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DETECTION AND MOLECULAR CHARACTERIZATION OF *THEILERIA OVIS* IN SHEEP AND GOATS WITH CLINICAL THE ILERIOSIS IN KURDISTAN, IRAQ

ZUBER **I**SMAEL **H**ASSEN[*] and **A**ZAD **A**BDULLAH **M**EERKHAN[**]
[*]Dept. of Medical Laboratory Techniques, Shaqlawa Technical Institute, Erbil Polytechnic University, Erbil, Kurdistan-Iraq
[**]Dept. of Nursing, Duhok Technical Institute, Duhok Polytechnic University, Duhok, Kurdistan-Iraq

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ABSTRACT

This study was carried out to detect *Theileria* infection in sheep and goats in Kurdistan region, Iraq from June 2019 to April 2020. Molecular method was used to identify *Theileria species.* Sixty-seven blood samples were taken from 45 sheep and 22 goats based on clinical signs of theileriosis during tick activating season. The 67 samples were PCR edm and as a result, 20 species-specific PCR were positives (26.67% (12/20) were *Theileria ovis* in sheep and 36.36% (8/20) were from goats). The results of the gene analysis in the current study were registered in NCBI under four accession numbers (MN889986, MN889987, MN889988 and MN889989), which shows that sheep and goats can be infected with *Theileria ovis.* This is the first report of *Theileria species* in goats with clinical theileriosis in Kurdistan, so the gene flow and disease transmission between sheep and goats is most expected. PCR is a useful diagnostic tool to detect ovine theileriosis with a single test and suggested that *T. ovis* is the dominant piroplasmid agent in Erbil. In addition, it revealed that sheep is very susceptible to theileriosis than goats.

KEYWORDS: *Theileria ovis*, Theileriosis, Zoonosis, Molecular parasitology

INTRODUCTION

Tick-borne diseases are the fundamental hindrance for the development of animal breeding and can lead to substantial economic losses of livestock as they manifest marked decrease in milk production and animal weight loss (Ahmed *et al*. 2002). The tick-borne diseases of livestock resemble a combination and complex of different disease entity with variable causative agents, including protozoa, rickettsia, bacteria and viruses. The only feature that is in common among these diseases is that all can be transmitted by biological vectors. Described as being attributed to six species of *Theileria*, ovine theileriosis is a serious disease caused by *Theleiria ovis* which reside in the blood cells of sheep and goats in tropical and subtropical areas (Altay *et al*. 2004).

In the susceptible hosts, mild to severe diseases manifestations can be noticed as a result of *Theileria species* infection which can be stated as tick-borne intracellular protozoan parasites (Bishop *et al*. 2004; Mans *et al*. 2015). *Theileria spp.* has a complex life cycle similar to other apicomplexan protozoa in which three variable stages-sporogony, merogony, and gametogony are observed. It has been documented that transmission of certain protozoal parasites to mammalian hosts is due to several species of ticks such as *Hyalomma spp.*, *Haemaphysalis spp.*, *Amblyomma spp.*, and *Rhipicephalus spp.* (Aktas *et al*. 2006). The diagnosis of theileriosis in small ruminant relies on clinical symptoms, blood smears (Samples with

azad.meerkhan@dpu.edu.krd

round, oval, ring and anaplasmoid forms were tentatively classified as *Theileria spp*. (Durrani *et al.,* 2013 and Rahmani-Varmale *et al.,* 2019)), serology, and molecular identification techniques (Inci *et al*. 2010).

Identification of species depending on DNA sequences forms the basis for DNA taxonomy. Genomic markers, including small subunit ribosomal RNA gene (18S), Major Piroplasma Surface Protein (MPSP), and rRNA internal transcribed spacer region (ITS), had been utilized for analyzing the phylogenetic relationships of *Theileria spp.* (Chae *et al*. 1999; Gubbels *et al*.; 2000 Gou *et al*. 2013). Nonetheless, the subject of precise taxonomy of *Theileria spp.* has been difficult to establish because it still causes a controversial debate (Gubbels *et al*. 2002).

Accurate identification of species/haplotypes of tick-borne parasites is an important avenue to alleviate many taxonomic discrepancies, adopting the perfect therapeutic approach and proceedings of the preventive policies (Mans *et al*. 2015). However, reclassification of several *Theileria spp.* by phylogenetic analysis and sequencing based on the allelic variability and the utilization of the well-recognized molecular marker (ssrRNA) are applied to identify new *Theileria variants* which were formerly classified as other protozoan spp. (Matjila *et al*. 2008). Therefore, this study aimed to determine the *Theileria spp*. by molecular characterization in sheep and goats in Kurdistan region, Iraq.

