



Molecular characterization and sequence phylogenetic studies on *Theileria annulata* Mathura isolate based on TAMS and 18S gene

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Bovine tropical theileriosis (BTT) is an economically important serious disease of dairy animals causing economic losses to the tune of US \$ 384.3 million annually in India (Minjauw and McLeod 2003). The disease is caused by an obligate intracellular apicomplexan protozoan parasite *Theileria annulata* and is transmitted mainly by tick vector—*Hyalomma anatolicum anatolicum* in India (Taylor *et al.* 2007). The pathogenicity of the disease can be evaluated based on alteration in various haematological and biochemical parameters (Dua *et al.* 2012). The disease is severe in calves (Gupta *et al.* 2018) and transplacental transmission reported in neonatal calves (Godhara *et al.* 2009, Sudan *et al.* 2015) has further added to the debate on characterization of virulent and pathogenic strains of *T. annulata* affecting calves. Such studies are very much significant as they will help in better understanding of the disease pattern and the distribution of the circulating pathogenic genotypes of the parasite (Tuli *et al.* 2015). Numerous marker genes like ribosomal 18S, ITS, cytochrome oxidase, TAMS, etc have been used over the period of time for genetic characterization studies of *T. annulata* (d'Oliveira *et al.* 1995, Gubbels *et al.* 2000, Azmi *et al.* 2016). However, when it comes to Indian scenario, literature on phylogenetic analysis of calf theileriosis is virtually very much limited. In present study, a local calf isolate of *T. annulata* from Mathura was characterized based on TAMS and 18S gene. Thereafter, analysis was made between the present isolate and other isolates based on these two gene loci to delineate phylogenetic association between various isolates across the world.

The study was in accordance with the ethical standards of the Institutional Animal Ethics Committee (Approval No. 34/2/IAEC/2016).

Blood samples (1 ml aliquot) was collected in clean

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sterile vacutainer, coated with EDTA anticoagulant, from the jugular vein of earlier confirmed theileriosis infected calf (through microscopic observation, clinical signs and PCR). DNA was isolated from blood using wizard DNA isolation kit (Promega, USA) following manufacturer's protocol.

Primer selection and PCR amplification: Oligonucleotide primers targeting the TAMS and 18S gene of *T. annulata* were custom synthesized (d'Oliveira *et al.* 1995, Azmi *et al.* 2016) from Imperial Life Sciences Pvt. Ltd., Gurugram, India. The PCR reactions were set up into 25 µl volume containing 12.5 µl of Green PCR Master Mix (0.05U/µl Taq DNA polymerase in reaction buffer, 4 mM MgCl₂, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, and 0.4 mM dTTP), 1.5 µl of each primer (15 pmol of each primer) and 5 µl of the extracted DNA template. The total volume of the PCR mix was made up to 25 µl using nuclease-free water. The thermocyclic profile alongside the primer sequence and expected amplicon size are given in Table 1. The respective amplified amplicons were analyzed by agarose gel electrophoresis in 1.25% agarose gel.

The two respective genes were purified using Gel extraction and PCR clean up kit (Fermentas) following the manufacturer's protocol and the purified PCR products were sent for DNA sequencing to Invitrogen Bio Sciences Pvt. Ltd., Gurugram, India. The sequences obtained were submitted in NCBI and corresponding accession numbers were obtained. Both the gene sequences were separately analyzed with other sequences available in NCBI using DNA star, Gene Tool and Mega 6 softwares.

The TAMS and 18S gene gave specific amplicons of size 721 and 542 bp, respectively. These respective amplicons were excised from 1.25% agarose gel and were purified using commercial kit. Thereafter, they were outsourced for custom sequencing and the respective sequences were submitted in NCBI and corresponding accession numbers MF384420 and MF384421 were obtained for TAMS and 18S gene, respectively.

Separate phylogenetic trees and divergence tables were

made for both the genes. The phylogenetic tree of both the genes revealed close association between Indian isolates in comparison to isolates across the globe (Fig. 1a, b). Based on TAMS gene, the present isolate showed per cent homologies in the range 65.8–97.2% with various isolates across India and abroad (Fig. 2a). Likewise, 18S gene based per cent homologies of the present isolate varied from 70.5 to 100% with other isolates across India and abroad (Fig. 2b).

