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Taqman based real time polymerase chain reaction (qPCR) for diagnosis of acute infections and carrier status due to *Babesia gibsoni* among dogs in Thrissur district, Kerala state

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Abstract
Canine babesiosis is a life-threatening disease caused by tick transmitted protozoan parasites that infect erythrocytes, belonging to genus *Babesia*. Important *Babesia* species responsible for canine infections include *B. gibsoni* & *B. canis*. Both the organisms are transmitted by *Rhipicephalus* or *Haemaphysalis* tick vectors and are reported worldwide. A total of 357dogs suspected for various tick transmitted infections presented to the veterinary surgeon, clinical laboratory during a six- year period from 2014 to 2020 were included in the study. Ticks (25 Nos) as a whole were collected randomly from dogs with severe infestation at the time of presentation and also processed as templates for molecular diagnosis. Two hundred and nine samples-209 (58%) out of 357canine samples and 20 (80%) out of 25 tick templates studied were found to harbor *B. gibsoni*. The results of the present study show that Taqman based Real Time PCR can be reliably used for the qualitative detection of *B. gibsoni* in EDTA blood samples and tick templates. To the authors knowledge this is the first report of RT PCR-based molecular detection of *B. gibsoni* among canine population and in ticks recovered from them in Thrissur district, Kerala state.

Keywords: *Babesia gibsoni*, dogs, real time PCR, 18SrRNA gene

1. Introduction
Cases of canine babesiosis due to *Babesia gibsoni* or *canis* may be present with a wide variation in clinical signs-ranging from a hyper acute, shock associated hemolytic crisis to an in apparent subclinical infection. Diagnosis of canine babesiosis is usually based on history, clinical signs, complete blood count and conventional microscopy. Conventional microscopy will detect acute infections but for identification of carrier status among dogs, it is highly essential to employ molecular diagnostic methods. There are lot of publications based on conventional PCR for diagnosis of *B. gibsoni*, but diagnostic methods based on Real Time PCR protocols is very less. This paper describes standardization of RT PCR protocols for detection of *B. gibsoni* from canine peripheral EDTA blood samples collected from dogs showing acute clinical signs, apparently normal dogs and in templates prepared from ticks as a whole.

2. Materials and Methods
2.1 Samples
A total of 382 samples were processed for the study (357 peripheral blood samples & 25 whole tick specimens). Three hundred and fifty seven (357) peripheral blood samples in EDTA were collected from dogs presented to the veterinary surgeon, clinical laboratory, with any one of the following clinical signs, suggestive of various tick transmitted diseases including *Babesia gibsoni*, *viz*; fever, anorexia, lethargy, pale mucus membrane, lymph node enlargement, convulsions, recurrent dermatological problems and infertility, brought for treatment purpose and diagnosis of etiological agent during a six year period from 2014 to 2020, formed the material for the study. Out of 357 dogs, 49 were police dogs who had undergone training at State Dog Training School (SDTS), Kerala Police Academy (KEPA), Thrissur district during the period (2014-15 & 2015-16), 66 police dogs presently (2020-2021) undergoing training at SDTS, KEPA under various trades like operations patrol, cadaver detection, search and rescue (SAR) and Kerala anti- terrorist squad (KATS), 6 police dogs

maintained with District Police Chief, Thrissur City during the period from 2014 to 2018, 6 police dogs maintained with India Reserve Battalion (IRB), 6 police dogs maintained at Central Prison, Viyyur (2014-18), Thrissur district, 8 pups belonging to Indian breeds selected to Mounted Police Unit, Thiruvananthapuram during 2018, and remaining 216 were domesticated or owned dogs brought for treatment. Out of 357 dogs only 80 (4 out of 49 from SDTS KEPA 2014-2016 batch, all 66 from SDTS KEPA 2020-21 batch, 2 out of 6 from prison dogs, and 8 out of 216 owned dogs) were showing acute clinical signs suggestive of *B. gibsoni viz*; high fever, pallor of mucus membrane and convulsions. All other dogs were not showing any acute or active clinical signs like rise in temperature but showing inapparent clinical signs like recurrent dermatological problems and infertility. About twenty-five (25) tick specimens collected randomly from dogs with severe tick infestation at the time of presentation, were also processed as templates for the study.

From each dog the following samples were collected, peripheral blood smear for Giemsa staining, peripheral blood in EDTA for preparation of template DNA and whole blood for hematology. All the dogs presented were sampled only once for the purpose, as and when they are presented with any one of the clinical signs described above. Hematological parameters were studied for all the 357 dogs on the same day as and when they are presented.

