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Phylogenetic study of theileria ovis and theileria lestoquardi in infected sheep and it is associated ticks in mosul city, Iraq

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***Abstract*---**This is a first molecular report investigate the phylogenic analysis of *Theileria spp.* in sheep and it's infested ticks in Mosul city-Iraq. A total of 185 blood samples were collected from sheep in different areas of Mosul city. A sixty five Ixodid ticks were also collected from different parts of infected animals. The overall prevalence of *Theileria spp.* was 52.4% (97 out of 185), for *Theileria ovis* it was 9.7%, *Theileria lestoquardi* it was 20% and mixed infection it was 22.7% in sheep in Mosul city, based on conventional-PCR and multiplex-PCR techniques. the infestation rat of Ixodid ticks on sheep was 11.8% (22 out of 185) and three species of Ixodid ticks (n=65) were identified and classified: *Hyalomma anatolicum anatolicum* 37 (56.9%), *Rhipicephalus sanguineus* 16 (24.6%), *Rh. turanicus* 12 (18.4%) based on microscopic examination. BLASTn individual sequencing analysis of six sequences of 18S rRNA gene, composing sequences of *T. ovis* (n=3) (One extracted from sheep blood and two extracted from engorged female ticks), and sequences of *T. lestoquardi* (n=3) (One extracted from sheep blood and two extracted from engorged female ticks). The sequences of *T. ovis* and *T. lestoquardi* were deposited in the GenBank under accession numbers (OP001587, OP001588, OP001589) and (OP001630 ,OP001631, OP001632) respectively. Sequences of *T. ovis* (n=3) were highly related (100% identity) to those sequences of Pakistan (MT498784, MN922940) and of India (MH819508), while sequences of *T. lestoquardi* (n=3) were highly related (100% identity) to those sequences of Sulaimani-Iraq (MN712472), Egypt (KY674519), Sudan (KY674519) and Italy (MT341858, MN944852). In conclusion *T. lestoquardi* and *T. ovis* are highly prevalence among sheep and *H. anatolicum anatolicum*, *Rh.*

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sanguineus and *Rh. turanicus* which probably the main vectors of these parasite in Mosul city, Iraq.

Keywords---*Phylogenetic Analysis, Theileria ovis, Theileria lestoquardi, Ixodid ticks, Mosul city-Iraq.*

Introduction

Ovine theileriosis (OT) is a worldwide distribution disease with highly morbidity and mortality rates, in spite of availability treatment and conducting tick control programs (Ozubek *et al.*, 2017). It is mainly caused by *Theileria lestoquardi*, previously called (*T. hirci*) and *Theileria ovis* previously called (*T. recondita*) (Niaz *et al.*, 2021; Qi *et al.*, 2018), which were biologically transmitted by hard ticks belonging to the Ixodidae family and the most common genus's are Haylomma, Rhipicephalus, Boophilus, Haemaphysalis and Amblyomma (M'ghirbi *et al.*, 2013; Gebrekidan *et al.*, 2014; Rjeibi *et al.*, 2016; Mamatha *et al.*, 2017).

The OT is reported in various geographical regions including the Central Asia, Eastern and Southern Europe, Middle East, China, India, and East and North Africa (Razmi *et al.*, 2019). The disease appears in different forms such as acute, subacute, chronic and subclinical (Naz *et al.*, 2012; Constable *et al.*, 2017), the acute form of the disease is more common in the case of *T. lestoquardi* infection, while the subclinical form of the disease is in the case of *T. ovis* infection (Yaghfoori *et al.*, 2017 ; Awad *et al.*, 2020). Furthermore, OT in small ruminants causes a high morbidity rate reach to 100%, with a mortality rate of more than 46% (Tageldin *et al.*, 2005; El Imam *et al.*, 2015). There are several genes used for molecular detection of the *T. ovis* and *T. lestoquardi* such as Major merozoite piroplasm surface antigen (MMPSA) gene (Esmaelizad *et al.*, 2011), 16S ribosomal ribonucleic acid (16S rRNA) gene (Ismael and Omer, 2020), 12S ribosomal ribonucleic acid (12S rRNA) gene (Azmi *et al.*, 2019), small subunit ribosomal RNA (SSU rRNA) gene (Benedicto *et al.*, 2020; Ceylan *et al.*, 2021), and the more common gene is 18S ribosomal ribonucleic acid (18S rRNA) (Eliwa *et al.*, 2021; Metwally *et al.*, 2021). Ovine theileriosis can be diagnosed based on polymerase chain reaction (PCR) assay, it is an important technology in epidemiological and phylogenetic studies of *Theileria spp.* in sheep, as it is highly sensitive, specific and accurate in detecting of the *T. lestoquardi* and *T. ovis* DNAs in infected cases with low parasitemia, in early, chronic and apparently healthy animals (Sharifi *et al.*, 2016; Hassen and Meerkhan, 2020; Metwally *et al.*, 2021). There were little molecular and phylogenetic researches about *T. lestoquardi* and *T. ovis* in Mosul city, Iraq. Therefore, The current study used 18S rRNA gene to determine the prevalence of *Theileria spp.* in sheep blood and engorged female ticks samples using conventional-PCR (c-PCR) and multiplex-PCR(m-PCR) techniques.

