RESEARCH ARTICLE

Canine Babesiosis among Indian Native Dog Breeds: Molecular Detection and Clinical Investigation

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Abstract

Canine babesiosis is a tick-borne disease of canines caused by *Babesia canis* (large piroplasm) and *Babesia gibsoni* (small piroplasm). This study aims to report an outbreak of babesiosis in a Kennel housing 51 dogs of 19 Indian native dog breeds acquired from various states of India. A comprehensive disease investigation was conducted following sudden death of puppies with haemorrhagic enteritis. Samples were collected for virology, bacteriology, clinical pathology, and parasitological examination. PCR and RT-PCR was carried out to identify canine enteric viruses. For the diagnosis of haemoprotozoa (*T. evansi, A. platys, E. canis* and *Babesia* spp), PCR targeting VSG, 16srRNA, virB and 18srRNA genes, respectively, was carried out. On clinical examination dogs had severe tick infestation, melena and blanched conjunctival mucous membrane. The samples were negative for canine enteric viruses and bacteria of etiological significance, but were positive for *B. canis, B. vogeli* and *B. gibsoni*. The dogs were found negative for *T. evansi, A. platys* and *E. canis*. The ticks were identified as *Rhipicephalus sanguineus sensu lato (s.l.)*. Haematological examination showed anaemia, leukocytosis and thrombocytopenia, while serum biochemistry revealed elevated total protein, albumin, and creatinine. The study confirmed the outbreak due to *B. canis, B. vogeli* and *B. gibsoni*. Despite haematological and biochemical profiles indicating acute babesiosis, the native dogs showed good survival rate, suggesting their better tolerance against babesiosis than crossbred dogs.

Keywords: Canine babesiosis, Molecular diagnosis, Native dogs, Treatment.

Ind J Vet Sci and Biotech (2025): 10.48165/ijvsbt.21.1.18

Introduction

Canine babesiosis is a life threatening haemoprotozoan disease of canines, caused by *Babesia canis* or *Babesia gibsoni*. Morphologically, *B. gibsoni* is classified as small piroplasm measuring about 1-2.5 µm in size. In contrast, the size of *Babesia canis* ranges from 2.5-5.0 µm and are classified as larger piroplasm (Torti *et al*., 2014). Based on vector specificity, pathogenicity and genomic data, the large piroplasm has been divided into three subspecies, *viz. B. canis canis, B. canis vogeli* and *B. canis rossi* (Laha *et al*., 2014). The smaller piroplasm group includes *B. gibsoni*, *B. conradae*, and *B. vulpes* (Karasova *et al*., 2022; Irwin, 2009). *B. rossi* is the most virulent one among the large piroplasm which occurs most predominantly in South Africa. *B. canis* has intermediate virulence and is widely prevalent in European and Asian continents. Whereas, *B. vogeli* is more virulent in puppies and less pathogenic in adult dogs (Abalaka *et al*., 2018). Both *B. gibsoni* and *B. canis* are widely prevalent in all the states of India (Viswakarma and Nandini, 2018).

Canine babesiosis is also known as "Malignant Jaundice" and can manifest in either acute or subclinical forms. The clinical signs of babesiosis vary with the breed, age, and immune status of the dogs. In the acute form of the disease, clinical signs include fever, lethargy, inappetence, weakness, generalised lymphadenopathy, splenomegaly, anaemia, haemoglobinuria and collapse associated with intra-and extravascular haemolysis, hypoxia, thrombocytopenia, pigmenturia, sepsis syndrome and multiple organ failure (Obeta *et al*., 2020). Canine babesiosis has been reported worldwide and was first reported in India in 1910. The prevalence of canine babesiosis in Southern India has been reported as 11.6 % and in North India as 8.9% (Kumar *et al*., 2009). Canine babesiosis is mainly transmitted by ticks. The ixodid hard ticks are the main vectors

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How to cite this article: Jaisree, S., Sridhar, R., Jayanthi, N., Azhahianambi, P., Kalaiselvi, G., Ramya, R., Balakrishnan, G., & Soundararajan, C. (2025). Canine Babesiosis Among Indian Native Dog Breeds: Molecular Detection and Clinical Investigation. Ind J Vet Sci and Biotech. 21(1), 88-93.

