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MOLECULAR STUDY AND EFFECT OF RISK FACTORS ON INFECTION RATES OF THEILERIOSIS IN CAMELS IN MIDDLE OF IRAQ

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Abstract

Molecular study was done to differentiate between *Theileria* species in 100 randomly selected out of 270 camel's blood samples. The results showed in this study, 39(39%) *Theileria spp.* and 34 (34%) *Theileria annulata* were diagnosed in camel's blood by using conventional PCR technique. The rate of infection with *Theileria* in camels affect with sex, male and female recorded 32.25 % and 42.02% in camels, respectively. There were significant differences in the rate of infection between age groups of camels, the highest rate of infection was observed at <6years age group 51.35%, and the lowest 30.76 % recorded in >12 years age group. DNA sequence analyses were done on 10 camel's isolates. All isolates of *Theileria* were *T. annulata* reported in middle of Iraq in camels. *Theileria annulata* were detected in camels didn't distinguished by morphology, but genetically (DNA sequence analysis) differentiated.
Keywords: Theileriosis, PCR, Phylogenetic tree, Camel.

Introduction

Theileriosis is a tick borne haemoprotozoan disease caused by intra erythrocytic protozoan parasites which infects cattle, sheep, horses, mules, zebra, dogs and camels (Beck *et al.*, 2009; Qablan *et al.*, 2012, 2013). The parasite forms in the erythrocytes are predominantly rod shaped and no schizonts are detected in the prescapular lymph node impression smears as reported by (Nassar, 1992).

Camel is physiological and anatomically adapted to survive harsh condition and play an important role in human's life how lived in desert and semi-desert and even in irrigate land. The ticks play an important role in the transmission of many different pathogenic organisms, but the most important are protozoan pathogens. Diseases caused by protozoan parasites in ruminant are known as piroplasmosis include Theileriosis (Makala *et al.*, 2003).

On other hand the Theileriosis one of the most important problems of the livestock industry in developing countries due to their responsible for significant economic losses to the livestock (Aktas *et al.*, 2012).

The a molecular technique is necessary for the diagnosis of *Theileria* spp. especially polymerase chain reaction which had been developed in order

to overcome the problems faced with conventional and serological techniques, In addition PCR is reliable method for diagnosis and epidemiological studies, as well as permits the identification of genetic variants and cryptic species (Qablan *et al.*, 2013).

Iraq is one of many countries complaining from Tropical Theileriosis and this disease is represented of the really challenge, many epidemiological studies was performed and indicated that the disease is endemic in many provinces such as, Al Muthanna and Basra (Jasim *et al.*, 2015).

Due to unavailable of epidemiological data about camel's theileriosis in middle of Iraq especially in Wassit, Qadissiyah and Al Najif Al Ashrif provinces, The present study was aimed to investigate the prevalence of theileriosis infection in camels in middle of Iraq utilizing molecular method, investigate effect of age and sex on infection rate, phylogenetic analysis of *Theileria annulata* local isolates.

Materials and Methods

The study will be started from 1st December, 2018 to 31th August, 2019. A total number of 100 camel's blood samples of different ages and sexes were collected from the jugular vein evenly of middle provinces of Iraq. Blood samples will draw by 5 ml sterile syringe from camels.

Blood samples collection

Collected (5 ml) with anticoagulant for DNA extraction as a target for PCR amplification. (Will store in -20°C till use for DNA extraction).All blood collection tubes with EDTA will be inverted gently five times for directed mixing after collection and before storage (Chaudhri and Gupta, 2003; Faraj *et al.*, 2019).

DNA extraction, PCR amplification, and DNA sequencing

PCR technique was performed for detection *Theileria* spp. And *Theileria annulata* from blood of camels, the method was carried out according to method described by (Alhassan *et al.*, 2005). Genomic DNA extracted by gSYAN DNA mini kit extraction kit (Geneaid, USA). The extracted blood genomic DNA was checked by using Nanodrop spectrophotometer (THERMO. USA), which measured DNA concentration (ng/µL) and check the DNA purity by reading the absorbance at (260 /280 nm).

