

Phylogenetic analysis of bovine *Theileria* spp. isolated in south India

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Abstract. The objective of the present study is to determine the phylogenetic position of the *Theileria* organisms in blood of cattle of southern India using molecular tools. *Theileria annulata* (Namakkal isolate, Tamil Nadu) and three *Theileria* field isolates (free of *T. annulata*) from Wayanad, Kerala (Wayanad 1, 2, 3) were used. The small subunit ribosomal RNA (SSU rRNA) and major piroplasm surface protein (MPSP) gene products were cloned, sequenced and the phylogenetic tree constructed. SSU rRNA gene of Wayanad 1 isolate (JQ706077) revealed maximum identity with *Theileria velifera* or *Theileria cervi*. The phylogenetic tree constructed based on SSU rRNA genes revealed that Wayanad 1 isolate belonged to a new type which share common ancestor with all the other theilerial species while Wayanad 2 and 3 isolates (JX294459, JX294460) were close to types A and C respectively. Based on MPSP gene sequences, Wayanad 2 and 3 (JQ706078, JX648208) isolates belonged to Type 1 and 3 (Chitose) respectively. When, the previously reported MPSP type 7 is also considered from the same study area, *Theileria orientalis* types 1, 3 and 7 are observed in south India. SSU rRNA sequence of South Indian *T. annulata* (JX294461) showed a maximum identity with Asian isolates while the Tams1 merozoite surface antigen (MSA) gene (JX648210) showed maximum identity with north Indian isolate.

INTRODUCTION

The taxonomic standings of *Theileria* species were based on morphology, clinical signs, host, tick vector and geographic area (Levine, 1985). The genus *Theileria* is currently classified under family Theileriidae (order: Piroplasmida, class: Aconoidasida, Phylum: Apicomplexa). The *Theileria* infecting domestic animals can be considered to fall into seven species or species groups, viz., *Theileria parva*,

Theileria mutans, *Theileria velifera*, *Theileria taurotragi*, *Theileria annulata*, *Theileria orientalis* and *Theileria* of small ruminants (Mehlhorn *et al.*, 1994). The pathogenic species of *Theileria* infecting cattle are *T. parva* and *T. annulata*.

In India *T. annulata* and *Theileria buffeli* / *orientalis* / *sergenti* group of parasites were previously described (Shastri *et al.*, 1985, 1988; Harikrishnan *et al.*, 2001; Ramesh *et al.*, 2003). *Theileria* which are of low pathogenicity or avirulent in nature occur in

all continents and cause benign theileriosis which are transmitted mainly by ticks of the genera *Amblyomma*, *Rhipicephalus* and *Haemaphysalis* (Uilenburg, 1981). For decades, there have been difficulties in the classification of the *T. buffeli / orientalis / sergenti* group of parasites, which are responsible for oriental theileriosis, due to the unreliability of distinguishing these parasites by morphological criterion (Fujisaki, 1994). The name *T. orientalis* was recommended (Uilenberg *et al.*, 1985) for the three benign species of *Theileria* (*T. buffeli / orientalis / sergenti*) based on morphological and serologic examination and experimental tick transmission. Previously, it was believed that these benign species of *Theileria* do not cause disease unless the cattle are affected by stress such as that caused by other pathogens or parturition (Kakuda *et al.*, 1998). However, outbreaks of theileriosis due to these organisms resulting in haemolytic anaemia and death are also increasingly reported from various parts of the world (Aparna *et al.*, 2011; Kamau *et al.*, 2011; McFadden *et al.*, 2011).

Little is known regarding the benign *Theileria* of Indian cattle and buffaloes and hence is to be studied further to ascertain their identity (Gill, 2004). A cross-sectional study using 150 blood samples collected from apparently healthy crossbred cattle of northern Kerala, southern India revealed that the prevalence of *Theileria* sp. other than *T. annulata* was 16 per cent (Nair *et al.*, 2001). Later, based on sequence analysis of p32/33 or major piroplasm surface protein (MPSP) gene, Aparna *et al.* (2011) reported that theilerial organisms causing fatal infection in crossbred adult bovines of south India showed maximum identity with *T. orientalis* type 7. However, detailed phylogenetic studies of theilerial organisms of bovines of south India were not previously undertaken. Hence, the present study focuses on the phylogeny of theilerial piroplasms of south India based on sequence analysis of the small subunit ribosomal RNA (SSU rRNA) and major piroplasm surface protein (MPSP) gene sequences.

