MOLECULAR DETECTION AND THERAPEUTIC MANAGEMENT OF CANINE BABESIOSIS

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(15-MVM-001)

THESIS

Submitted in partial fulfilment of the requirement for the degree of

MASTER OF VETERINARY SCIENCE

(Veterinary Epidemiology and Preventive Medicine)

2017

Faculty of Veterinary and Animal Sciences

Kerala Veterinary and Animal Sciences University

DEPARTMENT OF VETERINARY EPIDEMIOLOGY AND PREVENTIVE MEDICINE

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DECLARATION

I hereby declare that this thesis entitled **"Molecular Detection and Therapeutic Management of Canine 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 of any degree, diploma, fellowship or other similar title, of any other University or Society.

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ACKNOWLEDGEMENTS

First and foremost I wish to express my profound sense of gratitude and indebtedness to the Chairperson of the Advisory Committee, ***Dr. P. V. Tresamol****, Professor and Head, Department of Veterinary Epidemiology and Preventive Medicine. Her able and patient guidance, personal attention, moral support, creative and timely suggestions, motherly affection and unceasing encouragement during the course of my study was the major factor that helped me in my accomplishment. As a teacher and mentor, she has taught me more than I could ever give her credit for here. She has shown me, by her example, what a good scientist (and person) should be.*

I am grateful to ***Dr. K. Vinodkumar****, Assistant Professor, Department of Veterinary Epidemiology and Preventive Medicine and member of Advisory Committee for his valuable guidance, suggestions, advice and constructive criticism at every stage of this research work.*

I deem it my privilege to express my deep sense of gratitude to ***Dr. Sisilamma George****, Dean, College of Veterinary and Animal Sciences, Mannuthy for her inspiring professional guidance.*

I sincerely acknowledge ***Dr. K. Syamala****, Assistant Professor, Department of Veterinary Parasitology and member of Advisory Committee, for her expert advice, timely help and support for the successful completion of this work.*

I am deeply indebted to ***Dr. K. C. Bipin****, Assistant Professor, Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Sciences, Pookode, for his inspiring guidance and valuable advice given throughout this work.*

I sincerely thank ***Dr. S. Sulficar, Dr. Justin Davis K.*** *and* ***Dr. Shyma. V. H,*** *Assistant Professors, Department of Veterinary Epidemiology and Preventive Medicine for their constructive advice, profound encouragement and all possible help rendered during each and every phase of my research work.*

I sincerely acknowledge ***Dr. S. Ajith Kumar****, Professor and Head, Teaching Veterinary Clinical Complex for his expert advice and support for the successful completion of this work.*

I would like to pay special thankfulness, warmth and appreciation to the persons below who made my research successful and assisted me at every point to cherish my goal:

Professor ***Dr. Usha Narayana Pillai****, Head of the Department of Veterinary Clinical Medicine. Her encouragement made it possible to achieve the goal.*

Dr. Deepa Chirayath*, Assistant Professor, Department of Veterinary Clinical Medicine for her expert advice, vital support and valuable suggestions at the hospital.*

Dr. Sindhu K. Rajan*, whose help and sympathetic attitude at every point during my research helped me to work in time. I am indebted to her for the expert advice and valuable help given during the ultrasonographic examinations and inspired by her knowledge in the field of clinical medicine.*

Dr. Madhavan Unny, *Assistant Professor, Department of Veterinary Clinical Medicine, whose reminders and constant motivation encouraged me to meet the deadlines.*

Dr. S. Anoop, Dr. Sudheesh S. Nair, Dr. Laiju Philip, Dr. Ambily, Dr. K. Krithiga, Dr. Ramnath, Dr. C. Latha and Dr. R. Radhika *for their valuable guidance and help.*

I would like to place on record my heartfelt thanks and gratitude to ***Dr. V. L. Gleeja*** *Department of Statistics for the constructive help in the analysis of the research data. With great pleasure, I acknowledge* ***Mrs. Greeshma*** *for her valuable and constructive help rendered during statistical analysis.*

I treasure the understanding, concern, moral support, constant encouragement and selfless help rendered by my friends ***Drs. Azhaguraja, Lobo Andrea Robin, Aarsha Raj, Sukanya Sukumaran, Vineeta, Charlotte, Prasad*** *and* ***Vasanth.***

With great pleasure, I acknowledge my seniors ***Drs. Amulya, Amel Dev*** *and* ***Anju Sekar*** *for their expert advice and help. I also acknowledge my juniors* ***Drs. Jackson Benny, Revathi, Silpa Raj*** *and* ***Amrutha*** *for their support and help.*

I owe a lot to the research assistants ***Mrs. Suchithra, Mrs. Deepthi Joseph, Miss. Mery Rincy*** *and* ***Miss. Anu*** *for the technical assistance and patience taken to help me with my work.*

I am thankful to the laboratory technicians ***Mrs. Neethu, Mr. Vipin*** *and* ***Miss. Srubhi****, Teaching Veterinary Hospital Mannuthy and University Veterinary Hospital Kokkalai for all their earnest help during the course of my work.*

I am very much obliged to the staff members of library, ARIS cell, Teaching Veterinary Hospital, Mannuthy and University Veterinary Hospital, Kokkalai for all their co-operation.

I express with great pleasure my thankfulness to ***Hema Chechi, Sumesh Cheta, Manoj Cheta, Sojan Cheta, Saina Chechi, Renil Aby, Shameem, Sneha,***

Jomon, Srinivas, Jeevan, Anu *and* ***Shyama*** *for the co-operations rendered from time to time.*

The help and co-operation rendered by all the non-teaching staff of the Department of Veterinary Epidemiology and Preventive Medicine are duly acknowledged.

I am indebted to ***Kerala Veterinary and Animal Sciences University*** *for awarding me the fellowship for postgraduate study.*

No phrase or words can ever express my deep sense of gratitude to ***my Mother*** *who enriched my ignorant soul and taught me to survive in this world. I am deeply indebted to her for the constant support and immense inspiration all through my academic life.*

The affection and unstinted support by ***Ashwin, Chithi, Chithapa, Indira aunty, my friends Riyukta, Viji, Tarini*** *and* ***Barathi****, and all my family members was a constant source of encouragement in each phase of this tenure.*

I am deeply indebted to my beloved husband ***Mr. Shyam Karthik*** *for his understanding, unfailing patience and love.*

I am gratefully remembering all those people who put their hands together for me in this endeavour.

Above all, I bow my head before ***God Almighty*** *for the blessings showered on me.*

A. ARTHI

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1. INTRODUCTION

Pet animals like dogs are known for their social support and companionship. The canine-human bonding is essential to the health and well-being of both humans and dogs. The emotional and psychological interactions between the two have a significant impact in human lives. In India the prevalence of vector borne diseases like canine babesiosis has increased over the past few years. Despite this, very little information is available and many aspects of the disease are yet to be studied.

Canine babesiosis was first described in dogs by Piana and Galli-Valerio in 1895 and the organism was known as *Babesia canis. Babesia* piroplasms belong to two distinct species, the large forms are termed *B. canis* (4–5 µm) and the small piroplasms are termed *B. gibsoni* (1– 2.5 µm). Based on the differences in pathogenicity, vector specificity, geographical distribution and antigenic properties, three subspecies of *B. canis* are distinguished namely *B. canis vogeli, B. canis canis and B. canis rossi. Babesia gibsoni* is referred to as the Asian strain of *Babesia*, since it was first reported from a number of Asian countries.

Though parasite transmission to a susceptible host is mainly through the saliva of the infected tick, transplacental transmission and infection as a result of blood transfusion can also occur. *Babesia canis* that is transmitted by ticks of *Rhipicephalus* spp. and *B. gibsoni,* transmitted by *Haemaphysalis* spp. are endemic to the Indian sub-continent. In endemic areas, dogs are at a risk of getting infected simultaneously with more than one vector-borne agent.

Babesia canis vogeli infection manifests as a moderate and clinically inapparent infection whereas, *B. gibsoni* infection follows a hyper-acute, acute or chronic course. Recovered animals become sub-clinical carriers for life with low level parasitaemia. Canine babesiosis is characterised by regenerative haemolytic anaemia and thrombopaenia, usually accompanied by anorexia, fever, lethargy, pale

mucous membranes, jaundice, splenomegaly and increased pulse and respiratory rates. The complications occurring during the disease process depend on the nature of the parasite species, intensity of parasitaemia, host immunity and concurrent persisting infections. Infection with virulent strains (*B. canis rossi*) ensures complicated form of the disease and clinical manifestations depend on the particular organs involved.

Traditionally the disease is diagnosed by demonstration of the intra-cytoplasmic piroplasms in peripheral blood smears. As *Babesia* spp. organisms prefer to parasitize reticulocytes over mature red blood cells, a buffy coat smear can also be useful in diagnosis. Indirect Fluorescent Antibody Test (IFAT) and Enzyme Linked Immunosorbent Assay (ELISA) can also be used for diagnosis of canine babesiosis. Molecular biology is not only an effective tool to identify the subspecies but also provides definitive diagnosis when conventional methods give false negative results. As the virulence of the parasite and its response to anti-babesial therapy differ for each species, it is diagnostically important to determine the species and subspecies for a better prognosis.

The goal of treatment is ideally, elimination of parasites and reversal of anaemia. However a chronic state of premunition develops in most cases regardless of treatment. The standard treatment used for *B. canis* infection was diminazene aceturate. Eventhough imidocarb diproprionate is also claimed to be effective in the treatment of *B. canis* infection, no trials were conducted due to non-availability of the drug in Kerala. A variety of treatment protocols were suggested for *B. gibsoni* infection with variable cure rates. Relapses were reported frequently with the already used treatment protocols.

The climate of India favors survival and development of tick vectors facilitating increased incidence of tick borne parasitic diseases such as babesiosis.

Tick infestation can be prevented by regular visual inspection and acaricide therapy. Chronic carriers pose a risk of transmission or are susceptible in developing clinical piroplasmosis under stress conditions or concurrent infections.

Taking into consideration the aforementioned factors, the present study was undertaken with the following objectives:

1. Occurrence of canine babesiosis in and around Thrissur.
2. Assessment of haematological and biochemical parameters in infected dogs.
3. Comparing the efficacy of anti-babesial drugs in affected animals.

2. REVIEW OF LITERATURE

This chapter deals with the available literature pertaining to the prevalence, etiology, epidemiology, transmission, pathogenesis, clinical signs, diagnosis, therapeutic management, control and prevention of canine babesiosis.

2. 1. PREVALENCE

2. 1. 1. Global

Trapp *et al.* (2006) reported that antibodies against *B. canis vogeli* were found in 136 (36 per cent) dogs out of the 381 dogs that were examined in south Brazil.

A total of 68 blood samples collected between March and November 2010, from the Czech Republic revealed antibodies against *B. canis* in 12.21 per cent (five dogs) of dogs by indirect immunofluorescence test (Konvalinova *et al*., 2012).

Kamani *et al.* (2013) identified that out of 181 dogs tested from Nigeria between August and December 2011, the prevalence of *B. canis rossi* was 6.6 per cent and *B. canis vogeli* was 0.6 per cent.

A case–control study by Loftis *et al*. (2013) between 2009 and 2011 reported that from 55 clinically suspected cases of tick-borne diseases, 20 per cent cases were tested positive for *B. canis vogeli* and 18 per cent for *B. gibsoni* by PCR.

Wei *et al.* (2014) reported for the first time in Central America the presence of *B. gibsoni* and *B. canis vogeli* infection in four and six dogs respectively, a total of ten out of 39 apparently healthy dogs that was presented at a neutering program.

A study conducted in southern France by Rene-Martellet *et al*. (2015) from 2010 to 2012 revealed that among 140 blood samples screened by PCR, 19 (13.6 per cent) were positive for *B. canis vogeli* and 18 (12.9 per cent) for *B. canis canis.*

2. 1. 2. India

Sundar *et al*. (2004) reported a low incidence of *B. gibsoni* infection in Chennai during a one year period. Out of 5832 dogs screened only five (0.087 per cent) were infected with *B. gibsoni*.

The prevalence of *B. canis vogeli* between June to September 2008 in Delhi, Mumbai, Sikkim and Ladakh was reported to be 8.6 per cent, 7.4 per cent, two per cent and zero per cent respectively by molecular screening and the overall prevalence of *B. canis vogeli* from 525 dogs was 5.5 per cent (Rani *et al.,* 2011).

Singh *et al*. (2011) reported prevalence of *B. gibsoni* and *B. canis* as 4.51 per cent and 1.43 per cent respectively, among 488 canine blood samples examined in Punjab.

Bhattacharjee and Sarmah (2013) estimated the prevalence of *B. gibsoni* and *B. canis* as 47.16 per cent and 1.41 per cent amongst 424 dogs that were screened for haemoparasites by blood smear examination in Assam and north-east India from January 2009 to December 2010.

A study conducted in the College of Veterinary Science, Guwahati, Assam during the year 2010–2011 revealed that the overall prevalence of *B. gibsoni* was 48.64 per cent and *B. canis* was 54.05 per cent by PCR among 111 dogs that were sampled (Laha *et al*., 2014).

The incidence rate of canine babesiosis in and around Kolkata during a nine month period (November 2012 to July 2013) was found to be 31.86 per cent (Das *et al*., 2015).

A prevalence of 8.33 per cent of *Babesia* spp. infection was detected among 204 dogs sampled in Jalandhar District, Punjab during the year 2013-2014 (Vipan *et al.,* 2015).

Mahalingaiah *et al.* (2017) reported that out of 102 samples screened by PCR in Bengaluru, 23 (22.55 per cent) and 18 (17.65 per cent) were positive for *B. canis* and *B. gibsoni* respectively.

2. 1. 3. Kerala

The first report of canine babesiosis due to *B. gibsoni* and *B. canis* from Kerala were from Thrissur (Sabu *et al*., 2002; Sabu, 2005)

Augustine *et al*. (2013) reported prevalence of 29.31 per cent (34 dogs) with *Babesia* piroplasms in blood smears from a total of 116 dogs that were presented with symptoms suggestive of babesiosis. Out of the 34 positive cases, thirty (88.23 per cent) and four (11.76 per cent) of dogs were affected with *B. gibsoni* and *B. canis* respectively.

2. 2. ETIOLOGY

Large piroplasms (4-5µm) named *B. canis* and small piroplasms (1-2.5 µm) named *B. gibsoni* are known to cause canine babesiosis worldwide (Caccio *et al*., 2002).

2. 2. 1. Large *Babesia* spp.

2. 2. 1. 1. Babesia canis

The large piroplasm *Babesia canis* is sub-divided into *B. canis canis, B. canis rossi and B. canis vogeli* based on the geographical distribution, vector specificity and antigenic properties (Uilenberg *et al*., 1989; Taboada, 1998).

2. 2. 1. 1.1. *Babesia canis vogeli*

Ionita *et al.* (2012) reported the first molecular confirmation of *B. canis canis* and *B. canis vogeli* in dogs from Romania.

Kundu *et al*. (2012) identified the subspecies as *B. canis vogeli* in three cases one each from Sahajahanpur, Bareilly and Chandigarh by PCR based amplification.

Babesia canis vogeli is the least virulent species which is reported to cause subclinical infections with a low parasitaemia in adult dogs (Koster *et al*., 2015).

2. 2. 1. 1. 2. *Babesia canis canis*

The very first report of *B. canis canis* from Norway in a six year old male Irish setter with lethargy, anorexia, polydipsia, petechiae and pyrexia was confirmed by molecular diagnosis and the PCR products showed 100 per cent similarity to *B. canis canis*, isolate with accession number, AY072926 (Oines *et al*., 2010).

Adaszek *et al*. (2016) found that the DNA fragments of the 18S RNA B gene of *B. canis canis* isolated from the mother and puppies had 100 per cent similarily thus confirming the possibility of an *in utero* transmission.

2. 2. 1. 1. 3. *Babesia canis rossi*

Nuttall (1910) described *Piroplasma rossi,* an intraerythrocytic piroplasm in the blood of dead jackals in East Africa and also found them to be infested with the tick species *Haemaphysalis leachi.* Later this species was recognized as the African subspecies *B. canis rossi*.

Oyamada *et al*. (2005) reported that *B. canis rossi* occured only in sub-Saharan Africa and Eastern Sudan and was the most virulent species that was not susceptible to the currently available drugs.

Bohm *et al.* (2006) reported that significantly higher capillary and venous parasitaemias in *B. canis rossi* infections can lead to circulatory compromise.

2. 2. 1. 2. Babesia coco

Lehtinen *et al*. (2008) have cultured a large *Babesia* spp. from an infected dog by *in vitro* microaerophilous stationary phase culture methodology and this novel large *Babesia* spp. from North Carolina was termed *B. coco.*

2. 2. 2. Small *Babesia* spp.

2. 2. 2. 1. Babesia gibsoni

Kjemtrup *et al*. (2000) reported that there were three morphologically identical isolates of *B. gibsoni* and the Asian isolate found in India is considered *as B. gibsoni sensu stricto*. Molecular analysis of 18S rRNA show that these three isolates are distinct.

Babesia gibsoni was reported to be an emerging pathogen in the USA but with previous reports in the Middle East, south Asian countries, Japan, North Africa, South America and has been detected in regions like Italy, Hungary and Australia also (Muhlnickel *et al.*, 2002).

Thirty five (30.4 per cent) out of 115 dogs from various prefectures of Japan were found positive for *B. gibsoni* by PCR and ELISA in an epidemiological survey conducted by Miyama *et al.* (2005) in Eastern Japan from February to October 2003.

A higher prevalence of *B. gibsoni* (7.84 per cent) when compared to *B. canis* infection (0.49 per cent) was recorded in Jalandhar (Punjab), during a period of one year from April 2013 to March 2014 by Vipan *et al*. (2015).

2. 2. 2. 2. Theileria annae

The European isolate that was found to exist with a high frequency among Spanish dogs was named *Theileria annae* and was related to *B. microti.* (Camacho *et al*., 2003; Criado-Fornelio *et al*., 2003).

Kjemtrup *et al*. (2006) have proposed the name *B. conradae* to the California isolate which was a different species with some characteristics of the genus *Theileria*.

The first report of *T. annae* infection in a Swedish dog was by Falkeno *et al*. (2013).

2.3. EPIDEMIOLOGY

2. 3. 1. Host factors

2. 3. 1. 1. Age

Abdullahi *et al*. (1990) encountered about 70 cases of *B. canis* over a period of two years in Nigeria and found that the commonly affected age group was less than one year old.

In a study by Kumar *et al.* (2009) dogs above three years of age were found to be affected with blood parasites to a greater extent (63.1 per cent) and with respect to *B. gibsoni* positive cases, a higher percentage of incidence was amongst the adults (67.7 per cent).

Davitkov *et al*. (2015) reported that young and middle-aged dogs were most affected with babesiosis due to *B. canis* and *B. gibsoni* where the youngest dog to be infected was two and a half months and the oldest was thirteen years of age.

Vipan *et al*. (2015) reported that *B. canis* infection was recorded from the dogs above one year of age only. However comparable prevalence of *B. gibsoni* was noticed in all age groups.

Mahalingaiah *et al.* (2017) reported a higher incidence of canine babesiosis in Bengaluru in dogs between the age group of one to two years (23 per cent) and lesser incidence (3 per cent) in dogs between eight to ten years of age.

2. 3. 1. 2. Sex

Das *et al*. (2015) reported that the overall sex ratio (male/female) of incidence was 1:1.4 with females showing a higher level of infections compared to male.

Davitkov *et al*. (2015) reported higher prevalence of *B. canis and B. gibsoni* in male dogs than female dogs in Serbia.

Majority of the study subjects were males (64.5 per cent) out of 363 dogs in a retrospective analysis done using clinical records of dogs that were diagnosed with *Babesia* spp. infections in Lusaka, Zambia between the years 2000 to 2013 (Nalubamba *et al.,* 2015).

From a total of 204 canine blood samples screened for babesiosis in Jalandhar (Punjab), the prevalence of babesiosis was comparatively higher in males (6.47 per cent) than female dogs (3.52 per cent) (Vipan *et al*., 2015).

From a total of 102 samples screened for babesiosis in Hebbal, Bengaluru, Mahalingaiah *et al.* (2017) reported a higher incidence of babesiosis in male dogs (57.5 per cent) when compared to female dogs (42.5 per cent).

2. 3. 1. 3. Breed

Eighteen male Tosa breed of dogs in Japan were found to be sub-clinically infected with *B. gibsoni* which possibly would have been transmitted following dog fighting (Matsuu *et al.,* 2004).

In a study by Mathe *et al*. (2006) majority of Rottweilers (seven out of nine) that were included in the study developed complicated form of babesiosis.

Ayoob *et al*. (2010) reported a higher sero-prevalence of *B. canis subsp. vogeli* in Greyhound breed with seropositivity of up to 50.0 per cent in endemic areas compared with 3.8 to 13.0 per cent sero-positivity in general population.

Nalubamba *et al*. (2015) reported that from the records of 363 patients that were analyzed in Lusaka, Zambia between the years 2000 and 2013, the most represented dog breeds were mongrels (32.2 per cent) and Maltese poodles (16.3 per cent) while the Shar Pei and Boston Terrier breeds were the least represented.

Mahalingaiah *et al.* (2017) found that babesiosis has the highest occurrence in Labrador Retrievers (26 per cent) followed by other breeds which included German shepherd (17 per cent), Cocker Spaniel (10.5%), Non-descript (9.0 per cent), Golden Retriever (7.5 per cent), Pug (7.5 per cent), Rottweiler (4.5 per cent), Dalmatian (3 per cent), Great dane (3 per cent), Siberian huskey (1.5 per cent), Spitz (1.5 per cent), Mudhol hound (1.5 per cent), Irish setter (1.5 per cent), Boxer (1.5 per cent), Saint Bernard (1.5 per cent), Pit bull terrier (1.5 per cent) and Toy fox terrier (1.5 per cent).

2. 3. 2. Season

Mathe *et al*. (2006) observed more cases of babesiosis during spring and autumn in Hungary.

Porchet *et al*. (2007) recorded higher occurrence of babesiosis (11 cases) during the spring season between 2005 and 2006 and two cases in autumn 2005. This coincides with the activity period of *Dermacentor reticulatus*, the main vector of *B. canis canis.*

Konvalinova *et al*. (2012) opined that highest number of positive samples was found in the month of June.

A higher incidence of canine babesisosis was reported during hot humid summer weather in a study conducted by Das *et al*. (2015) during a nine month period in Kolkata. The incidence was found to be higher in summer season (19.02 per cent) followed by spring (13.71 per cent) and winter (3 per cent).

Babesiosis due to *B. canis vogeli* was observed mostly between the months of March and July with a rise in occurrence during April in the region Gard of Southern France (Rene-Martellet *et al*., 2015).

2. 3. 3. Transmission

The possibility of vertical transmission of *Babesia* spp. has been proposed by Fukumoto *et al*. (2005), Simoes *et al*. (2011) and Adaszek *et al*. (2016).

Miyama *et al.* (2005) reported a higher incidence of *B. gibsoni* infection in fighting dogs which had minimal exposure to the tick *Haemaphysalis longicornis*, the predominant species of eastern Japan.

Dantas-Torres *et al.* (2006) stated that *B. canis* and *B. gibsoni* parasites are transmitted to a susceptible host through the saliva of an infected tick, mainly *R. sanguineus*.

Jefferies *et al.* (2007a) proposed the possibility of direct blood to blood transmission of *B. gibsoni* via dog bites among fighting American Pitbull Terriers.

Solano-Gallego and Baneth (2011) stated that *B. gibsoni* was transmitted by *Haemaphysalis* spp. and *B. canis vogeli* by *Rhipicephalus* spp.

Babesia canis vogeli is transmitted by *R. sanguineus* the brown dog tick in tropical and subtropical regions and colder areas. *Dermacentor* spp. transmits *B. canis canis* in Europe and in South African countries. *Babesia canis rossi* was found to be transmitted by *Haemaphysalis* spp. ticks (Schnittger *et al*., 2012).

According to Kelly *et al*. (2013), *R. sanguineus* was the principal species transmitting 48 per cent of the *Babesia* spp. infections in St. Kitts, West Indies.

For the first time in Switzerland, Schaarschmidt *et al*. (2013) amplified the partial 18S rRNA gene of *B. canis* from the DNA isolated from 19 *Dermacentor reticulatus* ticks out of 23 collected.

Screening of 248 *R. sanguineus* specimens by PCR showed that 23 (9.3 per cent) were *B. canis vogeli* positive, 1 (0.4 per cent) was *B. canis canis* positive and 3 (1.2 per cent) were co-infected with *B. canis vogeli* and *B. canis canis* (Rene-Martellet *et al*., 2015).

2. 4. PATHOGENESIS

La Vier and Fombeure (1922) and Basson and Pienaar (1965) have observed eye lesions in *B. canis* infections. Bilateral thrombosis of the ophthalmic veins with parasitized erythrocyte within the thrombus was as remarkable finding.

Adachi and Makimura (1992) and Adachi *et al*. (1994) reported an increase in anti-erythrocyte membrane antibodies and erythrocyte bound immunoglobulin G concentrations in patients affected with *B. gibsoni,* suggesting a potential immune mediated haemolytic anemia.

Cerebral babesiosis was reported to cause very high mortality as parasitized erythrocytes adhere to microvasculature of the CNS, releasing inflammatory mediators and thereby cause tissue hypoxia that leads to neurological signs like seizures, altered consciousness, ataxia, paresis, tremors, anisocoria and vestibular signs (Schetters and Eling, 1999; Boozer and Macintire, 2003; Van de Maele *et al*., 2008).

In a retrospective study acute pancreatitis was confirmed histologically in four dogs with canine babesiosis (Mohr *et al*., 2000).

Leisewitz *et al.* (2001) reported that the degree of parasitaemia depends on the parasite's ability to replicate in the host's erythrocytes and cause cell lysis.

Liver followed by kidneys, muscles, lungs and then central nervous system were the most frequently affected organs in babesiosis caused by *B. canis rossi*. Both *B. canis rossi* and *B. canis canis* are documented to cause multiple organ dysfunction syndrome (MODS) (Welzl *et al*., 2001).

Babesia canis rossi is the most pathogenic among the three and causes haemolytic anaemia possibly due to immune mediated or an overwhelming inflammatory reaction. *Babesia canis canis* causes transient parasitaemia with congestion of internal organs and *B. canis vogeli* which is the least pathogenic causes a mild inapparent infection (Caccio *et al*., 2002).

Immune response is strain-specific and there is no cross-protective immunity between species and strains (Boozer and Macintire, 2003; Uilenberg, 2006).

Splenomegaly which was usually marked in canine babesiosis occurs because, up to one third of the body's total thrombocytes are stored here and excess sequestration results in concurrent thrombocytopaenia (Kettner *et al*., 2003).

In a study done by Furlanello *et al.* (2005), amongst 74 per cent (17 dogs) that were presented with anaemia, 70 per cent had haemolytic anaemia while the remaining 30 per cent had non-haemolytic anaemia. Mechanical injury, immune-mediated anaemia and toxic haemolytic factor produced by *B. gibsoni* are the three suggested mechanisms for haemolysis.

Bohm *et al*. (2006) compared capillary parasitaemia with venous parasitaemia in *B. canis rossi* infected dogs and found capillary parasitaemia to be more possibly because of a parasite-induced erythrocyte membrane rigidity, thus slowing down their movement through capillaries resulting in aggregation of parasitized erythrocytes towards the capillary side. Such a correlation between parasitaemia and circulatory disturbance showed that dogs with higher levels of parasitaemia suffered from septic shock.

Jacobson (2006) emphasized that erythrocyte depletion causing anaemia was attributed to parasite multiplication, haemodilution, accumulation in the spleen, autoimmune haemolysis, erythrophagocytosis and impaired production of the same.

Acute pancreatitis and disseminated intravascular coagulation (DIC) were the two complications reported from a retrospective study made by Matijatko *et al.* (2009). In the same study it was found that in patients with multiple organ dysfunction syndrome (MODS), the most common complications were acute renal failure (ARF), followed by hepatopathy, respiratory distress and cerebral babesiosis.

Ayoob *et al.* (2010) reported respiratory distress to be a common complication with more pathogenic strains resulting in acute respiratory distress syndrome (ARDS) and is manifested clinically by tachypnea, dyspnea, cough and frothy nasal discharge. Haemoconcentration occurs in conjunction with intravascular haemolysis and secondary to hypoalbuminaemia.

Out of 332 dogs naturally infected with *B. canis canis* in Croatia, 33 dogs (10 per cent) fulfilled the MODS criteria and among them 22 dogs had two-organ involvement, 10 dogs had three organ involvement and in only one dog four-organ dysfunction was present. Among these 33 MODS positive dogs 11 survived and 22 died (Matijatko *et al*., 2010).

