



Conventional and Molecular Diagnosis of Theileriosis (*Theileria annulata*) in Cattle in Sulaimani Province, Northern Iraq

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ABSTRACT

Theileria annulata has been identified as a major cause of bovine theileriosis in Iraq. The research was designed to reveal the prevalence of Theileriosis (*Theileria annulata*) in cattle in Sulaimani province, Kurdistan Region- Iraq from the 10th of March 2021 to the 10th of April 2021. Eighty blood samples were collected. Different techniques of parasitic detection, including Microscopic and PCR amplification were used. The results of microscopic examination and PCR assay revealed that 27.5% and 31.25% cattle were infected, respectively. The microscopic examination of the Giemsa-stained blood smears of infected erythrocytes revealed the typical morphological structure of piroplasm. According to the phylogenetic analysis of the partial 18S ribosomal RNA gene, *Theileria* field isolates were shown to be related to other *T. annulata* strains, including those from Turkey, Italy, and Pakistan. This study concludes that *T. annulata* is the causative agent of theileriosis of cattle in Sulaimani province and suggests that PCR technique is excellent tools for epidemiological researches and control programs.

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Keywords: *T. annulata*, Tropical theileriosis, Blood smear, PCR, Sulaimani province

1. Introduction

Theileriosis is a haemoprotozoan disease transmitted via biting of ticks and this disease leads to major economic losses in livestock including cattle [1]. Ticks (ixodid ticks) act as vectors in spreading of the disease in hot or moderate climates that inject *Theileria* spp. into the bovine blood [2].

Cattle are generally susceptible to *Theileria* [3], which includes mostly pathogenic species example, *Theileria annulata* that leads to tropical theileriosis. Also *Theileria parva* is another species that causes east coast fever [4].

Tropical theileriosis poses a huge impact on economic losses in dairy industry throughout the world [5]. Recent studies reported cattle infection with *T. annulata* in the Asian countries, including Iraq [6,7], Iran [8,9], Turkey [10], Saudi Arabia [11], and Egypt [12]. In the Kurdistan Region of Iraq, reliable and accurate diagnostic methods have rarely been used for epidemiological study; therefore, limited data is known on the epidemiology of tropical

theileriosis [6]. In general, clinical signs were somewhat trusted for *Theileria* diagnosis and it was confirmed by thin blood film stained by Giemsa looking microscopically for piroplasms in erythrocytes or lymph node smear looking for macroschizonts in lymphocyte. However, clinical signs cannot be trustful for diagnosis in many cases because similarity in symptoms are seen between *T. annulata* and other blood parasitic diseases [13,14]. In addition, diagnostic method microscopically for sub-clinical and chronic cases needs expertise for reading of the slides. For instance, in case of parasitemias finding of piroplasms may difficult because of their extremely low ratio in the stained blood smears. Therefore, performing of this conventional method for diagnosis of carrier animals is difficult and it requires more time to identify the form of piroplasms in the RBCs [14]. To detect of different *Theileria* species in infected cattle, molecular technique was developed which have a high degree of sensitivity and specificity [15]. The prevalence rate of tropical theileriosis caused by of *T. annulata* needs to be determined in cattle in the various areas of Kurdistan Region, therefore the present research was carried out to assess the spread of *T. annulata* in cattle during spring (10th March – 10th April) using conventional microscopy and PCR technique, in Sulaimani, Kurdistan Region of Iraq.

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2. Material and Methods

The study was undertaken in the Sulaimani province, Kurdistan Region, Iraq during spring (10th March – 10th April). The climate of Sulaimani is variable according to its seasons; temperature in spring is relatively moderate (20° - 40° Celsius). The clinical manifestations of theileriosis were noted on some animals during sampling such as high temperature, weakness, lymphnodes swelling in some cases, increased respiration, and nasal discharge. In addition, tick infestations were seen on some examined animals.

