



## Clinical, microscopical and molecular detection of caprine theileriosis

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### Abstract

The study investigated the detection of caprine theileriosis using Giemsa-stained direct blood smears and conventional PCR techniques, with a sample size of 150 goats of both gender and various ages were collected between July 7, 2023, and October 20, 2023, in Erbil governorate, Iraq. The infection rates of theileriosis were determined as 29.3% and 65.3%, respectively. Clinical symptoms in infected goats included dullness, depression, fever, increased respiratory rate, anorexia, pale conjunctival mucous membrane, lethargy, rough hairy skin with tick infestations, cough, nasal discharge, and prescapular lymph node enlargement. Gender-based analysis showed no significant difference in infection rates between females 17.76% and males 11.81%. However, a higher infection rate 21.8% was observed in goats aged 1-5 years compared to other age groups. Imported goats had the highest infection rate 50%, followed by imported black goats 27.3% and Merzi goats 21.4%. PCR analysis using 'catch-all' primers detected a 1098 bp band, indicating *Theileria* spp. infection. Specific primers for *T. ovis* revealed a 520 bp band, confirming *T. ovis* infection. All goats identified as positive through microscopic examination were also positive in PCR. Phylogenetic analysis based on 18S rRNA sequences indicated a 98-100% similarity to *T. ovis*. Sequences were isolated from Iraq, Turkey, and China. In conclusion, the study provides insights into the prevalence, clinical manifestations, and molecular characteristics of caprine theileriosis. The findings contribute valuable information for understanding and managing theileriosis in goat populations.

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### Introduction

Goats (*Capra aegagrus hircus*) are the most important species of food animals that have a unique capacity to adapt to harsh situations with multiple economically relevant traits such as short reproduction cycles, higher rates of reproduction and high consumer acceptability worldwide, there are nearly 1 billion goats (1,2). In Iraq, the goat farming sector plays an essential role to the nation's food security. There have been an estimated 1.5-2.0 million goats dispersed across the nation, with 2.5% in the south, 44.2% in the center, and 43.3% in the north, providing a vital source of meat and milk production (3). According to historical records, the two distinct Iraqi traditional goat breeds are Black (Native or Rashoky) and Meriz goats (4,5). The small ruminant industry in Iraq has a considerable challenge provided by the

challenging animal disease circumstances (4,6). Theileriosis is a blood parasitic disease of domestic and wild ruminants caused by the parasite's apicomplexan (Apicomplexan parasites) of the genus *Theileria*. This parasite is protozoa are obligate parasites living inside erythrocytes and lymphocytes (7), and transmitted through by ixodid tick species related to the genera (*Hyalomma*, *Haemaphysalis*, and *Rhipicephalus*) and have complex life cycles in both vertebrates and invertebrates (8). It represents a significant pathogenic condition affecting both small and large ruminants, manifesting in acute, subacute, or chronic forms (9,10). Theileriosis causes significant economic losses in goats because to high mortality and morbidity rates (11). Caprine theileriosis (CT) can be caused by a minimum of six different species of *Theileria* (*T. luwenshuni*, *T. lestoquardi*, *T. uilenbergi*, *T. ovis*, *T. separata*, and *T. recondite*). the

three species are *T. luwenshuni*, *T. lestoquardi* and *T. uilenbergi* are all extremely virulent goat species (12,13). The disease in small ruminants results in a significant morbidity rate of up to 100% and a fatality rate of more than 46%, which is a very high percentage (14). They may include fever, loss of appetite, weight loss, lymphadenopathy (15), respiratory signs (cough, nasal discharge, shortness of breath) (6,16), anemia, jaundice, diarrhea. Reproductive losses including abortion can be seen (17). Various laboratory techniques are employed for diagnosing the disease. The direct microscopic examination of parasites in blood smears, dyed with Giemsa stain, only useful during the acute stage of infection. However, it proves unsatisfactory for diagnosing carrier animals and is impractical on a large scale (18,19). In contrast, serological testing is indicated as the best technique of diagnosis during the latent stage of disease and in epidemiological studies. It is essential to highlight that serological evaluations do not distinguish between recent and old infections (20). Later, multiple reliable diagnostic molecular tools, such as conventional polymerase chain reaction (c-PCR), multiplex (m-PCR), nested (n-PCR), and real-time (r-PCR) (21,22), have been established for the direct detection of haemoprotozoan infections. These PCR assays are considered reliable diagnostic tools and are advised to detect latent infections. Additionally, they may be used to assess genetic diversity and evolutionary relationships among emerging haemoprotozoan species (23,24).