Then for DNA amplification used AccuPower PCR PreMix Kit (Bioneer, Korea). The extracted DNA was kept at -20 °C. PCR was performed using two sets of primers were designed in this study by using NCBI Gene-Bank and Primer 3 online and provided by (Macrogen Company, Korea), (5'-

TGGCGGCGTTTATTAGTTCG-3' and 5'-ATTGGAGGGCAAGTCTGGTG-3') forward primers, (5'-CCACGCTTGAAGCACAGGA-3' and 5'-TCCACCAACTAAGAACGGCC-3') reverse primers and amplicon 426 bp and 722 bp for *Theileria* spp. And *T. annulata,* respectively in a final reaction volume 20 µl contain 5 µl of (KCl, Tris–HCl pH 9.0, MgCl2, Stabilizer and Tracking dye, deoxyNucleoside TriPhosphates dNTPs, Taq polymerase) and 5 µl of DNA template and 1 µl of Forward primer (10pmol) and 1 µl of Reveres primer (10pmol) and complete the final volume with PCR water (Luna, 1992). The reactions were performed in an automatic PCR Thermocycler (MJ-Mini BioRad. USA) for 30 cycles. Each cycle consisted of a denaturing step of 30sec. at 95 °C, and annealing step of 30sec. at 58 °C and 1 min of extension step at 72 °C, according to AccuPower PCR PreMix Kit (Bioneer, Korea).

DNA sequencing method was performed for confirmative detection of local *Theileria annulata* and study of phylogenetic relationship tree analysis between local *Theileria annulata* isolates and NCBI-Blast submission local *Theileria annulata* as well as submission of our isolates in NCBI-Genbank. 10 PCR positive products of local *Theileria annulata* blood were sent to Macrogen Company in Korea in ice bag by DHL for performed the DNA sequencing by AB DNA sequencing system. The PCR sequence results were edited and analyzed by (Mega 6.0) program and using nucleotide collection (nt/nr) database, also to specify the parasite blasted sequences(Neighbor-joining method) were used for aligned sequences. phylogenetic analysis were carried out to find the relationship of sequences determined in the study obtained along with relevant sequences deposited in Genbank DNA Sequencing carried out by (Suol–Korea).

Statistical analysis

Statistical analysis for this study was achieved by using Chi Square tests (X^2) at $p \leq 0.05$ was used to analyze differences in *Theileria* spp. rate among samples, sex and different studied ages (Leech *et al.*, 2014).

Results and Discussion

PCR product analysis

One hundred blood samples were randomly isolated from 270 samples collected from camels and screened for *Theileria* infection. In this study, 39(39%) *Theileria* spp. and 34 (34%) *Theileria annulata* were diagnosed in camel blood by using PCR technique as in table (1) and figure (1) and (2). The present study demonstrated that the infection rate in females was 42.02% (29/69) more than in males 32.25% (10/31) with significant differences (P<0.05) Table (2). The study showed that all age groups were infected with *Theileria* parasite with variable rates. A significant differences P<0.05 was recorded between old and young age groups Table (3).

Table 1: Show the diagnosis *Theileria* infection by PCR

Parasite	No. of samples examined	No. of positive by PCR	Percentage %
Theileria spp.	100	39	39
Theileria annulata	100	34	34

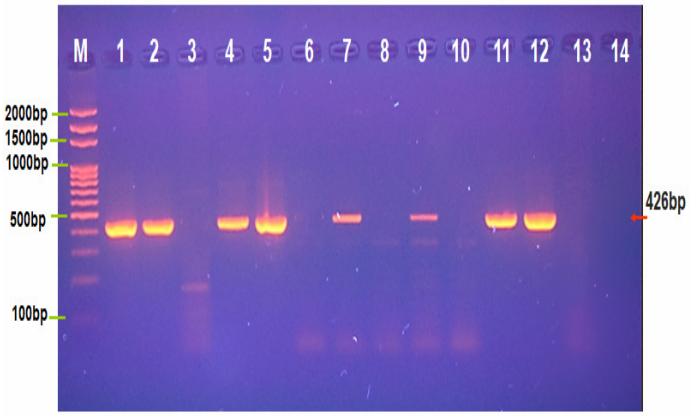

Fig. 1: Agarose gel electrophoresis image that showed the PCR product analysis of 18S ribosomal RNA gene in *Theileria* spp. from camel blood samples. Where M: Marker (2000-100bp), lane (1-14) showed some positive *Theileria* spp. samples at (426bp) PCR product size, 1.5% agarose gel.

