Archives of Razi Institute, Vol. 76, No. 2 (2021) 243-252

Copyright © 2021 by
Razi Vaccine & Serum Research Institute
DOI: 10.22092/ari.2020.341390.1431

Original Article

Molecular Characterization and Phylogenetic Analysis of Pathogenic *Theileria* spp. Isolated from Cattle and Sheep based on Cytochrome *b* Gene in Iran

Afshari, A[1, 2], Tavassoli, M[1 *], Esmaeilnejad, B[1], Habibi, Gh[2], Esmaeilnia, K[2]

1. Department of Pathobiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
2. Department of Parasitic Vaccines Research and Production, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

Received 4 January 2020; Accepted 15 February 2020
Corresponding Author: m.tavassoli@urmia.ac.ir

Abstract

The present study investigated the phylogenetic relationship based on cytochrome *b* gene sequences among pathogenic *Theileria* species (spp.) in Iran, including *Theileria annulata* and *Theileria lestoquardi*, along with other data available in GenBank. A total of 136 (cattle) and 80 (sheep) blood samples suspected of piroplasm infection were obtained from six different provinces of Iran. Both microscopic and molecular methods using species-specific primers were used for screening *T. annulata* and *T. lestoquardi* positive samples. Finally, the partial cytochrome *b* gene of 30 *T. annulata* and 5 *T .lestoquardi* were amplified, sequenced, and deposited in GenBank. The results indicated that there were 12 different genotypes among *T. annulata* isolates, while only one genotype was observed among *T. lestoquardi* isolates. *T. lestoquardi* infection in cattle was detected in one sample, and no *T. annulata* and *T. lestoquardi* coinfection were detected in sheep and cattle. In the phylogenetic tree, different *Theileria* spp. were placed in separate clades, and the reliability of depicted tree and monophyly of *T. annulata* and *T. lestoquardi* ingroups were supported by the bootstrap value of 94% which significantly indicated that these two species evolved from a common ancestor. The tree also showed that these two pathogenic spp. shared a more recent common ancestor, compared to another species of *Theileria* parasites. To the best of our knowledge, this study is the first phylogenic analysis of pathogenic *Theileria* spp. in Iran based on the cytochrome *b* gene sequences. In addition, the first *T. lestoquardi* cytochrome *b* gene was sequenced and deposited in GenBank.

Keywords: Cytochrome b, Iran, Multiplex PCR, Phylogeny, Theileria

Caractérisation Moléculaire et Analyse Phylogénétique de *Theileria spp.* Isolé des Bovins et des Moutons sur la base du Gène du Cytochrome b en Iran

Résumé: La présente étude a examiné la relation phylogénétique basée sur les séquences du gène du cytochrome *b* parmi les espèces pathogènes de *Theileria* (*spp.*) en Iran, y compris *Theileria annulata* et *Theileria lestoquardi*, ainsi que d'autres données disponibles dans la GenBank. Les échantillons de sang de 136 bovins et 80 moutons suspectés d'infection par le piroplasme ont été prélevés dans six provinces différentes d'Iran. Des méthodes microscopiques et moléculaires utilisant des amorces spécifiques à l'espèce ont été utilisées pour cribler des échantillons positifs pour *T. annulata* et *T. lestoquardi*. Enfin, le gène partiel du cytochrome *b* de 30 *T. annulata* et 5 *T. lestoquardi* ont été amplifiés, séquencés et déposés dans la GenBank. Les résultats ont indiqué qu'il y avait 12 génotypes différents parmi les isolats de *T. annulata*, alors qu'un seul génotype a été observé parmi les isolats de *T. lestoquardi*. Une infection à*T. lestoquardi* chez les bovins a été détectée dans un échantillon, et aucune co-infection à *T. annulata* et *T. lestoquardi* n'a été détectée chez les moutons et les bovins.

Dans l'arbre phylogénétique, différentes *Theileria spp.* Ont été placées dans des clades séparés, et la fiabilité de l'arbre représenté et de la monophylie des endogroupes de *T. annulata* et de *T. lestoquardi* a été soutenue par la valeur de bootstrap de 94% qui a indiqué de manière significative que ces deux espèces ont évolué d'un ancêtre commun. L'arbre a également montré que ces deux *spp.* pathogènes partageaient un ancêtre commun plus récent, comparé à une autre espèce de parasites *Theileria*. À notre connaissance, cette étude est la première analyse phylogénique de *Theileria spp.* pathogène en Iran basée sur les séquences du gène du cytochrome *b*. De plus, le premier gène du cytochrome *b* de *T. lestoquardi* a été séquencé et déposé dans la GenBank.
Mots-clés: Cytochrome *b*, Iran, PCR multiplex, phylogénie, *Theileria*