A study by Lobetti *et al*. (2012) demonstrated that myocardial injury occurred with babesiosis. Dogs with myocardial lesions and higher concentrations of cardiac troponins in plasma died naturally of disease than the survivors. Dogs with immune mediated haemolytic anaemia and complicated babesiosis had higher cardiac troponin concentrations than the control group. The concentration of plasma cardiac troponin I (cTnI) increases with the severity of the disease.

Matijatko *et al.* (2012) proposed that the process of sequestration, where parasitized erythrocytes adhere to the capillary endothelium of particular organs will result in the host maintaining immunity with a low-level chronic infection.

Wei *et al.* (2014) reported that dogs with heavy parasitaemia that survive after therapy can remain subclinically infected and can act as chronic carriers with a risk of relapse following any stress condition.

2. 5. CLINICAL SIGNS

Carlos *et al.* (1972) found bilateral keratitis in a dog with *B. canis* infection.

A hyperacute form of babesiosis manifested by sub-normal temperature, shock, jaundice, twitching of muscles, incoordination and convulsions was encountered by Abdullahi *et al*. (1990).

Carlotti *et al*. (1992) have reported gross lesions like cutaneous haemorrhagic macules, urticaria and necrosis of the extremities in dogs infected with *B.canis* infection.

Kettner *et al*. (2003) emphasized that there was no spontaneous bleeding in babesiosis although thrombocytopaenia was evident. This is because platelets were still functional, whereas in immune-mediated thrombocytopaenia or disseminated intravascular coagulation (DIC) there are other non-platelet associated coagulatory disturbances resulting in bleeding.

Furlanello *et al*. (2005) observed dehydration, apathy, anorexia, fever, icterus, generalized lymphadenomegaly, epistaxis and cervical pain in dogs having *B. canis* infection in northeastern Italy.

Bourdoiseau (2006) reported that atypical forms of babesiosis caused by *B. canis* involving the locomotor, cerebral, ocular, gastrointestinal and vascular systems compromise diagnosis.

Mathe *et al*. (2006) opined that clinical signs like diarrhoea, abdominal pain, tachypnea or dyspnea, tremors and lymphadenopathy were rarely observed in clinical cases of babesiosis.

An acute atypical form of babesiosis caused by *B. canis* in Belgium was reported by Van de Maele *et al*. (2008) in a 10 year old Akita Inu which manifested with grand mal' seizures and weakness.

Erythema, crust formation, desquamation, generalized alopecia and pruritus were observed in a Satsuma dog in Japan that was positive for *B. gibsoni.* Histopathological examinations of skin biopsies were suggestive of early leukocytoclastic vasculitis or ischemic vasculopathy (Tasaki *et al.,* 2013).

Gonde *et al*. (2016) reported paraplegia in a *B. gibsoni* infected dog, which recovered after treatment with diminazene aceturate (single dose) followed by a combination of enrofloxacin, doxycycline and metronidazole for three weeks.

2. 6. HAEMATOLOGICAL ALTERATIONS

In a study that included 34 dogs suffering from severe babesiosis caused by *B. canis rossi,* 29 dogs had severe anaemia (VPRC < 15 per cent), two dogs showed mild anaemia (VPRC 25 to 35 per cent) and the remaining three dogs were haemoconcentrated (Leisewitz *et al.,* 2001).

Kettner *et al*. (2003) noticed variations in the degree of thrombocytopaenia caused by different *Babesia* spp. in the same host species. Such differences in pathogenecity are governed by the nature of the parasite.

Profound thrombocytopaenia was reported in a dog with concurrent babesiosis caused by *B. canis* and bartonellosis (Tuttle *et al*., 2003).

Normocytic–hypochromic anaemia, polychromasia, anisocytosis, leukocytosis, monocytosis, lymphopaenia, and thrombocytopaenia were the

haematological alterations observed in proven cases of babesiosis by Guimaraes *et al*. (2004) in a study conducted with 500 dogs from Rio de Janiero.

Among 23 dogs with babesiosis that were evaluated, 16 dogs showed leucopaenia, 17 dogs showed neutropaenia, 15 dogs showed lymphopaenia, one dog showed leucocytosis and 14 dogs had activated lymphocytes (Furlanello *et al*., 2005).

Thrombocytopaenia and macrocytic anaemia were reported by Vishnurahav *et al.* (2014) in six dogs affected with *B. gibsoni* infection.

2. 7. SERUM BIOCHEMICAL ALTERATIONS

Boozer and Macintire (2005) reported hyperbilirubinaemia to be a common feature of *B. canis* infection but not *B. gibsoni* infection.

Furlanello *et al*. (2005) noted in majority of dogs with *B. canis* infection, a mild elevation of aspartate transaminases, alanine transaminases and total bilirubin concentration. Hyperfibrinogenaemia was also observed in all the dogs evaluated suggesting an acute inflammatory response.

Mathe *et al*. (2006) reported that elevation in blood urea nitrogen (BUN) values was a common finding in babesiosis patients, but this did not coincide with creatinine concentrations. Dehydration and increased degradation of haemoglobin can both raise BUN concentrations.

Zygner *et al*. (2006) found that the most common biochemical changes in affected dogs were an increase in activity of transaminases and alkaline phosphatase, elevated creatinine concentration, hypoalbuminaemia and hypoglycaemia, all resulting from hepatopathy, renal failure and fasting.

de Gopegui *et al*. (2007) encountered hyperglycemia in 21 clinical cases of babesiosis from a total of 45 cases, in a retrospective study done in Spain. It was opined that hyperglycemia can be caused by increased glucose mobilization, stress

and by increased cortisol secretion, a common physiological event that occurs during critical illness. Though the serum creatinine levels were within the normal range, serum urea levels were increased in majority of the dogs. This is mainly because of the haemolysis and subsequent loading of ammonia resulting in non-renal elevations in serum urea levels.

Hypoproteinaemia, hyperglobulinaemia and an elevation in total and direct bilirubin were reported in dogs suffering from babesiosis (Vishnurahav *et al.,* 2014).

An elevation in bilirubin levels with visible icterus was reported to occur in advanced stages of canine babesiosis and a high alanine transaminase activity can result in poor prognosis. Decreased renal perfusion or hypovolaemia could result in an increase in the serum urea and creatinine levels in complicated babesiosis (Koster *et al.,* 2015).

Konto *et al*. (2014) reported elevated alkaline phosphatase and alanine transaminase and reduced blood glucose in dogs after experimental inoculation with *B. canis.*

2. 8. DIAGNOSIS

2. 8. 1. Staining techniques

Gay *et al.* (1996) reported that acridine orange (AO) staining technique was more sensitive than Giemsa staining technique for detecting a low parasitaemia.

Furlanello *et al*. (2005) ranked the degree of parasitaemia based on the number of erythrocytes parasitized in one blood smear as +, ++, +++ and ++++ for 1 to 5, 6 to 20, 21 to 50 and >50 erythrocytes parasitized per blood smear respectively.

While comparing capillary and venous parasitaemias, Bohm *et al*. (2006) found that capillary parasitaemias were usually higher making them more appropriate diagnostic samples. This was because the larger piroplasms aggregate in capillaries.

Ravindran *et al*. (2007) reported that the (AO) staining technique was simple, rapid and accurate for screening large number of samples.

A variety of morphological forms of *Babesia* spp. are detectable inside erythrocytes like oval, pyriform or elongate and this is mainly because of the disintegration of host erythrocyte membrane following endocytosis of the parasite (Ayoob *et al,*. 2010).

Babesia canis has been reported by Balachandran *et al*. (2010) from a ten day old puppy on postmortem examination of Leishmann stained peripheral blood smears.

Several intraerythrocytic forms of *B. canis* like, ovoid, amoeboid, elongated and umbrella-like piroplasms were reported by Eljadar *et al*. (2012) using the DP2-BSW software (Olympus).

2. 8. 2. Molecular diagnosis

Birkenheuer *et al.* (2003) developed a seminested PCR to discriminate *B. gibsoni* (Asian genotype) and the three subspecies of *B. canis* DNA from infected dog blood.

More number of positive cases of *B. canis* and *B. gibsoni* were detected by PCR than blood smear examination in a study done by Inokuma *et al.* (2004) at Okinawa Island, Japan.

Though Matjila *et al*. (2005) made the first report of *B. canis vogeli* in South Africa, the most prevalent sub-species here was *B. canis rossi*.

de Sa *et al*. (2006) used a genus specific primer pair, PIRO A and PIRO B to amplify a 400bp region of 18S rRNA gene of *Babesia*.

Trapp *et al*. (2006) used the primer pair PIRO-F (5^{o}-AGTCATATGCTTGTCTCA-3^{o}) and PIRO-R (5^{o}-CCATCATTCCAATTACAA-3^{o}) to amplify a 460bp fragment of the 18 S ribosomal RNA gene of *B. gibsoni.*

Wang *et al*. (2010) used quantitative fluorescent resonance energy transfer (FRET)-PCR to amplify 18S rRNA gene fragments of *Babesia* spp. for species differentiation and was considered to have more sensitivity and specificity.

Kundu *et al*. (2012) used the specific primer pair BAB1/BAB4 which amplified a 600 bp fragment of 18S rRNA and identified the subspecies to be *B. canis vogeli* in three dogs.

According to Schaarschmidt *et al*. (2013) PCR was more sensitive than staining techniques as it detected *Babesia* DNA in all 28 animals that were tested whereas eight cases among them were found to be blood smear negative.

2. 8. 3. Serological diagnosis

Verdida *et al*. (2004) suggested an ELISA with GST-P50t (Glutathione S-transferase with surface antigen P50) as a reliable tool for diagnosis of piroplasmosis due to *B. gibsoni* as it can clearly differentiate between infected and uninfected dog sera and show no cross - reactivity with similar piroplasms.

Ulutas *et al.* (2005) reported an increase in the plasma levels of C-reactive protein (CRP) and Ceruloplasmin (*Cp*) in complicated babesiosis and hence suggested that in severe forms of the disease condition these acute phase proteins would be elevated.

A recombinant BgTRAP ELISA was found to be more sensitive than recombinant BgP50 ELISA to detect *B. gibsoni* antibodies in dogs and thus can detect previous infection as well as current infection (Konishi *et al*., 2008).

Mandal *et al*. (2016) reported that indirect ELISA and dot ELISA using recombinant BgP12 (rBgP12) can be used for large scale epidemiological investigation as they have high sensitivity and reliable specificity for detection of specific antibodies to *B. gibsoni* without showing cross reactivity.

2. 8. 4. Ultrasonography

Hepatomegaly, renomegaly, increased echogenicity of the liver and kidneys and increase in the corticomedullary ratio was a consistent finding in canine babesiosis noted by Mathe *et al*. (2006).

Fraga *et al*. (2011) reported that the mechanism of splenomegaly in canine babesiosis may be due to acute splenitis, haemolytic anaemia, toxaemia, extramedullary haematopoiesis or amyloidosis. Hepatomegaly and splenomegaly with diffuse, hypoechoic, heterogeneity were the consistent finding in dogs harbouring *Babesia* spp.

Reduced echogenicity of liver along with a distended gall bladder was the most prominent finding observed by Sarma *et al*. (2016).

2. 9. THERAPEUTIC MANAGEMENT OF BABESIOSIS

Penzhorn *et al.* (1995) claimed that a recommended therapeutic dose of diminazene aceturate on day one followed by a therapeutic dose of Imidocarb dipropionate on the next day prevents relapse of *B. canis* infection.

Milner *et al.* (1997) reported that the side effects that occured following administration of diminazene aceturate which included vomition, nausea, urination, nervous signs and anaphylactic shock were not mediated by inhibiting cholinesterase enzyme.

Complete elimination of infection caused by the Asian genotype of *B. gibsoni* was not possible with any of the currently available anti-babesial drugs because of its

virulent nature in dogs. The California genotype of *B. gibsoni* is also virulent and it's susceptibility to anti-babesial therapy is yet to be characterized (Birkenheuer *et al.,* 2003).

Milner *et al.* (1997) reported the alternative use of drugs like Trypan blue an azonaphthalene dye which has lesser side effects. An aqueous solution of Trypan blue at a dose of 10 mg/kg BW can be given intravenously. The choice of drug used for babesiosis is determined by the severity of the condition.

Boozer and Macintire (2003) suggested that although a dose of 3.5mg/kg body weight of diminazene aceturate was effective for *B. canis* infection, for *B. gibsoni* infection a higher dose of 7.5 to 10 mg/kg BW was needed.

Chaudhuri and Varshney (2007) studied the efficacy of homeopathic Crotalus horridus 200C for moderate cases of *B. gibsoni* infection and found that it was as effective as diminazene aceturate in providing clinical recovery.

Imidocarb at a dose rate of 5 to 6.6 mg/kg body weight was reported to resolve clinical signs of babesiosis and had a prophylactic effect for a period of four week post injection (Ayoob *et al*., 2010).

Lin *et al.* (2012) compared the combined efficacy of clindamycin, diminazene aceturate and imidocarb (CDI) against that of atovaquone and azithromycin (AA) combination for *B.* gibsoni infection. The CDI protocol was found to be more efficacious than AA protocol.

Imidocarb dipropionate was reported to be safe for both puppies and geriatric patients (Adaszek *et al.,* 2016; Kumara, 2016).

Kumara *et al*. (2016) treated a concurrent case of *B. gibsoni* and *E. ewingi* with a combination of injections metronidazole, clindamycin, oxytetracycline and supportive blood transfusion.

Six dogs positive for *B. gibsoni* on blood smear examination were treated with clindamycin at the rate of 11 mg/kg BW intravenously q24h for 10 days and this monotherapy was found to be effective for the successful management of clinical case of *B. gibsoni* infection (Vishnurahav *et al* ., 2017).

2. 10. CONCURRENT INFECTIONS

Concurrent ehrlichiosis and babesiosis with predominant clinical finding like anaemia and thrombocytopaenia was reported by Klag *et al.* (1991).

Tuttle *et al.* (2003) reported the presence of persistent parasitaemia in a 12 year old male West Highland White Terrier with concurrent babesiosis and bartonellosis.

Co-infection with *Ehrlichia* spp. *Babesia* spp. and *Hepatozoon* spp. was reported by Sasanelli *et al.* (2009) in a 3.5 months old crossbreed dog that was presented with mild depression, pyrexia, anorexia, diarrhoea, cough and ascites.

Concurrent *Babesia* and *Ehrlichia* infections have been described under the name Lahore Canine Fever dating back to 1938 (Abbas *et al.*, 2015).

Concurrent infection with *B. canis rossi* and *B. gibsoni* was reported in 59.7 per cent of dogs from a total of 363 babesiosis confirmed cases in Lusaka, Zambia between the years 2000 to 2013 (Nalubamba *et al*., 2015).

A case of concurrent Leptospirosis, microfilariosis and babesiosis caused by *B. gibsoni* was successfully treated with Metronidazole, Clindamycin, Doxycycline combination and Ivermectin (Ajith *et al.*, 2016).

Kumara *et al*. (2016) observed concurrent infections of *B. gibsoni* and *E. ewingi* in a German Shepherd breed that had anaemia and thrombocytopaenia.

2. 11. CONTROL AND PREVENTION

Less severe anaemia, with reduced clinical signs and morbidity were the outcomes of the culture-derived vaccine that contained soluble parasite antigens from the European homologous *B. canis* (Schetters *et al*., 1995).

Schetters *et al*. (2001) studied that when a mixture of soluble parasite antigens from both a *B. canis canis* isolate and a *B. canis rossi* isolate were used, protective immunity was induced against a heterologous *B. canis* infection indicating the absence of cross-protection when challenged with a different strain.

Vaccine containing soluble antigens derived from a culture of *B. canis* (Pirodog®) can be administered subcutaneously to healthy dogs when they are at least 5 months old followed by a booster after a minimum interval of 2 weeks (Bourdoiseau, 2006)

Vaccination with soluble parasite antigens (SPA) along with saponin provided protective immunity against clinical babesiosis caused by *B.* canis *rossi* and *B.* canis *canis*. Protection was conferred from three weeks after booster vaccination and lasted for a period of six months (Schetters *et al*., 2006).

The use of amitraz-impregnated collars was found to significantly reduce the number of new infections of *B. canis rossi* in endemic areas (Last *et al*., 2007).

Dogs treated with a top-spot preparation of fipronil or selamectin and dogs in which an amitraz impregnated collars were used had lesser tick infestation rates when compared to dogs treated with topical flumethrin, fipronil spray and over the counter (OTC) shampoos and powders (Loftis *et al*., 2013).

3. MATERIALS AND METHODS

The study was conducted at the department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Sciences, Mannuthy during the period of April 2016 to March 2017. Prevalence of babesiosis was studied among the dogs presented to the Kerala Veterinary and Animal Sciences University, Veterinary Hospitals at Mannuthy and Kokkalai and hospitals attached to the Department of Animal Husbandry in Thrissur district.

3. 1. MATERIALS FOR THE STUDY

3. 1. 1. Animals

Dogs presented with clinical signs suggestive of babesiosis such as pyrexia, haemoglobinuria, pale mucous membranes, jaundice, general weakness, convulsions, limb edema, scrotal oedema, anorexia and tick infestation formed the study material. A brief clinical history of the cases was also recorded, along with factors such as age, sex and breed of the suspected animals. Detailed clinical examinations were made on each case and the same was recorded in a performa as given in the appendix. Ten apparently healthy blood smear negative dogs were taken as control groups.

3. 1. 2. Chemicals, Glassware and Plastic ware

Chemicals used for the study were of analytical grade. Glassware like microscopic glass slides, economy grade, rough ground edge were procured from Hi-media. Plastic wares procured from Tarson Company were used for the study.

3. 1. 3. Staining solutions

3. 1. 3. 1. Giemsa stain

Geimsa’s staining solution from Nice chemicals, Edapally, Kochi was used.

3. 1. 3. 2. Field stain

1. Field stain A powder - 5 grams
2. Field stain B powder - 4.8 grams
3. Distilled water (at 60°C) - 1200 ml

Field stain A

Five gram of Field stain A powder (5 grams) was dissolved in distilled water (at 60°C), filtered using a filter paper and stored it in a sterile container until use.

Field stain B

Field stain B powder (4.8 grams) was dissolved in distilled water (at 60°C), filtered using a filter paper and stored it in a sterile container until use.

3. 1. 3. 3. Acridine orange stain

1. Acridine orange powder (Hi-media) - 20mg
2. Hydrochloric acid (1 N) (Hi-media) - 13.6 g
3. Sodium acetate (Hi-media) - 90 ml
4. Distilled water - 100 ml

Stock solution of sodium acetate buffer was prepared by adding 13.6 g of sodium acetate to 100 ml of distilled water and 90 ml of 1N HCl. The pH was adjusted to 3.5 by adding 1N HCl. Finally, 20 mg of acridine orange powder was added to 190 ml of sodium acetate buffer.

3. 1. 4. Polymerase chain reaction

3. 1. 4. 1. Tris EDTA borate buffer (TBE) - 5 X

Tris base (Hi-media) - 54 g
Boric acid (SRL, AR) - 27.5 g

0.5 M EDTA (SRL, AR) - 20 ml

Made up the volume to one litre by adding distilled water (DW) and pH was adjusted to 8.0. Autoclaved and stored at room temperature.

Two hundred millilitre 5X TBE buffer was added to 800 ml of distilled water to make a working solution of 1X TBE buffer. This buffer was used as a tank buffer during electrophoresis.

3. 1. 4. 2. Ethidium bromide stock solution

Ethidium bromide (Sigma Aldrich Chemicals, Bangalore) - 100 mg / 10 ml
Triple distilled water - 10 ml

Dissolved 100 mg ethidium bromide in 10 ml distilled water. Solution stored at - 4ºC in amber coloured bottle.

3. 1. 4. 3. Gel loading buffer (6X) (Fermentas, Lithuania)

Bromophenol blue - 0.25 per cent
Xylene - 0.25 percent
Sucrose - 40 percent (w/v) in water

3. 1. 4. 4. Preparation of DNA from blood

QIAGEN, DNA extraction kit (DNeasy Spin-Column), procured from Vision Scientific, Angamaly was used for extraction of DNA from blood, which contained the following reagents.

a) Proteinase K
b) Phosphate Buffered Saline (PBS), pH-7.2
c) Buffer AL

d) Absolute ethanol (96 – 100 per cent)
e) Wash Buffer 1 (AW1) (Concentrate)
f) Wash Buffer 2 (AW2) (Concentrate)
g) Buffer AE (Elution buffer)

3. 1. 4. 5. PCR amplification

3. 1. 4. 5. 1. Primers

Genus, species and sub-species specific primers procured from Sigma Aldrich Chemicals, Bangalore were selected for PCR amplification as per Table 3.1.

3. 1. 4. 5. 2. PCR Reaction buffer (10 X)

This includes 500mM KCl and 100mM Tris hydrochloride.

3. 1. 4. 5. 3. Deoxy Ribonucleotide Triphosphates (dNTP)

Mixed 2.5 mM each dGTP/dCTP/ dATP/ dTTP in equal volumes.

dGTP	-	Deoxy guanosine triphosphate
dCTP	-	Deoxy cytosine triphosphate
dATP	-	Deoxy adenosine triphosphate
dTTP	-	Deoxy thymidine triphosphate

3. 1. 4. 5. 4. Taq DNA polymerase

Taq DNA polymerase enzyme with a concentration of 3 U/µl was used.

3. 1. 4. 5. 5. Magnesium chloride

Magnesium chloride with strength of 25 mM was used for PCR.

Table 4.9. Haematological parameters of *Babesia gibsoni* affected groups before and after treatment

Haematological parameters	Treatment groups								
	Group I (n=11)			Group II (n=11)			Group III (n=11)		
	BT	AT		BT	AT		BT	AT	
		Day 3	Day 14		Day 3	Day 14		Day 3	Day 14
Total Erythrocyte count (x 10^6/mm^3)	2.655 ± 0.401 [a]	2.430±0.387 [a]	3.790±0.358 [b]	3.254± 0.401 [a]	2.824±0.387 [b]	3.603±0.358 [a]	3.350± 0.401 [a]	3.579±0.387 [a]	3.430±0.358 [a]
Haemoglobin (g/dl)	7.009 ± 1.094 [a]	6.491±0.982 [a]	9.555±0.875 [b]	8.355± 1.094 [a]	7.055±0.982 [b]	9.482±0.875 [a]	9.409± 1.094 [a]	9.864±0.982 [a]	9.845±0.875 [a]
VPRC (per cent)	18.018±2.362 [a]	17.464±2.430 [a]	25.355±1.918 [b]	22.864±2.362 [a]	19.945±2.430 [b]	25.145±1.918 [a]	23.400±2.362 [a]	24.855±2.430 [a]	23.800±1.918 [a]
MCV (fl)	73.500±3.833 [a]	76.136±3.790 [ab]	69.773±3.114 [ac]	74.300±3.833 [a]	74.218±3.790 [ab]	71.555±3.114 [ac]	71.536±3.833 [a]	71.155±3.790 [a]	71.745±3.114 [a]
MCH (pg)	27.291±1.389 [a]	27.727±1.298 [a]	25.809±1.543 [a]	25.664±1.389 [a]	25.045±1.298 [a]	26.573±1.543 [a]	28.409±1.389 [a]	28.136±1.298 [a]	29.609±1.543 [a]
MCHC per cent	37.536±1.712 [a]	36.545±1.250 [a]	36.945±1.591 [a]	35.300±1.712 [a]	34.364±1.250 [a]	37.400±1.591 [a]	39.545±1.712 [a]	39.555±1.250 [a]	41.155±1.591 [a]
Platelet count (x 10^3/ µl)	60.000±22.887[a]	81.909±24.211[a]	175.364±33.454[b]	77.182±22.887[a]	82.455±24.211[a]	191.636±33.454[b]	138.364±22.887[a]	162.818±24.211[a]	184.364±33.454[a]
Total leucocyte count (x 10^3/mm^3)	18.991±2.516 [a]	20.164±4.402 [a]	19.782±4.404 [a]	15.345±2.516 [a]	14.664±4.402 [a]	12.073±4.404 [a]	13.191±2.516 [a]	11.555±4.402 [a]	11.027±4.404 [a]
Monocyte (x 10^3/mm^3)	0.973±0.136 [a]	0.973±0.189 [a]	0.800±0.358 [a]	0.927±0.136 [a]	0.782±0.189 [a]	0.664±0.358 [a]	0.691±0.136 [a]	0.600±0.189 [a]	1.227±0.358 [a]
Lymphocyte (x 10^3/mm^3)	4.627±0.588 [a]	3.827±0.639 [a]	3.573±1.214 [a]	3.809±0.588 [a]	2.936±0.639 [a]	2.455±1.214 [a]	3.073±0.588 [a]	2.364±0.639 [a]	4.527±1.214 [a]
Granulocyte (x 10^3/mm^3)	12.900±2.086 [a]	15.364±3.714 [a]	15.382±5.050 [a]	10.600±2.086 [a]	10.836±3.714 [a]	8.482±5.050 [a]	9.445±2.086 [a]	8.582±3.714 [a]	13.364±5.050 [a]

BT - Before Treatment, AT- After Treatment,

For each parameter, mean with same superscript (small letters in rows) does not vary significantly

Table 4.8. Haematological parameters of *Babesia canis* affected groups before and after treatment

Haematological parameters	Treatment groups					
	Group I (n=10)			Group II (n=10)		
	BT	AT		BT	AT	
		Day 3	Day 14		Day 3	Day 14
Total Erythrocyte count (x 10^6/mm^3)	3.988±0.265 [a]	4.281±0.224 [a]	4.109±0.229 [a]	4.126±0.265 [a]	4.011±0.224 [a]	4.415±0.229 [a]
Haemoglobin (g/dl)	10.930±0.651 [a]	10.990±0.506 [a]	11.470±0.516 [a]	11.330±0.651 [a]	11.260±0.506 [a]	12.050±0.516 [a]
VPRC (per cent)	27.790±1.670 [a]	29.540±1.612 [a]	29.000±1.629 [a]	28.780±1.670 [a]	28.620±1.612 [a]	30.900±1.629 [a]
MCV (fl)	70.000±1.782 [a]	69.270±1.697 [a]	70.830±1.939 [a]	70.540±1.782 [a]	71.320±1.697 [a]	70.230±1.939 [a]
MCH (pg)	27.730±1.229 [a]	25.850±0.825 [b]	28.250±1.052 [a]	27.800±1.229 [a]	28.290±0.825 [a]	27.540±1.052 [a]
MCHC per cent	39.560±1.767 [a]	37.360±1.404 [a]	39.990±1.378 [a]	39.690±1.767 [a]	39.910±1.404 [a]	39.340±1.378 [a]
Platelet count (x 10^3/ µl)	60.000±20.376 [a]	126.200±25.238 [b]	203.800±23.471 [c]	73.300±20.376 [a]	133.600±25.238 [b]	205.300±23.471 [c]
Total leucocyte count (x 10^3/mm^3)	7.790±1.441 [a]	10.560±1.466 [ab]	11.640±1.256 [b]	9.880±1.441 [a]	11.690±1.466 [a]	12.140±1.256 [a]
Monocyte (x 10^3/mm^3)	0.600±0.168 [a]	0.920±0.168 [a]	0.780±0.71 [a]	0.670±0.168 [a]	0.750±0.168 [a]	0.760±0.71 [a]
Lymphocyte (x 10^3/mm^3)	1.710±0.350 [a]	2.900±0.636 [ab]	2.600±0.359 [b]	2.250±0.350 [a]	2.500±0.636 [a]	2.720±0.359 [a]
Granulocyte (x 10^3/mm^3)	5.490±0.972 [a]	7.340±1.076 [ab]	8.260±1.003 [b]	6.960±0.972 [a]	8.430±1.076 [a]	8.630±1.003 [a]

BT - Before Treatment, AT- After Treatment

For each parameter, mean with same superscript (small letters in rows) does not vary significantly

Table 4.4. Haematological parameters of *B. canis* infected dogs

Haematological parameters	Mean Values ± SD		z - value	p – value
	Infected group (n = 34)	Control (n = 10)		
Total Erythrocyte count (x 10^6/mm^3)	3.9750 ± 0.179	4.5320 ± 0.302	1.505 ^{ns}	0.140
Haemoglobin (g/dl)	10.197 ± 0.49	13.07 ± 0.48	3.011**	0.004
VPRC (per cent)	27.888 ± 1.188	32.350 ± 1.45	2.250*	0.030
MCV (fl)	68.385 ± 1.28	72.290 ± 1.73	1.530 ^{ns}	0.134
MCH (pg)	25.671 ± 0.96	29.550 ± 1.41	2.006 ^{ns}	0.051
MCHC (per cent)	37.312 ± 1.166	40.71 ± 1.320	1.492 ^{ns}	0.143
Platelet count (x 10^3/ µl)	102.12 ± 26.35	239.10 ± 27.88	2.677 *	0.011
Total leucocyte count (x 10^3/mm^3)	10.197 ± 1.269	11.780 ± 0.813	1.050 ^{ns}	0.300
Monocyte (x 10^3/mm^3)	0.621 ± 0.0847	0.710 ± 0.0722	0.552 ^{ns}	0.584
Lymphocyte (x 10^3/mm^3)	2.109 ± 0.2943	2.380 ± 0.2529	0.482 ^{ns}	0.633
Granulocyte (x 10^3/mm^3)	7.468 ± 0.9791	8.700 ± 0.7292	1.009 ^{ns}	0.319