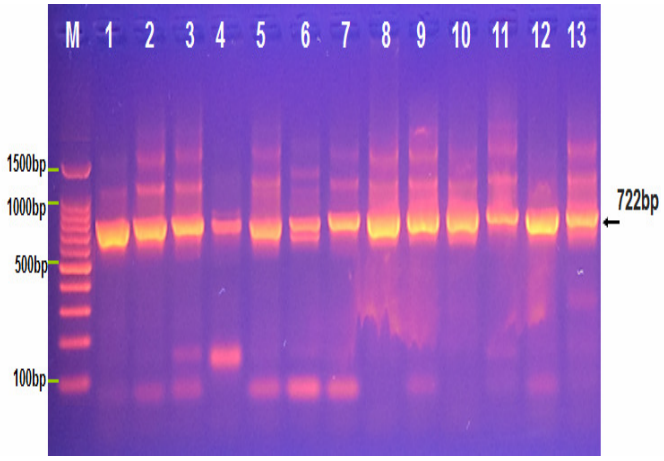

Fig. 2: Agarose gel electrophoresis image that showed the PCR product analysis of 18S ribosomal RNA gene in *Theileria annulata* from camel blood samples. Where M: Marker (1500-100bp), lane (1-13) showed some positive *Theileria annulata* samples at (722bp) PCR product size, 1% agarose gel.

Table 2: Prevalence of *Theileria* infection by PCR in relation to sex of camels

Sex groups	No. of samples examined	No. of positive	Percentage (%)
Male	31	10	32.25 B
Female	69	29	42.02 A
Total	100	39	39

Different letter = significant difference at $p \leq 0.05$

Table 3: Prevalence of *Theileria* infection by PCR in relation to age groups of camels

Age groups	No. of samples examined	No. of positive	Percentage (%)
< 6 Years	37	19	51.35 A
6- 12Years	37	12	32.43 B
> 12 Years	26	8	30.76 B
Total	100	39	39

Similar letter = no significant difference at $p \geq 0.05$
Different letter = significant difference at $p \leq 0.05$

DNA sequencing result: Ten samples purified by PCR technique analyzed by sequencing to get nucleotide sets of (18s ribosomal RNA) gene (426 bp) for *Theileria* spp. And (cytochrom b) gene (722 bp) for *Theileria annulata* isolated

from camels in different parts of middle of Iraq and recorded in gene bank with accession numbers: MK855082, MK855083, MK855084, MK855085, MK855086, MK855087, MK855088, MK855089, MK855090 and MK855091. (Table 4 and 5) (Figure 3 and 4)

The analysis of phylogenetic tree based on sequences of (426 bp) 18s ribosomal RNA gene, and (722 bp) cytochrom b gene for *Theileria* spp. and *Theileria annulata* respectively. Phylogenetic tree using constructed neighbor joining bootstrab1000 radiation tree. Analysis result showed that homology of nucleotides sequences between local isolate of Iraqi *Theileria* spp. was nearly closed to Khuzestan Iran isolates (MK 183000) with homology sequence identity 99% (Figure 5 and 6).

Table 4: The NCBI-BLAST Homology Sequence identity (%) between local *Theileria spp.* camels isolates and NCBI-BLAST submitted *Theileria spp.* isolates.

***Theileria spp.* isolates Nos.**	**Genbank Accession number**	**NCBI-BLAST Homology Sequence identity (%)**		
		Identical *Theileria spp.*	**Genbank Access. number**	**Identity %**
No.1	MK855082.1	*Theileria annulata*	MF287951.1	99.27%
No.2	MK855083.1	*Theileria annulata*	MF287951.1	99.11%
No.3	MK855084.1	*Theileria annulata*	MF287951.1	99.71%
No.4	MK855085.1	*Theileria annulata*	MF287951.1	99.85%
No.5	MK855086.1	*Theileria annulata*	MF287951.1	98.98%
No.6	MK855087.1	*Theileria annulata*	MF287951.1	99.71%
No.7	MK855088.1	*Theileria annulata*	MF287951.1	99.27%
No.8	MK855089.1	*Theileria annulata*	MF287951.1	99.56%
No.9	MK855090.1	*Theileria annulata*	MF287951.1	99.26%
No.10	MK855091.1	*Theileria annulata*	MF287951.1	99.26%

Table 5: The NCBI-BLAST Homology Sequence identity (%) between local *Theileria annulata* Camels isolates and NCBI-BLAST submitted *Theileria annulata* isolates related to country.