1. Introduction

Theileriosis is a tick-borne disease of ruminants caused by apicomplexan parasites belonging to the genus *Theileria*. The disease is transmitted by ixodid ticks and distributed in endemic regions from tropical and subtropical countries, including Iran (Hashemi-Fesharki, 1988). The life cycle of *Theileria* is complex and involves asexual and sexual replications in both vertebrate and invertebrate hosts. *Theileria* parasites replicate asexually in three different stages (sporogony in the tick vector, as well as schizogony and merogony in bovine host cells). Sexual reproduction of the parasite occurs within the intestinal lumen of the tick vector (Conrad et al., 1985; Sivakumar et al., 2014). Despite several *Theileria* spp. which infect ruminants in the endemic area, only two *Theileria* spp. are highly pathogenic in Iran, including *T. annulata* and *T. lestoquardi* that infect large and small ruminants, respectively (Hooshmand-Rad and Hawa, 1973; Hashemi-Fesharki, 1988). However, there is also a report of cattle being infected with *T. lestoquardi* (Namavari et al., 2015). Although indigenous cattle are resistant to tropical theileriosis, exotic and crossbred cattle are very susceptible to disease; moreover, the mortality rate can reach up to 40%-60% if untreated (Habibi, 2012).

T. lestoquardi (formerly *T. hirci*) is the most virulent species in sheep and goats, followed by a mortality rate up to 90% (Hooshmand-Rad and Hawa, 1973). These high rates of mortality demonstrate the economic impact of theileriosis in the livestock industry. Traditionally, *Theileria* parasites are diagnosed by the microscopic inspection of Giemsa stained blood smears. However, differentiation of some *Theileria* parasites, such as *T. annulata* and *T. lestoquardi*, is difficult using this method. Different types of molecular markers have been used for diagnostic and phylogenetic studies previously, some of them are small subunit ribosomal RNA (18S rRNA) (Habibi, 2012), large subunit ribosomal RNA (28S rRNA) (Gou et al., 2013), *T. annulata* merozoite surface antigen 1 (Tams1) (Kirvar et al., 2000), sporozoite *T. lestoquardi* antigen-1 (Slag-1) (Skilton et al., 2000), cytochrome *b* (*Cytb*) gene (Sharifiyazdi et al., 2012; Mhadhbi et al., 2015), and rRNA internal transcribed spacers (ITS1 and 2) (Aktas et al., 2007). Among these molecular markers, the *Cytb* gene, due to its sequence variability, is commonly used as a region of mitochondrial DNA for determining phylogenetic relationships between organisms within families and genera levels (Castresana, 2001). Additionally, the role of mutation in *Cytb* ubiquinol/ubiquinone binding sites (Qo1, Qo2) associated with drug resistance has been shown in some studies (Sharifiyazdi et al., 2012; Mhadhbi et al., 2015).

Moreover, polymorphism of the *Cytb* gene of *T. annulata* has been studied previously, and the utility of this genetic marker has been proposed as a tool to discriminate between different *T. annulata* genotypes (Mhadhbi et al., 2015). Theileriosis is endemic in Iran due to two highly pathogenic *Theileria* spp.; accordingly, different genotypes are required to be sequenced and identified based on cytochrome *b* gene sequences in order to conduct phylogenetic analysis

and drug resistance studies. This study aimed at setting up a new multiplex polymerase chain reaction (mPCR) method to simultaneously detect the pathogenic *Theileria* spp. Additionally, sequenced cytochrome *b* gene sequences derived from two pathogenic *T. annulata* and *T. lestoquardi* from different areas in Iran were used for molecular phylogenetic analysis based on both nucleotide and amino acid sequences of the *Cytb* gene.

2. Material and Methods

2.1. Blood Samples

A total of 216 EDTA whole blood samples suspected to piroplasmosis were obtained from cattle (n=136), sheep (n=69), and goats (n=11) of six different provinces in Iran, including, East Azerbaijan, Khorasan Razavi, Fars, Khuzestan, Alborz, and Qazvin during 2017-2019. Furthermore, the *T. annulata* and *T. lestoquardi* cell lines and *Babesia sp.* positive blood were obtained from the Department of Parasitic Vaccines Research and Production, Razi Vaccine and Serum Research Institute and were used as positive controls in all PCR reactions.

2.2. Screening for Piroplasms

Initially, all the samples were screened for the presence of piroplasms by a microscopic examination of prepared (Barcia, 2007) thin blood smears using the mPCR method for simultaneous detection and differentiation of *T. annulata* and *T. lestoquardi* from other piroplasms.

2.3. DNA Extraction

The phenol-chloroform method was used for genomic DNA extraction from blood and cell line samples. Following that, the extracted DNAs were kept at -20ºC till further use in PCR (Sambrook, 1989).

