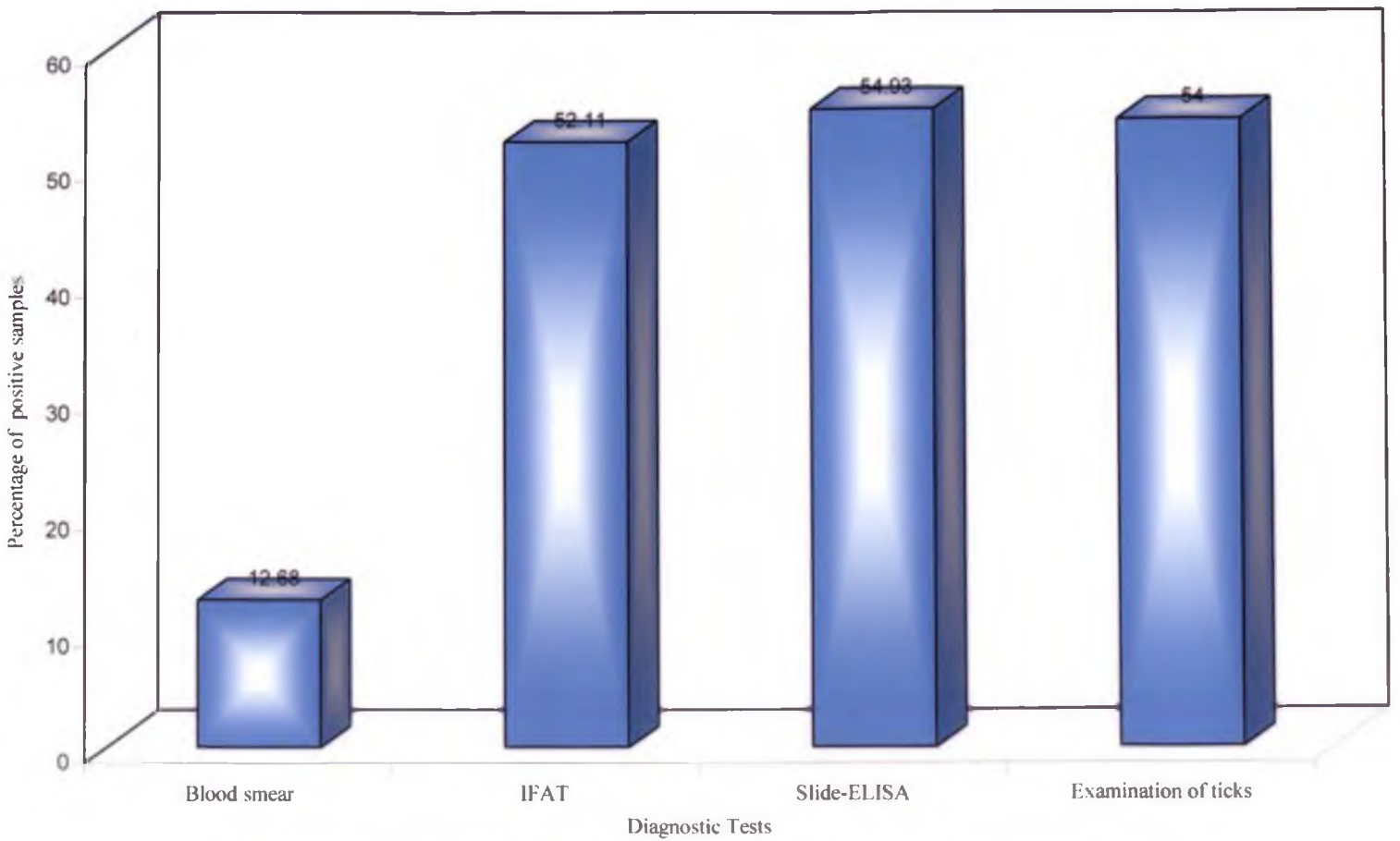


Fig1:Performance of different diagnostic tests to detect *B.bigemina* infections in cattle

4.5.1 Comparison of Blood Smear and Serological Tests

4.5.1.1 Blood Smear and IFAT

A total of 71 animals were screened by blood smear examination and by IFAT. The blood smears revealed babesial organisms in only nine animals (12.68 per cent) while 37 animals (52.11 per cent) were detected seropositive by IFAT.