Material and Methods

Ethical approval

This work has been permitted ethically by the Institutional Animal Care and Use Committee of the College of Veterinary Medicine, University of Mosul, (UM.VET.2021.18) on 6th of September 2021.

Animal and Samples collection

This study was conducted on 185 sheep, from both sexes, different ages, breed, origins and from different regions of Mosul city-Iraq, During the period from October 2021 to May 2022, A one-handed eighty five blood samples (2.5ml) were withdrawn via the jugular vein using 3ml sterile syringe, then dispended into tubes with anticoagulant ethylene diamine tetraacetic acid (EDTA) which kept in the freezer at -20°C until the molecular examination was performed using c-PCR and m-PCR techniques for the purpose of distinguish species of Theileria species. Moreover, when the samples were taken, 65 ticks (males and females) infested sheep were collected from deferent parts of the animals body, then preserved in 70% ethanol at 4°C until classification and detection of the *Theileria spp.* inside them using the c-PCR and m-PCR techniques.

Morphological identification and classification of ticks

The identification and classification Ixodid tick (n=65) at the genus and species is based on the morphological characterization using stereoscopic microscope and according to taxonomic keys (Wall and Shearer, 2001; Walker *et al.*, 2014; Estrada-Pena, 2015).

Molecular diagnosis of *Theileria spp.* in sheep blood and ticks samples

DNA extraction

A ready kits were used to extract genomic DNA from 185 sheep whole blood and 9 engorged female ticks samples: FavorPrepTM Blood and Tissue / Cultured cells Genomic DNA Extraction Mini Kit (FAVORGEN Biotech Corporation, Taiwan). Using Nano-drop (BioDrop, England), the concentration of extracted DNA was regarded at wavelength 260 nm, ranged between 22.6 – 322.7 ng/µl. While, by calculating the ratio of (A260 nm to A280 nm), the purity was found to be between 1.5 and 1.9.

DNA amplification

Two reactions were used to amplify the 18S rRNA gene of *Theileria spp.:* The first reaction by c-PCR technique, to identify the positive sheep and Ixodid ticks for all *Theileria spp.* in approximately band size 1098bp, using universal primers (989-F and 990-R). While the second reaction by m-PCR technique was done to differentiate between *T. lestoquardi* and *T. ovis* in all positive samples in the first reaction, using specific primers (T170-F and T670-R) for *T. ovis* in approximately band size 520bp and (TF2 and TR2) for *T. lestoquardi* in approximately band size 230bp, all primers were provided by (Macrogen Inc. South Korea), (Table 1).

Conventional PCR technique was done with a total volume of 25µl composing 12.5µl of master mix (2X), 1µl (10 pmol) of each primer (989-F and 990-R), 3µl of template DNA, and 7.5µl of PCR–Grade water. At the same time, the m-PCR technique was done with a total volume of 25µl consisting of 12.5µl of master mix(2X), 1µl (10 pmol) of each primer (T170-F and T670-R) for *T. ovis*, and (TF2 and TR2) for *T. lestoquardi*, 3µl of template DNA and 5.5µl of PCR –Grade water. In the m-PCR technique, positive control was consisting the same components above. Instead of the extracted DNA sample, the DNA sample of the known parasite type was placed, obtained from a clinically infected laboratory sheep. Moreover, a negative control consistend of the same components without template DNA.

The thermocycler (BIO-RAD/ USA) was set as follows: 5min at 95°C for the predenaturation step (1 cycle), 1 min at 95°C for the denaturation step, 45s at 55°C for the annealing step, and 1 min at 72°C for extension step (35 cycles), with a 5 min at 72°C for final extension step (1cycle), according to Radwan and El Kelesh, (2009) with some modification in annealing step. PCR yields were electrophoresed in a 1.5% agarose gel stained with Midori green. UV transillumination (BIO-RAD/USA) was used to visualize the resulting bands.