***Theileria annulata* isolate Nos**	**Genbank Accession number**	**NCBI-BLAST Homology Sequence identity (%)**		
		Identical *Theileria annulata*	**Genbank Accession number**	**Identity (%)**
No.1	MK855082.1	Khuzestan Iran isolate	MK183000.1	99.42%
No.2	MK855083.1	Khuzestan Iran isolate	MK183000.1	99.26%
No.3	MK855084.1	Khuzestan Iran isolate	MK183000.1	99.85%
No.4	MK855085.1	Khuzestan Iran isolate	MK183000.1	100.00%
No.5	MK855086.1	Khuzestan Iran isolate	MK183000.1	99.12%
No.6	MK855087.1	Khuzestan Iran isolate	MK183000.1	99.85%
No.7	MK855088.1	Khuzestan Iran isolate	MK183000.1	99.42%
No.8	MK855089.1	Khuzestan Iran isolate	MK183000.1	99.70%
No.9	MK855090.1	Khuzestan Iran isolate	MK183000.1	99.41%
No.10	MK855091.1	Khuzestan Iran isolate	MK183000.1	99.41%

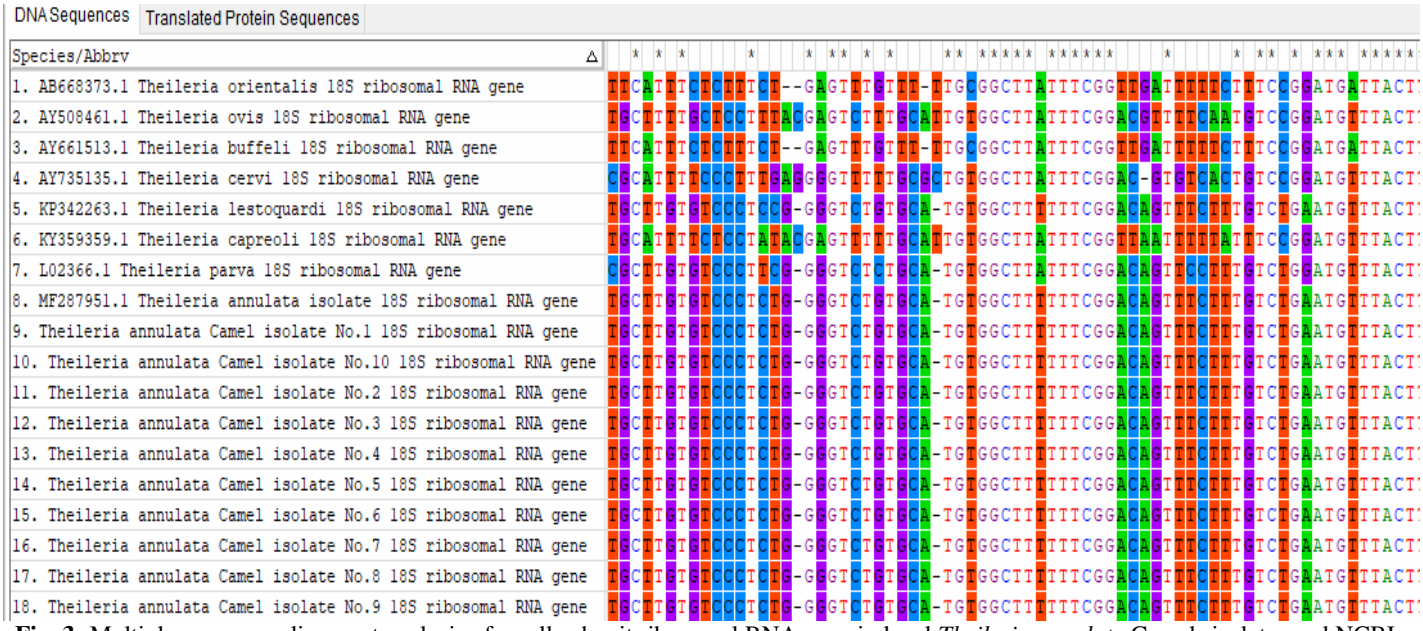

Fig. 3: Multiple sequence alignment analysis of small subunit ribosomal RNA gene in local *Theileria annulata* Camels isolates and NCBI-Genbank *Theileria* spp. isolates. The multiple alignment analysis was constructed using ClustalW alignment tool in (MEGA 6.0 version). That show the nucleotide alignment similarity as (*) with substitution mutations in small subunit ribosomal RNA gene.

