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Original article

Molecular investigation and phylogeny of *Theileria* spp. from naturally infected sheep and the first report of *Theileria* sp. OT3 in Sulaymaniyah governorate/Iraq

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Abstract

Theileriosis is a significant hemoprotozoal disease of domestic and wild ruminants in tropical and subtropical regions of the world. Ovines are mainly infected by *T. ovis* and *T. lestoquardi*, causing economic losses. Due to data scarceness in the Sulaymaniyah governorate, north of Iraq, this study was conducted to investigate subclinical theileriosis using microscopic examination and PCR. A total of 450 blood samples were collected from eight districts in Sulaymaniyah. The samples were randomly taken from clinically healthy sheep in 40 farms from April to October 2017. Following the organism verification, PCR products were sequenced and aligned. The study results revealed that 76.0% (n=342) and 58.0% (n=261) of the examined samples were positive for *Theileria* spp. by PCR and microscopic examination. *T. ovis* was reported in 76.0% (n=342) of all tested samples, while *T. lestoquardi* was present in 28.4% (n=97) of the positive specimens. Higher infection rates were observed during July and August. Analysis of the 18S rRNA gene partial sequence of the studied isolates with corresponding sequences in GenBank showed high degrees of identities with *T. ovis* and *T. lestoquardi* isolates reported from Iraq and other countries. *T. uilenbergi* and *T. sp. OT3* were detected only through analysis of obtained partial sequences from *Theileria*-positive samples. Following analysis, *T. uilenbergi* isolates represented a high homology degree with *Theileria* isolates from Iraq and China. The newly identified *T. sp. OT3* showed >99% identity with *T. sp. OT3* isolates of Chinese and Spanish origin.

Key words: *Theileria ovis*, *T. lestoquardi*, *T. uilenbergi*, PCR, sheep parasites

Introduction

Theileriosis is a widespread infection of livestock caused by apicomplexan protozoan parasites of the genus *Theileria* that infect leukocytes and erythrocytes (Jenkins 2018).

Sheep- and goat-rearing areas have significant risks of ovine theileriosis, causing significant economic losses (Ullah et al. 2018). Most of the infected animals are found mainly in the tropical and subtropical regions, including the Middle East, Asia, Southern Europe, and Africa (Razmi et al. 2019).

Different Ixodid ticks belonging to the genera *Hyalomma*, *Rhipicephalus*, *Haemaphysalis*, and *Amblyomma* are regarded as vectors for *Theileria* organisms. Transmission takes place via the bite of infected ticks. Theileriosis is characterized by establishing schizonts inside the cytoplasm of lymphocytes in the host, where the asexual division results in merozoites' development. Afterward, merozoites infect the red blood cells, and piroplasms are developed. The sexual part of the life cycle occurs in the intermediate host's gut, leading to the development of motile kinetes that settle in the tick's salivary gland. More divisions in sporoblasts result in infective sporozoites formation (Watts et al. 2016).

Historically, attention was directed toward bovine *Piroplasma* infection, while tick-borne diseases acquire less attention in small ruminants. However, due to sheep and goats' economic importance in several countries, interest has been ascended in ovine and caprine piroplasmiasis, particularly *Theileria* infection (Adil Khan et al. 2020).

Theileriosis in small ruminants is caused by *T. lestoquardi*, *T. luwenshuni*, and *T. uilenbergi*, which cause high mortalities, while *T. ovis* and *T. separata* are less pathogenic. Recently, *Theileria* sp. OT1, *Theileria* sp. OT3, and *Theileria* sp. MK have also been described in sheep and goats (Goudarzi et al. 2019).

Infections with some *Theileria* spp. are benign and cause minor or no clinical signs. Simultaneously, some other species can cause fever, anemia, hemoglobinuria, and death in severe cases. In general, animals that recover from acute or primary infections remain persistently infected and may act as reservoirs for tick vectors (Ahmed et al. 2008). The presence of theileriosis in sheep has been confirmed in different regions of Iraq and neighboring countries like Iran and Turkey (Dhaim and Aaiz 2014).