Nine animals were positive by both blood smear examination and IFAT whereas no positive result could be obtained in 34 animals by either test. Twenty eight animals, which were negative by blood smear examination revealed antibodies by IFAT. Samples in which antibodies were not detected serologically did not reveal any organisms either, in the blood smear.

	Blood smear (+)	Blood smear (-)	Total
IFAT (+)	9	28	37
IFAT (-)	0	34	34
Total	9	62	71

4.5.1.2 Blood Smear and Slide-ELISA

On blood smear examination and Slide-ELISA performed in 71 animals to detect *B. bigemina* infection, nine animals (12.68 per cent) were microscopically detected as positive and 39 animals (59.43 per cent) as seropositive by Slide-ELISA.

The nine animals which were detected positive for *Babesia* spp. by blood smear examination, were also positive for the antibodies in a Slide-ELISA.

Thirty two animals in which organisms were not detected microscopically, were seronegative by Slide-ELISA too. A positive reaction was obtained by Slide-ELISA in 30 samples, which were negative for the organisms in the blood smears. Organisms were not detected in the blood smears of any sample that showed a negative reaction by Slide-ELISA.

	Blood smear (+)	Blood smear (-)	Total
Slide-ELISA (+)	9	30	39
Slide-ELISA (-)	0	32	32
Total	9	62	71

4.5.2 Comparison of IFAT and Slide-ELISA

The presence of babesial antibodies in 71 sera was detected serologically, by IFAT and Slide-ELISA resulting in a total of 37 animals (52.11 per cent) being seropositive to IFAT and 39 (54.93 per cent) to Slide-ELISA.

Antibodies were successfully detected in 34 sera by both tests. While, 29 samples were seronegative to the antibodies by both. Three of the sera which were found positive by IFAT showed a negative reaction in a Slide-ELISA while five of them which were negative in IFAT, indicated a positive reaction by Slide-ELISA.

	Slide-ELISA (+)	Slide-ELISA (-)	Total
IFAT (+)	34	3	37
IFAT (-)	5	29	34
Total	39	32	71

4.5.3 Comparison of Examination of Ticks with Other Techniques

Results of examination of ticks from 50 animals were compared with those of blood smear and serological examination of the same number of animals.

4.5.3.1 Examination of Ticks and Blood Smear Examination

Five animals out of 50, which were positive for babesiosis by tick examination were also positive for the organisms in the blood smear. Meanwhile, 22 animals were negative for the organisms in the blood smears as in the ticks collected from them. Another 22 of the total animals which rendered negative results by blood smear examination, were actually detected as positive upon tick examination. There was only one animal from which infected ticks were not obtained that rendered a positive blood smear.

	Blood smear (+)	Blood smear (-)	Total
Tick examination (+)	5	22	27
Tick examination (-)	1	22	23
Total	6	44	50

4.5.3.2 Examination of Ticks and IFAT

From a total of 50 animals, 18 were found positive for the infected ticks and antibodies by IFAT. In 16 animals, no results were obtained with regard to the infection upon tick examination and use of IFAT. Nine animals whose sera did not show any antibodies by IFAT were positive for the organisms, when the ticks were examined. Seven sera of animals which did not carry any infected tick were found to be seropositive to babesial antibodies in an IFAT.

	IFAT (+)	IFAT (-)	Total
Tick examination (+)	18	9	27
Tick examination (-)	7	16	23
Total	25	25	50

4.5.3.3 Examination of Ticks and Slide-ELISA

On comparing the results of tick examination for developmental stages of *Babesia* spp. and Slide-ELISA for the detection of antibodies, it was found that 18 out of 50 samples were positive for the organisms by both the methods, while 16 samples were obtained as negative. Slide-ELISA failed to reveal antibodies in eight samples of serum collected from animals that were actually maintaining ticks infected with babesial organism. However, Slide-ELISA succeeded in detecting eight animals as seropositive, where from no parasitic stages could be identified in the tick tissues collected from their surfaces.