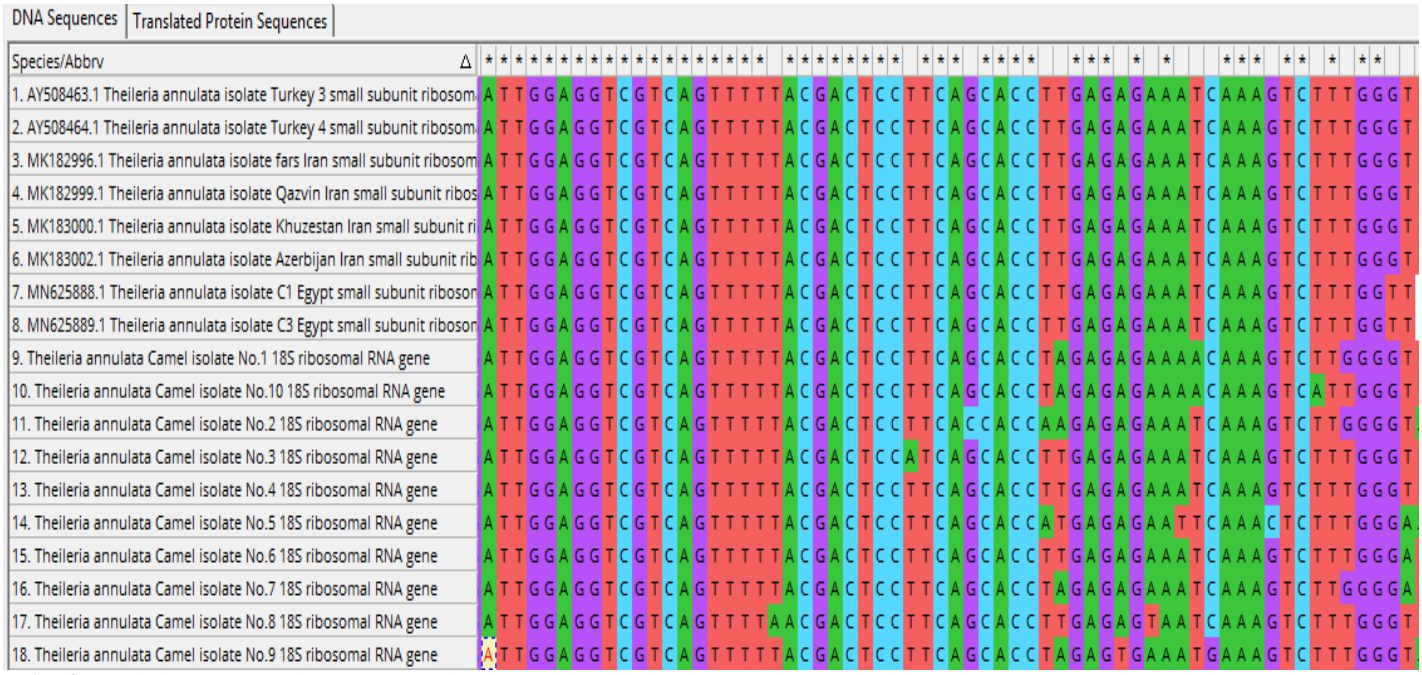

Fig. 4: Multiple sequence alignment analysis of small subunit ribosomal RNA gene in local *Theileria annulata* camels isolates and NCBI-Genbank *Theileria annulata* related country isolates. The multiple alignment analysis was constructed using ClustalW alignment tool in (MEGA 6.0 version). That show the nucleotide alignment similarity as (*) and substitution mutations in small subunit ribosomal RNA gene.