**** - Highly Significant (P < 0.01), * - Significant at (P < 0.05), ^{ns} - Non - Significant**

Table 4.5. Haematological parameters of *B. gibsoni* infected dogs

Haematological parameters	Mean Values ± SD		z - value	p – value
	Infected group (n = 109)	Control (n = 10)		
Total Erythrocyte count (x 10^6/mm^3)	3.1545 ± 0.138	4.5320 ± 0.302	4.148 **	0.001
Haemoglobin (g/dl)	7.911 ± 0.348	13.07 ± 0.489	8.591**	0.000
VPRC (per cent)	21.012 ± 0.830	32.350 ± 1.45	6.766**	0.000
MCV (fl)	69.193 ± 1.059	72.290 ± 1.73	1.524 ^{ns}	0.146
MCH (pg)	25.307 ± 0.475	29.550 ± 1.41	2.598 *	0.011
MCHC (per cent)	36.716 ± 0.568	40.71 ± 1.32	2.069 *	0.041
Platelet count (x 10^3/ µl)	115.39 ± 9.537	239.10 ± 27.88	3.777 **	0.000
Total leucocyte count (x 10^3/mm^3)	17.150 ± 0.967	11.780 ± 0.813	4.248 **	0.000
Monocyte (x 10^3/mm^3)	0.931 ± 0.658	0.710 ± 0.0722	1.004 ^{ns}	0.317
Lymphocyte (x 10^3/mm^3)	3.553 ± 0.2325	2.380 ± 0.2529	3.414 **	0.002
Granulocyte (x 10^3/mm^3)	12.543 ± 0.79	8.700 ± 0.7292	3.566 **	0.001

**** - Highly Significant (P < 0.01), * - Significant at (P < 0.05), ^{ns} - Non - Significant**

Table 4.6. Serum biochemical parameters of *B. canis* infected dogs

Serum Biochemical parameters	Mean Values ± SD		z - value	p – value
	Infected group (n = 34)	Control (n = 10)		
Total Bilirubin (mg/dl)	1.3434±0.2238	0.7919±0.1086	2.242*	0.030
Direct Bilirubin (mg/dl)	0.5624±0.1303	0.3679±0.0555	1.373^{ns}	0.177
Indirect Bilirubin (mg/dl)	0.7870±0.1370	0.4240±0.0875	2.233*	0.031
Creatinine (mg/ dl)	1.27 ± 0.064	1.01 ± 0.67	2.058*	0.046
Blood Urea Nitrogen (mg/ dl)	18.74 ± 2.169	14.76 ± 1.60	0.965^{ns}	0.034
Alanine Aminotransferase (U/L)	33.52 ± 3.319	46.75 ± 10.40	1.212 ^{ns}	0.251
Alkaline Phosphatase (U/L)	144.30 ± 19.98	95.16 ± 13.55	1.299 ^{ns}	0.201
Aspartate Aminotransferase (U/L)	40.47 ± 4.88	36.88 ± 4.94	0.380 ^{ns}	0.706
Total protein (g/dl)	6.672 ± 0.183	7.437 ± 0.304	2.027*	0.049
Serum Albumin (g/dl)	2.652 ± 0.985	3.742 ± 0.254	4.798 **	0.000
Serum Globulin (g/dl)	4.019 ± 0.183	3.695 ± 0.241	0.889 ^{ns}	0.379
Albumin: Globulin	0.731± 0.056	1.06 ± 0.113	2.740**	0.009
Glucose (g/ dl)	94.349 ± 3.79	101.63 ± 4.88	0.970 ^{ns}	0.338
Gamma Glutamyl Transferase (U/L)	7.081±1.455	4.141±0.659	1.078 ^{ns}	0.287

**** - Highly Significant (P < 0.01), * - Significant at (P < 0.05), ^{ns} - Non - Significant**

Table 4.7. Serum biochemical parameters of *B. gibsoni* infected dogs

Serum Biochemical parameters	Mean Values ± SD		z - value	p – value
	Infected group (n = 109)	Control (n = 10)		
Total Bilirubin (mg/dl)	2.2896±0.2883	0.7919 ± 0.1086	4.862 **	0.001
Direct Bilirubin (mg/dl)	1.2679±0.21084	0.3679 ± 0.055	4.136**	0.001
Indirect Bilirubin (mg/dl)	1.0199±0.1347	0.4240±0.0875	3.711**	0.001
Creatinine (mg/ dl)	1.65 ± 0.13	1.011 ± 0.067	1. 467^{ns}	0.145
Blood Urea Nitrogen (mg/ dl)	27.26 ± 3.12	14.7 ± 1.60	3.564**	0.001
Alanine Aminotransferase (U/L)	61.16±12.65	46.75 ± 10.400	0.343 ^{ns}	0.733
Alkaline Phosphatase (U/L)	158.49± 10.81	95.16 ± 13.55	3.653**	0.001
Aspartate Aminotransferase (U/L)	54.47±7.67	36.88 ± 4.94	0.690^{ns}	0.492
Total protein (g/dl)	6.75 ± 0.155	7.43 ± 0.304	1.303 ^{ns}	0.195
Serum Albumin (g/dl)	2.53± 0.076	3.74 ± 0.25	4.580 **	0.000
Serum Globulin (g/dl)	4.222±0.149	3.695 ± 0.241	1.856 ^{ns}	0.081
Albumin: Globulin	0.722±0.052	1.064±0.113	1.936 ^{ns}	0.055
Glucose (g/ dl)	87.12±3.38	101.63 ± 4.88	1.282 ^{ns}	0.202
Gamma Glutamyl Transferase (U/L)	8.201±0.878	4.14±0.659	1.806 ^{ns}	0.075

**** - Highly Significant (P < 0.01), * - Significant at (P < 0.05), ^{ns} - Non - Significant**

Table 3.2. List of Medicines for the Therapeutic Study

S.no	GENERIC NAME	TRADE NAME	DOSAGE	PHARMACEUTICAL	QUANTITY	PRICE
1.	Diminazene aceturate	Inj. BERENIL® RTU	70 mg/ml	INTERVET INDIA	20 ml	Rs. 82
2.	Imidocarb dipropionate	Inj. Imicarb	120 mg/ml	SAVAVET	10 ml	Rs. 500
3.	Clindamycin	Dalacin C® Clindamycin Injection USP	150 mg/ml	PFIZER	2ml	Rs. 157
		Dalacin C® Clindamycin Capsules I.P	300 mg	PFIZER	10 capsules	Rs. 220
4.	Doxycycline	Tab. DOXRID (Doxycycline Hyclate)	100 mg	RIDLEY	8 tablets	Rs. 31
		Tab. Dr. DOXY	300 mg	TTK	10 tablets	Rs. 150
5.	Metronidazole	FLAGYL	100 mg /ml	Abbott	100 ml	Rs. 12.60
		Tab. METROGYL®	400 mg	Unique's (J.D Chemicals)	15 tablets	Rs. 11.81

Table 3.1. Genus, species and sub-species specific primers selected for PCR amplification

ORGANISM	PRIMER NAME		GENE SEQUENCE 18S rRNA	PRODUCT SIZE	REFERENCE
Babesia genus	Forward	**PIRO A**	5'-AAT-ACC-CAA-TCC-TGA-CAC-AGG-G -3'	400 bp	de Sa *et al*. (2006)
	Reverse	**PIRO B**	5'-TTA-AAT-ACG-AAT-GCC-CCC-AAC -3'		
Babesia gibsoni	Forward	**PIRO-F**	5'-AGTCATATGCTTGTCTCA- 3'	460 bp	Trap *et al.* (2006)
	Reverse	**PIRO-R**	5'-CCATCATTCCAATTACAA-3'		
Babesia canis	Forward	**Can172F**	5'-GTT-TAT-TAG-TTT-GAA-ACC-CGC 3'	454 bp	Inokuma *et al.* (2004)
	Reverse	**Can626R**	GAA-CTC-GAA-AAA-GCC-AAA-CGA 3'		
Babesia canis vogeli	Forward	**BAB1**	5'-GTG AAC CTT ATC ACT TAA AGG-3'	590 bp	Duarte *et al*. (2008)
	Reverse	**BAB4**	5'-CAA CTC CTC CAC GCA ATC G-3'		
Babesia canis canis	Forward	**BAB1**	5'-GTG AAC CTT ATC ACT TAAAGG-3'	746 bp	Duarte *et al*. (2008)
	Reverse	**BAB3**	5'-CTA CAC AGA GCA CAC AGC C-3'		
Babesia canis rossi	Forward	**BAB1**	5'-GTG AAC CTT ATC ACT TAAAGG-3'	342 bp	Duarte *et al*. (2008)
	Reverse	**BAB5**	5'-AGG AGT TGC TTA CGC ACT CA-3'		

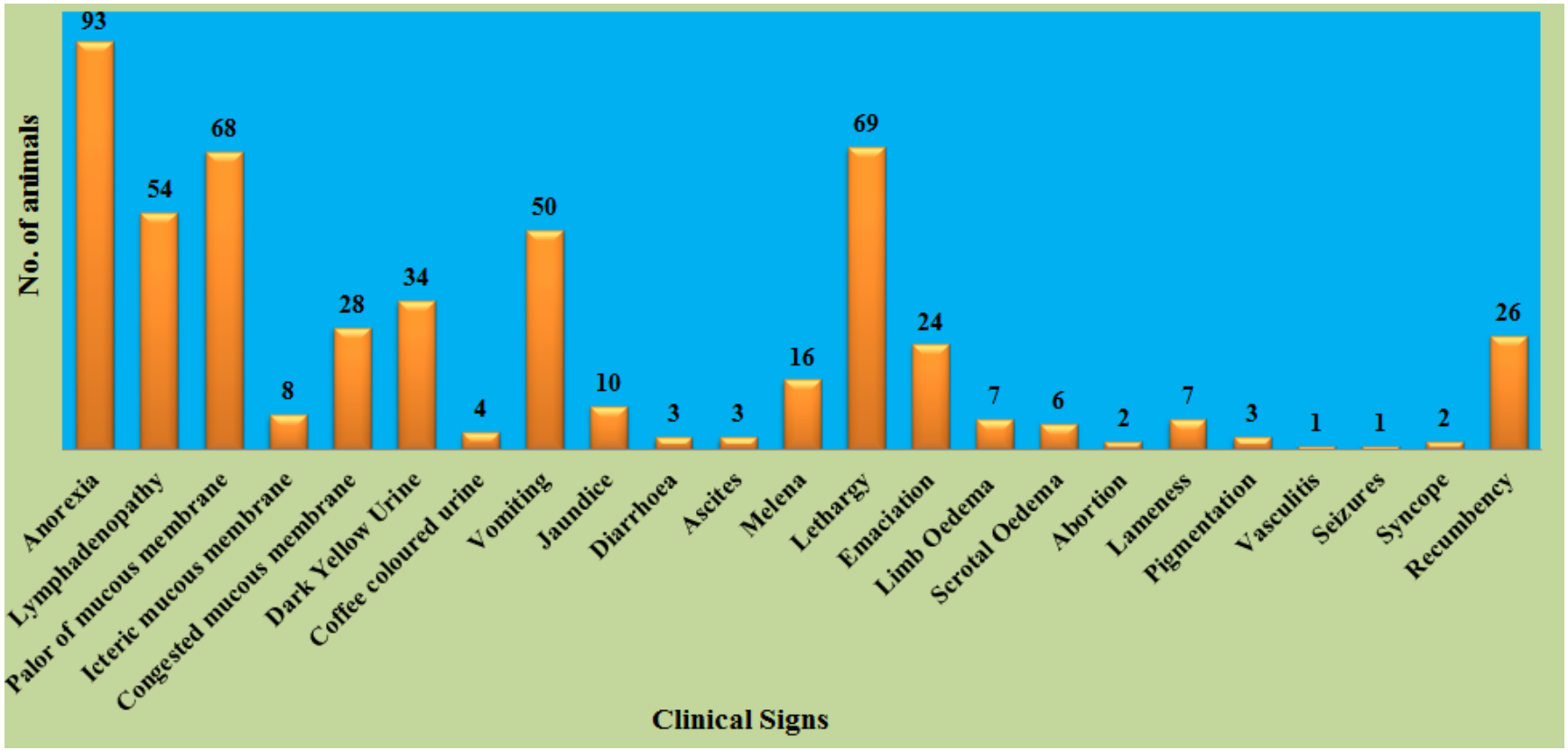


Fig. 3: Variable clinical manifestations of *Babesia gibsoni* affected dogs

3. 1. 5. Haematological analysis

An Automatic Haematology Analyzer (Orphee, Mythic Vet 18) was used to assess the complete blood count.

3. 1. 6. Serum biochemical analysis

Diagnostic kits to estimate serum levels of bilirubin total, bilirubin direct and gamma glutamyl transferase (SPINREACT) were procured. Diagnostic kits to estimate serum levels of alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, creatinine, blood urea nitrogen, albumin, total protein and glucose were procured from Cormay group. To check blood glucose levels a blood glucose meter (Safe - Accu) procured from SINOCARE (Changsha) was utilized.

3. 1. 7. Treatment

Anti-protozoal drugs and antibiotics were used for the treatment of positive cases, which were procured from various pharmaceutical companies as given in Table 3.2.

3. 1. 8. Ultrasonography

Ultrasound scanner E saote MyLab was used for the sonographic examination of 20 dogs with *B. canis* infection and 33 dogs with *B. gibsoni* infection.

3. 2. METHODS

3. 2. 1. Screening of Dogs for Canine Babesiosis

3. 2. 1. 1. Collection of samples

Thin peripheral blood smears were prepared on two slides, air-dried and fixed in methanol. About six millilitre of blood was collected aseptically from either the cephalic vein or the saphenous vein and two millilitre was subsequently transferred to a sterile EDTA coated vial and the remaining four millilitre was transferred to a sterile vial with clot activator.

3. 2. 1. 2. Staining of blood smears

The blood smears were stained following the standard Giemsa's/ Field's staining method and 50 samples were subjected to acridine orange staining technique.

3. 2. 1. 2. 1. Giemsa Staining Technique

One set of the methanol fixed smears were stained using 1: 7 dilution of Giemsa stain, for 20 minutes. Stained blood smears were examined under oil immersion objective of a light microscope (Olympus, CH20i and Leica, DM 500).

3. 2. 1. 2. 2. Field's Staining Technique

Two coupling jars were filled with Field's stain A and B solutions separately. Peripheral blood smears were fixed in methanol for 45 sec to one min and air dried. This was followed by flooding of stain B and allowing it to act for 30 seconds. After washing the slides in running tap water, stain A was added and allowed to act for 30 seconds. The slides were further washed, air dried and observed under 100X objective of a light microscope.

3. 2. 1. 2. 3. Staining by Acridine Orange

Methanol fixed smears were flooded with acridine orange stain solution for two minutes and then rinsed under tap water. A cover slip was placed over the slide and examined under 100 X objective of a fluorescent microscope (Olympus CX 41 Fluorescent Microscope)

3. 2. 1. 3. Polymerase Chain Reaction

Whole blood was collected aseptically in EDTA containing vacutainers and stored at -4ºC initially and then transferred to -20ºC until used for DNA extraction.

3. 2. 1. 3. 1. Protocol for Genomic DNA Extraction

The DNA was extracted from the stored EDTA added whole blood with the Qiagen DNeasy Blood and Tissue Kit according to the manufacturer's protocol which was as follows:

1) Twenty microlitre of proteinase K was pipetted into a microcentrifuge tube. 100µl of anticoagulated blood was added and the volume was adjusted to 220 µl by adding 100µl PBS.
2) Two hundred microlitre of Buffer AL (without added ethanol) was added to the sample, mixed thoroughly by pulse vortexing for 10 sec and then incubated at 56ºC for 10 min.
3) Two hundred microlitre of absolute ethanol was added to the sample and mixed thoroughly by pulse vortexing for 10 sec.
4) The mixture from step three was pipetted into a DNeasy Mini spin column placed in a two ml collection tube provided with the kit and centrifuged at 8,000 rpm for one minute. The spin column was retained and the flow-through with collection tube was discarded.
5) The DNeasy Mini spin column was placed in a new two millilitre collection tube provided with the kit. Five hundred microlitre of Buffer AW1 was added and centrifuged for one minute at 8,000 rpm. The spin column was retained and the flow- through with collection tube was discarded.
6) The DNeasy Mini spin column was placed in a new two millilitre collection tube provided with the kit. Five hundred microlitre of Buffer AW2 was added and centrifuged for three minutes at 14,000 rpm to dry the DNeasy membrane. The spin column was retained and the flow-through with collection tube was discarded.
7) The DNeasy Mini spin column was placed in a clean 1.5 ml microcentrifuge tube. Two hundred microlitre of Buffer AE was pipetted directly onto the

DNeasy membrane. The solution was incubated at room temperature for one minute and then centrifuged for one minute at 8,000 rpm for elution.

8) The extracted DNA was stored at -20ºC until use.

3. 2. 1. 3. 2. DNA Amplification

Extracted DNA (5µl) was used as a template to amplify a fragment of the 18S rRNA gene using the following reaction mix.

Sl. No	**REAGENTS**	**VOLUME**
1.	Nuclease Free Water (NFW)	11.25 µl
2.	$MgCl_2$ solution (25 mM)	3 µl
3.	dNTP mix (2.5 mM each)	0.75 µl
4.	Forward Primer (25 pmol/µl)	1 µl
5.	Reverse Primer (25 pmol/µl)	1 µl
6.	Taq Buffer (10 X)	2.5 µl
7.	Taq DNA Polymerase (3U/µl)	0.5 µl
8.	Template	5 µl
TOTAL VOLUME		25 µl

A master mix containing all the above constituents except template DNA and DNA polymerase was freshly prepared every time. Template DNA was added after the master mix was distributed to individually marked 0.2 ml PCR tubes. Contents were mixed by spinning to collect the constituents at the bottom. The PCR programmes were carried out as in Table 3.3.

3. 2. 1. 3. 3. Preparation and casting of 1.2 per cent agarose gel

1. Weighed out 0.48 g of agarose powder (Agarose low EEO, Sisco Research Laboratories Pvt. Ltd.) in a beaker.

2. Added 40 ml of 1X TBE buffer to the beaker.
3. The solution was heated in a microwave oven until a clear, homogenous solution was formed and no suspended particles could be visualized.
4. Allowed to cool to a bearable handling temperature.
5. Three microlitre of ethidium bromide solution (10 mg/ml in distilled water) (Sigma) was added and mixed well.
6. The solution was then poured into a casting tray (BIO-RAD) fitted with a gel comb and allowed to solidify for about 30 minutes.
7. The comb was removed after solidification and the casting tray with gel was placed in an electrophoresis tank (BIO-RAD).

3. 2. 1. 3. 4. Loading of PCR products and electrophoresis

Five microlitre of the PCR product were mixed with one microliter of gel loading dye (6X DNA Loading Dye, Thermo Scientific) and electrophoresed on 1.2 per cent agarose gel, at 70 Volts for 60 minutes (BIO-RAD). One microlitre of O'GeneRuler 100 bp DNA Ladder (Thermo Scientific) was added along with each set of samples.

Subsequent to electrophoresis, the gel was transferred to a UV transilluminator (GeNei^{TM}) for visualization of bands. The gel was analysed in Gel Documentation System (BIO-RAD, USA).

8. Table 3.3. PCR programmes for *Babesia* spp. DNA amplification

Amplification of 400 bp fragment of 18S rRNA gene of *Babesia*	**PCR Programme**	**TEMPERATURE (°C)**	**TIME**	**CYCLES**
	Initial denaturation	94	5 min	
	Denaturation	94	1 min	30 cycles
	Annealing	55	1 min	
	Extension	72	1 min	
	Final extension	72	5 min	
Amplification of 460 bp fragment of 18S rDNA sequence of the *B. gibsoni*	**PCR Programme**	**TEMPERATURE (°C)**	**TIME**	**CYCLES**
	Initial denaturation	95	10 min	
	Denaturation	95	45 sec	40 cycles
	Annealing	52	45 sec	
	Extension	72	1 min	
	Final extension	72	10 min	
Amplification of 454 bp fragment of 18S rDNA sequence of the *B. canis*	**PCR Programme** Ulutas *et al*. (2005)	**TEMPERATURE (°C)**	**TIME**	**CYCLES**
	Initial denaturation	95	5 min	
	Denaturation	95	30 sec	40 cycles
	Annealing	55	30 sec	
	Extension	72	90 sec	
	Final extension	72	10 min	
Amplification of 590 bp fragment of 18S rDNA sequence of the *B. canis vogeli*	**PCR programme**	**TEMPERATURE (°C)**	**TIME**	**CYCLES**
	Initial denaturation	94	2 min	
	Denaturation	94	30 sec	35 cycles
	Annealing	56	30 sec	
	Extension	72	1 min	
	Final extension	72	6 min	
Amplification of 746 bp fragment of 18S rDNA sequence of the *B. canis canis*	**PCR programme**	**TEMPERATURE (°C)**	**TIME**	**CYCLES**
	Initial denaturation	94	2 min	
	Denaturation	94	30 sec	35 cycles
	Annealing	56	30 sec	
	Extension	72	1 min	
	Final extension	72	6 min	
Amplification of 342 bp	**PCR programme**	**TEMPERATURE (°C)**	**TIME**	**CYCLES**

fragment of 18S rDNA sequence of the *B. canis rossi*	Initial denaturation	94	2 min	
	Denaturation	94	30 sec	35 cycles
	Annealing	56	30 sec	
	Extension	72	1 min	
	Final extension	72	6 min	

3. 2. 1. 4. Sequencing and procurement of Accession Number

DNA nucleotide sequence determination was carried out by the Sequencing Service, Sci-genome, Ernakulam with the same primers that were used for amplification. Sequences were edited and aligned by using Emboss Merger Website and global sequence comparisons were performed by using basic local alignment search tool (BLAST) hosted by the National Center for Biotechnology Information (NCBI). New sequences were submitted using the Sequin software (Version 15.50) and deposited in the Gen Bank database under the procured accession numbers.

3. 2. 2. Complete Blood Count

Two millilitre of blood collected in EDTA vials from infected and control groups were analyzed within half an hour of collection.

3. 2. 3. Serum Biochemistry

Serum from dogs of infected and control groups were separated and stored in sterile microcentrifuge tubes at - 20^{o}C. Serum biochemical parameters were estimated using commercial kits supplied by Cormay Group and Spinreact company in a semi-automatic analyser (Erba Manheim, Chem-5 Plus v2) (Table 3.4). The serum albumin values were subtracted from the corresponding values obtained for total protein in order to estimate the globulin value. The ratio of albumin: globulin (A/G) was estimated from the values obtained for the respective biochemical parameters, i.e., serum albumin and globulin.

3. 2. 5. Treatment

3. 2. 5. 1. Babesia canis

The dogs diagnosed with *B. canis* were divided into two groups and were given the treatment as follows:

GROUP I (n = 10)		
DRUG	**DOSE AND ROUTE**	**DURATION**
Inj. Diminazene aceturate (Berenil ^{RTU})	3.5mg/kg body weight I/M	Single dose

GROUP II (n = 10)		
DRUG	**DOSE AND ROUTE**	**DURATION**
Inj. Imidocarb (Imicarb)	6 mg/kg body weight S/C	Two doses with a two weeks interval

3. 2. 5. 2. Babesia gibsoni

The dogs with *B. gibsoni* were divided into three groups and were given the treatment as follows:

GROUP I (n = 11)		
DRUG	**DOSE AND ROUTE**	**DURATION**
Inj. Diminazene aceturate (Berenil ^{RTU})	3.5 mg/kg body weight I/M	Day one (single dose)
Inj. Imidocarb (Imicarb)	6 mg/kg body weight S/C	Day two (single dose)
Tab. Clindamycin (Dalacin)	30 mg/kg body weight, P.O.	q12h for two weeks

GROUP II (n = 11)		
DRUG	**DOSE AND ROUTE**	**DURATION**
Inj. Clindamycin (Dalacin)	11 mg/kg body weight I/V or P.O.	Once daily for ten days
Inj. Metronidazole (Flagyl)	25 mg/kg body weight I/V or P.O.	Once daily for ten days
Tab. Doxycycline (Doxrid/ Dr. DOXY)	10 mg/kg body weight P.O.	Once daily for ten days

GROUP III (n = 11)		
DRUG	**DOSE AND ROUTE**	**DURATION**
Inj. Imidocarb (Imicarb)	6 mg/kg body weight S/C	Single dose

3. 2. 6. Ultrasonography

Ultrasonography was performed before and after treatment to evaluate the liver and spleen. Abdominal area was shaved and acoustic gel was applied on the abdomen before sonogram to ensure an intimate contact of the scan head with the body surface. Sonograms were evaluated for liver size, shape, contour and its internal architecture. The spleen was visualized for its size, echogenicity and shape. Any kind of organ abnormalities in kidney and liver were also analysed.

3. 2. 7. Evaluation of Treatment

Parasitological examination by microscopy was performed on days three and fourteen to evaluate the treatment outcome. The parasites were identified on the basis of characteristic morphology. Blood samples were declared as microscopically negative if there was failure to detect parasite in the smear after evaluating at least 20 different oil immersion fields for about 20-30 mins time.

Haematology was performed on day three and day fourteen of initiating treatment to evaluate treatment response. Therapeutic outcomes were also evaluated based on the regression of symptoms. Recurrence of disease was also recovered.

3. 2. 8. Data handling and statistical analysis

Data were entered into a Microsoft Excel spreadsheet, verified for correctness, and imported into IBM-SPSS software for statistical analysis and graphing.

The results were analyzed statistically as per the principles of Snedecor and Cochran (1985).

A repeated measures ANOVA was performed to compare hematological parameters between and within treatment groups.

Z - test was performed to statistically analyze the hemato-biochemical parameters.

4. RESULTS

A total of 200 dogs brought to the Kerala Veterinary and Animal Sciences University, Veterinary Hospitals at Mannuthy and Kokkalai, and hospitals attached to the Department of Animal Husbandry in Thrissur district with signs suggestive of blood parasite infection during the period from April 2016 to March 2017 were included in the study.

4. 1. SCREENING FOR BABESIOSIS

4. 1. 1. Microscopic Examination of Blood smear

4. 1. 1. 1. Giemsa's Staining / Field Staining

Single and multiple forms of *Babesia gibsoni* organisms with a signet-ring shaped appearance were observed inside the erythrocytes in 112 cases (56 per cent) (Plate 1).

Examination of Giemsa/ Field stained blood smears revealed intra-erythrocytic pear-shaped parasites, in pairs typical of *B. canis* in 38 cases (19 per cent) (Plate 2). Sometimes pleomorphic amoeboid forms were also observed.

Concurrent infections with other blood parasites were detected during peripheral blood smear examination. Mixed infection with *B. gibsoni* and *E. canis* was diagnosed in four dogs, whereas five dogs with *B. gibsoni* had concurrent infection with haemotropic mycoplasma. Similarly four dogs had *E. canis* as a concurrent infection among the *B. canis* infected dogs.

4. 1. 1. 2. Acridine Orange Staining

Acridine orange staining of blood smears from 50 positive dogs revealed the presence of apple green coloured pear shaped piroplasms of *B. canis* in 12 samples (Plate 3) and signet ring shaped *B. gibsoni* piroplasms in 38 samples (Plate 4).

4. 1. 2. Polymerase Chain Reaction

The DNA extracted from blood samples of 200 dogs screened for babesiosis were also subjected to genus specific PCR for molecular identification of the organism. One hundred and fifty six samples positive by genus specific PCR for *Babesia* spp. were further subjected to species and sub-species specific PCR for molecular confirmation of the organism.

4. 1. 2. 1. Extraction of Genomic DNA

The genomic DNA was extracted from 200 blood samples of dogs that were screened for babesiosis using the High Pure PCR Template preparation kit (Qiagen DNeasy Blood and Tissue kit). The DNA concentration for the 200 samples were checked using Nanodrop Spectrophotometer 2000C and was within the concentration range of 13 to 18.5 ng/µl.

4. 1. 2. 2. Polymerase Chain Reaction Assay

The *Babesia* genus specific PCR with primer pair PIRO A and PIRO B yielded a specific PCR product of 400 bp from 156 samples (Plate 5). No bands were observed with negative control.