MATERIALS AND METHODS

The peripheral blood smears of 30 animals (selected randomly) from Wayanad district (Kerala) were screened microscopically after staining with giemsa to detect the presence of *Theileria* sp.. All these animals were apparently healthy and did not reveal any clinical symptoms suggestive of theileriosis. Heparinized blood samples were collected from three *Theileria* sp. infected adult crossbred Holstein cows. These isolates were designated as Wayanad 1, 2 and 3. They were confirmed negative for *T. annulata* using a specific PCR (d'Oliveira *et al.*, 1995).

Heparinized blood sample collected from an infected (a clinical case) crossbred Jersey cow from Namakkal, Tamil Nadu showing heavy *T. annulata* parasitaemia was designated as Namakkal isolate. The presence of the parasite was confirmed based on specific PCR (d'Oliveira *et al.*, 1995).

The blood samples were processed for crude DNA isolation following the method described for detection of *Plasmodium* sp. from human blood (Tirasophon *et al.*, 1991) with slight modifications. Heparinized blood samples (150 µL) were treated with 1300 µL dehaemoglobinization buffer (0.015% saponin, 3.5 mM NaCl and 1 mM EDTA) and then centrifuged at 12,000 rpm for 2 min. The supernatant was discarded. The pellet was washed once with 750 µL of reaction mixture buffer (10 mM Tris HCl, 50 mM MgCl₂ and 0.01% gelatin) and centrifuged at 6000 rpm for one min. Again, the supernatant was discarded carefully. The final pellet was resuspended in 75 µL of distilled water, boiled for 10 min and 10 µL from this was used as template for PCR. The blood sample of a day old calf processed by the similar technique was used as negative control in all PCR reactions.

Amplification of the 1098 bp small subunit ribosomal RNA (SSU rRNA) gene was performed based on d'Oliviera *et al.* (1995). The primers (Forward 5'-AGTTTCCTGA CCT ATC AG-3' and Reverse 5'-TTG CCT TAA ACT TTC TTG-3') were custom synthesized from Sigma Aldrich, Bangalore, India and used at 10 pmol concentration. Cycling

conditions were as follows: Initial denaturation at 94°C for five min followed by 30 cycles, each cycle consisting of a denaturation step of one min at 94°C, an annealing step of one min at 60°C and an extension step of one min at 72°C. Final extension was performed at 72°C for five min.

The amplification of major piroplasm surface protein (MPSP) or 32 KDa protein gene (~876 bp) of *T. orientalis* was performed (Tanaka *et al.*, 1993) using a pair of oligonucleotide primers custom synthesized from Sigma Aldrich, Bangalore, India (Forward 5'-CAC GCT ATG TTG TTC AAG AG- 3' and Reverse 5'-TGT GAG ACT CAA TGC GCC TA-3') at concentration of 25 pmol. The reactions were carried out in 30 cycles, each consisting of a 2 min denaturation at 95°C (3 min for first cycle), 2 min of annealing at 60°C and two and half min of extension at 72°C, with an additional 7 min elongation at 72°C after the last cycle. *Theileria annulata* specific PCR which amplify 721 bp of merozoite surface antigen gene was performed according to the protocol described by d'Oliviera *et al.* (1995). Primers (Forward 5'- GTA ACC TTT AAA AAC GT-3' and Reverse 5'-GTT ACG AAC ATG GGT TT-3') were custom synthesized from Sigma Aldrich, Bangalore, India and used at the rate of 40 pmol for 25 µl reaction. Cycling conditions were as follows: initial denaturation of 94°C for 5 min followed by 30 cycles with each cycle consisting of a denaturation for one min at 94°C, an annealing for one min at 55°C and an extension for one minute at 72°C. Final extension at 72°C for five min was also provided at the end of 30 cycles.