Source of support: Nil

Conflict of interest: None

Submitted 22/11/2024 **Accepted** 29/12/2024 **Published** 10/01/2025

for *Babesia* spp. *Rhipicephalus sanguineus*, *Haemaphysalis ellipticum*, and *Dermacentor* spp. are capable of transmitting large Babesia in dogs. In contrast, *Haemaphysalis longicornis Haemaphysalis bispinosa* and *Rhipicephalus sanguineus* are well known vectors for *B. gibsoni* (Megat Abd Rani *et al*., 2010). Studies on the occurrence of babesiosis among native breed of dogs in India are limited. This study aims to report the occurrence of babesiosis in native breeds of dogs in India that have been maintained in an organized kennel.

Materials and Methods

History and Samples Collection

Samples were collected from an organized native dog kennel located at Chennai during October 2022. The kennel had 51 dogs belonged to 19 Indian native breeds acquired from different states of India. Initially the kennel reported death of two puppies with vomiting and haemorrhagic enteritis. Clinical examination revealed that some dogs were active and in fair body condition, while others were lethargic and poor in body condition with icteric mucous membrane. Further, the dogs had severe tick infestation. Blood samples, faecal samples, faecal swabs, and ticks were collected from 11 dogs for viral, bacterial, pathological and parasitological examination. The ticks were processed and mounted as per the standard procedure (Soundararajan *et al*., 2016) and examined under a stereo zoom microscope (Labomed®).

Haemato-Biochemistry Analysis

Haematological parameters were estimated using blood autoanalyzer (Mindray BC-2800). Serum biochemical parameters were estimated using commercial kits (BioSystems) in an auto-biochemical analyzer (BioSystems A15)

Microbial Examination

Isolation of bacteria from feacal swabs was carried out to identify enteric bacterial pathogens (Quinn *et al*., 1994). Canine enteric viruses, *viz.* canine parvovirus (CPV), canine circovirus (CCV), canine distemper virus (CDV), canine coronavirus (CCoV), canine adenovirus (CAV) were screened by PCR (Hao *et al*., 2019). DNA was synthesized from faecal swab and blood samples using the QIAmp Fast DNA stool mini kit and DNeasy blood and tissue kit (Qiagen), respectively, as per manufacturer's instruction. RNA was extracted using RNAisoplus (TAKKARA) and converted into cDNA using Verso cDNA synthesis kit (Thermo Scientific). The primers utilized in this study along with their cycling conditions were as given in Table 1. Faecal samples were examined microscopically for the presence of parasites. Blood smears were prepared from whole blood, fixed with methanol, and stained with Giemsa stain for microscopic examination under oil immersion objective (100X) (Soulsby, 1982).

Polymerase Chain Reaction for Canine Haemoprotozoa

Extraction of DNA from blood samples of dogs was carried out using DNeasy blood and tissue kit (Qiagen) as per manufacturer's protocol. Extracted DNA was used for the screening of canine haemoprotozoa, including *Babesia spp., E. canis,* and *T. evansi*. The genes VirB9 and VSG genes were targeted for the detection of *E. canis* and *T. evansi,* respectively (Ravindran *et al*., 2008; Kledmanee *et al*., 2009). Semi-nested PCR was carried out for the detection of canine babesiosis (Birkenheuer *et al*., 2003). For the initial detection of canine babesiosis the primer pair 455 F and 793 R targeting 18S rRNA gene were used. The reverse primer 793 R was kept common for the detection of subspecies. The forward primers BG-F, BCV-F and BCC-F were used for the specific detection of *B. gibsoni , B. canis subsp. vogeli* and *B. canis subsp. canis*, respectively. The list of primers used for the detection of canine haemoprotozoa is listed in Table 2. The PCR reaction mixture was prepared by mixing 12.5 µL of red dye master mix (Ampliqon®), 1 µL (10 pmol) of each forward and reverse primer, 1 µL of DNA, 9.5 µL of DEPC water. The reaction mixture was placed in a BioRad® C1000 Thermal cycler with the following conditions: initial denaturation at 94 °C, 5 min; followed by 35 cycles each of denaturation at 94˚C, 30 s, annealing at 56˚C, 45 s, extension at 72˚C, 45 s,