Notably, there has been no prior study on the occurrence of theileriosis infection in caprine in Erbil governorate, north Iraq. Consequently, the present study aims to assess the detection of caprine theileriosis using direct microscopic examination and molecular tools. It also aims to document the phylogenetic diversity of theileriosis among different caprine species based on 18S rRNA sequences. This study represents the first exploration of these aspects in the Erbil of Iraq.

## Materials and methods

### Ethical approval

The study was carried out in compliance with the ethical norms provided by the Institutional Animal Care and Use Committee at Mosul University's College of Veterinary Medicine, and all samples were gathered with owner agreement under the approved of 10<sup>th</sup> congress of Department of Microbiology at 11/6/2023.

### Sample collection

A total of 150 samples from diverse goat breeds, spanning various ages and both genders, were randomly collected between July 7, 2023, and October 20, 2023, in Erbil governorate, Iraq. These samples were examined to confirm the presence of caprine theileriosis infection.

### Clinical examination of animals

A thorough clinical examination, including assessment of temperature, mucous membrane condition, lymph nodes, heart rate, and respiration, was performed on all potentially affected animals.

### Hematological examination

Blood samples were collected from the jugular vein using a 5 ml disposable syringe with an 18-gauge needle, and they were collected in 2.5 ml tubes containing EDTA. Subsequently, smears were prepared, air-drying, fixed in absolute methanol for five minutes, and stained with ten percent Giemsa for a thirty-minute period to identify the presence of parasites in erythrocytes (25). The slides underwent examination using an oil immersion lens at a power of  $\times 1000$  to detect *Theileria* parasites. All the remaining blood samples have been kept at  $-20^{\circ}\text{C}$  for subsequent DNA extraction.

### Molecular examination and processing

The Prime Prep<sup>TM</sup> DNA Genome Extraction Kit from Genet Bio in South Korea (SK) was employed to extract genomic material from 150 goat blood samples, following the manufacturer's instructions. Subsequently, two amplification reactions were carried out to target the 18S rRNA gene of *Theileria* spp. The first reaction, utilizing the c-PCR technique, aimed to identify positive sheep for all *Theileria* spp. using universal primers (989-F AGTTTCTGACCTATCAG and 990-R TTGCCTTAAACTTCCTTG), resulting in an approximate band size of 1098bp. The second reaction, employing the m-PCR technique, aimed to differentiate *T. ovis* in all positive samples from the first reaction, utilizing specific primers (T170-F TCGAGACCTTCGGGT and T670-R TCCGGACATTGTAAAACAAA) for *T. ovis*, yielding an approximate band size of 520bp. All primers used were supplied by Macrogen Inc., South Korea.

In brief, a 25  $\mu\text{l}$  mixture, add 2  $\mu\text{l}$  of target DNA, 1  $\mu\text{l}$  (10 pmol) for every of the reverse primers (990-R) for *Theileria*, 2  $\mu\text{l}$  of a common forward primer (989-F), 6.5 dH<sub>2</sub>O, and 12.5  $\mu\text{l}$  of ready-to-use 2X PCR master-mix (GeNet Bio. Laboratory, Korea). The mixes were cycled using a BioRad thermocycler, which included an initial denaturing phase at  $96^{\circ}\text{C}$  for 10 min., followed by 35 cycles of denaturing at  $96^{\circ}\text{C}$  for 1 min., annealing at  $54^{\circ}\text{C}$  for 1 min., and extension at  $72^{\circ}\text{C}$  for 1 min. The procedure was completed with a 10-minute extension at  $72^{\circ}\text{C}$ . The products were then kept at  $4^{\circ}\text{C}$  for future analysis.