	Slide-ELISA (+)	Slide ELISA (-)	Total
Tick examination (+)	18	8	26
Tick examination (-)	8	16	24
Total	26	24	50

Discussion

5. DISCUSSION

The present study was undertaken to evaluate different tests for the diagnosis of *B. bigemina* infections in cattle. The tests employed were examination of blood smear, examination of ticks and serological tests like IFAT and Slide-ELISA.

A total of 71 blood samples collected from cattle exhibiting symptoms suggestive of babesiosis were screened. Identification of ticks collected from 50 infested animals and their processing was also carried out in the present study.

5.1 PREVALENCE

Description of clinical signs of bovine babesiosis by Dwivedi *et al.* (1976), Roychoudhury *et al.* (1976), Pandey and Mishra (1978 and 1979), Saha and Das (1978), Knowles *et al.* (1982), Gupta and Sinha (1988), Kasaralikar *et al.* (1996) and Bhikane *et al.* (2001) were found to be beneficial as animals showing any one of these symptoms were considered as carriers of the organisms and were screened by the tests stated above.

A total of 71 cattle were tested out of which 11 (15.49) per cent were found to suffer from an acute illness. The common clinical signs manifested by these animals were haemoglobinuria, fever (40 to 41.6°C), pallor of mucosa and anorexia. Similar signs were also described in acute bovine babesiosis by Dwivedi *et al.* (1976), Roychoudhury *et al.* (1976), Pandey and Mishra (1978 and 1979), Saha and Das (1978), Knowles *et al.* (1982), Gupta and Sinha (1988), Kasaralikar *et al.* (1996) and Bhikane *et al.* (2001).

Reduction in milk yield was observed in five acutely infected animals affirming the observations of Dwivedi *et al.* (1976), Pandey and Mishra (1978) and Bhikane *et al.* (2001).

In the present study, out of 71 cases, subclinical parasitaemia was noted in majority i.e., 60(84.5 per cent). The possible explanation for the existence of subclinical form of babesiosis, observed in the present study, is that cattle reared in the enzootic areas of babesiosis usually become infected while being relatively young and suffer only mild reactions, subsequently becoming resistant to severe clinical manifestation of babesiosis. As such the recovered animals become resistant carriers of the disease. The clinical signs noticed in subclinical babesiosis were similar to those observed by Pandey and Mishra (1979).

In the present study, acute infections were observed only in animals above three years of age, similar to the observations of Dwivedi *et al.* (1976), Pandey and Mishra (1978), Hostis *et al.* (1995) and Bhikane *et al.* (2001). Although there are reports of acute babesiosis in calves by Roychoudhury *et al.* (1976), Saha and Das (1978), James *et al.* (1985) and Guglielmone (1995), no acute disease was encountered in calves during the present study. However, Saha and Das (1978) also opined that, clinical cases of babesiosis in calves were uncommon.

As per the results of blood smear examination also, the prevalence was more in adult animals rather than in calves. The number of animals with ticks bearing babesial developmental stages was greater in the group above three years of age, followed by those of one to three years of age, while a less percentage of the same was detected in animals below one year.

Similarly, the prevalence of antibodies to *B. bigemina* was found to be higher in animals above one year of age by IFAT and Slide-ELISA in the work conducted.

The antibody pattern to *B. bigemina* detected by both the serological tests was in accordance to that reported by Woodford *et al.* (1990). A lower rate of prevalence of antibodies was detected in animals below six months of age, which was followed by an increase in the prevalence, in animals of six months to one year. Higher rate of prevalence was observed in animals above one year of age, with not much significant difference between those of one to three years and above three years in the number determined as seropositive.

These results provide further evidence that the resistance of animals to babesiosis decreases with age and that the presence of antibodies may be ascribed to the initial infection and subsequent resistance acquired by the animal.

Information as to the influence of sex on the incidence of bovine babesiosis is limited except for one by Bhikane *et al.* (2001) who reported that males were more susceptible than females to *Babesia spp* which was accounted to the heavy workload, adverse climatic conditions and under nutrition.

In the present study, all acute cases were observed only in females. Out of 60 animals suspected for a subclinical infection, 56 (93.33 per cent) were females. None of the male animals screened, revealed *B. bigemina* either in the blood smears or tick tissues. But it was possible to detect antibodies in 25 per cent males by IFAT. Yet, none of them revealed the presence of antibodies in a slide-ELISA.

