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PROTOZOOLOGY - ORIGINAL PAPER

Molecular characterization of South Indian field isolates of bovine *Babesia* spp. and *Anaplasma* spp.

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Abstract

Ticks and tick-borne diseases (TTBDs) are considered major causes of economic loss in the livestock sector which incur an annual control cost estimated at US$ 498.7 million in India. Among these diseases, babesiosis, theileriosis and anaplasmosis are listed among the top ten livestock diseases in India and cause significant mortality and morbidity among cattle. However, molecular characterization of bovine *Babesia* and *Anaplasma* species are scant; thus, the aim of this study is to perform molecular characterization of field isolates of *Babesia* spp. and *Anaplasma* spp. infecting bovines in Kerala, South India. Blood smears and whole blood samples were collected from a total of 199 apparently healthy adult female cattle in Kerala. Based on microscopy, *Babesia* spp., *Theileria orientalis* and *Anaplasma* spp. organisms were detected in 9 (4.5%), 40 (20%) and 6 (3%) samples, respectively. Genus-specific polymerase chain reactions for amplification of 18S rRNA of *Babesia* spp. and 16S rRNA of *Anaplasma* spp. revealed positive results with 18 (9%) and 14 (7%) samples. The phylogenetic analysis of 18S rRNA gene sequences of *Babesia* spp. confirmed the existence of two different populations of *Babesia* spp. circulating in the blood of infected cattle viz., *Babesia bigemina* and a *Babesia* sp. genetically related to *Babesia ovata*. Further phylogenetic analysis using *rap-1a* sequences of isolates of *B. bigemina* revealed higher levels of genetic heterogeneity. However, the field isolates of *B. bigemina* displayed only slight heterogeneity when the *rap-1c* gene was examined. Polymerase chain reaction followed by sequencing and phylogenetic analysis of 16S rRNA gene of *Anaplasma* spp. revealed the existence of *Anaplasma marginale*, *Anaplasma bovis* and *Anaplasma platys* in bovines in South India. Based on *msp4* gene sequences, all the field isolates of *A. marginale* from Kerala were clustered in a single clade with others isolated from around the world. To our knowledge, this study forms the first report on occurrence of *B. ovata*-like parasites and *A. platys* in cattle from India.

Keywords *Babesia bigemina* · *B. ovata* like · *Anaplasma marginale* · *A. platys* · *A. bovis* · Phylogeny · South India

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Introduction

Among all animal diseases, ticks and tick-borne diseases (TTBDs) are considered major causes of economic loss in the livestock sector which directly or indirectly hamper the development of the dairy industry in India (Ghosh et al. 2006). It is estimated that approximately 80% of the world's cattle population is at risk of infection with TTBDs (de Castro 1997). During 2014–15, babesiosis, theileriosis and anaplasmosis were listed among the top ten livestock diseases in India

(Annual Report, PD-ADMAS 2014–15) with the annual control cost estimated at US$ 498.7 million, of which babesiosis alone attributed to approximately US$ 57.2 million (Minjauw and McLeod 2003).

Babesia spp. are the tick-transmitted intra-erythrocytic apicomplexan haemoparasites that are the second most prevalent blood-borne parasites of mammals in the world after trypanosomes (Telford et al. 1993). In cattle, *Babesia* spp. cause substantial mortality and morbidity and large economic loss due to infection with the causative agents *Babesia bovis*, *Babesia bigemina*, *Babesia divergens*, *Babesia major*, *Babesia ovata*, *Babesia occultans* and *Babesia jakimovi*. In India, babesiosis was first reported by Walker and Edward (1927) and it is thought that *B. bigemina* is the predominant pathogenic species in the country (Kolte et al. 2017; Ravindran and Ghosh 2017). However, there are also a few reports of *B. bovis*-associated babesiosis in Indian cattle and buffaloes (Indani 1938; Setty and Rao 1972; Gautam and Chhabra 1983; Muraleedharan et al. 1984, 1991; Shastri et al. 1991; Kolte et al. 2017).

The bovine anaplasmosis is a tick-borne haemo-rickettsial disease of ruminants caused by *Anaplasma* spp. (order Rickettsiales and family Anaplasmataceae), an obligate intracellular, Gram-negative bacteria parasitizing mammalian blood cells (Dumler et al. 2001). Infection due to *Anaplasma marginale* can result in the development of mild to severe anaemia and icterus without haemoglobinuria and haemoglobinaemia. Clinical symptoms may include fever, weight loss, abortion, lethargy, icterus and often death (Kocan et al. 2003). Anaplasmosis in cattle was first reported by Patnaik (1963) in Odisha, India, and subsequently, the pathogenic species *A. marginale* and *Anaplasma bovis* have been identified affecting cattle and buffaloes in India (Nair et al. 2013; Sharma et al. 2015; George et al. 2017).

Traditionally, the identification, description and classification of *Babesia* and *Anaplasma* species are based on the morphological characteristics using microscopic examination of Giemsa-stained thin or thick blood smears with or without serology. However, recent studies have demonstrated that *Babesia* and related piroplasmids are not monophyletic and can co-infect hosts; therefore, they require the use of molecular tools for the identification and characterization of these cryptic and polyphyletic species (Lack et al. 2012). To date, the small subunit ribosomal RNA (18S rRNA) gene is widely used for detection and phylogenetic analysis of protozoan organisms due to its high level of conservation (Van de Peer et al. 2000). Molecular phylogeny of *Babesia* spp. based on the sequence information of the 18S rRNA gene has been useful for the identification of major clade groups (Lack et al. 2012). Additionally, the conserved rhoptry-associated protein-1 (RAP-1) gene, which plays a key role in parasite invasion (Mosqueda et al. 2002), is considered as an informative marker for a phylogenetic group which includes *Babesia motasi*, *B. bigemina*, *B. major*, *B. ovata* and *Babesia crassa* (Niu et al. 2014). The RAP-1 locus is encoded by a family of 11 genes and is characterized by five tandemly arranged copies of *rap-1a* and *rap-1b* and a single polymorphic gene *rap-1c* (Suarez et al. 2003). Previous studies have determined that the multiple-copy *rap*-1a gene is highly conserved and is an informative marker for a broader phylogenetic analysis within the *Babesia* genus. Conversely, the highly conserved *rap-1b* and the single-copy polymorphic *rap-1c* gene are present only in *B. bigemina* and *B. motasi* (Niu et al. 2015). Hence, *rap-1c* is considered an eligible candidate for the molecular characterization of *B. bigemina* from the field isolates of different geographical areas (Hilpertshauser et al. 2007; Thompson et al. 2014).