Theileriosis, due to *T. lestoquardi* and *T. ovis*, had been reported by other researchers. *Theileria lestoquardi*, a causal agent of malignant ovine theileriosis, has been distributed in the middle and south regions (Alkhaled et al. 2016).

Theileria spp. detection is performed through different diagnostic methods with various specificity and usefulness in identifying the full spectrum of organisms present in an area. Microscopically, parasitic detection can be difficult with low parasitemia and does not permit species differentiation. Serological tests are sensitive and relatively easy to use but might result in cross-reactivity between different *Theileria* spp. Hence, the specificity of these tests is low. With the development of molecular techniques, several studies were carried out on sheep that focused on detecting blood parasites by PCR and RLB. Detection of parasite DNA by PCR and sequencing of amplified products for verification is a highly sensitive method for diagnosing pathogenic species (Altay et al. 2008). PCR is also a more specific method than a blood smear examination (Khattoon et al. 2015).

The 18S rRNA gene has been successfully applied to identify and classify the known and several previously unknown *Theileria* and *Babesia* species (Barbosa et al. 2019).

The occurrence and extent of theileriosis have not been studied sufficiently in Sulaymaniyah governorate/north Iraq previously. Hence, this study was the first attempt to investigate *Theileria* spp. in naturally infected sheep in this area by molecular methods.

Materials and Methods

Study site and sample collection

Theileriosis was investigated from April to October 2017 in eight locations throughout the Sulaymaniyah governorate, north Iraq (Fig. 1). Forty small ruminant farms with a previous history of theileriosis were selected for sampling. About 10-15 sheep were chosen randomly from each farm, and a total of 450 blood samples were collected from 1-to-6-year-old sheep. About 5 mL of blood was drawn from the jugular vein and put in vacutainer tubes coated with EDTA. After labeling, the tubes were transported in a cool box to the lab.

Thin smears were prepared from each blood sample, and the remainder was frozen at -20°C for DNA extraction. Blood smears were stained with diluted Giemsa stain and examined under a light microscope for *Theileria* using 100× objective magnification.

DNA extraction

DNA was extracted from aliquots of 200 µL of whole blood using a DNA extraction kit (GeNet Bio, South Korea) according to the manufacturer's instructions. Extracted DNA samples were stored at -80°C till use.