The present observations might be due to the low number of males (four) screened during the study which might have overtly affected the results or it can also be attributed to the low population of male animals reared in the field for domestic purposes.

In the present study conducted, different responses were obtained on the distribution of infection among crossbreds and indigenous animals. Since the

number of animals in these two breeds tested were too uneven, a breed related comparison could not be made.

It is worthy to mention that all the acute infections and all the blood smear positive animals belonged to crossbreds tested, in the present study. Dwivedi *et al.* (1976) observed cardinal signs of clinical babesiosis in pure as well as crossbreds. In contrast, Bhikane *et al.* (2001) reported a high prevalence of clinical babesiosis in indigenous animals. It should be noted that the indigenous animals showed a cent per cent positive response upon tick examination, while only 52.08 per cent of crossbreds were found to be positive by the same. The serological tests viz., IFAT and Slide-ELISA were able to detect 36 (52.17 per cent) and 38 (55.07 per cent) out of 69 crossbreds respectively and one (50 per cent) each out of two indigenous breeds as seropositive. Any way, percentage of positive animals detected in both the breeds by serology was more or less equal.

As the indigenous breeds were very few in the field, only two of them were available for the diagnostic tests. Hence results with regard to tick examination and serology in these breeds could not be considered as conclusive.

5.2 SCREENING OF BLOOD SMEAR

The examination of Giemsa-stained blood smears was found to be the method of choice to detect parasites during acute phase of the disease as claimed by Bose *et al.* (1995). The results of the present study, wherein eight animals out of 11 with an acute disease were screened positively by Giemsa-stained blood smears confirm this finding. That is to say all animals suffering from an acute disease revealed babesial organisms in the blood smear with an exception of three. The failure to detect organisms in the three acutely infected animals might be due to faulty preparation of smears obtained from the field or an error in the staining procedure. Smears too

thick could foreshadow the distinct morphology of the organisms, making them indistinguishable.

In this study, blood smear examination failed to confirm the subclinical status of the disease in animals, except in one. This finding further demonstrates the low reliability and sensitivity of blood smear examination in animals, in detecting subclinical infections of *Babesia spp.* This fact is supported by similar studies conducted by Akinboade and Dipeolu (1984).

5.3 EXAMINATION OF TICKS

5.3.1 Collection

While Roychoudhury *et al.* (1976), Gautam and Chhabra (1983) and Bhikane *et al.* (2001) recovered *B. microplus* as the main vector of bovine babesiosis, all the 200 ticks collected during the period of research were identified as *B. annulatus* concurring with the observations of Rajamohanan (1980).

5.3.2 Dissection and Staining

Examination of ticks was undertaken to assess *B. bigemina* infections in cattle suspected for babesiosis. Methyl green pyronine staining of salivary glands, gut and ovaries of the dissected ticks was used to assess the extent of infection. Twenty seven (54 per cent) animals out of 50, were found to carry the infected ticks.

The stain was found stable and easy to prepare. The staining pattern of developmental stages of *Theileria spp* in the salivary glands of other Ixodid ticks recorded by Walker *et al.* (1979), Irvin *et al.* (1981), Das and Sharma (1991 and 1994) and Sundar *et al.* (1993) were referred to, in this study as reports of methyl green pyronine staining of salivary glands of *Boophilus spp* to detect babesial developmental stages were not available. But, as described, immature forms of the

parasite could not be determined in the salivary glands. Since, the literature on methyl green pyromine staining of gut and ovaries was also scarce, the interpretation of the results turned to be difficult. However, ticks with vacant spaces in the gut epithelial cells left behind by the parasite and those with spherical masses inside the oocytes were considered as positive.

Contrary to the vivid demonstrations of Achuthan *et al.* (1980), elongate or pyriform bodies were not recovered from the gut tissues of any tick identified as *B. annulatus* in the present work.

Distribution of the parasitic stages in different tissues had a tendency to vary between ticks. Hence, it was necessary to examine all the tick tissues, before interpreting the conclusive results.