2.4. Primer Design

This study designed four sets of primers and utilized them in the PCR and mPCR. Species-specific Tams and Slag primers were also used for specific detection of *T. annulata* and *T. lestoquardi*, respectively. Furthermore, a common 18S rRNA primer pair was also designed to increase the specificity of mPCR. It should be noted that the common 18S rRNA primers produce 256 and 266 bp fragment lengths on *Theileria* and *Babesia* positive specimens, respectively. Finally, a pair of primers (TcytbF, R) was used to amplify the partial sequence of cytochrome *b* gene for two above-mentioned *Theileria* spp. All the primers were designed using Gene Runner and Oligo 7 software and checked for complementarity and primer dimers. The specificity of primers was checked by the Basic Local Alignment Search Toll (BLAST) of the national center for biotechnology information (NCBI) before primer synthesis. Table 1 summarizes some characteristics of the primers.

Table 1. Characteristics of the PCR primers used in this study

Primer sets	Accession number	Length and position	Sequence (5' to 3')	PCR product$_{(bp)}$
18SF 18SR	KF429795	17nt$_{(1151-1167)}$ 20nt$_{(1416-1397)}$	TAATTTGACTCAACACG ATCACAGACCTGTTATTGCC	256-266
TamsF TamsR	Z48739	23nt$_{(407-429)}$ 25nt$_{(1003-979)}$	GAGACAAGGAATATTCTGAGTCC TTAAGTGGCATATAATGACTTAAGC	597
SlagF SlagR	AF128526	18nt$_{(138-155)}$ 19nt$_{(537-519)}$	ATCAGCGGCAACACAACC TTCCTGGTCATGAGAACCG	400
TCytbF TCytbR	M63015	20nt$_{(118-137)}$ 20nt$_{(1100-1081)}$	GTGCCAGCAAAAGGTATGGC CCCCTCCACTAAGCGTCTTT	983

2.5. PCR and mPCR

Single PCR was carried out using Tams and Slag primers in a final volume of 20 µl, which included 10µl of master mix (Yekta Tajhiz, IRAN) 2X, 7 µl of DEPC water, 1µl of each primer (10 pmol/ µl), and 1µl of DNA template. The PCR conditions were performed as follows: Initial denaturation for 3 min at 95ºC, followed by 33 cycles of denaturation (10S at 95ºC), annealing (40S at 54ºC), and extension (40S at 72ºC), followed by final extension for 5 min at 72ºC. In addition, the PCR was performed using a Gradient Palm-Cycler™ (Corbett Research, Sydney, Australia), and 10 µl of each PCR product was electrophoresed on 1.5% agarose gel contained GelRed™ (Biotium, USA) and visualized under UV light. The mPCR was carried out in final volume of 25 µl containing 11 µl of master mix 2X, 7.5 µl of nuclease-free DEPC water, 0.5µl (10 pmol/µl) of each Slag F/R and 18S F/R, 1.5 µl (10 pmol/µl) of Tams F/R primers, and 1.5 µl of template DNA. The mPCR conditions were performed as described in the case of single PCR.

2.6. *Cytb* Gene Amplification and Sequencing

T. annulata and *T. lestoquardi* positive samples obtained from mPCR reaction of different specimens were used for the amplification of partial *Cytb* gene sequences of two aforementioned pathogenic *Theileria* spp. Each reaction was performed as the same condition of single PCR that mentioned above, except for annealing (45S at 61ºC) and final volume of 50 µl containing 25 µl of master mix 2X, 17.5 µl of nuclease-free DEPC water, 2.5 µl of each Tcytb F/R primers (10 pmol/µl), and 2.5 µl of template DNA. The expected 983 bp amplicons of the *Cytb* gene were analyzed by electrophoresis on 1.5% agarose gel. Afterward, the amplicons were purified using Gel extraction and PCR clean-up kit (Roche, USA) according to the manufacturer's instruction. Subsequently, the purified PCR products along with forward and reverse primers were sent for both directional DNA sequencing by the Sanger method on an ABI 3730 sequencer (Bioneer, Daejeon, South Korea).

2.7. Sequence Assembly, Alignment, and Analysis

The consensus partial *Cytb* gene nucleotide and predicted amino acid sequences were generated by editing and alignment of their forward and reverse reads using Mega 7 software; moreover, the similarity of sequences was compared using the BLAST program (NCBI). Multiple alignments were performed using the Clustal W program of Mega 7 software, and the resulted sequences were submitted to the NCBI GenBank database. The phylogenetic tree based on the *Cytb* gene was constructed with the Neighbor-Joining method using Mega 7 software (Kumar et al., 2016).

3. Results

3.1. Microscopic Examination of Blood Films

The presence of piroplasms was assessed by the observation of 50 microscopic fields under a light microscope. Totally, 216 smears were examined, 155 of which were positive for piroplasms (Figure 1 and Table 2).