Table 1: Primers and cycling condition used in the diagnosis of canine enteric viruses (Hao *et al*., 2019)

Virus	Primer Name	Primer sequence (5'- 3')	Cycling condition	Product size
Canine Adeno virus	CAV-F	CGCTGARCAYTACTACCTTGTCTATATTTATG	94 °C, 5 min 35 cycles of 94˚C, 30 s 55˚C, 45 s, 72˚C, 1 min Final elongation at 72˚C, 10 min	1020 bp
	CAV-R	GGTAGAGCWCTTCGTGTCCGCTT		
Canine Parvo virus	CPV-F	AAGACGTGCAAGCGAGTCC		337 bp
	CPV-R	GAGCGAAGATAAGCAGCGTAA		
Canine Circo virus	CCV-F	GCGTTTACCTGTTCACCC		819 bp
	CCV-R	AACTGTTTCATCTGCGRCTG		
Canine Corona virus	CCoV-F	AGGAAGGCAACAATCCAATA		477 bp
	CCoV-R	GCCACCTCTGATGGACGA		
Canine Distemper	CDV-F	AGATTCAGCCATTTGTAGCCA		794 bp
	CDV-R	GTTGGACTACCTGAGCCCTA		

and final elongation at 72˚C for 10 min. PCR products were run on 2% agarose gel and documented with Bio-Rad Gel doc XR+ imager.

Results and Discussion

This study reports the occurrence of babesiosis in Indian native dog breeds. The history of vomiting, haemorrhagic enteritis and death of puppies suggested the involvement of canine enteric pathogens. Clinical examination revealed that some dogs were lethargic, had icteric conjunctival mucous membranes, and tick infestation raising the suspicion of haemoprotozoan diseases. Pyrexia is not a cardinal sign in canine babesiosis, and most of the affected dogs exhibit normal body temperature (Bilwal *et al*., 2017). The clinical signs in canine babesiosis are variable. Lethargy, anorexia, pale conjunctival mucous membrane and haemolytic aneamia are routinely observed in uncomplicated cases of babesiosis. In complicated cases, the disease is characterized by severe haemolytic anaemia with secondary multiple organ dysfunction syndrome (MODS) due to host's systemic inflammatory response (Ombowale *et al*., 2017). Hence, samples were collected to rule out bacterial, viral, and parasitic diseases. Blood and serum samples were also collected to assess the health status of the animal.

In haematological examination most of the dogs had low level of PCV, Hb concentration, thrombocytopenia, and leukocytosis (Table 3). Babesiosis is primarily a disease of RBCs. Low PCV, Hb and thrombocytopenias have been reported as characteristic features by several authors in canine babesiosis (Yogeshpriya *et al*., 2018). Anaemia has been reported due to mechanical damage caused by the parasite, intravascular haemolysis, immune and non-immune mediated destruction of RBCs (Karasova *et al*., 2022). Thrombocytopenia is due to sequestration of platelets bound with IgG in spleen or lysis of platelets by the inflammatory mediators released during the erythrocyte lysis. Leukocytosis is due to severe inflammatory reaction and said to be a major mechanism in the pathogenesis of complicated canine babesiosis (Welzl *et al*., 2001; Ombowale *et al*., 2017). Dogs that tested negative for *Babesia spp*. by PCR had thrombocytosis and these dogs may be in the recovery stage.

Serum biochemical analysis indicated elevated levels of total protein, albumin, and creatinine while, ALT and AST values remained within normal ranges (Table 4). Elevated protein levels may be due to haemoconcentration. Elevated creatinine levels might be due to inflammatory mediators-induced damage to the kidney cells (Welzl *et al*., 2001).