The *B. canis* species specific PCR with primer pair Can172F and Can626R yielded a specific PCR product of 454 bp from 38 samples (Plate 6). No bands were observed with negative control.

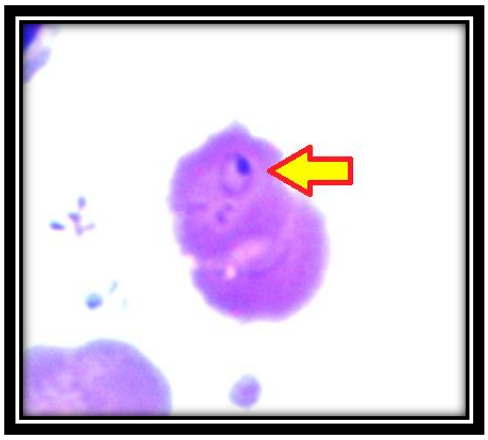


Plate 1. Signet ring shaped *Babesia gibsoni* within the erythrocyte (Giemsa staining X1000)

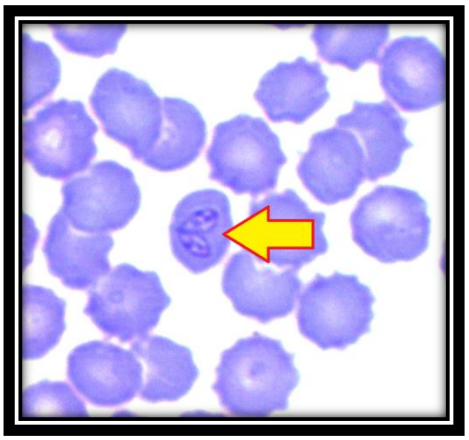


Plate 2. Pear shaped *Babesia canis* in pairs within the erythrocyte (Giemsa staining X1000)

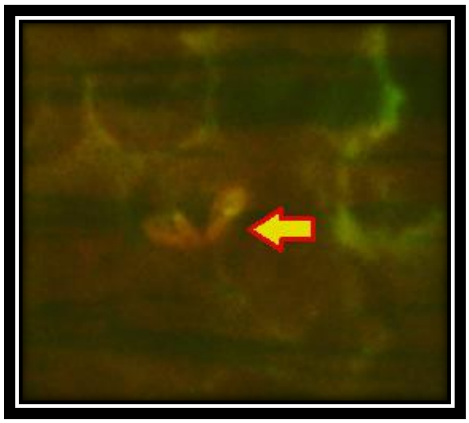


Plate 3. Pear shaped *Babesia canis* in pairs within the erythrocyte with apple green fluorescence (Acridine Orange staining X1000)

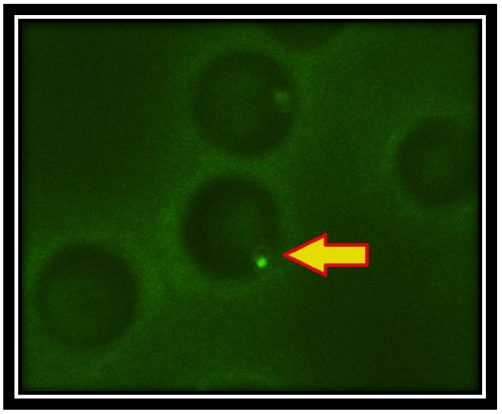


Plate 4. Signet ring shaped *Babesia gibsoni* within the erythrocyte with apple green fluorescence (Acridine Orange staining X1000)

The *B. canis vogeli* sub-species specific PCR with primer pair BAB 1 and BAB 4 yielded a specific PCR product of 590 bp from 38 samples. The result of the amplification is shown in Plate 7. No bands were observed with negative control.

The *B. canis canis* and *B. canis rossi* sub-species specific PCR with primer pair BAB 1 and BAB 3, and BAB 1 and BAB 5 did not yield amplicons of 746 bp and 342 bp suggestive of *B. canis canis* and *B. canis rossi* respectively, from any of the samples.

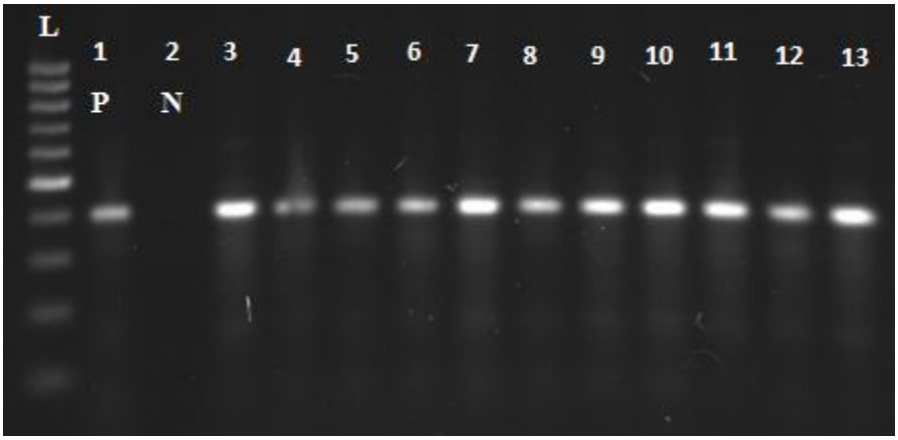
The *B. gibsoni* species specific PCR with primer pair PIRO F and PIRO R yielded a specific PCR product of 460 bp from 118 samples (Plate 8). No bands were observed with negative control.

A BLAST search against GenBank revealed the highest similarity score (99 per cent) with a *Babesia gibsoni* genotype Asia 2 small subunit ribosomal RNA gene, partial 1646 bp long sequence accession No. AF175301. The BLAST analysis also confirmed 99 per cent similarity of the sequences of *B. gibsoni* in Genbank database (India-accession No. KF171471 and Japan-AB478328.1). The nucleotide sequences isolated from the two dogs are available in GenBank database (accession numbers: *Babesia gibsoni*, MF409016/460 bp (Plate 9); *Babesia gibsoni*, MF409017/460 bp) (Plate 10).

The BLAST analysis confirmed 100 per cent similarity of the sequences of *B. canis vogeli* with that available in GenBank database (France-KF953983 and Tunisia-KT445940).

Comparison between staining technique and PCR are depicted in Fig. 1.

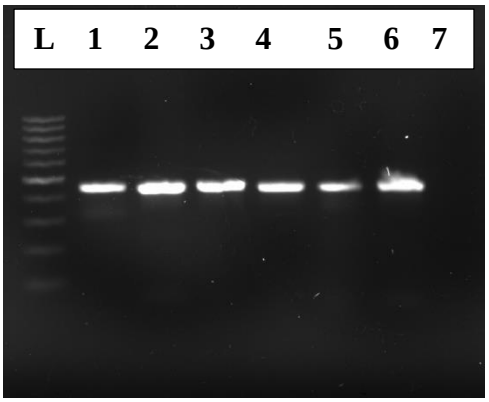
Plate 5. Agarose gel electrophoresis of PCR products of *Babesia* spp.



≈ 400 bp

L - 100 bp DNA ladder
Lane 1 - Positive Control
Lane 2 - Negative Control
Lane 3 to 13 - Positive samples of *Babesia* spp.

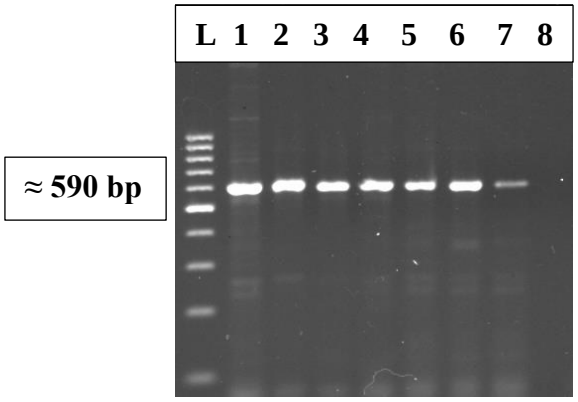
Plate 6. Agarose gel electrophoresis of PCR products of *Babesia canis*



≈ 454 bp

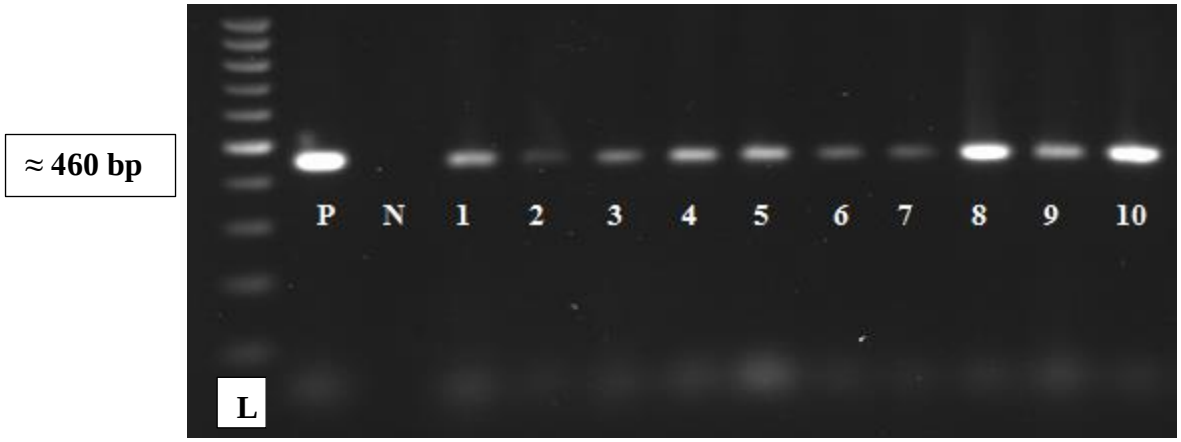
L - 100 bp DNA ladder
Lane 6 - Positive Control
Lane 7 - Negative Control
Lane 1 to 5 - Positive samples of *Babesia canis*

Plate 7. Agarose gel electrophoresis of PCR products of *Babesia canis vogeli*

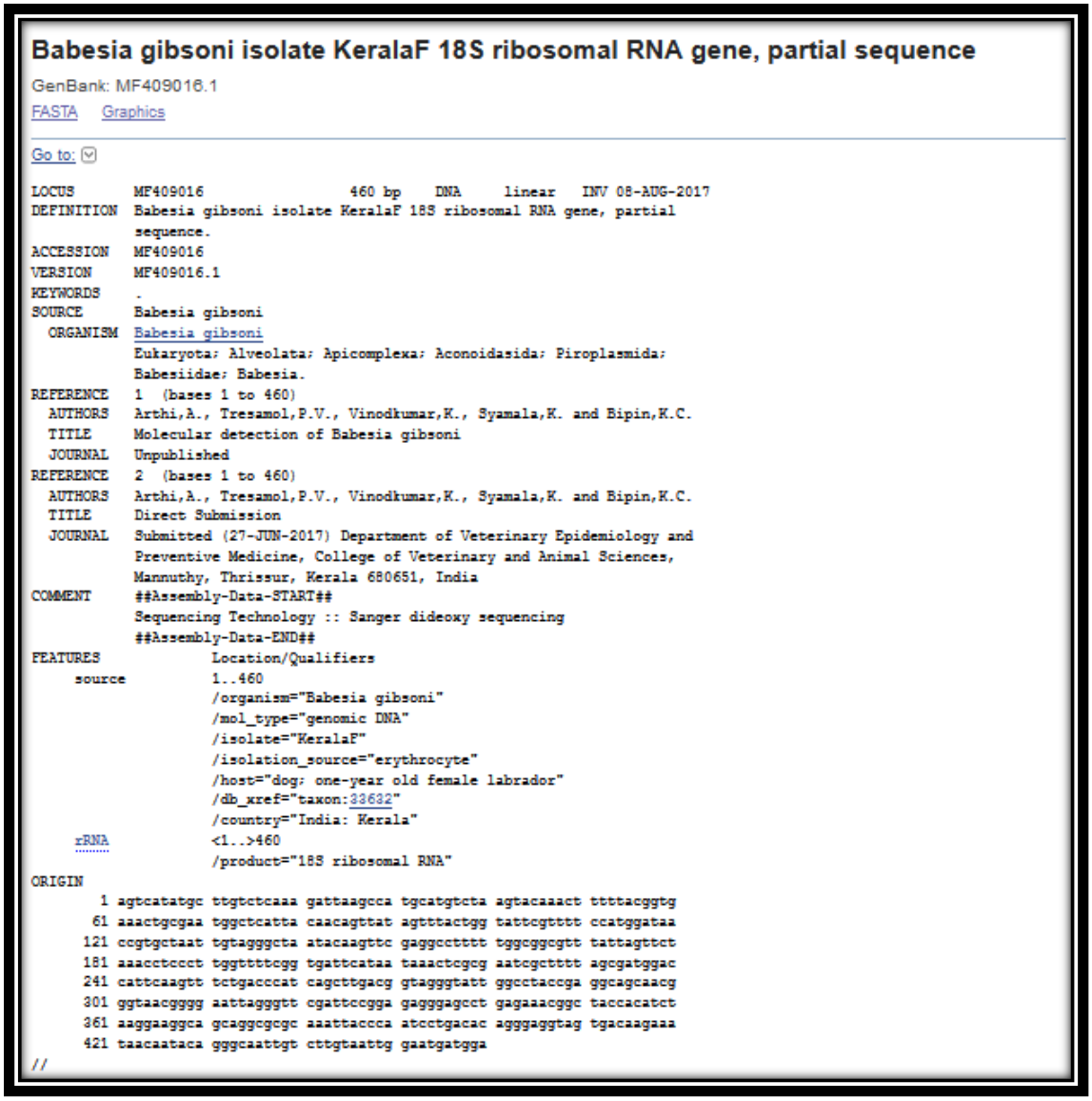


L - 100 bp DNA ladder
Lane 1 - Positive Control
Lane 8 - Negative Control
Lane 2 to 7 - Positive samples of *Babesia canis vogeli*

Plate 8. Agarose gel electrophoresis of PCR products of *Babesia gibsoni*



L - 100 bp DNA ladder
P - Positive Control
N - Negative Control
Lane 1 to 10 - Positive samples of *Babesia gibsoni*



Babesia gibsoni isolate KeralaF 18S ribosomal RNA gene, partial sequence

GenBank: MF409016.1

FASTA Graphics

Go to:

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  TITLE     Molecular detection of Babesia gibsoni
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 460)
  AUTHORS   Arthi,A., Tresamol,P.V., Vinodkumar,K., Syamala,K. and Bipin,K.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (27-JUN-2017) Department of Veterinary Epidemiology and
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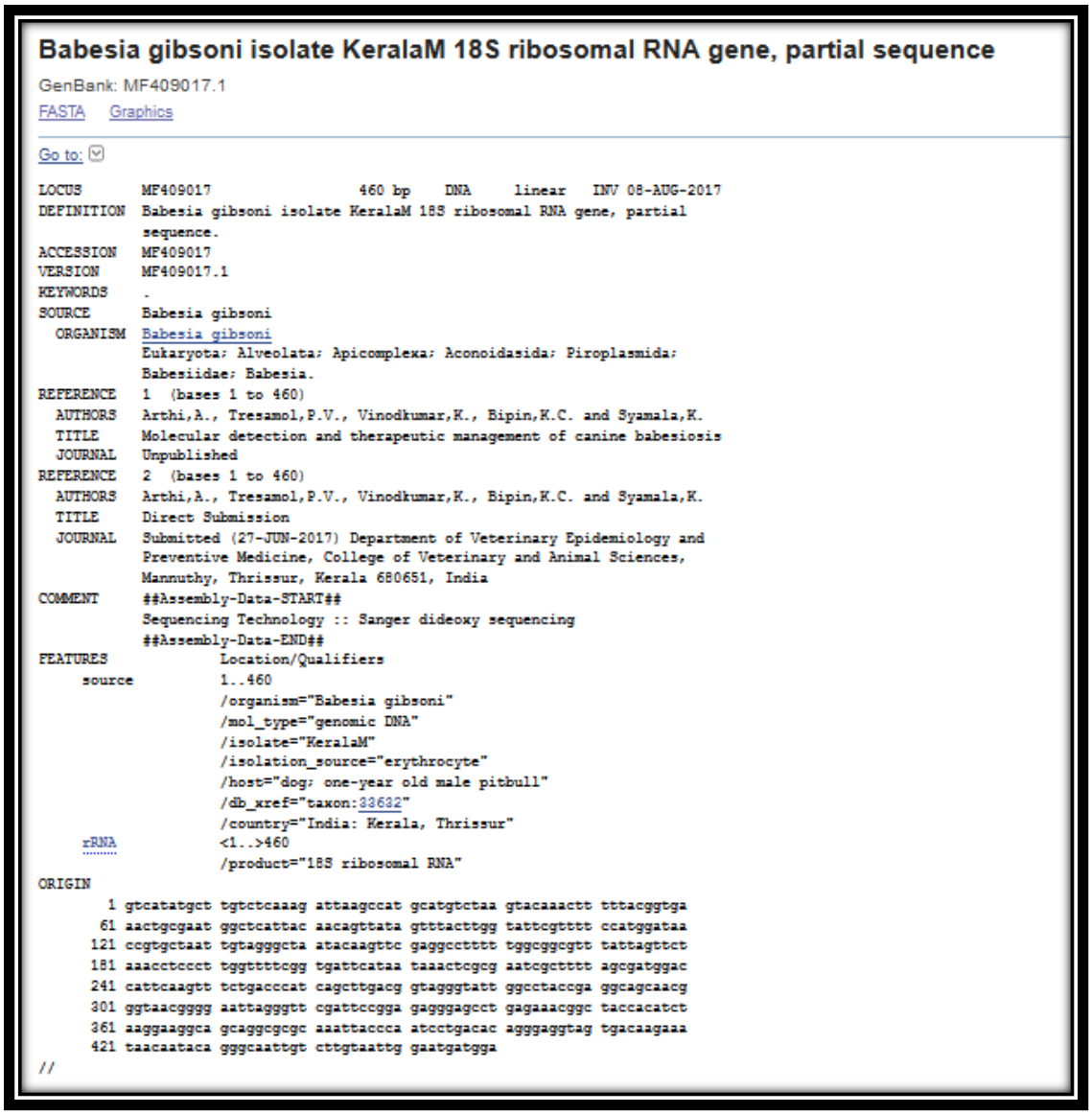
Plate 9. Accession Number - MF409016 for *Babesia gibsoni* isolate in GenBank database



Babesia gibsoni isolate KeralaM 18S ribosomal RNA gene, partial sequence

GenBank: MF409017.1

FASTA Graphics

Go to:

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  AUTHORS   Arthi,A., Tresamol,P.V., Vinodkumar,K., Bipin,K.C. and Syamala,K.
  TITLE     Molecular detection and therapeutic management of canine babesiosis
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 460)
  AUTHORS   Arthi,A., Tresamol,P.V., Vinodkumar,K., Bipin,K.C. and Syamala,K.
  TITLE     Direct Submission
  JOURNAL   Submitted (27-JUN-2017) Department of Veterinary Epidemiology and
            Preventive Medicine, College of Veterinary and Animal Sciences,
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      361 aaggaaggca gcaggcgcgc aaatttaccca atcctgacac agggaggtag tgacaagaaa
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Plate 10. Accession Number - MF409017 for *Babesia gibsoni* isolate in GenBank database

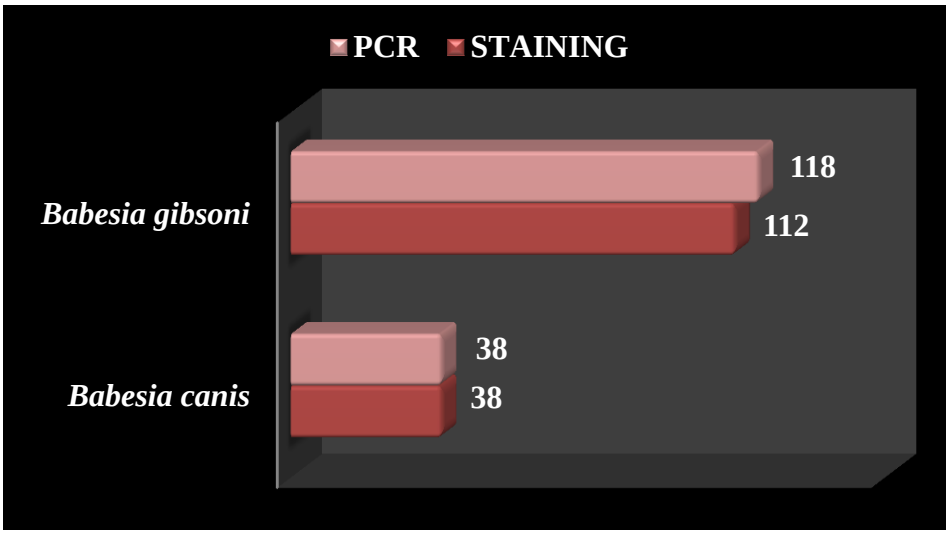


Fig. 1: Comparison of results of PCR and blood smear examination

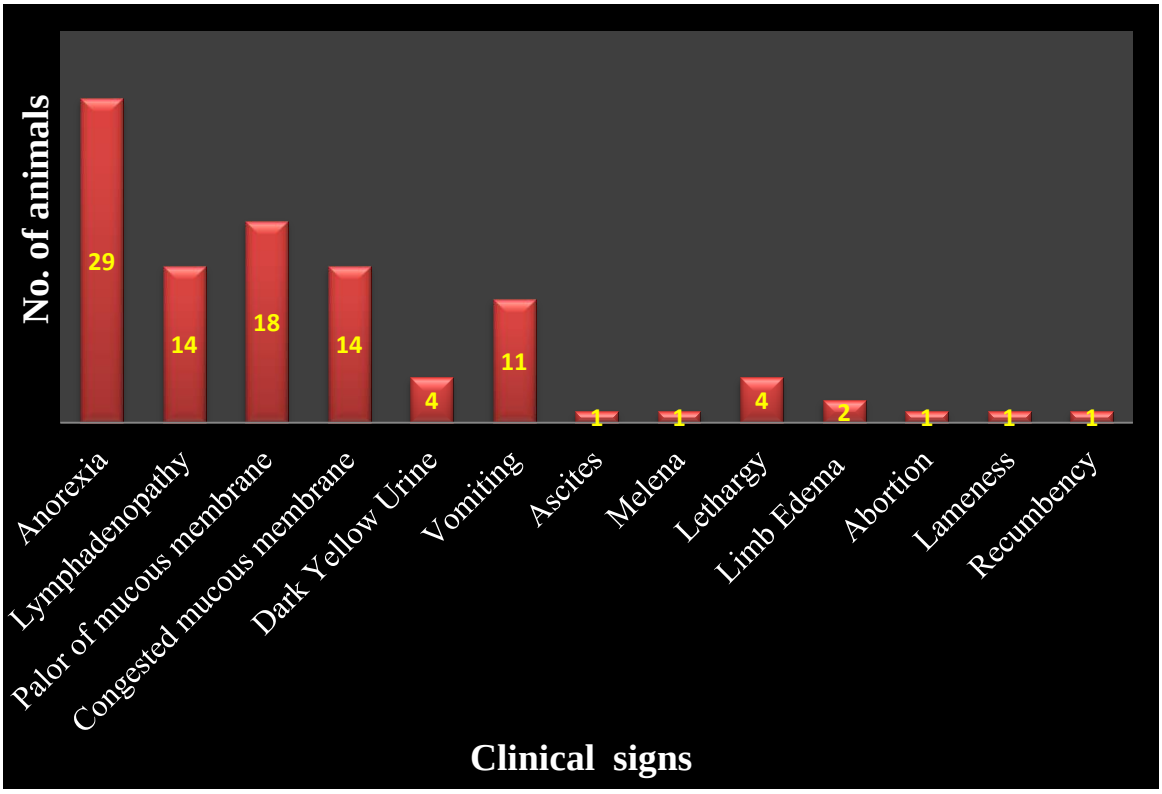


Fig. 2: Variable clinical manifestations of *Babesia canis* affected dogs

4. 2. CLINICAL SIGNS

The varied clinical manifestations of *B. canis* infected animals are depicted in Table 4.1 and Fig. 2. On analyzing clinical manifestations of positive cases, the most common signs exhibited by the *B. canis* infected animals were pyrexia, anorexia, followed by pallor of mucous membrane, congestion of mucous membranes and lymphadenopathy (Plate 11). Gastrointestinal disturbances like vomiting and melena were observed in 11 and one animal respectively. Marked lethargy and voiding of dark yellow urine was also noticed in four animals. Haemoglobinuria or voiding of coffee coloured urine and jaundice were not reported in any of the cases. Limb oedema was observed in two animals. Unusual signs like ascites, abortion, lameness and recumbency were reported in one animal each.

The varied clinical manifestations of *B. gibsoni* infected animals are depicted in Table 4.1 and Fig. 3. On analyzing clinical manifestation of positive cases, the most common signs exhibited by the *B. gibsoni* infected animals were pyrexia, anorexia, lethargy, pallor of mucous membrane (Plate 12 and Plate 13) and lymphadenopathy. Gastrointestinal disturbances like vomiting, diarrhoea and melena were observed in 69 animals. Icteric sclera (Plate 14) / mucous membranes (Plate 15, 16 and 17) and visible yellow pigmentation of skin (jaundice) were reported in eight and ten cases respectively. Voiding of dark yellow urine was also noticed in thirty four animals (Plate 18). Haemoglobinuria or voiding of coffee coloured urine was reported in four cases. Emaciation (Plate 19) or poor body condition was noted in 24 dogs. Unusual signs like syncope and abortion (Plate 20) were reported in two animals each. Ascites was observed in three animals (Plate 21). Oedema of limb (Plate 22) and scrotum (Plate 23) was observed in seven and six animals respectively. Seizures (Plate 24) and vasculitis (Plate 25) were also reported in one animal each.

Table 4.1. Number of animals with different clinical signs of *B. gibsoni* and *B. canis* infection

CLINICAL SIGNS	***Babesia canis***	***Babesia gibsoni***
Pyrexia	19	44
Anorexia	29	93
Lymphadenopathy	14	54
Pallor of mucous membrane	18	68
Icteric mucous membrane	0	8
Congested mucous membrane	14	28
Dark Yellow Urine	4	34
Haemoglobinuria/ Coffee coloured urine	0	4
Vomiting	11	50
Jaundice	0	10
Diarrhoea	0	3
Ascites	1	3
Melena	1	16
Lethargy	4	69
Emaciation	0	24
Limb Oedema	2	7
Scrotal Oedema	0	6
Abortion	1	2
Lameness	1	7
Pigmentation	0	3
Vasculitis	0	1
Seizures	0	1
Syncope	0	2
Recumbency	1	26

Lameness was reported in seven animals. Twenty six animals were recumbent when presented.

Babesiosis was recorded in puppies (five affected with *B. gibsoni* and three affected with *B. canis*), bitches in estrous (four affected with *B. gibsoni*) and lactating dams (five affected with *B. gibsoni* and three affected with *B. canis*). One *B. canis* and two *B. gibsoni* infected dogs were presented with a history of abortion.

4. 3. ECTOPARASITES

Presence of ectoparasites was reported in 92 cases. Out of these, 23 cases were reported with a past history of tick infestation and 69 cases had visible ticks at the time of clinical examination. In 15 cases severe tick infestation was noticed (Plate 26 and Plate 27). The ticks were identified as *Rhipicephalus* spp. and *Haemaphysalis* spp. Presence of fleas was reported in 11 cases.

4. 4. HISTORY OF PREVIOUS ILLNESS

Out of the 38 *B. canis* cases studied, one dog had a previous history of babesiosis due to *B. gibsoni* and the remaining 37 were affected for the first time. Out of the 118 *B. gibsoni* cases studied, one dog had a previous history of babesiosis due to *B. canis*.

