Molecular Detection of *Theileria*, *Babesia*, and *Anaplasma* in Livestock of Erbil, Iraq: Prevalence and Seasonal Trends Revealed by Real-Time PCR

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Abstract. *This investigation sought to determine the seasonal fluctuations in the occurrence of Theileria spp., Babesia spp., and Anaplasma spp. within herbivorous farm animals, encompassing various species such as cattle, sheep, goats, and lambs, within the Erbil region. A total of 244 animals were assessed throughout four distinct seasons: winter, spring, summer, and autumn. Seasonal prevalence rates were computed by dividing the number of infected animals by the total number of animals examined within each season. Polymerase chain reaction (PCR) was employed to identify and quantify parasitic infections present in the blood samples. In general, 84.2% of the animals exhibited positive results for one or more blood parasites, with Theileria spp. demonstrating the highest prevalence at 41.4%. Seasonal fluctuations in parasite prevalence were documented, Theileria prevalence peaks in summer at 59.72% and declines to its lowest point in autumn (21.66%). Babesia spp. and Anaplasma spp. exhibited comparable seasonal trends, characterized by elevated prevalence rates in spring and summer. This investigation underscores substantial seasonal and species-specific disparities in the prevalence of Theileria spp., Babesia spp., and Anaplasma spp., thereby implying the necessity for focused parasite control measures within the context of livestock management.*

Introduction

Farm animals, notably cows, sheep, and goats, are a significant source of meat, milk, and dairy products. These animals have several diseases that harm them both directly and indirectly, leading to high mortality rates, sterility, and abortion of numerous embryos, as well as a decline in protein synthesis, which results in significant economic losses to humans (Ponnampalam et al., 2022). Blood parasites, including *Theileria*, *Babesia*, and *Anaplasma*, which cause anemia, weakness, weight loss, overall animal fatigue, inflammation of mucous membranes, and many other symptoms that harm the animal's health and productivity, are prevalent in livestock and sheep (Hussen, 2020). The infection spreads swiftly across the entire herd unless it is decreased and controlled because these parasites are conveyed by a tick-related vector that lives momentarily ectoparasitically on the skin and spreads the infection from one animal to another (Villanueva-Saz et al., 2022). The multiplicity and high frequency of hemoparasite infection in farm animals for various years and locations in Iraq provide the strongest evidence (Ali and Alyasiri, 2024, Abdullah et al., 2019). The most crucial technique for determining whether the animal has a blood parasite infection is microscopic inspection. Although the basic, approved method of microscopic examination of a blood smear is still used because it is inexpensive and does not require complicated procedures to prepare and stain blood, it takes time and effort and requires enough practice and experience to distinguish and diagnose the tiny parasite (Willard and Tvedten, 2011, Rosenblatt et al., 2009).

The availability and variety of serological tests make them a good approach for diagnosis, but they are not without drawbacks that are typically associated with serological tests, such as cross-reactivity, difficulties obtaining antigens, their cost, and issues with sensitivity and specificity (Momčilović et al., 2019). Yet even though molecular tests are sensitive and exact, not all laboratories provide them as a diagnostic tool, and they are also expensive. So far, real-time PCR can be utilized as an accepted technique in both central and private laboratories for precise and swift diagnosis and to get over the drawbacks seen in other laboratory techniques. The aim of this study was to determine the prevalence of *Theileria* spp., *Babesia* spp., and *Anaplasma* spp. in livestock in the Erbil region using RT-PCR, and to evaluate seasonal variation and the effectiveness of this molecular method for early detection and disease management.