DNA sequencing

From a total of 106 PCR amplicons from sheep blood and engorged female ticks that were positive for *Theileria spp.* using c-PCR and m-PCR. A six amplicons composing sequences of *T. ovis* (n=3) (One extracted from sheep blood and two extracted from engorged female ticks), and sequences of *T. lestoquardi* (n=3) (One extracted from sheep blood and two extracted from engorged female ticks) were sent to a commercial company for purification and sequencing (Macrogen company, South Korea). These sequences of 18S rRNA gene were blasted against other published *T. ovis* and *T. lestoquardi* sequences from the Genbank using NCBI BLAST (BLASTn) from NCBI (available at http://www.ncbi.nlm.nih.gov), and analyzed using online multiple sequences alignment-CLUSTALW (GenomeNet) (available at http://www.gen ome.Jp/tools/clustalw/). Phylogenetic trees was generated using the same CLUSTALW (GenomeNet) and Neighbor-joining (NJ) programs. The 18S rRNA gene sequences of the B. bovis (L19077) was used as outgroup in the construction of phylogenetic trees (100 replicates).

Table 1: Oligonucleotide primers of *Theileria spp.*, *T. lestoquardi* and *T. ovis* used in this work

Type of Parasite	Primers	Sequences 5’-3’	Amount of oligo (nMoles)	Expected size (bp)	References
Theileria spp. (Universal)	989-F	AGTTTCTGACCTATCAG	32	1098	d’Oliveira *et al.*, 1995
	990-R	TTGCCTTAAACTTCCTTG	32		

T. lestoquardi (Specific)	TF2	GACACAGGGAGGTAGTGACAAG	30	230	Altay *et al.*, 2005
	TR2	CTAAAGAATTTCACCTTTCTGACA	30		
T. ovis (Specific)	T170-F	TCGAGACCTTCGGGT	32	520	Spitalska *et al.*, 2004
	T670-R	TCCGGACATTGTAAAACAAA	30		

Statistical analysis

In this study's data were analyzed using IBM-SPSS Version 19 (Inc., Chicago, USA), which included the Chi-square 2x2 table. When the P value was (0.05), the data was deemed statistically significant.

Results

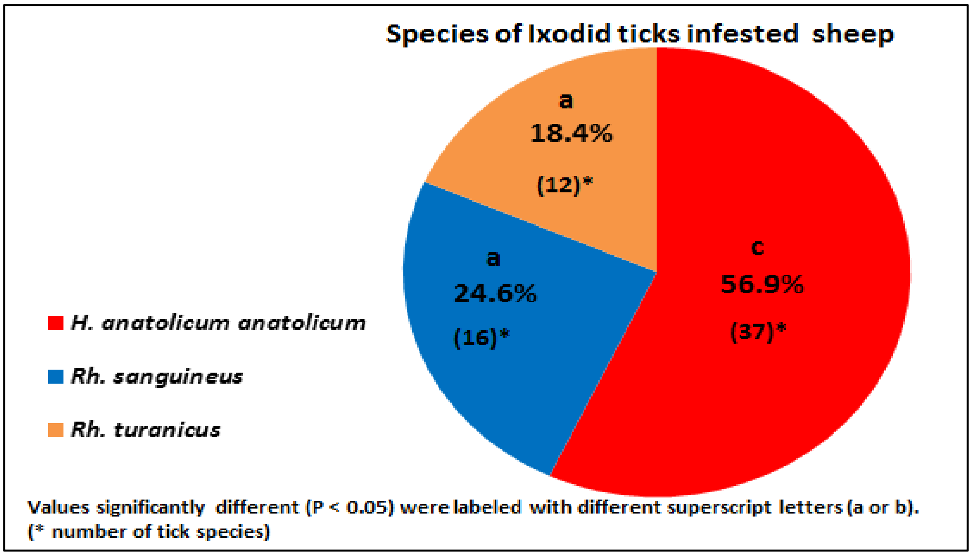
In the current study, the overall prevalence of *Theileria spp.* was 52.4% (97 out of 185), for *T. ovis* it was 9.7%, *T. lestoquardi* it was 20% and mixed infection it was 22.7% in sheep in Mosul city based on c-PCR and m-PCR techniques. This indicated that significantly ($P<0.05$) higher prevalence of *T. lestoquardi* infection among sheep in compared to *T. ovis* infection (Table 2). The infestation rat of Ixodid ticks on sheep was 11.8% (22 out of 185) and three species of Ixodid ticks (n=65) were identified and classified: *Hyalomma anatolicum anatolicum* 37 (56.9%), *Rhipicephalus sanguineus* 16 (24.6%), and *Rh. turanicus* 12 (18.4%) based on microscopic examination. *Hyalomma anatolicum anatolicum* was significantly ($P<0.05$) predominant species in compared to other species diagnosed in this study (Figure 1). In addition, using m-PCR technique for extracted DNA of the engorged female ticks (n=9) was observed *T. ovis* and *T. lestoquardi* were detected in *H. anatolicum anatolicum* and *Rh. sanguineus*, while only *T. lestoquardi* was detected in *Rh. turanicus* (Table 3).