2.1. Samples Collection

A total number of 80 (male and female) Arabic, local, Simmental and crossbred cattle older than 6 months reared in open management system were screened for haemoprotozoan parasite from various area of Sulaimani province (Tanjaro, Kalar, Smud, Said Sadiq), Kurdistan Region, Iraq. Approximately 6ml of blood samples from Jugular vein of cattle randomly were collected, 2ml of the blood were put in tube containing EDTA. The samples were stored and transfer to laboratory in a special designed cool box. Giemsa's stain was done for the blood samples of EDTA tubes to determine of piroplasms microscopically. Then the blood samples were stored at - 70 °C for PCR test. Ethical standards were considered during sampling.

2.2. Microscopic Examination (ME)

After preparation of peripheral blood smears on glass slides, the slides were dried and fixed with methanol for about 5 minutes, then stained by 10% Giemsa stain for 30 minutes and examined under Oil immersion lens (100 x magnifications).

2.3. DNA extraction

The DNA was extracted used (200 µl) of the whole blood samples with DNA extraction Kit (ADD BIO INC, DAEGEON Republic of Korea) according to the manufacturer's instructions. The DNAs were eluted into 100 µl Elution buffer at room temperature where they were frozen at -20°C until PCRs performed.

2.4. PCR amplification and sequencing

The PCR reactions were performed using specific primer which was designed by [16] to amplify a 1098bp fragment of highly conserved regions of 18S ribosomal RNA gene in all *Theileria* spp. 18SrRNA forward primer (AGTTTCTGACCTA TCAG) and 18SrRNA Reverse primer (TTGCCTTAA ACTTCCTTG) were provided by (Macrogen company, Korea). The 18S ribosomal RNA gene was amplified using PCR Premix (2X). This master mix provides an entire system for fast, high yield, and reliable single-tube PCR contains: prime taq polymerase unit, tris HCL (PH 9.0), PCR enhancer, (NH₄)₂so₄, 4 mM Mgcl, enzyme stabilizer sediment, Loading dye, and 2.0 mM dNTPs mixture (Genetbio, Korea). The reactions were carried out in a 0.2 mL PCR tube that met the following requirements: 10 µL supreme script PCR premix, 5 µL DNA, 1 µL forward (10 pmol), 1 µL reverse primers (10 pmol), and three µL ultra-pure water to form up a final volume of 20 µL. the traditional PCR machine (Hercuvan, USA) was programmed as followed: initial denaturation at 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s; annealing at 55°C for 30s, and extension at 72 °C for 60s and a final extension at 72 °C for 10-min. After electrophoresis on 1% agarose gels stained with prime safe dye (Genetbio, Korea) at 100 volt for 60 minutes, the obtained PCR products were

visualized under UV illumination. The PCR product for *T. annulata* was estimated to be 1098 bp. Two positive samples' PCR products were sequenced (Macrogen, Republic of Korea) and sent to the national center for bioinformatics and information (NCBI) GenBank.

2.5. Accession numbers:

For this analysis, the partial 18SrRNA gene sequences of two field sequences were deposited under the GenBank accession numbers (MW94053 and MW94054).

2.6. Bioinformatics and sequences analysis

MEGA.X program was used to bring the sequences together. The ClustaW Multiple Sequence Alignment tool was used to trim and arrange the ribosomal RNA sequences [17]. The Neighbor-Joining approach was used to infer the phylogenetic tree and evolutionary relationships [18]. In the bootstrap test (1000 replicates), the percentage of duplicate trees in which the related taxa clustered together [19]. The Kimura 2-parameter method [20] was used to calculate the evolutionary distances.

3. Results

Out of 80 samples, 22 (27.5 %) were found positive for tropical theileriosis by conventional microscopic technique using Giemsa staining method; whereas, 25 (31.25 %) samples were detected positive by PCR assay (Table, 1)

Table 1: The prevalence rates of *T. annulata* infection in cattle using Giemsa staine and PCR methods.