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Materials and methods

Samples collection

In 2018 and 2019, a total of 244 animals, ranging in age from one month to four years, were sampled across all four seasons. The animals were randomly selected from four farms located in villages and suburbs to the south (Makhmur), north (Soran), east (Koya), and west (Khabat) of Erbil, northern Iraq (Fig. 1). These farms were chosen to represent different weather conditions and topographical features across the region. Within each farm, animals suspected of being infected with *Theileria* spp., *Babesia* spp., or *Anaplasma* spp. were selected for inclusion in the study. The selected animals represented various livestock species from both small- and medium-scale operations, ensuring a comprehensive sample of the local farming environment. The study design achieved a balanced geographical and climatic distribution by sampling 45 animals in winter, 67 in spring, 72 in summer, and 60 in autumn.

For each animal, 3 mL of blood was drawn from the jugular vein and placed in an EDTA tube. The tubes were gently mixed to ensure that the blood and anticoagulant reagent were well combined. DNA isolation was performed immediately after the blood samples were processed. The animals sampled represented a broad age range, from one month to four years old, and the selection from different seasons helped ensure a comprehensive analysis of the variables under varying environmental conditions.

DNA purification

The anticipated DNA purification was carried out using the DNA/RNA Prep NA (Nucleic acid extraction kit to extract and purify total RNA and DNA) from clinical materials (Sacace Biotechnologies Srl via Scalabrini, Como, Italy). As instructed by the kit, 300 μL of cell lysis solution was first put in an Eppendorf tube before 100 μL of anticoagulant blood was introduced to it. The tubes were vortexed and heated to 65°C for 15 minutes. Afterwards, 400 μL of Prec buffer was added and vortexed after being centrifuged for 7–10 seconds. Using a micropipette with an aerosol barrier tip blocked, the supernatant from each tube was carefully collected and discarded without disturbing the pellet after all tubes had been centrifuged at 13 000 rpm for 5 minutes. The tube tips were switched. With 500 μL of Wash Sol 1 and 300 μL of Wash Sol 2, the wishing phase was performed twice. All tubes were then incubated at 65°C for 5 minutes with open covers. In 50 μL of dilution buffer, the particle was resuspended. The tubes were incubated at 65°C for 5 minutes with intermittent vortexing, and then centrifuged for 60 seconds at 13 000 g. The RNA/DNA in the supernatant was then prepared for amplification. If amplification was not done on the same day as extraction, the processed samples were frozen at −20°/−80°C or stored at 2–8°C for a maximum of 5 days.

Primer design and PCR amplification

IDT (Integrated DNA Technologies, Coralville, Iowa, United States) designated and provided the oligonucleotide primers (Table 1) based on the mitochondrial and 18S and 16S rRNA gene sequences of *Babesia*, *Theileria*, and *Anaplasma*, respectively. The DNA and RNA Nucleotide Databases from Genebank, China, NCBI, and the complete relevant gene sequences and primer locations were all confirmed to be present in the genomes. A specific TaqMan hydrolysis probe was used in a real-time PCR with applied biosystems step one PCR in the