2.2 Peripheral blood smear

Peripheral blood smear was stained with Giemsa stain as per the standard protocols and examined for the presence of any inclusions inside the RBC's.

2.3 DNA extraction

Peripheral blood in EDTA was stored at- 80°C until usage and processed for preparation of template DNA as per the protocols described in DNA isolation kit for mammalian blood (Version7) and High Pure PCR template preparation kit (Version 23-Roche Diagnostics). Ticks collected from infected dogs were stored at -80°C and processed for preparation of template DNA as per protocols described in DNA isolation kit for cells and tissues (Version7&23-Roche Diagnostics). The DNA templates prepared from whole blood and ticks were stored at -80°C until usage.

2.4 Estimation of concentration of Template DNA

The DNA templates prepared from whole blood were processed for estimation of DNA concentration as per protocols described for (Invitrogen-Qbit ds DNA High Sensitivity Assay Kit) and estimated in Qbit.3.0 Fluorometer (Thermo Fischer Scientific, USA).

2.5 Primers and Probes for PCR

Two pairs of forward, reverse, sense and antisense primers and probe sequences targeting a specific region of *B. gibsoni* 18SrRNA gene partial sequence (Accession number KF171473) were designed and used in the study. Primers targeting specific region of *B. gibsoni* 18SrRNA gene-which produces a 144 bp amplicon with a Taqman probe labeled at the 5' and 3' ends with dye FAM (5'Caboxy Fluorescein) and BBQ respectively were used (Table.1)

Table 1: Details of primers and probes used for qPCR

Oligo Name	Primer Sequence (5'-3')	Product Size	Target Genome
B.g_F,	F-TggAATgATggTgACgTAAAATCT	144bp	18SrRNA
B.g S	S-gTgACgTAAAATCTCACCAgAgTAACA		
B.g A	A-AAggCAAgTAgCCgAgTCg- R-AgACAAggCAAgTAgCCgAgT		
B.g_R, Probe-B.gP	F-ACgCTATTggAgCTggAATTACggC-BBQ		

2.6 Optimized conditions for Real Time PCR

Format for Real Time PCR followed– with total reaction volume of 20 µl, using Fast Start Essential DNA Probes Master 2X concentration -10µl, 1 µl each of forward, sense, antisense and reverse primer (10µM), 2 µl of probe (10µM), 1 µl of water and 3 µl of template DNA extracted from whole blood and ticks. Negative and positive controls were included in all the runs.

Babesia gibsoni positive control was prepared by custom synthesizing entire sequence of specific region of *B. gibsoni* 18SrRNA gene and cloned into *pUGM* Plasmid (SciGenom Labs PVT Ltd, Kakkanad, Cochin). Custom synthesized positive clone in *pUGM* plasmid was used for primer efficiency testing and standardization of cycling conditions, optimal for detection of 18S rRNA gene from suspected samples. Five (5µl) of clone (initial concentration-10ng/µl) was diluted to serial one in tenths concentration and 5µl from each 1:10 dilution was used as positive control in reaction mixture. Six serial dilutions in duplicate were prepared for the experiment and all the dilutions were tested in RT PCR assay to find out the limit of detection and efficiency of the primers designed. Cycling conditions optimized for primer efficiency testing and for testing of samples after standardization with positive control, included an initial activation (preincubation) at 95 °C for 10 min followed by 40 cycles of 15s denaturation at 95 °C followed by a 1 min annealing - extension step at 60 °C.

3. Results

3.1 Peripheral blood smear

Freshly prepared peripheral blood smears prepared from the ear tip of dogs, stained with overnight Giemsa stain revealed the presence of small, single, signet ring shaped trophozoites of the parasite in more than 50% of erythrocytes were considered as positive. The trophozoites could be demonstrated by peripheral blood smear examination only in 80 dogs (22%) which were also showing acute clinical signs suggestive of *Babesia gibsoni*.

3.2 Estimation of concentration of Template DNA

DNA concentrations were estimated for all the template DNA samples prepared from whole blood prior to Real Time PCR experiments.

3.3 Primers and Probes and Optimized conditions for Real Time PCR

Real Time PCR for amplifying specific fragments of the 18SrRNA gene of *B. gibsoni* was used for qualitative detection of target genome in blood samples collected from 357 canine cases and in 25 tick templates collected randomly from infected dogs. The cycling conditions followed was optimal and successfully amplified *Babesia gibsoni* 18SrRNA

gene with specific primers B.g_F, B.g_S, B.g_A, B.g_R, along with a Taqman probe B.g_P (Fig.1). Amplified products after sequencing and identity checking with NCBI Blast showed 100% identity to *Babesia gibsoni* small subunit RNA sequences reported earlier and available in data base.