Table 2: Primers and cycling condition used in the diagnosis of canine babesiosis

Hemoprotozoa	Primer Name	Primer sequence (5'- 3')	Product size	Reference
Babesia sp.	455 F	GTCTTGTAATTGGAATGATGGTGAC	340 bp	Birkenheuer *et al*. (2003)
	793 R	ATGCCCCCAACCGTTCCTATTA		
B. gibsoni	BG - F	AAGACGTGCAAGCGAGTCC	185 bp	
B. canis subsp. vogeli	BCV-F	GCGTTTACCTGTTCACCC	192 bp	
B. canis subsp. canis	BGV-F	AGGAAGGCAACAATCCAATA	198 bp	
T. evansi	Tev-F	TGCAGACGACCTGACGCTACT	227 bp	Azhahianambi *et al*. (2018), Kledmanee *et al*. (2009)
	Tev-R	CTCCTAGAAGCTTCGGTGTCCT		
E. canis	Ehr1401F	CCATAAGCATAGCTGATAACCCTGTTACAA	377 bp	
	Ehr1780R	TGGATAATAAAACCGTACTATGTATGCTAG		

Table 3: Heamatological findings in dogs of different breeds with canine babesiosis

Breed	Dog name	Hb (g/dL)	PCV (%)	RBC (10^6/µl)	PLT 10^5/µl)	WBC 10^3/µl)	Inference
Reference range		12-19	37-57	5.0-10.0	160-510	5.0-15.0	
Chippiparai	Chippi	3.8	13.5	3.68	108	8.1	Severe anemic, thrombocytopenia
Gutkal	Bhokal	10.3	35.3	6.03	162	21.5	Leukocytosis
Bully kutta	Venba	6.8	25.6	6.61	747	15.0	Anemia and thrombocytosis
Indian Pariah	Ariyan	8.5	33.0	5.74	137	24.0	Anemia, leukocytosis, thrombocytopenia
Jonangi	Jonangi	10.3	42.9	7.26	372	16.8	Mild leukocytosis
Marwari sheep dog	Summer	5.1	17.9	4.90	432	15.0	Severe anemic changes
Indian Pariah	Adan	15.9	42.0	8.23	405	20.2	Normal
Kanni	Kuyil	6.7	21.5	4.93	182	17.0	Anemia
Mudhol	Jappy	12.8	41.3	5.91	588	15.7	Thrombocytosis
Chippiparai	Whity	12.4	40.2	6.65	1168	14.7	Severe thrombocytosis

Table 4: Biochemical findings in dogs of different breeds with canine babesiosis

Breed	Dog	Total Protein (g/dL)	Albumin (g/dL)	ALT (IU)	BUN (mg/dL)	Creatinine (mg/dL)
Reference range		5.4 – 7.1	2.3 - 3.3	10-109	8-28	0.5 – 1.8
Chippiparai	Chippi	9.9	5.6	37	14.4	1.94
Gutkal	Bhokal	8.6	3.4	25	11.2	1.73
Bully kutta	Venba	9.0	3.6	54	12.1	1.61
Indian Pariah	Ariyan	10.0	3.7	51	12.61	0.92
Jonangi	Jonangi	8.7	3.6	58	15.8	1.94
Marwari sheep dog	Summer	10.4	3.4	50	13.55	0.86
Indian Pariah	Adan	7.5	2.9	58	15.4	0.49
Kanni	Kuyil	8.9	3.1	39	12.2	1.41
Mudhol	Jappy	8.3	2.9	44	16.8	0.39
Chippiparai	Whity	9.3	3.2	54	16.8	0.62

Table 5: Microscopic examination and PCR analysis of blood samples collected from native dogs

Breed	Dog name	Microscopic examination of blood smears	PCR screening results				
			T.evansi	*E. canis*	*B. canis*	*B. vogeli*	*B.gibsoni*
Chippiparai	Chippi	+	-	-	+	+	-
Gutkal	Bhokal	-	-	-	+	+	-
Bully kutta	Venba	-	-	-	+	+	+
Indian Pariah	Ariyan	-	-	-	+	+	+
Jonangi	Jonangi	-	-	-	+	+	+
Marwari sheep dog	Summer	-	-	-	+	+	+
Indian Pariah	Adan	-	-	-	-	-	-
Kanni	Kuyil	-	-	-	-	-	-
Mudhol	Jappy	-	-	-	-	-	-
Chippiparai	Whity	-	-	-	-	-	-
Marwari sheep	Kulir	-	-	-	-	-	-

Out of ten Giemsa stained blood smears examined, only one blood smear was found positive for *Babesia* spp. In PCR examination, all the dogs tested negative for *E. canis* and *T. evansi*. In *Babesia* genus specific PCR, six dogs were found positive with an amplicon size of 340 bp. In species-specific singular PCR examination, *B. canis, B. vogeli* and *B. gibsoni* were detected in two dogs with amplicon sizes of 198 bp, 192 bp and 185 bp, respectively. In four dogs, *B. canis* and *B. vogeli* alone were detected (Fig. 1-3). PCR results are represented in Table 5. Thus, PCR examination identified co-infections with three species of babesiosis: *B. gibsoni, B. canis subsp. vogeli* and *B. canis subsp. canis*. PCR was highly sensitive and specific compared to microscopic detection in the diagnosis of babesiosis.

