Research Article

Genotyping Study for Infections of *Theileria* and *Babesia* in Cattle and Sheep in AL-Najaf Governorate Iraq

Maytham Askar Alwan Al-shabbani[1], Hawraa Nasser rufaish[1], Hassan Hachim Naser[2], Murtadha Abbas[3]*, Hazem Almhanna[4]

[1]Department of Microbiology, Faculty of Veterinary Medicine, University of Kufa, Iraq; [2]Department of Microbiology, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Qadisiyah, Iraq; [3]Department of public health, Faculty of Veterinary Medicine, University of Kufa, Iraq; [4]Department of Anatomy, and Histology, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Qadisiyah, Iraq.

Abstract | *Theileria* and *Babesia* species are blood parasites that commonly cause illness in both juvenile and mature animals. The primary objective of this study was to identify the specific genotypes of *Theileria* and *Babesia* species responsible for infections in cattle and sheep. Blood samples were collected from male and female cattle and sheep slaughtered at the Al-Najaf slaughterhouse. Identification of *Theileria* and *Babesia* spp. was conducted using conventional PCR, sequencing, and phylogenetic techniques based on the 18S rRNA gene. The blood isolates from cattle and sheep were subjected to PCR-based molecular assays, which detected *Theileria* and *Babesia* infections at 694 bp and 383 bp, respectively. PCR products from positive isolates were then used for DNA sequencing to construct the phylogenetic tree of *Theileria* and *Babesia* species using partial 18S rRNA gene sequences. NCBI-Blast analysis revealed that the *Theileria* and *Babesia* species in the sheep isolates (No. 1 and No. 3) were closely related to *Theileria ovis* (AY508461.1) based on sequence similarity. Additionally, the sheep isolate of *Theileria* spp. (No. 2) was identified as *Theileria lestoquardi* (KP342263.1). The analysis of bovine isolates showed that the *Theileria* spp. isolates (No. 1 and No. 2) were highly similar to *Theileria annulata* (MF287951.1). Further examination of sheep isolates confirmed that the *Babesia* spp. isolates (No. 1 and No. 2) corresponded to *Babesia ovis* (MG569902.1). The genetic sequence analysis of cattle *Babesia* isolates (No. 1 and No. 2) revealed a 100% match with *Babesia bovis* (HQ264126.1). In conclusion, this study identifies *Theileria ovis*, *Theileria lestoquardi*, and *Babesia ovis* as the primary infecting species in sheep, while *Theileria annulata* and *Babesia bovis* primarily infect cattle. However, *Theileria* and *Babesia* species exhibit significant genetic similarities within their respective genera, suggesting the need for alternative identification techniques to accurately determine the specific species of these parasites.

Keywords | Genotype , *Theileria*, *Babesia*, PCR, DNA sequencing, 18S rRNA

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***Correspondence** | Murtadha Abbas, Department of public health, Faculty of Veterinary Medicine. University of Kufa, Iraq; **Email:** murtadhaa.alghazali@uokufa.edu.iq
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INTRODUCTION

Babesia and *Theileria* species are well-known blood parasites that invade the red blood cells of their hosts and cause the destruction of these cells. As a result, affected domesticated animals experience severe clinical symptoms and have a significant risk of death (Almazán *et al*., 2022; Karasová *et al*., 2022).

Babesia is a highly virulent blood parasite that affects both small and large ruminants, and is the primary cause of babesiosis, a disease that has been documented in over 100 species worldwide. It is a contagious disease that can affect various animals, including mammals and birds (Ganzinelli *et al*., 2018). This protozoan displays an intricate life cycle and has the ability to infect mammals and birds through ticks that are carrying the infection. When *Babesia* enters a blood cell that is already infected, it is referred to as sporozoites, which are infectious parasite bodies. Previously, *Babesia* would undergo conversion within erythrocytes, transitioning from trophozoites to merozoites, and then back to trophozoites and merozoites again. After the rupture of erythrocytes, which is induced by hemolytic blood cells, merozoites are released directly into the bloodstream and subsequently produce gametocytes. Subsequently, gametocytes undergo fertilization within the tick's gut and transform into sporozoite forms, initiating a new cycle of life for the parasites (Wright and Goodger, 2018; Elsworth and Duraisingh, 2021).