The PCR products were eluted from the gel using QIAquick gel extraction kit (QIAGen, Germany) based on manufacturer's protocol. The eluted product (8 µL) was used for cloning in to a pTZ57R/T vector (InsTAclone PCR cloning kit, Fermentas, U.S.A) and transformed using JM107 strain of *Escherichia coli*. The transformed cells were plated on a LB agar with ampicillin (50 µg/mL) as antibiotic marker. After overnight incubation at 37°C, positive white colonies were selected and subcultured in LB broth overnight. The presence of the insert

was confirmed by restriction digestion using *Eco*R1 and *Sal* I enzymes of the plasmid isolated (GeneJET plasmid mini prep kit) from the subculture. The stab culture of the colony containing the insert was sent for automated sequencing to SciGenome Labs, Cochin. In order to verify mixed infection or recombination, two clones from each PCR products were sequenced. The sequences were compared with sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST).

Phylogenetic tree was plotted based on ClustalV method of MegAlign programme (DNASTAR) using the following SSU rRNA gene sequences [L02366 (*T. parva*), L19080 (*Cytauxzoon felis*), L19081 (*Theileria* sp., Sable type, South Africa), L19082 (*T. taurotragi*, South Africa), M64243 (*T. annulata*), U97047 (*Theileria* type A, Korea, Cattle), U97048 (*Theileria* sp. type B, Korea, cattle), U97049 (*Theileria* type B1, Korea, cattle), U97050 (*Theileria* sp. type H, Korea, cattle), U97051 (*Theileria* sp. type C, Korea, Cattle), U97052 (*Theileria* type D, Korea, Cattle), U97053 (*Theileria* sp. type E, Korea, Cattle), U97054 (*Theileria* sp. type F, USA, White tailed deer), U97055 (*Theileria* sp. type G, USA, White tailed deer), U97056 (*Theileria* type G1, USA, White tailed deer), Z15105 (*Babesia equi*), Z15106 (*T. buffeli*, Marula, Bovine), AF086804 (*Theileria cervi* subtype G2, USA, Deer), AF086805 (*T. cervi* subtype G3, USA, Deer), L19077 (*Babesia bovis*), L19079 (*Babesia canis*), U09833 (*Babesia microti*), U09834 (*Babesia* sp.), U16369 (*Babesia odocoilei*, USA, Deer), U16370 (*Babesia divergens*, USA, Cattle), X59604 (*Babesia bigemina*), X68523 (*Toxoplasma gondii*), Z15104 (*Babesia caballi*)] (Chae *et al.*, 1998; Chae *et al.*, 1999 a, b; Chansiri *et al.*, 1999).

The phylogenetic tree was also plotted using the MPSP gene sequences based on Ota *et al.*, 2009 and Jeong *et al.*, 2010. The following accessions *viz.*, AB008369 (*T. orientalis*, Essex, Cattle), AB010702 (*Theileria* sp., Texas, USA, Cattle), AB010703 (*Theileria* sp., Kamphaeng Saen, Thailand, Cattle), AB016276 (*Theileria* sp., Nha-Trang, Vietnam, Buffalo), AB016277 (*Theileria* sp., Nha-Trang, Vietnam, buffalo), AB016278 (*T.*

buffeli, Marula, Buffalo), AB016279 (*T. sergenti*, Russia, Buffalo), AB081329 (*Theileria* sp., Narathiwat, Thailand, cattle), AB218430 (*Theileria* sp., Okinawa, Japan, Cattle), AB218431 (*Theileria* sp., Okinawa, Japan, Cattle), AB218436 (*Theileria* sp., Okinawa, Japan, Cattle), AB218442 (*Theileria* sp., Okinawa, Japan, Cattle), AB218443 (*Theileria* sp., Okinawa, Japan, Cattle), AB218444 (*Theileria* sp., Okinawa, Japan, Cattle), AB491342 (*T. orientalis*, Okinawa, Japan, Cattle), AB491343 (*T. orientalis*, Aso, Japan, Cattle), AB491347 (*T. orientalis*, Aso, Japan, Cattle), AB560822 (*T. orientalis*, Hue, Viet Nam, Cattle), AB560831 (*T. orientalis*, Hue, Viet Nam, Buffalo), AB560832 (*T. orientalis*, Hue, Viet Nam, Buffalo), AB560833 (*T. orientalis*, Hue, Viet Nam, Sheep), AB571884 (*T. orientalis*, Hue, Viet Nam, *Rhipicephalus* tick), AB571885 (*T. orientalis*, Hue, Viet Nam, *Rhipicephalus*), AF102500 (*T. orientalis*, Jonggol-1, Indonesia, Cattle), AF236095 (*T. buffeli*, Brisbane, Australia, Buffalo), AF236096 (*T. buffeli*, China, Buffalo), D11046 (*T. sergenti*, Ikeda stock, Japan), D11047 (*T. buffeli*, Warwick stock, Japan, Buffalo), D12689 (*T. sergenti*, Japan, calf), D87190 (*T. sergenti*, Chonju, Korea, Bovine), D87192 (*T. sergenti*, Chonju and Cheju, Korea, Bovine), D87193 (*T. sergenti*, Chitose, Japan, Bovine), D87201 (*Theileria* sp. CJ48-28, Cheju island, Korea, Cattle), D87202 (*Theileria* sp. DJ2, Dangjin, Korea, Bovine), D87205 (*Theileria* sp. OK3, Okushiri Island, Japan, Bovine), and EU584234 (*Theileria* sp., Hubei Province, Hongan, China, Buffalo) were used for plotting the phylogenetic tree. The gene sequences of *T. annulata* Tams1 merozoite surface antigen (MSA) gene of from Ankara (Z48738), *T. parva* Tpms1 merozoite surface glycoprotein from Muguga (Z48740) were used as outgroups for the analysis.

