RESEARCH ARTICLE

Molecular Detection of *Babesia motasi* in a Goat – First Report from Kerala

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Abstract

Caprine babesiosis is a tick borne haemoprotozoan infection less frequently reported from Kerala. In the present report, we document a confirmed case of *Babesia motasi* for the first time in the state. A Sirohi Kathiavari crossbred goat was presented to the University Veterinary Hospital (UVH), Kokkalai with symptoms of anorexia and pyrexia. On blood smear examination, large and small forms of *Babesia* organisms in Giemsa's stain could be detected. Complete blood count revealed hypochromic microcytic anaemia with thrombocytopenia. Molecular confirmation was done with polymerase chain reaction (PCR) targeting 18S rRNA gene. A 390 bp fragment was obtained and sequenced which was confirmed as *Babesia motasi*. *Babesia ovis* or *Theileria* spp., could not be detected by PCR. This study reports the molecular confirmation of two forms of *B. motasi* in a crossbred goat from Kerala.

Key words: *Babesia*, goat, Morphometry, Polymerase chain reaction (PCR), Tick-borne disease.

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Introduction

Among tick-borne haemoprotozoan diseases, babesiosis is an economically important disease of small ruminants that affects the livestock production (Galon *et al*., 2022). It is caused by the intraerythrocytic protozoan parasite of the genus *Babesia* spp. such as *B. ovis, B.motasi* and *Babesia crassa* (Friedhoff, 1988). Among them, *B. motasi* and *B. ovis* are considered to be pathogenic in small ruminants (Kaufmann, 1996). In India, the most pathogenic species in goats is *B. motasi* (Ajith *et al*., 2017). *Babesia motasi,* the large (2.5-4 x 2µm size) pyriform-shaped organisms found in pairs at acute angles inside RBCs cause severe fever and haemolytic anaemia, while *B. ovis* (1-2.5µm size), the small round organisms found towards the margin of RBCs cause mild signs of jaundice, anaemia and haemoglobinuria (Taylor *et al*., 2007).Ixodid ticks like *Rhipicephalus* spp. and *Haemaphysalis* spp. are the main vectors for *B. ovis* and *B. motasi* respectively (Fakhar *et al*., 2012).

Identification of *Babesia* spp. in stained blood smears is considered as ''gold standard'' in the diagnosis of babesiosis (Kage *et al*., 2019). However, the low sensitivity of this method does not permit its use in epidemiological investigations (Aktas *et al*., 2005). Serological methods are frequently used to detect subclinical infections of *Babesia* spp. These methods lack specificity due to cross-reactivity with other species of *Babesia* leading to false-positive results (Aktas *et al*., 2005). Based on morphology, large and small forms of *Babesia* spp., have been described in blood smears by several authors. Christensson and Thunegard (1981) reported *B. motasi* as large (3.1 × 1.9 µm) and small forms (2.2 × 1.8 µm) in sheep on the Gotland Island in Sweden. Bai *et al*. (2002) reported two *Babesia* spp. *viz*., *B. ovis* and large form of *Babesia* (1.8 - 2.5 × 0.9 - 1.8 µm) from sheep and goats in the eastern part of Gansu province, China. Shayan *et al*. (2008) reported a large form of *B. ovis* (2.7 × 0.4 µm) in sheep in the Northwest of Iran.

Molecular methods such as PCR have become the preferred method for the diagnosis and differentiation of *Babesia* infections in the present scenario. Besides it can also detect early stages of infection as they are more sensitive and specific than the conventional methods (Esmaeilnejad *et al*., 2015). Moreover, detecting *Babesia* infection in small ruminants using traditional PCR was a powerful tool for epidemiological investigation because these animals are a major source of infection in Ixodid ticks (Aktas *et al*., 2005). Therefore, this technique was used to identify and characterize the 18S rRNA gene of *Babesia* spp. in goat.