Similarly, detection and characterization of *Anaplasma* spp. are performed using molecular tools. Most of these techniques target the 16S rRNA (Parola et al. 2003; Strik et al. 2007), 23S rRNA (Dahmani et al. 2015), major surface protein (*msp*) (de la Fuente et al. 2001, 2002; Shimada et al. 2004), heat-shock protein *groEL* (Park et al. 2005) and citrate synthase *gltA* (Inokuma et al. 2002b) genes for the detection of *Anaplasma* spp. Further differentiation of various *Anaplasma* spp. and strains typically relays on the genes *msp1α*, *msp1β*, *msp4* and *groEL* with the *msp4* gene and protein sequences proving the most useful for the genetic characterization due to high levels of inter-species variation (Quiroz-Castañeda et al. 2016). These specific major surface proteins (*msp1α*, *msp1β*, *msp2* and *msp4*) are of further interest as they are expressed on infected erythrocytes, and therefore, variation in these *msp* genes could provide valuable information that could be used to identify potential targets for vaccine development (de la Fuente et al. 2005a, b; Contreras et al. 2017).

Even though babesiosis and anaplasmosis have a great impact on the livestock sector in India, previous studies on the molecular characterization of bovine *Babesia* and *Anaplasma* species are scant. Hence, the present communication focuses on the molecular characterization and phylogenetic analysis of the field isolates of *Babesia* spp. and *Anaplasma* spp. of Kerala, South India, based on the sequence polymorphism of 18S rRNA, rhoptry-associated proteins (*rap-1a*, *rap-1c*) of *Babesia* and 16S rRNA, *msp4* genes of *Anaplasma*.

Materials and methods

Study area, animals and blood samples

Thin peripheral blood smears (from tip of the ear) and heparinized blood samples (~2 mL, from the jugular vein using 18-gauge needle) were randomly collected from a total of 199 apparently healthy adult female cattle from three different zones of Kerala, South India, viz., Northern Kerala (44 from Wayanad, 10 from Kozhikode), Central Kerala (30 from

Thrissur) and Southern Kerala (28 from Thiruvananthapuram, 87 from Idukki). All the blood samples were collected as part of a normal farming/veterinary practice for regular disease screening. A map showing different locations within Kerala where blood samples were collected is depicted in Fig. 1.

Staining technique

Thin peripheral blood smears were fixed in methanol and stained using 1:10 dilution (stain:water) of Giemsa's stain (Merck Life Science, Mumbai) for 45 min. Stained blood smears were examined microscopically for the presence of parasites under the oil immersion (100× magnification) objective of the light microscope (Leica, Germany). The presence of a single piroplasm was considered as a positive case, and a minimum of 5000 red blood cells (RBCs) was screened before proclaiming the sample negative for any blood parasites.

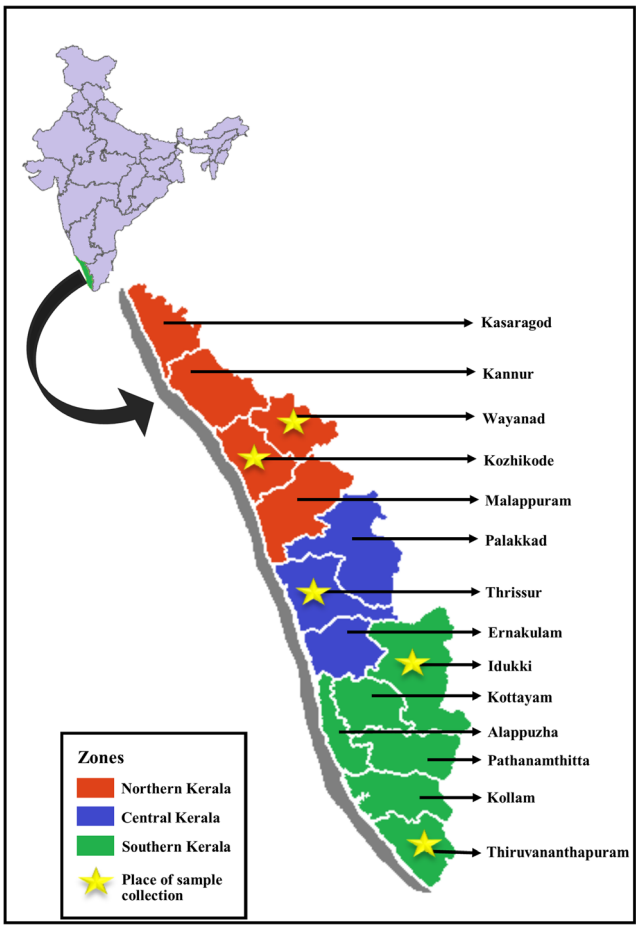

Fig. 1 Map of Kerala showing different places of collection of blood samples from three zones of Kerala viz., Northern Kerala (Kozhikode and Wayanad districts), Central Kerala (Thrissur district) and Southern Kerala (Thiruvananthapuram and Idukki districts) (National Agricultural Research Project Zones, Kerala Agro-Climatic Zones)

Genomic DNA extraction and quantification

Genomic DNA was isolated from the heparinized blood samples (100 μL) using DNeasy® blood and tissue kit (Qiagen, Germany), according to the manufacturer's protocol. Extracted DNA was eluted in 100 μL of DNA elution buffer and stored at − 20 °C for further analysis. DNA concentration was determined using a NanoDrop® 2000C spectrophotometer (Thermo Scientific, USA).

Positive and negative control

DNA isolated from the whole blood of a *B. bigemina*-infected bovine animal (from Teaching Veterinary Clinical Complex, College of Veterinary and Animal Sciences, Pookode, Kerala) was used as the positive control for *B. bigemina*-specific and *Babesia* genus-specific PCRs. Similarly, the DNA isolated from the whole blood of a *A. marginale*-infected bovine animal (from Teaching Veterinary Clinical Complex, College of Veterinary and Animal Sciences, Pookode, Kerala) was used as a positive control for *A. marginale*-specific and *Anaplasma* genus-specific PCR.

The leucocyte DNA from a 3-day old calf served as negative control for both *Babesia* and *Anaplasma* species.

Polymerase chain reaction for *Babesia* and *Anaplasma* species

All polymerase chain reactions (PCRs) for *Babesia* and *Anaplasma* spp. were carried out in a final reaction volume of 25 μL containing 0.25 mM dNTPs (Genei, Bangalore), 1 U DyNAzyme II DNA polymerase (Thermo Scientific, USA), 1× PCR buffer (containing $MgCl_2$ at a final concentration of 1.5 mM), 20–40 ng of template DNA and 20 pmol each of forward and reverse primers. The details of primers used for the amplification of different genes of *Babesia* and *Anaplasma* species are shown in Table 1. All the reactions were conducted as previously described (Table 1), without modification, in an automated thermal cycler with heated lid (Eppendorf, Germany). The PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide (0.5 μg/mL) and visualized under UV light using a gel documentation system (Uvitech, Cambridge). All the blood samples of host genomic DNA positive for 18S rRNA (*Babesia* spp.) and 16S rRNA (*Anaplasma* spp.) were represented as isolates (Table 2).