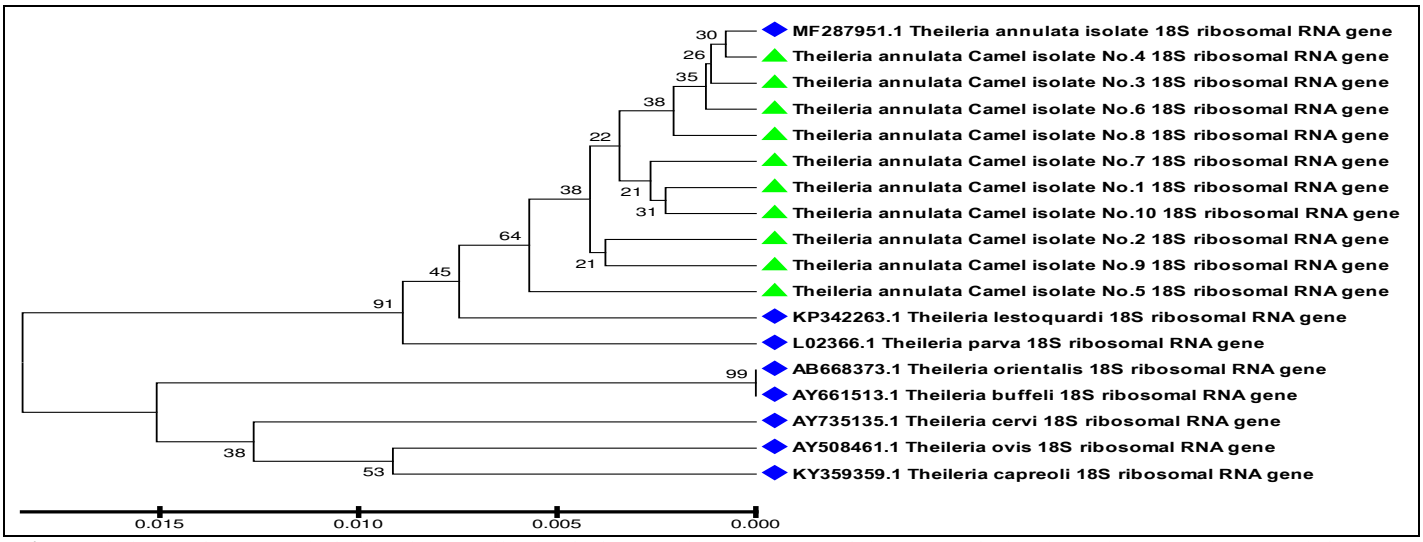

Fig. 5: Phylogenetic tree analysis based on small subunit ribosomal RNA gene partial sequence in local *Theileria annulata* camels isolates that used for genetic *Theileria* species identification . The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local *Theileria annulata* isolate (No.1- No.10) were showed closed related to NCBI-BLAST *Theileria annulata* isolate (MF287951.1). at total genetic changes (0.005-0.015%).