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Materials and Methods

A one-year-old Sirohi Kathiavari crossbred female goat was presented to the University Veterinary Hospital (UVH), Kokkalai, Thrissur, Kerala. The infected goat showed varying clinical signs of anorexia, inappetence, depression, weakness, body temperature of 41.2°C, cough, nasal discharge and ocular discharge. Peripheral blood smear was stained with Giemsa Stain and examined under the oil immersion objective (100X) of the light microscope (Leica DM1000 LED, Germany) for the presence of piroplasms. The morphological characteristics and morphometery of piroplasms in infected erythrocytes were studied and morphometry was studied. About 2 mL of whole blood sample was collected from the jugular vein and analysed for haematological parameters including the total erythrocyte count, haemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), total leukocyte count (TLC) and differential leukocyte count by automatic haematological analyser (Orphee Mythic Vet 18).

Polymerase Chain Reaction

Blood sample was collected in 5 mL EDTA vacutainer tubes and transported to the lab on ice for PCR analysis. The genomic DNA of whole blood was extracted using DNeasy® Blood and Tissue kit (Qiagen, Germany), according to the manufacturer's instruction. The extracted DNA was stored at -20°C.

Polymerase chain reaction was performed with specific primers (P1B-F & P2B-R) flanking the hypervariable region V_4 of 18s rRNA (Table 1). for simultaneous differentiation between *Theileria* and *Babesia* spp. (Bazmani *et al*., 2018). The expected size of PCR products for *Theileria* and *Babesia* spp. were 426-430 bp and 389-402 bp, respectively. The variation of approximately 30 bp in the sequence of PCR products can be easily differentiated. The PCR was optimized at an initial denaturation of 94°C for 3 min, followed by 34 cycles of 94°C for 45s, annealing at 60°C for 45s and 72°C for 45s with a final extension of 72°C for 3 min. The reaction was carried out in a total volume of 12.5 µL containing 2 µL genomic DNA, 6.25 µL of master mix (Green dye PCR master mix (2x), TaKaRa Emerald, Japan), 2.25 µL of nucleus-free water and 1 µL (10 pmol) of each forward and reverse primers. PCR products were electrophoresed on 2 % agarose gel stained with ethidium bromide and amplified fragment size was compared using a 100 bp DNA marker in a gel-documentation system (Bio-Rad Laboratories T100, USA). The positive PCR product obtained was sent for nucleotide sequencing at the DNA sequencing facility available at GeneSpec Biosciences, Cochin. The obtained sequence was blasted with other published sequences available in the NCBI to analyse their similarity.

Phylogenetic Analysis

The obtained sequence was aligned with other previously published sequences from the Gen Bank using Clustal W. The aligned sequences were trimmed to the same length from which the phylogenetic tree was constructed based on the Maximum Likelihood method, using the MEGA version 11.0 program with the Tamura-Nei model. A bootstrap value of 1000 replicates to maintain the reliability of the topology.

Results and Discussion

Giemsa's Staining

Microscopic examination of Giemsa's-stained blood smears revealed the occurrence of large and small forms of pleomorphic babesial organisms *viz*., single, paired pyriform with an acute or obtuse angle, amoeboid, oval, elongated and ring forms (Fig. 1). Different morphological forms of *Babesia* piroplasms observed in the present study were in agreement with Shayan *et al*. (2008) from Iran; Sevinc *et al*. (2013) from Turkey and Kage *et al*. (2019) from India. The routinely used technique for detection of babesiosis in small ruminants is Giemsa staining of blood smears (Razmi *et al*., 2003; Shayan *et al*., 2008). This method is inappropriate for identifying different species of *Babesia* and also subclinical babesiosis due to lack of sensitivity and specificity (Esmaeilnejad *et al*., 2015). The morphometric studies identified large (78%) and small forms (22%) of *Babesia* organisms in goat with predominantly large forms (Fig. 2). A large form of *Babesia* which measured 2.5 to 3 µm in length and 1 to 2 µm in breadth, suggestive of *B. motasi* while the small form of *Babesia* measured 1.5 to 2 µm by 0.5 to 1.5 µm, indicative of *B. ovis*. These findings were in agreement with Soulsby (1982) who observed *B. ovis* (1-2.5 µm) to be much smaller compared to *B. motasi* (2.5-4×2 µm). Kage *et al*. (2019) identified small and large forms of *B. ovis* organisms, smaller one of 1.0 to 1.31 µm in length by 0.5 to 2.5 µm in breadth and larger one of 1.8 to 2.7 µm in length by 0.9 to 1.5 µm in breadth. Based on morphological and micrometrical data, the small *Babesia* was routinely diagnosed as *B. ovis* and the large *Babesia* as *B. motasi* in blood smears. In contrast to this, Lewis *et al*. (1981) reported a small *B. motasi* in Wales as double pyriform being 2.23 µm mean length. In another study, large *Babesia* piroplasm with mean dimensions of 2.21±0.12×1.17±0.18 µm was reported with different morphological forms including ring form, rod-like, three-leafed, budding form, single and double pyriforms (Bai *et al*., 2002). It is concluded that