Initially, amplification of the 18S rRNA gene (Hilpertshauser et al. 2007) specific for the genus *Babesia* was carried out. All the samples which revealed PCR amplification for *Babesia* genus-specific 18S rRNA gene were then used for PCR amplification of the *SpeI-AvaI* restriction fragment specific for *B. bigemina* (Figueroa et al. 1992), the apocytochrome *b* gene specific for *B. bovis* (Fahrimal et al. 1992), the apical membrane

Table 1 Primers used for the amplification of different genes of *Babesia* and *Anaplasma* species

Specificity	Locus	PCR assay	Sequence (5′–3′)	Amplicon size (bp)	Reference
Babesia sp.	18S rRNA	PCR	F-GTTTCTGMCCCA TCAGCTTGAC R-CAAGACAAAAG TCTGCTTGAAAC	440	Hilpertshauser et al. (2007)
B. bigemina	*SpeI-AvaI* restriction fragment	PCR	F-CATCTAATTTCT CTCCATACCCCTCC R-CCTCGGCTTCAA CTCTGATGCCAAAG	278	Figueroa et al. (1992)
	rap-1a	PCR	F-TATGGCACATTG GCGCATA R-TCGCTGTTAACC TCCTGAGTA GT	1501	Molad et al. (2015)
		nPCR	F-TTCTTGGGTGTG TGTTTTGGA R-ATCATGTACTC GCCGTAGCC	758	
	rap-1c	PCR	F-CTGTTGAGGCG CAACCTGT R-AAGTACTTCGG GAACCTG	1010	
		nPCR	F-CTACCGTTTCTC CTCGAT R-AGTACTTCTTG GGAACCT	698	
B. bovis	Apocytochrome *b*	*PCR*	F-GGGTTTATAGTCG GTTTTGT R-ACCATTCTGGTAC TATATGC	711	Fahrimal et al. (1992)
B. ovata	β- Tubulin	PCR	F-ACACTGTGCATC CTCACCGTCATAT R-CTCGCGGATCTT GCTGATCAGCAGA	444	Sivakumar et al. (2014)
	AMA-1	PCR	F-GATACGAGGCTG TCGGTAGC R-AGTATAGGTGAG CATCAGTG	504	Sivakumar et al. (2014)
		PCR	F1-GGCAGGTGCCT GCGTGGCGATCG R1-GAGCAACAACA GCGCCGTAGTAATCACG	600	Niu et al. (2015)
		nPCR	F2-TGATATCGATA TCGACCTTGATTC R2-GAGCTGTCACC ATTGTCCTTAACAC	310	
		PCR	F-GATACGAGGCT GTCGGTAGC R1-GAGCAACAACA GCGCCGTAGTAATCACG	1234	
		nPCR	F-GATACGAGGCT GTCGGTAGC R2-GAGCTGTCACC ATTGTCCTTAACAC	1041	
Anaplsma spp.	16S rRNA gene	PCR	F-GGA ATT CAG AGT TGG ATC MTG GYT CAG R-CGG GAT CCC GAG TTT GCC GGG ACT TCT TCT	492–498	Aktas et al. (2011)
Anaplasma marginale	*msp 4*	PCR	F-GGG AGC TCC TAT GAA TTA CAG AGA ATT GTT TAC R-CCG GAT CCT TAG CTG AAC AGG AAT CTT GC	854	de la Fuente et al. (2001, 2002)

antigen-1 (*ama-1*) gene and the β-tubulin gene specific for *B. ovata* (Sivakumar et al. 2014), in order to confirm respective parasites. The confirmed *B. bigemina* isolates were further used for the molecular characterization by nested PCR amplification for *rap-1a* and *rap-1c* (Molad et al. 2015).

Additionally, for the identification of *Anaplasma* spp., PCR amplification of the 16S rRNA gene (Aktas et al. 2011) specific for the genus *Anaplasma* was carried out on all samples. All the positive samples were further characterized by PCR amplification of the *A. marginale*-specific *msp4* gene (de la Fuente et al. 2001, 2002).

Sequencing and sequence analysis

Positive polymerase chain reaction products (18S rRNA, 16S rRNA, *rap-1a*, *rap-1c*, *msp4*) were purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany) as per the manufacturer's protocol. Each sample was sent to the AgriGenome Labs Private Ltd., Cochin, Kerala, for automated nucleotide sequencing by Sanger dideoxy method with both the forward and reverse primers. The resulting sequences were examined for overlapping peaks suggestive of co-infection using Bioedit software (Hall 1999) before consensus sequences of each isolate were compared to other published sequences available in GenBank using NCBI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Unique sequences were deposited in GenBank database (Table 2).

Phylogenetic analysis

For the phylogenetic analysis of the *Babesia* and *Anaplasma* isolates, the nucleotide sequences were aligned using ClustalW (Thompson et al. 1994) with the previously published sequences in the GenBank. Aligned sequences were trimmed to the same length (with gaps) from which phylogenetic trees were constructed based on the maximum likelihood method, using the programme MEGA 7.0 (Kumar et al. 2016) with the suitable models [18S rRNA: Kimura 2-parameter (K2); *rap-1a*: Kimura 2-parameter (K2); *rap-1c*: Jukes-Cantor (JC); 16S rRNA: Kimura 2-parameter (K2) and

Table 2 Details of field isolates of *Babesia* and *Anaplasma* species from Kerala, South India, and their GenBank accessions

Sl. no.	Zone	Place (district)	Isolate	*Babesia* spp.			*Anaplasma* spp.	
				18S rRNA	*rap 1a*	*rap 1c*	16SrRNA	*msp4*
1	Northern Kerala	Wayanad	Wayanad-1	MF784355	MG191283	MG191289	–	–
2		Wayanad	Wayanad-2	MG062770	–	–	–	–
3		Wayanad	Wayanad-3	MF784354	MG191284	MG191290	–	–
4		Wayanad	Wayanad-4	MG062771	–	–	–	–
5		Wayanad	Wayanad-5	MF784389	–	–	–	–
6		Wayanad	Wayanad-6	MG062772	–	–	–	–
7		Wayanad	Wayanad-7	MG062773	–	–	–	–
8		Wayanad	Wayanad-8	MF784357	MG191285	MG191291	–	–
9		Wayanad	Wayanad-9	MF784391	–	–	–	–
10		Wayanad	Wayanad-10	MF784396	–	–	–	–
11		Wayanad	Wayanad-11	MF784431	–	–	–	–
12		Wayanad	Wayanad-12	MF784390	MG191286	MG191292	–	–
13		Wayanad	Wayanad-13	MF784435	–	–	–	–
14		Wayanad	Wayanad-14	–	–	–	MG711853	–
15		Wayanad	Wayanad-15	–	–	–	MG660443	MG676451
16		Wayanad	Wayanad-16	–	–	–	MG712286	–
17		Wayanad	Wayanad-18	–	–	–	MG660826	MG676453
18		Wayanad	Wayanad-19	–	–	–	MG711855	–
19		Wayanad	Wayanad-20	–	–	–	MG660830	MG676454
20	Central Kerala	Thrissur	Thrissur-1	MG062769	–	–	–	–
21		Thrissur	Thrissur-2	MF784356	MG191281	MG191287	–	–
22		Thrissur	Thrissur-3	–	–	–	MG711856	–
23		Thrissur	Thrissur-4	–	–	–	MG709033	MG676455
24		Thrissur	Thrissur-5	–	–	–	MG709053	MG676456
25		Thrissur	Thrissur-6	–	–	–	MG709054	MG676457
26		Thrissur	Thrissur-7	–	–	–	MG701130	MG676458
27		Thrissur	Thrissur-8	–	–	–	MG709056	MG676459
28	Southern Kerala	Thiruvanan-thapuram	TVM-1	MF784387	MG191282	MG191288	–	–
29		Thiruvanan-thapuram	TVM-2	–	–	–	MG654735	MG676450
30		Thiruvanan-thapuram	TVM-3	–	–	–	MG709131	MG676460
31		Idukki	Idukki-1	MF784446	–	–	–	–
32		Idukki	Idukki-2	MG062768	–	–	–	–