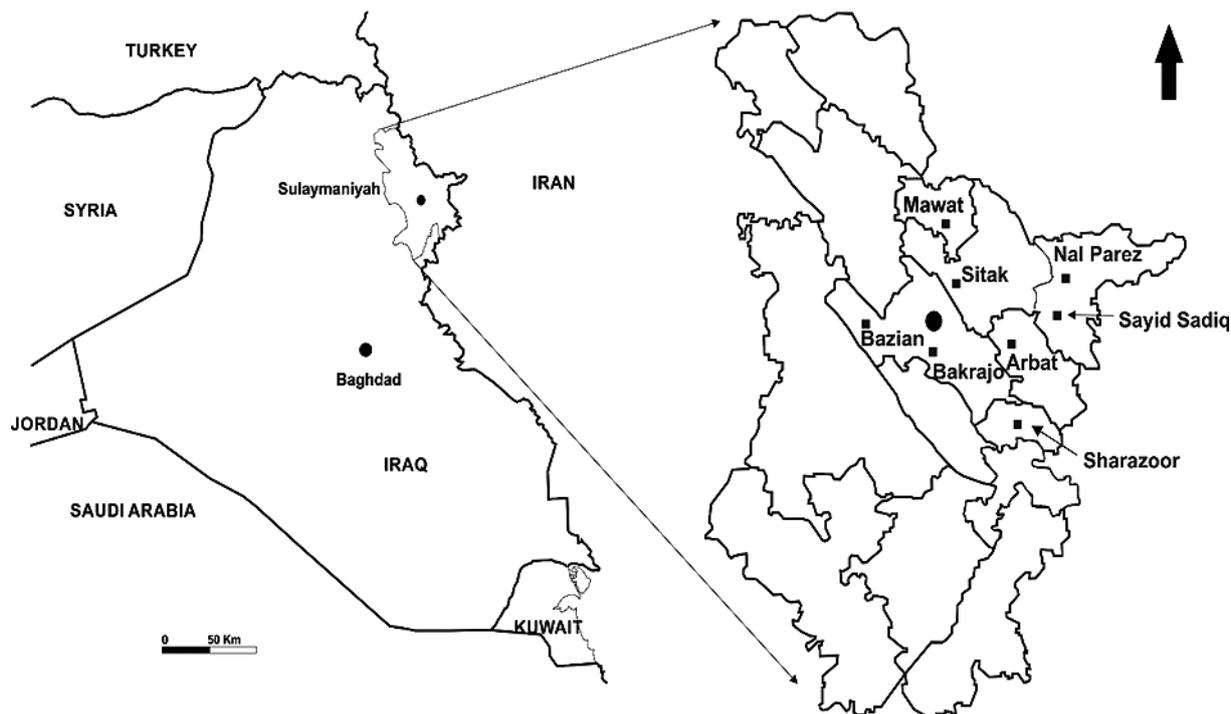


Fig. 1. A map of locations in the Sulaymaniyah governorate where samples were collected.

Molecular detection

PCR was run to identify *Theileria* by amplifying a fragment of the 18S rRNA gene. Samples were screened with primer sets of oligonucleotides designed for this study. The forward 5'-CCTGGTTGATCCTGCCAGT AGTC-3' and reverse 5'-CCTTCTGCAGGTTCCACC TACGG-3' primers were used to amplify a nearly 1700 bp of piroplasma organisms (*Theileria* and *Babesia* spp.). Also, other primers from previous studies were used for confirmation purposes.

The forward primer 5'-AGTTTCTGACCTATCAG-3' and reverse primer 5'-TTGCCTTAAACTTCCTTG-3' were used to amplify a 1098 bp gene to identify *Theileria* (Allsopp et al. 1993). Two primers (forward: 5'-GTGCCGCAAGTGAGTCA-3', and reverse 5'-GGACTGATGAGAAGACGATGAG-3') were used to obtain 785 bp amplicon of *T. lestoquardi* (Aktaş et al. 2005). Moreover, *T. ovis* was identified by amplifying a 520 bp with the forward 5'-TCGAGACCTTCGGGT-3' and reverse 5'-TCCGGACATTGTAAAACAAA-3' primers (Altay et al. 2005).

All PCR reactions were performed in 200 µL capacity PCR tubes. The content comprised 10.0 µL of 2.0× Prime Tag Premix (GeNet Bio), 5.0 µL of template genomic DNA, and 10.0 µg of each primer. The volume was completed with nuclear-free water to 20.0 µL.

DNA amplification was carried out in Prime Thermal cycler (UK), and different thermo-profiles were used. For *Piroplasma* organisms, the program was start-

ed with 95°C/5 min, followed by 38 cycles, 95°C/1 min for denaturation, 60°C/1 min for annealing, and 72°C/90 sec, with a final extension at 72°C for five minutes. A thermo-profile described by Allsopp et al. (1993) was followed to obtain a 1098 bp amplicon of *Theileria* spp.

Other PCR thermo-profiles used for *T. lestoquardi* and *T. ovis* detection were used following Aktaş et al. (2005) and Altay et al. (2005), respectively.

The amplified PCR products were visualized on an agarose gel to document PCR amplified fragments from extracted DNA of blood samples. 7 µL of PCR-amplified products were run in 1× TBE loading buffer. The result was visualized on a 1% gel stained with ethidium bromide and observed under a UV illuminator.

