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The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; SP-10(12): 1167-1170

www.thepharmajournal.com
Received: 23-09-2021
Accepted: 03-11-2021

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Molecular detection of *Babesia gibsoni* in stray dogs of Southern Kerala

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Abstract
A molecular study was conducted to know the prevalence of *Babesia gibsoni* in stray dogs from Southern zone of Kerala. A total of (n=50) blood samples and smears were collected from Animal Birth Control Centers (ABCs) from Southern Zone of Kerala. Peripheral blood smears revealed *B. gibsoni* piroplasms in 20 per cent (10/50) of the samples. Primary PCR targeting 18S rRNA gene showed amplification at ~1665 bp in 42 per cent (21/50) of samples whereas by nested PCR using a set of internal primers which targets primary PCR product showed amplification at ~308 bp fragment in 56 per cent (28/50) of samples. This study showed a higher prevalence of *B. gibsoni* in stray dogs compared to previous studies on the prevalence of *B. gibsoni* in pet dogs. The nested PCR targeting 18S rRNA was more sensitive in detecting *B. gibsoni* compared to primary PCR.

Keywords: *Babesia gibsoni,* stray dogs, PCR, 18S rRNA

Introduction
India's diverse climatic zones, make it ideal for a wide range of vectors and pathogens, whose transmission and geographical distribution are closely linked to regional temperature, humidity and rainfall (Patz *et al*., 2005) [17]. Babesiosis, hepatozoonosis, trypanosomosis, ehrlichiosis and anaplasmosis are the major vector borne parasitic diseases prevalent in dogs in India. Co-infections of *Babesia* with *Ehrlichia*, *Hepatozoon*, *Bartonella*, *Anaplasma* and *Leishmania* have been reported in dogs (O'Dwyer *et al*., 2001) [16]. Babesiosis is one of the widespread haemoprotozoan diseases in dogs world-wide (Homer *et al*. 2000) [6]. Taxonomically *Babesia* is under the phylum Apicomplexa, class Aconoidasida, order Piroplasmida and family Babesidae (Taylor *et al*., 2016) [28]. The babesiosis causing organisms are classified based on the morphology of piroplasm within red blood cell as large (4-5 µm) (Eg: *B. canis*) or small (1-2.5 µm) forms (Eg: *B. gibsoni*) (Gallego *et al*., 2016) [25]. The *B. gibsoni* was reported first in India by Patton in 1910 (Patton, 1910). *Babesia gibsoni* is the most pathogenic species and is transmitted mainly by ticks (Jefferies *et al*. 2007; Schnittger *et al*. 2012) [9, 21]. There are different modes of transmission for canine babesiosis which include tick bite, blood transfusion, direct contact between dogs through wounds (fighting dogs), saliva or blood ingestion and transplacental transmission (Stegeman *et al*., 2003; Birkenheuer *et al*., 2005; Jefferies *et al*., 2007; Yeagley *et al*., 2009; Fukumoto *et al*., 2005, Adaszek *et al*., 2016) [26,10, 9, 5, 31]. Canine babesiosis can range from chronic or sub-clinical to per acute and fatal infection (Schoeman 2009) [22]. The clinical signs include fever, anaemia, icterus, thrombocytopenia and splenomegaly. The microscopic examination of peripheral blood smears does not allow for reliable identification of the parasites in sub-clinical and asymptomatic carriers (Rani *et al*., 2011) [18]. Parasite morphology being a poor guide to speciation, polymerase chain reaction (PCR) differentiates piroplasm species with higher specificity and sensitivity. Highly conserved 18S rRNA is used to differentiate genotype or sub-species of canine *Babesia* (Kjemtrup *et al*., 2000) [10].