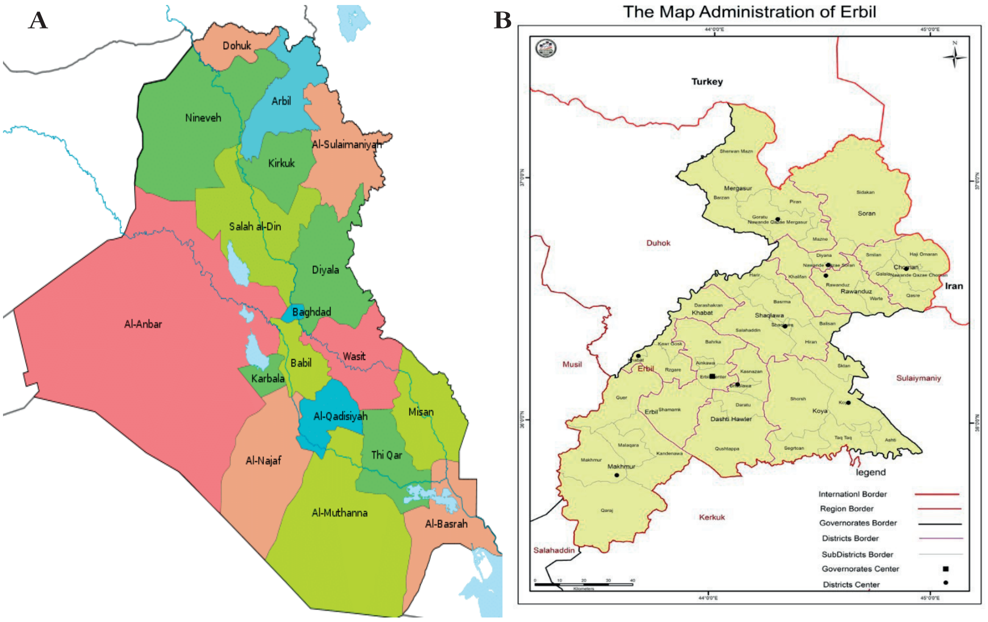

Fig. 1. (A) Map of Iraq showing the location of Erbil Governorate in northern Iraq. (B) Administrative map of Erbil Governorate indicating the sample site locations

Table 1. Primer sequences used for parasite detection

No.	Blood parasite	Primer sequence
1	*Babesia*	FWD 5'- ACG ATA GCC TTC CTA AAC TTC C -3' REV 5'- CAA GAG CTG CTA GTC CAG TTA T -3'
2	*Theileria*	FWD 5'- GCT TTG GAC GGT AGG GTA TT -3' REV 5'-TTA GAT GTG GTA GCC GTT TCT C -3'
3	*Anaplasma*	AM-F 5'-TTG GCA AGG CAG CTT -3' AM-R 5'- TTC CGC GAG CAT GTG CAT -3'

Thermal Cycler to amplify and detect the DNA by the prescribed procedure (Applied Biosystems, Foster City, California, USA). The reaction mixture contained 20 µL (10 µL of precision PLUS 2X Master Mix, 1 µL primer / probe Mix, and 4 µL RNase/DNase) and 5 µL of DNA template of blood DNA extracts. Enzyme activation took place for two minutes at 95°C, then there were 50 cycles of 10 seconds each at 95°C, 60°C, and 72°C. Throughout the procedure, barrier pipette tips were utilized to avoid cross-contamination between samples. For each sample, two replicate PCRs were conducted. After amplification, we used melting curve analysis to interpret the results. In all PCR experiments, positive control DNA and negative controls, in which the DNA templates were switched out for sterile water, were used.

Statistical analyses

The data were analyzed using Microsoft Excel, and chi-square (χ^2) tests were applied to compare infection prevalence among animal species and across seasons. A P value < 0.05 was considered statistically significant.

Results

Despite the observed differences in the occurrence of infections caused by *Theileria* spp., *Babesia* spp., and *Anaplasma* spp. across various animal species, including cattle, calves, sheep, lambs, and goats, statistical analysis using the Chi-square test ($\chi^2 = 1.494$, df = 8, $P = 0.9928$) showed no significant relationship between the type of animal and the specific parasitic infection. The observed variances in infection rates among animal groups show that these variations are likely due to chance, rather than any underlying biological or environmental variables. The data in Table 2 reveal that the overall infection rate in the animals studied was 73.77% (180 of 244 animals). *Theileria* spp. was the most common, followed by *Babesia* spp. and *Anaplasma* spp.

Figs. 2–4 illustrate the seasonal incidence of *Theileria* spp., *Babesia* spp., and *Anaplasma* spp. in the examined animals. For all three hemoparasites, infection prevalence was lowest in winter, increased markedly in spring, peaked in summer, and declined again in autumn, with *Theileria* spp. showing the highest overall rates, followed by *Babesia* spp. and *Anaplasma* spp. This consistent pattern across parasites indicates that transmission is strongly influenced by seasonal environmental conditions and associated vector activity.