4. 5. ULTRASONOGRAPHIC OBERVATIONS

4. 5. 1. *Babesia canis* affected dogs

The sonographic evaluation of liver, gall bladder, spleen and kidneys of 20 *Babesia canis* affected dogs on the day of presentation is depicted in Table 4.2. Mild to moderately enlarged spleen characterized by diffuse hypoechoic parenchyma (Plate 28) was found in 16 dogs affected with *B. canis.* Mild to moderate hepatomegaly was identified in five dogs (Plate 29). The sonographic evaluation of

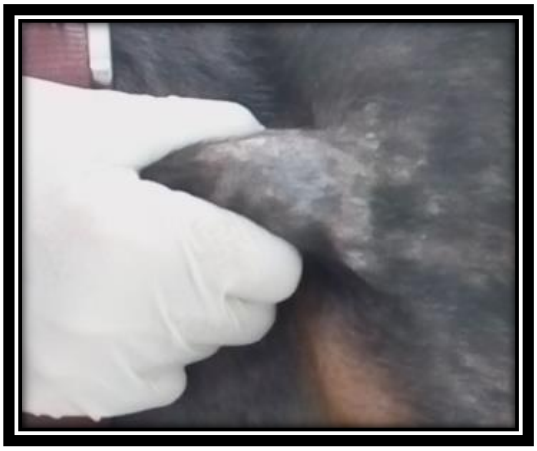
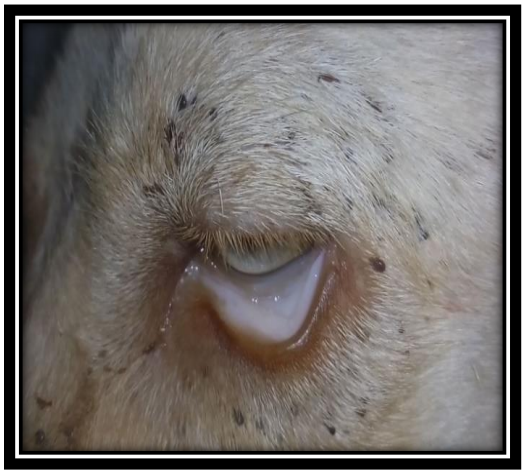


Plate 11. Lymphadenopathy in a *Babesia canis* infected dog



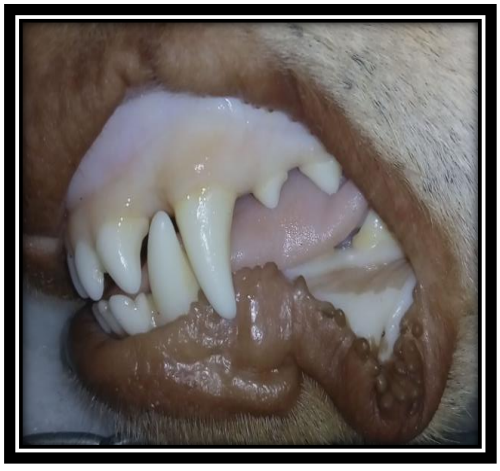


Plate 12. Pallor of conjunctival mucous membrane in a *Babesia gibsoni* infected dog

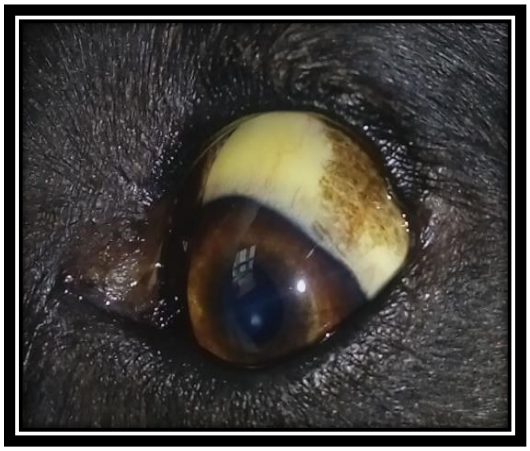


Plate 13. Pallor of oral mucous membrane in a *Babesia gibsoni* infected dog

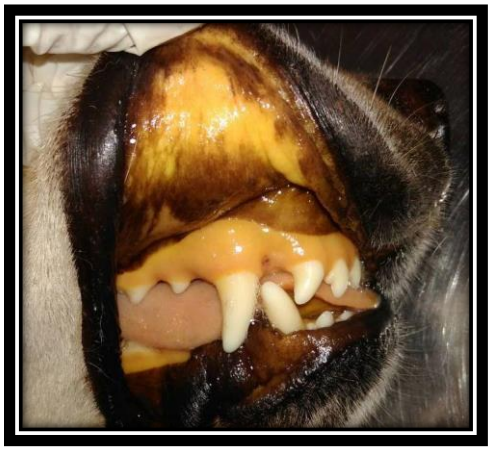


Plate 14. Icteric sclera in a *Babesia gibsoni* infected dog

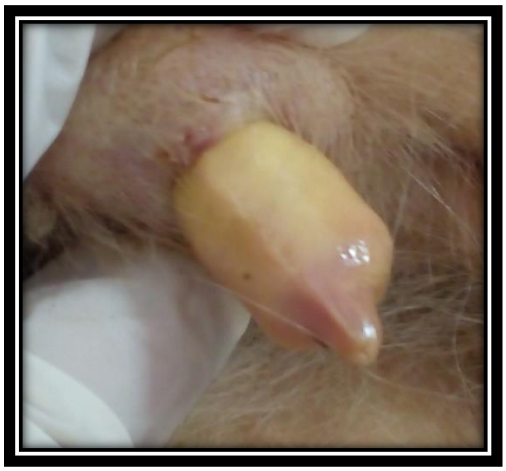


Plate 15. Icteric oral mucosa in a *Babesia gibsoni* infected dog

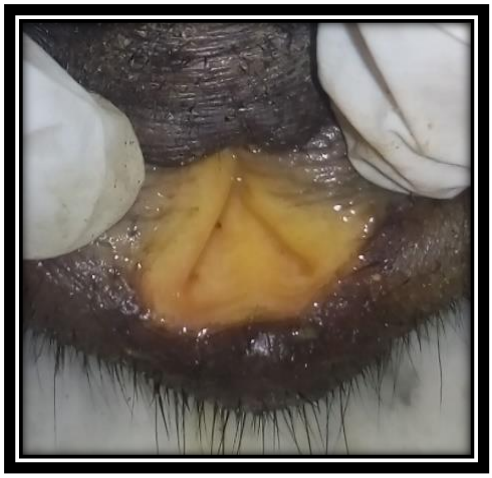


Plate 16. Icteric penile mucous membrane in a *Babesia gibsoni* infected dog

Plate 17. Icteric vaginal mucous membrane in a *Babesia gibsoni* infected dog

Plate 18. Voiding of dark yellow urine by a dog with *Babesia gibsoni* infection

Plate 19. Emaciation in a dog with *Babesia gibsoni* infection

Plate 20. Aborted fetuses expelled by a dog infected with *Babesia gibsoni*

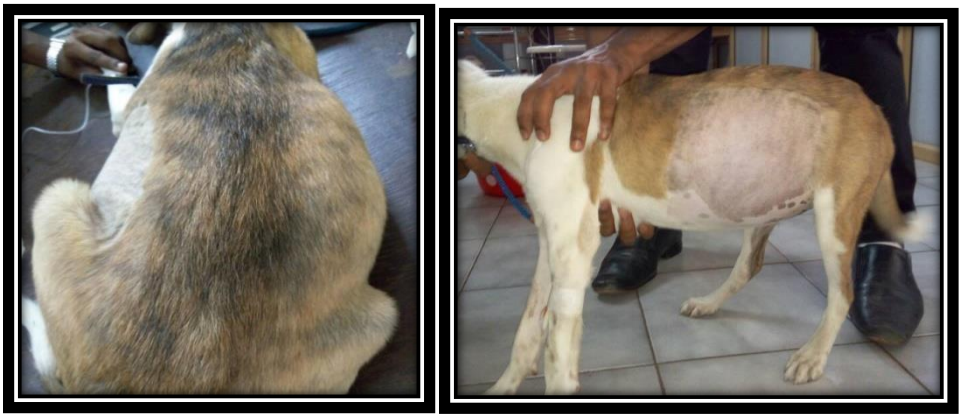


Plate 21. Ascites in a dog with *Babesia gibsoni* infection

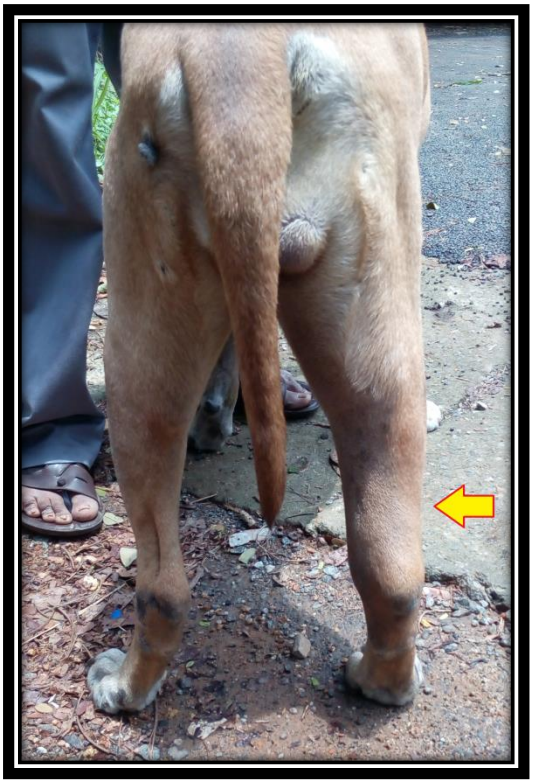


Plate 22. Limb oedema (right leg) in a dog with *Babesia gibsoni* infection

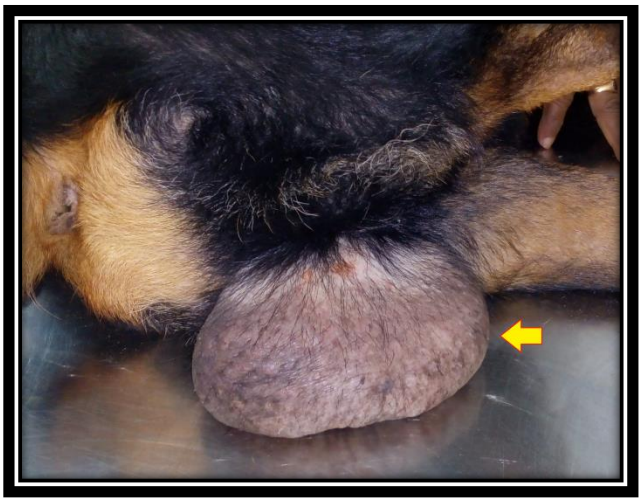


Plate 23. Scrotal oedema in a dog affected with *Babesia gibsoni*

Plate 24. Neurological symptoms (Seizures) in a *Babesia gibsoni* infected dog

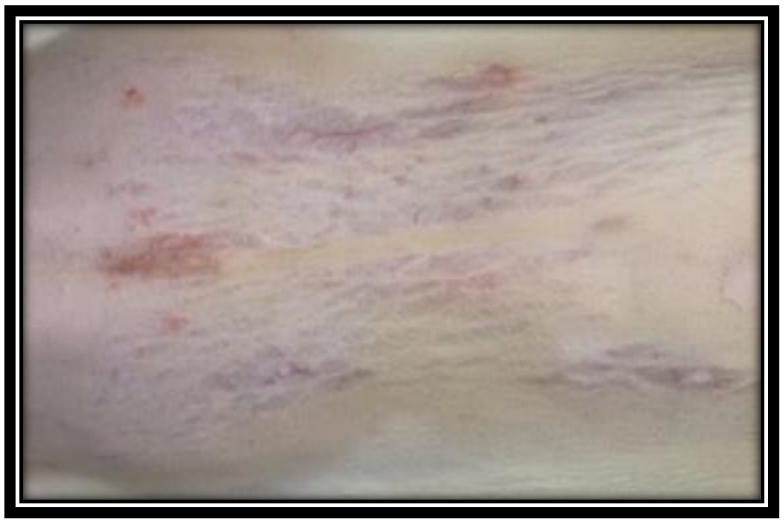


Plate 25. Vasculitis on the ventral abdomen in a dog with *Babesia gibsoni* infection

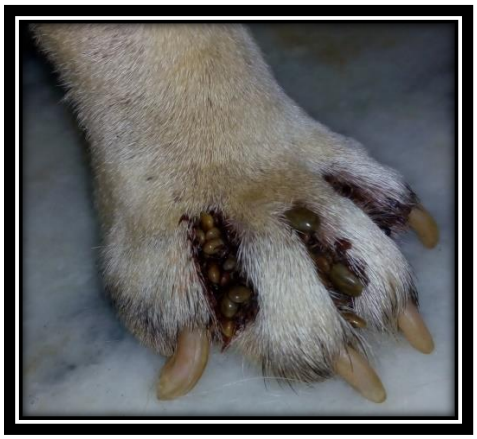


Plate 26. Tick infestation in the interdigital spaces in a dog with *B. gibsoni* infection

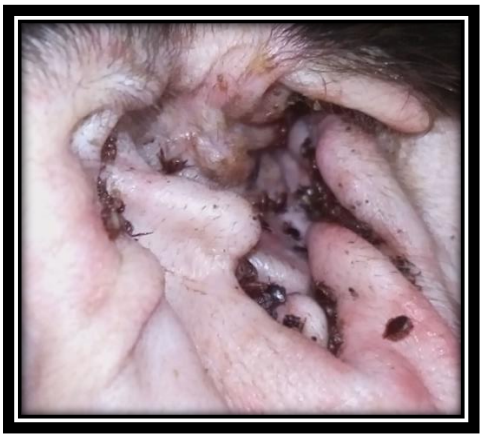
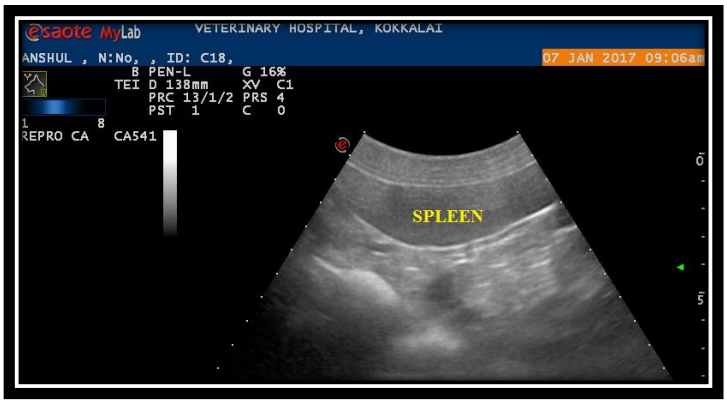


Plate 27. Tick infestation in the external ear canal in a dog with *B. gibsoni* infection



moderate splenomegaly in *Babesia canis* infected dogs

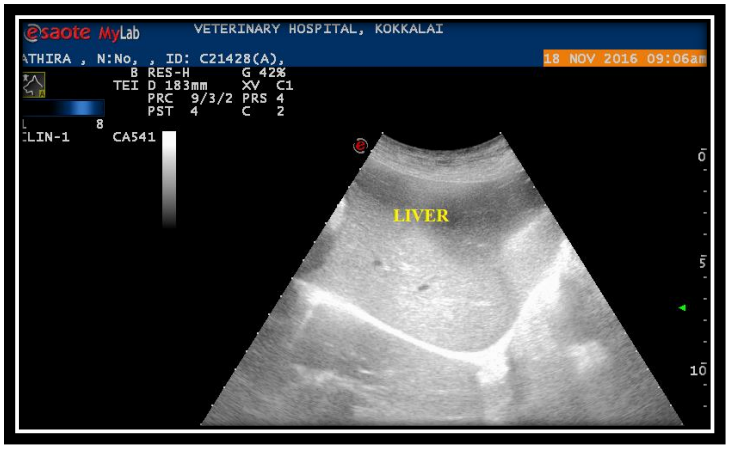


Plate 29. Mild to moderate hepatomegaly in *Babesia canis* infected dogs

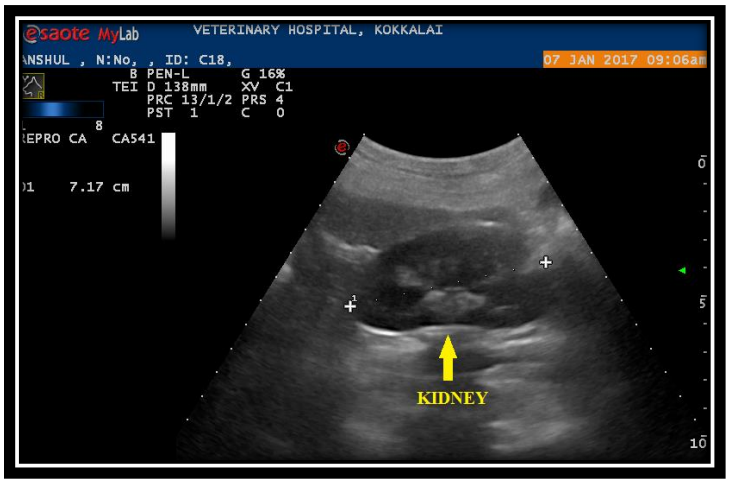


Plate 30. Renomegaly in a *Babesia canis* infected dog

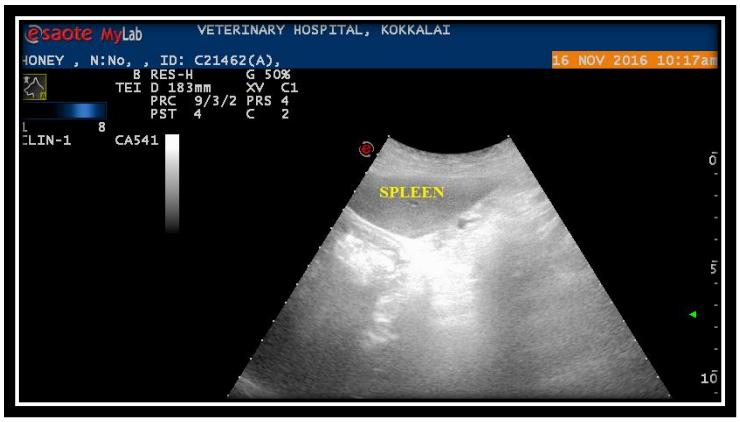


Plate 31. Mild splenomegaly in a *Babesia gibsoni* infected dog

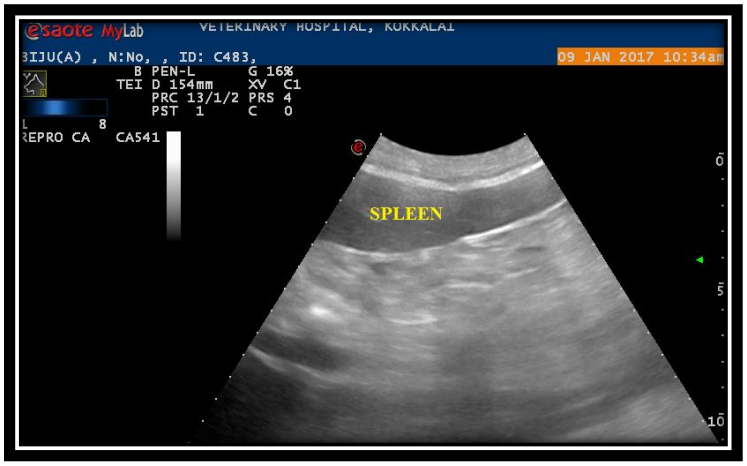


Plate 32. Moderate splenomegaly in a *Babesia gibsoni* infected dog

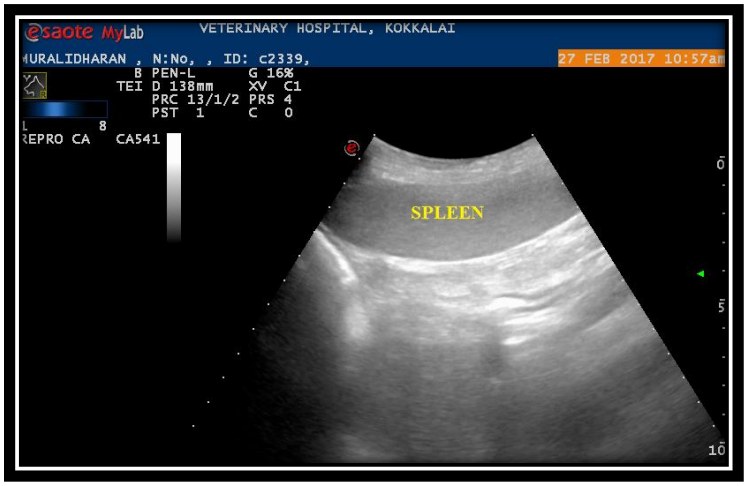


Plate 33. Severe splenomegaly in a *Babesia gibsoni* infected dog

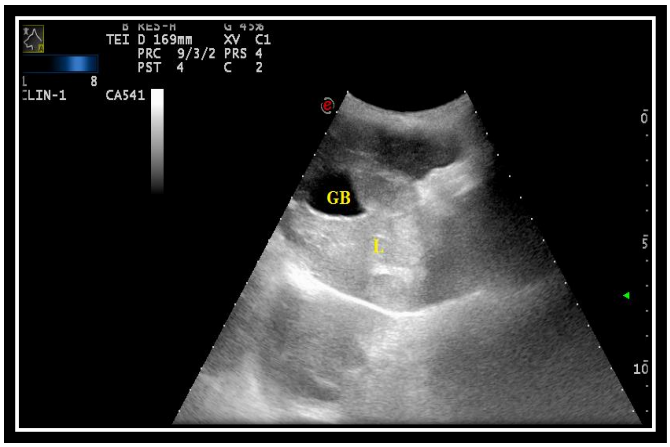


Plate 34. Severe hepatomegaly with gall bladder distension in a *Babesia gibsoni* infected dog

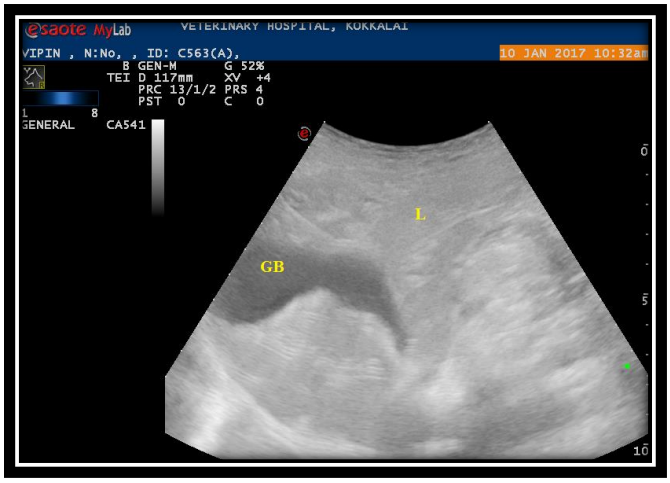


Plate 35. Severe hepatomegaly with a distended and tortous gall bladder in a *Babesia gibsoni* infected dog

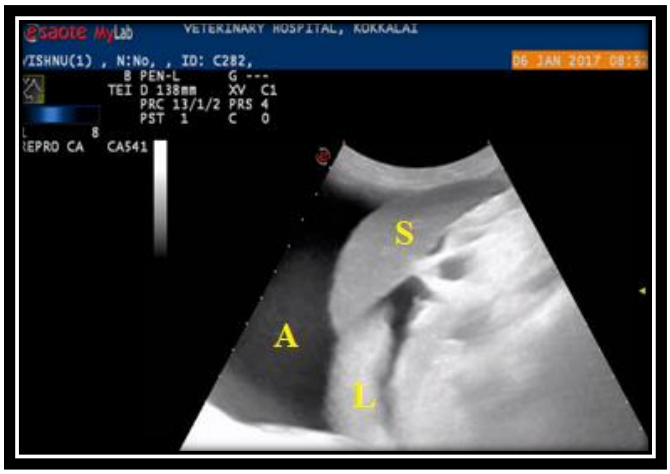


Plate 36. Splenomegaly with ascites in a *Babesia gibsoni* infected dog

KEY:
L - Liver
S - Spleen
GB - Gall bladder
A - Ascitic fluid

4. 5. 2. *Babesia gibsoni* affected dogs

The sonographic evaluation of liver, gall bladder, spleen and kidneys of 33 *Babesia gibsoni* afftected dogs on the day of presentation is depicted in Table 4.3. Mild enlargement of spleen was noticed in 7 dogs affected with *B. gibsoni* (Plate 31). Moderate splenomegaly was observed in 15 dogs (Plate 32). Severe splenomegaly was seen in ten dogs (Plate 33). No splenic changes were evident in one dog. Mild to moderate hepatomegaly was identified in 18 dogs, whereas four dogs had severe hepatomegaly (Plate 34). Eleven dogs had no hepatic involvement. The sonographic evaluation of gall bladder in five dogs showed a distended and tortous gall bladder with clear anechoiec bile (Plate 35) in one dog. Slight distention with presence of anechoeic clear bile was observed in 17 dogs. In dogs with ascites splenomegaly with surrounding anechoiec clear fluid was observed (Plate 36). Renomegaly was observed in seven dogs, but without affecting the architecture and cortico-medullary distinction.

4. 6. HAEMATOLOGICAL STUDIES

4. 6. 1. *Babesia canis* affected dogs

The haematological parameters of the dogs infected with *Babesia canis* are presented in Table 4.4.

4. 6. 1. 1. Total Erythrocyte Count

Mean value of total erythrocyte count of the *B. canis* affected dogs was lower ($3.975 \pm 0.179 \times 10^6/mm^3$) when compared to the control group ($4.532 \pm 0.302 \times 10^6/mm^3$), but statistical analysis revealed no significant difference between the two groups.

Table 4.4. Haematological parameters of *B. canis* infected dogs

4. 6. 1. 2. Haemoglobin

Mean value of haemoglobin of the dogs affected with *B. canis* was 10.197 ± 0.49 g/dl, which was significantly lower ($p < 0.01$) when compared to that of control group (13.07 ± 0.48 g/dl).

4. 6. 1. 3. Volume of Packed Red Blood Cells (VPRC)

The mean value of VPRC for the dogs affected with *B. canis* was 27.888 ± 1.188 per cent, which was significantly lower ($p< 0.05$) when compared to that of control group (32.350 ± 1.45 per cent).

4. 6. 1. 4. Mean Corpuscular Volume (MCV)

Mean value of MCV for the *B. canis* affected dogs was lower (68.385 ± 1.28 fl) when compared to the control group (72.290 ± 1.73 fl), but no significant variation was found statistically.

4. 6. 1. 5. Mean Corpuscular Haemoglobin (MCH)

Mean value of MCH for the *B. canis* affected dogs was lower (25.671 ± 0.96 pg) when compared to the control group (29.550 ± 1.41 pg), but no significant variation was found statistically.

4. 6. 1. 6. Mean Corpuscular Haemoglobin Concentration (MCHC)

Mean value of MCHC for the *B. canis* affected dogs was lower (37.312 ± 1.166 per cent) when compared to the control group (40.71 ± 1.320 per cent) but no significant variation was found statistically.

4. 6. 1. 7. Platelet Count

A significant decrease ($p < 0.05$) was noticed in the platelet count of the *B. canis* affected animals ($102.12 \pm 26.35 \times 10^3/\mu l$) compared to the control group ($239.10 \pm 27.88 \times 10^3/\mu l$).

4. 6. 1. 8. Total Leucocyte Count

There was no significant difference between the mean leukocyte counts of infected group ($10.197 \pm 1.269 \times 10^3/mm^3$) and control group ($11.780 \pm 0.813 \times 10^3/mm^3$).

4. 6. 1. 9. Differential Leucocyte Count

When the differential leukocyte counts of *B. canis* affected dogs were compared with that of the controls, the mean monocyte ($0.621 \pm 0.0847 \times 10^3/mm^3$), lymphocyte ($2.109 \pm 0.2943 \times 10^3/mm^3$) and granulocyte ($7.468 \pm 0.9791 \times 10^3/mm^3$) values were lower than the control group ($0.710 \pm 0.0722 \times 10^3/mm^3$, $2.380 \pm 0.2529 \times 10^3/mm^3$ and $8.700 \pm 0.7292 \times 10^3/mm^3$ respectively, but no significant variation was found statistically.

4. 6. 2. *Babesia gibsoni* affected dogs

The haematological parameters of the dogs infected with *Babesia gibsoni* are presented in Table 4.5.

4. 6. 2. 1. Total Erythrocyte Count

Mean value of total erythrocyte count for the *B. gibsoni* affected dogs was lower ($3.1545 \pm 0.138 \times 10^6/mm^3$) when compared to the control group ($4.5320 \pm 0.302 \times 10^6/mm^3$). Reduction in the mean total erythrocyte count of *B. gibsoni* affected dogs was highly significant ($p<0.01$) when compared to the controls.

4. 6. 2. 2. Haemoglobin

Mean value of haemoglobin for the dogs affected with *B. gibsoni* was 7.911 ± 0.348 g/dl, which was significantly lower ($p < 0.01$) when compared to the control group (13.07 ± 0.485 g/dl).

4. 6. 2. 3. Volume of Packed Red Blood Cells (VPRC)

The classification of anaemia based on the VPRC in *B. gibsoni* affected dogs is represented in Fig. 4. The mean value of VPRC for the dogs affected with *B. gibsoni* was 21.012 ± 0.830 per cent, which was significantly lower ($p < 0.01$) when compared to the control group (32.350 ± 1.45 per cent).