Materials and Methods
A total of (n=50) peripheral blood smears and whole blood samples from saphenous vein of stray dogs in EDTA (ethylene diamine tetra acetic acid) vials were collected from apparently healthy animals brought to the Animal Birth Control centers and stored at -20°C until further processing. Peripheral blood smears were stained with Giemsa stain for 45 min. Genomic DNA was isolated from the blood samples using DNeasy blood and tissue kit (Qiagen,

Germany) according to the manufacture's protocol. The isolated DNA was stored at -20 ºC. All the PCR reactions were conducted in an automated thermal cycler with heated lid (M/s. Eppendorf, Hamburg, Germany). The PCR reaction was set up in a total volume of 25 µL reaction mixture as follows 12.5 µL of Master-mix, 1 µL of forward and reverse primer, 1.5 µL of template DNA and 9 µL of Nucleus free water. The primers targeting 18S rRNA gene were showed in (Table 1).

Primary PCR was performed based on the protocol described by Jefferies *et al*. (2007) [9] for the amplification of ~1665 bp fragment of 18S ribosomal RNA gene. The cycling conditions are as follows: initial denaturation at 94 ºC for 5 min followed by 35 cycles, consisting of a denaturation step of 1 min at 92ºC, an annealing temperature of 45sec at 52 ºC and an extension step of 2 min at 72 ºC. The final extension was performed at 72 ºC for 10 min.

The primary PCR products were used as a template for nested PCR. *B. gibsoni* species specific PCR based on the protocol described by Jefferies *et al*. (2007) [9] for the amplification of ~330 bp fragment of 18S ribosomal RNA gene. The cycling conditions are as follows: initial denaturation at 92 ºC for 2 min followed by 35 cycles, each consisting of denaturation at 92 ºC for 45sec, annealing temperature of 45 sec at 52 ºC and an extension step of 5 min at 72 ºC. the final extension was performed at 72 ºC for 5 min.

Results

The Peripheral blood smears (n=50) were examined under 100X objective of a compound microscope (Leica, Germany) of which (10/50) samples (20 per cent) were positive for *B. gibsoni* organisms (Fig. 1). The genus specific primers targeting 18S rRNA gene of *Babesia* spp. amplified at ~1665 bp fragment during primary PCR in 21/50 (42 per cent) samples (Fig. 2). The nested PCR using a set of internal primers amplified at ~308 bp fragment of 18S rRNA gene when the product of the primary PCR ~1665 bp was used as the template (Fig. 3). A total of 28/50 (56 per cent) samples were positive for *B. gibsoni* by nested PCR.

Discussion

Ticks and tick-borne diseases (TTBDs) cause severe impediments to human and animal health. The blood sucking habits of ticks complement the transmission of various pathogens like bacteria, virus, rickettsia and haemoprotozoans. Among the haemoparasitic infections in mammals, babesiosis caused by the organisms of the genus *Babesia*, occupies the second place after trypanosomosis (Schnittger *et al*., 2012) [21]. Both large and small forms of *Babesia* can cause considerable morbidity and mortality if they are not diagnosed and treated at the appropriate time. *Babesia vogeli* and *B. gibsoni* are the commonly distributed canine *Babesia* in the tropical and subtropical areas of the world. *Babesia gibsoni* is widely distributed in the Indian subcontinent and east Asian countries (Lee *et al*., 2010; Mandal *et al*., 2014; Laha *et al*., 2014; Terao *et al*., 2015) [12, 14, 11, 29]. The distribution of these organisms not only depend on the presence of ticks but also can be transmitted by dog bite, transplacental transmission and blood transfusion (Birkenheuer *et al*., 2005; Fukumoto *et al*., 2005; Adaszek *et al*., 2016) [5, 10].

In the present study, the prevalence of *B. gibsoni* in stray dogs was very high compared to that of previous reports from pet dogs of Kerala (Jain *et al*., 2017, Bora *et al*., 2021) [8, 3] as well as from other states of India (Rani *et al*., 2011; Singh *et al*., 2016; Mahalingaiah *et al*., 2017; Chandra *et al*., 2018; Sarma *et al*., 2019, Manoj *et al*., 2020, Sindhu *et al*., 2020) [18,4, 23, 15]. The reasons could be due to the abundance of tick vectors (Rani *et al*., 2011, Sahu *et al*., 2014) [18] and absence of treatment in community owned dogs (Traub *et al*., 2014; Sudan *et al*., 2015) [27]. Moreover, there are reports for possible transmission *B. gibsoni* through direct contact through wounds, saliva or blood ingestion (Irizarry-Rovira *et al*., 2001) [7]. These findings corrobates with the high prevalence of *B. gibsoni* in community owned dogs in the present study.