Table 3 presents the distribution of *Theileria* spp., *Babesia* spp., and *Anaplasma* spp. infections across various infection classifications, including both singular and co-infections in the animal subjects. It details the number of infected animals, the proportion of infected animals (n = 180), and the percentage of the total examined population (n = 244), thereby illustrating the overall infection prevalence within the sampled cohort. The table incorporates P values to evaluate the statistical significance of the observed disparities. Single infections accounted for 70.0% of the infected animals (n = 180) and 51.64% of the entire population under study (n = 244). *Theileria* spp. showed a higher prevalence in infected animals (43.3%), although this difference was not statistically significant ($P = 0.32$). Mixed infections (with two or

Table 2. Incidence of *Theileria* spp., *Babesia* spp., and *Anaplasma* spp. in examined animals

Type of animal	Total examined animals	Positive number (%)		
		Theileria spp.	*Babesia* spp.	*Anaplasma* spp.
Cattle	31	12 (38.7)	8 (25.8)	4 (12.9)
Calf	26	11 (42.3)	5 (19.2)	2 (7.6)
Sheep	91	43 (46.2)	24 (26.3)	8 (8.8)
Lamb	29	10 (34.4)	7 (24.1)	3 (10.3)
Goat	67	25 (37.3)	12 (17.9)	6 (8.9)
Total	244 (180 were positive) (73.77%)	101 (41.4)	56 (22.95)	23 (9.43)