4. 6. 2. 4. Mean Corpuscular Volume (MCV)

Mean value of MCV for the *B. gibsoni* affected dogs was lower (69.193 ± 1.059 fl) when compared to the control group (72.290 ± 1.73 fl), but no significant variation was found statistically.

4. 6. 2. 5. Mean Corpuscular Haemoglobin (MCH)

Mean value of MCH for the *B. gibsoni* affected dogs was lower (25.307 ± 0.475 pg) when compared to the control group (29.550 ± 1.41 pg), and was statistically significant ($p < 0.05$).

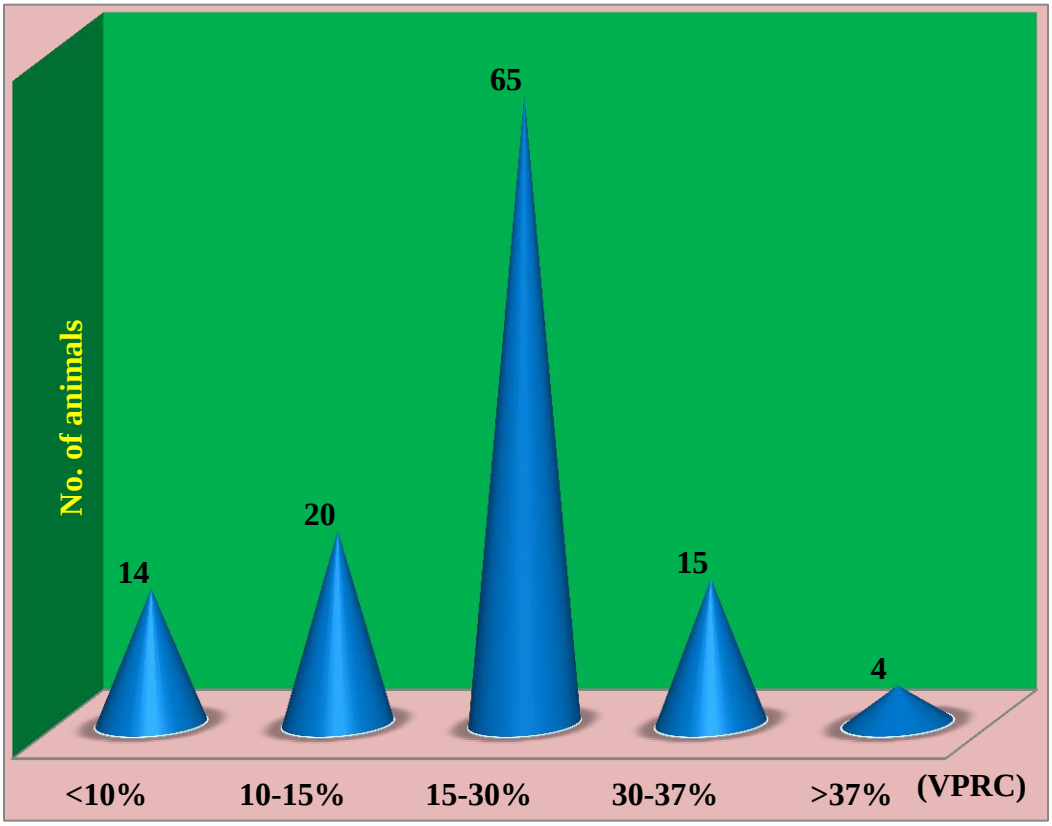


Fig. 4: Classification of anaemia in *Babesia gibsoni* affected dogs

VPRC < 15% - Severely anaemic

VPRC 15–30 % - Moderately anaemic

VPRC > 30–37 % - Mildly anaemic

VPRC > 37% - Non-anaemic

(VPRC – Volume of Packed red cells)

4. 6. 2. 6. Mean Corpuscular Haemoglobin Concentration (MCHC)

Mean value of MCHC for the *B. gibsoni* affected dogs was significantly lower (36.716 ± 0.568 per cent) when compared to the control group (40.71 ± 1.320 per cent) ($p < 0.05$).

4. 6. 2. 7. Platelet Count

A significant decrease ($p < 0.01$) was noticed in the platelet count of the *B. gibsoni* affected animals (115.39 ± 9.537) compared to the control group (239.10 ± 27.88).

4. 6. 2. 8. Total Leucocyte Count

There was significant difference ($p < 0.01$) between the mean leukocyte counts of *B. gibsoni* infected group (17.150 ± 0.967 x $10^3/mm^3$) and control group (11.780 ± 0.813 x $10^3/mm^3$).

4. 6. 2. 9. Differential Leucocyte Count

When the differential leukocyte counts of *B. gibsoni* affected dogs were compared with that of the controls, the mean values of monocyte (0.931 ± 0.658 x $10^3/mm^3$), lymphocyte (3.553 ± 0.2325 x $10^3/mm^3$) and granulocyte (12.543 ± 0.79 x $10^3/mm^3$) values were higher than the control group (0.710 ± 0.0722 x $10^3/mm^3$, 2.380 ± 0.2529 x $10^3/mm^3$ and 8.700 ± 0.7292 x $10^3/mm^3$) respectively. No significant variation was found statistically between the mean values of monocyte count but there was significant difference between the mean lymphocyte and granulocyte counts ($p < 0.01$).

4. 7. SERUM BIOCHEMICAL ANALYSIS

4. 7. 1. Biochemical analysis of *Babesia canis* affected animals

The mean serum biochemical values of dogs affected with *B. canis* are depicted in Table 4.6.

4. 7. 1. 1. Total Bilirubin

The mean total bilirubin level of infected group (1.3434 ± 0.2238 mg/dl) was significantly higher ($p<0.05$) than that of the control group (0.7919 ± 0.1086 mg/dl).

4. 7. 1. 2. Direct Bilirubin

The mean serum direct bilirubin level of diseased dogs (0.5624 ± 0.1303 mg/dl) was higher than the control group of dogs (0.3679 ± 0.0555 mg/dl), but no significant variation was found statistically.

4. 7. 1. 3. Indirect Bilirubin

The mean serum indirect bilirubin level of diseased dogs was 0.7870 ± 0.1370 mg/dl, which was significantly higher ($p<0.05$) when compared to that of the control group of dogs (0.4240 ± 0.0875 mg/dl).

4. 7. 1. 4. Creatinine

The mean serum creatinine was higher in diseased dogs (1.27 ± 0.064 mg/dl) than control group (1.01 ± 0.67 mg/dl) which was statistically significant ($p<0.05$).

4. 7. 1. 5. Blood Urea Nitrogen (BUN)

The mean value of blood urea nitrogen (BUN) level of affected dogs was 18.74 ± 2.169 mg/dl which was higher when compared to that of control group (14.76 ± 1.60 mg/dl). But differences were not statistically significant.

4. 7. 1. 6. Alanine Aminotransferase (ALT)

There was no significant difference between the mean values of ALT of infected group (33.52 ± 3.319 U/L) and control group (46.75 ± 10.40 U/L).

4. 7. 1. 7. Alkaline Phosphatase (ALP)

There was no significant difference between the mean values of ALP of infected group (144.30 ± 19.98 U/L) and control group (95.16 ± 13.55 U/L).

4. 7. 1. 8. Aspartate Aminotransferase (AST)

There was no significant difference between the mean values of AST of infected group (40.47 ± 4.88 U/L) and control group (36.88 ± 4.94 U/L).

4. 7. 1. 9. Total protein

The mean serum total protein was 6.672 ± 0.183 g/dl and 7.437 ± 0.304 g/dl respectively in the diseased and control groups. The mean value of diseased group was significantly lower than that of the control group ($p<0.05$).

4. 7. 1. 10. Serum Albumin

The difference between mean serum albumin of diseased and control group was statistically significant ($p<0.01$). Mean values of diseased (2.652 ± 0.985 g/dl) was lower than that of control group (3.742 ± 0.254 g/dl).

4. 7. 1. 11. Serum Globulin

There was no significant difference between the mean value of globulin of infected group (4.019 ± 0.183 g/dl) and control group (3.695 ± 0.241 g/dl).

4. 7. 1. 12. Albumin : Globulin Ratio (A: G)

The mean Albumin - globulin ratio of the diseased and control groups were 0.731± 0.056 and 1.06 ± 0.113 respectively, with the former being lower than the latter. Difference between the diseased and control were statistically significant ($p<0.01$).

4. 7. 1. 13. Serum Glucose

The mean glucose level of infected group (94.349 ± 3.79 g/dl) was lower than the control group (101.63 ± 4.88 g/dl) without any statistically significant difference between the two groups.

4. 7. 1. 14. Gamma Glutamyl Transferase (GGT)

There was no significant difference between the mean value of GGT of infected group (7.081 ± 1.455U/L) and control group (4.141 ± 0.659 U/L).

4. 7. 2. Biochemical analysis of *Babesia gibsoni* affected animals

The mean serum biochemical values of *Babesia gibsoni* affected animals are depicted in Table 4.7.

4. 7. 2. 1. Total Bilirubin

There was significant increase ($p<0.01$) in the mean total bilirubin levels of infected group (2.2896 ± 0.2883 mg/dl) when compared to the control group (0.7919 ± 0.1086 mg/dl).

4. 7. 2. 2. Direct Bilirubin

The mean serum direct bilirubin level of diseased dogs (1.2679 ± 0.21084 mg/dl) was higher than the control group of dogs (0.3679 ± 0.0555 mg/dl) which was significant statistically ($p<0.01$).

4. 7. 2. 3. Indirect Bilirubin

The mean serum indirect bilirubin level of diseased dogs was 1.02 ± 0.135 mg/dl, which was significantly higher ($p<0.01$) when compared to that of the control group of dogs (0.4240 ± 0.0875 mg/dl).

4. 7. 2. 4. Creatinine

The mean serum creatinine was apparently higher in diseased dogs (1.65 ± 0.13 mg/dl) than in control group of dogs (1.011 ± 0.067 mg/dl). But difference was not statistically significant.

4. 7. 2. 5. Blood Urea Nitrogen (BUN)

The mean value of blood urea nitrogen level of affected dogs was 27.26 ± 3.12 mg/dl which was higher than that of control group (14.7 ± 1.60 mg/dl). The difference was statistically significant ($p < 0.01$).

4. 7. 2. 6. Alanine Aminotransferase (ALT)

Even though a higher level of ALT was found in affected dogs, there was no significant difference between the mean value of ALT of infected group (61.16 ± 12.65 U/L) and control group (46.75 ± 10.400 U/L), on statistical analysis.

4. 7. 2. 7. Alkaline Phosphatase (ALP)

The mean value of serum ALP level of affected dogs was 158.49 ± 10.81 U/L, which was significantly higher ($p<0.01$) when compared to that of the control group (95.16 ± 13.55 U/L).

4. 7. 2. 8. Aspartate Aminotransferase (AST)

The mean value of AST was higher in infected group (54.47 ± 7.67 U/L) when compared to control group (36.88 ± 4.94 U/L). But no significant difference was observed on statistical analysis.

4. 7. 2. 9. Total protein

There was no significant difference between the mean serum total protein values of diseased (6.75 ± 0.155 g/dl) and control groups (7.43 ± 0.304 g/dl).

4. 7. 2. 10. Serum Albumin

The mean value of serum albumin level of affected dogs was 2.53 ± 0.076 g/dl, was significantly lower ($p<0.01$) when compared to that of the control group (3.74 ± 0.25 g/dl).

4. 8. 2. 11. Serum Globulin

There was no significant difference between the mean value of serum globulin of infected group (4.222 ± 0.149 g/dl) and control group (3.695 ± 0.241 g/dl).

4. 7. 2. 12. Albumin : Globulin Ratio (A: G)

The mean albumin - globulin ratio of the diseased group was lower (0.722 ± 0.052) when compared to that of the control group (1.064 ± 0.113). But there was no difference of statistical significance.

4. 7. 2. 13. Serum Glucose

Mean value of serum glucose of infected group was lower than that of control group. But there was no significant difference between the mean value of glucose of infected group (87.12 ± 3.38 g/dl) and control group (101.63 ± 4.88 g/dl) on statistical analysis.

4. 7. 2. 14. Gamma Glutamyl Transferase (GGT)

There was no significant difference between the mean value of GGT of infected group (8.201 ± 0.878 U/L) and control group (4.141 ± 0.659 U/L), though the former was higher than the latter.

4. 8. TREATMENT RESPONSE

Effects of various treatments were assessed based on remission of clinical signs, evaluation of peripheral blood smears and haematological parameters on days three and 14 after onset of the treatment protocols and changes observed in ultrasonography.

The means values of the haematological parameters before and after treatment in each group for *B. canis* and *B. gibsoni* infected dogs are depicted in Table 4.8 and Table 4.9 respectively.

Table 4.9. Haematological parameters of *Babesia gibsoni* affected groups before and after treatment

Haematological parameters	Treatment groups						
	Group I (n=11)			Group II (n=11)			
	BT	AT		BT	AT		
		Day 3	Day 14		Day 3	Day 14	
Total Erythrocyte count (x 10^6/mm^3)	2.655 ± 0.401 [a]	2.430±0.387 [a]	3.790±0.358 [b]	3.254± 0.401 [a]	2.824±0.387 [b]	3.603±0.358 [a]	3.3
Haemoglobin (g/dl)	7.009 ± 1.094 [a]	6.491±0.982 [a]	9.555±0.875 [b]	8.355± 1.094 [a]	7.055±0.982 [b]	9.482±0.875 [a]	9.4
VPRC (per cent)	18.018±2.362 [a]	17.464±2.430 [a]	25.355±1.918 [b]	22.864±2.362 [a]	19.945±2.430 [b]	25.145±1.918 [a]	23.4
MCV (fl)	73.500±3.833 [a]	76.136±3.790[ab]	69.773±3.114 [ac]	74.300±3.833 [a]	74.218±3.790[ab]	71.555±3.114 [ac]	71.5
MCH (pg)	27.291±1.389 [a]	27.727±1.298 [a]	25.809±1.543 [a]	25.664±1.389 [a]	25.045±1.298 [a]	26.573±1.543 [a]	28.4

Table 3.4. List of biochemical parameters and analytic procedures

S. NO	PARAMETER	PURCHASED FROM	ANALYTICAL METHOD	REFERENCE
1.	**Total Bilirubin and Direct Bilirubin**	SPINREACT	Bilirubin react with sulphodiazonium salt and form azobilirubin (Malloy method)	Malloy and Evelyn (1937)
2.	**Creatinine**	CORMAY	Jaffe's method without deproteinization	Fabiny and Ertingshausen (1971)
3.	**Urea**	CORMAY	Enzymatic kinetic method with urease and glutamate dehydrogenase	Talke and Schubert (1965)
4.	**Alkaline Phosphatase (ALP)**	CORMAY	IFCC kinetic method	Bowers and McComb (1966)
5.	**Alanine Aminotransferase (ALT)**	CORMAY	IFCC method without pyridoxal phosphate	Bergmeyer *et al.* (1986a)
6.	**Aspartate Aminotransferase (AST)**	CORMAY	IFCC method without pyridoxal phosphate	Bergmeyer *et al*. (1986b)
7.	**Total Protein**	CORMAY	Biuret reaction	Gomall *et al*. (1949)
8.	**Albumin**	CORMAY	Bromocresol green method	Doumas *et al*. (1971)
9.	**Glucose**	CORMAY	Enzymatic colorimetric method with glucose oxidase	Barham and Trinder (1972)
10.	**γ- Glutamyl Transpeptidase (GGT)**	SPINREACT	Kinetic method with L-γ-glutamyl-3-carboxy-4-nitroanilide	Persijn and van der Slik (1976)

Table 4.3 (Contd.) Ultrasonographic observations of *B. gibsoni* affected dogs (n=33)

SL. NO	CASE NUMBER	SPLEEN	LIVER	GALL BLADDER	KIDNEY
23.	4697	Mild splenomegaly	No hepatomegaly	NAD	Normal size, cortico-medullary distinction present, NAD
24.	5308	Moderate splenomegaly	No hepatomegaly	NAD	
25.	5401	Moderate splenomegaly	Severe hepatomegaly	Distension with clear bile	Renomegaly, cortico-medullary distinction present
26.	4306	Severe splenomegaly	Severe hepatomegaly	Distension with clear bile	
27.	6282	Mild splenomegaly	Mild hepatomegaly	Slight distension	Normal size, cortico-medullary distinction present, NAD
28.	6305	Moderate splenomegaly	No hepatomegaly	NAD	
29.	6306	Mild splenomegaly	Mild hepatomegaly	Distension with clear bile	
30.	6679	Severe splenomegaly	Moderate hepatomegaly	Distension with clear bile	
31.	6611	Moderate splenomegaly	No hepatomegaly	NAD	
32.	6669	No splenomegaly	No hepatomegaly	NAD	
33.	5106	Mild splenomegaly	Mild hepatomegaly	Distension with clear bile	

Table 4.3 (Contd.) Ultrasonographic observations of *B. gibsoni* affected dogs (n=33)

SL. NO	CASE NUMBER	SPLEEN	LIVER	GALL BLADDER	KIDNEY
23.	4697	Mild splenomegaly	No hepatomegaly	NAD	Normal size, cortico-medullary distinction present, NAD
24.	5308	Moderate splenomegaly	No hepatomegaly	NAD	
25.	5401	Moderate splenomegaly	Severe hepatomegaly	Distension with clear bile	Renomegaly, cortico-medullary distinction present
26.	4306	Severe splenomegaly	Severe hepatomegaly	Distension with clear bile	
27.	6282	Mild splenomegaly	Mild hepatomegaly	Slight distension	Normal size, cortico-medullary distinction present, NAD
28.	6305	Moderate splenomegaly	No hepatomegaly	NAD	
29.	6306	Mild splenomegaly	Mild hepatomegaly	Distension with clear bile	
30.	6679	Severe splenomegaly	Moderate hepatomegaly	Distension with clear bile	
31.	6611	Moderate splenomegaly	No hepatomegaly	NAD	
32.	6669	No splenomegaly	No hepatomegaly	NAD	
33.	5106	Mild splenomegaly	Mild hepatomegaly	Distension with clear bile	

NAD –No abnormalities detected

Table 4.3 (Contd.) Ultrasonographic observations of *B. gibsoni* affected dogs (n=33)

SL. NO	CASE NUMBER	SPLEEN	LIVER	GALL BLADDER	KIDNEY
12.	25185	Severe splenomegaly	No hepatomegaly	NAD	Renomegaly, cortico-medullary distinction present
13.	924	Severe splenomegaly	Moderate hepatomegaly	Slight distension	Normal size, cortico-medullary distinction present, NAD
14.	2789	Moderate splenomegaly	No hepatomegaly	NAD	
15.	25595	Hypoechoeic spleen Moderate splenomegaly	Hypoechoeic Parenchyma Moderate hepatomegaly	Slight distension	
16.	18636	Moderate splenomegaly	Mild hepatomegaly	Slight distension	
17.	22860	Moderate splenomegaly	No hepatomegaly	NAD	
18.	282	Severe splenomegaly	Severe hepatomegaly	Distension with clear bile	Renomegaly, cortico-medullary distinction present
19.	5997	Severe splenomegaly	Moderate hepatomegaly	Distension with clear bile	
20.	21426	Moderate splenomegaly	Moderate hepatomegaly	Distension with clear bile	
21.	7788	Moderate splenomegaly	Mild hepatomegaly surrounded by anechoeic acsitic fluid	NAD	Normal size, cortico-medullary distinction present, NAD
22.	7189	Mild splenomegaly	No hepatomegaly	NAD	

Table 4.3. Ultrasonographic observations of *B. gibsoni* affected dogs (n=33)

SL. NO	CASE NUMBER	SPLEEN	LIVER	GALL BLADDER	KIDNEY
1	23174	Congested spleen , Mild splenomegaly	No hepatomegaly	NAD	Normal size, cortico-medullary distinction present, NAD
2	17935	Severe splenomegaly	Moderate hepatomegaly	Slight distension	
3	17819	Hypoecheoic spleen, Moderate splenomegaly	No hepatomegaly	NAD	
4	17005	Moderate splenomegaly	Moderate hepatomegaly	NAD	Normal size, cortico-medullary distinction present Focal Hyperechoiec area at cortico-medullary junction
5	563	Severe splenomegaly	Hypercheoic Parenchyma, Severe hepatomegaly	Distended and tortous gall bladder with clear bile	Renomegaly cortico-medullary distinction present, achitecture retained
6	1251	Moderate splenomegaly	Mild hepatomegaly	NAD	Normal size, cortico-medullary distinction present, NAD
7	15302	Severe splenomegaly	Mild hepatomegaly	Slight distension	
8	6746	Moderate splenomegaly	Mild hepatomegaly	Slight distension	
9	6588	Mild splenomegaly	Moderate hepatomegaly	Slight distension	
10	7115	Moderate splenomegaly	Mild hepatomegaly	NAD	
11	6001	Severe splenomegaly	Moderate hepatomegaly	Slight distension	

NAD –No abnormalities detected

Table 4.2. Ultrasonographic Observations of *B. canis* affected dogs (n=20)

SL. NO	CASE NUMBER	SPLEEN	LIVER	GALL BLADDER	KIDNEY
1	15728	No splenomegaly	No hepatomegaly	NAD	Normal size, cortico-medullary distinction present, NAD
2	17024	Moderate splenomegaly	No hepatomegaly	NAD	Normal size, cortico-medullary distinction present, NAD
3	21428	Hypoecheoic and congested No splenomegaly	Moderate hepatomegaly	Slight wall thickening	Renomegaly, normal architecture Cortico-medullary distinction present
4	18319	Moderate splenomegaly	Moderate hepatomegaly	Slight distension	Renomegaly, normal architecture Cortico-medullary distinction present
5	3276	Mild splenomegaly	No hepatomegaly	NAD	Normal size, cortico-medullary distinction present, NAD
6	893	No splenomegaly	No hepatomegaly	NAD	Normal size, cortico-medullary distinction present, NAD
7	4490	Moderate splenomegaly	No hepatomegaly	NAD	Normal size, cortico-medullary distinction present, NAD
8	6792	Moderate splenomegaly	Moderate hepatomegaly	Slight distension	Normal size, cortico-medullary distinction present, NAD
9	7684	Moderate splenomegaly	No hepatomegaly	NAD	Normal size, cortico-medullary distinction present, NAD
10	6189	Mild splenomegaly	No hepatomegaly	NAD	Normal size, cortico-medullary distinction present, NAD

NAD –No abnormalities detected

Table 4.2. (Contd.) Ultrasonographic Observations of *B. canis* affected dogs (n=20)

SL. NO	CASE NUMBER	SPLEEN	LIVER	GALL BLADDER	KIDNEY
11.	20576	Moderate splenomegaly	No hepatomegaly	NAD	Normal size, cortico-medullary distinction present, NAD
12.	13248	Moderate splenomegaly	No hepatomegaly	NAD	Normal size, cortico-medullary distinction present, NAD
13.	20574	Mild splenomegaly	No hepatomegaly	NAD	Normal size, cortico-medullary distinction present, NAD
14.	21426	Mild splenomegaly	No hepatomegaly	NAD	Normal size, cortico-medullary distinction present, NAD
15.	23370	Moderate splenomegaly	No hepatomegaly	NAD	Normal size, cortico-medullary distinction present, NAD
16.	24972	Moderate splenomegaly	Mild hepatomegaly	Slight distension	Normal size, cortico-medullary distinction present, NAD
17.	15957	No splenomegaly	No hepatomegaly	NAD	Normal size, cortico-medullary distinction present, NAD
18.	15255	Moderate splenomegaly	Moderate hepatomegaly	Slight distension	Normal size, cortico-medullary distinction present, NAD
19.	21005	Mild splenomegaly	No hepatomegaly	NAD	Normal size, cortico-medullary distinction present, NAD
20.	18	Moderate splenomegaly	No hepatomegaly	NAD	Renomegaly, architecture retained Cortico-medullary distinction present

NAD –No abnormalities detected

MCHC per cent	39.560±1.767 [a]	37.360±1.404 [a]	39.990±1.378 [a]	39.690±1.767 [a]	
Platelet count (x 10^3/ µl)	60.000±20.376 [a]	126.200±25.238 [b]	203.800±23.471 [c]	73.300±20.376 [a]	
Total leucocyte count (x 10^3/mm^3)	7.790±1.441 [a]	10.560±1.466 [ab]	11.640±1.256 [b]	9.880±1.441 [a]	
Monocyte (x 10^3/mm^3)	0.600±0.168 [a]	0.920±0.168 [a]	0.780±0.71 [a]	0.670±0.168 [a]	
Lymphocyte (x 10^3/mm^3)	1.710±0.350 [a]	2.900±0.636 [ab]	2.600±0.359 [b]	2.250±0.350 [a]	
Granulocyte (x 10^3/mm^3)	5.490±0.972 [a]	7.340±1.076 [ab]	8.260±1.003 [b]	6.960±0.972 [a]	

BT - Before Treatment, AT- After Treatment

For each parameter, mean with same superscript (small letters in rows) does not vary

4. 8. 1. Treatment response of *Babesia canis* infected dogs

4. 8. 1. 1. Group I

4. 8. 1. 1. 1. Evaluation of peripheral blood smears

Blood smears from all the dogs in the group were negative on day three except one dog which was positive on day three but became negative on day fourteen.

4. 8. 1. 1. 2. Haematology

Serial haematological examinations were also performed on days three and 14. The *B. canis* affected dogs that were treated with Inj. Diminazene aceturate (BERENIL^{RTU}) showed marked improvement at the time of evaluation on days three and fourteen. Among the haematological parameters significant increase in the

platelet count was observed on days three (126.200 ± 25.238 x 10^3/ µl) and fourteen (203.800 ± 23.471 x 10^3/ µl). There was increase in the levels of total erythrocyte count and haemoglobin values on days three and fourteen, but was not statistically significant. The mean value of VPRC and MCV also improved at the time of evaluation, but was not statistically significant. The mean value of MCH and MCHC showed a reduction on day three but then increased on day fourteen after treatment. An increase in the mean value of total leucocyte count, lymphocyte and granulocyte counts were observed post-treatment, but were not statistically significant (Table 4.8).

4. 8. 1. 1. 3. Clinical Response

With an exception to three dogs that remained anorexic up to two days post treatment, others showed marked improvement in appetite and overall condition after day one. Remission was noticed in two dogs that had vomiting. Reduction in body temperature was noticed at the time of evaluation on day three. There was change in colour of urine from dark yellow to clear urine from day four following treatment in two patients that were voiding dark yellow urine.

4. 8. 1. 1. 4. Changes in Ultrasonography

Sonographic evaluation showed no variation in the degrees of splenomegaly and/or hepatomegaly on day fourteen after commencing treatment.

4. 8. 1. 2. Group II

4. 8. 1. 2. 1. Evaluation of peripheral blood smears

All animals in group II were blood smear negative on day three and day fourteen after the treatment with Imidocarb dipropionate.

4. 8. 1. 2. 2. Haematology

The *B. canis* affected dogs that were treated with Inj. Imidocarb dipropionate (IMICARB) showed marked improvement at the time of evaluation on day fourteen. There was a slight decrease in the mean erythrocyte count on day three (4.011±0.224 x 10^6/mm^3), which then increased by day 14 (4.415 ± 0.229 x 10^6/mm^3). There was increase in the mean haemoglobin values on day fourteen (12.050 ± 0.516 g/dl), but was not statistically significant. There was a mild decrease in the mean value of VPRC on day three followed by an increase on day fourteen, but was not statistically significant. The mean value of MCV, MCH and MCHC increased on day three but decreased on day fourteen, but was not statistically significant. Among the haematological parameters significant increase in the platelet count was observed on days three (133.600±25.238 x 10^3/ µl) and fourteen (205.300 ± 23.471 x 10^3/ µl). A significant increase in the mean value of total leucocyte count, lymphocyte and granulocyte levels were observed post-treatment, but were not statistically significant (Table 4.8).

4. 8. 1. 2. 3. Clinical Response

Marked improvement in appetite, with remission of vomiting was noticed in six dogs that were presented with emesis. In all dogs reduction in pyrexia was noticed at the time of evaluation on day three. Considerable improvements with remission of clinical signs were observed in all animals in group II after 14 days of treatment.

4. 8. 1. 2. 4. Changes in Ultrasonography

Sonographic evaluation showed no variation in the degrees of splenomegaly and/or hepatomegaly on day fourteen after commencing treatment.

4. 8. 1. 3. Comparison between group I and group II

There was no statistical significance between treatment groups I and II with respect to the values of mean erythrocyte count, haemoglobin, VPRC, MCV, MCH, MCHC, platelet count and monocyte value on days zero (before treatment), three and fourteen.

4. 8. 2. Treatment response of *Babesia gibsoni* infected dogs

4. 8. 2. 1. Group I

4. 8. 2. 1. 1. Evaluation of peripheral blood smears

Blood smears from one dog in group I was positive for *B. gibsoni* on day three following treatment. However, all animals in group I were found blood smear negative on day fourteen.