5.4 IMMUNODIAGNOSTIC TESTS

5.4.1 Preparation of Antigen-coated Slides

In this study, antigen-coated slides were prepared from the erythrocytes of animals which demonstrated the organisms in the blood smears. But the slides were not found suitable for performing the serological tests. The reasons might be the lack of sufficient parasitised cells and improper separation of erythrocytes. Madden and Holbrook (1968) considered these two factors as the prime in the preparation of antigen-coated slides in the serodiagnosis of babesiosis. Besides, the antigen-coated slides should have been prepared from animals with atleast ten per cent parasitaemia as carried out successfully by Johnston *et al.* (1973), Kuttler *et al.* (1977) and Kungu and Goodger (1990) in spleenectomised calves.

Eventhough organisms were clearly demonstratable in the blood smears of the acutely infected animals, the parasitaemia was less than ten per cent in all these cases, a fact that could not be ignored.

5.4.2 Indirect Fluorescent Antibody Test

Bose *et al.* (1995) opined that IFAT could be used routinely for the diagnosis of *B. bigemina* infections and it was well suited to the low technology environment. In this study also, IFAT was found effective for detecting antibodies to *B. bigemina* in 52.11 per cent of 71 animals screened. The present work confirms the observations of Akinboade and Dipeolu (1984), Alonso *et al.* (1988), Linhares *et al.* (1992), Basalo *et al.* (1995), Mishra *et al.* (2001) and Ravindran *et al.* (2002), who applied IFAT for screening *B. bigemina* infections in cattle. They agreed that the test was accurate, sensitive and reliable and could be effectively utilised as a diagnostic test for bovine babesiosis. It is indicated from the present research findings that IFAT could be applied as a suitable diagnostic tool for serological surveys. But, provision for a sophisticated fluorescent microscope curtails its application at a field level.

5.4.3 Slide-Enzyme Linked Immunosorbent Assay

In this study, Slide-ELISA was effectively utilised for the diagnosis of *B. bigemina* antibodies in cattle. An overall seropositive reaction of 54.93 per cent was obtained by screening of 71 cattle sera. The present work confirms the observations of Kungu and Goodger (1990) and Ravindran *et al.* (2001) who applied Slide-ELISA for the diagnosis of *B. bovis* and *B. bigemina* infections in cattle respectively. Inspite of the less number of samples which can be charged in a single Slide-ELISA, it is found to be a very simple, economical and convenient test to perform and easy to interpret. It is evident from this study, that Slide-ELISA proves to be a suitable field diagnostic test as an ordinary light microscope is sufficient to read the results.

5.5 COMPARISON OF DIFFERENT DIAGNOSTIC TESTS

On comparing the results obtained from the different tests, the blood smear examination was found to be the least efficient than the others. This is in conformity with the finding of Akinboade and Dipeolu (1984), eventhough they compared blood smear with IFAT only. They concluded that the level of infection could be monitored better serologically rather than microscopically. Still, the blood smear examination remains as a good technique in detecting organisms in cases of acute infections.

Examination of ticks came out with equal efficiency with serological tests and as more efficient than the blood smear examination. But, for interpreting a tick as positive, all the tissues should be examined with expertise and further, it requires a skill to dissect the ticks. So this method can only be combined with other tests so as to rule out the presence of infection, in studies related to the epidemiology of the disease.

The two serological tests viz., IFAT and Slide-ELISA were performed simultaneously during the period of research. The results indicated that both of them performed quite well and could be applied in epidemiological surveys. Bose *et al.* (1995) reported that IFAT was the most common serological test used for the diagnosis of babesial antibodies owing to its high sensitivity and specificity. Similarly, Slide-ELISA was also reported as a sensitive and specific test by Kungu and Goodger (1990).

Slide-ELISA was found to satisfy all the pre-requisites for serological tests put forward by Todorovic (1975), especially its applicability in the field and case of interpretation of results than the IFAT. The test has been also recommended for field application by Kungu and Goodger (1990) and Ravindran *et al.* (2001). Kungu and Goodger (1990) also mentioned that, the completed test slides of Slide-ELISA could

be stored for atleast one week at room temperature for future use which was proved true in this study also.