Multiple studies have confirmed the presence of numerous species of *Babesia* that infect cattle. The majority of these species are infected with *B. bigemina* and *B. divergens*, which are responsible for causing bovine babesiosis (BB). Nevertheless, the species that are most commonly observed in sheep and goats are *B. ovis* and *B. motasi*, as reported by Alvarez *et al*. (2019) and Calleja-Bueno *et al*. (2022).*Babesia* can be spread among mammalian hosts by many tick species, such as *Rhipicephalus, Hyalomma, Haemophysalis, Dermacentor, Ixodes,* and *Boophilus*. The incubation period of the disease varies across animals depending on the individual *Babesia* species that infects them (Kuttler, 2018; Martínez-García *et al*., 2021; Friedhoff, 2018; Gray *et al*., 2019). From a diagnostic perspective, *Babesia* spp. can exhibit comparable clinical signs and symptoms to *Theileria* and other pathogenic bacteria that infect ruminants.

Various techniques can be used to distinguish *Babesia*, such as microscopic inspection, serological tests, conventional polymerase chain reaction (PCR), and other approaches (Tavassoli *et al*., 2013; Weiland and Reiter, 2018; Alvarez *et al*., 2019). Furthermore, *Theileria* spp. is a prevalent blood parasite that invades the cells of several vertebrate mammals, particularly small and big ruminants. Likewise, this single-celled organism specifically attacks the red blood cells of infected animals and moves to other areas within the host's body, such as lymphocytes, immature red blood cells, bone marrow, and cells of internal organs (Ngetich, 2019; Naik *et al*., 2016). Therefore, this protozoan undergoes the development of Theileriosis, a condition that is marked by severe clinical symptoms such as elevated body temperature, sudden decrease in red blood cells, yellowing of the skin, loss of appetite, excessive drooling, swelling of superficial lymph nodes, difficulty in breathing, and a high rate of illness (Kho *et al*., 2017). During the life cycle of *Theileria*, the sporozoites of *Theileria* undergo replication of schizonts in leukocytes, whereas piroplasms survive in the erythrocytes of hosts (Ganaie *et al*., 2019).

Recent investigations have shown that *Theileria* spp. infect large ruminants internationally, with the most harmful species being *T. parva*, *T. annulata*, and *T. orientalis* (Zhou *et al*., 2016; Oakes *et al*., 2019). Young ruminants are infected with *T. lestoquardi* (*T. hirci*), *T. uilenbergi*, and *T. luwenshuni*, as stated by Ahmed *et al*. (2011) and Altay *et al*. (2012). In addition, *Theileria* species often utilize multiple vectors depending on the geo-environmental conditions, ticks belonging to the *Rhipicephalus appendiculatus, Hyalomma* and *Haemaphysalis* consider as important vectors (Sivakumar *et al*., 2014; Zeb *et al*., 2022). The most prevalent approaches for diagnosing *Theileria* spp. infections are clinical symptoms and laboratory testing, as they contribute to the management of sick animals (Maharana *et al*., 2016; Nayel *et al*., 2012). Given the economic importance of cattle and sheep and the losses caused by theileriosis and babesiosis, this study seeks to identify the various genotypes of *Theileria* and *Babesia* in large and small ruminants in Al-Najaf province. This will be accomplished through the utilization of molecular techniques and DNA partial Nucleotide sequencing and phylogenetic tree analysis for the 18S rRNA gene.

MATERIALS AND METHODS

Collection of Blood Samples

A total of 150 blood samples were obtained from cattle and sheep in various parts of Al-Najaf Governorate between March 2020 and August 2020. The samples were collected using anticoagulant tubes. The samples comprised cattle and sheep of various ages and sexes. The samples were promptly stored in ice boxes and subsequently analyzed using direct smear techniques.