msp4: Kimura 2-parameter (K2)]. The reliability of the topologies was tested by bootstrapping with 1000 replications. Genetic divergence analysis among the field isolates of *Babesia* and *Anaplasma* species was performed using MEGA7 (Kumar et al. 2016).

Results

Microscopical examination

The piroplasms of *Babesia* spp. were observed in nine (4.5%) of 199 blood smears with morphological variations like paired pear-shaped or larger single pear-shaped piroplasms or single oval/irregular amoeboid forms within the erythrocytes (Fig. 2). The piroplasms of *Theileria orientalis* and inclusions of *A. marginale* were observed in 40 (20%) and 6 (3%) out of 199 blood smears (images not shown).

Molecular characterization of *Babesia* spp.

The *Babesia* genus-specific PCR amplifying the 18S rRNA gene revealed specific amplification in 18 (9%) of 199 samples tested. The specific PCRs targeting *SpeI-AvaI* restriction fragment, *rap-1a* and *rap-1c* genes of *B. bigemina*, were amplified in six (33%) of 18 samples. The PCRs targeting apocytochrome *b* gene of *B. bovis*, apical membrane

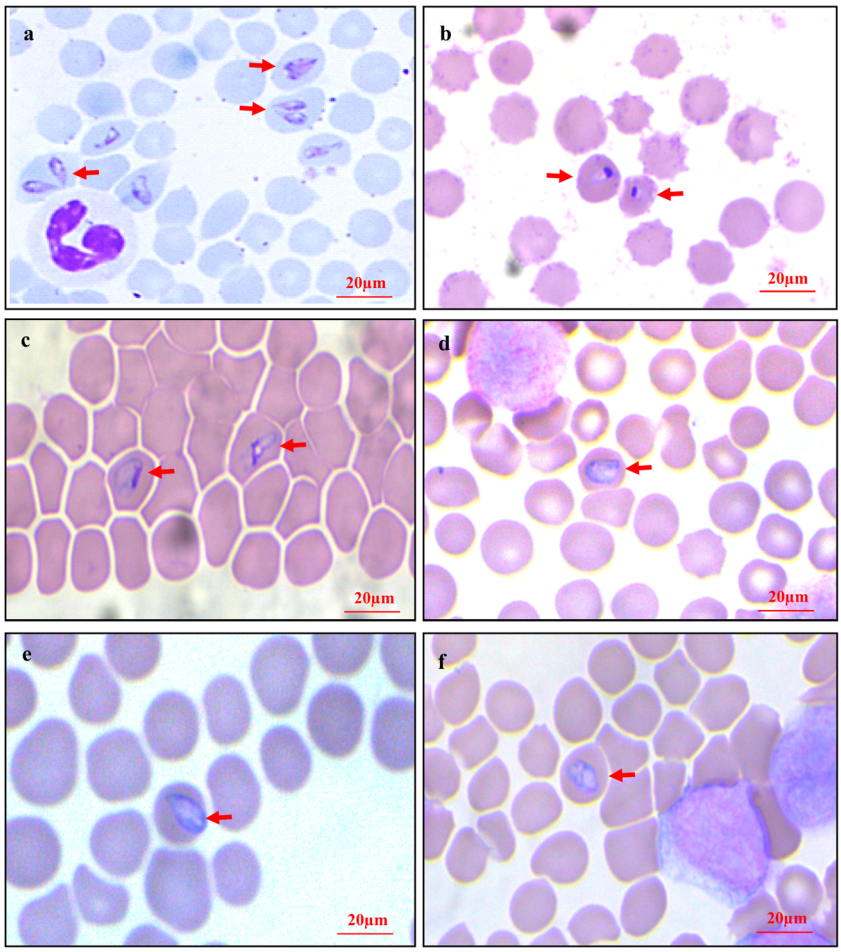

Fig. 2 Blood smears with various morphological appearances of the *Babesia* spp. piroplasms (1000×). **a** Paired pear-shaped piroplasms; **b** single pear-shaped piroplasms; **c**–**f** single oval/irregularly shaped amoeboid-form piroplasms (indicated by red arrows)

antigen-1 (*ama-1*) and β-tubulin genes of *B. ovata* failed to amplify specific products in the present study.

The NCBI-BLAST and phylogenetic analysis of 18S rRNA gene sequences of the field isolates of *Babesia* spp. revealed that the South Indian field isolates occupied three different clades out of total six clades (Fig. 3). Twelve of our isolates showed 91–99% identity to *B. bigemina* (GenBank KU206297, KU206296) with five of these isolates clustered in clade 1, which also comprised *B. bigemina* isolates from other countries (India, Uganda, Argentina, Kenya, Mozambique, China, Brazil, Australia and Switzerland), and the further seven isolates clustered together forming a separate unique clade 2 (Fig. 5). The remaining isolates showed 98–99% identity to *B. ovata* (GenBank KU947081) and a *Babesia* sp. Hue-1 strain (GenBank LC125456) and clustered in clade 3 (Fig. 3). It should be noted that the amplicon sequence was too short for one isolate, Thrissur-1 (GenBank MG062769); therefore, it was not included in the phylogenetic tree. The *B. major*, *B. occultans* and *B. bovis* 18S rRNA sequences formed the clades 4, 5 and 6, respectively, in which there were no isolates from this study represented. The intraspecies divergence among *B. bigemina* isolates in clades 1 and 2 ranged between 1.3 and 3.4%. Similarly, the intraspecies divergence among *B. ovata* isolates within clade 3 was 0–7.6%.