Molecular characterization studies primarily involve selective targets. *T. annulata* merozoite surface antigen (TAMS) is widely used for phylogenetic analysis throughout the globe. TAMS is highly specific for *T. annulata* and it does not show cross reactivity with other *Theileria* spp. like *T. parva*, *T. mutans*, *T. sergenti*, *T. buffali*, *T. velifera* and *T. taurotragi* (d'Oliveira *et al.* 1995). Likewise, 18S ribosomal RNA is a universal gene employed for phylogenetic studies of a number of parasites including

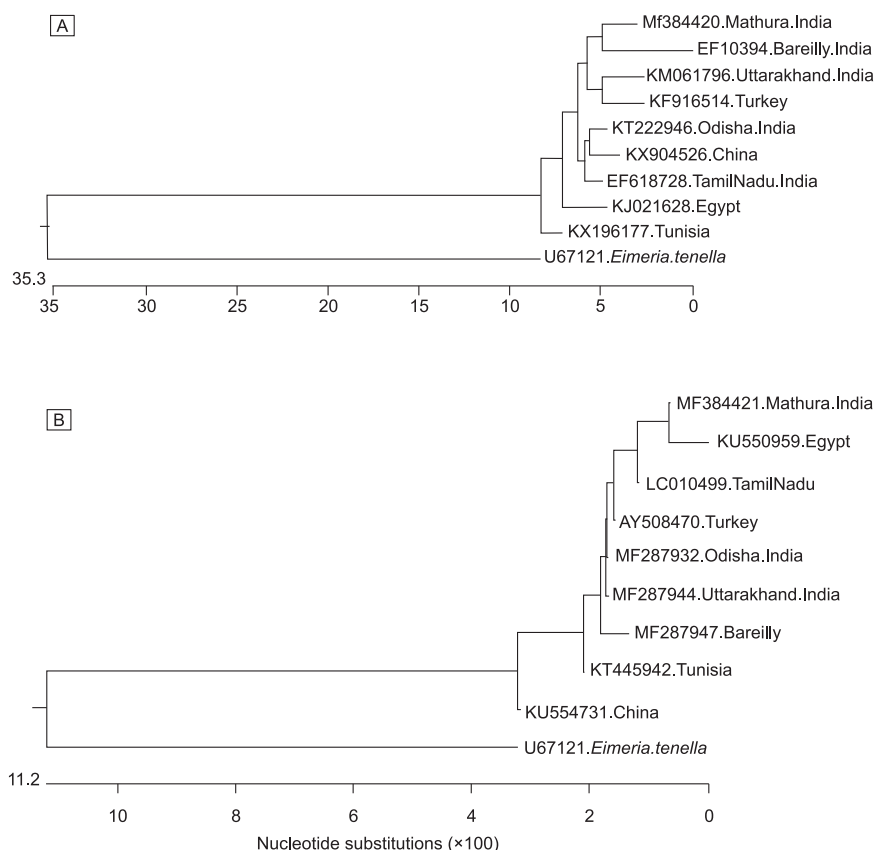


Fig. 1. Phylogenetic tree of studied isolate of *Theileria annulata* in comparison with other isolate across India and abroad based on TAMS (A) and 18S (B) gene. The accession number of individual isolate is followed by respective place of origin.

Table 1. Primer sequences, expected amplicon size and thermo cyclic profile for TAMS and 18S gene of *Theileria annulata*

Primer	Primer sequence	Amplicon size	Reference		
TAMS F	5'ATG AGG ATG AAA AGA AA	721 bp	d'Oliveira <i>et al.</i> (1995)		
TAMS R	A AGG AGG AAA AAA AAG ATG T 3'				
18S F	5'GCG AAGACT GCA AGG GG	542 bp	Azmi <i>et al.</i> (2016)		
18S R	G GAG AAC T3'				
	5'GTC TTG TAA TTG GAA				
	TGA TGG3'				
	5'TAG TTT ATG GTT				
	AGG ACT ACG3'				
Thermal cycling profile					
	Initial denaturation	Denaturation	Hybridization	Extension	Termination
PCR with TAMS gene	95°C for 5 min	94°C for 45 sec	55°C for 1 min × 30 cycles	72°C for 1 min	72°C for 5 min
PCR with 18S gene	94°C for 5 min	94°C for 1 min	55°C for 30 sec × 35 cycles	72°C for 45 sec	72°C for 5 min