Table 1: Primers used for the detection of *Babesia gibsoni*

Target gene	Primer name	Oligonucleotide sequence (5' 3')	Product size (bp)	Reference
18S rRNA	Bg18S F (Primary)	TGGTTGATCCTGCCAG TA	1665 bp	(Jefferies *et al*. 2007) [9]
	Bg18S R (Primary)	CTTCTCCTTCCTTTAAGTGA		
	Bg18S F (Nested)	ATAACCGTGCTAATTGTAGG	308 bp	
	Bg18S R (Nested)	TGTTATTTCTTGTCACTACC		

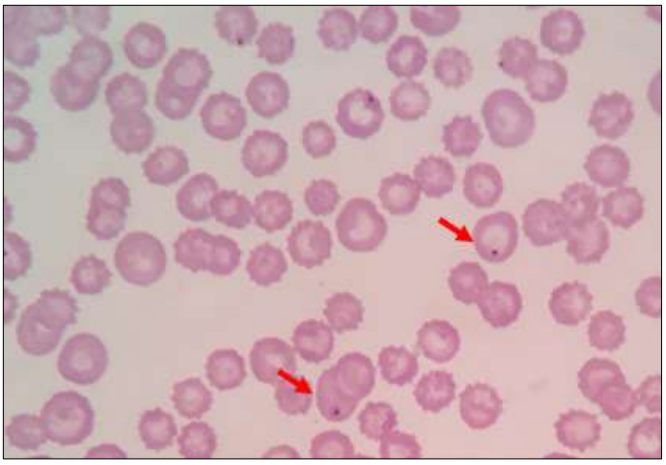
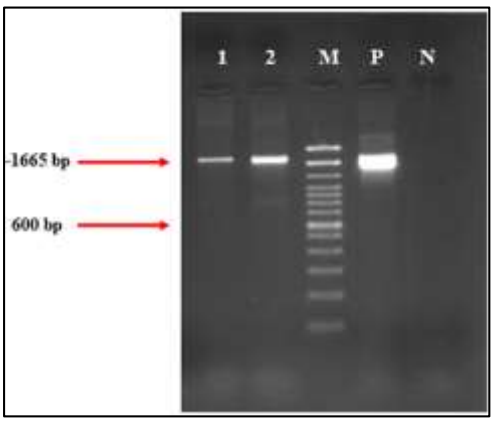
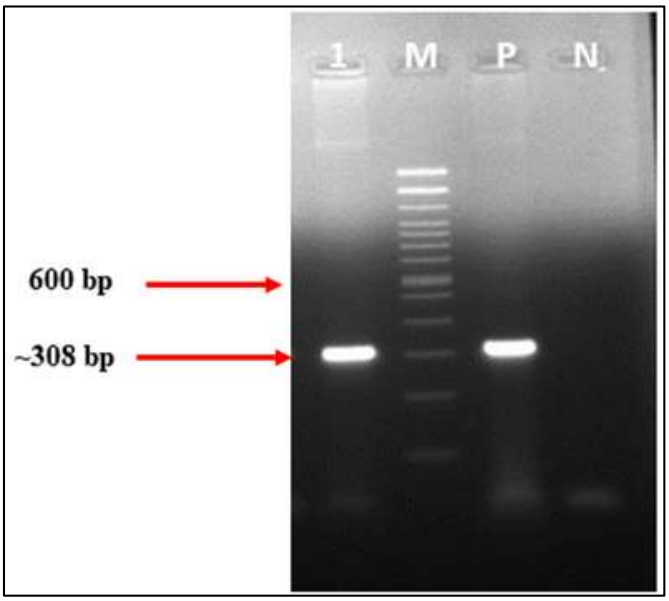


Fig 1: Blood smear of a stray dog showing ring shaped piroplasms of *B. gibsoni*



Lane 1,2: Samples, Lane M: 100 bp plus ladder, Lane P: Positive control, Lane N: Negative control

Fig 2: PCR amplification of 18S rRNA gene (~1665 bp) of *Babesia* spp.



