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Microscopic and molecular detection of *Theileria annulata* in cattle from Babylon province of Iraq

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Abstract

Objective

This study aimed to investigate the detection of theileriosis caused by *Theileria annulata* in cattle applying microscopic and molecular methods. The other goal of the study was to evaluate the effects of geographical region, age, and sex on infection rates.

Materials and methods

In this study, 300 bovine blood samples were collected from different areas of Babil province, Iraq. The study was conducted between September 2025 and March 2026. Giemsa-stained blood smears were prepared for 200 samples for microscopic examination. 100 samples were analyzed by PCR to target the mitochondrial cytochrome b gene of T. annulata. Chi-square test was used in SPSS version 27 for statistical analysis.

Results

The results showed that 66 (33%) of 200 bovine blood samples examined microscopically were positive for *Theileria annulata*. In the PCR method, 54 (54%) of 100 samples that studied the mitochondrial cytochrome b gene were positive. This could indicate a higher sensitivity of PCR than the microscopic method. The prevalence of infection was 53.3% in women and 55% in men, which was not significant. Also, the age of the individuals did not report a significant difference in the infection rate ($\chi^2 = 0.34$, $p = 0.84$). However, the geographical regions showed a significant difference. So that the highest infection rate was related to Al-Shumali (73.9%) and the lowest rate was related to Al-Qasim (35%) ($\chi^2 = 8.922$, $p < 0.01$). These results indicate that there is a significant relationship between the region and the infection rate. Phylogenetic analysis of ten T.

annulata isolates (PX849363-PX849372) confirmed their genetic relationship with reference strains available in GenBank.

Conclusion

PCR-based molecular detection demonstrated higher sensitivity than microscopy for identifying *T. annulata* infection in subclinical infections that may contribute to parasite transmission within herds. These findings highlight the need for integrated control strategies, including regular tick management, molecular surveillance, and improved animal husbandry, to decrease the impact of bovine theileriosis and enhance cattle health and productivity in endemic areas.

Keywords: bovine theileriosis, cytochrome b gene, molecular detection, PCR, *Theileria annulata*

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Introduction

Animal breeding in the economy of a country is considered one of the most important economic branches and is of special importance (Javanmard et al., 2008; Ahsani et al., 2022). Animal breeding is a very profitable job and it is considered as a means of raising the economy of countries (Rohallah et al., 2007; Mohammadabadi et al., 2024c). Most of the people of the world are engaged in cattle breeding and use its products. In addition, cattle breeding has an important role (Eghtedari et al., 2024). Since the creation of mankind, food and nutrition have been one of the major issues for humans, whether when mankind was living wildly in deserts or now, with the help of technology, it has conquered the infinite space (Mohammadabadi et al., 2010; Mohammadinejad et al., 2024). The issue of food and nutrition has been one of the main issues that have occupied human thought, and even though in the new era, mankind has been able to make significant progress in different stages of his life, still the issue of food and nutrition in human societies is a special priority from an economic and social point of view (Mohammadabadi et al., 2024a; Mohammadabadi et al., 2023). Today, the importance of nutrition is such that it is considered one of the important criteria of the level of civilization and progress of any society