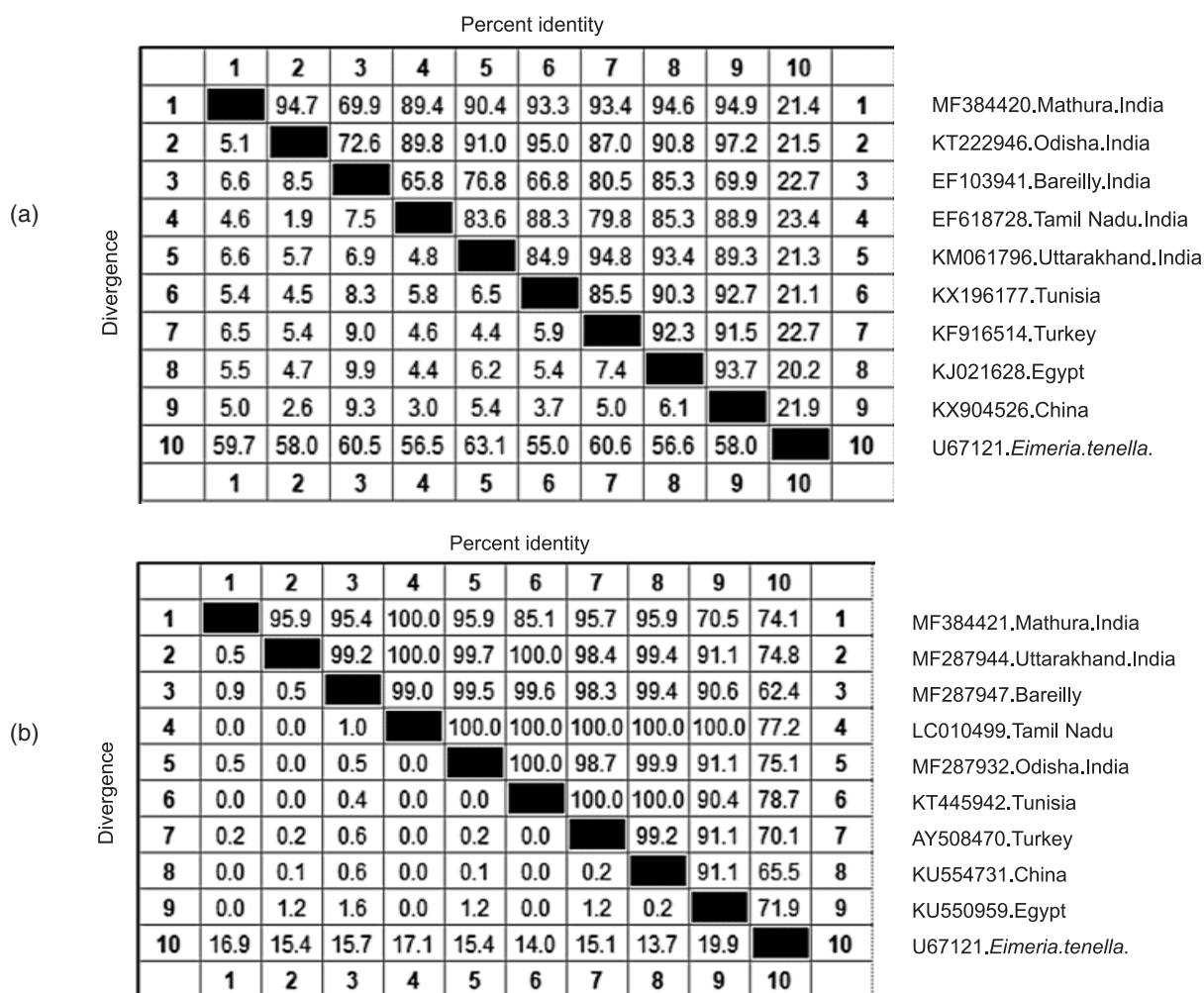


Fig. 2. Per cent identity of studied isolate of *Theileria annulata* in comparison with other isolate across India and abroad based on TAMS (2a) and 18S (2b) gene. The accession number of individual isolate is followed by respective place of origin.

piroplasms (Sudan *et al.* 2017) although there are records of variations at 5' end of the gene in certain protozans (Sudan and Shanker 2018).

Based on TAMS loci, the present isolate was closer with isolate from Bareilly and Uttarakhand than the isolate from Odhisa and Tamil Nadu. Interestingly, isolates from North India, viz. Mathura, Bareilly and Uttarakhand formed a separate clade on phylogenetic tree and isolates from Southern India, viz. Tamil Nadu and Odhisa formed other clade. Globally, the isolate was to be closer to isolates from Turkey than to isolates from China, Egypt and Tunisia (Fig. 1a). So far as 18S loci is concerned, the present isolate was closer to isolate from Tamil Nadu followed by Odhisa and Bareilly. All the Indian isolates formed a single clade on phylogenetic tree. Globally, on the basis of 18S loci, the present isolate was close to Egypt isolate followed by Turkey, Tunisia and China isolates (Fig. 1b). Based on TAMS gene, the present isolate showed per cent homologies in the range 65.8–97.2% with various isolates across India and abroad (Fig. 2a). Likewise, 18S gene based per cent homologies of the present isolate varied from 70.5 to 100%

with other isolates across India and abroad (Fig. 2b).

In conclusion, for molecular characterization, multiple gene loci are required for better understanding of the phylogenetic position of the given isolate. This compensates the limitation of one gene loci by another one so that a true picture comes into preposition. The characterization of Mathura calf isolate of *T. annulata* based on TAMS and 18S gene revealed that Indian isolates formed separate clade differentiating from most of the other isolates across the globe. Wide variation was noticed in terms of per cent homologies of studied isolate in comparison to other isolates across world and India.

SUMMARY

The TAMS and 18S gene of local calf isolate from semi-arid Mathura were amplified and used for phylogenetic analysis after custom sequencing. Results revealed phylogenetic association between Indian isolates in comparison to isolates across the world. Based on TAMS gene, the present isolates showed per cent homologies in the range 65.8–97.2% with various isolates across India

and abroad. Likewise, 18S gene based per cent homologies of the present isolates varied from 70.5–100% with other isolates across India and abroad. The findings are important from molecular evolutionary point of view.

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