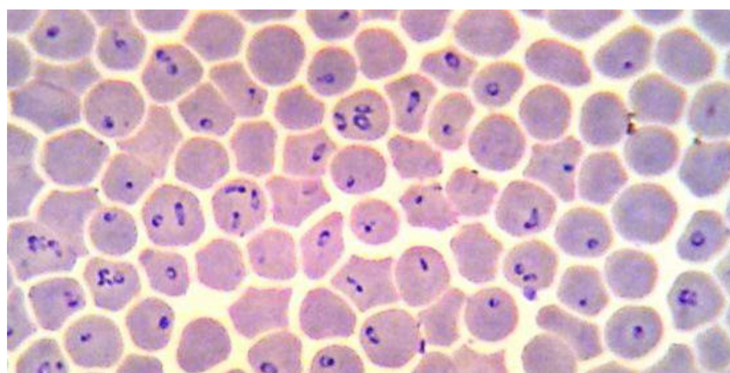

Figure 1. Piroplasm of *Theileria annulata* in red blood cells

Table 2. Results of microscopy and multiplex PCR screening

	Number of the examined sample	Methods					
		Microscopy		Multiplex PCR			
		+	-	*T. annulata*	*T. lestoquardi*	Other piroplasms	Negative
Cattle	136	98	38	104	1	6	25
Sheep and Goat	80	57	23	0	9	58	13
Total	216	155	61	104	10	64	38

3.2. Single and Multiplex PCR Optimization

The expected PCR products corresponding to Tams (597 bp) and Slag (400 bp) genes were separately amplified using *T. annulata* and *T. lestoquardi* DNA derived from available schizont infected cell lines as positive controls in the PCR reaction (Figure 2a). Furthermore, the 983 bp PCR product resultant to the partial *Cytb* gene sequences of *T. annulata* and *T. lestoquardi* was successfully amplified (Figure 2b). The optimization of mPCR was successfully performed using species-specific (Tams and Slag) primers along with a common 18S rRNA primer as well as DNAs of *T.annulata*, *T. lestoquardi*, and *Babesia sp.* as positive controls (Figure 3).

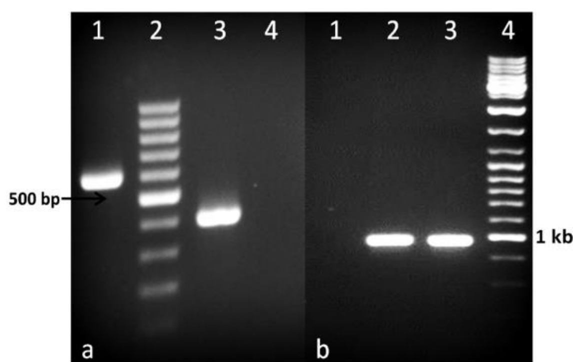

Figure 2. Agarose gel electrophoresis of single PCR products. **a)** lane 1: Tams, lane 2: DNA size marker (100bp), lane 3: Slag, and lane 4: negative control. **b)** lane 1: negative control, lanes 2-3: *T. annulata* and *T. lestoquardi* partial *Cytb* gene, and lane 4: DNA size marker (1kb).

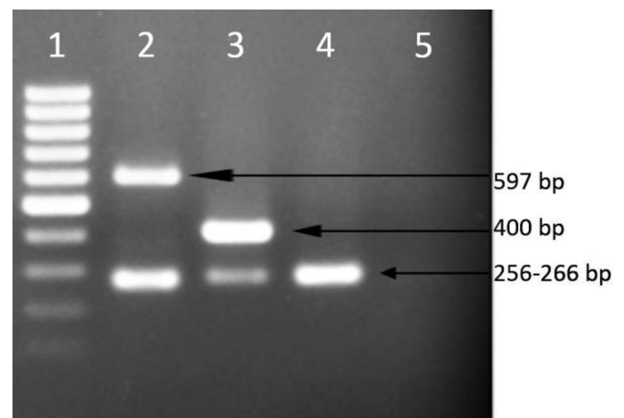

Figure 3. Agarose gel electrophoresis of the multiplex PCR amplification products. Lane 1: DNA size marker 100 bp, lane 2: *T. annulata* (597 and 256 bp), lane 3: *T. lestoquardi* (400 and 256 bp), lane 4: *Babesia sp.* (266 bp), and lane 5: no DNA as a negative control

3.3. mPCR Screening

Out of 216 examined samples, 104, 10, and 64 positive samples were found for *T. annulata*, *T. lestoquardi*, and other piroplasm spp., respectively. Neither *T. annulata* and *T. lestoquardi* coinfection nor sheep and goat *T. annulata* infection was detected. More details of microscopy and mPCR screening results are described in Table 2.