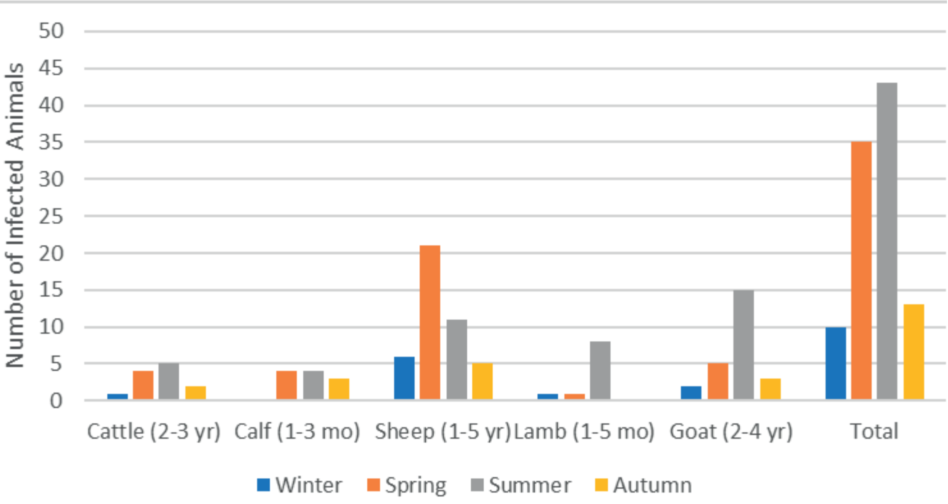

Fig. 2. Incidence of *Theileria* spp. in examined animals according to seasons

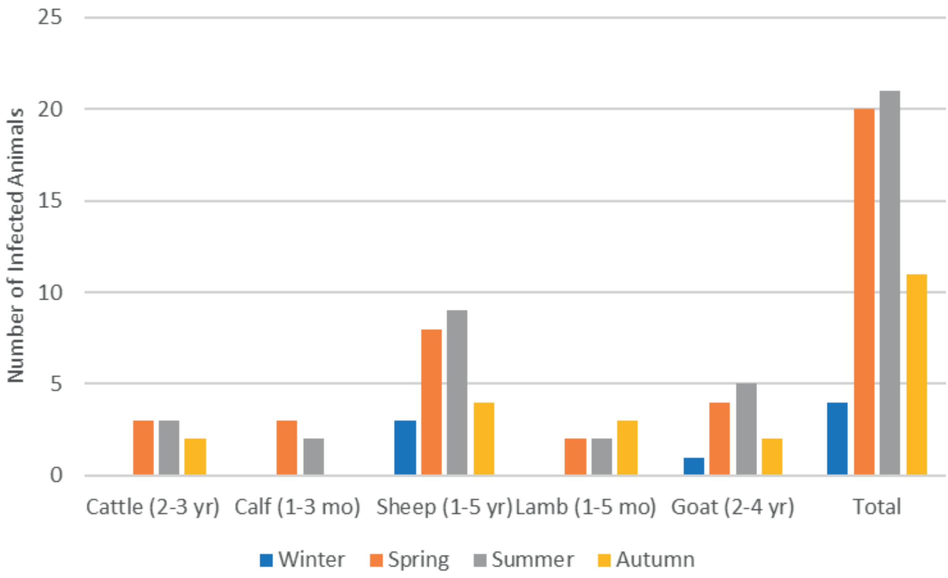

Fig. 3. Incidence of *Babesia* spp. in examined animals according to seasons

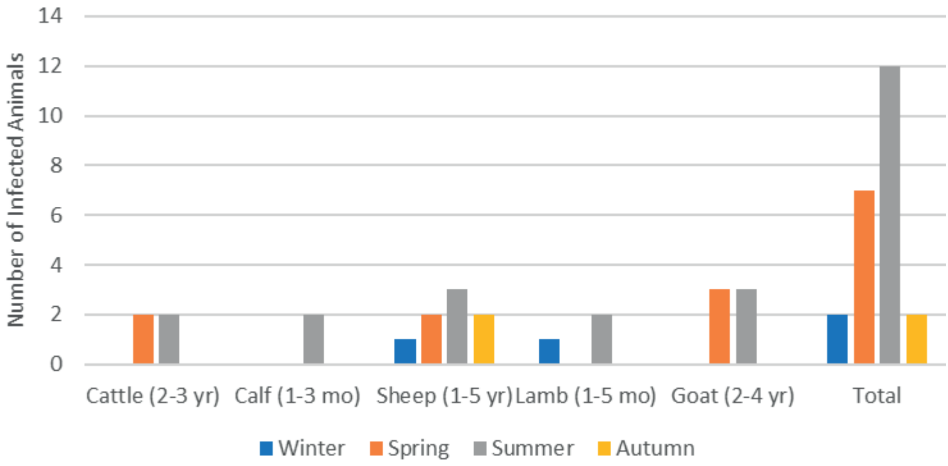

Fig. 4 Incidence of *Anaplasma* spp. in examined animals according to seasons

Table 3. Age-stratified prevalence of *Theileria* spp., *Babesia* spp., and *Anaplasma* spp. in different animal species

Species	Age group	*Theileria* spp. (%)	*Babesia* spp. (%)	*Anaplasma* spp. (%)	*P* value
Cattle	Adult (31)	12 (38.7)	8 (25.8)	4 (12.9)	
	Calf (26)	11 (42.3)	5 (19.2)	2 (7.6)	*P* = 0.85
Sheep	Adult (91)	43 (46.2)	24 (26.3)	8 (8.8)	
	Lamb (29)	10 (34.4)	7 (24.1)	3 (10.3)	*P* = 0.56
Goat	Adult (67)	25 (37.3)	12 (17.9)	6 (8.9)	

more parasites) were found in 30.0% of the infected animals (n = 180) and 22.13% of all animals studied (n = 244). The Theileria + Babesia combination had a prevalence of 16.7% in infected animals, but this was not statistically significant (*P* = 0.32). This data helps us understand the distribution of single and mixed infections and the importance of different parasite combinations in determining infection rates within the community.