Following the process of amplification, gel separation was carried out in a 1.5% agarose gel, with 1X TBE buffer. The amplicons (5  $\mu\text{l}$ ) were run for 45 minutes and examined under UV illumination (Proxima 2500 Isogene LifeScience, Netherlands), revealing distinct bands of *Theileria* (1098 bp). Positive controls for *Theileria* species have been obtained from the blood of infected goats with

verified clinical theileriosis, whereas negative controls were derived from piroplasm-free goat DNA. Furthermore, the second reaction utilizing *T. ovis*-specific primers (T170-F and T670-R) produced DNA bands 520 bp, confirming the samples' positive for *T. ovis*.

### Statistical analysis

The present study's data was analyzed using Chi-square with a  $P < 0.05$  (10).

## Results

### Clinical hematological detection of *Theileriosis*

The overall infection rate of caprine theileriosis, determined through Giemsa-stained blood smears and c-PCR technique among 150 samples, was 29.3% (44/150) and 65.3% (98/150), respectively. The identification of *Theileria* species was based on the morphological characteristics of the merozoites observed in infected red blood cells (RBCs). Notably, typical *Theileria* spp exhibited a small parasite morphology characterized by a single round or double pyriform shape with acute or obtuse angles. Additionally, these parasites manifested various morphological configurations within RBCs, including oval, anaplasmod, spherical, and single or double pear shapes. The double pear shape was particularly prominent, as illustrated in figure 1. The major observations found in clinically infected goats were suffered from dull, depressed, fever, increased respiratory rate, anorexia, pale conjunctival mucous membrane, lethargy, the goat had rough hairy skin and coat with tick infestations. cough and nasal discharge, enlargement of prescapular lymph nodes as shown in (Table 1). In this present study, the detection of *Theileria* spp. did not exhibit a significant difference concerning the gender and age of goats. Female and male goats showed infection rates of 32.9% (27/82) and 25% (17/68), respectively, with no statistically significant distinction ( $P \leq 0.05$ ). A higher infection rate with caprine theileriosis was observed in the age group exceeding 5 years, reaching 32% (13/44), in comparison to the other two groups, with no significant differences (Tables 2 and 3). In the current study, the incidence of theileriosis in caprine populations revealed that imported goats had the highest infection rate 50% with significant differences, followed by imported black goats 27.3%, and Merzi goats 21.4% (Table 4).

### Molecular detection of *Theileria* species using PCR

The outcomes of the amplified PCR product using general or universal primers (Macrogen Inc, South Korea) revealed that the DNA band size for the initial reaction was 1098 bp, indicating a positive presence of *Theileria* spp. (Figure 2). In contrast, the second reaction using specific primers for *T. ovis* displayed a DNA band size of 520 bp, signifying a positive detection of *T. ovis* (Figures 3). The findings indicated that 44 goats, constituting 29.3% of the

sample, were positive by microscopical examination. Notably, all goats that tested positive through microscopic examination were also confirmed as positive through PCR. These results provide insights into the detection of *Theileria* catch-all in goats, using numerous criteria that describe both the features of the animals and the flocks.

Phylogenetic trees of 18S rRNA sequences analyses based on neighbor-joining program have shown in figures 4 showed that *Theileria* spp. have a relation to *Theileria ovis*. The three sequences were blasted with prior GenBank registration, and found that OR854546 showed 99.54% identity with MN544931/Sheep/Sulaimani-Iraq, While OR854547 showed 99.44% identity with KT851437/*Hyalomma excavatum*/ Turkey, AY260172 /Sheep/Turkey. Moreover, sequences OR854548 were 98.15% identity to those sequences previously published *T. ovis* in NCBI GenBank for Turkey (OR652382/ *Canis lupus familiaris*, KT851436/ *Rhipicephalus bursa*) and China (FJ603460/ Sheep).