3.4. Cytochrome *b* Gene Amplification, Sequencing, and Analysis

The partial 983bp fragment of the *Cytb* gene was subjected to sequence analysis. The fragments of the mitochondrial *Cytb* gene were amplified and sequenced from 30 *T. annulata* isolates (from Alborz, Qazvin, Fars, Khuzestan, East Azerbaijan, and Khorasan provinces along with *T. annulata* vaccine strain) and

five *T. lestoquardi* isolates (from Qazvin, Fars, and Khuzestan provinces) using PCR. After sequence editing and assembly, the sequences between 918 and 936 bp in size of the *Cytb* gene were obtained and submitted to GenBank for sequence deposition (Accession numbers: MN422301-5, MN432494-518, and MN481235-9).

A total of 918 positions were used for analysis in the final dataset, and among 30 sequences of the *T. annulata Cytb* gene, 12 different genotypes were detected after multiple alignments using previously registered *T. annulata Cytb* gene sequence in GenBank (accession number CR940346) as a reference sequence. The nucleotide transition substitutions were detected in 14 positions, and nonsynonymous substitutions were detected in five positions and indicated in bold in Figure 4. Among five *T. lestoquardi Cytb* sequences obtained from sheep, goats, and cattle, only one genotype was detected. The phylogenetic tree was constructed by the Neighbor-Joining method using partial *Cytb* gene of *T. annulata* and *T. lestoquardi* sequences obtained in this study along with the *Cytb* sequences of *T. annulata*, *T. parva* (AB499089, NC_011005), *T. orientalis* (AB499090), and *Theileria* spp. Sri Lanka and *Babesia bovis* (GQ214235) available in GenBank (Figure 5). As can be observed in Figure 5, different *Theileria* spp. were grouped in separate clades supported by bootstrap values higher than 90%. The studied *T. annulata* isolates were divided into three subclades of A, B, and C. In total, 10 out of 12 different *T. annulata Cytb* genotypes obtained in this study along with *T. annulata* from other countries (Turkey, India, and Sudan) were grouped in subclade A, and two other genotypes (Fars and Azerbaijan genotypes) were grouped in subclades B and C, respectively, which were close to African isolates (Figure 5).

Codon	78	116	129	139	143	146	203	210	227	237	290	330		
Accession No.	Ser	Thr	Ser	Leu	Phe	Ala	Ile	Val	Val	Phe	Val	Lys	n	GT
CR940346(5543-4452)	TCG	ACT	AGC	TTA	TTC	GCT	ATA	GTA	GTG	TTT	GTA	AAG	-	-
MN432518 Vaccine	..A	..C	...	..G	..T	...	...	...	...	...	..G	...	2	1
MN432496 Alborz	...	...	...	...	..T	...	...	..G	...	...	...	...	1	2
MN432497 Azerbaijan	...	...	..T	..G	..T	...	...	...	A..	...	..G	...	3	3
MN422302 Qazvin	..A	...	...	..G	..T	...	...	...	...	...	..G	...	6	4
MN422301 Alborz	..A	...	...	..G	..T	...	...	...	...	..C	..G	...	3	5
MN432510 Khuzestan	..A	...	...	..G	..T	A..	...	...	...	...	..G	...	3	6
MN432502 Fars	..A	...	G..	..G	..T	A..	...	...	...	..C	..G	..A	1	7
MN432504 Fars	...	...	G..	..G	..T	A..	...	...	...	...	..G	...	1	8
MN432503 Fars	..A	..C	G..	..G	..T	A..	G..	...	...	...	..G	...	1	9
MN422303 Qazvin	..A	...	...	..G	..T	A..	G..	...	...	...	..G	...	1	10
MN422305 Khorasan	..A	..C	...	..G	..T	A..	...	...	...	...	..G	...	6	11
MN422304 Khorasan	..A	..C	...	..G	C.T	A..	...	...	...	...	..G	...	2	12

Figure 4. Cytochrome *b* sequence diversity among Iranian isolates, compared to *T. annulata* strain Ankara (CR940346) as a reference sequence. Totally, 12 different genotypes were characterized from 30 total *T. annulata* samples collected from cattle with theileriosis. Furthermore, 14 nucleotide substitutions were discovered throughout the 918 bp fragment of the *Cytb* gene, five of which were nonsynonymous (gray shading).