(Mohammadabadi et al., 2024b; Ghasemi et al., 2010). Because in the all-round development of a society, the level of mental and physical health of the people of that society is the determining factor of animal breeding (Badakhshan and Mohammadabadi 2015; Nejad et al., 2024). Theileriosis is a tick-borne hemoprotozoan disease caused by parasites of the genus *Theileria*, which primarily infects cattle in tropical and subtropical regions (Mor et al., 2024; Kareem et al., 2025). The parasite has a complex life cycle involving both vertebrate hosts and ixodid ticks as vectors, making its transmission dependent on the interaction between host, vector, and environment. Clinically, tropical theileriosis manifests with fever, enlargement of superficial lymph nodes, profuse ocular and nasal discharge, hypersalivation, respiratory distress, icterus, and in severe cases, death due to asphyxiation or secondary infections (Omer et al., 2003). Subclinical infections are also common, where animals carry the parasite without showing obvious signs, yet they contribute to disease transmission within herds. *Theileria* parasites belong to the phylum Apicomplexa, class Sporozoea, order Proplasmida, and family Theileriidae (Knowles et al., 2018). Morphologically, the parasites appear in various forms, including circular, oval, irregular, or bacillary shapes, with rhoptries in the apical region that facilitate host cell invasion. The classification of *Theileria* subspecies is based on a combination of parasite morphology, host species, tick vector, clinical manifestations, and geographic distribution (Jalovecka et al., 2019). Among them, *Theileria annulata* is considered the most pathogenic in tropical regions, causing severe losses in local and crossbred cattle. The economic impact of theileriosis is substantial. Infected animals suffer reduced milk and meat production, loss of draft power, increased treatment costs, and high mortality rates, leading to global annual economic losses estimated to exceed $7 billion (Ahmed et al., 2002). In endemic areas, the disease continues to pose a major threat to livestock production and food security. Effective disease management depends on timely detection and intervention to prevent outbreaks. Traditional diagnostic methods for theileriosis primarily rely on clinical observation and microscopic examination of stained lymph node and blood smears to detect schizonts and piroplasms (Alnahass et al., 2026). However, these methods have significant limitations, especially in detecting subclinical infections or animals with low parasitemia. This has prompted the adoption of molecular techniques, such as polymerase chain reaction (PCR), which offer higher sensitivity and specificity for detecting *T. annulata* DNA. PCR not only allows early diagnosis but also facilitates molecular characterization and phylogenetic analysis, providing insights into the genetic diversity and evolutionary relationships of local isolates. Given the economic and veterinary significance of theileriosis, this study was conducted to determine the prevalence of *T. annulata* in cattle using both microscopic and molecular methods. The study also aimed to evaluate the influence of host factors, including age, sex, and geographic location, on infection rates, and to perform molecular

characterization and phylogenetic analysis of local isolates based on the mitochondrial cytochrome b gene. By combining traditional and molecular approaches, this research seeks to provide a comprehensive understanding of the epidemiology of *T. annulata* in the study area, which may inform future control and prevention strategies.

Materials and methods

Sample collection: A total of 300 blood samples were randomly collected from cattle from south of Babylon (Al-Shomali, A-Musayyib, Al-Hamaza Al-Gharbi, A-Talia, Al-Hilla, and A-Qasim centers), during the period from September 2025 to March 2026. Blood was collected from jugular vein of each cattle using vacuum tubes. Then, 5 mL of blood was drawn by sterile needle with a vacutainer EDTA sterile tubes and immediately transported in the ice box for both laboratory diagnosis and molecular diagnosis.

Microscopic examination-Blood smear: Peripheral blood samples were used to prepare thin and thick smears (Patel et al., 2025). For staining and differentiation of parasites, Giemsa staining method was used.

Staining method: Blood slides were first air-dried. Then, they were fixed with 100% methyl alcohol (methanol) for 15 minutes and then stained with 10% Giemsa solution for 30 minutes. After appropriate washing with distilled water, the slides were dried and examined using a light microscope equipped with an oil immersion lens (100×).

Thin blood slide preparation method: A thin layer of blood was prepared by placing a drop of blood on the slide and spreading it at an appropriate angle. The slide was dried at room temperature, then fixed in pure methanol for 3 to 5 minutes. Giemsa staining was performed for 15 to 30 minutes at room temperature. Finally, the slides were washed well with running water and, after drying, were prepared for microscopic examination (Vu et al., 2021).

Molecular detection: A total of one hundred blood samples were taken from one hundred cattle before extracting their DNA. The DNA was extracted from the blood using a standard commercial extraction kit according to the manufacturer's instructions. The primers used in this study target the mitochondrial cytochrome b gene of *Theileria annulata* to amplify a fragment of 1092 bp for molecular detection. These primers enable specific and sensitive identification of the parasite using PCR technique (Table 1).