4. 8. 2. 1. 2. Haematology

The *B. gibsoni* affected dogs that were treated with a combination of diminazene aceturate, imidocarb and clindamycin showed marked improvement at the time of evaluation on days three and fourteen. There was a slight decrease in the mean values of erythrocyte count, haemoglobin values and VPRC on day three, followed by significant increase on day fourteen. Among the haematological parameters significant increase in the mean value of platelet count was observed on days three (81.909 ± 24.211 x 10^3/µl) and fourteen (175.364 ± 33.454 x 10^3/µl). The mean value of haemoglobin on day zero (7.009 ± 1.094 g/dl) and days three (6.491 ± 0.982 g/dl) showed a significant increase on day fourteen (9.555 ± 0.875 g/dl) following treatment. The mean value of VPRC which was 18.018 ± 2.362 per cent (on day zero) and 17.464±2.430 per cent (on day three) showed a significant increase on day fourteen (25.355 ± 1.918 per cent). The mean value of MCV and MCH showed an increase on day three but decreased on day fourteen. The mean value of

MCHC on day zero (37.536 ± 1.712 per cent) decreased on days three (36.545 ± 1.250 per cent) and fourteen (36.945 ± 1.591 per cent) (Table 4.9.)

4. 8. 2. 1. 3. Clinical Response

Reduction in body temperature was noticed in six dogs from day three of initiating treatment. Among the six dogs that had vomiting as presenting sign, two dogs had remission from day three. Marked improvement in appetite, change in urine colour from dark yellow to normal clear urine and betterment in overall condition was reported in all dogs at the time of evaluation on day fourteen.

4. 8. 2. 1. 4. Changes in Ultrasonography

Sonographic evaluation showed no variation in the degrees of splenomegaly and/or hepatomegaly on day fourteen after commencing treatment.

4. 8. 2. 2. Group II

4. 8. 2. 2. 1. Evaluation of peripheral blood smears

Blood smears from two dogs in group II were positive on day three following treatment and one dog remained blood smear positive on day fourteen after treatment. The same treatment was continued for five more days.

4. 8. 2. 2. 2. Haematology

The *B. gibsoni* affected dogs that were treated with a three drug protocol (Inj. Metronidazole, Inj. Clindamycin and Tab. Doxycycline) showed marked improvement at the time of evaluation on day fourteen. There was a significant decrease in mean erythrocyte count, haemoglobin and VPRC on day three. But, an increase in these values was found on day fourteen. The mean value of MCV on day zero was 74.300 ± 3.833 fl which showed a reduction on day three (74.218 ± 3.790 fl) and day fourteen (71.555 ± 3.114 fl), but was statistically significant. The mean value

of MCH on day zero was 25.664 ± 1.389 pg, which showed a reduction on day three (25.045 ± 1.298 pg) and increase on day fourteen (26.573 ± 1.543 pg), but was not statistically significant. The mean value of MCHC decreased by day three (34.364 ± 1.250 per cent) and increased by day fourteen (37.400 ± 1.591 per cent), but was not statistically significant. Among the haematological parameters, significant increase in the mean platelet count was observed on days three (82.455 ± 24.211 x 10^3/µl) and fourteen (191.636 ± 33.454 x 10^3/µl) when compared to day zero (77.182 ± 22.887 x 10^3/µl) (Table 4.9).

4. 8. 2. 2. 3. Clinical Response

Among the six dogs that had anorexia, marked improvement in appetite was noticed in four dogs that were presented for evaluation on day three. Reduction in body temperature was noticed in all dogs from day three of initiating treatment. Among the seven dogs that had vomiting as presenting sign, four dogs had remission from day three. Urine colour changed from dark yellow to normal clear urine in one dog from day three. Betterment in overall condition was reported at the time of evaluation on day fourteen in all cases. However, relapse was noticed in four dogs after a period of two to four months.

4. 8. 2. 2. 4. Changes in Ultrasonography

Sonographic evaluation showed no variation in the degrees of splenomegaly and/or hepatomegaly on day fourteen after commencing treatment.

4. 8. 2. 3. Group III

4. 8. 2. 3. 1. Evaluation of peripheral blood smears

Blood smears from two dogs in group II was positive for *B. gibsoni* on day three and day fourteen following treatment. The current combination therapy

(Inj. Metronidazole, Inj. Clindamycin and Tab. Doxycycline) was given for 10 more days, for the two dogs that remained blood smear positive.

4. 8. 2. 3. 2. Haematology

The *B. gibsoni* affected dogs that were treated with Inj. Imidocarb showed marked improvement at the time of evaluation on day three and day fourteen. The mean values of erythrocyte count, haemoglobin and VPRC were higher on day three and fourteen when compared to day zero, but was not statistically significant. The mean value of MCH and MCHC decreased on day three and increased on day fourteen, but was not statistically significant. The mean value of platelet count which was 138.364 ± 22.887 x 10^3/µl (on day zero) increased on day three (162.818 ± 24.211 x 10^3/µl) and day fourteen (184.364 ± 33.454 x 10^3/µl), but was not statistically significant (Table 4.9).

4. 8. 2. 3. 3. Clinical Response

Out of four dogs with anorexia as the presenting sign, only one dog had remission following treatment on day one. Three other dogs remained anorectic up to four days following treatment. Only one dog had vomiting as presenting sign in this group which resolved from day two. Reduction in body temperature was noticed in all dogs from day three of initiating treatment. Voiding of dark yellow urine that was reported in three dogs resolved after initiation of treatment. Though improvement in overall condition was reported in these cases at the time of evaluation on day fourteen in all cases, there was a delay in response when compared to other two groups.

4. 8. 2. 3. 4. Changes in Ultrasonography

Sonographic evaluation showed no variation in the degrees of splenomegaly and/or hepatomegaly on day fourteen after commencing treatment.

4. 9. PREVALENCE

Out of the 200 dogs, 156 (78 per cent) were found to be infected with *Babesia* organisms. Among the 156 *Babesia* infected dogs, 118 (75.64 per cent) were found to be infected with *Babesia gibsoni* and 38 (24.35 per cent) with *Babesia canis.*

4. 9. 1. Age-wise prevalence

In the present study, babesiosis was diagnosed in animals of various age groups from two months to twelve years. Percentage of *Babesia canis* and *Babesia gibsoni* positive cases among different age groups is shown in Table 4.10.

A higher rate of occurrence of *B. canis* infection was noticed in dogs between the age group of six months to one year (26.08 per cent), followed by dogs between the age group of one to five years (21.24 per cent). Three dogs between the age group of six years and ten years and three dogs below six months of age were affected. The lowest incidence was in dogs more than ten years of age (two dogs) (Fig .5).

A higher rate of occurrence of *B. gibsoni* infection was noticed in dogs between the age group of one to five years (71.68 per cent), followed by dogs between the age group of six to ten year (51.43 per cent). Ten dogs between six months to one year of age were affected. The lowest incidence was in dogs less than six months (five dogs) and more than ten years of age (four dogs) (Fig .6).

However, statistical analysis showed no significant difference in the proportion of positive cases among different age groups of dogs.

4. 9. 2. Sex-wise prevalence

Out of 200 dogs that were screened for babesiosis, 106 dogs were males and 94 dogs were females. From a total of 156 dogs diagnosed with babesiosis 77 dogs were male and 79 dogs were female (Table 4.11). Out of 38 positive *B. canis* 16 were males and 22 were females. But the per cent positivity based on gender of screened

dogs was higher among females (23.40 per cent) than males (15.09 per cent). Out of 118 dogs positive for *B. gibsoni*, 61 were males and 57 were females. But the percent positivity based on gender of screened dogs was higher among females (60.63 per cent) than males (57.54 per cent) (Table 4.11) (Fig .7).

4. 9. 3. Breed-wise prevalence

Babesiosis was observed in different breeds of dogs, viz., Rottweiler, Labrador Retriever, German Shepherd, Cocker Spaniel, Pug, Great Dane, Spitz, St. Bernard, Dachshund, Doberman Pinscher, Non - descript, Pit bull Terrier, British Bulldog, Miniature Pinscher, Bull Mastiff, Dalmatian, Rajapalayam, Golden Retriver, Terrier, Lhasa Apso, Beagle and Boxer.

Distribution of *B. gibsoni* and *B. canis* positive cases among different breeds of dogs screened by blood smear examination is shown in Table 4.12. Among the dogs positive for *B. canis* prevalence was found to be more in Rottweilers followed by Labrador retrievers and Spitz (Fig .8). The prevalence of *B. gibsoni* was found to be more in Labrador retrievers followed by Rottweiler, Dachshund and Spitz (Fig .9).

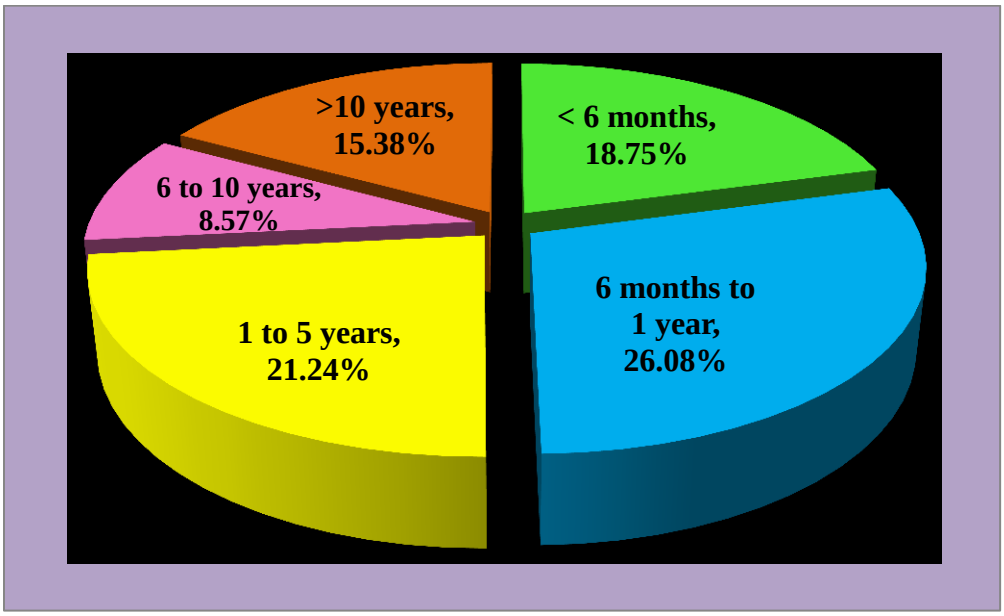


Fig. 5: Age-wise distribution of *Babesia canis* among dogs

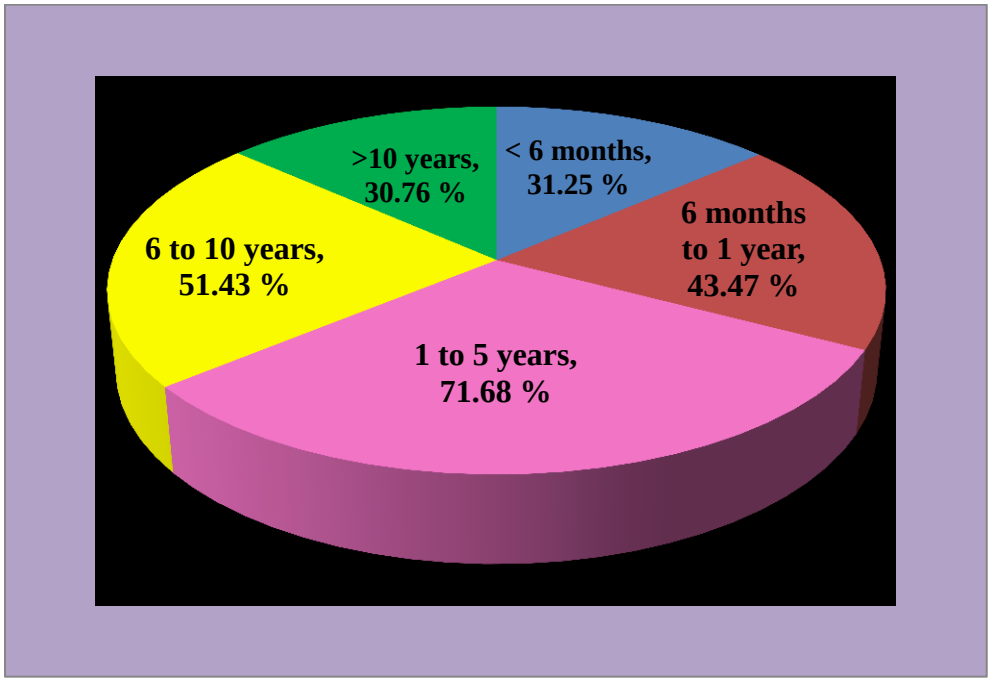


Fig. 6: Age-wise distribution of *Babesia gibsoni* among dogs

Table 4.10. Age-wise distribution of *B. gibsoni* and *B. canis* among dogs

Organism	Age group	No. examined	No. positive	Percentage positives (%)
B. canis	< 6 months	16	3	18.75
	6 months to 1 year	23	6	26.08
	1 to 5 years	113	24	21.24
	6 to 10 years	35	3	8.57
	>10 years	13	2	15.38
B. gibsoni	< 6 months	16	5	31.25
	6 months to 1 year	23	10	43.47
	1 to 5 years	113	81	71.68
	6 to 10 years	35	18	51.43
	>10 years	13	4	30.76

Table 4. 11. Sex-wise distribution of *B. canis* and *B. gibsoni* among dogs

Organism	Sex	No. examined	No. positive	Percentage positives (%)
B. canis	Male	106	16	15.09
	Female	94	22	23.40
B. gibsoni	Male	106	61	57.54
	Female	94	57	60.63

Fig. 7: Sex-wise distribution of *Babesia canis* and *Babesia gibsoni* among dogs

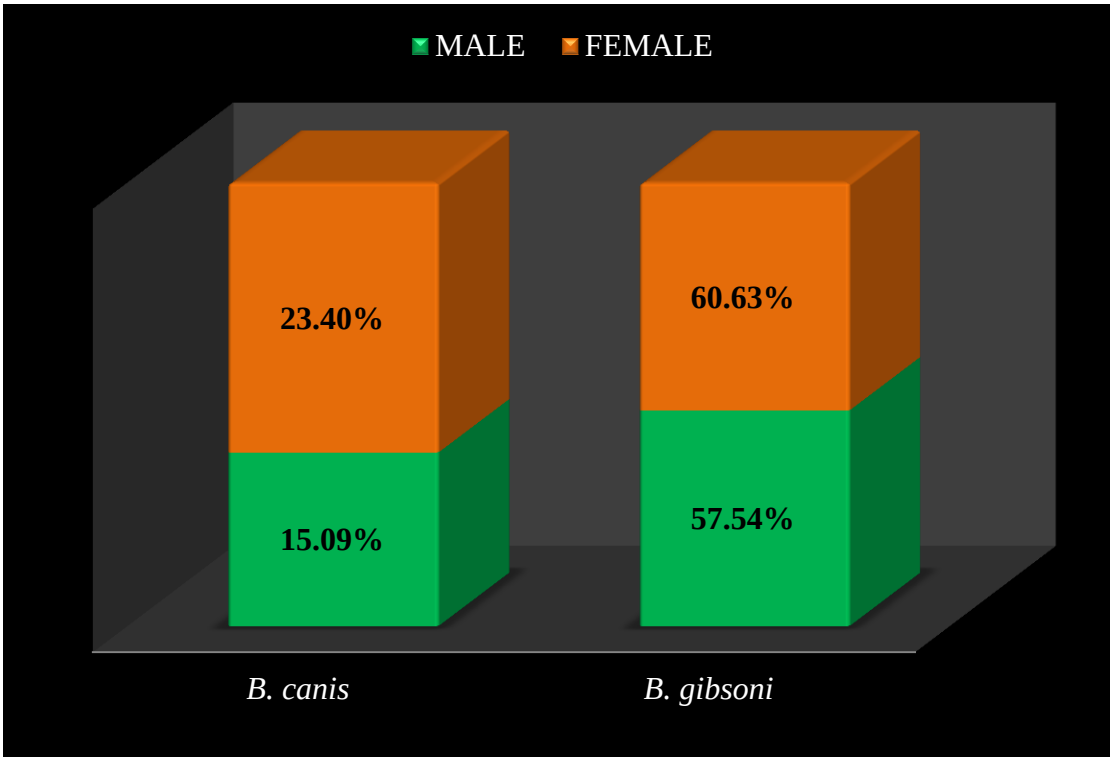


Table 4.12. Breed-wise distribution of *B. gibsoni* and *B. canis* among dogs

Sl. no	Name of the breed	No.Examined	No. and per cent positive for *B. gibsoni*	No. and per cent positive for *B. canis*
1.	Rottweiler	38	20 (52.63)	11 (28.94)
2.	Labrador Retriever	43	31 (72.09)	8 (18.60)
3.	German Shepherd	11	7 (63.63)	2 (18.18)
4.	Cocker Spaniel	5	2 (40.00)	1(20.00)
5.	Pug	11	6 (54.54)	2 (18.18)
6.	Great Dane	3	1 (33.33)	0 (0.00)
7.	Spitz	17	10 (58.82)	4 (23.53)
8.	St. Bernard	2	1 (50.00)	0 (0.00)
9.	Dachshund	14	10 (71.42)	2 (14.28)
10.	Doberman Pinscher	9	6 (66.67)	2 (22.22)
11.	Non- descript	12	6 (50.00)	2(16.67)
12.	Pit bull Terrier	5	1 (20.00)	1 (20.00)
13.	British Bulldog	1	0 (0.00)	1(100.00)
14.	Miniature Pinscher	3	2(66.67)	1(33.33)
15.	Bull Mastiff	5	2(40.00)	1(20.00)
16.	Dalmatian	2	1 (50.00)	0 (0.00)
17.	Rajapalayam	1	1(100.00)	0 (0.00)
18.	Golden Retriever	5	3(60.00)	0 (0.00)
19.	Terrier	3	1(33.33)	0 (0.00)
20.	Lhasa Apso	5	4(80.00)	0 (0.00)
21.	Beagle	2	1(50.00)	0 (0.00)
22.	Boxer	3	2(66.67)	0 (0.00)
	Total	**200**	**118 (59 per cent)**	**38 (19 per cent)**

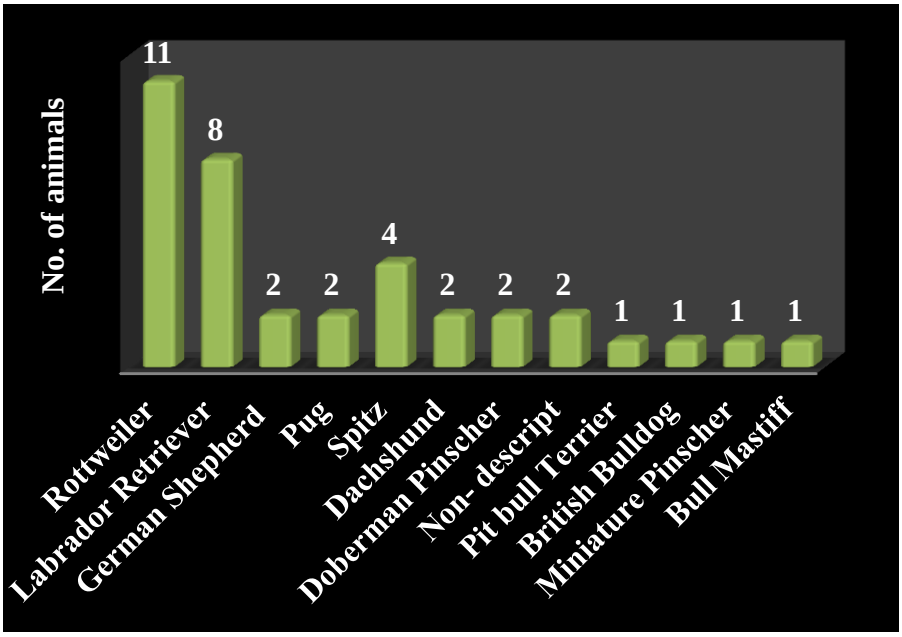


Fig. 8: Breed-wise distribution of *Babesia canis* among dogs

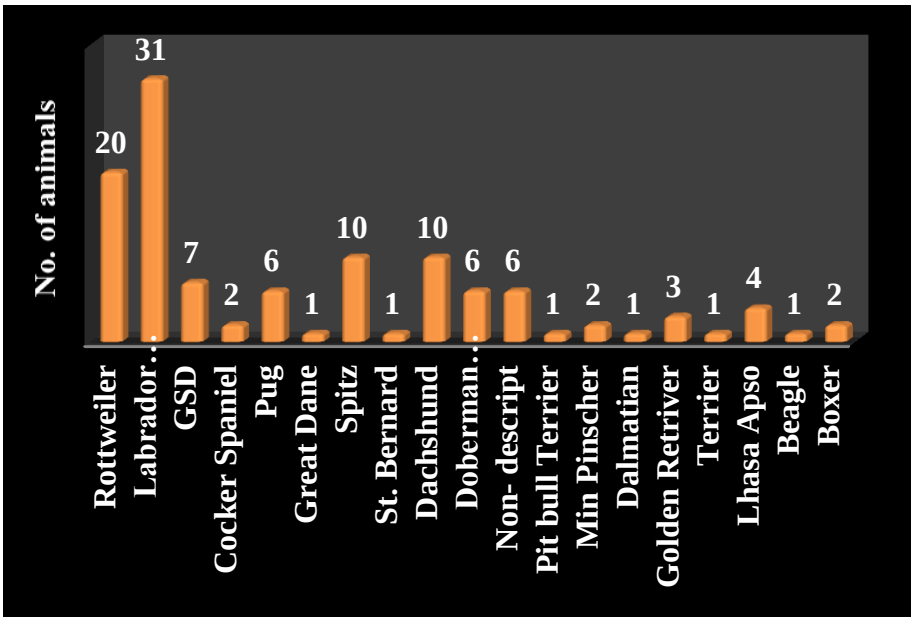


Fig. 9: Breed-wise distribution of *Babesia gibsoni* among dog

5. DISCUSSION

The present study was carried out in clinically suspected cases of babesiosis in dogs. These animals were screened using conventional blood smear examination and molecular technique (PCR). Haemato-biochemical evaluation was performed in all positive cases. Among the infected animals, dogs were selected at random for each treatment group. Treatment was done using single and combination anti-babesial drugs. Ultrasound evaluation was done before and after treatment of dogs included in the clinical trials. Treatment response was evaluated and analyzed statistically.

5. 1. SCREENING FOR BABESIOSIS

5. 1. 1. Microscopic Examination of Blood smear

5. 1. 1. 1. Giemsa's Staining / Field Staining

Blood smears of 200 dogs were examined by Giemsa or Field's stain. Microscopic examination of Field's or Giemsa stained blood smears therefore revealed 38 (19 per cent) and 112 (56 per cent) dogs positive to *B. canis* and *B. gibsoni* respectively, with a total of 75 per cent incidence. Microscopic examination of blood smears being rapid and cost effective has become the widely used test for the detection of canine haemoparasites. Several epidemiological studies have been conducted based on blood smear examination (Augustine *et al*., 2013; Bhattacharjee and Sarmah, 2013; Laha *et al*., 2014; Das *et al*., 2015; Mahalingaiah *et al.,* 2017). Examination of stained blood smears is not only time consuming but requires technical expertise as parasites can be missed with significantly low parasitaemia and multiple infections in the same host may get undiagnosed. However, it is the simplest and most accessible diagnostic test for veterinarians under field conditions and is considered to be a reasonably sensitive diagnostic tool during acute

or clinical cases (Rani *et al*., 2011). Co-infections with haemotropic mycoplasma and *E. canis* were found in five and four *B. gibsoni* infected dogs respectively. Four dogs with *B. canis* were concurrently infected with *E. canis.* Similarly, several authors have reported concomitant tick borne infections in dogs (Klag *et al.,* 1991; Tuttle *et al.,* 2003; Rani *et al*., 2011; Laha *et al*., 2014; Abbas *et al.,* 2015).

5. 1. 1. 2. Acridine Orange Staining

Acridine Orange (AO) staining detected *B. gibsoni* piroplasms in 38 samples and *B. canis* in 12 samples. As Richards *et al.* (1969) mentioned, parasites rich in RNA like *Babesia* spp. fluoresce red with AO while the other elements of the smear appear green or unstained. Although AO technique requires sophisticated equipment, it is less time consuming and is more effective to screen large number of samples because of its sensitivity when compared to Giemsa staining technique for detecting a low parasitaemia (Ravindran *et al*., 2007; Nair *et al.,* 2011).

5. 1. 2. Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) using genus specific primers detected *Babesia* DNA in 156 blood samples out of which 38 samples were positive for *B. canis* and 118 were positive for *B. gibsoni* using species specifc primers. All the 38 samples positive for *B. canis* by PCR were also diagnosed by blood smear examination. But out of 118 samples positive for *B. gibsoni* by PCR, only 112 were diagnosed by blood smear examination. Hence, blood smear examination is equally sensitive for detection for large *Babesia* spp. i.e., *B. canis.* But it is less sensitive for detection of small *Babesia* spp. i.e., *B. gibsoni* when compared to PCR.

On the basis of the clinical findings in the present study, microscopy could detect 75 per cent cases as *Babesia* positive against 78 per cent detection by PCR. So, three per cent cases remained false negative to *Babesia* spp. during microscopic examination. Similarly, higher detection of *Babesia* by PCR in comparison to

microscopy was also observed by several workers (Macintire *et al*., 2002; Porchet *et al*., 2007; Laha *et al*., 2014; Salem and Farag, 2014).

The presence of *B. canis vogeli* and absence of *B. canis rossi* and *B. canis canis* in infected dogs is in agreement with the known distribution of the vectors of the two sub-species. However, this situation might change in future with the expanding international mobility of pet dogs, and the presence of competent tick vectors leading to spread of parasites into previously non-endemic areas.

A combination of cytology, haematology and molecular diagnosis is therefore essential to avoid misdiagnoses.

5. 2. CLINICAL SIGNS

A high proportion of dogs in this study, presented with a fever of more than 103.5^{o}F. Pyrexia was reported by most of the workers (Yamane *et al*., 1993; Miyama *et al*., 2005; Abbas *et al*., 2015; Mahalingaiah *et al*., 2017) but on the contrary, Loftis *et al*. (2013) found majority of the *Babesia* affected dogs to be afebrile. The most common clinical manifestations dogs with *B. canis* in this study were fever, anorexia, pallor, lymphadenopathy, and the presence of ticks. None of the *B. canis vogeli* infected dogs had icterus in the present study. However, Salem and Farag (2014) reported haematuria and icterus associated within *B. canis vogeli* infection in two out of 13 cases studied. The most common clinical signs in dogs with *B. gibsoni* in this study were fever, anorexia, depression, pallor, lymphadenopathy, emesis, haemoglobinuria and presence of ticks. Similar observations were reported by Dantas-Torres and Figueredo (2006) and Vishnurahav *et al*. (2017). Vichova *et al*. (2016) observed massive haemoglubinuria in a three years old mixed pit bull dog affected with *B. gibsoni*. An acute form of *B. gibsoni* infection comprising of signs like fever, lethargy, haemolytic anaemia, thrombocytopaenia, lymphadenopathy and splenomegaly as mentioned by Schoeman (2009) was evident in a considerable number of dogs in the present study. Icteric sclera and mucous membranes and

visible yellow pigmentation of skin were reported in *B. gibsoni* infected dogs. Among the *B. gibsoni* infected dogs 16 dogs had exaggerated breathing on auscultation of the lung area and 18 dogs had dyspnea during presentation. Although there was a report by Iournals *et al*. (2011) on the association between respiratory distress syndrome and *B. canis* infection, molecular confirmation of the sub-species was not done.

Although transmission of *B. gibsoni* through biting wounds is a possibility (Jefferies *et al.*, 2007a; Miyama *et al.*, 2005), in the present study there was no such mention about recent dog attacks.

Fukumoto *et al*. (2005), Simoes *et al*. (2011) and Adaszek *et al*. (2016) have reported the possibility of transplacental transmission of both *B. canis* and *B. gibsoni* to puppies. Abortions were reported in the present study in two *B. gibsoni* affected dogs and one *B. canis* affected dog. However, failure to test the aborted foetuses could not confirm the significance of transplacental transmission in the three cases.