Species	Positive sample by Giemsa staine		Positive sample by PCR	
Cattle	22	27.5 %	25	31.25 %

3.1. Morphology of *Theileria annulata*

The microscopic examination of the Giemsa-stained blood smears of infected erythrocytes revealed the typical morphological structure inside erythrocytes included common shape (ring, Oval, and parachute forms). They were observed as single, double, triple and tetra-forms like inclusion bodies (Figure 1).

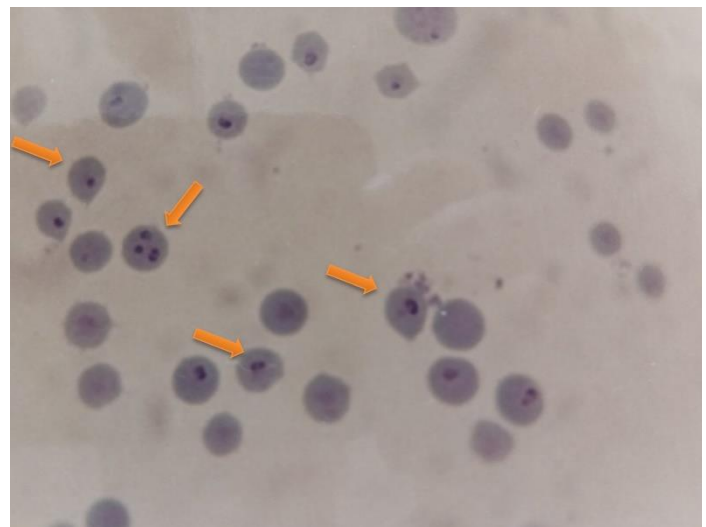


Figure 1: Erythrocytic form of theileria annulata, giemsa stain X 1000 (arrows)

3.2. Molecular detection

PCR is used to identify theileria. PCR analysis showed that 25 of

the 80 samples tested were positive, with a band of 1098 bp corresponding to the partial amplification of the 18S rRNA gene on agarose gel electrophoresis (Figure 2).