MATERIALS AND METHODS

Collection of blood samples

The present study was conducted in Erbil Province, located in the North of Iraq. Sampling was carried out through June 2019 to April 2020. Sheep and goats with clinical signs of theileriosis were selected from the animals referred to Shaqlawa Veterinary Hospital. The disease was diagnosed based on clinical examination (temperature, heart rate, respiration rate, lymph node, mucus membrane) and laboratory confirmation by microscopic examination of blood smears and species-specific PCR analysis.

Sixty-seven sheep and goats with clinical theileriosis were sampled during the study period. The age of animals ranged from 6 to 78 months with a mean of 19 months. After recording signalmen's of each animal, body temperature, presence of pale or icteric mucous membranes and lymphadenopathy, blood samples were collected from jugular veins into anticoagulant (EDTA) containing tubes. Lymph node aspirations of 13 acutely infected animals involving 8 Sheep and 5 goats were also performed to examine for the presence of *Theileria schizonts.*

Microscopic examination

Fixed thin blood smear and lymph node Smears (Lymph samples were collected from the enlarged superficial lymph node especially prescapular lymph node by puncture and diatheses the lymph to making lymph film) were stained with Giemsa stain for 30 minutes:

1-Blood smears were examined for intra erythrocytic forms of *Theileria* piroplasm under 100×objective magnifications. About 20 microscopic fields, per slide, were observed to view the parasite. The presence of single piroplasms was recorded as positive for *Theileria*.

2-Lymph node smears were examined under 100×magnifications in the search for characteristic Koch blue bodies, the presence of which is confirmatory diagnosis of theileriosis.

DNA extraction

DNA was extracted only from whole blood samples using the DNeasy Blood & Tissue Kit (Qiagen, Germany) and stored at –20 °C until subsequent analysis (Hassan *et al*. 2017). Regarding the concentration of the extracted DNA, the highest concentration of amplicons was 18.4 ng/ µl, whereas, the lowest concentration was 7 ng/ µl.

PCR Amplification

The conventional PCR method was employed to specifically identify and differentiate *Theileria ovis* based on the method previously explained by Aktas *et al.* (2006). Concisely, a pair of primers targeting small subunit ribosomal RNA (ssu rRNA) gene of *T. ovis* were utilized for amplification. The outer primers were forward strand primer

azad.meerkhan@dpu.edu.krd

5′-TCGAGACCTTCGGGT-3′ and reverse strand primer 5′-TCCGGACATTGTAAAACAAA -3′. The PCR was carried out in a total reaction volume of 25 µl containing 12.5µl of (2X) Go-Tag master mix, 20 pmol (2µl) of each primer (forward and reverse), 2 µl of template DNA and 6.5µl nuclease-free water. PCR reactions included a negative control, consisting of the reaction mix and 2 µl of DNase/ RNase-free water. The amplification was performed in a thermocycler (Eppendorf, Germany) under the following conditions: an initial denaturation step at 95°C for 3min followed by 39 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 2 min, with a final extension step at 72°C for 10 minutes. PCR products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. The resulting bands of amplification were subsequently visualized by UV transillumination (Bio-Rad Gel Doc.) and commercially sequenced in both directions (Macrogen Inc. South Korea).

Nucleotide sequence accession numbers

In this study *Theileria ovis* sequences were identified and deposited in GenBank database of the National Center for Biotechnology Information (https://submit.ncbi.nlm.nih.gov/) under the accession number (MN889986-MN889989).

Sequence homology and phylogenetic analysis

According to sequence data, the sequences of all *Theileria species* (20 samples) were aligned. The final determination of the species was based on the comparison of the sequences of the isolates with the reference sequences (MN922940 (Pakistan), MN625886 (Egypt), MG203886 (South African), LC430938 (Iran), AY508455 (Turkey) and MG203885 (South African)) in the GenBank database, using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/). Multiple alignments were done using MEGA7 software for drowning the phylogenetic tree.

Statistical Analysis

The data obtained from small ruminants through microscopic examination of thin blood smears and DNA amplification (PCR) were compared by Pearson chi-square test in the SPSS 17.00 software for correlated proportions. P-value <0.05 were considered statistically significant.