The NCBI-BLAST analysis of *rap-1a* gene sequences of *B. bigemina* field isolates in the present study revealed 98 to 100% homology to the published *B. bigemina* isolates from Israel (GenBank KP822955, KP671751). The phylogenetic tree constructed based on *rap-1a* gene sequences of *B. bigemina* formed two distinct clades (Fig. 4). The isolates of *B. bigemina* from Mexico, Africa (Nigeria and Kenya), Egypt, Thailand, Philippines and Turkey were clustered in clade 1. All the six *B. bigemina rap-1a* sequences of field isolates from South India in the present study were clustered in clade 2 which also comprised isolates from USA and Israel (field and vaccine isolates). Of note, the isolates within clade 2 showed two subclades with *Babesia bigemina* isolates viz., Wayanad-1 and -8, forming a distinct subclade from the other isolates.

The NCBI-BLAST and phylogenetic tree based on *rap-1c* sequences indicated that the field isolates of *B. bigemina* from South India in the present study were included in two separate clades viz., clade 1 and clade 2 (Fig. 5). Among the five *rap-1c* isolates included in the phylogenetic analysis, two isolates (Wayanad-1 and TVM-1) had 98% homology with

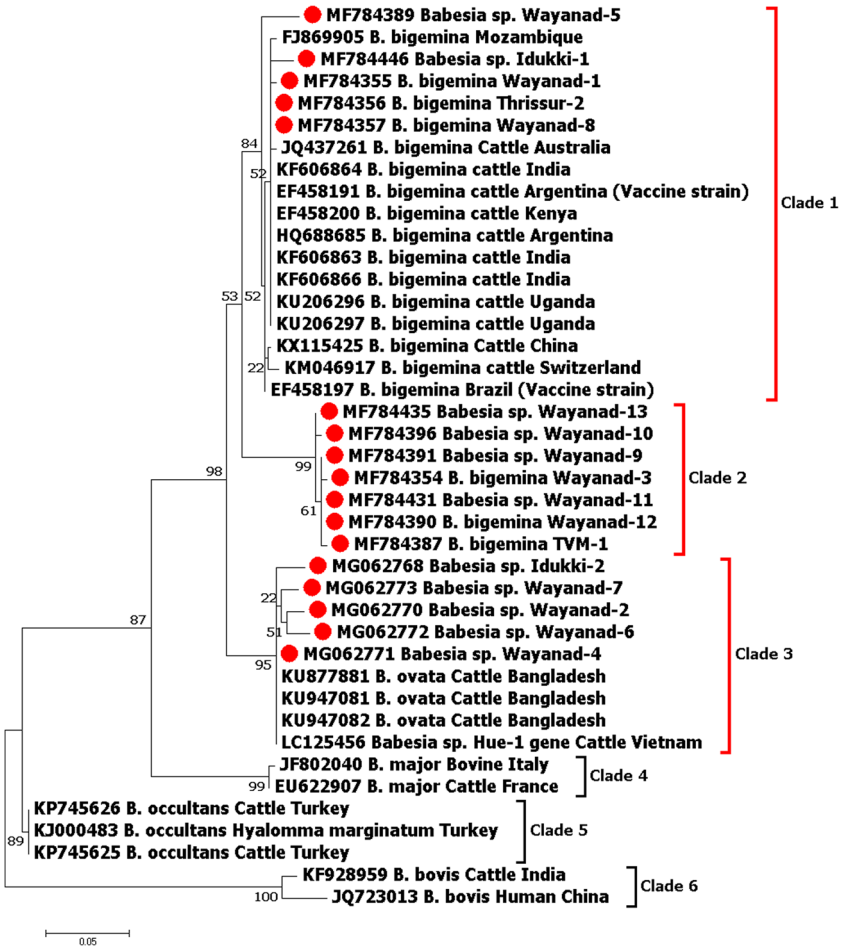

Fig. 3 Phylogenetic tree constructed using the 18S rRNA gene sequences of *Babesia* spp. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter (K2) model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3096). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 41 nucleotide sequences comprising of field isolates from the current study (indicated by red circle) and previously published sequences in the GenBank. Evolutionary analyses were conducted in MEGA7

B. bigemina rap-1c sequences from China (GenBank KT312802) and clustered in clade 1, while three isolates (Wayanad-3, Wayanad-12 and Thrissu-2) showed 98–99% identity with *rap-1c* sequence of *B. bigemina* from Brazil (GenBank AY146985) and were distributed in clade 2 comprising isolates from Brazil (GenBank AY146985), Mexico

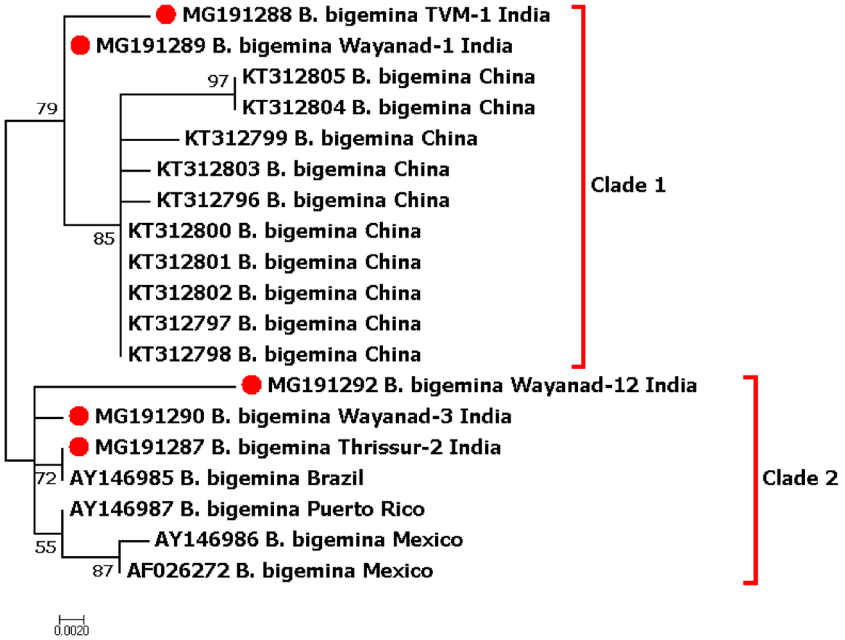

Fig. 5 Phylogenetic tree constructed using the *rap-1c* gene sequences of *Babesia bigemina.* The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor (JC) model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The analysis involved 19 nucleotide sequences comprising of field isolates from the current study (indicated by red circle) and previously published sequences in the GenBank. Evolutionary analyses were conducted in MEGA7