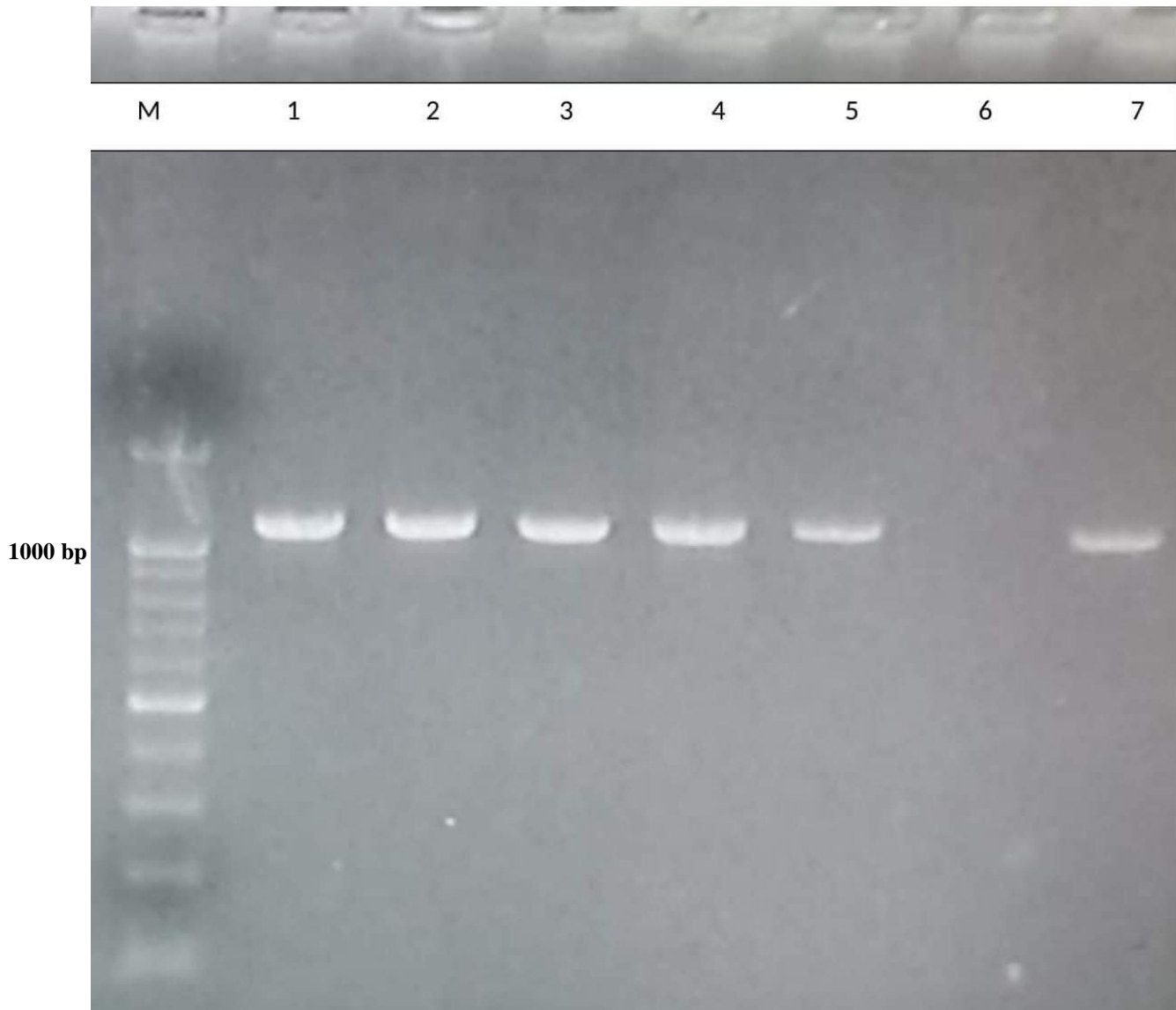


Figure 2: Agarose electrophoresis image, which show the PCR product results for *Theileria* spp of 18S rRNA gene at 1098bp PCR product size, Lane M: Marker 100bp, Lane (1-5+ 7) are positive samples. Lane 6 Negative sample.

3.3. Sequencing results

Two samples of PCR products were sequenced. After sequencing, the strains were given the names (MW940543-*T.annulata*/Sh/3/21, and MW940544-*T.annulata*/Sh2/3/21). For genetic relatedness, these sequences were linked to *T. annulata* references found in GenBank.

3.4. Alignment of sequences and phylogenetic analysis

The two nucleotide sequences of the detected *T. annulata* were 100% identical and had no diversification. The field sequences isolated with *T. annulata* Turkey and Italy strains with accession numbers (AY508463 and MT341858) exhibited the highest identities of 99.24% and 99.24%, respectively (Table 2).

When field parasite DNA sequences were compared to other *T. spp* strains in GenBank, *T. parva* was the most closely related, with a similarity of 96.72%, while *T. sergenti* had a similarity of 94.04%. In addition, comparisons of *T. annulata*/Sh/21 sequences with those of an Iraqi strain (Iraq/AN10) indicated no significant differences (Table 2, Figure 3). A partial sequences of the 18S rRNA gene from the field strains and representative strains from GenBank were used in phylogenetic analysis to compare and verify the evolutionary ancestry of the field samples. Furthermore, the two field strains were shown to be related to other *T. annulata* in different countries (Turkey, Italy, and Pakistan). Field isolate sequences are heterologous with *T. spp* strains like (like *T. parva*, *T. bovis*, *T. taurotragi*, *T. capreoli*, *T. sergenti*, and *T. buffeli*), but only homologous with (*T. annulata*), according to a phylogenetic tree (Figure 4).

Table 2: The 18S rRNA gene sequence homology of field *T. annulata* strains with other *Theileria* spp. Available in Genbank.