Extraction of DNA of Blood Samples

The genomic DNA of *Theileria* and *Babesia* species was extracted from blood samples of sheep and cattle using the gSYAN DNA mini kit from Geneaid, USA, following the instructions provided with the kit. The concentration and

purity of the extracted DNA were assessed using a nanodrop spectrophotometer (THERMO, USA) by measuring the absorbance at 260/280 nm. Subsequently, the DNA was stored at -20 ˚C until it is used in the PCR procedure.

The Polymerase Chain Reaction (PCR) Method

The PCR approach was utilized to directly identify *Theileria* and *Babesia* species by amplifying the 18S rRNA gene in the extracted DNA of sheep and cattle. The polymerase chain reaction (PCR) was conducted on all genomic samples, adhering to the detailed procedures outlined by (Kundave, 2014). The selection of PCR primers for *Theileria* and *Babesia* species was based on the NCBI Genbank database and the primer3 plus tool provided by Macrogen Company, Korea. The nucleotide sequence of the 18SrRNA gene for *Theileria* spp. was determined to be 694 base pairs long. The forward primer sequence is ATTGGAGGGCAAGTCTGGTG and the reverse primer sequence is TGCACCACCACCCAAAGAAT. The corresponding NCBI-Genbank code for this sequence is AF078816.1. The 18SrRNA gene sequence for *Babesia* spp. was obtained using the primers F: AGGAATTGACGGAAGGGCAC and R: CTAGTGATTACCCGCGCCTT. The sequence is 383 base pairs long, and its NCBI-Genbank code is KY867436.1. The AccuPower PCR PreMix Kit was utilized to create a PCR master mix as per the instructions provided with the kit, with a total volume of 20µL. Subsequently, the PCR mixture comprised Taq DNA polymerase, dNTPs, 10µL of PCR buffers, 2 µL of DNA samples, and 2µL each of forward and reverse primers for both species. Subsequently, the PCR mixture tubes were thoroughly agitated for a duration of 3 minutes. Meanwhile, PCR tubes were inserted into a thermocycler (MJ-Mini BioRad, USA). The thermocycler conditions for the conventional PCR machines were established as follows: an initial denaturation at 95 ˚C for 5 minutes for one cycle, followed by denaturation at 95 ˚C for 30 seconds, annealing at 58 ˚C for 30 seconds, extension at 72 ˚C for 1 minute for 30 cycles, and a final extension at 72 ˚C for 5 minutes for one cycle.

Analysis of the PCR Products

The PCR products, measured 383 and 694 bp, were examined using 1% agarose gel electrophoresis and 1X TBE buffer. In addition, the PCR results were treated with ethidium bromide dye and visualized using a UV transilluminator.

The Nucleotide Sequencing Method

A PCR was used to amplify and DNA was sequenced to confirm the molecular detection of some isolates that tested positive for *Theileria* and *Babesia* species of cattle and sheep blood samples. The phylogenetic tree was identified and examined using isolates for the local in comparison with the global isolates deposited on NCBI/GenBank to analyze the genetic relationship between isolates of local *Theileria* and *Babesia* spp. and international isolates submitted on NCBI-Blast. In addition, the local isolates that were identified had been recorded in the NCBI-GenBank as novel data originating from Iraq. To prevent degradation, the PCR products were stored in ice-filled containers prior to being shipped to the Macrogen Company, Korea. The AB DNA sequencing machine was utilized for DNA sequencing with the assistance of Molecular Evolutionary Genetics Analysis version 6.0 (Mega 6.0). In addition, the phylogenetic tree of *Babesia* and *Theileria* spp. was calculated using the sequence alignment of the partial 18S ribosomal rRNA gene and the UPGMA method to determine the evolutionary distances (Zhu *et al*., 2010).