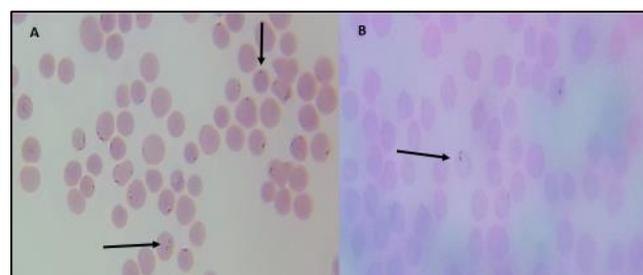


Figure 1: Displays a Giemsa-stained blood smear, where A) reveals multiple blood cells infected with *Theileria* spp., exhibiting double pear shapes with acute and obtuse angles, single pear shapes, and round configurations. B) *Theileria* spp. Are depicted with a pyriform shape (a pair of joints), observed at a magnification of x1000.

Table 1: Clinical signs of infected goats with theileriosis

| Clinical signs                     | Infected n (%) |
|------------------------------------|----------------|
| Fever                              | 52 (86.6)      |
| Dehydration                        | 22 (36.6)      |
| Anorexia                           | 31 (51.6)      |
| Yellowish soft diarrhea            | 18 (30.0)      |
| Pale mucous mm.                    | 42 (70.0)      |
| Edema                              | 7 (11.6)       |
| Respiratory signs                  | 37 (61.6)      |
| Swelling of prescapular lymph node | 45 (75.0)      |

Table 2: Sex detection of *Theileriosis* in goats

| Factor | Total (n) | No positive (%) | P value |
|--------|-----------|-----------------|---------|
| Female | 82        | 27 (32.9)       | 0.29    |
| Male   | 68        | 17 (25.0)       |         |

Table 3: Age-wise detection of *Theileriosis* in goats

| Factor    | Total (n) | No positive (%) | P value |
|-----------|-----------|-----------------|---------|
| <1 year   | 32        | 8 (25.0)        | 0.78    |
| 1—5 years | 78        | 23 (29.5)       |         |
| >5 years  | 40        | 13 (32.5)       |         |

Table 4: The prevalence of *Theileria* species in goats according to the breeds

| Factor   | Examined (n) | No positive (%) | P value |
|----------|--------------|-----------------|---------|
| Merzi    | 42           | 9 (21.4)        | 0.41    |
| Black    | 84           | 23 (27.3)       |         |
| Imported | 24           | 12 (50.0)       |         |
| Total    | 150          | 44 (29.3)       |         |

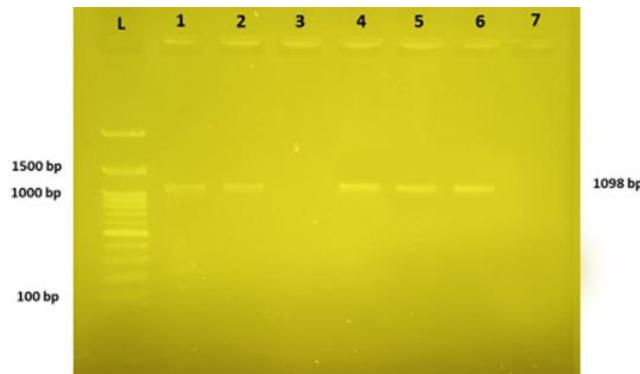


Figure 2: Gel electrophoresis image showing; PCR detection of *Theileria* spp. with a pair of universal primers (Macrogen Inc, South Korea): Lanes L) 100 bp ladder DNA marker; Lane 1,2,4,5,6) *Theileria* spp. in approximately band size 1098 bp; lane 3 and 7) negative control.