RESULTS

Clinical examination for 45 sheep and 22 goats were subjected to this study. The infected sheep (n=12) showed different signs graduated from temperature (39.7 ± 0.93), heart rate (98.23 ± 17.72), and respiratory rate (42.81 ± 8.42). While in goats (n=8), the signs parameters of temperature, heart rate and respiratory rate were 40.25 ± 0.77, 93.94 ± 15.33 and 32.33 ±9.11, respectively (Table 1).

Table(1): The effect of *Theileria **ovis*** on the respiratory rate, heart rate and the temperature in infected sheep and goats in Erbil province.

Parameters	Infected sheep Means ± SE	Infected goats Means ± S.E.
Temperature °C	39.7 ± 0.93	40.25 ± 0.77
Heart rate/minute	98.23 ± 17.72	93.94 ± 15.33
Respiratory rate/minute	42.81 ± 8.42	32.33 ± 9.11

Furthermore, other clinical signs of theileriosis in sheep and goats were found, like the loss of appetite (83.33% of sheep, 50.00% of goats and 70% as a total), gross enlargement of the prescapular lymph nodes (91.67% of sheep, 87.50% of goats and 90% as a total), pale mucous membranes (75.00% of sheep, 62.50% of goats and 70% as a total), diarrhea (16.67% of sheep, 37.50% of goats and 25% as a total) and coughing (75.00% of sheep, 75.00% of goats and 75% as a total) (table 2).

Table (2): Percentage of clinical signs in *Theileria **ovis*** infected sheep and goats

Clinical signs	Infected sheep (n=12)		Infected goats (n=8)		Total (n 20)	
	No.	%	No.	%	No.	%
Loss of appetite	10	83.33	4	50	14	70
Enlarged of the prescapular lymph node	11	91.67	7	87.5	18	90
Pale of mucous membranes	9	75.00	5	62.5	14	70
Diarrhea	2	16.67	3	37.5	5	25
Coughing	9	75.00	6	75	15	75

The prevalence of *Theileria spp.* was found to be (13.43%, 17.91%) and (7.46%, 11.94%) in sheep and goats by microscopic and PCR technique, respectively (Table 1). Investigation of 67 samples (14 positive and 53 were negative in microscopic examination). Samples with round, oval, ring and anaplasmoid forms were tentatively classified as *Theileria* spp.. Using PCR *Theileria* species-specific primer sets, the results indicated that all of the samples that were positive on blood smears were also positive via PCR and six from the negative samples by microscopic examination gives positive by PCR (30%). There is no schizont forms detection in 8 Sheep and 5 goats' by lymph node puncture.

Table (3): The comparison between the numbers of animals clinically affected with *Theileria spp.* and PCR positive samples of sheep and goats.

Species	No. Of Animals Clinically Affected		Positive Sample by Microscopy						Positive Sample by PCR					
	Male	Female	Male	%	Female	%	Total	%	Male	%	Female	%	Total	%
Sheep (45)	9	36	3	6.67	6	13.33	9	13.43	4	8.89	8	17.78	12	17.91
Goats (22)	6	16	1	4.55	4	18.18	5	7.46	2	9.09	6	27.27	8	11.94
Total (67)	15	52	4	11.21	10	31.52	14	20.90	6	17.98	14	45.05	20	29.85

The PCR products (20 positive samples) are separately subjected for ssu rRNA gene and electrophoresed to obtained 520 bp band size on 1.5% agarose gel after staining with ethidium bromide as shown in Figure (1.a.b) which were the same bands generated by primer measured for DNA size marker 100bp DNA ladder.