RESULTS AND DISCUSSION

This study utilized the PCR technique, which relies on the 18S rRNA gene, to detect *Theileria* and *Babesia* species in blood samples from cattle and sheep. The results of the investigation verified the existence of infections with *Theileria* and *Babesia* spp. specifically in the cattle. The genomic DNA extracted from the blood samples of sheep and cattle was subjected to agarose gel electrophoresis using a 1% concentration. This was done to identify the presence of the 18S rRNA gene in *Theileria* spp. As a result, the samples from sheep and cattle tested positive for *Theileria* spp. at 694bp, as shown in Figure 1A. The polymerase chain reaction (PCR) was used to identify the presence of *Babesia* spp. in the blood samples of sheep and cattle. Specifically, the PCR product size was determined by targeting the 18S rRNA gene, resulting in a fragment of 383 base pairs (bp) (Figure 1B). The DNA sequences of *Theileria* and *Babesia* species were analyzed utilizing phylogenetic tree and ClustalW alignment analysis with the MEGA 6.0 version. The local species isolates were subjected to NCBI-Blast analysis and compared to isolates from various countries. The results suggested a strong resemblance in the multiple alignment analysis and mutation of the nucleotide sequences in the 18S ribosomal rRNA gene (Figure 2). The genetic link between local and global *Theileria* and *Babesia* spp. was determined by comparing the 18S rRNA gene sequences using NCBI-BLAST homology analysis. Sheep isolates No. 1 and No. 3 from NCBI-Blast are genetically identical to *Theileria ovis* (AY508461.1), a strain found in Turkish sheep. The sheep isolate No. 2 from NCBI-Blast is genetically identical to *Theileria lestoquardi* (KP342263.1), a strain found in Iraqi sheep. Cattle isolates No. 1 and No. 2 from NCBI-Blast are genetically identical to *Theileria annulata* (MF287951.1), a strain found in Bengal cattle (Figure 2 and Table 1). The homology sequence identity between *Babesia* spp. sheep isolates (No.1 and No. 2) and *Babesia* ovis (MG569902.1), a Turkey isolate, is 100%

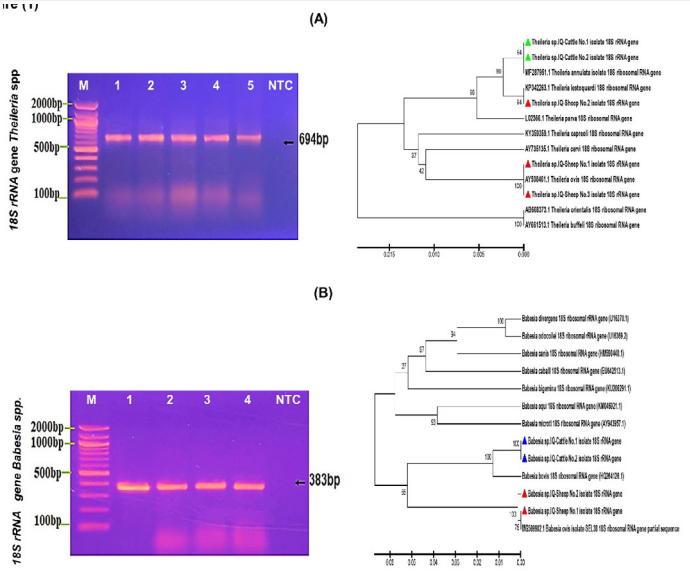

Figure 1: (A) This figure illustrates the PCR product of the 18*S rRNA* gene in *Theileria* spp. of sheep and cattle blood samples at 694bp. M: DNA marker ladder (2000-100bp), and Lane (1-3) showed some positive samples of *Theileria* spp. from Sheep and Lane (4-5) positive samples of *Theileria* spp. from Cattle, and lane (NTC) Non-template negative control. Besides, the analysis of the phylogenetic tree of *Theileria* spp. is based on the 18S ribosomal rRNA gene partial sequence and genetic identification. The phylogenetic tree was constructed using the Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in the MEGA 6.0 version. *Theileria* spp. Sheep isolates (No. 1 and No. 3) were found to be closely related to NCBI-Blast *Theileria ovis* (AY508461.1), and *Theileria* spp. sheep isolates (No. 2) were identified to be closely related to NCBI-Blast *Theileria lestoquardi* (KP342263.1). *Theileria* spp. Cattle isolates (No. 1 and No. 2) were found to be closely related to NCBI-Blast *Theileria annulata* (MF287951.1) at a total genetic change (0.005-0.015%). **(B):** This figure illustrates the PCR product run on 1% Agarose gel electrophoresis and detected the *18S rRNA* gene in *Babesia* spp. of sheep and cattle blood samples M: DNA marker ladder (2000-100bp), and Lane (1-2) showed some positive samples of *Babesia* spp. from Sheep and Lane (3-4) some positive samples of *Babesia* spp. from Cattle at 383bp PCR product size and lane (NTC) Non-template negative control. Besides, the analysis of the phylogenetic tree is based on 18S ribosomal rRNA gene partial sequence in the local *Babesia* species sheep and cattle isolates that were used for *Babesia* species genetic identification. The phylogenetic tree was analyzed using the Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in the MEGA 6.0 version. *Babesia* spp. sheep isolates (No.1 and No.2) were found to be closely related to NCBI-Blast *Babesia ovis* (MG569902.1) and *Babesia* spp. cattle isolates (No.1 and No. 2) were found to be closely related to NCBI-Blast *Babesia bovis* (HQ264126.1) at a total genetic change (0.01-0.06%).