Table 4 shows that analysis of co-infection patterns among the 180 PCR-positive animals revealed that a single parasite species caused the majority of infections. Single infections accounted for 70.0% of all positive cases (126/180). Among these, *Theileria* spp. only was the most frequent pattern, representing 43.3% of infected animals (78/180), followed by *Babesia* spp. only (20.6%, 37/180) and *Anaplasma spp.* only (6.1%, 11/180). Mixed infections were also recorded, comprising 30.0% of all positive animals (54/180). The most common mixed pattern was *Theileria* + *Babesia*, identified in 16.7% of infected animals (30/180), whereas Theileria + Anaplasma and Babesia + Anaplasma were detected in 8.9% (16/180) and 4.4% (8/180) of animals, respectively. Triple co-infection involving all three genera was not observed (0/180). Comparison of mixed-infection patterns showed no statistically significant differences between animal species (χ^2, *P* = 0.32). Overall, these findings demonstrate that although mixed infections are relatively common, *Theileria* spp. remains the dominant pathogen when present either alone or in combination with other hemoparasites.

Table 5 details the seasonal prevalence of infections caused by *Theileria* spp., *Babesia* spp., and *Anaplasma* spp. across the four seasons: winter, spring, summer, and autumn. The table provides the percentage of infected animals for each parasite within each season, with the number of positive animals relative to the total examined animals presented in parentheses. As an illustration, during winter, 22.22% of the animals exhibited *Theileria* spp. infection (10 of 45), 8.88% were infected with *Babesia* spp. (4 of 45), and 4.44% were infected with *Anaplasma* spp. (2 of 45). The overall infection rate across all seasons was

Table 4. Co-infection patterns of *Theileria* spp., *Babesia* spp., and *Anaplasma* spp. (single vs. mixed infections)

Type of infection	Number of infected animals	% of infected (n = 180)	% of all examined (n = 244)	*P* value
Single infections (any one parasite)	126	70.0%	51.64%	None
Theileria spp. only	78	43.3%	31.97%	*P* = 0.32*
Babesia spp. only	37	20.6%	15.16%	None
Anaplasma spp. only	11	6.1%	4.51%	None
Mixed infections (⩾ 2 parasites)	54	30.0%	22.13%	None
Theileria + Babesia	30	16.7%	12.30%	*P* = 0.32*
Theileria+ Anaplasma	16	8.9%	6.56%	None
Babesia + Anaplasma	8	4.4%	3.28%	None
Theileria + Babesia + Anaplasma	0	0.0%	0.00%	None

Table 5. Seasonal variation in prevalence of *Theileria* spp., *Babesia* spp., and *Anaplasma* spp.

Season	*Theileria* spp. (%)	*Babesia* spp. (%)	*Anaplasma* spp. (%)	Total infected (%)	*P* value
Winter (45)	22.22% (10/45)	8.88% (4/45)	4.44% (2/45)	73.77% (180/244)	*P* < 0.05
Spring (67)	52.23% (35/67)	29.85% (20/67)	10.44% (7/67)		None
Summer (72)	59.72% (43/72)	29.16% (21/72)	16.66% (12/72)		None
Autumn (60)	21.66% (13/60)	18.33% (11/60)	3.33% (2/60)		None

73.77% (180 of 244 animals), a figure that remained consistent throughout the observed periods. The *P* value associated with the winter season suggests that the observed differences are statistically significant ($P < 0.05$), whereas no significant variations were detected in other seasons. This table offers crucial information regarding the seasonal fluctuations in infection prevalence for each parasite, thereby serving as a resource for informing season-specific control strategies within livestock management practices.