azad.meerkhan@dpu.edu.krd

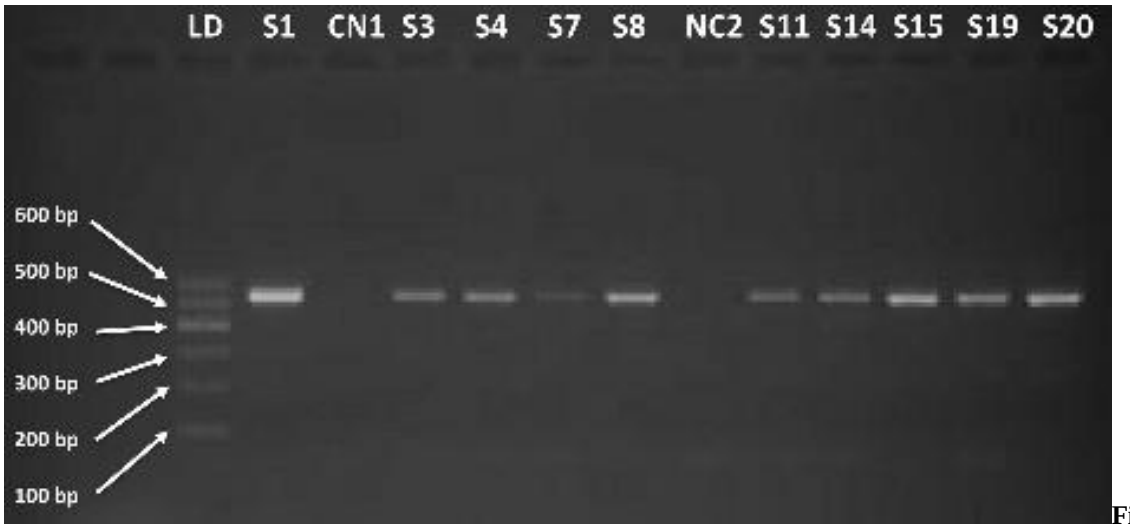

Fig. (1a): Agarose gel electrophoresis of amplified *Theileria ovis* in sheep.

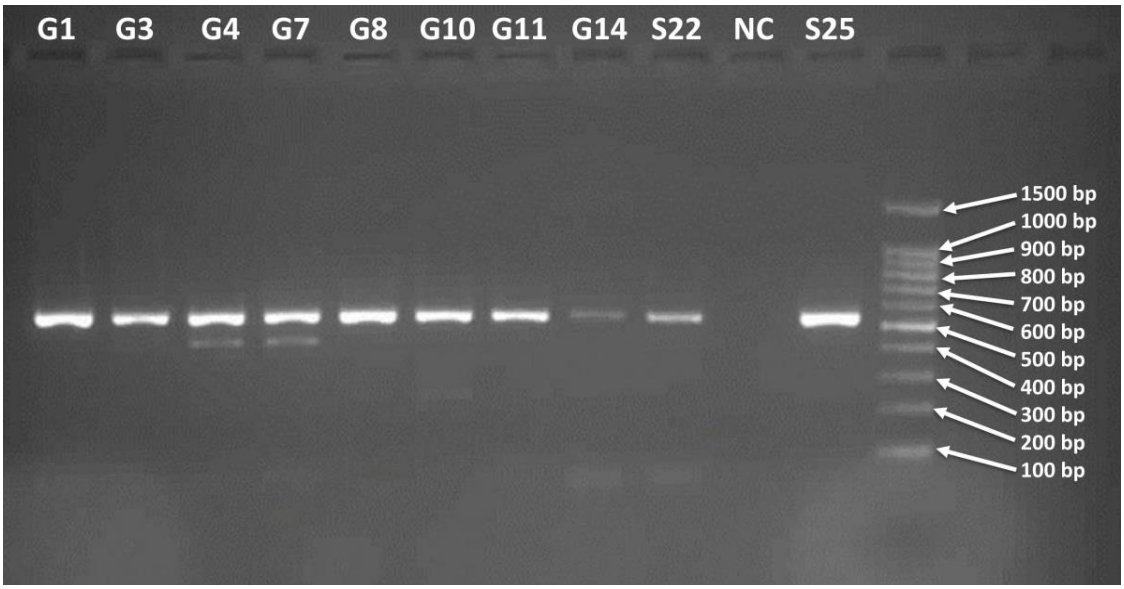

Fig. (1b): Agarose gel electrophoresis of amplified *Theileria ovis* in goats and sheep.

The forward and the reverse of each sequenced sample were assembled by using SeqMan laser gene software version 11 and saved in one FASTA file. All the united (FASTA) files were collected, aligned and restricted from both ends to obtain a matched part of 485 bps divided into 4 groups using Megalign phylogenetic tree and then registered in the GenBank under accession numbers MN889986, MN889987, MN889988, MN889989 then named as MN889986/ $Sheep_1$/ $Erbil_1$, MN889987/ $Sheep_3$/ $Erbil_2$, MN889988/ $Goat_1$/ $Erbil_3$ and MN889989/ $Goat_8$/ $Erbil_4$ respectively.