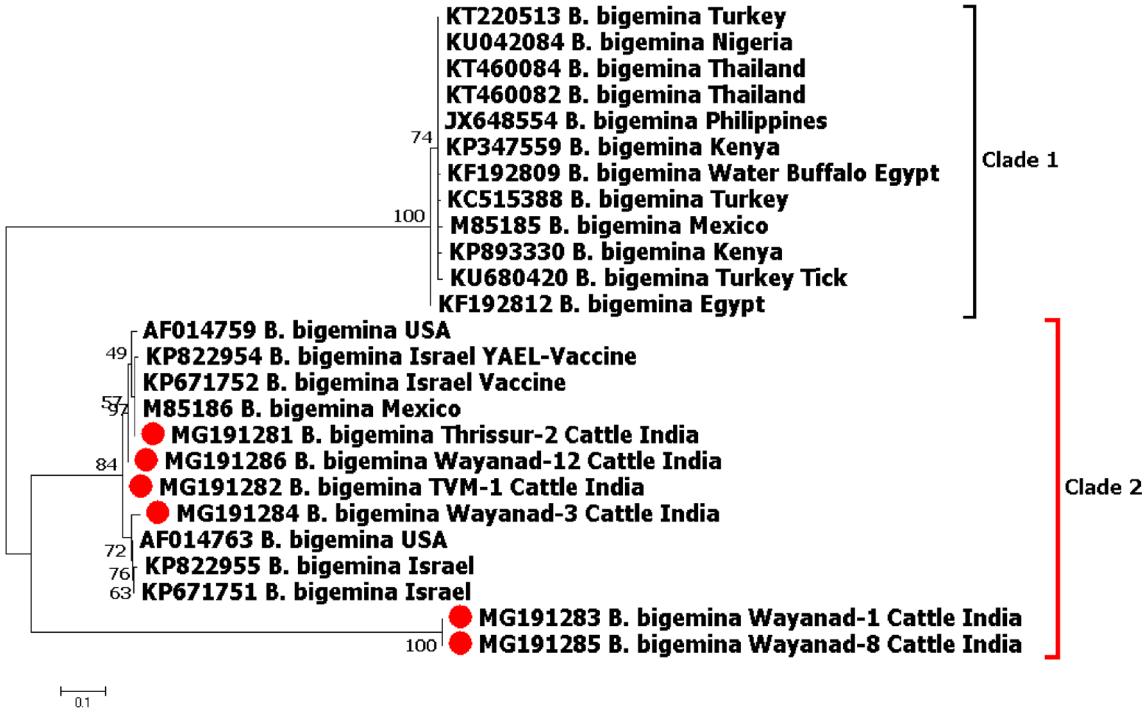

Fig. 4 Phylogenetic tree constructed using the *rap-1a* gene sequences of *Babesia bigemina*. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter (K2) model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 25 nucleotide sequences comprising of field isolates from the current study (indicated by red circle) and previously published sequences in the GenBank. Evolutionary analyses were conducted in MEGA7

(GenBank AY146986, AF026272) and Puerto Rico (GenBank AY146987) (Fig. 5). The Wayanad-8 isolate (GenBank MG191291) was not included for making tree as sequence length was too short despite showing BLAST homology with Wayanad-1 (GenBank MG191289) and Chinese isolates (GenBank KT312800) clustered in clade 1. The interclade divergence between *B. bigemina* isolates belonging to clades 1 and 2 was 0–3%.

Molecular characterization of *Anaplasma* spp.

Of the 199 samples tested, 14 (7%) were positive for the presence of the *Anaplasma* spp. based on successful amplification of the 16S rRNA genus-specific primers. The NCBI-BLAST and phylogenetic analysis of 16S rRNA gene sequences revealed that the sequences clustered into one of three distinct clades. Ten isolates were in clade 1 with 99–100% identity to *A. marginale* (GenBank KT264188, KU585985), one isolate was in clade 3 with 99% identity to *A. bovis* (GenBank KP314251) and three isolates were in clade 5 with 98–99% identity to *A. platys* (GenBank KU586124, KU586006) (Fig. 6). Intraspecific divergence of 0–0.7% and 1–1.2% was observed among the field isolates of *A. marginale* ($n = 10$) and *A. platys* ($n = 3$), respectively.

The phylogenetic analysis using NCBI-BLAST of *A. marginale msp4* gene sequences confirmed the presence of *A. marginale* in the 10 isolates. However, only eight sequences could be analyzed further as two of the sequence products for TVM-3 and Wayanad-15 were too short. The remaining eight field isolates (Trissur-4, -5, -6, -7, -8, Wayanad-18, -20 and TVM-2) of Kerala clustered together along with isolates from around the world, including isolates from North America, South America and Asia, and revealed only minor levels of genetic diversity with intra-clade divergence of 0–0.9% within the *msp4* gene (Fig. 7).

Discussion

Babesia spp.

Species differentiation is challenging among the large *Babesia* parasites of bovines because of the morphological similarity of *B. bigemina* and *B. ovata*. *Babesia bigemina* organisms appear as larger pear-shaped piroplasms in pairs joined at their narrow ends, placed centrally in the erythrocytes at acute angle, occupying at least 3/4 of the area within the cell or single larger pear-shaped, oval and ring forms (Muraleedharan 2015). *Theileria orientalis* are the most prevalent haemoprotozoan parasites in Kerala, which could be easily differentiated from *B. bigemina*; they are smaller with thin and thick rod shaped or annular with light staining trailing

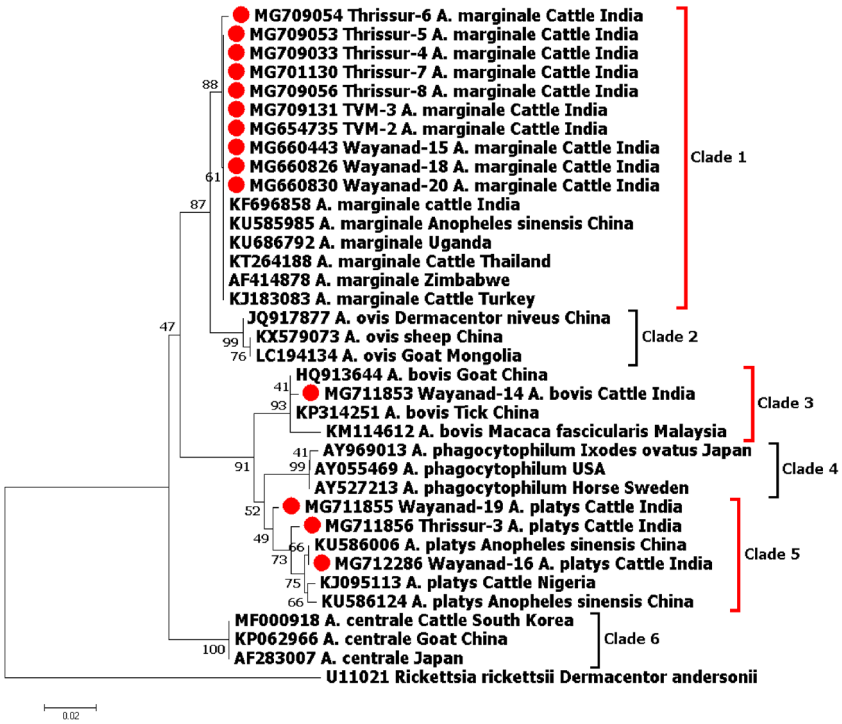

Fig. 6 Phylogenetic tree constructed using the *16S rRNA* gene sequences of *Anaplasma* spp. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (K2). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The analysis involved 36 nucleotide sequences comprising of field isolates from the current study (indicated by red circle) and previously published sequences in the GenBank. Evolutionary analyses were conducted in MEGA7

cytoplasm. In the present study, microscopy revealed that 40 samples contained *T. orientalis*, while *Babesia* piroplasms were detected in nine out of 199 samples.