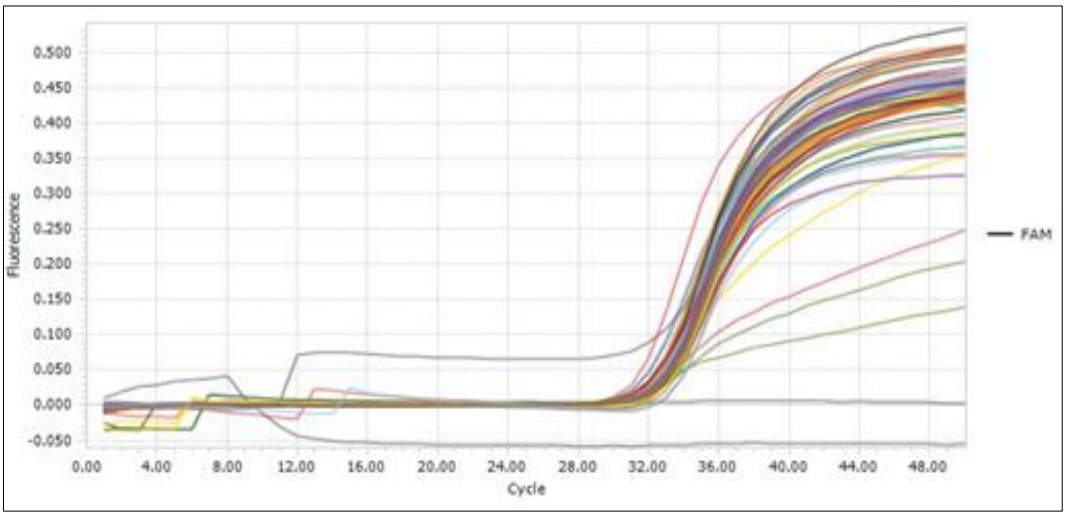

Fig 1: Amplification curve of positive samples of *Babesia gibsoni*

3.4 Primer efficiency testing

Primer efficiency testing was conducted by plotting a standard curve, custom synthesized positive clone in *pUGM* plasmid, with initial concentration 10ng/µl was used as template for the plotting of standard curve. Six tubes in duplicate with serial 1:10 dilutions prepared for the experiment showed, change of approximately 3.3 cycles between 10fold dilutions of the template. The slope of the standard curve generated after primer efficiency testing showed an efficiency of 1.97 and R value 1.00 (Fig.2).