Accession no.	country	<i>Theileria</i> spp.	DNA Identities
L19082	South Africa	<i>T. taurotragi</i>	96.30
AY260172	Turkey	<i>T. ovis</i>	94.37
AY726011	USA	<i>T. capreoli</i>	94.06
AY661515	JAPAN	<i>T. sergenti</i>	94.05
AY661512	USA/texas	<i>T. buffeli</i>	94.45
AF013418	Kenya	<i>T. parva</i>	96.72
	Iraq/AN10	<i>T. annulata</i>	99.61
MT341858	Italy	<i>T. annulata</i>	99.24
AY508463	Turkey	<i>T. annulata</i>	99.24

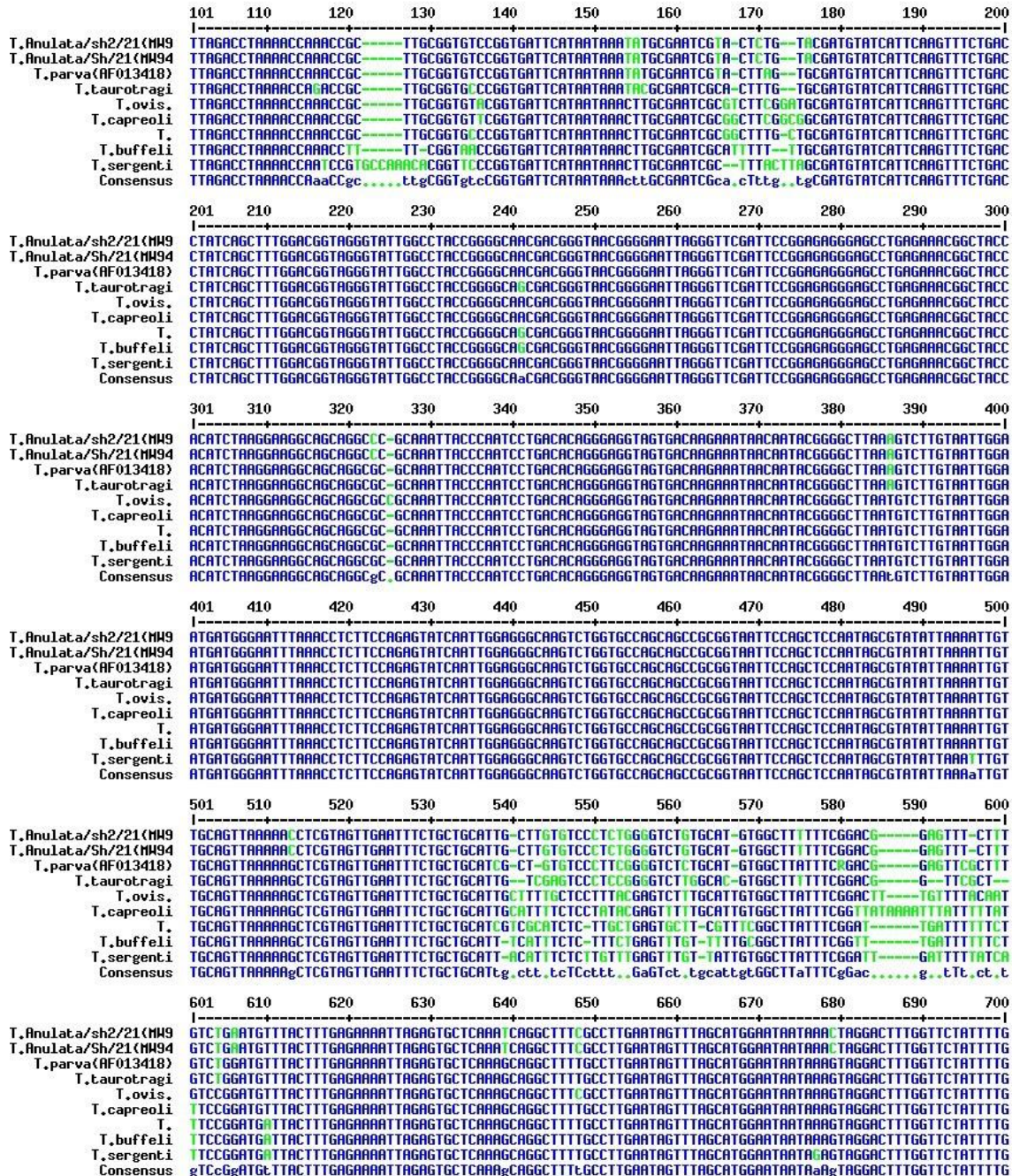


Figure 3: CLUSTAL W2.1 multiple sequence alignment of 18S rRNA gene 1098bp PCR product of two field isolate (MW940543 & MW940544) with other *Theileria* spp. Strains. *T. ovis* (AY260172), *T. taurotragi* (L19082), *T. capreoli* (AY726011), *T. sinensis* (EU277003), *T. buffel*