PCR was then performed on each of the samples for the mitochondrial cytochrome b gene (Al-Musawi et al., 2022). The PCR reaction was carried out in 25 µL volumes for each sample, with 12.5 µL of 2× PCR Master Mix, 1µL forward primer (10 µM), 1 µL reverse primer (10 µM), 5 µL template DNA, and 5.5 µL of nuclease-free water. PCR amplification was performed under the following cycling conditions. Initial denaturation was performed at 95°C for 5 minutes,

followed by 35 cycles of denaturation at 95°C 30 seconds, annealing at 58°C (45 seconds), and extension at 72°C for (1 minute), with a final extension at 72 °C for 7 minutes. PCR products were visualized under UV light after electrophoresis on 1.5% agarose gel. Ten positive PCR products were subsequently sent to Macrogen Company (South Korea) for Sanger sequencing. The sequences received from this procedure were proceeded to be trimmed of any unwanted noise, and submitted to the NCBI data base in order to obtain the accession number of the sequences.

Table 1. The primer used in this study targeting the mitochondrial gene (Cytochrome b gene) (Mhadhbi et al., 2015)

Primer	Sequence (5'-3')	Amplicon size	Target gene
Thiel-cytoF	CAGGGCTTTAACCTACAAATTAAC	1092 bp	Cytochrome b gene (mitochondrial)
Thiel-cytoR	CCCCTCCACTAAGCGTCTTTCGACAC		

Phylogenetic tree: In order to evaluate these strains' evolutionary relationship and to classify them to evaluate the genetic relationships among local isolates, a phylogenetic analysis of their genome based on the genetic similarity was performed. The Nucleotide sequences were aligned with Clustal W and then used to create a phylogenetic tree based on the Likelihood method with MEGA X software. The evolutionary model used to generate this tree was Tamura-Nei, and the robustness of the tree topology was evaluated through 1000 bootstrap replicates. The resulting sequences were compared with the reference sequences available from the NCBI database via BLASTn to confirm their taxonomic identities and the degree of genetic similarity.

Statistical analysis: We used Microsoft Excel 2010 and SPSS for Windows version 27 to do the statistical analysis. The chi-squared test was utilized to analyze the differences between groups. A p-value < 0.05 was considered statistically significant.

Results

The objective of this study was to compare molecular diagnosis and traditional diagnosis regarding the prevalence of *Theileria annulata* in blood samples. There were 200 samples for traditional diagnosis and 100 samples for molecular diagnosis. The results indicate 54% (54/100) and 33% (66/200) for molecular diagnosis and traditional diagnosis, respectively (Table 2). The results showed that the infection rate detected by the molecular method was higher than that detected by the microscopic examination. The molecular test recorded 54% (54/100) positive samples, whereas the microscopic method detected 33% (66/200) positive samples. Statistical analysis using the Chi-square test revealed a χ^2 value of 11.39 with a p-value of 0.0007, indicating

a highly significant difference ($p < 0.05$) between the two diagnostic methods. This suggests that the molecular technique is more sensitive and capable of detecting infections that may not be identified by microscopic examination.

Table 2. Comparative study between molecular diagnosis and traditional diagnosis and infected rate of *Theileria annulata*

Test	Examined samples	Positive samples	Percentage %
Molecular	100	54	54%
Microscopic	200	66	33%
X^2		11.39	
p-value		0.0007	

The results showed that out of 100 examined animals, 54 were infected with *Theileria annulata*, giving an overall prevalence of 54%. When analyzed by sex, females (60 animals) had 32 positive cases (53.3%), while males (40 animals) had 22 positive cases (55%), indicating that infection rates were very similar between sexes (Table 3). Considering age groups, the highest number of infected animals was in the <1 year group (20/36, 55.6%), followed by 1-3 years (19/34, 55.9%), and the lowest in the >3 years group (15/30, 50%). These findings indicate that no statistically significant association was observed between age or sex and infection prevalence in the examined cattle population. The relatively high prevalence across all categories indicates that *Theileria annulata* is widely distributed among cattle regardless of sex or age, highlighting the importance of control measures in all age groups and both sexes**.**

Table 3. Molecular detection of *Theileria annulata* according to sex and age groups

Age / Sex	Less than 1 year	1-3 years	More than 3 years	Total	Percentage %
Female (60)	13	11	8	32	(53.3%)
Male (40)	7	8	7	22	(55%)
Total (100)	20/36	19/34	15/30	54/100	(54%)
X^2			0.34		
P-value			0.84		

Non-significant ($p>0.05$).