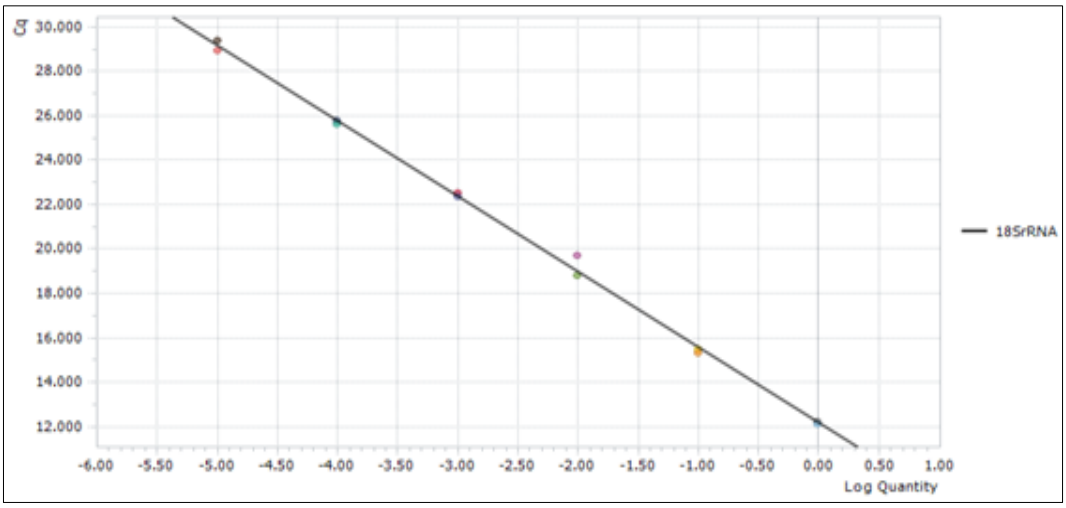

Fig 2: Slope of Standard Curve -Primer efficiency

Table 2: Slope of Standard Curve -Primer efficiency

Gene Name	Slope	Efficiency	R^2	Y Intercept
18SrRNA	-3.4021	1.97	1.00	12.16

3.5 Results of the test samples

The samples were considered positive with threshold cycle (Ct/Cq) levels less than 40 (Fig.1). The average Ct value observed for positive samples was 29.12+0.5 cycles ranging from 1.25 to 40.06 cycles. Out of the 49 Police Dogs from SDTS, KEPA (2014-16) 33 dogs (67%) were found to harbor *B. gibsoni* 18SrRNA gene, Out of 66 dogs from SDTS KEPA (2020-21) all (100%) were found to be positive, all 6 dogs (100%), maintained with District Police Chief, Thrissur City, India Reserve Battalion (100%) and Central Prison, Viyyur (100%), were found to be positive, all 8 Indian breed pups selected for police force during 2018 turned out to be positive (100%) and 85out of 216 domesticated dogs screened were found to be positive (39%). All together 210- 58% (125 police dogs &85 owned dogs) out of total 357 dogs and 20 (80%) out of 25 tick specimens tested were found to harbor *B. gibsoni* indicating a very high prevalence among canine population studied.

3.6 Hematological and serum biochemical parameters

There were abnormalities in hematological parameters studied, in 80 dogs which were showing acute canine babesiosis due to *Babesia gibsoni.* These dogs had significantly reduced hemoglobin values (2-4g%), low PCV (<=10%) and reduced platelet count (50- 100 X 109/L). There was not much abnormality in serum biochemical parameters- AST, ALT, ALP, total bilirubin, but there were elevated levels of TP, ALB & Globulin, low serum Ca, elevated levels

of P. Most of the dogs presented with acute clinical signs were treated immediately for the cause with triple antibiotic therapy-clindamycin @11mg/kg, metronidazole@22mg/kg intravenously for 10 days and oral doxycycline@10mg/kg SID for 28 days along with liver correctives, hematinic and supplements containing all essential amino acids/vitamins orally. All the animals responded very well to the treatment protocol followed and recovered fully, except for one dog (Labrador) belonging to prison dog squad dead in between treatment schedule (3rd day of treatment schedule). Dog had splenomegaly which was evident from ultrasonography of abdomen and PM examination after death (Fig.3,4). All other dogs were given same treatment orally to eliminate carrier status and appropriate measures were taken for long term tick control and educating pet owners that they may suffer relapse of disease in future.

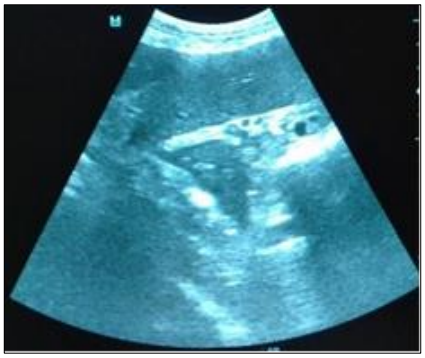

Fig 3: Enlarged spleen-Trans abdominal ultrasonography

Fig 4: Same spleen in USG- splenomegaly after PM-Extramedullary hematopoiesis, megakaryocytes and other hemopoietic cells. The greyish white areas correspond to foci of infarction

The clinical signs described previously *viz*; fever, anorexia, lethargy, pale/papery white mucus membrane, lymph node enlargement, convulsions, recurrent dermatological problems not responding to routine treatment and infertility in canines can be suggestive of acute clinical signs or carrier status of *B. gibsoni* and the results of present study indicate that the Taqman based qualitative Real Time PCR (qPCR)can be used as an accurate and confirmatory diagnostic method to detect the presence of *B. gibsoni* in the peripheral blood samples. This can be used as a diagnostic tool not only for canines presented with acute clinical signs but also helpful to identify carrier status. Identification of carrier status will be helpful for preventing disease in future and appropriate education of pet owners.

4. Discussion

Babesia gibsoni and *B. canis* comprise the two main species causing natural infections in dogs in India. *B. canis* is grouped into three phylogenetic groups varying in their geographical distribution, vector specificity and antigenic properties. These subspecies include *Babesia canis canis*, found in Europe; *B. canis vogeli*, in North and South Africa, North America and Brazil; and *B. canis rossi*, in South Africa [1, 2, 3, 4].