The success of detecting *Babesia* spp. by microscopic examination depends on several factors, including the level of parasitaemia, staining techniques and the quality of smear (Azhahianambi *et al*., 2018). In this study the clinical signs, PCR results and haemogram were indicative of an acute stage of infection, but microscopic examination failed to detect parasitaemia in blood smears. The erythrocytes were highly fragile, and erythrolysis was observed in most of the blood smears. The increased osmotic fragility and erythrolysis might be the reason for low level of positivity in blood smear examination. Furthermore, no canine enteric pathogens or bacteria of etiological significance were detected in affected dogs. Previous studies from Southern India indicated a higher prevalence of *B. gibsoni* than *B. canis* (Kumar *et al*., 2009; Latha *et al*., 2016). However, in this study, the prevalence of *B. canis* was found to be higher than that of *B. gibsoni*.

The ticks collected from the ailing dogs were identified as *Rhipicephalus sanguineus sensu lato (s.l.)* (Fig. 4). These ticks are the potential vector in the transmission of *B. gibsoni, B. canis,* and *B. vogeli* (Chao *et al*., 2017). Clinical signs, hemogram and serum biochemical profiles revealed that the dogs were suffering from an acute form of babesiosis, but the native dogs of India were survived the acute infection, indicating that the native dogs are susceptible to the *Babesia spp*. Infection, but are tolerant to babesiosis.

The dogs were treated with atovaquone (13.4 mg/kg, t.i.d, p/o) and azithromycin (10 mg /kg, s.i.d). It has been reported that no single drug is efficient in the treatment of canine babesiosis (Obeta *et al*., 2020). Hence, this combination was used to treat the dogs. Apart from antimicrobial therapy, the dogs were also treated with haematinics and oral fluralaner tablet for tick control. All the dogs recovered completely after two weeks of treatment.

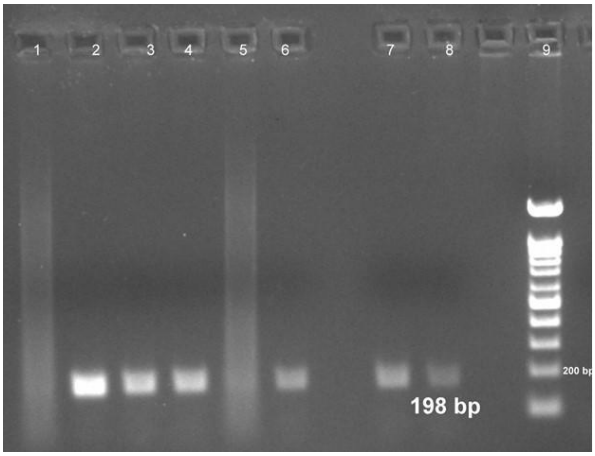

Fig. 1: Agarose gel electrophoresis image showing amplicons positive for *B. canis subsp. canis* (198 bp) . Lane 1 to 8 - samples; Lane 9 - 100 bp DNA ladder

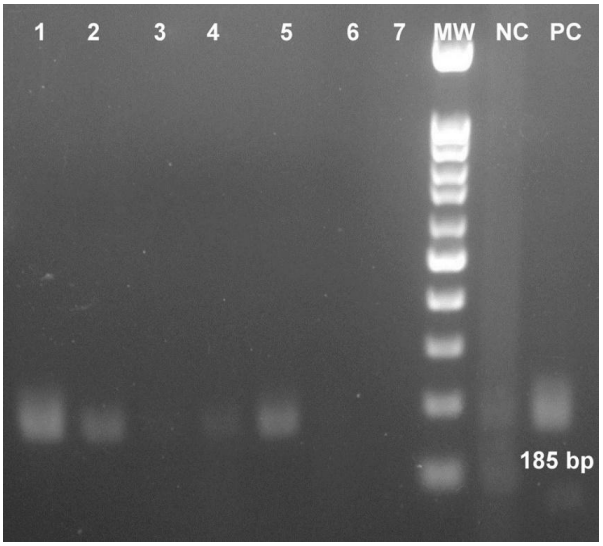

Fig. 2: Agarose gel electrophoresis image showing amplicons positive for *B. gibsoni* (185 bp). Lane 1-7 samples; MW- 100 bp DNA ladder, PC- Positive control; NC- Negative control