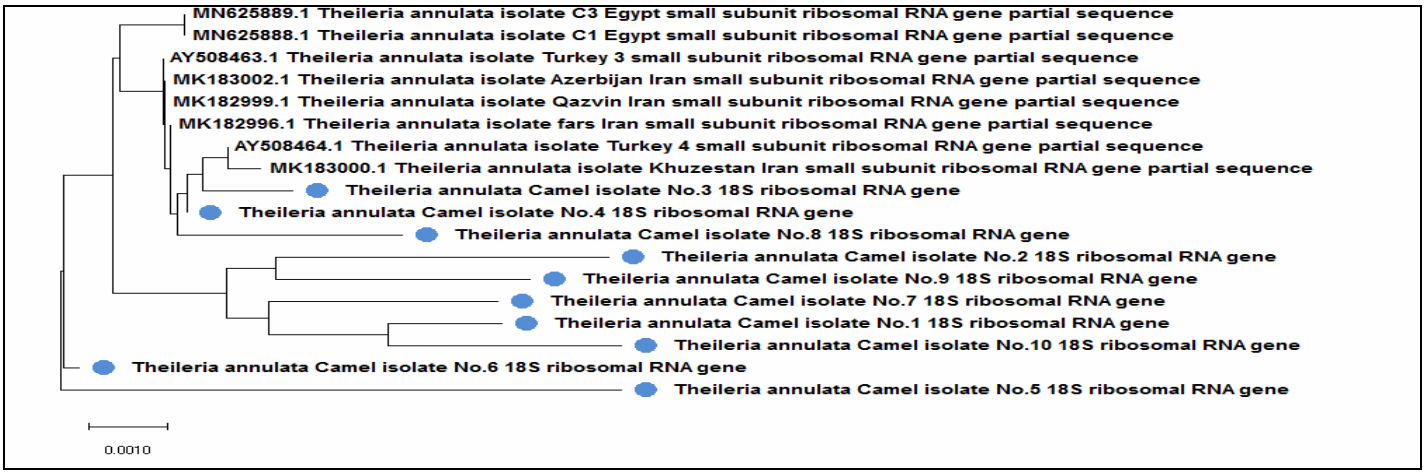

Fig. 5: Phylogenetic tree analysis based on small subunit ribosomal RNA gene partial sequence in local *Theileria annulata* Camels isolates that used for genetic country relationship analysis . The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local *Theileria annulata* isolate (No.1- No.10) were showed closed related to NCBI-BLAST *Theileria annulata* isolate Khuzestan Iran isolate (MK183000.1). at total genetic changes (0.001%).

Theileria species can be differentiate by Molecular tools, this technique have proved to be highly sensitive and specific in detecting blood parasite DNA (Tarimo, 2013). Piroplasm in carrier animals determine by using diagnostic Molecular species–specific assay in veterinary parasitology (Gul *et al.*, 2015; Yusufmia *et al.*, 2010). PCR have been used to detect and differentiate *Theileria* spp. In carrier and clinically infected animals, even with very low amount of parasite DNA. PCR technique showed low rate of infection with *Theileria* spp. and in different parts of middle of Iraq, using specific primers: 18s ribosomal RNA gene (426 bp) and 18s ribosomal RNA gene (722 bp), The results were lower than that recorded by (Al-Saeed *et al.*, 2010) 68.9 %, (Al-Emarah *et al.*, 2012) 88.23 %, (Kawan, 2019) 88% and (Hassan *et al.*, 2012) 70% in Kurdistan, Basra, Baghdad and Sulaimaniyah province/Iraq respectively and higher than (Ziam *et al.*, 2015) in Algeria 30.16 %, (Nayel *et al.*, 2012) in Egypt 24.05% and (Jasim *et al.*, 2015) in Basra and Muthana provinces 23.68%.

Female animals were at higher risk of infection (30.57%) as compared to male counterparts (23.11%) at 5% level of significance. Higher prevalence in female may be attributed to the fact that the milch animals have higher hormonal stress, carry more ticks and thus are at higher risk of exposure to the infection (Kabir *et al.*, 2011; Sutherst *et al.*, 1983). (Lloyd, 1983) reported that higher level of prolactin and progesterone hormones make the individual more susceptible to any infection. A higher infection rate in females as compared to male was in agreement with the results of (Durrani, 2007) and (Tuli *et al.*, 2015).

In our study, least prevalence was recorded in >6 year of age (least risk of 13.69% which can be correlated with the facts that the age groups less than 6 years are not competent to resist any type of infection due to underdeveloped immune system (Subramanian *et al.*, 1989). Findings of (Singh, 2012) provide the information that among the different age group that screened, the maximum tick infestation was recorded in age less than 6 years. Animals of this age groups stay in close association with the adults who are mainly considered to be harboring carrier phase of the infection resulting from their prior exposures to *Theileria* infection in life. Adult (> 6year of age) also start exhibiting different types of stress entity viz., cycling heat, production, vaccination and reproduction stress. Our findings are in concordance to those of (Irvin and Morrison, 1987) whom found that one-month old animals from non-immune dams were more susceptible to *Theileria* infection than older.

Phylogenetic analysis confirmed low differences between Iraqi strains of *Theileria annulata* and other countries .The genetic variation that recorded may be due to variation on area size of reference sequence and differences in geographical areas where isolates collected. Using different methods like PCR based on gene sequencing of partial or complete genes, Phylogenetic have been used for genetic analysis. When environment of parasite change, genetic diversity plays important role in survival and makes accurate analysis of variation applicable for studies on taxonomy, biology, epidemiology and pathogenesis of parasites. Result of this study agreed with some studies that reported the existence of genetic variation and phylogenetic relationships based on mitochondrial and nuclear gene sequences among populations of parasite that analyze genetic variations of *Theilerial* protozoa in the world .

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