Abbreviations:

n: number of sequences with same genotypes

GT: genotype number

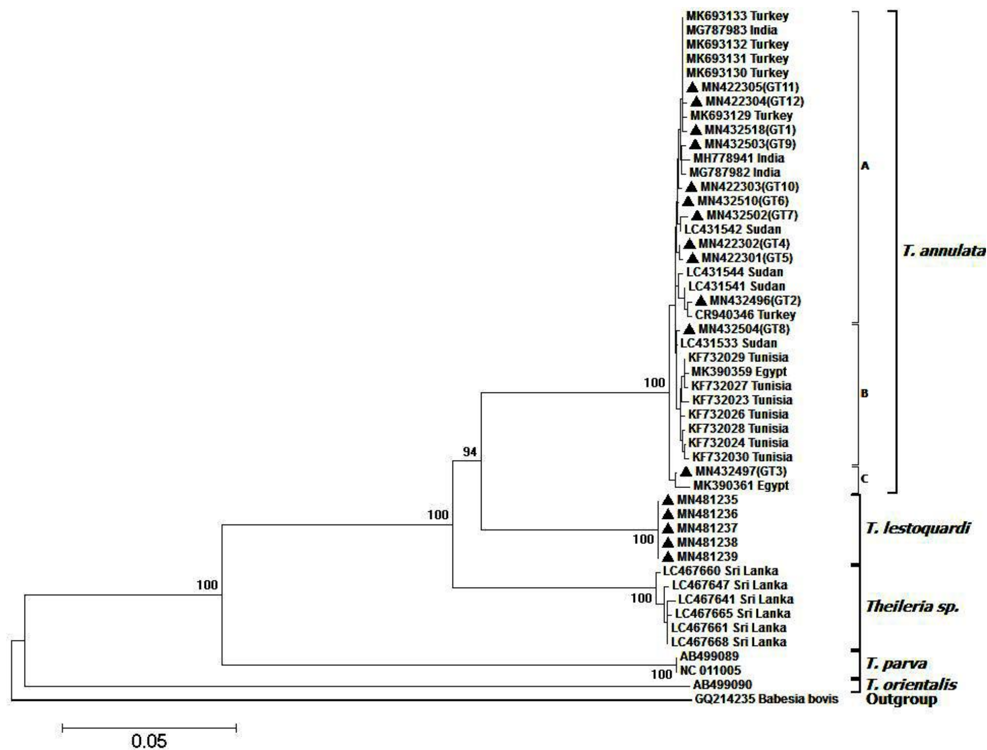

Figure 5. Phylogenetic tree based on partial *Cytb* gene sequences of *Theileria* spp. using the Neighbor-Joining method in MEGA7. Black triangles denote Iranian *T. annulata* (GT1-GT12) and *T. lestoquardi*. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches and those with bootstrap values lower than 90% were eliminated. There were a total of 879 positions in the final dataset. The analysis involved 49 Cytb nucleotide sequences.

Table 3. Nucleotide and amino acid *Cytb* sequence identity scores (%) shared within and among *T. annulata*, *T. lestoquardi*, and *Theileria* spp. Sri Lanka based on partial (879 bp) *Cytb* gene sequences

	Nucleotide sequences			Amino acid sequences		
	T. annulata	*T. lestoquardi*	*Theileria* sp. Sri lanka	*T. annulata*	*T. lestoquardi*	*Theileria* sp. Sri lanka
T. annulata	98.6-100			97.3-100		
T. lestoquardi	88.6-89.1	100		87.1-88.8	100	
Theileria sp. Sri lanka	87-87.5	87.5-87.9	99.4-100	82.5-83.5	84.8-85.1	99.6-100

4. Discussion

T. annulata and *T. lestoquardi* are the main pathogenic *Theileria* spp. in large and small ruminants in Iran. Although case history, clinical signs, as well as lesions and microscopic examination of thin blood smears can lead to the diagnosis of infection with *Theileria* parasites, there are some difficulties associated with microscopic detection, including morphological similarity of piroplasms, false-negative diagnosis due to the low number of the parasite (particularly in carrier animals), and difficulty in the detection of mixed infections. Therefore, a comprehensive diagnosis and differentiation of various species of piroplasms could be achieved by sensitive and specific diagnostic assays, such as PCR. Although *T. lestoquardi* is the causative agent of malignant theileriosis in sheep and goats, there are some reports of *T. lestoquardi* infection in cattle (Namavari et al., 2015; Jalali et al., 2016). In the present study, an mPCR was designed for simultaneous detection and differentiation of *T. annulata* and *T. lestoquardi* from other piroplasms. Moreover, the species-specific Tams, Slag primers, and common 18S rRNA primers were used to increase the specificity of the mPCR reaction. As indicated in Figure 3, the mPCR method has been successfully utilized for the differentiation of these two pathogenic *Theileria* spp.

In total, among 216 examined blood samples, 155 (71.7%) and 178 (82.5%) samples were positive for piroplasms using microscopy and mPCR method, respectively (Table 2).