The diagnosis of babesiosis especially the subclinical infections has always been problematic because of the variety and nonspecificity of symptoms. A rapid and simple diagnostic method is needed in order to conduct epidemiological surveys successfully. For these reasons, serological tests play an important role in the diagnosis and epidemiology of the disease. It is understood from the present research that both IFAT and Slide-ELISA perform almost equally in the detection of antibodies against *B. bigemina* in cattle. Yet, the need of a fluorescent microscope for immunofluorescence assays limits its use as a field diagnostic tool. This putsforth the Slide-ELISA as a suitable field tool for the diagnosis of babesiosis. It is evident from the present study that, it will be beneficial to include the examination of vectors along with serological and parasitological surveys of bovine babesiosis. Still, the conventional method of examination of blood smears cannot be fully ignored as it has been already proved as a method of choice in acute infections.

Summary

6. SUMMARY

A study was undertaken to determine the suitability of different diagnostic tests in the detection of babesial infection in cattle. The samples for screening were collected from cattle showing symptoms suggestive of babesiosis, that were presented at various Veterinary hospitals of Thrissur and Ernakulam districts and from those maintained at the University Livestock Farm, Mannuthy and the Cattle Breeding Farm, Thumburmuzhi. A total of 71 animals were screened by the examination of blood smears, examination of ticks and by serological tests viz., IFAT and Slide-ELISA.

An acute form of the disease was observed in 11 animals, while the remaining 60 maintained a subclinical infection. All the acutely infected animals manifested symptoms like haemoglobinuria, fever (40 to 41.6°C), anorexia and pallor of the mucosa in common. A few of them showed a drastic reduction of milk yield along with tick infestation.

In the subclinically infected animals, the symptoms noted were a mild temperature elevation, pale mucous membranes, general weakness, rough hair coat, anorexia etc. Tick infestation either alone or along with other symptoms was shown by some of them.

Blood smear examination by Giemsa revealed piroplasms similar to *B. bigemina* in nine (12.68 per cent) out of 71 suspected animals. Out of the nine positive blood smears, eight (88.89 per cent) were from acutely infected animals and one (11.11 per cent) was from a subclinically infected calf. Majority of animals that were detected positive by this method was above one year of age. Positive smears were obtained only from female animals and crossbreds. The present study implies that blood smear examination is a technique of choice for detecting *Babesia* spp in acute phase of the infection in cattle.

Two hundred ticks were collected approximately from 50 suspected cattle. The ticks were identified as *B. annulatus*. Partially engorged ticks were dissected to separate the salivary glands, gut and ovaries which were stained with methyl green pyronine for the presence of parasitic stages of Babesia. Considering the ticks as positive on detecting the parasitic stages in any one of the tissues, 27 (54 per cent) out of 50 animals with tick infestation were designated as positive for babesiosis.

The parasitic stages in the different tissues were distinguished by the hypertrophy of acinar cells with a deep blue coloured mass indicative of the parasite in the salivary gland acini, hypertrophy of the infected epithelial cells and vacuolation in the cell cytoplasm in the gut and blue coloured spherical masses inside the oocytes in the ovaries.

All the animals that exhibited acute symptoms of the disease were found positive for the parasitic stages in the ticks. Only 20 animals with a subclinical infection carried infected ticks.

It was seen that most of the animals that were detected positive by this method were above one year of age.

Although the developmental stages were identified in the ticks collected from only female animals, infected ticks were recovered from both crossbreds and indigenous cattle.

As the dissection of the ticks and identification of parasitic stages is fraught with difficulties, this method may be combined with other diagnostic tests for eventually coming to a conclusion. However, examination of ticks in studies related to the epidemiology of babesiosis, looks promising, as subclinical infections could be easily diagnosed by this method.

Sera collected from 71 animals suspected for babesiosis were screened by immuno diagnostic tests like IFAT and Slide-ELISA. The babesial antigen-

coated slides procured from the Indian Veterinary Research Institute (IVRI), Izatnagar were used for the serological examination in the present study.

Thirty seven sera (52.11 per cent) out of 71 were detected positive by IFAT. A positive reaction in the test sera was indicated by a specific fluorescence of either the infected erythrocytes or, the piroplasms.

All the sera obtained from the 11 acutely infected cattle were detected positive for *B. bigemina* antibodies. Out of 60 animals that showed subclinical infection 26 (43.33 per cent) were seropositive.