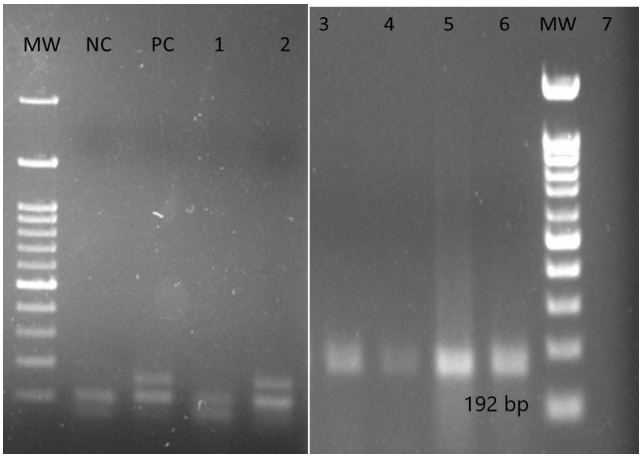

Fig 3: Agarose gel electrophoresis image showing amplicons positive for *B. canis subsp. vogeli* (192 bp). Lane 1-7 samples; MW- 100 bp DNA ladder, PC- Positive control; NC- Negative control

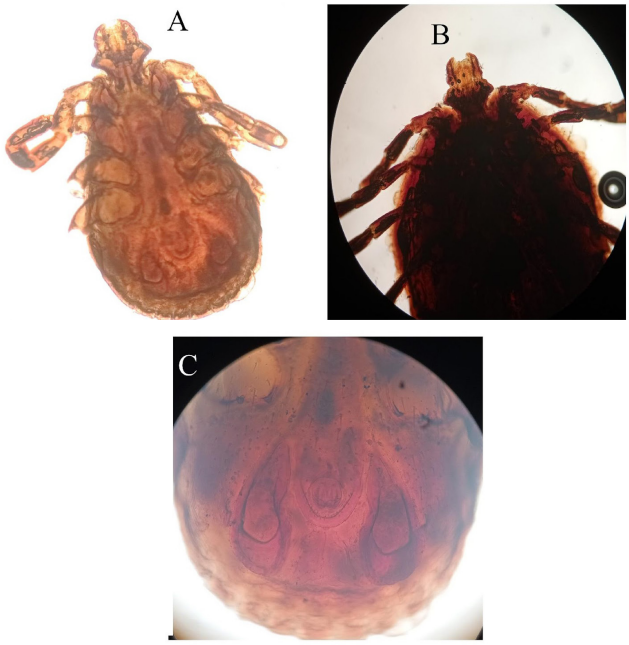

Fig. 4: Microscopic view of *Rhipicephalus sanguineus* s.l. tick collected from the native dogs. A. Ventral view of male tick showing hexagonal basis capitulum with festoons and bifid 1st coxa; B. Dorsal view of engorged female tick C. Anus of male tick showing adanal plate

Conclusion

This study reports an outbreak of canine babesiosis among native dog breeds in a kennel. PCR results confirmed that the outbreak was caused by co-infection with *B. gibsoni, B. canis subsp. vogeli* and *B. canis subsp. canis*. These findings were further supported by haemato-biochemical findings. PCR was more sensitive than microscopic examination in detecting Babesia *spp*. Despite the acute form of babesiosis, the native dogs survived well, indicating their tolerance to babesiosis. Moreover, this study found a higher prevalence of *B. canis* than *B. gibsoni*. The combination therapy with atovaquone and azithromycin was found to be highly effective in the treatment of canine babesiosis.

Acknowledgement

The authors thank Tamil Nadu Veterinary and Animal Sciences University for providing necessary facilities and financial support to carry out this study.

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