Previous studies from Kerala, South India, have reported a very high seroprevalence of 67.6% based on indirect fluorescent antibody test (IFAT) (Ravindran et al. 2002) and 70.9% with slide enzyme-linked immunosorbent assay (SELISA) (Ravindran et al. 2007) against bovine babesiosis. However, more recently, Nair et al. (2011) reported a very low incidence (0.6%) of *B. bigemina* based on molecular tools in the same region. This difference in prevalence could be attributed to the cross-reactivity of antibodies against piroplasms of *Babesia* spp. and *Theileria* spp. present in the same geographical area in serological tests. In addition, genetic variation among the *B. bigemina* field isolates of Southern and Northern India has been reported (Ravindran et al. 2010), although it is unclear whether the observed genetic heterogeneity impacts on the virulence of the *B. bigemina* isolates.

Based on 18S rRNA sequences, all the *B. bigemina* field isolates used in the present study were grouped into two different clades, in which, clade 1 and clade 2 comprised of five and seven field isolates, respectively. *Babesia bigemina*-specific *SpeI-AvaI* restriction fragment, *rap 1a* and *rap 1c* genes were amplified from only three isolates of clade 1 (Wayanad-1, Wayanad-8 and Thrissur-2) and three isolates of clade 2 (Wayanad-3, Wayanad-12 and TVM-1). This may be due to the low template concentration or lack of sensitivity of the PCR methods. Despite this technical difficulty, *B. bigemina* types do appear to be clustered in two clades (18Sr RNA), although analysis of full-length gene sequences will give a more complete picture of the diversity of *B. bigemina* in India. However, this genetic heterogeneity observed among the *B. bigemina* isolates of the present study could be explained based on the heterogeneity among the *rap-1a* and *1c* sequences of these isolates except Thrissur 2 (for *rap-1a*) and TVM-1 (for *rap-1c*). The reason for their altered behaviour could not be explained based on the results of the present study.

The phylogenetic classification of *B. bigemina rap-1c* gene sequences in the present study is in agreement with the previously described phylogenetic tree of *B. bigemina rap-1c* gene (Niu et al. 2015). Thus, a lower rate of polymorphism/heterogeneity of *B. bigemina rap-1c* gene sequences exists among the South Indian isolates and with the other isolates from different geographical locations of the world. Niu et al. (2015) reported that, due to the low genetic variation in *B. bigemina rap-1c* gene sequence, RAP-1C protein is often considered as a potential antigen for developing immunodiagnostic techniques (like ELISA) for specific detection of *B. bigemina*. However, when RAP-1c protein is used as an antigen for diagnosis or as a vaccine candidate in South India, care should be taken to include both types of *rap-1c* variants (belonging to clades 1 and 2).

The existence of genetic diversity among *B. bigemina* isolates may be explained due to the varying geographical and climatic conditions affecting hosts carrying these parasites and the parasite's interaction with different hosts (domestic and wild bovines). Among the places of sample collection, Wayanad and Idukki districts are hilly areas with lower

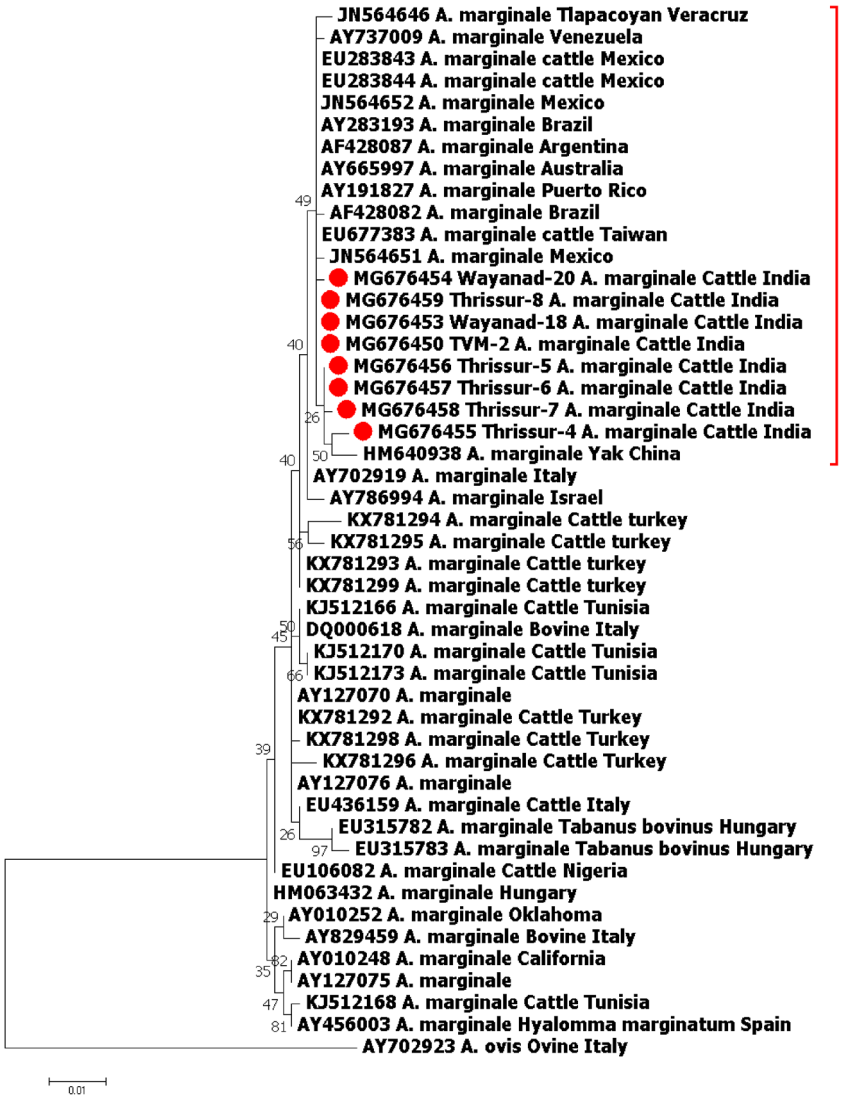

Fig. 7 Phylogenetic tree constructed using the *msp4* gene sequences of *Anaplasma marginale*. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter (K2) model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.1702)). The analysis involved 48 nucleotide sequences comprising of field isolates from the current study (indicated by red circle) and previously published sequences in the GenBank. There were a total of 714 positions in the final dataset. Evolutionary analyses were conducted in MEGA7

environmental temperature compared to Kozhikode, Thrissur and Thiruvananthapuram, which are in plains with higher atmospheric temperature. All the districts surveyed have very good forest cover especially Wayanad and Idukki, where sizable deer population in the forest interacts with domestic livestock population frequently. Detailed studies are required for confirming these associations.