Table 2: Prevalence of the type of *Theileria spp.* in sheep in Mosul city using c-PCR and m-PCR technique (n*=185).

Type infection	Multiplex PCR technique	
	No. of positive	Percentage (%)
T. ovis	18	a9.7
T. lestoquardi	37	b 20
T. ovis with *T. lestoquardi*	42	b22.7
Total	97	52.4

Different superscript letters (a,b) were assigned to values that were significantly different ($P<0.05$)

n= Total number of animals used for calculating the prevalence of *Theileria spp.*


Species of Ixodid ticks infested sheep

Values significantly different (P < 0.05) were labeled with different superscript letters (a or b).
(* number of tick species)

Figure 1: Species and percentage of Ixodid ticks infested sheep (n=65)

Table 3: *Theileria* species detected in engorged female ticks using multiplex polymerase chain reaction technique

Tick species	No. of exanimated ticks	No. of positive	*Theileria spp.*
H. anatolicum anatolicum	3	2	*T. ovis* & *T. lestoquardi*
Rh. sanguineus	3	2	*T. ovis* & *T. lestoquardi*
Rh. turanicus	3	1	*T. lestoquardi*

In this work, the sequences of the 18S rRNA gene for *T. ovis* and *T. lestoquardi* detected in sheep blood and engorged female ticks were deposited in the GenBank under accession numbers (OP001587, OP001588, OP001589) and (OP001630, OP001631, OP001632) respectively (Table 4). Moreover, phylogenetic trees analyses based on neighbor-joining program have showed that the sequences of *T. ovis* (n=3) were highly related (100% identity) to those sequences previously reported *T. ovis* in NCBI GenBank for Pakistan (MT498784, MN922940) and India (MH819508) (Figure 2). While, the sequences of the *T. lestoquardi* (n=3) were highly related (100% identity) to those sequences previously reported *T. lestoquardi* in NCBI GenBank for Sulaimani-Iraq (MN712472), Egypt (KY674519), Sudan (KY674519) and to Theileria annulata in Italy (MT341858, MN944852) (Figure 3).

Table 4: GenBank accession numbers of *T. ovis* and *T. lestoquardi* sequences in the sheep and engorged female ticks.

Accession No. of 18S rRNA gene	*Theileria spp.*	Type of sample
OP001587	*T. ovis*	Sheep blood
OP001588	*T. ovis*	Engorged female tick
OP001589	*T. ovis*	Engorged female tick

OP001630	*T. lestoquardi*	Sheep blood
OP001631	*T. lestoquardi*	Engorged female tick
OP001632	*T. lestoquardi*	Engorged female tick