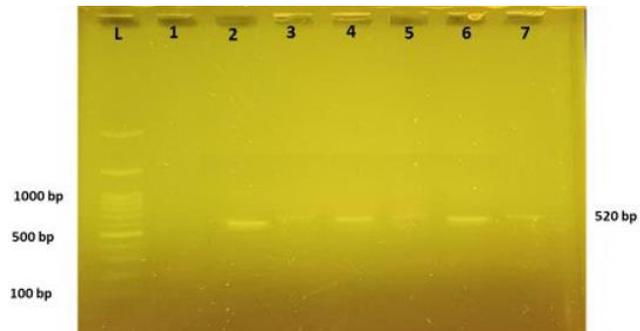
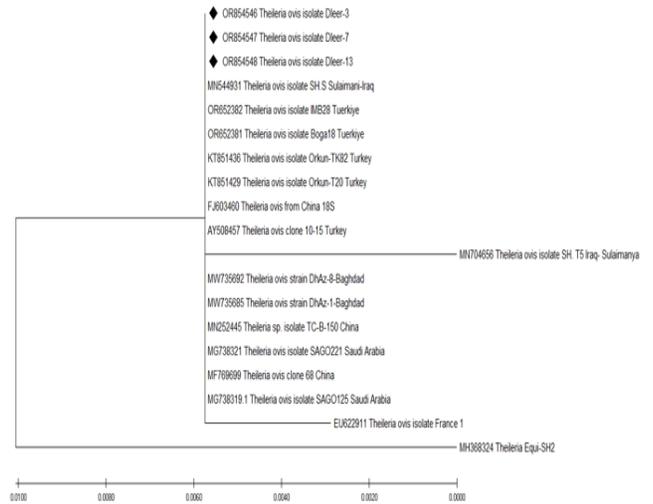


Figure 3: The gel- electrophoresis picture shows; PCR detection of *T. ovis*. with a pair of specific primers (Macrogen Inc, South Korea): Lanes L) 100 bp ladder DNA marker; Lane 2-7 *T. ovis*. in approximately band size 520 bp; lane 1) negative control.



Figures 4: Phylogenetic tree of 18S rRNA *T. ovis* partial sequences by maximum likelihood. The black diamond indicates sequences derived from the present investigation; the others reflect sequences from Genbank.

**Discussion**

Caprine theileriosis has been reported previously in Iraq by a variety of direct and indirect approaches, such as Giemsa-stained blood smears (26,27). However, these methods are primarily suitable for detecting infections during the acute stage and may not effectively identify presymptomatic or carrier animals with low parasitemia (28,29). Thus, the purpose of this study is to assess the incidence of *Theileria* infection in several goat species from Erbil region, North Iraq, employing both blood smears and molecular techniques. Additionally, it seeks to document the phylogenetic sequencing of c-PCR products for the first time.

In the current investigation, the infection rate was 29.3% (44/150) and 65.3% (98/150) by Giemsa-stained blood smears and the conventional PCR technique, respectively. The lower infection rate observed through microscopic methods is attributed to their limited sensitivity and specificity, particularly in detecting infections during the latent or carrier stage with low parasitemia (30,31).

Molecular techniques, known for their high sensitivity and specificity, have been widely employed for detecting and differentiating caprine theileriosis, particularly in carrier animals (32). In this study, molecular study of goat blood samples from several regions in Erbil governorate, Iraq, revealed that 29.3% (44/150) of clinical goats were infected with *Theileria*. *Theileria* spp. had an infection rate of 29.3%. The significantly high incidence identified in this study might be due to the extensive tick vector population. Previous studies in Duhok reported prevalence rates of 20.8% for *Theileria* species (33), and in Baghdad, infection

rates were reported at 26.6% for *Theileria* species (34-36). Higher prevalence rates were noted in Sulaimani city 71.7% by PCR (37,38), and Mosul city 22.7% by microscopic examination and 52.4% by PCR (37).

The study found no significant differences within goat genders and age categories, suggesting that caprine theileriosis is widespread in Erbil. This could be attributed to physical stressors temporarily compromising the immune system, rendering animals more susceptible to infection (39). The study utilized the 18S *rRNA* gene for genetic identification and phylogenetic evaluation of caprine theileriosis (40), marking the first of its kind in the Erbil province. The research provides initial insights into the genetic variety of theileriosis in the goat herds of Iraq based on 18S-*rRNA* sequencing data. Despite six genotypes of *Theileria* previously identified in goat populations from various countries (41), the study's discovery of a new sequence from Erbil emphasizes the need for continued research to better understand the prevalence of the disease, especially considering unregulated animal trade with neighboring countries. PCR emerged as an important method in epidemiological research, allowing the identification of both carrier and diseased animals. Based to the molecular investigation, *Theileria ovis* is considered the main species infecting goats in Erbil city. Future studies on caprine theileriosis should focus on aspects related to the vector (ticks), aligning with findings from previous research (42-46).