Sequencing and phylogenetic analysis

Sixteen samples positive for *Theileria* organism were chosen randomly and sequenced by Sanger DNA Sequencing System in South Korea. All partial nucleotide sequences identified in the study were deposited in the GenBank database. The nucleotide sequences identities and similarities were studied by nucleotide sequence homology analysis using BLASTn at NCBI's network server (National Center for Biotechnology Information).

The pairwise nucleotide percent identity of the new sequence isolates was calculated using MEGA X software (Kumar et al. 2018). The maximum likelihood method was used to construct phylogenetic trees for *Theileria* spp., and the evolutionary history was

Table 1. Regional distribution of theileriosis in sheep from different locations in Sulaymaniyah governorate, North Iraq.

| Locations | Sample no. | Diagnostic test | | | |
|-------------|------------|-----------------|-------|-----|-------|
| | | ME | % | PCR | % |
| Arbat | 65 | 43 | 66.15 | 50 | 76.92 |
| Bakrajo | 57 | 29 | 50.88 | 45 | 78.95 |
| Bazian | 68 | 39 | 54.00 | 55 | 80.88 |
| Mawat | 45 | 26 | 57.78 | 30 | 66.67 |
| Nal Perez | 50 | 27 | 60.00 | 38 | 76.00 |
| Sayid Sadiq | 50 | 33 | 66.00 | 43 | 86.00 |
| Sharazoor | 63 | 39 | 61.90 | 49 | 77.78 |
| Sitak | 52 | 25 | 48.07 | 32 | 61.53 |
| Total | 450 | 261 | 58.00 | 342 | 76.00 |

Table 2. Theileriosis distribution in sheep sampled from April to October 2017 in Sulaymaniyah governorate, using microscopic examination and PCR.

| Months | Sample no. | Diagnostic test | | | |
|-----------|------------|-----------------|-------|-----|-------|
| | | ME | % | PCR | % |
| April | 36 | 19 | 52.78 | 25 | 69.44 |
| May | 55 | 32 | 58.18 | 42 | 76.36 |
| June | 52 | 35 | 67.30 | 42 | 80.76 |
| July | 90 | 62 | 68.89 | 76 | 84.44 |
| August | 91 | 61 | 67.00 | 73 | 80.21 |
| September | 72 | 31 | 43.00 | 48 | 66.67 |
| October | 54 | 21 | 38.89 | 36 | 66.67 |
| Total | 450 | 261 | 58.00 | 342 | 76.00 |

inferred based on the Kimura 2-parameter model (Kimura 1980). Bootstrap analysis with 1000 replications was used to estimate the confidence of the trees' nodes and branches.

Statistical analysis

Distribution of theileriosis in different sampling areas and according to the months were tested for homogeneity using version 24.0 of Statistical Package for Social Sciences (SPSS) software (IBM, USA).

Results

Occurrence of theileriosis

The result of PCR assay for investigation of theileriosis in naturally infected sheep revealed that 76.0% (n=342) of the collected samples were infected with one or more *Theileria* spp. PCR amplification revealed that 76.0% (n=342) of the total *Theileria*-positive samples were DNA-positive for *T. ovis*. This result means that *T. ovis* was detected in all positive samples, and *T. lestoquardi* was reported in 28.4% (n=97) of the tested samples as co-infections with *T. ovis*.

The observed results of 450 stained blood smears revealed that 58.0% (n=261) of examined sheep were infected with *Theileria* organism. All the blood smears positive by microscopic examination were also positive when confirmed by PCR.

Distribution of *Theileria* in different sampling sites showed variation between districts, although it was statistically insignificant ($p>0.05$). PCR amplification showed a higher infection rate of theileriosis (86.0%) in Sayid Sadiq, while lower in Sitak (61.5%). However, microscopic examination revealed a higher occurrence rate of 66.2% in Arbat, and the lowest positivity rate of 48.1% was observed in animals from Sitak (Table 1).