according to NCBI-Blast. Similarly, the homology sequence identity between *Babesia* spp. cattle isolates (No.1 and No. 2) and *Babesia* bovis (HQ264126.1), a USA isolate, is also 100% according to NCBI-Blast (Table 2). The analysis of the phylogenetic tree revealed that the sheep isolates (No. 1 and No. 3) of *Theileria* spp. were genetically similar to the NCBI-Blast *Theileria ovis* (AY508461.1), while the sheep isolate (No. 2) of *Theileria* spp. was genetically similar to the NCBI-Blast *Theileria lestoquardi* (KP342263.1).

Table 1: NCBI-BLAST homology sequence identity between local *Theileria* spp. isolates and closely related *Theileria* spp. isolates in NCBI-BLAST.

Isolate No.	Genbank Accession number	NCBI-BLAST Homology Sequence Identity		
		Identical Theileria sp.	Genbank Accession number	Identity (%)
Theileria sp. Sheep No.1	OQ818311	Theileria ovis	AY508461.1	100%
Theileria sp. Sheep No.2	OQ818312	Theileria lestoquardi	KP342263.1	100%
Theileria sp. Sheep No.3	OQ818313	Theileria ovis	AY508461.1	100%
Theileria sp. Cattle No.1	OR438632	Theileria annulata	MF287951.1	100%
Theileria sp. Cattle No.2	OR438633	Theileria annulata	MF287951.1	100%

Table 2: NCBI-BLAST homology sequence identity between local *Babesia* spp. isolates and closely related *Babesia* spp. isolates in NCBI-BLAST.

Isolate No.	Genbank Accession number	NCBI-BLAST Homology Sequence Identity		
		Identical Babesia sp.	Genbank Accession number	Identity (%)
Babesia sp. Sheep No.1	OQ818624	Babesia ovis	MG569902.1	100%
Babesia sp. Sheep No.2	OQ818625	Babesia ovis	MG569902.1	100%
Babesia sp. Cattle No.1	OQ818626	Babesia bovis	HQ264126.1	100%
Babesia sp. Cattle No.2	OQ818627	Babesia bovis	HQ264126.1	100%

Likewise, the cattle isolates (No. 1 and No. 2) of *Theileria* spp. were genetically similar to the NCBI-Blast *Theileria annulata* (MF287951.1), with a total genetic change ranging from 0.005% to 0.015% (Figure 1). The *Babesia* spp. sheep isolates (No.1 and No. 2) were found to have a close genetic relationship with NCBI-Blast *Babesia ovis* (MG569902.1). Similarly, the *Babesia* spp. cattle isolates