Results of the present study support those of Nair (2008) who speculated the occurrence of unidentified piroplasms in the cattle of Northern Kerala. In the present study, the evidence for the existence of *Babesia* species other than *B. bigemina* was provided by phylogenetic analysis of 18S rRNA gene sequences, which showed that six isolates were 98–99% identical to and cluster with *B. ovata*. Until now, *B. ovata* was not reported from the Indian subcontinent. *Babesia ovata* was reported only from the eastern Asian countries viz., China (Bai et al. 1990; Niu et al. 2015; Tian et al. 2015), Japan (Minami and Ishihara 1980; Yoshinari et al. 2013), Thailand and Mongolia (Yoshinari et al. 2013), including Vietnam (Weerasooriya et al. 2016) and Korea (Suh 1987). Indeed, the phylogenetic analysis of 18S rRNA gene sequences revealed that the five field isolates under this study shared the same clade 3 along with *B. ovata* isolates from Bangladesh and *Babesia* sp. Hue-1 strain from Vietnam. The PCR assay using *B. ovata*-specific primers targeting *ama-1* and β-tubulin genes did not amplify the specific products. Hence, further study is warranted for the confirmation of the existence of *B. ovata* in South India.

In this study, *Babesia bovis* parasites could not be detected based on 18S rRNA gene sequence analysis or by *B. bovis*-specific PCR. However, *B. bovis* has been previously reported in India from Karnataka (Indani 1938; Setty and Rao 1972; Muraleedharan et al. 1984, 1991; Shastri et al. 1991) based on microscopical examination and from Maharashtra (Kolte et al. 2017) based on PCR technique.

Anaplasma spp.

Anaplasmosis is one of the most prevalent and economically important rickettsial diseases throughout the country. The higher prevalence of anaplasmosis in clinically normal animals indicates that there are subclinical or carrier animals in Kerala, South India (Nair et al. 2013). These carrier animals can act as the source of infection for naive hosts (Radostits et al. 2000). Anaplasmosis was previously reported from different regions of the country (Garg et al. 2004; Muraleedharan et al. 2005; Harish et al. 2006; Soundararajan and Rajavelu 2006), including Kerala (Devada et al. 1996; Gopinath 2004; Nair 2008; Nair et al. 2013). Though anaplasmosis is highly prevalent in India, reports on the molecular characterization of *Anaplasma* spp. in cattle are scarce. Hence, molecular characterization of *Anaplasma* spp. was carried out in the present study.

In the present study, microscopy revealed *Anaplasma* organisms only in six out of 199 samples, while PCR targeting 16S rRNA gene revealed 14 samples positive. The NCBI BLAST analysis and phylogenetic analysis of 16S rRNA gene sequences of field isolates of *Anaplasma* spp. confirmed the existence of *A. marginale*, *A. bovis* and *A. platys* infecting bovines in Kerala, South India. There have been no previous reports on *A. platys* infecting bovines in India. However, *A. marginale* and *A. bovis* have been previously reported in India based on both microscopy and molecular techniques (Devada et al. 1996; Sreekumar et al. 2000; Gopinath 2004; Nair 2008; Nair et al. 2013).

Genetic heterogeneity among the population of *A. marginale* was previously studied from two South Indian states viz., Seemandhra and Telangana (George et al. 2017). Based on phylogeny using *msp4*, it was reported that most of the strains (21/24) from these South Indian states showed close proximity with strains from Mexico and Brazil. In the present study, it was revealed that minimal heterogeneity exists within 16S rRNA and *msp4* genes among the field isolates from Kerala. The results of phylogeny using *msp4* in the present study are similar to those of George et al. (2017). Hence, it could be inferred that isolates of *A. marginale* of South India are genetically closer.

Anaplasma bovis (formerly referred as *Ehrlichia bovis*, Dumler et al. 2001) is a monocytotrophic *Ehrlichia* sp. which was first described in 1931 (Donatien and Lestoquard 1936) and later in cattle and buffalo mainly from Africa, Asia and Middle East countries. *Rhipicephalus appendiculatus*, *Amblyomma variegatum*, *Hyalomma anatolicum* and *Hyalomma truncatum* may act as vectors for *A. bovis* (Radostits et al. 2000). *Anaplasma bovis* was previously reported from various different states in the country mainly by microscopical examination (Devada et al. 1996; Sreekumar et al. 1996; Vijayalakshmi and Sreekrishnan 2005; Prasath et al. 2016) and by molecular techniques (Nair et al. 2013). However, the full extent of genotypic variability and phylogenetic relationships of the Indian *A. bovis* strains is still to be assessed.

Anaplasma platys is assumed to be transmitted by *Rhipicephalus sanguineus* (Inokuma et al. 2000; Dyachenko et al. 2012), which is the common dog tick in India (Abd-Rani et al. 2011). Previously, *A. platys* was described in dogs from USA (French and Harvey 1983), Brazil (Ferreira et al. 2007), China (Hua et al. 2000), Thailand (Suksawat et al. 2001), Taiwan (Chang et al. 1996), Japan (Inokuma et al. 2002a), Australia (Brown et al. 2006), Africa (Sanogo et al. 2003), Italy (de la Fuente et al. 2006), Spain (Aguirre et al. 2006), France (Beaufils et al. 2002), Turkey (Ulutas et al. 2007) and many other countries. *Anaplasma platys*-like parasites were also reported from wild ruminants viz., Brazilian marsh deer (Sacchi et al. 2012), white-tailed deer (Munderloh et al. 2003) and brown brocket deer and marsh deer (Silveira et al. 2012). However, this is the first study to identify *A. platys* in ruminants in India and there is no conclusive evidence for the presence of *A. platys* in dogs. Thus, a detailed systematic study is essential for confirming *A. platys* prevalence and pathogenicity in both carnivores and herbivores.

Conclusion

The present study confirmed the existence of two different populations of *Babesia* spp. circulating in the blood of bovines in Kerala viz., *B. bigemina* and *Babesia* sp., closely related to *B. ovata*/*Babesia* sp. Hue-1 strain (unidentified piroplasms). The *B. bovis* parasites could not be detected in cattle from Kerala, South India. The phylogenetic analysis based on *rap-1a* revealed the existence of higher levels of genetic heterogeneity among *B. bigemina* isolates while using *rap-1c* only, slight heterogeneity was observed. The present study also confirmed the existence of *A. marginale*, *A. bovis* and *A. platys* infecting bovines in Kerala. *Anaplasma marginale* isolates of Kerala were genetically conserved based on phylogenetic analysis of *msp4*. To our knowledge, this study forms the first report of *B. ovata* and *A. platys* in Kerala, South India. Further confirmation on the existence and pathogenicity of unconfirmed *B. bigemina*, *B. ovata*/*B. ovata*-like parasites, *A. bovis* and *A. platys* is urgently warranted.

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