Months-wise occurrence revealed that 84.4% of samples collected in July were infected, followed by 80.8% and 80.2% in June and August, respectively. On the other hand, a lower occurrence rate of 66.7% was detected in October samples. However, the differences were not statistically significant (Table 2).

Sequence and phylogenetic analysis

The obtained partial sequences of 18S rRNA genes from the study isolates were deposited in the GenBank database of the National Center for Biotechnology

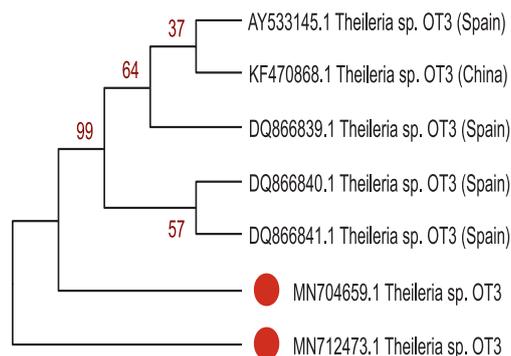


Fig. 2. Phylogenetic tree based on the 18S rRNA gene's partial sequences isolates of *Theileria* sp. OT3 with previously registered sequences in the GenBank database. The tree shows genotypes relationship by maximum likelihood, based on the Kimura 2-parameter model, in the bootstrap test (1000 replicates) in MEGA X. Red circles differentiate the study's isolates.

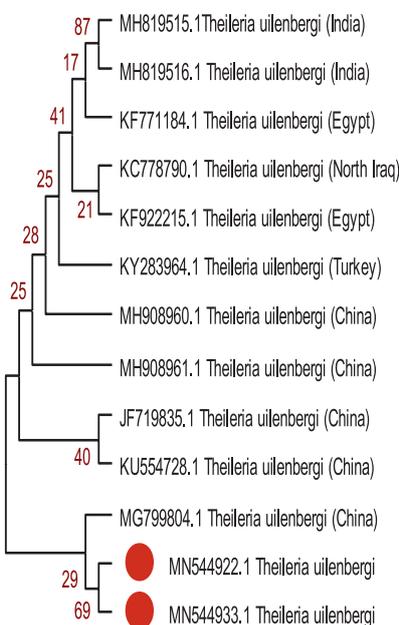


Fig. 3. Phylogenetic tree based on the 18S rRNA gene's partial sequences isolates of *T. uilenbergi* with previously registered sequences in the GenBank database. The tree shows genotypes relationship by maximum likelihood, based on the Kimura 2-parameter model, in the bootstrap test (1000 replicates) in MEGA X. Red circles differentiate the study's isolates.

Information, and revealed different *Theileria* spp. The assigned accession numbers of *T. ovis* isolates were MN544895, MN544915, MN544931, MN560040, MN560042, MN704656T, and MN712508. The *T. lestoquardi* isolates received accession numbers MN544921, MN544936, MN560043, MN704657, and MN712472. Furthermore, *T. uilenbergi* and *T. sp. OT3* were detected only through analysis of obtained partial sequences of 18S rRNA genes from *Theileria* positive samples.

T. uilenbergi was obtained from two samples, which received the accession numbers MN544922 and MN544933. Also, *T. sp. OT3* was present in two samples, which received the accession numbers MN712473 and MN704659.

Phylogenetic analysis and sequence identity

Theileria isolates from the current study are shown in four phylogenetic trees. Isolates from this study were clustered in different subgroups of phylogenetic branches, with corresponding *Theileria* spp., based on a partial sequence of the 18S rRNA gene (Figs. 2-5).