Babesia gibsoni is a much smaller parasite which has a more limited distribution and characteristically causes a chronic disease with progressive, severe anemia that is not readily treated with normal babesia ides. Naturally occurring cases of babesiosis in dogs were manifested by a wide variety of nonspecific vague clinical signs [5]. Animals that survived babesiosis may become sub clinically infected and suffer a relapse of disease in future and may serve as a point source for further spread of disease in a given area [6]. In the present study also only 80 out of 357 dogs were showing acute clinical signs and majority were showing subclinical infection or carrier status which is very well detected by Taqman based RT PCR protocols used in present study.

Babesia infection causes disease with clinical manifestations that may vary with the different species and strains involved and their specific virulence, and also with factors that determine the host's response to infection such as age, individual immune status, and the presence of concurrent infections or other diseases [5, 7, 8]. During the acute stage of the disease the number of parasites inside the erythrocytes increases in such a way that they can be detected microscopically, however, in chronically infected animals where a subclinical form of the disease occurs, this method is useless and other, more sophisticated methods must be employed. In the present study also only in those animals showing acute clinical signs (80/357) *B. gibsoni* inclusions could be demonstrated by direct microscopy, which is in accordance with the finding that in a subclinical form of disease more sophisticated methods like RT PCR must be employed [10].

Immunological methods to detect *Babesia* have the disadvantage of relying on the presence of specific antibodies against those parasites, which may take days or weeks to develop in an infected animal or they are present for months after the infection has disappeared, making their usefulness very limited in acute disease cases, vaccinated animals or cleared-by- treatment animals. Molecular methods aimed to detect nucleic acids have been very useful when immunological methods do not work [9].

The first descriptions of the polymerase chain reaction to detect *Babesia* were reported in 1992 for *B. bovis* [10], *B. bigemina* [11] and *B. microti* [12]. The first RTPCR method reported for the quantification of *Babesia* was in 2003 when SYBR Green was used to quantify the transcription of the *B. bigemina* rap-1 locus genes [13]. Since then, several protocols have been published for the quantification of *B. gibsoni*, *B. microti*, *B. bovis*, *B. bigemina*, *B. caballi*, *B. canis*, and *B. orientalis*, [14-21]. The sensitivity of RT-PCR has been reported to be also higher than that of conventional PCR, for *B. bovis* and *B. bigemina*, it was reported to detect 0.75 copies of DNA per µl of blood [18]. As per reports of Augustine *et al*., 57.5% prevalence of *Babesia* species infections among dogs through

a molecular survey using semi nested PCR protocol [22] was earlier published. Their study revealed a very high prevalence of *B. gibsoni* (50%) infections when compared with *B. canis vogeli* (7%) infections in the state of Kerala. In the present study also 58% prevalence of the *B. gibsoni* was observed among canine population studied in and around Thrissur District, Kerala state using real time PCR protocols when compared to semi nested PCR protocols described previously [22].

Canine babesiosis is a tick-borne disease caused by several Babesia spp. which have different susceptibility to anti-protozoal drugs. A few drugs and drug combinations are used in the treatment of canine babesiosis often without complete parasite elimination leaving treated dogs as carriers which could relapse with clinical disease and also transmit infection further. Although the large form canine babesia species *B canis*, *B vogeli* and *B. rossi* are sensitive to the aromatic diamidines imidocarb dipropionate and diminazene aceturate, small form species such as *B gibsoni*, *B conradae* and *B. vulpes* (*Theileria annae*) are relatively resistant to these drugs and are treated with the combination of the hydroxynaphthoquinone atovaquone and the antibiotic azithromycin. The triple combination of clindamycin, diminazene aceturate and imidocarb dipropionate is also effective against *B. gibsoni* and used to treat atovaquone-resistant strains of this species. Antibiotics with some anti-protozoal activity such as doxycycline, minocycline, clindamycin, enrofloxacin and metronidazole have been evaluated for the treatment of canine babesiosis with various levels of efficacy [23]. In the present study also triple combination of antibiotics like clindamycin, metronidazole and doxycycline were found very much effective in treating acute cases of canine babesiosis and all cases treated had uneventful recovery except for one dog which had splenomegaly.

To conclude RT PCR protocols developed in present study is a very accurate and sensitive method not only to identify acute infections but also to determine carrier status among canine population which will be of great help to clinicians in canine practice as well as to pet owners. Direct Microscopy alone in routine diagnostics can be misleading because parasite can be identified only in those dogs with acute clinical signs and demonstration of parasite in peripheral blood smear requires good staining techniques, good microscope and expertise/experience in identifying the parasite. To authors knowledge this is the first report of qualitative RTPCR (qPCR) based diagnostic protocols for detection of *Babesia gibsoni* among canine population in Kerala state.

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Competing interests

The authors declare that they have no competing interests.

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