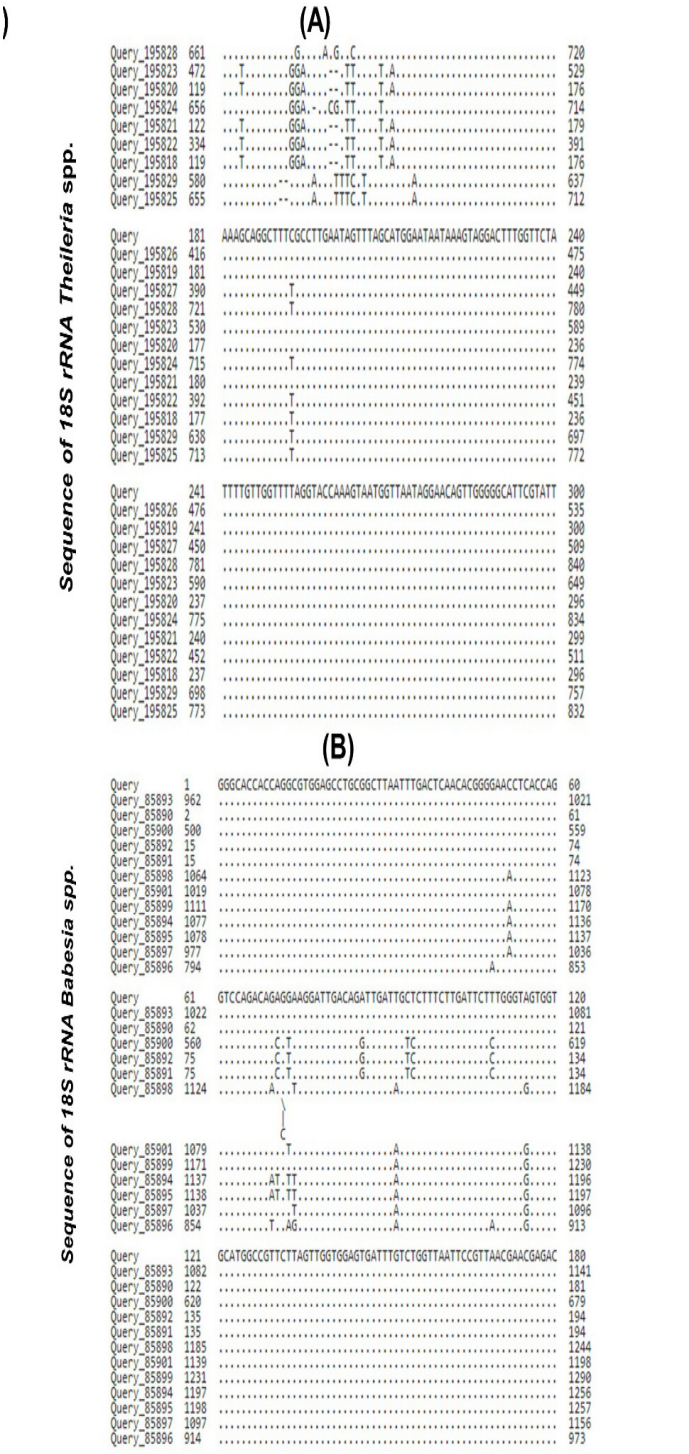

Figure 2: (A) Multiple sequence alignment analysis for 18S ribosomal rRNA gene partial sequence in the local *Theileria* spp. isolates and the GenBank located *Theileria* spp. isolates based on the NCBI-BLAST Alignment tool. The figure shows similarity in the multiple alignment analysis (*) and mutation in the nucleotide sequences of the 18S ribosomal rRNA gene. **(B):** Multiple sequence alignment analysis for 18S ribosomal rRNA gene partial sequence in the local *Babesia* spp. isolates and the GenBank located *Babesia* spp. isolates based on the NCBI-BLAST Alignment tool. The figure shows similarity in the multiple alignment analysis (*) and mutation in the nucleotide sequences of the 18S ribosomal rRNA gene.

(No.1 and No. 2) were found to be closely related to NCBI-Blast *Babesia bovis* (HQ264126.1). This genetic similarity was observed to have a total genetic change ranging from 0.01% to 0.06%, as depicted in Figure 2. Ultimately, the isolated specimens of locally identified species were sent to the NCBI-GenBank, and specific GenBank accession numbers were assigned to each isolate of *Theileria* and *Babesia* species.