Only an experimentally infection of sheep and goats with *T. annulata* has already been reported (Brown et al., 1998), and no natural infection by this parasite was reported in small ruminants to date. In the present study, no *T.annulata* and *T.lestoquardi* coinfection was noted in any sample; moreover, *T. annulata* infection was not detected in sheep and goats; however, *T. lestoquardi* infection was detected in cattle from Khuzestan province (Table 2). Since all of *T. lestoquardi* have been previously reported in cattle from Khuzestan province (Namavari et al., 2015; Jalali et al., 2016), cross-infection of this parasite among sheep, goats, and cattle should be considered in the control and prevention of the disease, particularly in this region.

Over the past years, several molecular markers, such as 18S rRNA, ITS1, ITS2, and *Cytb* genes have been used to determine the phylogenetic relationship among the piroplasms population (Aktas et al., 2007; Habibi, 2012; Tian et al., 2013). The mitochondrial cytochrome *b* gene is commonly employed for analyzing phylogenetic relationships between organisms within families and genera levels (Castresana, 2001).

In the present study, the cytochrome *b* gene sequence was used for the construction of the phylogenetic tree among different species of *Theileria* parasites. It should be noted that the reliability of the phylogenetic tree is measured by a bootstrap value, whereas bootstrap values higher than 90% are considered highly significant and values between 70% and 89% are slightly significant; moreover, the ones below 70% have less value and consistency (Shaffer et al., 1997). As indicated in Figure 5, different *Theileria* spp. were placed in separate clades, and the reliability of depicted tree and monophyly of *T. annulata* and *T. lestoquardi* ingroups were supported by a bootstrap value of 94%, which significantly indicated that these two species evolved from a common ancestor.

Based on the drawn phylogenetic tree, the main *T. annulata* clade was divided into three subclades of A, B, and C, and the most of Iranian studied isolates were fitted along with *T. annulata Cytb* gene sequences from Turkey, India, and Sudan in subclade A. However, the *T. annulata* sequences from Africa were classified in the subclades of B and C, except for the sequences from Fars and Azerbaijan provinces, both of which were placed into the subclades of B and C, respectively. Molecular phylogenetic studies based on the 18S rRNA sequence clearly indicated that *T. lestoquardi* was more closely related to *T. annulata*, compared to *T. parva* (Katzer et al., 1998).

As indicated in Figure 5, similar results were obtained for the differentiation of two species by the *Cytb* gene

sequences in this study. As indicated in Table 3, the degrees of similarities between *T. annulata Cytb* gene sequences were determined at 98.6% to 100%; however, the similarities between *T. annulata* and *T. lestoquardi* isolates were estimated at 88.6% to 89.1%. These values show clearly that the similarity between *T. annulata* isolates is 10% more than that between two *T. annulata* and *T. lestoquardi* species. Nonetheless, the calculated similarity between the new species (Theileria spp. Sri Lanka) (Sivakumar et al., 2019) and *T. annulata* was obtained at 87%-87.5%, and similarity between new *Theileria* spp. and *T. lestoquardi* was determined to be 87.5%-87.9%. These findings show clearly that the similarity value between two *Theileria* spp., namely *T. annulata* and *T. lestoquardi*, is higher than that between the new *Theileria* spp. and *T. annulata* or *T. lestoquardi*. In other words, the results indicate that the *T. lestoquardi* spp. is more close to *T. annulata*, compared to *Theileria* spp. Sri Lanka. Additionally, the phylogenetic tree shows that *T. annulata* and *T. lestoquardi* share a more recent common ancestor, compared to the Sri Lankan species of *Theileria*.

Although most of the phylogenetic analyses have used the 18S rRNA gene in the studies of *Theileria*, there is a vast region of conserved sequences that do not always differentiate among closely related species (Li et al., 2011); however, our results indicated that the *Cytb* sequence could distinguish different *Thieleria* spp. in distinct clades effectively (Figure 5).

To the best of our knowledge, this study is the first phylogenic analysis of pathogenic *Theileria* spp. in Iran based on cytochrome *b* gene sequences. In addition, the first *T. lestoquardi Cytb* gene was sequenced and deposited in GenBank (MN481235-9).

Authors' Contribution

Study concept and design: M. T., Gh. H., K. E. and A. A.
Acquisition of data: A. A. and B. E.
Analysis and interpretation of data: A. A. and Gh. H.
Drafting of the manuscript:A. A., B. E. and Gh. H.
Critical revision of the manuscript for important intellectual content: M. T., Gh. H. and A. A.
Statistical analysis: A. A.
Administrative, technical, and material support: M. T., Gh. H. and A. A.

Ethics

The authors hereby declare that all ethical standards have been respected in the preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

Grant Support

This study was financially supported by Deputy for research at Urmia University, Iran (Grant No. D99-212)

Acknowledgment

This study was supported by the Research Fund of Urmia University, Urmia, Iran. The authors are grateful to all members of the Department of Parasitic Vaccines Research and Production, Razi Vaccine and Serum Research Institute for their technical assistance.