Lane 1: Sample, Lane M: 100 bp plus ladder, Lane P: Positive control, Lane N: Negative control

Fig 3: Nested PCR amplification of 18S rRNA gene (~308 bp) of *Babesia gibsoni*.

Conclusion

In the present investigation, the prevalence of *B. gibsoni* in stray dogs was very high compared to that of previous reports from pet dogs of Kerala. The nested PCR targeting 18S rRNA was more sensitive in detecting *B. gibsoni* compared to primary PCR.

Acknowledgements

This work was supported financially by Kerala Veterinary and Animal Sciences University, RKVY-RAFTAAR-2019-20 project (KE/RKVY-ANHB/2019/1422) and State plan project- (2021-22) (RSP/21-22/VI–7).

References

1. Adaszek L, Obara-Galek J, Piech T, Winiarczyk M, Kalinowski M, Winiarczyk S. Possible vertical transmission of *Babesia canis canis* from a bitch to her puppies: a case report. Vet Med. 2016;61:263-266.
2. Birkenheuer AJ, Correa MT, Levy MG, Breitschwerdt, EB. Geographic distribution of babesiosis among dogs in the United States and association with dog bites: 150 cases (2000–2003). J. Am. Vet. Med. Assoc. 2005;227:942-947.
3. Bora CAF, Varghese A, Deepa CK, Nandini A, Malangmei L, Kumar KGA, *et al*. Sequence and phylogenetic analysis of the thrombospondin-related adhesive protein gene of *Babesia gibsoni* isolates in dogs in South India. Parasitol. Int. 2021;86:102-477.
4. Chandra BS, Rajkumar K, Das SS, Vijayalakshmi P, Abiramy Prabavathy A. Prevalence of *Babesia gibsoni* Infection in Dogs of Puducherry Region. Int. J Curr. Microbiol. App. Sci. 2018;3:1275-1278.
5. Fukumoto S, Suzuki H, Igarashi I, Xuan X. Fatal experimental transplacental *Babesia gibsoni* infections in dogs. Int. J Parasitol. 2005;35:1031- 1035.
6. Homer MJ, Aguilar-Delfin I, Telford SR, Krause PJ, Persing DH. Babesiosis. Clin. Microbiol. Rev. 2000;13:451-469.
7. Irizarry-Rovira AR, Stephens J, Christian J, Kjemtrup A, DeNicola DB, Widmer WR. *Babesia gibsoni* infection in a dog from Indiana. Vet. Clin. Pathol. 2001;30:180-188.
8. Jain KJ, Lakshmanan B, Syamala K, Praveena JE, Aravindakshan T. High prevalence of small Babesia species in canines of Kerala, South India. Vet. World. 2017;10:1319-1323.
9. Jefferies R, Ryan UM, Irwin PJ. PCR–RFLP for the detection and differentiation of the canine piroplasm species and its use with filter paper-based technologies. Vet. Parasitol. 2007;144:20-27.
10. Kjemtrup AM, Kocan AA, Whitworth L, Meinkoth J, Birkenheuer AJ, Cummings J, *et al*. There are at least three genetically distinct small piroplasms from dogs. Int. J Parasitol. 2000;30:1501–1505.
11. Laha R, Bhattacharjee K, Sarmah PC, Das M, Goswami A, Sarma D. *Babesia* infection in naturally exposed pet dogs from a north-eastern state (Assam) of India: detection by microscopy and polymerase chain reaction. J. Parasit. Dis. 2014;38:389-393.
12. Lee CC, Hsieh YC, Huang CC, Tsang CL, Chung YT. Sequence and phylogenetic analysis of the thrombospondin-related adhesive protein (*TRAP*) gene of *Babesia gibsoni* isolates from dogs in Taiwan. J. Vet. Med. Sci. 2010;72:1329 -1335
13. Mahalingaiah MKC, Asoor M, Thimmaiah RP, Narayanaswamy HD, Mukartal SY, *et al*. Prevalence of canine babesiosis in different breeds of dogs in and around Bengaluru. Adv. Anim. Vet. Sci. 2017;5:140-144.
14. Mandal M, Banerjee PS, Garg R, Ram H, Kundu K, Kumar S, *et al*. Genetic characterization and phylogenetic relationships based on 18S rRNA and ITS1 region of small form of canine *Babesia* spp. from India. Infect. Genet. Evolution. 2014;27:325-331.
15. Manoj RRS, Iatta R, Latrofa MS, Capozzi L, Raman M, Colella V. Canine vector-borne pathogens from dogs and ticks from Tamil Nadu, India. Acta tropica. 2020;203:105-308.
16. O'Dwyer LH., Massard CL, de Souza JCP. *Hepatozoon canis* infection associated with dog ticks of rural areas of Rio de Janeiro State, Brazil. Vet. Parasitol. 2001;94:143-150.
17. Patz JA, Campbell-Lendrum D, Holloway T, Foley JA: Impact of regional climate change on human health**.** Nature. 2005;438:310-317.
18. Rani PAMA, Irwin PJ, Coleman GT, Gatne M, Traub RJ. A survey of canine tick-borne diseases in India. Parasit. Vectors. 2011;4:141.
19. Sahu A, Mohanty B, Panda MR, Sardar KK. Incidence of haemoprotozoan parasites in dogs in and around Bhubaneswar, Odisha. Indian vet. J. 2014;91:93-95.
20. Sarma K, Nachum-Bial Y, Kumar M, Baneth G. Molecular investigation of vector-borne parasitic infections in dogs in Northeast India. Parasit Vectors. 2019;12:1-8.
21. Schnittger L, Rodriguez AE, Florin-Christensen M, Morrison DA. *Babesia*: a world emerging. Infection, Genet. Evolution. 2012;12:1788-1809.
22. Schoeman JP. Canine babesiosis. *Onderstepoort* J. Vet. Res. 2009;76:59-66.
23. Sindhu BS, Shobhamani B, Suresh K, Chengalva V. Clinico-haemato biochemical alterations and Electrocardiograhy findings in *Babesia* infected dogs. 2020.