Table 1: Primer sequences used in PCR

Organism	Primer name and sequence	Gene	PCR product
Theileria and *Babesia* spp.	P1B-F 5'-CACAGGGAGGTAGTGACAAG-3' P2B-R 5'-CTAAGAATTTCAC CTCTGACAGT-3'	*18S rRNA* gene	390-442 bp

morphometric studies could not be considered as a gold standard method in the differential diagnosis of *Babesia* spp. which was as per the report of Shayan *et al*. (2008).

In the present study, *Babesia* caused a severe infection associated with a parasitaemia of 0.5 to 5%, ++++; (more than 1 parasite in 1 field) by Ishihara's method. Papadopoulos *et al*. (1996) could also detect 1% parasitaemia in *Babesia* infected sheep and goats from the Macedonia region of Greece. Kage *et al*. (2019) also detected parasitaemia ranging from 1.0 to 1.8% in microscopically confirmed *Babesia* infection of sheep and goats from India. However, a higher parasitaemia of 23.7% with a high mortality rate in both sheep and goats infected with *Babesia* from Northwest Iran was recorded by Shayan *et al*. (2008). The observed changes in the percentage of parasitaemia may be attributed to the timing of blood smears taken from animals and also to the stage of disease. Higher blood parasitaemia levels were typically observed during the clinical or acute stage, while lower parasitaemia levels are a characteristic feature of the carrier or chronic phase of the disease (Yin *et al*., 2007).

In this study, a Sirohi Kathiavari crossbred goat showed varying clinical signs of anorexia, inappetence, depression,