Discussion

Blood parasite infections severely hamper the management and welfare of domestic farm animals in Iraq. One of these infections with the highest prevalence and economic impact is piroplasmosis (Alabbody, 2024; Alali et al., 2022). Furthermore, in many parts of Iraq, serological tests, stained blood smear examinations, and clinical signs are used to diagnose blood parasites (Gharban et al., 2022). To evaluate the epidemiological aspects of hemoparasites, however, these procedures are not sufficiently reliable and efficient. Nevertheless, taking into account the shortcomings of microscopy, serology, and molecular analysis, as well as the urgent need for an accurate and general diagnostic kit that diagnoses all or most infections regardless of the causative species, RT-PCR-based molecular detection and identification of blood parasites in some farm animals were conducted in Erbil province in northern Iraq. Of all non-randomly chosen animals tested for the parasites, 73.77% were infected. *Theleria* had the highest infection rate at 41.4%, which was significantly higher than the prevalence rates of Babesia (22.95%) and Anaplasma (9.43%). Using the PCR approach, similar outcomes were seen for the prevalence rates of these parasites. When compared with the PCR technique, Giemsa-stained smear microscopic analysis produced negative results in 8 (16%) blood samples, demonstrating that the PCR is more sensitive in the detection of tropical theileriosis (Hassan et al., 2012). A study using molecular diagnostic techniques identified multiple hemoparasitic agents that were affecting sheep in the Kurdistan region of Iraq. A total of 195 samples from the three governorates of Duhok, Erbil, and Sulaimaniya were examined. The pathogens discovered were *Anaplasma ovis* (62.6%), *Theileria ovis* (14.35%), *T. lestoquardi* (7.7%), *T. uilenbergi* (5.6%), and *Babesia ovis* (1.5%) (Renneker et al., 2013). According to the PCR results, 12 (31.57%) and 9 (23.68%) of the 38 camels were solitary and positive for *B. caballi* and *T. equi*, respectively (Jasim et al., 2015).

Seroprevalence of *B. bigemina* and *A. marginale* in cattle, sheep, goats, and wild goats was studied in the Erbil district, between January to December 2010. A total of 184 blood samples were collected from 44 cattle, 59 sheep, 70 goats, and 11 wild goats. The overall prevalence of *B. bigemina* infection was 12 (27.27%), 4 (6.77%), 5 (7.14%) and 1 (9.09%) in cattle, sheep, goats and wild goats and for *A. marginale* 4 (9.09%), 2 (3.38%), 3 (4.28%) and 1 (9.09%) respectively (Ameen et al., 2012).

The findings of the current study showed the effect and the difference in infection rates according to the different seasons of the year. The highest rates of infection were in the hot seasons of the year, such as summer and autumn, and the lowest were in the cold and temperate seasons, such as winter and spring. These findings were consistent with prior research on the impact of seasons on infection rates (Alimam et al., 2022). The highest rate of infection recorded in April was 45% (9/20) with highly significant differences between months of study (Arwa and Kawan, 2022). *Anaplasma* spp., *Babesia* spp., and *Theileria* spp. infections tend to follow a pattern that is influenced by precipitation, temperature, and relative humidity (Abdullah et al., 2019). The impact of environmental conditions may be to blame for the fluctuation in infection rates between seasons. Due to the seasonality of the vector, it is clear that animals are susceptible to blood parasite infection(Yadav and Upadhyay, 2023).

Conclusions

This study demonstrated a high prevalence (73.77%) of hemoparasitic infections – *Theileria* spp., *Babesia* spp., and *Anaplasma* spp. – in livestock from the Erbil region of Iraq, with *Theileria* spp. being the most frequently detected (41.4%). Seasonal patterns were evident, with peak infection rates occurring during spring and summer, likely due to increased vector activity. Although variations in infection rates were noted among livestock species, no statistically significant associations were detected. RT-PCR proved more sensitive than conventional microscopy, confirming its reliability as a diagnostic tool. These results emphasize the importance of seasonal parasite control measures and highlight the potential of developing cost-effective PCR-based diagnostics to improve early detection, limit herd-level transmission, and reinforce veterinary surveillance systems.