24. Singh MN, Raina OK, Sankar M, Rialch A, Tigg MN, Kumar GR, *et al*. Molecular detection and genetic diversity of *Babesia gibsoni* in dogs in India. Infect. Genet. Evol. 2016;41:100-106.
25. Solano-Gallego L, Sainz Á, Roura X, Estrada-Peña A, Miró G. A review of canine babesiosis: the European perspective. Parasit. Vectors. 2016;9:1-18.
26. Stegeman JR, Birkenheuer AJ, Kruger JM, Breitschwerdt EB. Transfusion-associated *Babesia gibsoni* infection in a dog. J Am. Vet. Med. Assoc. 2003;222:959-963.
27. Sudan V, Jaiswal AK, Shanker D, Kanojiya D, Sachan A. Prevalence of endoparasitic infections of non-descript dogs in Mathura, Uttar Pradesh. Parasite. 2015;39:491-494.
28. Taylor MA, Coop RL, Wall RL. *Veterinary Parasitology* (2nd Ed.). Set in 9/11pt Minion Pro by Aptara Inc., New Delhi, India. Terao M, Akter S, Yasin MG, Nakao R, Kato H, Alam MZ. Molecular detection and genetic diversity of *Babesia gibsoni* in dogs in Bangladesh. Infect. Genet. Evol. 2016;31**:**53-60.
29. Terao M, Akter S, Yasin MG, Nakao R, Kato H, Alam MZ. Molecular detection and genetic diversity of *Babesia gibsoni* in dogs in Bangladesh. Infect. Genet. Evol. 2015;31:53-60.
30. Traub RJ, Padnekar RP, Cuttell L, Porter RB, Abd Megat Rani PA, Gatne ML. The prevalence and distribution of gastro intestinal parasites of stray and refuge dogs in four locations in India. Vet. Parasitol. 2014;205:233-2.
31. Yeagley TJ, Reichard MV, Hempstead JE, Allen KE, Parsons LM, White MA *et al*. Detection of *Babesia gibsoni* and the canine small Babesia 'Spanish isolate' in blood samples obtained from dogs confiscated from dogfighting operations. J Am. Vet. Med. Assoc. 2009;235:535-539.