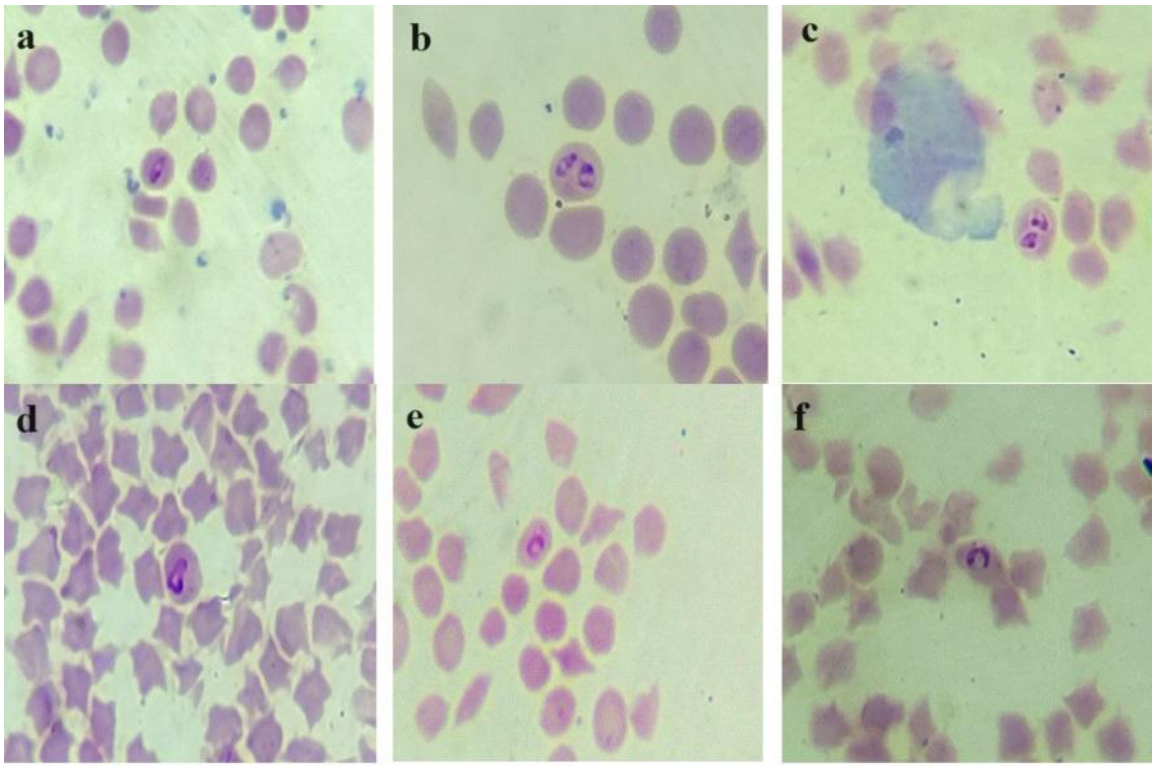

Fig. 1: Pleomorphic forms of *Babesia* piroplasms in blood smears. **a** single pyriform, **b** paired pyriforms with acute angle, **c** paired pyriforms with obtuse angle, **d** elongated form, **e** amoeboid form, **f** ring form

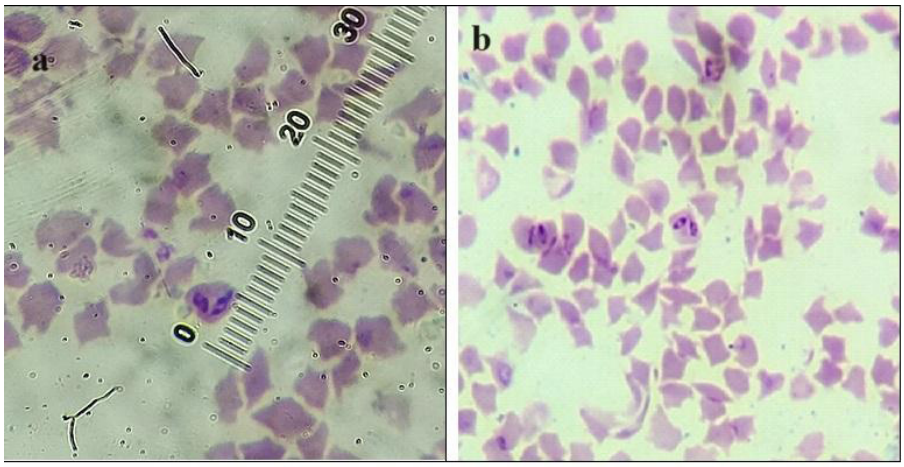

Fig. 2: a Large and **b** small forms of *Babesia* piroplasms

weakness, body temperature of 41.2°C, watery diarrhoea, cough, nasal discharge and ocular discharge. Similar clinical signs were detected by Muthuramalingam *et al*. (2014) in 34.5 % of newly purchased Tellicherry goats from Kerala which were infected by *B. ovis*. Ajith *et al*. (2017) also reported similar clinical signs along with icteric sclera and coffee-coloured urine in a Malabari goat suffering from an acute febrile hemolytic form of *B. ovis* infection from Kerala. In these studies, species were ascertained based on size of piroplasms.

Haemtological Analysis

Hematological analysis of affected goat revealed leukopenia, lymphocytopenia, thrombocytopaenia, granulocytopenia, monocytosis and hypochromic microcytic anaemia (Table 2). Fall in blood MCH, MCHC values in infected goat was the significant haematological change recorded during this study. In accordance with Muthuramalingam *et al*. (2014), *Babesia* infected Tellichery goats from Kerala exhibited a reduction in platelet count, consistent with our current findings. Conversely, there were no observable alterations detected in key blood parameters, including total red blood cell (RBC) count, heamoglobin concentration (Hb) and volume of packed red cells (VPRC). Another study by Ajith *et al*. (2017), on a Malabari goat infected with babesiosis from Kerala revealed anaemia, thrombocytopenia and leukocytosis with neutrophilia.