RESULTS

The primers specific for SSU rRNA gene amplified 1098bp product from all isolates including *T. annulata*. The two clones from each isolates revealed similar sequences. Wayanad 1 isolate (JQ706077) revealed a

maximum identity of 98 per cent with bovine *T. velifera* isolate from Tanzania (AF097993) followed by 97 per cent with sika deer isolates of *T. cervi* from China (HQ184411, HQ184406, HQ184410, HQ184407), deer isolates of *Theileria* sp. from Japan (AF529271, AF529272, AF5229273, AB602882 to AB602889, AB012194, AB012196, AB012197) and white tailed deer isolates from USA (AY735131, AY735133, AY735136, AB012199). The Wayanad 2 (JX294459) isolate demonstrated 99-100 per cent identity with bovine *T. sergenti* from China (EU083804, HM538195, FJ822144), Japan (AY661514) and *T. buffeli* isolates from USA (AY661511, AY661512). Wayanad 3 isolate (JX294460) showed a maximum identity of 100 per cent with bovine *T. sergenti* isolate from China (EU083804) followed by 99 per cent with bovine isolates of *T. orientalis* form Australia (AB520956), China (HM538221, HM538222) and bovine isolate of *T. buffeli* from China (HM538197). The phylogenetic tree constructed based on SSU rRNA gene (Fig. 1) revealed that Wayanad 2 and 3 isolates were phylogenetically close to Type A and Type C respectively. The Wayanad 1 isolate belonged to a new type which share common ancestor with all the other theilerial species (Fig. 1).

Expected product size of MPSP gene specific PCR was ~876 bp. Wayanad 1 isolate did not reveal any amplification. Wynanad 2 and 3 isolates (JQ706078, JX648208) showed 871 and 868 bp products respectively. The two clones from each isolate revealed similar sequences. Wynanad 2 shared 97 per cent identity with ovine *Theileria luwenshuni* from China (GQ281044) and bovine *T. sergenti* isolates from China (FJ515688, FJ515689, FJ515690). For Wynanad 3, 96 per cent identity was observed with *T. buffeli* isolates from Marula (AB016278), Warwick (D11047), Australia (D87189), Taiwan (D87207) and Essex (AB008369). The phylogenetic tree constructed using the MPSP gene sequences (Fig. 2) revealed that Wayanad 2 isolate belonged to Type 1 while Wayanad 3 belonged to type 3 *T. orientalis*.

On BLAST search analysis, SSU rRNA sequence of *T. annulata* (Namakkal) isolate (JX294461) showed a maximum identity of

94 per cent with bovine *T. annulata* isolates from Pakistan (JQ743630, JQ743633), Iran (HM628581), China (EU083799) and Turkey (AY524666, AY508472, AY508471, AY508463, AY508463). The phylogenetic tree revealed that *T. annulata* (Namakkal) isolate (JX294461) was closely related to the reference (M64243) isolate (Fig. 1). Similarly, on BLAST search analysis, the gene sequences of *T. annulata* Tams1 merozoite surface antigen (MSA) gene of Namakkal isolate (JX648210) showed maximum identity of 98 per cent with north Indian (Hissar) isolates (AF214840, AF214841, AF214843). The phylogenetic tree revealed that the isolate was close to Ankara (Z48738) isolate (Fig. 2).