Prior research on *Theileria* spp. has demonstrated that there are six distinct species of *Theileria* that are capable of infecting various animal species. The key species identified in cattle are *T. annulata*, which is responsible for causing tropical theileriosis, and *T. parva*, which causes East Coast fever (Gul *et al.*, 2015; Morrison, 2015). The conventional PCR technique was employed to analyze blood samples from cattle, leading to the identification of *Theileria* spp. at 694bp. The size of the PCR product was determined based on the partial sequence of the 18S ribosomal rRNA gene of *Theileria* spp., which was previously confirmed by studies conducted by (Zaeemi *et al.*, 2011; Jalali *et al.*, 2014; Saeed *et al.*, 2015). Furthermore, our research has validated the use of DNA sequencing analysis to identify *Theileria annulata* in positive blood samples from cattle, aligning with previous studies (Nourollahi-Fard *et al.*, 2015) that also identified *Theileria annulata* and *Theileria parva* in cows (Nene and Morrison, 2016). However, our analyses have not detected the presence of *Theileria parva*, most likely because *Theileria annulata* is more prevalent in Iraqi cattle. Therefore, it is imperative to do additional research by examining various blood samples from cattle in order to gain deeper insights. Furthermore, it is crucial to examine the impact of seasons and the geographical distribution of cattle in order to ascertain the degree to which environmental factors can affect the transmission and occurrence of certain illnesses in cattle. This study differentiated between the sheep species *Theileria ovis* and *Theileria lestoquardi* by analyzing the phylogenetic tree of *Theileria* spp. This finding aligns with a previous study conducted by (Al-Hosary *et al.*, 2021; Zaeemi *et al.*, 2011), which used a nested PCR–restriction fragment length polymorphism (RFLP) approach. Similarly, this study discovered that *Theileria annulata* did not induce any infections in sheep. This finding is consistent with the study conducted by Zaeemi *et al.* (2011), which suggested that the identification of *Theileria annulata* in sheep using RFLP-based methods could be more efficient depending on aspects such as sample size, season, and geographical location. In addition, this study detected *Babesia* spp. at a length of 383 base pairs (bp) in both cattle and sheep. This finding is consistent with prior studies conducted by (Esmaeilnejad *et al.*, 2014; Khamesipour *et al.*, 2015) for both species. Furthermore, the veracity of this discovery was validated through the examination of the DNA sequence and the study of the phylogenetic tree of *Babesia* species. Conse-

quently, the presence of *Babesia ovis* was detected in the samples taken from the infected sheep, whereas *Babesia bovis* was identified in the infected cattle. These findings are consistent with previous research conducted on *Babesia* species in sheep and cattle, as reported by (Ranjbar-Bahadori *et al*., 2012; Shahzad *et al*., 2013). Notwithstanding these studies and research, this study found no further species of *Babesia*. Future investigations on the infections of cattle and sheep with *Babesia* spp. should take into account additional variables, including sample size, seasonal variations, and alternative methodologies. Moreover, this study indicates that *T. annulata* is the prevailing species causing infection in cattle, while *T.* ovis and *T.* lestoquardi are more commonly found in sheep. Moreover, *Babesia bovis* and *Babesia ovis* are the predominant pathogenic species found in cattle and sheep. Hence, further examination employing diverse methodologies should consider distinct seasons and diverse environmental conditions in order to identify all the species of *Theileria* and *Babesia* spp.

CONCLUSIONS AND RECOMMENDATIONS

Overall, the present study reveals that *Theileria ovis*, *Theileria lestoquardi*, and *Babesia ovis* predominantly infect sheep, while *Theileria annulata* and *Babesia bovis* primarily infect cattle during this period. Although there are distinct differences between *Theileria* and *Babesia* species, they also share considerable similarities with each other and with other related taxa. Therefore, future research should adopt innovative methodologies to accurately identify the species of these parasites.

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NOVELTY STATEMENTS

This study is one of the rare studies in the genetic diagnosis of these pathogens.

AUTHOR'S CONTRIBUTIONS

All the research team for this work have equal roles.

Conflict of Interest

The authors have declared no conflict of interest.

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