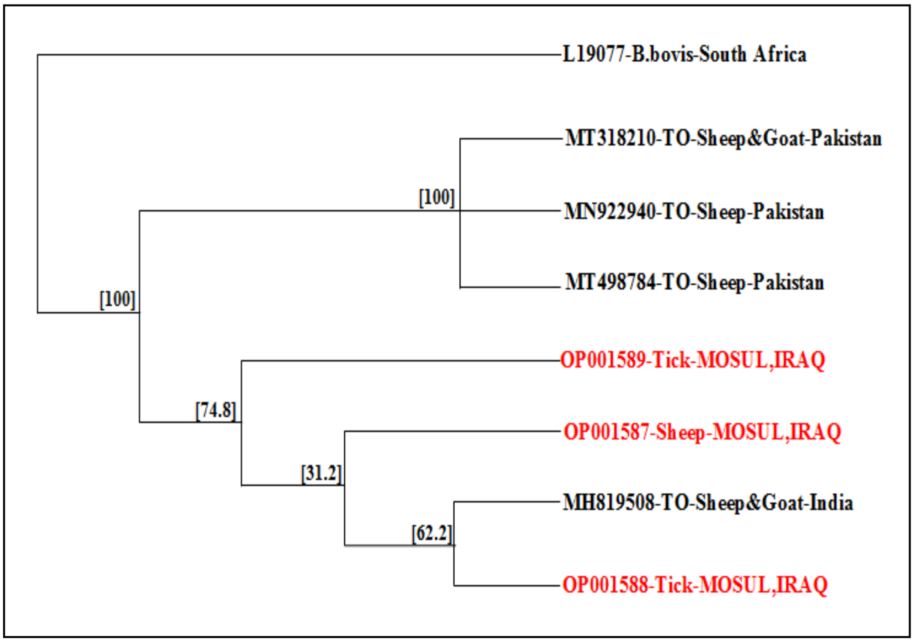


Figure 2: Phylogenetic tree of the partial sequence of the 18S rRNA gene of the local sequences of the *T. ovis* with the sequences of the *T. ovis* reported in the NCBI GenBank, The numbers at the branches indicate bootstrap supports (100 replications), and (L19077-B. bovis-South Africa) represents the Outer group.

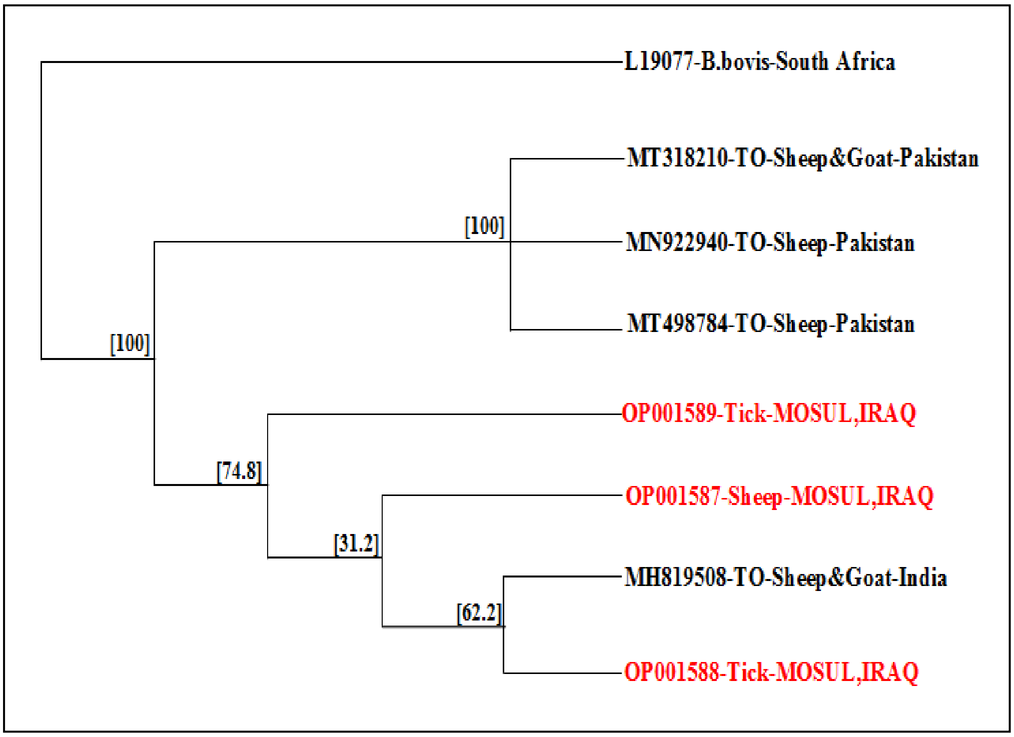


Figure 2: Phylogenetic tree of the partial sequence of the 18S rRNA gene of the local sequences of the *T. lestoquardi* with the sequences of the *T. lestoquardi* reported in the NCBI GenBank, The numbers at the branches indicate bootstrap supports (100 replications), and (L19077-B. bovis-South Africa) represents the Outer group.