Polymerase Chain Reaction

In this study, DNA was analyzed by PCR using common specific primers (P1B-F & P2B-R) derived from the flanking part of hypervariable region of 18S rRNA that could easily and simultaneously differentiate between *Theileria* spp. and *Babesia* spp.(Shayan *et al*., 2008; Bazmani *et al*., 2018). The PCR products of *Babesia* spp. and *Theilera* spp. are reported to be 389–402 and 426–430 bp, respectively. The difference of 30 bp approximately in the nucleotide sequence of the PCR products could be detected in agarose gel (Shayan and Rahbari, 2005). Results from this study produced a 390 bp fragment, indicative of *Babesia* spp. (Fig. 3). The 390 bp obtained was sequenced and the sequence was analysed using NCBI BLAST which revealed 93.55% identity with a query coverage of 97% with the already published partial sequence of 18S rRNA gene of *B. motasi* (MH899762.1) and was subsequently submitted to the Genbank database at the National Centre for Biotechnology Information and assigned accession number PP087913.1. There was no significant similarity with any other *Babesia* species or *Theileria*.

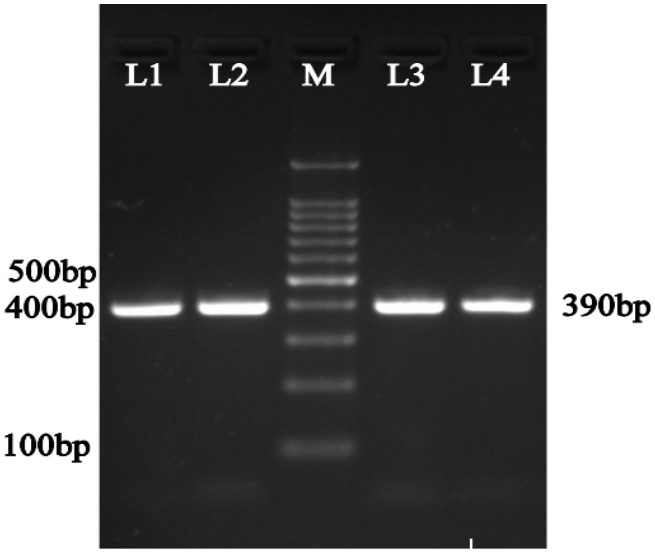

Fig. 3: Amplified product of 18S rRNA gene of the *Babesia* spp. from the goat. M: Marker; Lane L1, L2, L3, L4: Amplified product

Table 2: Haematological parameters of the goat infected by *Babesia* spp.

Blood Parameters	Animal value	Normal range	Remarks
WBC (thousand cells/µL)	2.56 x 10^3/µL	4.0-13.0	Low
Lymphocytes (thousand cells/µL)	1.1 x 10^3/µL	2.0-9.1	Low
Monocytes (thousand cells/µL)	0.5 x 10^3/µL	0.0-0.5	-
Granulocytes (thousand cells/µL)	1.0 x 10^3/µL	1.7-5.4	Low
Lymphocytes%	40.9%	50.0-70.0	Low
Monocytes%	19.8%	0.0-4.0	High
Granulocytes%	39.3%	35.0-41.5	-
RBC (million cells/µL)	15.42 x 10^6/µL	8.0-18.00	-
Haemoglobin (g/dL)	9.8g/dL	8.0-14.0	-
HCT%	28.7%	19.0-38.0	-
MCV (fl)	18.6	15.0-30.0	-
MCH (pg)	6.4pg	10.0-12.6	Low
MCHC (g/dL)	34.1 g/dL	35.0-42.0	Low
Platelet (thousand cells/µL)	234 x 10^3/µL	247-912	Low

