DETECTION AND MOLECULAR CHARACTERIZATION OF
THEILERIA OVIS IN SHEEP AND GOATS WITH CLINICAL THEILERIOSIS IN 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
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 Shaqawwa 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 100xobjective 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 100xmagnifications 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

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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 3 min 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 drawing 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).

| Parameters          | Infected sheep | Infected goats |
|---------------------|----------------|----------------|
| 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).
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 (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     |

**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.
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/ Sheep1_ Eribil1, MN889987/ Sheep2_ Eribil2, MN889988/ Goat1_ Eribil3 and MN889989/ Goat8_ Eribil4 respectively.

The four sequences were arranged and found that MN889986 Sheep1_ Eribil1, MN889988 Goat1_ Eribil3 and MN889989 Goat8_ Eribil4 were similar 100% (Figure 2), while MN889987_Sheep1_ Eribil2 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_ Sheep1_ Eribil1 showed 100% identity with MG203885/ Sheep/ South African (Ringo et al. 2018), while
MN889987/ Sheep/Erbil, MN889988/ Goat/Erbil, and MN889989/ Goat/Erbil, 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 |
|-------------------------|------------------------|------------------------|------------------------|------------------------|-------------------|
| 1.44                    | 0.15                   | 1.00                   | 1.00                   | 1.00                   | 1.00              |

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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% and 22%) 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 metazoanosis 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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