## Conclusion

The findings of this study indicate a notable infection rate of theileriosis, suggesting that the disease is endemic in Erbil city, possibly linked to unregulated animal trade with neighboring countries. During the last decade, there has been a significant increase in the imported of goats into the Erbil. These results underscore the need for the invention of additional diagnostic methods capable of identifying various genotypes of *Theileria* species in different animals, aiming to mitigate the risk associated with importing carrier goats. PCR emerged as an important method in epidemiological investigations, allowing the identification of both carriers and diseased animals. According to the molecular analysis, *Theileria ovis* is the main species infecting goats in Erbil city. Future studies on caprine theileriosis should concentrate on aspects related to the vector, particularly ticks.

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## Conflict of Interest

The researchers had no conflict of interest.

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## الكشف الجزيئي والتوصيف الوراثي لداء الثيليريا في الماعز المحلي

دلير أنور توفيق و هيثم صديق البكري

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### الخلاصة

تحررت هذه الدراسة عن مدى انتشار داء الثيليريا في الماعز باستخدام مسحات الدم المصبوغة بصبغة كيمزا وتقنيات تفاعل البلمرة المتسلسل التقليدية، تم جمع 150 عينة دم من الماعز. وتم تحديد معدلات الإصابة بـ 29,3% و 65,3% على التوالي. وأظهرت الأعراض السريرية في الماعز المصاب كالحمول والحمى وزيادة معدل التنفس وفقدان الشهية وشحوب غشاء الملتحمة المخاطي وخشونة الجلد مع الإصابة بالقراد والسعال وإفرازات الأنف وتضخم العقد الليمفاوية أمام الكتف. وأظهر التحليل المبني على جنس الحيوان عدم وجود اختلاف كبير في معدلات الإصابة بين الإناث 17,76% والذكور 11,81%. ومع ذلك لوحظ ارتفاع معدل الإصابة 21,8% في الماعز بعمر 1-5

أشار التحليل الوراثي المعتمد على تسلسل الحمض النووي الريبوزي الريبوسومي س ١٨ إلى وجود علاقة بين أجناس ثايليريا الماعز و ثايليريا الأغنام. أظهرت التسلسلات تطابق عالي مع إدخالات في بنك الجينات في العراق وتركيا والصين. وفرت نتائج هذه الدراسة نظرة شاملة حول مدى انتشار داء الثايليريا للماعز، والمظاهر السريرية، والخصائص الجزيئية. تم تحديد ترابط قوي بين أجناس ثليريا الأغنام من خلال التحليل الجيني لهذه العلاقة. تساهم نتائج هذه الدراسة لفهم الإدارة والسيطرة على داء الثايليريا في قطعان الماعز.

سنوات مقارنة بالفئات العمرية الأخرى. كانت نسبة الإصابة في الماعز المستورد أعلى نسبة ٥٠%، تلاها الماعز الأسود المحلي ٢٧,٣% وماعز المرعز ٢١,٤%. أظهر تحليل تفاعل البلمرة المتسلسل باستخدام بادئات حزمة يبلغ ١٠٩٨ زوجاً من القواعد النيتروجينية، مما يشير إلى الإصابة بداء الثايليريا. وكشفت البادئات المحددة في ثليريا الأغنام عن ٥٢٠ زوجاً من القواعد النيتروجينية مما يؤكد الإصابة بثليريا الأغنام وأظهرت جميع عينات الماعز التي كانت إيجابية من خلال الفحص المجهرى وحيث كانت إيجابية أيضاً في اختبار تفاعل البلمرة المتسلسل.