DISCUSSION

Theileria isolates from different geographic locations of the world were classified into seven (A-G) types based on a 200 bp fragment of the variable (V4) region of the SSU rRNA gene (Chae *et al.*, 1998). The type G infecting white-tailed deer and elk, was reclassified into three subtypes (Chae *et al.*, 1999b). The benign and moderately pathogenic *Theileria* species infecting cattle and deer were classified by Chae *et al.* (1999a) into two major divisions each with a common ancestor. One major division consisted of two groups, one group (benign) being composed of the closely related isolates of *T. buffeli* Warwick, *T. buffeli* Marula and type A

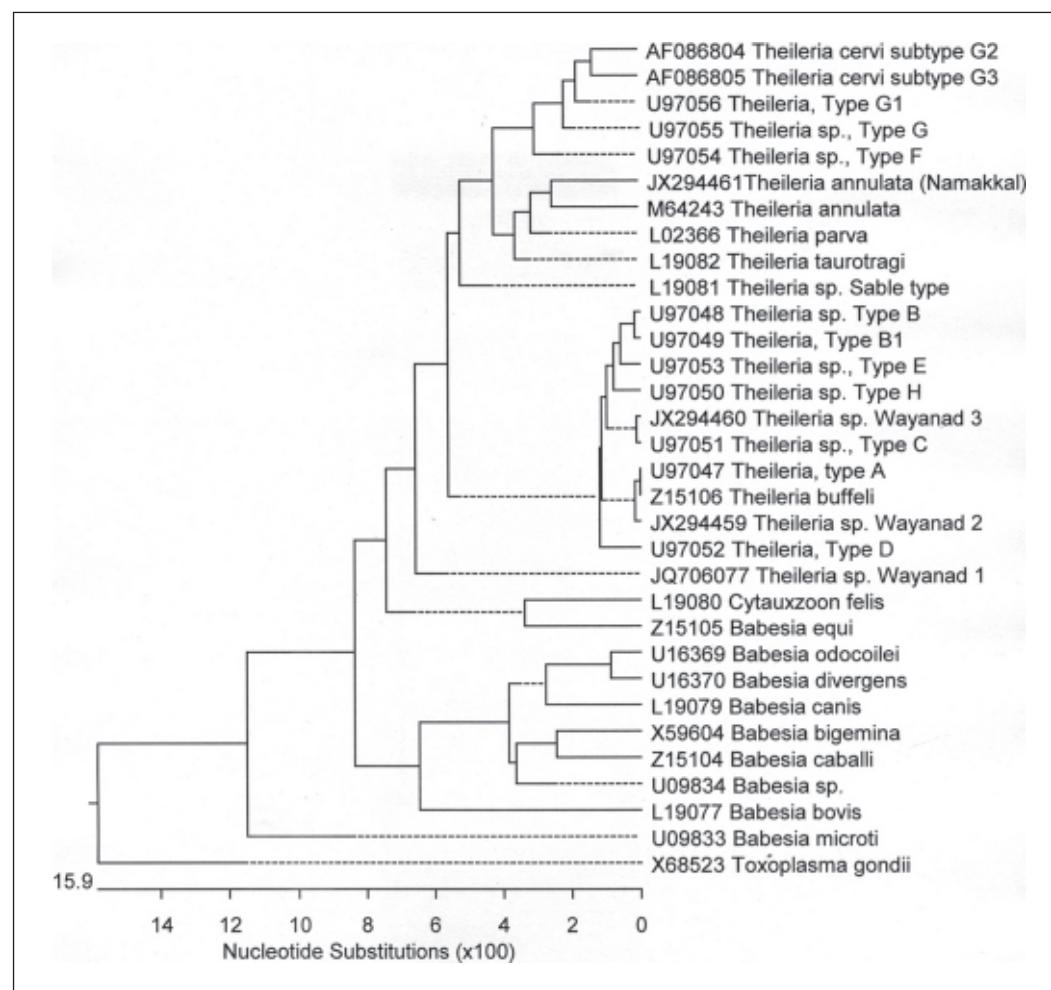


Figure 1. Phylogenetic tree constructed using the SSU rRNA gene sequences

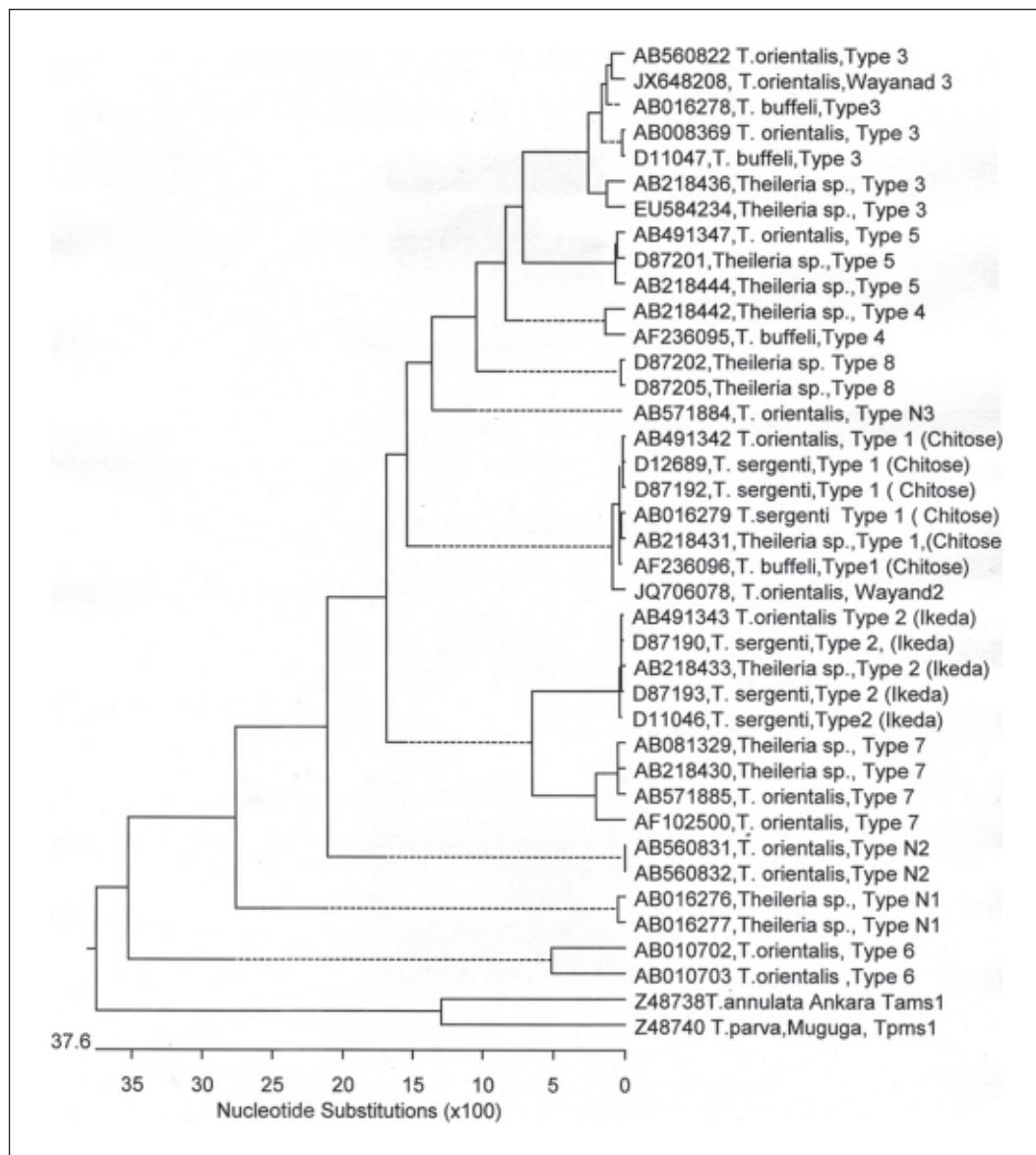


Figure 2. Phylogenetic tree constructed using the MPSP gene sequences

isolates. The other group (moderately pathogenic) consisted of types B (including subtype B1), C, E and H. The other major division is composed of four relatively divergent groups. Group one consisted of pathogenic *Theileria* species, *T. annulata*, *T. parva*, *T. taurotragi* and *Theileria* species (antelope). The groups, two to four consisted of *T. cervi*, *T. mutans* (Intona) and type D. Sequence analysis of SSU rRNA gene was used previously for phylogenetic analysis of *T. buffeli* (Chansiri *et al.*, 1999), *T.*

annulata (Cossio-Bayugar *et al.*, 2002) and *T. orientalis* (Cradio-Fornelio *et al.*, 2003). In the present study, the Wayanad 2 and 3 isolates were identified as type A and C respectively based on SSUrRNA sequences analysis. The type C parasites were considered moderately pathogenic.