The BLAST analysis showed that our sequences shared a high degree of identity with the GenBank's published sequences. The analysis revealed that the new isolate of *Theileria* sp. OT3 with accession no. MN712473 shared 99.8% identity with isolate DQ866841 from Spain, and 99.6% with the Chinese and Spanish isolates KF470868 and AY533145, respectively. The other newly reported *Theileria* sp. OT3 isolate with accession no. MN704659 shares 98.4% identity with the Chinese isolate KF470868 and Spanish isolate AY533145, and 98.3% with other Spanish iso-

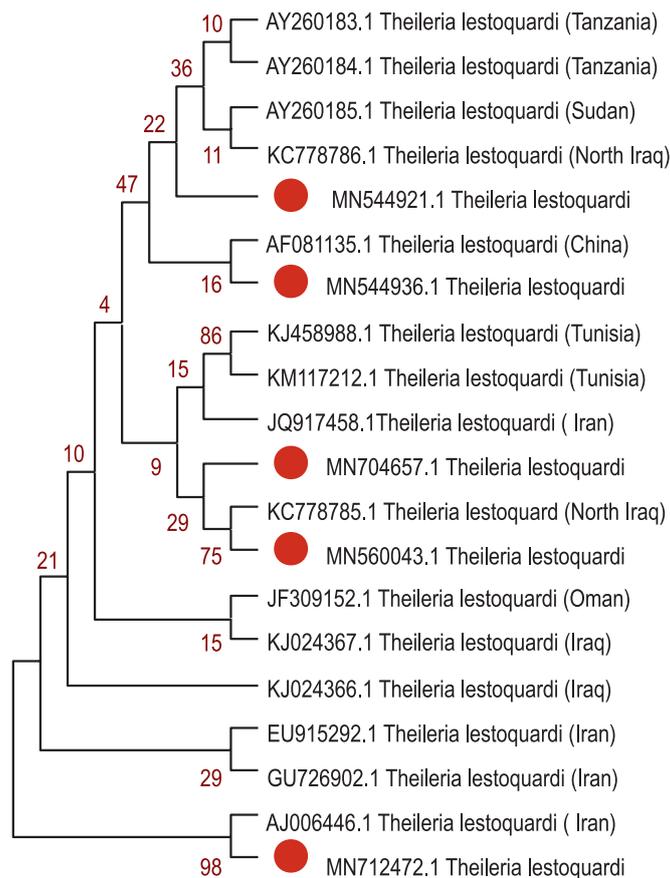


Fig. 4. Phylogenetic tree based on the 18S rRNA gene's partial sequences isolates of *T. lestoquardi* with previously registered sequences in the GenBank database. The tree shows genotypes relationship by maximum likelihood, based on the Kimura 2-parameter model, in the bootstrap test (1000 replicates) in MEGA X. Red circles differentiate the study's isolates.

lates (DQ866839, DQ866840). Furthermore, *T. uilenbergi* isolates with accession no. MN544933 and MN544922 shared 99.2% and 98.4% homology respectively with *T. uilenbergi* isolate KC778790 from Iraq. The isolates shared 99.2% and 99.1% respectively similarity with the Chinese isolates JF719835, AY262120, and AY262121.

The isolates of *T. lestoquardi* with accession numbers MN544921, MN544936, MN704657, and MN712472 shared 99.3% similarity with *T. lestoquardi* isolates KC778785 and KC778786 from northern Iraq, and their similarity ranged between 99.1% and 99.9%, with isolates KJ024366 and KJ024367 from the middle region of Iraq.

Theileria ovis isolates with GenBank no. MN544931, MN704656, MN712508, MN544915, and MN560040 were >99.1% identical to corresponding *T. ovis* isolates from other countries. The identity of isolate MN544895 was >98.38% with published sequence isolates. Furthermore, the identity of >96.28 was reported for the current study's isolate MN560042 with previously reported isolates of *T. ovis*.