The four sequences were arranged and found that MN889986 $Sheep_1$ $Erbil_1$, MN889988 $Goat_1Erbil_3$ and MN889989 $Goat_8$ $Erbil_4$ were similar 100% (Figure 2), while MN889987_$Sheep_3$_$Erbil_2$ showed substitution of T→C at position 114 and C→A at position 188.

Phylogenetic analyses revealed that the *Theileria* spp. is genetically related to *Theileria ovis*. The four sequences blasted with previous GenBank registration and found that MN889986_ $Sheep_1$_$Erbil_1$ showed 100% identity with MG203885/ Sheep/ South African (Ringo *et al*. 2018), while

MN889987/ $Sheep_3$/ $Erbil_2$, MN889988/ $Goat_1$/$Erbil_3$ and MN889989/ $Goat_8$/ $Erbil_4$ together showed 100% identity with AY508455/ Sheep/ Turkey (Altay *et al*. 2005), LC430938/ Goat/ Iran (Hakimi *et al*. 2019), MG203886/ Sheep/ South African (Ringo *et al*. 2018), MN922940/ Sheep/ Pakistan (Iqbal *et al*. 2013) and MN625886/ Sheep/ Egypt (table 4 and figure 3).

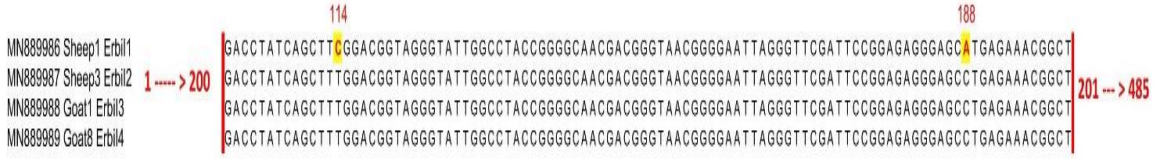

Fig. (2): Comparison of sequences of RNA gene in identified isolates (MN889986, MN889987, MN889988 and MN889989)

Table (4): The percentage of diversion and identity between the new identified *Theileria ovis* and selected references' sequences circulating globally from GenBank database.

	MN889986 Sheep1 Erbil1	MN889987 Sheep3 Erbil2	MN889988 Goat1 Erbil3	MN889989 Goat8 Erbil4	AY508455 Sheep Turkey	LC430938 Goat Iran	MG203886 Sheep S. African	MG203885 Sheep S. African	MN922940 Sheep Pakistan	MN625886 Sheep Egypt
MN889986_Sheep1_Erbil1		1.44	1.44	1.44	1.44	1.44	1.44	0.00	1.44	1.44
MN889987_Sheep3_Erbil2	0.15		0.00	0.00	0.00	0.00	0.00	1.44	0.00	0.00
MN889988_Goat1_Erbil3	0.15	1.00		0.00	0.00	0.00	0.00	1.44	0.00	0.00
MN889989_Goat8_Erbil4	0.15	1.00	1.00		0.00	0.00	0.00	1.44	0.00	0.00
AY508455_Sheep_Turkey	0.15	1.00	1.00	1.00		0.00	0.00	1.44	0.00	0.00
LC430938_Goat_Iran	0.15	1.00	1.00	1.00	1.00		0.00	1.44	0.00	0.00
MG203886_Sheep_SouthAfrican	0.15	1.00	1.00	1.00	1.00	1.00		1.44	0.00	0.00
MG203885_Sheep_SouthAfrican	1.00	0.15	0.15	0.15	0.15	0.15	0.15		1.44	1.44
MN922940_Sheep_Pakistan	0.15	1.00	1.00	1.00	1.00	1.00	1.00	0.15		0.00
MN625886_Sheep_Egypt	0.15	1.00	1.00	1.00	1.00	1.00	1.00	0.15	1.00	