Theileria spp. parasites close to Wayanad 1 were not previously reported from India. Wayanad 1 isolate revealed a maximum identity with *T. velifera* / *T. cervi*. We presume that the host species for this isolate

is deer, as the present study was conducted in an area with a good forest cover that harbours a good population of deer including sambar deer (*Cervus unicolor*), barking deer (*Muntjac muntiacus*) and spotted deer (*Axis axis*). *Amblyomma integrum* are very common in deer population in this area (Ajith Kumar, 2011). Deer that are infected with this tick and *Theileria* spp. can transmit the infections to domestic animals. Additional investigations are required on the prevalence, characterization, pathogenicity and transmissibility of the *Theileria* spp. sharing their hosts as wild deer and domestic ruminants in this area.

Major piroplasm surface protein (MPSP or p³²) is a major target of host humoral immune response because of its surface location (Shirakata *et al.*, 1989) and its high immunogenicity (Ohgitami *et al.*, 1987; Zhuang *et al.*, 1994). The Major piroplasm surface protein (MPSP) homologues ranging from 30 to 34 kDa are conserved among *Theileria* species (Shiels *et al.*, 1995). Immunity against MPSP molecule interferes with parasite growth, but do not completely inhibit it which suggested its biological significance in parasite growth (Onuma *et al.*, 1998). MPSP (p^{32/33}) and p²³ of *T. orientalis* are major immunodominant proteins expressed on the surface of the parasite during its intraerythrocytic (piroplasm) stage and show significant sequence diversities among field isolates of *T. orientalis* (Kawazu *et al.*, 1992; Zhuang *et al.*, 1994; Kakuda *et al.*, 1998; Sako *et al.*, 1999(a,b); Ota *et al.*, 2009). Yokoyama *et al.* (2011) observed genetic diversity among *T. orientalis* organisms present in cattle of Kumamoto and Okinawa prefectures of Japan based on MPSP gene. Based on nucleotide sequencing of MPSP gene, Kamau *et al.* (2011) identified four *T. orientalis* genotypes in Australia.

Theileria orientalis populations are currently known to consist of 8 genotypes worldwide, based on all registered MPSP gene sequences (Kim *et al.*, 2004; Ota *et al.*, 2009; Jeong *et al.*, 2010). According to Khukhue *et al.* (2011), *T. orientalis* piroplasms in cattle can be generally divided into the Ikeda group consisting of types 2 (Ikeda) and 7 and the Chitose group

consisting of types 1 (Chitose), 3, 4, 5, 8 and N3. Type N1 and N2 may form separate groups (Buffalo group) consisting of *Theileria* populations distributed in water buffaloes and other ruminants.

Based on MPSP gene analysis, Wayanad 2 isolate belonged to Type 1 while Wayanad 3 belonged to type 3 *T. orientalis*. Both of them belong to Chitose group (Khukhue *et al.*, 2011). These isolates were further characterized as Type A and Type C respectively based on molecular phylogeny of SSU rRNA gene. The animals from which these isolates derived, did not reveal any symptoms of theileriosis. However, the presence of type C parasites in the animals of study area clearly indicate the potentiality of these parasites for pathogenicity. Aparna *et al.* (2011) identified *T. orientalis* type 7 from the same study area based on MPSP sequence which causes fatal disease in periparturient cattle. It is clear that there exists a mixed population of theilerial isolates other than *T. annulata* belonging to *T. buffeli* / *orientalis* / *sergenti* group (usually designated as *T. orientalis*) belonging to types 1, 3 and 7 in southern India. Recently, Sivakumar *et al.* (2013) reported four genotypes based on MPSP sequence analysis *viz.*, types 1, 3, 5 and 7 from Sri Lanka.

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