Discussion

Sheep become chronic carriers and act reservoirs for tick-borne infection. Therefore, latent infections are critical in the epidemiology of diseases. Traditionally, *Piroplasma* infection is diagnosed based on clinical findings and examination of Giemsa-stained blood smears. However, in chronic carriers, they are less valuable, mainly in the presence of mixed infection. Molecular techniques, on the other hand, enable sensitive and specific detection of the parasites.

The current study results showed a superiority of PCR over the microscopic examination to detect carrier sheep with *Theileria*. Such a result was expected because carrier animals with very low parasitemia were challenging to be detected microscopically.

The PCR diagnostic method is also highly sensitive and more specific than routinely diagnosing by serological and microscopic examination (Chauhan et al. 2015).

The overall occurrence rate of 76.0% by PCR amplification was higher than that reported in the neighboring countries. The occurrence rate of theileriosis in Iran was 62.5% (Zarei et al. 2019). In Turkey, theileriosis was reported in 38.7% of the examined cases

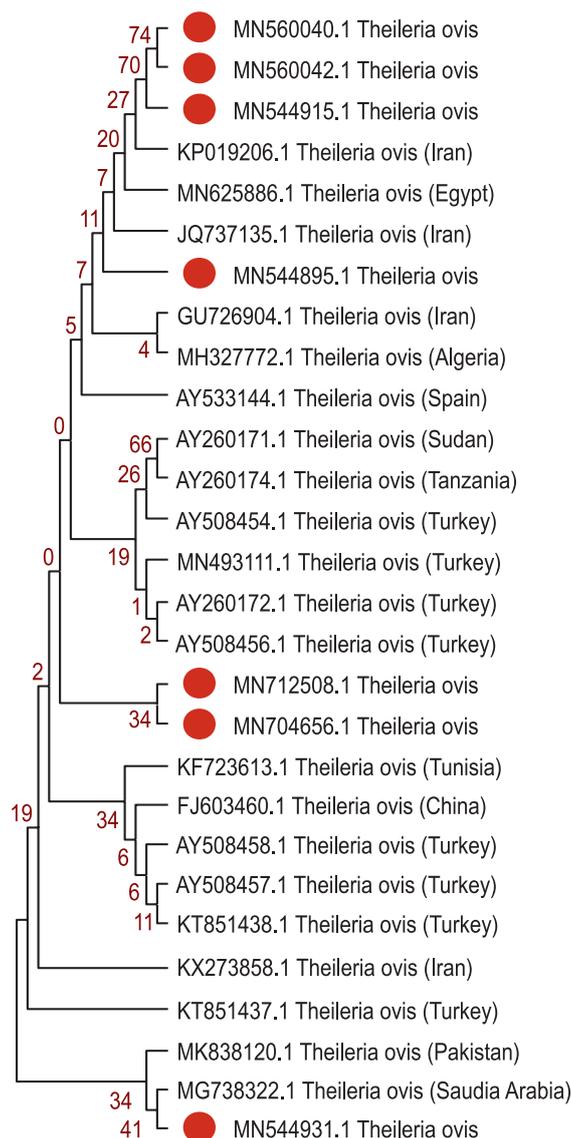


Fig. 5. Phylogenetic tree based on the 18S rRNA gene partial sequences isolates of *T. ovis* with registered sequences in the GenBank database. The tree shows genotypes relationship by maximum likelihood, based on the Kimura 2-parameter model, in the bootstrap test (1000 replicates) in MEGA X. Red circles differentiate the study's isolates.

(Altay et al. 2007). In our study area, individual farmers raise livestock in villages, and the management procedures followed are considered preliminary by today's standards. Sheep are raised on a pasture shared by several flocks, and new animals are continuously added to the herd without being tested for blood protozoa. Most cases were subclinical, and the farmers are often unaware of the infection. These factors may be the reason for the high occurrence rate of theileriosis in our study.