Most of the animals that were detected as positive for the antibodies were above one year.

The test was able to detect more number of females and almost an equal number of animals in both the breeds.

Although the IFAT is said to be a specific, sensitive and reliable test in detecting subclinical infections widely, its use in the field becomes limited as it requires a fluorescent microscope. However, epidemiological surveys comprising of large numbers of samples may be undertaken by employing the IFAT in well-equipped laboratories.

Slide-ELISA was able to detect 39 animals (54.93 per cent) as seropositive. A positive reaction was indicated by a dark brown staining of infected erythrocytes or piroplasms.

All the 11 sera collected from the acutely infected animals, showed a positive reaction, while 28 out of 60 animals with a subclinical infection were determined as positive.

The age of the animals found positive for babesial antibodies by Slide-ELISA was above one year as detected by the other three techniques.

Only female animals were detected positive by this test which recorded a similar per cent of seropositive animals in the breeds studied.

The Slide-ELISA is well known for its simplicity and ease to perform and interpret. These qualities corroborate the suitability of Slide-ELISA as a diagnostic measure in the field. The ability to retain the test slides for a longer duration (one week) and the need for only an ordinary light microscope adds to the usefulness of the test.

On comparing the performance of the different diagnostic tests namely blood smear examination, examination of ticks, IFAT and Slide-ELISA for the diagnosis of babesiosis in cattle, it was seen that nine (12.68 per cent), 37 (52.11 per cent), 39 (54.93 per cent) animals out of 71, were found positive for the organism by blood smear examination, IFAT and Slide-ELISA respectively. Examination of ticks collected from 50 animals denoted 27 (54 per cent) as positive based on the detection of parasitic stages in the tick tissues. On analysis of the data by test for proportion, it was found that there was no considerable variation between IFAT, Slide-ELISA and tick examination with regard to its performance. But all the three tests differed significantly when compared with the results of blood smear examination.

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EVALUATION OF DIFFERENT TECHNIQUES FOR THE DIAGNOSIS OF BOVINE BABESIOSIS

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ABSTRACT

Different techniques to diagnose bovine babesiosis were evaluated by screening 71 cattle presented at the various Veterinary Hospitals of Thrissur and Ernakulam districts and those maintained at the University Livestock Farm, Mannuthy and the Cattle Breeding Farm, Thumburmuzhi with symptoms suggestive of the disease. The techniques employed were examination of Giemsa stained blood smears, examination of ticks collected from suspected animals and serological tests like the Indirect Fluorescent Antibody Test (IFAT) and Slide-Enzyme Linked Immunosorbent Assay (Slide-ELISA).

Among the 71 cattle, 11 were suffering from an acute illness whereas 60 showed only subclinical symptoms.

Examination of Giemsa stained blood smear could detect *Babesia bigemina* organisms in 9 (12.68 per cent) of the total samples, which were mainly from the acutely infected cattle, except for one, that was obtained from a subclinically infected calf.

The principal vector of babesiosis was identified as *Boophilus annulatus* in the present study. Parasitic masses indicative of the organisms were identified from the various tick tissues dissected and stained with methyl green pyronine. Considering the ticks as positive on detection of developmental stages of the parasite in any of the tissues, ticks from 27 (54 per cent) out of total 50 animals were interpreted as positive.

The IFAT detected *B. bigemina* antibodies in 37 (52.11 per cent) and Slide-ELISA in 39 (54.93 per cent) sera out of 71 samples that were screened. These two serological techniques were found to be equally efficient, with Slide-ELISA proving more suitable for use as a field diagnostic test.

Most of the animals that were detected positive for the organisms by all the tests, were above one year of age. As the number of male and female animals was too uneven, a sex related comparison could not be appropriately done. Similarly, a comparison on the performance of the different tests between the breeds was also not feasible owing to the wide variation between the numbers of breeds studied.

On evaluation of the different diagnostic techniques, serology and examination of ticks were found equally effective in detecting both the clinical and subclinical forms of the disease over and above the conventional method i.e., the blood smear examination. However, examination of Giemsa stained blood smears continues to be the method of choice to detect acute infections.