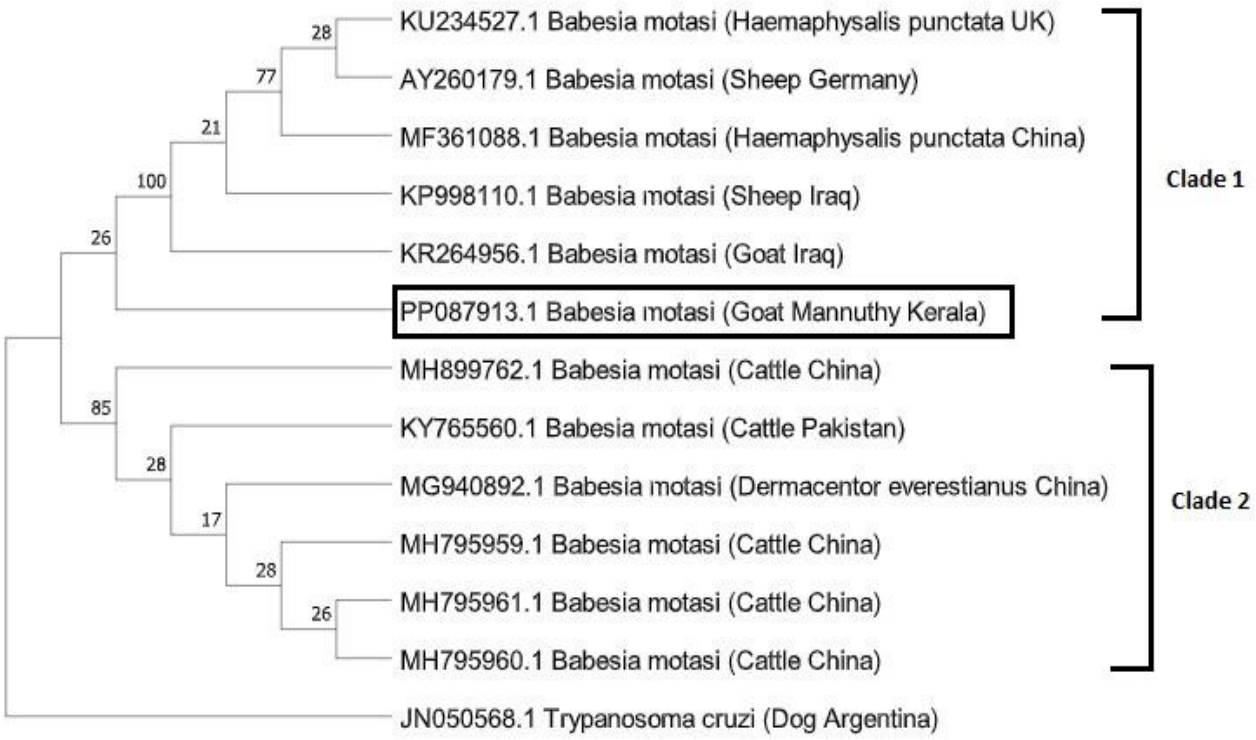

Fig. 4: Phylogenetic tree constructed using 18S rRNA gene sequences of *B. motasi*. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. *Trypanosomacruzi* represents the outgroup and sequence in the present study indicated in the box.

Phylogenetic Analysis

The phylogenetic tree was constructed based on the Maximum Likelihood method and Tamura-Nei model in MEGA 11 using the 18S rRNA sequence of *B. motasi* (PP087913.1) in the present study along with other *B. motasi* sequences deposited in Gen Bank with *Trypanosoma cruzi* as out group (Fig. 4). The isolates of *B. motasi* from Kerala (India), Iraq, China, Germany, UK and Pakistan were included. Results of present study revealed that *B. motasi* (PP087913.1), was included in clade-1 along with other isolates from Iraq, China, Germany and UK.

Conclusion

In the present study, we utilized molecular techniques to successfully identify both large and small variants of *B. motasi* for the first time in Kerala. This method proved effective for distinguishing between *Babesia* spp. Additionally, examination of Giemsa-stained blood smears from infected goat revealed large and small forms of *Babesia* organisms, conventionally recognized as *B. motasi* and *B. ovis*, respectively. Through PCR and sequencing analysis, we confirmed the presence of *B. motasi* in our study. Our findings suggest the presence of biometrical polymorphisms within *B. motasi* populations in Kerala, which could pose challenges in differentiating between *B. motasi* and *B. ovis* via Giemsa staining alone. Further investigation is warranted to ascertain the prevalence of *B. motasi* in Kerala's goat population and to identify the specific tick species involved in disease transmission.

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