References

Aktas, M., Bendele, K.G., Altay, K., Dumanli, N., Tsuji, M., Holman, P.J., 2007. Sequence polymorphism in the ribosomal DNA internal transcribed spacers differs among Theileria species. Vet Parasitol 147, 221-230.

Barcia, J.J., 2007. The Giemsa stain: its history and applications. Int J Surg Pathol 15, 292-296.

Brown, C.G., Ilhan, T., Kirvar, E., Thomas, M., Wilkie, G., Leemans, I., *et al.*, 1998. Theileria lestoquardi and T. annulata in cattle, sheep, and goats. In vitro and in vivo studies. Ann N Y Acad Sci 849, 44-51.

Castresana, J., 2001. Cytochrome b phylogeny and the taxonomy of great apes and mammals. Mol Biol Evol 18, 465-471.

Conrad, P.A., Kelly, B.G., Brown, C.G., 1985. Intraerythrocytic schizogony of Theileria annulata. Parasitology 91, 67-82.

Gou, H., Guan, G., Ma, M., Liu, A., Liu, Z., Xu, Z., *et al.*, 2013. Phylogenetic analysis of ruminant Theileria spp. from China based on 28S ribosomal RNA gene. Korean J Parasitol 51, 511-517.

Habibi, G., 2012. Phylogenetic Analysis of Theileria annulata Infected Cell Line S15 Iran Vaccine Strain. Iran J Parasitol 7, 73-81.

Hashemi-Fesharki, R., 1988. Control of Theileria annulata in Iran. Parasitol Today 4, 36-40.

Hooshmand-Rad, P., Hawa, N.J., 1973. Malignant theileriosis of sheep and goats. Trop Anim Health Prod 5, 97-102.

Jalali, S.M., Jolodar, A., Rasooli, A., Darabifard, A., 2016. Detection of Theileria lestoquardi cross infection in cattle with clinical theileriosis in Iran. Acta Parasitol 61, 756-761.

Katzer, F., McKellar, S., Kirvar, E., Shiels, B., 1998. Phylogenetic analysis of Theileria and Babesia equi in relation to the establishment of parasite populations within novel host species and the development of diagnostic tests. Mol Biochem Parasitol 95, 33-44.

Kirvar, E., Ilhan, T., Katzer, F., Hooshmand-Rad, P., Zweygarth, E., Gerstenberg, C., *et al.*, 2000. Detection of Theileria annulata in cattle and vector ticks by PCR using the Tams1 gene sequences. Parasitology 120 (Pt 3), 245-254.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33, 1870-1874.

Li, Y., Guan, G., Ma, M., Liu, J., Ren, Q., Luo, J., *et al.*, 2011. Theileria ovis discovered in China. Exp Parasitol 127, 304-307.

Mhadhbi, M., Chaouch, M., Ajroud, K., Darghouth, M.A., BenAbderrazak, S., 2015. Sequence Polymorphism of Cytochrome b Gene in Theileria annulata Tunisian Isolates and Its Association with Buparvaquone Treatment Failure. PLoS One 10, e0129678.

Namavari, M., Ezhdehakosh-Pour, S., Habibi, G.R., Rahimian, A., Namazi, F., 2015. First isolation and establishment of in vitro culture of Theileria lestoquardi from a naturally infected cow. J Parasit Dis 39, 328-331.

Sambrook, H.C., 1989. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY.

Shaffer, H.B., Meylan, P., McKnight, M.L., 1997. Tests of turtle phylogeny: molecular, morphological, and paleontological approaches. Syst Biol 46, 235-268.

Sharifiyazdi, H., Namazi, F., Oryan, A., Shahriari, R., Razavi, M., 2012. Point mutations in the Theileria annulata cytochrome b gene is associated with buparvaquone treatment failure. Vet Parasitol 187, 431-435.

Sivakumar, T., Fujita, S., Tuvshintulga, B., Kothalawala, H., Silva, S.S.P., Yokoyama, N., 2019. Discovery of a new Theileria sp. closely related to Theileria annulata in cattle from Sri Lanka. Sci Rep 9, 16132.

Sivakumar, T., Hayashida, K., Sugimoto, C., Yokoyama, N., 2014. Evolution and genetic diversity of Theileria. Infect Genet Evol 27, 250-263.

Skilton, R.A., Musoke, A.J., Nene, V., Wasawo, D.P.S., Wells, C.W., Spooner, P.R., *et al.*, 2000. Molecular characterisation of a Theileria lestoquardi gene encoding a candidate sporozoite vaccine antigen. Mol Biochem Parasitol 107, 309-314.

Tian, Z., Luo, J., Zheng, J., Xie, J., Shen, H., Yin, H., *et al.*, 2013. Phylogenetic analysis of Babesia species in China based on cytochrome b (COB) gene. Infect Genet Evol 13, 36-40.