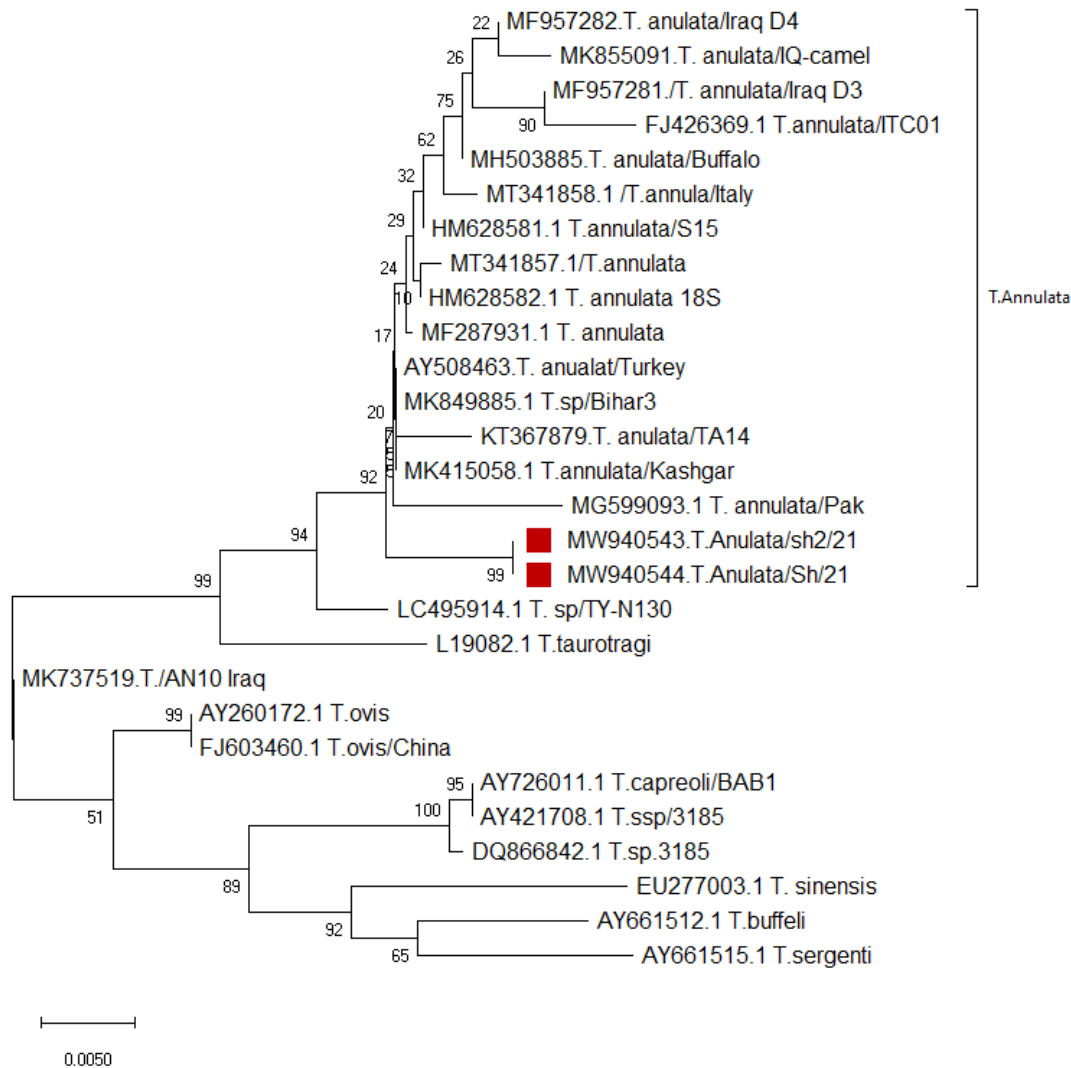


Figure 4: Neighbor-Joining method. Phylogenetic tree of *Theileria* spp. based on 18S rRNA gene sequences (*T.annulata*, *T. ovis*, *T. taurotragi*, *T. capreoli*, *T. sinensis*, *T. buffeli*, and *T. sergenti*). The Iraqi sequences obtained in the present study are red square.

4. Discussion

Theileriosis is a significant clinical illness of cattle in many countries. It also causes major economic losses as well as reduced production [21, 22, 23, 24]. Several studies in Iraq revealed that tropical theileriosis are endemic in cattle in different parts of the country [7, 16, 25, 26, 27]. The traditional identification methods are based on morphological structure inside erythrocytes. In the current study, different shapes were found inside infected erythrocytes by direct microscopic examination including ring, Oval, and parachute forms. Giemsa-stained blood smears showed that cattle erythrocytes infected with the piroplasm forms of *Theileria annulata* pathogens showed round-shaped appearance and irregular thorn-like protrusions (figure 1). These erythrocyte morphologic disorders are attributed to presence of the parasites in the erythrocytes [28]. They are generally in agreement with findings of [26]. However, carrier animals are sometime difficult to identify using traditional Giemsa staining method. Although, this method uses as convenient technique in the labs for the diagnosis of clinical cases, but for carrier animals' determination having low parasitemia. Thus, alternative techniques, for instance PCR can be used as it is more accurate

technique to identify of persistently infected cattle with *Theileria* spp. [27, 29].

According to the current study's sequence analysis, *T. annulata* was confirmed to be the source of Theileriosis in the field. The phylogenetic tree shows that two field isolates are homologous and identical to the most of other *T.annulata*. This demonstrates that the 18S rRNA is suitable for identification of this parasite. The disease' prevalence