Discussin

In the present study the overall prevalence of *Theileria spp.* in sheep in Mosul city was 52.4%. using c-PCR technique. This result is higher to the prevalence has been reported in other studies such as: in Mosul city and Basrah province, Iraq it was 0.03%, 17.5% in sheep using microscopic examination of stained blood smears respectively (Abood *et al.*, 2005; Abdullah *et al.*, 2019), in Kurdistan areas, Iraq it was 13.43% and 17.91% in sheep using microscopic examination and PCR techniques respectively (Hassen and Meerkhan, 2020), in Iran, Turkey, Sudan, and Pakistan it was 47.27%, 7%, 12.9%, and 24.6% respectively using PCR techniques (Khezri *et al.*, 2016; Riaz and Tasawar, 2017; Lee *et al.*, 2018; Benedicto *et al.*, 2020), and in Egypt it was 21.7% using microscopic examination method (Eliwa *et al.*, 2021). The reasons for the variance in the prevalence of *Theileria spp.* among regions in the same country and among countries including: diverse management strategies, used efficient diagnostics techniques, effective ticks control programs, presence of competent tick vectors, and environmental variations (Maharana *et al.*, 2016; Abdullah *et al.*, 2019; Karatepe *et al.*, 2019).
This study observed that higher prevalence of *T. lestoquardi* infection among sheep in compared to *T. ovis* infection. This result corresponds with Ali *et al.* (2017) and

Zhao *et al.* (2020), they mentioned that infection with *T. lestoquardi* causes malignant form of the disease, while *T. ovis* causes benign form and it is considered non-pathogenic to animals. The reason may also be attributed to the host immune response being resistant to some types of *Theileria spp.* (Hassen and Meerkhan, 2020).

The present study reported three species of Ixodid ticks infested sheep including *Hyalomma anatolicum anatolicum*, *Rhipicephalus sanguineus* and *Rh. turanicus*. This result is consistent with that reported by Kadir *et al.* (2012); Mohammad, (2016) and Ismael and Omer, (2020) in different governorates of Iraq. Razmi and Yaghfoori (2013) and Ramzan *et al.* (2019) and Al-Hamidhi *et al.* (2021) reported these species Ixodid ticks infested sheep in other countries such as Iran, and Saudi Arabia, respectively. The study showed that the H. anatolicum anatolicum is predominant species in compared to other species diagnosed in this study. The reason may be attributed to its ability to tolerate dry and harsh environment, this result agreed with Sultana *et al.* (2015) and Al-Fahdi *et al.* (2017).

Various targeting genes has been used for identifications of *T. ovis* and *T. lestoquardi*, in sheep and it's infested ticks, in this study 18S rRNA gene was used, due to the sequences of this gene at the species level are commonly conserved, available in molecular databases, present in diverse copies in the complete gene, and this gene contains highly frequented sequences (Al-Hamidhi *et al.*, 2021; Al-Hosary *et al.*, 2021).

In this study, *T. ovis* and *T. lestoquardi* were detected in engorged female ticks (*H. anatolicum anatolicum*, *Rh. sanguineus*, and *Rh. turanicus*) base on m-PCR technique. these finding agrees with Rahmani-Varmale *et al.* (2019) and Al-Hamidhi *et al.* (2021) They mention that these Ixodid ticks species is the main biological vector for *Theileria spp.*

The results of the phylogenetic tree of the local sequences of *T. ovis* was showed that it possesses common phylogenetic characteristics and a high evolutionary relationship with the sequences of *T. ovis* recorded in the NCBI GenBank for different countries including Pakistan and India, with the 100% Identity, after performing 100 nucleotide sequence reconstructing using Bootstrap analysis (Abid *et al.*, 2021; Eliwa *et al.*, 2021; Niaz *et al.*, 2021). In addition, results of the phylogenetic tree of the local sequences of *T. lestoquardi* was also showed that it possesses common phylogenetic characteristics and a high evolutionary relationship with the sequences of *T. lestoquardi* recorded in the NCBI GenBank. for different countries, including Sulaymaniyah governorate, Iraq and Kurdistan regions, Iraq and Italy, with the 100% Identity, after performing 100 nucleotide sequence reconstructing using Bootstrap analysis (Abdullah and Ali, 2021; Eliwa *et al.*, 2021).

Conclusion

This study is the first molecular report provide the phylogenic analysis of *Theileria spp.* in sheep in Mosul city, indicates *T. lestoquardi* and *T. ovis* are widespread among sheep in Mosul city, *H. anatolicum anatolicum*, *Rh. sanguineus* and *Rh.*

turanicus which probably the main vectors of these parasite and a tick control program should be conducted to prevent the transmission of parasites.

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Conflict of Interest

The authors declare that there are no conflicting interests in the article.

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