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References

1. Abdullah D. A., Ali M. S., Omer S. G., Ola-Fadunsin S. D., Ali F. F., Gimba F. I. Prevalence and climatic influence on hemoparasites of cattle and sheep in Mosul, Iraq. Journal of Advanced Veterinary and Animal Research. 2019; 6: 492.
2. Alabbody H. H. K. Assessment the owner's awareness towards livestock diseases at rural of Iraq. Cahiers Magellanes-NS. 2024; 6: 749–757.
3. Alali F., Jawad M., Al-Obaidi Q. T. A review of endo and ecto parasites of equids in Iraq. Journal of Advances in VetBio Science and Techniques. 2022; 7: 115–129.
4. Ali H. H., Alyasiri A. J. Zoonosis in Iraq from 2014–2018: An epidemiological study. Journal of Applied Health Sciences and Medicine. 2024; 4: 4–13.
5. Alimam H. M., Moosa D. A., Ajaj E. A., Dahl M. O., Al-Robaiee I. A., Allah S. F. H., Al-Jumaa Z. M., Hadi E. D. Proportion and seasonality of blood parasites in animals in Mosul using the Veterinary Teaching Hospital Lab data. PLoS One. 2022; 17: e0264121.
6. Ameen K., Abdullah B., Abdul-Razaq R. Seroprevalence of Babesia bigemina and Anaplasma marginale in domestic animals in Erbil, Iraq. 2012.
7. Arwa R., Kawan M. Microscopic examination of ovine babesiosis at Baghdad city/Iraq. Iraqi Journal of Agricultural Sciences. 2022; 53: 798–801.
8. Gharban A., Al-Shaeli S., Al-Abedi G., Abbas Z. R., Jassim A. F. Microscopic investigation of bovine haemoparasites in Wasit Province, Iraq. Annals of the Romanian Society for Cell Biology. 2022; 26: 1143–1159.
9. Hassan A. H., Salmo N. A., Jabbar S. A. Pathological and molecular diagnostic study of theileriosis in cattle in Sulaimaniyah province, Iraq. The Iraqi Journal of Veterinary Medicine. 2012; 36: 306–314.
10. Hussen S. M. A review on intestinal and blood parasitic (haemoparasite) protozoans. International Journal of Veterinary Science and Research. 2020; 6: 164–172.
11. Jasim H. J., Azzal G. Y., Othman R. M. Conventional and molecular detection of Babesia caballi and Theileria equi parasites in infected camels in south of Iraq. Basrah Journal of Veterinary Research. 2015; 14: 110–121.
12. Momčilović S., Cantacessi C., Arsić-Arsenijević V., Otranto D., Tasić-Otašević S. Rapid diagnosis of parasitic diseases: Current scenario and future needs. Clinical Microbiology and Infection. 2019; 25: 290–309.
13. Ponnampalam E. N., Kiani A., Santhiravel S., Holman B. W., Lauridsen C., Dunshea F. R. The importance of dietary antioxidants on oxidative stress, meat and milk production, and their preservative aspects in farm animals: Antioxidant action, animal health, and product quality—Invited review. Animals. 2022; 12: 3279.
14. Renneker S., Abdo J., Bakheit M., Kullmann B., Beyer D., Ahmed J., Seitzer U. Coinfection of sheep with Anaplasma, Theileria and Babesia species in the Kurdistan Region, Iraq. Transboundary and Emerging Diseases. 2013; 60: 113–118.
15. Rosenblatt J. E., Reller L. B., Weinstein M. P. Laboratory diagnosis of infections due to blood and tissue parasites. Clinical Infectious Diseases. 2009; 49: 1103–1108.
16. Villanueva-Saz S., Borobia M., Fernández A., Jiménez C., Yzuel A., Verde M. T., Ramo M. Á., Figueras L., Ruíz H. Anaemia in sheep caused by Babesia and Theileria haemoparasites. Animals. 2022; 12: 3341.
17. Willard M. D., Tvedten H. Small Animal Clinical Diagnosis by Laboratory Methods. Elsevier Health Sciences; 2011.
18. Yadav N., Upadhyay R. K. Global effect of climate change on seasonal cycles, vector population and rising challenges of communicable diseases: A review. Journal of Atmospheric Science Research. 2023; 6.