Theileria ovis was the most widespread species; it was reported in all *Theileria*-positive samples in our study. It was also the most abundant species in Iran, at the rate of 91.0% (Jalali et al. 2014). In Pakistan, *T. ovis* accounts for 79.0% of *Theileria* infections (Iqbal et al. 2013). Additionally, *T. ovis* accounted for 91.2%

and 78.0% of theileriosis cases in Ethiopia and China, respectively (Li et al. 2011, Gebrekidan et al. 2014).

Out of the total 342 *Theileria*-positive samples, 28.4% was infected with *T. lestoquardi* as shown by PCR. Previous studies confirmed the presence of pathogenic *T. lestoquardi* in the southern part of the country, with an occurrence rate of 93.1% (Minnat and Abdulwadood 2012). However, the occurrence was reported to be much lower in the middle and northern parts of Iraq being 23.3%, and 7.7%, respectively (Alkhaled et al. 2016). Indifferently to our result, the infection rate of *T. lestoquardi* in Iran was 32.6% (Mohammadi et al. 2017).

The highest occurrence rates of theileriosis were reported during June, July, and August. This result is attributed to the abundance of tick vector. Hemo-

protozoan parasites are often observed in the warmer months of the year due to the availability of a favorable environment for the vectors at that time (Atif et al. 2012).

Blast analysis of 18S rRNA gene partial sequences of new isolates from Sulaymaniyah has shown a high homology degree with reported isolates from GenBank.

Theileria sp. OT3 (new isolate MN712473) shared 99.8% identity with isolate DQ866841 from Spain and 99.6% with the Chinese isolate KF470868. The other newly reported *Theileria* sp. OT3 isolate MN704659 shares 98.4% identity with the Chinese isolate KF470868 and 98.3% with other Spanish isolates (DQ866839, DQ866840).

Furthermore, *T. uilenbergi* isolates with accession no. MN544933 and MN544922 shared the identity of 99.2% and 98.4% respectively with the isolate KC778790 of *T. uilenbergi* from Iraq. They also shared 99.2% and 99.1% respectively similarity with the Chinese isolates JF719835, AY262120, and AY262121.

The isolates of *T. lestoquardi* with accession no. MN544921, MN544936, MN704657, and MN712472 shared 99.3% similarity with *T. lestoquardi* isolates KC778785 and KC778786 from northern Iraq. Their similarity ranged between 99.1% and 99.9%, with isolates KJ024366 and KJ024367 from Iraq's middle region.

Theileria ovis isolates with GenBank no. MN544931, MN704656, MN712508, MN544915, and MN560040 were >99.1% identical to corresponding *T. ovis* isolates from other countries.

The identity of MN544895 isolate was >98.38% with published sequence isolates. Furthermore, an identity >96.28 was reported for isolate MN560042 with previously reported *T. ovis* isolates from GenBank.

Despite the presence of previously mentioned *Theileria* spp. in the studied area, all sampled sheep were free from clinical signs of disease. Asymptomatic development of infections could give evidence of their endemic stability in the area. Such a state was defined as relationships between host, vector, and pathogen in the environment when clinical cases of the disease were absent or rarely occurred (Bock et al. 2004).

Various predisposing factors increase animals' susceptibility to infection with *Theileria* spp., such as a favorable environment for tick vector proliferation and transmission. Additionally, Osman et al. (2017) reported that a high prevalence rate of theileriosis might be due to the in and out movements of animals or the introduction of tick vectors with exotic breeds. Besides, mixed grazing of cattle and small ruminants could affect the spread of the disease among small ruminants.

Conclusions

This study revealed a high occurrence rate of sub-clinical ovine theileriosis in Sulaimani province, caused by different *Theileria* spp. as a single or mixed infection. The current study also revealed that high proportions of sheep in the sampling areas harbor *T. ovis*. Furthermore, *T. sp.* OT3 infection was reported for the first time in apparently healthy sheep in northern Iraq. Further investigation should be directed toward the source and vector ticks responsible for transmitting the newly recorded *Theileria* spp. in the area.

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