is lower in Kurdistan Region of Iraq (31.25 % in cattle) in comparison to other countries those used PCR technique. In Sudia Arabia, the disease' prevalence was 76.5 % in cattle [11]. In Turkey, the rate of the disease was 37.8 % [10]. Other research have shown similar prevalence rates, for instance, the rate of the disease in Iran using PCR was 31.5 % in cattle [8]. On contrast to our results, according to the results of other studies, the prevalence of theileriosis was 14.32 % In Dera Ismail Khan, Khyber Pakhtunkhwa Province, Pakistan [30]. In Egypt, the prevalence rate was 24.05 % in cattle by PCR assay [12], while in the Sulaihiya area of Kuwait, prevalence rate of theileriosis recorded was 7.6 % [31], and in Spain, the prevalence rate was 22.4 % by using PCR technique [32]. These differences in the

prevalence of *T. annulata*-infection reported from various studies can be attributed to many factors such as climatic conditions, breeds' susceptibility, vectors' distribution, breeding system, immunization, prophylactic strategy and systems of treatment. However, season is one of the most common considerable differences because in summer highest rate of the disease was recorded [30].

5. Conclusions

The findings of the current study illustrate that traditional method could be a suitable technique to determine tropical theileriosis. Proper formulation of control strategies and reduction of economic loss caused by theileriosis in cattle can be performed via further identification trial of *T. annulata* based on 18S rRNA gene sequences. In addition, the study showed that *T. annulata* infection was dominant among other *Theileria* species in cattle of the studied area. PCR technique accepted as an accurate tool to determine *Theileria* even in asymptomatic carrier animals which is quite necessary for carrying out of control programs successfully. Therefore, further field studies to verify these results are suggested.

Conflict of interests

None.

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