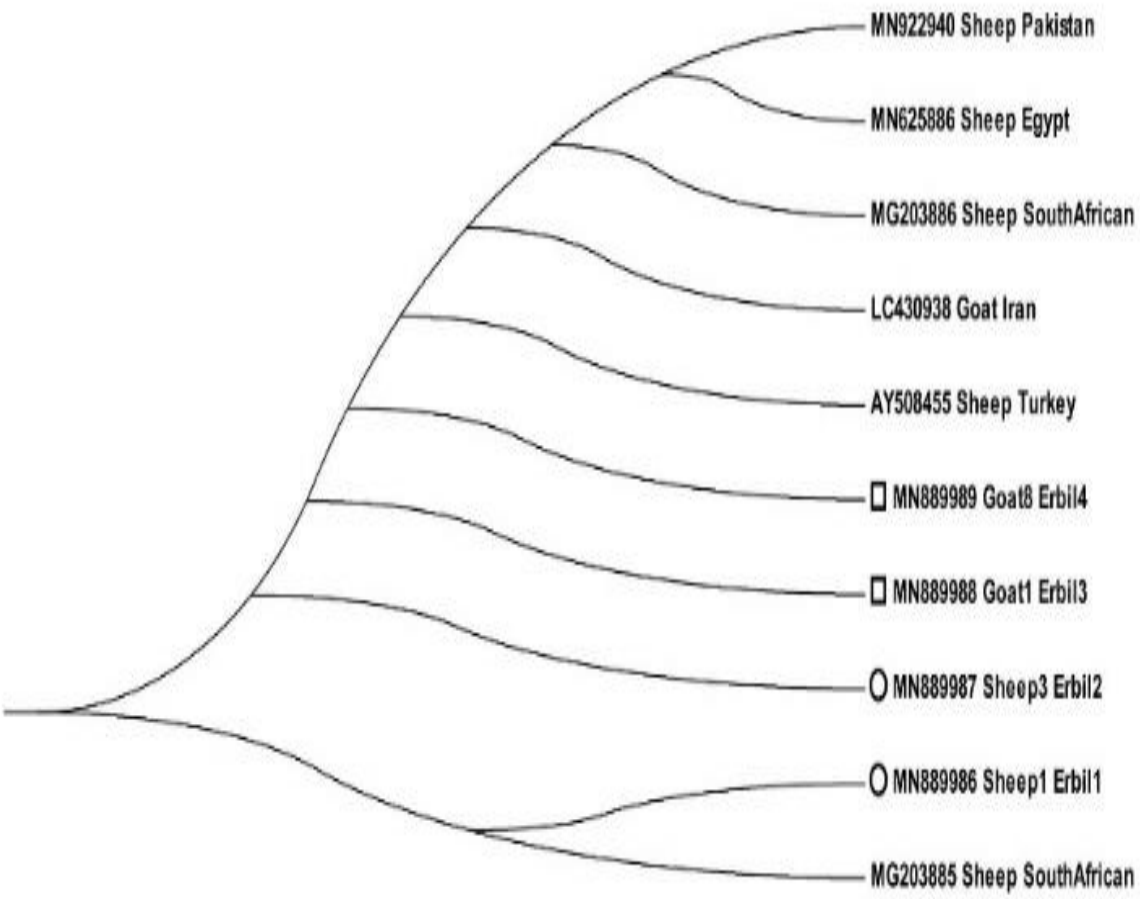

Fig. 3: Maximum likelihood bootstrap tree showing the relationships of the haplotypes of RNA gene fragment for four Theileria isolates (accession numbers; MN889986, MN889987, MN889988 and MN889989) with those submitted in GenBank using MEGA v7 software.

DISCUSSION

Several studies in Iraq revealed that *T. ovis* are endemic in sheep and goats in different parts of the country (Al-Fetly *et al.,* 2012, Renneker *et al.,* 2013 and Mahmoud *et al.,* 2019). The results of this study showed that the clinical signs observed in infected sheep and goats were in agreement with (Hassan *et al.,* 2013) In Iran, who showed that, the clinical signs of theileriosis in sheep (with more prominent signs) and goats were diagnosable. The reliable clinical signs in sheep and goats included fever, tachycardia, cough, increased respiratory rate, mucosal pallor, anorexia and lymph node enlargement. As well as, Tageldin *et al.,* 2005 in sultanate of Oman, expressed that, all the affected sheep and goats showed pyrexia, pale of mucous membrane, lymph node enlargement, respiratory distress, decumbency and death. The increase in respiration rate and pulse rate occur as a compensatory mechanism to balance the level of oxygenated blood due to decrease of RBCs levels (Mahmoud *et al.,* 2019). The cause of pale mucous membrane was development of anemia and decrease in erythrocyte count and haemoglobin, while jaundice due to increase in the total bilirubine (Sulaiman *et al.,* 2010)