5. 3. ECTOPARASITES

Tick infestation was observed in 92 cases and ticks were identified as *Rhipicephalus* spp. and *Haemaphysalis* spp. Sundar *et al*. (2004) identified *Rhipicephalus sanguineus* ticks in four out of five dogs diagnosed with *B. gibsoni* infection. Konishi *et al*. (2008) reported tick infestation to be the most dominant risk factor for *B. gibsoni* infection. Based on morphological characteristics, Salem and Farag (2014) identified the ticks as *R. sanguineus* affecting dogs diagnosed with *B. canis vogeli* in Egypt. *Rhipicephalus sanguineus* has been incriminated in transmission of both *B. canis* and *B. gibsoni* and is considered as the major risk factor during outbreaks (Dantas-Torres *et al.*, 2006; Solano-Gallego and Baneth, 2011; Kelly *et al*., 2013; Rene-Martellet *et al*., 2015).

5. 4. HISTORY OF PREVIOUS ILLNESS

Among 156 dogs diagnosed with babesiosis in the presented study, two dogs had previous histories with similar infections. This can be because of failure in

maintaining a solid immunity. Repeated exposure to ticks can be a major risk factor. Animals with repeated relapses and recurrences will mount a weak immune response that leads to multiplication of parasites (Bourdoiseau, 2006).

5. 5. ULTRASONOGRAPHIC OBSERVATIONS

The most common splenic finding was generalized splenomegaly without focal or multifocal splenic lesions. An enlarged spleen characterised by a diffuse hypoechoic parenchyma was found in most of the *B. gibsoni* affected dogs. Fraga *et al.* (2011) and Sarma *et al.* (2016) observed similar changes in the spleen of babesia affected dogs. The cause of splenomegaly is attributed to reactive lymphoid hyperplasia and concurrent extramedullary haematopoiesis.

None of the dogs in our study had hepatic lesions, but in dogs with high levels of alanine aminotransferase (ALT) and alkaline phosphatase (ALP) diffuse hepatomegaly was noticed. This is similar to the finding of Fraga *et al.* (2011) who reported hepatomegaly with reduced parenchymal echotexture and correlated these observations with elevations in ALP and ALT to describe the condition as complicated babesiosis due to *B. canis canis* infection. Jacobson and Clark (1994), Lobetti (2012) and Meyer and Twedt (2000) suggested that reticuloendothelial hyperplasia, acute inflammation or passive congestion may be the possible reasons for hepatomegaly to occur in dogs infected with *Babesia* spp. The sonographic evaluation of gall bladder showed distention with presence of anechoeic clear bile which is in agreement with the reports of Adaszek *et al*. (2016) and Sarma *et al.* (2016). Prolonged anorexia could be the possible reason for gall bladder distension.

Renomegaly was observed in seven dogs from a total of 33 *B. gibsoni* affected dogs that were scanned. Renomegaly was observed in three dogs from a total of 20 *B. canis* affected dogs that were scanned. Though bilaterally enlarged kidneys were

observed in these cases, the kidneys retained its normal architecture with distinct cortico-medullary differentiation. A study was done by Fraga *et al.* (2011) to understand the *B. canis* related ultrasonographic lesions and found increased cortical echogenicity with an enhanced cortico-medullary junction suggestive of renal dysfunction.

5. 6. HAEMATOLOGICAL STUDIES

5. 6. 1. *Babesia canis* affected dogs

Mean values of haemoglobin, VPRC and platelet count of *B. canis* affected dogs were lower when compared to that of the control group indicating variable degrees of anaemia and thrombocytopaenia. Similar observations were also made by Dantas-Torres and Figueredo (2006) and de Gopegui *et al*. (2007). Van Heerden *et al*. (1983), Kettner *et al*. (2003) and Sikorski *et al*. (2010) noted severe thrombocytopaenia in majority of the cases, but without apparent bleeding diatheses and thrombocytopaenia here was ascribed to disseminated intravascular coagulopathy. Bourdoiseau (2006) observed early onset of thrombocytopaenia with absence of anaemia to severe anaemia in *B. canis* affected dogs. The erythrocyte indices such as MCV, MCH and MCHC were lower than that of control group, but were within the normal range indicating normocytic normochromic anaemia. No significant variation was observed in the values of mean total erythrocyte count, leukocyte count and differential leukocyte count in the present study. Although non-regenerative anaemia, leukocytosis and leucopaenia are reported by Solano-Gallego *et al*. (2011) in dogs affected with *B. canis vogeli*, in the present study none were observed. Severe haemolytic anaemia was observed in this study in a four month old dog and this was in accordance with reports by Solano-Gallego *et al*. (2008) that mentions the possibility of fatal haemolysis in puppies and young dogs. Salem and Farag (2014) noticed neutropaenia, lymphocytosis and monocytosis with significant reduction in erythrocyte count, haemoglobin content and VPRC percentage in dogs

affected with *B. canis vogeli*. Leucocytosis was reported by Iournals *et al*. (2011) in dogs affected with *B. canis*.

5. 6. 2. *Babesia gisboni* affected dogs

Haematological analysis revealed marked reduction in total erythrocyte count, haemoglobin and VPRC suggestive of anaemia in *B. gibsoni* affected dogs. This finding is in agreement with Irizzary- Rovira *et al*. (2001) who reported regenerative anaemia in dogs affected with *B. gibsoni* in Indiana. Anaemia in affected dogs might be due to an increase in the circulating IgG - bound erythrocytes that resulted in severe erythrocyte depletion due to immunological phagocytosis secondary to macrophages activation (Adachi and Makimura, 1992; Adachi *et al*., 1994; Otsuka *et al*., 2002). Free radicals initiate oxidative stress to the erythrocytes thereby enabling production of anti-erythrocyte membrane antibodies. This will further enhance the susceptibility to macrophage-mediated bone marrow phagocytosis (Murase *et al*., 1996). Direct parasitic activity destroys erythrocyte membrane intergrity resulting in intravascular haemolysis. The mean value of haemoglobin and VPRC was significantly low in the diseased group. A reduction in erythrocyte count, haemoglobin and VPRC values was also reported by Meinkoth *et al*. (2002), Nalubamba *et al*. (2015) and Vichova *et al*. (2016). Onishi *et al*. (1990) described the presence of a haemolytic factor in the serum that could result in haemolytic anaemia. The mean value of MCV of infected group was within the reference range. The mean value of MCH and MCHC of infected group was significantly lower when compared to the control group, but was within the reference range. Hence, anaemia was normocytic normochromic type.

Thrombocytopaenia was the most consistent finding in most of the *B. gibsoni* affected dogs. Thrombocytopaenia in babesiosis is attributed to increased platelet destruction which in turn may be due to immunological or non-immunological,

decreased production and increased utilization or sequestration (Kettner *et al*., 2003). But on the contrary no bleeding disorders were reported in these dogs. In contradiction to this Tasaki *et al.* (2013) observed no thrombocytopaenia in a chronic case of *B. gibsoni* infection.

The total leucocyte count of the study group was significantly higher than the control group. Similar observations were also made by Tasaki *et al.* (2013) and Gonde *et al*. (2016).

5. 7. SERUM BIOCHEMISTRY

5. 7. 1. *Babesia canis* affected dogs

Serum biochemical estimations in *B. canis* affected dogs revealed hypoproteinaemia, hypoalbuminaemia, a decrease in A/G ratio and an increase in creatinine, total and indirect bilirubin levels. No significant changes were noticed in the mean values of ALT, AST, ALP, BUN, globulin and glucose. Zygner *et al.* (2006) reported a reduction in serum albumin and total protein and an elevation in creatinine values among dogs affected with *B. canis*. On the contrary de Gopegui *et al*. (2007) found an increase in BUN values while creatinine values remained within the normal limits, suggesting pre-renal elevations in BUN following ammonia overload that resulted due to haemolysis. Hypoproteinaemia, hypoalbuminaemia and significant elevations in ALP and ALT were reported in *B. canis* affected dogs by Konto *et al.* (2014). Eventhough hypoproteinaemia and hypoalbuminaemia was observed, no change in ALT and ALP values indicative of hepatic impairment could be observed in the present study. Anorexia may have been the possible cause for impaired synthesis of plasma proteins by the liver. Mierzejewska *et al.* (2014) observed no significant changes in the biochemical profile of *B. canis* affected dogs.

5. 7. 2. *Babesia gibsoni* affected dogs

Serum biochemical estimations in *B. gibsoni* affected dogs revealed hypoalbuminaemia, an increase in serum BUN, ALP and total, direct and indirect bilirubin levels. No significant changes were noticed in the mean values of ALT, AST, creatinine, glucose, total protein, globulin and A/G ratio. Irizarry-Rovira *et al*. (2001) reported hypoalbuminaemia which might be due to inflammation, hepatic dysfunction, ascites or starvation in affected dogs. In the same study unconjugated hyperbilirubinaemia was also observed and was attributed to increased erythrocyte destruction. Elevation in serum ALP and BUN values was reported by Tasaki *et al.* (2013). In the present study a significant increase in BUN values was observed but without concurrent increase in creatinine which is considered as reliable indicator of renal dysfunction. An explanation to this variation according to de Scally *et al*. (2004) is haemolysis induced non-renal azotaemia and interference of haemoglobinaemia in the estimation of serum creatinine thereby obscuring azotemia. Eventhough de Gopegui *et al*. (2007) has reported hyperglycaemia in *Babesia* infected dogs, in the present study only two dogs with *B. gibsoni* infection had hyperglycaemia, possibly due to glucose mobilization and stress. Gonde *et al*. (2016) observed hypoglycaemia with elevations in ALP, ALT, bilirubin and creatinine. Eventhough the mean creatinine and ALP values in the present study were lower than that of the control group, organ dysfunction was present in a few *B. gibsoni* affected dogs and was correlated with the ultrasonographic findings.

5. 8. TREATMENT RESPONSE

5. 8. 1. Treatment response of *Babesia canis* infected dogs

The present clinical trial was designed to compare the efficacy of two treatments (Diminazene aceturate and Imidocarb dipropionate) in an effort to improve the current clinical management of canine piroplasmosis caused by *B. canis.* Schoeman (2009) emphasized that the primary goal of treatment is to eliminate the

blood parasite and to reverse life-threatening anaemia by using one of the following anti-protozoal drugs viz., Diminazene aceturate or imidocarb dipropionate against *B. canis.*

5. 8. 1. 1. Group I

Treatment with Inj. Diminazene aceturate was effective in all the ten animals which resulted in remission of clinical signs with marked improvement of condition. Certain breeds like Dachshund, Golden Retriever, Labrador, Doberman and Rottweiler were reported by Kumara (2016) to be more susceptible to diminazene aceturate toxicity. Though several reports suggest that this drug has a low therapeutic index with a propensity to cause cerebral toxicity with classic cerebellar sulci haemorrhages in certain breeds of dogs (Donghyun *et al.,* 2014), this complication was fortunately not observed in the present study. The youngest dog to be treated in the present study was four months old.

5. 8. 1. 2. Group II

All the animals in group II showed improvement and remission of clinical signs following treatment with Inj. Imidocarb dipropionate. Similar observations were previously mentioned by Vial and Gorenflot (2006). Although there was a reduction in the mean erythrocyte count and mean value of VPRC on day three, remarkable increase in these values were noted on day 14. Undesirable effects of imidocarb dipropionate that occurs due to excessive acetylcholine action including pain at the injection site, vomiting, colic, diarrhoea and salivation was not observed in the present study. Routine blood smear analysis for all the ten dogs did not reveal the presence of *B. canis* on day 14 indicating clearance of parasitaemia.

To our knowledge, this is the first study done in Kerala to compare the use of imidocarb dipropionate with that of diminazene aceturate against *B. canis* in naturally

infected dogs. On comparison of the two treatment groups in this study it is observed that both the anti-babesial drugs were found to be equally effective against *B. canis.*

5. 8. 2. Treatment response of *Babesia gibsoni* infected dogs

The present clinical trial was designed to compare the efficacy of three treatments (Diminazene aceturate + Imidocarb dipropionate + Clindamycin), (Clindamycin + Metronidazole + Doxycycline) and (Imidocarb dipropionate) in an effort to improve the current clinical management of canine piroplasmosis caused by *B. gibsoni.*

5. 8. 2. 1. Group I

All the eleven dogs in group I responded well to treatment in terms of clinical improvement. There was improvement in haematocrit values and platelet count following treatment. Overall, the disease course showed clinical improvement in response to treatment. The group I regimen was tolerated by all 11 dogs with no product related adverse events being reported during the study period. Similarly Lin *et al*. (2012) observed a higher recovery rate of 84.6 per cent and lower relapse rate of 15.2 per cent in dogs treated with diminazene aceturate, imidocarb dipropionate and clindamycin combination. Development of resistance in *Babesia* parasites towards diminazene aceturate has been reported by Collett, (2000) and Matsuu *et al.* (2004) which may be the reason for relapse to occur as reported by previous workers, Wulansari *et al.* (2003) and Matsuu *et al.* (2004). This has lead to its limitations in usage against *B. gibsoni*. Vishnurahav *et al*. (2017) observed good response when a monotherapy using Clindamycin at 11 mg/kg body weight IV for 10 days was used against *B. gibsoni* infection. Lin and Huang (2010) and Gonde *et al*. (2016) suggested that an oral administration of a doxycycline–enrofloxacin–metronidazole combination with a single injection of diminazene aceturate was found to be very

effective against *B. gibsoni*. Sundar *et al*., (2004) observed a varied therapeutic potency of diminazene aceturate against *B. gibsoni* infection.

5. 8. 2. 2. Group II

Following treatment, the clindamycin, metronidazole and doxycycline (CDM) group showed two dogs with blood smears testing positive on day three and one dog with blood smears testing positive on day 14. Clinical signs like anorexia, vomiting and fever present in several dogs at the time of inclusion were reduced in frequency and severity in response to treatment. The Marshall Protocol that includes a combination of therapies with oral clindamycin-metronidazole-doxycycline at dose rates of 25 mg/kg, 15 mg/kg and 5 mg/kg respectively with frequency of 12 hours for 10 days is known to show clinical and haematological improvement without causing adverse effects by boosting the innate immunity (Kumara *et al.*, 2016).

5. 8. 2. 3. Group III

Though the platelet values increased on days three and 14 following treatment, the mean values of total erythrocyte count, VPRC and hemoglobin did not show significant increase following treatment. Insufficient clearance of parasitaemia was evident on routine blood smear evaluation for two dogs that remained positive on days three and 14 indicating the reduced efficacy of single anti-babesial therapy against *B. gibsoni* infection. Similarly, Vichova *et al*. (2016) also reported failure of remission of clinical signs in a dog affected with *B. gibsoni* after two doses of imidocarb dipropionate (Imizol) that was given at the rate of 6 mg/kg intramuscularly. Though this therapy was found to be less effective against severe *B. gibsoni* infection, there is the possibility to limit the use of macrolide antibiotics in animals and can thus help minimize antibiotic resistance.

To our knowledge, this is the first study done in Kerala to access the efficacy of imidocarb dipropionate against *B. gibsoni* in naturally infected dogs. Some dogs

treated with imidocarb dipropionate in group I and group III experienced vomiting after starting treatment. Thereafter for those dogs that had vomiting as a pre-existing complaint, atropine at 0.02 mg/kg S/C was given to avoid cholinergic effects.

Of the three treatment trials tested, the single dose of imidocarb dipropionate showed the least clinical and parasitological efficacy against *B. gibsoni* piroplasm infection. The combination of diminazene aceturate, imidocarb dipropionate and clindamycin showed the best clinical and parasitological efficacy, even though it is not capable of completely eliminating the infection at the recommended dose.

Majority of dogs are unlikely to be cured of *B. gibsoni* infection inspite of receiving the specific anti-babesial therapy. Hence, recovered animals are regarded as potentially infected for life, despite treatment and remission of clinico-pathological signs. Combination therapy of atovaquone (anti-malarial) and azithromycin (macrolide antibiotic) has been recommended by several workers to be a more effective therapy against *B. gibsoni* (Birkenheuer *et al*., 2003; Jefferies *et al*., 2007b; Ayoob *et al*., 2010; Vichova *et al*., 2016).

5. 9. PREVALENCE

The overall prevalence of babesiosis among 200 clinically suspected dogs in the present study was 78 per cent. The result is not comparable with the previous reports of Augustine *et al*. (2013), Das *et al*. (2015) and Vipan *et al.* (2015) who reported the overall prevalence to be 29.31 per cent, 31.86 per cent and 8.33 per cent respectively in clinically suspected cases of babesiosis. Higher incidence in the present study might be due to variation in the sampled animals. A higher individual prevalence of *B. canis* (54.05 per cent) was reported by Laha *et al*. (2014), however in the present study the individual prevalence of *B. canis* was only 24.35 per cent. The incidence of *B. canis* and *B. gibsoni* in the present study was higher than that reported by Singh *et al*. (2011) in Punjab and Bhattacharjee and Sarmah (2013) in

Assam and north-east India. Highest infection rate with *B. gibsoni* (84.9 per cent) has been reported by Senthil Kumar *et al.* (2009).

5. 9. 1. Age-wise Occurrence

In the presented study babesiosis was diagnosed in animals of different age groups from three months to twelve years. Analysis of percentage of dogs suffering from *B. canis* in various age groups revealed that dogs between six months to one year had the highest incidence followed by dogs between one to five years. Abdullahi *et al*. (1990) also found a higher infection rate of *B. canis* in dogs less than one year old. In the presented study babesiosis was found to occur in puppies and a similar finding was reported by Adaszek *et al*. (2016) who diagnosed *B. canis* infection in puppies that were eight weeks of age.

Analysis of per cent positivity of dogs suffering from *B. gibsoni* in various age groups revealed that dogs between one to five years had the highest incidence followed by dogs between six to ten years. This result was comparable with that of Kumar *et al.* (2008) who found a higher occurrence of *B. gibsoni* in adults.

In this study least incidence of babesiosis was noted in dogs above ten years of age. A similar finding has been reported by Nalubamba *et al*. (2015). On the contrary Konishi *et al*. (2008) noticed a higher sero-prevalence of *B. gibsoni* infection in dogs above 14 years of age.

5. 9. 2. Sex-wise occurrence

Sex-wise distribution of babesiosis showed more number of cases of *B. canis* and *B. gibsoni* in females than males. A similar finding was reported by Konishi *et al*. (2008). In contradictory to the finding in the present study higher prevalence of *B. canis and B. gibsoni* in male dogs in comparison to females was reported by several authors (Salem and Farag 2014; Davitkov *et al*., 2015; Nalubamba *et al*., 2015; Vipan *et al*., 2015; Mahalingaiah *et al*., 2017).

5. 9. 3. Breed-wise Occurrence

In this study, the per cent positivity of *B. canis* was observed to be more in Rottweiler, Labrador Retriever and Spitz compared to other breeds. *Babesia gibsoni* positive cases were more in Labrador Retriever, Rottweiler, Spitz and Dachshund followed by German Shepherd, Pug, Doberman Pinscher and Non-descript, but no significant difference was observed between the breeds by statistical analysis. More number of infections with *B. canis. vogeli* were reported among German Shepherd breed in Cairo, Egypt by Salem and Farag (2014). Konishi *et al*. (2008) observed a higher sero-prevalence of *B. gibsoni* infection among mixed breed followed by Golden Retrievers, Maltese, Shetland Sheepdog and Labrador Retrievers in Japan. Rottweiler and Labrador Retrievers breeds were over presented in the present study during this period. However, Mahalingaiah *et al*. (2017) had observed that Labrador Retrievers were more affected (26 per cent) followed by German shepherd (17 per cent) and Rottweilers were least affected (4.5 per cent) in Bengaluru. The highest incidence of babesiosis in Rottweiler breed (seven out of nine) was reported by Mathe *et al*. (2006) from Hungary.

6. SUMMARY

The present project "Molecular Detection and Therapeutic Management of Canine Babesiosis" was carried out to study the epidemiology, protozoal etiology, haemato-biochemical alterations and ultrasonographic changes in liver and spleen, in canine babesiosis, to diagnose babesiosis using PCR and to compare the efficacy of various treatment protocols.

Two hundred dogs brought to the Kerala Veterinary and Animal Sciences University, Veterinary Hospitals at Mannuthy and Kokkalai, and Veterinary hospitals attached to the Department of Animal Husbandry in Thrissur district, with clinical signs suggestive of babesiosis were screened by conventional and molecular methods. Microscopical examination of Giemsa and Field's stained smears revealed pyriform shaped piroplasms in pairs suggestive of *B. canis* in 38 dogs and signet-ring shaped piroplasms within erythrocytes suggestive of *B. gibsoni* in 112 dogs. Concomitant infection with *Ehrlichia canis* and haemotropic mycoplasma organisms were noted in both *B. gibsoni* and *B. canis* affected dogs. Acridine orange staining of blood smears from 50 positive dogs revealed characteristic organisms with apple green fluorescence in 12 cases of *B. canis* and 38 cases of *B. gibsoni.*

The PCR assay was carried out for the detection of 18S rRNA gene fragment of *Babesia* organism from the blood of dogs, using genus, species and sub-species specific primers. The genus specific primers (PIRO A and PIRO B) amplified a 400-bp product of *Babesia* spp. in 156 animals (78 per cent). The species specific primers (PIRO-F and PIRO-R) amplified a 460-bp product of *B. gibsoni* in 118 dogs (75.64 per cent) out of 156 *Babesia* positive dogs. The nucleotide sequences of two *B. gibsoni* isolates from the present study were found to have 99 per cent similarity with the *B. gibsoni* Asia isolate (Accession No. AF175301). The species specific primers (Can172F and Can626R) amplified a 454-bp product of *B. canis* in 38 dogs (24.36 per cent) out of 156 *Babesia* positive dogs. The PCR using sub-species

specific primers amplified a 590- bp product of *B. canis vogeli* in all *B. canis* positive cases. The nucleotide sequence of *B. canis vogeli* isolates from the present study was found to have 100 per cent similarity with already existing nucleotide sequence in GenBank. No sample was found to be positive for *B. canis canis* or *B. canis rossi*. Out of the 200 blood samples screened, 156 (78 per cent) were positive by PCR while Giemsa and Field's stain blood smear examination revealed *Babesia* organisms in 150 (75 per cent) samples only.

The highest percentage of *B. gibsoni* positive dogs were Labrador retrievers while *B. canis* infection was detected more in Rottweilers. Age-wise prevalence studies revealed a higher percentage of *B. canis* positive dogs of age group between six months to one year of age and a higher percentage *B. gibsoni* infection among dogs between one to five years of age. Females were more affected when compared to males in both *B. canis* and *B. gibsoni* affected dogs.

The frequently observed clinical signs in *B. gibsoni* and *B. canis* affected dogs include pyrexia, anorexia, vomiting, pallor or congestion of mucous membrane, enlargement of lymph nodes, dark yellow urine or haemoglobinuria, lethargy and tick infestation. Severe loss of body condition, jaundice, respiratory distress, seizures, syncope and vasculitis were observed only in *B. gibsoni* infected dogs. Ascites, abortion and oedema of limb and scrotum were some of the unusual presenting signs in both infections. Clinical manifestations in complicated cases were indicative of a multisystemic involvement.

Haematological parameters revealed significant reduction in the volume of packed red cells, haemoglobin and platelet count in *B. canis* infected dogs. Significant reduction in total erythrocyte count, haemoglobin, volume of packed red cells, MCH and MCHC along with thrombocytopaenia, leucopaenia, lymphocytopaenia and granulocytopaenia were observed in *B. gibsoni* infected dogs. Analysis of serum biochemical parameters of *B. canis* infected dogs revealed

hyperbilirubinaemia, hypoalbuminaemia, elevated creatinine and reduction in albumin: globulin ratio. Analysis of serum biochemical parameters of *B. gibsoni* infected dogs revealed hyperbilirubinaemia, hypoalbuminaemia and elevations in BUN and ALP values.

Among the *B. canis* affected dogs excellent clinical and haematological response was observed in all dogs treated under the group I (diminazene aceturate) and group II (imidocarb dipropionate) regimen. Clinical improvement and absence of piroplasms in the blood smears post treatment were indicative of therapeutic response. Significant increase in haemoglobin, total erythrocyte count and platelet count was observed in both groups following treatment on day 14, indicating efficacy of diminazene aceturate and imidocarb for the treatment of *B. canis vogeli*.

Among the *B. gibsoni* affected dogs clinical improvement along with absence of piroplasms in the blood smears and improvement in the haemotological parameters were observed in all the eleven dogs treated under the group I regimen which received a combination of diminazene aceturate, imidocarb dipropionate and clindamycin. Significant increase was noted in total erythrocyte count, haemoglobin, volume of packed red cells and platelet count by day 14 after treatment.

Good clinical and hematological response was observed in ten out of 11 dogs treated under the group II regimen (clindamycin, metronidazole, doxycycline). Significant increase in total erythrocyte count, haemoglobin, volume of packed red cells and platelet count was observed after 14 days of treatment. But relapse was reported in four cases within a period of six months indicating insufficient elimination of parasitaemia.

Among the *B. gibsoni* affected dogs that received group III regimen (imidocarb dipropionate), delay in clinical improvement along with presence of organism in the blood smears on days three and 14 in two dogs indicate poor therapeutic response. No statistically significant improvement was observed in total

erythrocyte count, haemoglobin, volume of packed red cells and platelet count by 14 days after treatment. This indicates that a single drug against *B. gibsoni* infection is not sufficient enough to clear parasitaemia. A good survival rate was noted in cases with an early intervention.

Ultrasonography of 33 dogs with *B. gibsoni* infection revealed hypo and hyper echogenicity of liver, gall bladder distension with clear bile, splenomegaly, hepatomegaly and ascites. Ultrasonographic findings in 20 *B. canis* positive dogs include hypoechogenic congested spleen with mild splenomegaly. No significant changes were observed day 14 after treatment. Abdominal ultrasonographic studies enabled the better understanding on the role of spleen in controlling the infection and in extra-medullary haematopoiesis.

The higher susceptibility of dogs with the presence of ticks might have resulted in wide spread occurrence of the condition. Attempts to control tick population have to be done to reduce the risk of infection. Prophylaxis in the form of vaccination or a prophylactic dose of Inj. Imidocarb dipropionate will protect the high risk groups.

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ABSTRACT

Canine piroplasmosis caused by *Babesia canis* and *B. gibsoni* is increasingly being detected among dogs in Kerala. The present work was carried out to study the epidemiology, protozoal etiology, haemato-biochemical alterations and ultrasonographic changes in liver and spleen, in canine babesiosis, to diagnose babesiosis using blood smear examination and PCR and to compare the efficacy of various treatment protocols. Diagnosis of babesiosis was performed by direct microscopic observation of intra-erythrocytic piroplasms in stained blood smears and by PCR. A total of 200 blood samples from dogs suspected for canine babesiosis were collected from different breeds, gender and age group of animals. Blood smear examination revealed *B. canis* piroplasms in 38 dogs and *B. gibsoni* piroplasms in 112 dogs. Polymerase chain reaction using genus specific PCR yielded amplicons specific for *Babesia* spp. in 156 samples. Species specific PCR for *B. canis* yielded positive results in 38 samples and that of *B. gibsoni* in 118 samples. The sub-species specific primers confirmed the *B. canis* organism as *B. canis vogeli,* in all 38 positive samples. Overall prevalence of canine babesiosis was estimated to be 78 per cent and 75 per cent by conventional staining technique and PCR respectively. Higher incidence of *B. canis* infection was seen in the age group of six months to one year in breed Rottweilers and in female dogs. Higher incidence of *B. gibsoni* infection was seen in the age group of one to five years in breed Labrador retrievers and in female dogs. The most frequent clinical signs recorded in *B. canis* infection include pyrexia, anorexia, vomiting, pallor or congestion of conjunctival mucous membrane and lymphadenopathy whereas, in *B. gibsoni* infection signs include pyrexia, anorexia, lethargy, pallor of mucous membrane, emaciation, jaundice, voiding of dark yellow or coffee coloured urine. Atypical signs like syncope, abortion, ascites, vasculitis, limb oedema and scrotal oedema were also observed. Tick infestation was observed in both infections. Ultrasonographic evaluation in 20 *B. canis* affected dogs and 33

B. gibsoni affected dogs revealed mild to moderate to severe hepato-splenomegaly, gall bladder distension and renomegaly. The haematological parameters associated with canine babesiosis are anaemia and thrombocytopaenia. Among the serum-biochemical alterations hyperbilirubinaemia and hypoalbuminaemia were common to both *B. canis* and *B. gibsoni* infections, whereas elevations in BUN and ALP values were noted in *B. gibsoni* infected dogs and an elevated creatinine was noted in *B. canis* infected dogs. Among the treatment regimens used against *B. gibsoni*, a combination therapy consisting of diminazene aceturate, imidocarb and clindamycin showed better efficacy in terms of resolution of clinical signs, improvement in haematological and clinico-pathological abnormalities and absence of parasitaemia. The study also showed that both diminazene aceturate and imidocarb dipropionate had equal efficacy against *B. canis vogeli* infection. Higher incidence of canine babesiosis among dogs in Thrissur district warrants the need for early and accurate diagnosis, prompt treatment and control of ticks in order to maintain a healthy canine population.