Out of the 67 samples, 14 (20.9%) were positive in the blood smear showing *Theileria spp.*, 9 sheep out of 45 (13.43%) and 5 goats out of 22 (7.46%). These agreed with Naz *et al.,* 2012 who showed that the prevalence of *Theileria spp*. was found to be 13.9% and 8.2% in sheep (38/273) and goats (21/256). The results revealed percentage of infection was the higher; this may be due to the animals undergo from environmental stresses, overcrowding, starvation, and may be due to mixed living of different animal species that were facilitate the

transmission of ticks between animals which were lead to transmission of disease (Karatepe *et al.*, 2019). Also, the goat has thin skin that seems to be more resistant for the tick compared to sheep. The ticks may easily get entangled in wool of sheep and subsequently may cause infestation (Durrani *et al.*, 2012). Results of PCR amplification revealed that 20 blood samples (29.85% of total), produced the 520 base pairs DNA fragment specific for small subunit Ribosomal RNA (ssu rRNA) gene of *T. ovis*. 12 (17.91%) of the *T. ovis* positive were sheep and 8 (11.94%) were goats indicating that sheep are more significantly to this parasite than goats. This agreed with Gebrekidan *et al.*, 2014 in northern Ethiopia, who expressed that 150 (93.8%) of the sheep and 5 (1.9%) of the goats, in total 235 (44.8%) of the ruminants, were positive for infection with at least one species of *Theileria* based on PCR followed by DNA sequencing. Sheep had a significantly higher rate of infection with *Theileria spp.* as compared to goats. The variation in infection rates of domestic ruminants with tick-borne pathogens is related to several factors including the presence and abundance of tick species which act as vectors for specific pathogens, genetic variation among animals and breeds in resistance (Yang *et al.*, 2014). PCR is more sensitive than microscopic examination. because the second one is simple to perform, quick and cost effective techniques and remains the most rapid confirmatory method for detecting this infection in acute phase of the disease. However, less sensitivity makes it difficult to detect carrier cases or chronic phases of piroplasmosis (Maharana *et al.*, 2016). As well as, PCR is much more sensitive in clinically infected, apparently healthy animals (carriers) and in the early phase of infection. This agree with Charaya *et al.* (2016) reported that PCR assay showed no cross-reactivity with *theileria* sp. and can be used specifically to diagnose theileriosis in herd and differentiate it from other hemoprotozoan diseases. However, the PCR method enables us to detect clinical, subclinical and chronic infections (Sharifi *et al.* 2016). This result agreed with Durrani *et al.* 2011 and Yaghfoori *et al*. 2013, who revealed that (15.5% and22%) of blood smears while (41.2% and 35%) by PCR, respectively. Although there are limited molecular studies carried out in Kurdistan Region concerning sheep *T. ovis* identifications, Ameen *et al*. 2012; Renneker *et al.* 2013 primarily considered the identification of *T. ovis* infections along with their different heterogeneity ranges was unequivocally isolated and typed based on phylogenetic exploration in goats, then.

Theileria ovis in this study is shown that there is a potential metazoonosis transmission among ruminants. High haplotype diversity (0.71–0.89) identified in sheep and goats populations are alerted to pathogenicity range of *T. spp.* complex, the creation of emergent genotypes in the areas and also the resistance of *Theileria species* against host immunity responses. This observation that *T. ovis* isolates originating from Erbil Province suggests that *T. ovis* does represent a single species. Most closely related to *T. ovis* is the sequence of a *Theileria species* isolated Turkey, Iran, Pakistan, and South Africa (Aktas *et al.*, 2006; Iqbal *et al.*, 2013; Ringo *et al.*, 2018 and Hakimi *et al.*, 2019). The reason of the identity among the study genotypes and the genotypes from other areas can be the gene migration due to the variation in the sources and origin of the livestock. Furthermore, due to non-controlled boarders and the huge migration of refugees from Syria and internal displaced people from some parts of Iraq accompanied with their livestock.

CONCLUSION

PCR is a useful diagnostic tool as it will enable diagnostic laboratories to detect ovine theileriosis in all domestic species with a single test and suggested that *T. ovis* is the dominant piroplasmid agent in Erbil. In addition, it revealed that sheep is very much susceptible to theileriosis than goats.

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azad.meerkhan@dpu.edu.krd

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azad.meerkhan@dpu.edu.krd

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azad.meerkhan@dpu.edu.krd