The results (Table 4) show that the prevalence of *Theileria annulata* is not uniform across regions, with the highest infection observed in Al-shomali 17/23 (73.9%) and the lowest in Al-Qassim 7/20 (35%). These results suggest that region-specific factors, such as tick density, cattle management practices, and environmental conditions, influence infection rates. Therefore,

control and preventive measures should focus more on high-risk regions to reduce the spread of theileriosis.

Table 4. Molecular detection of *Theileria annulata* according to geographic area

Region	No. of sample	No. of positive	Percentage %
Al-Qasim	20	7	35%
Al-Hamaza al-gharbi	12	8	66.7%
Al-shomali	23	17	73.9%
Al-Talia	20	8	40%
AL-Hilla center	10	5	50%
AL-Musayyib	15	9	60%
Total	100	54	54%
X^2		8.922	
P-Value		<0.01	

Conventional PCR: Conventional PCR was performed to amplify (Cytochrome b gene). The amplification results showed product sizes of approximately 1092 bp in cattle host as illustrated in Figure 1. Gel electrophoresis on 1.5 % agarose gel showed positive samples (lane 1-17) of *Theileria annulata* targeting cytochrome b gene in cattle host. The size of PCR amplicon was 1092 bp. A phylogenetic tree analysis of the identified *Theileria annulata* (cytochrome b gene) was performed (Figure 2). The bootstrap values obtained in the phylogenetic tree supported the stability of the major clades and confirmed the close evolutionary relationship among the local isolates. The evolutionary history was inferred using the maximum likelihood method and the Tamura-Nei model, and evolutionary analyses were performed in MEGA11. The analysis included 20 nucleotide sequences. There was a total of 726 positions in the final dataset.

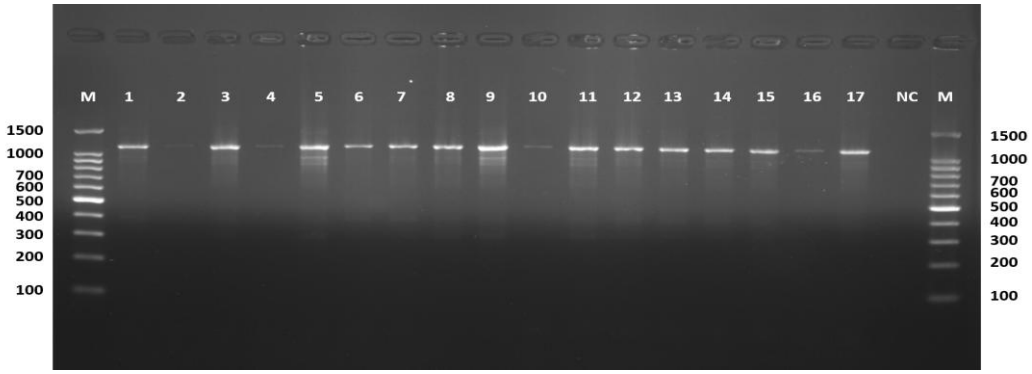

Figure 1. Gel electrophoresis of some samples on 1.5 % agarose gel. Lanes 1- 17 are positive sample of *Theileria annulata.* PCR amplicon size of target cytochrome b gene in cattle host is 1092 bp. NC is negative control. M is molecular marker, M100 (GeneDirex, South Korea)

Phylogenetic sequences analysis of *Theileria annulata*: Ten *T. annulata* isolates were sequenced and submitted to GenBank under accession numbers PX849363-PX849372. Phylogenetic analysis confirmed close genetic relationships among the local isolates and reference strains. Comparison of the mitochondrial cytochrome b gene sequences of local isolates of Theileria annulata with previously published isolates available in GenBank showed that there is a high nucleotide similarity (from 98% to 100%). The closest genetic relationship was observed between isolates from Iraq and regional countries, including Turkey, Pakistan, and India. This could indicate a regional genetic connection. Phylogenetic analysis showed that local isolates were placed in the same main clade as other Asian and Middle Eastern isolates. This could indicate limited genetic divergence between regional strains. From this clustering pattern, it is possible to understand animal movement, tick distribution, and common ecological conditions that exist in the countries of the region.

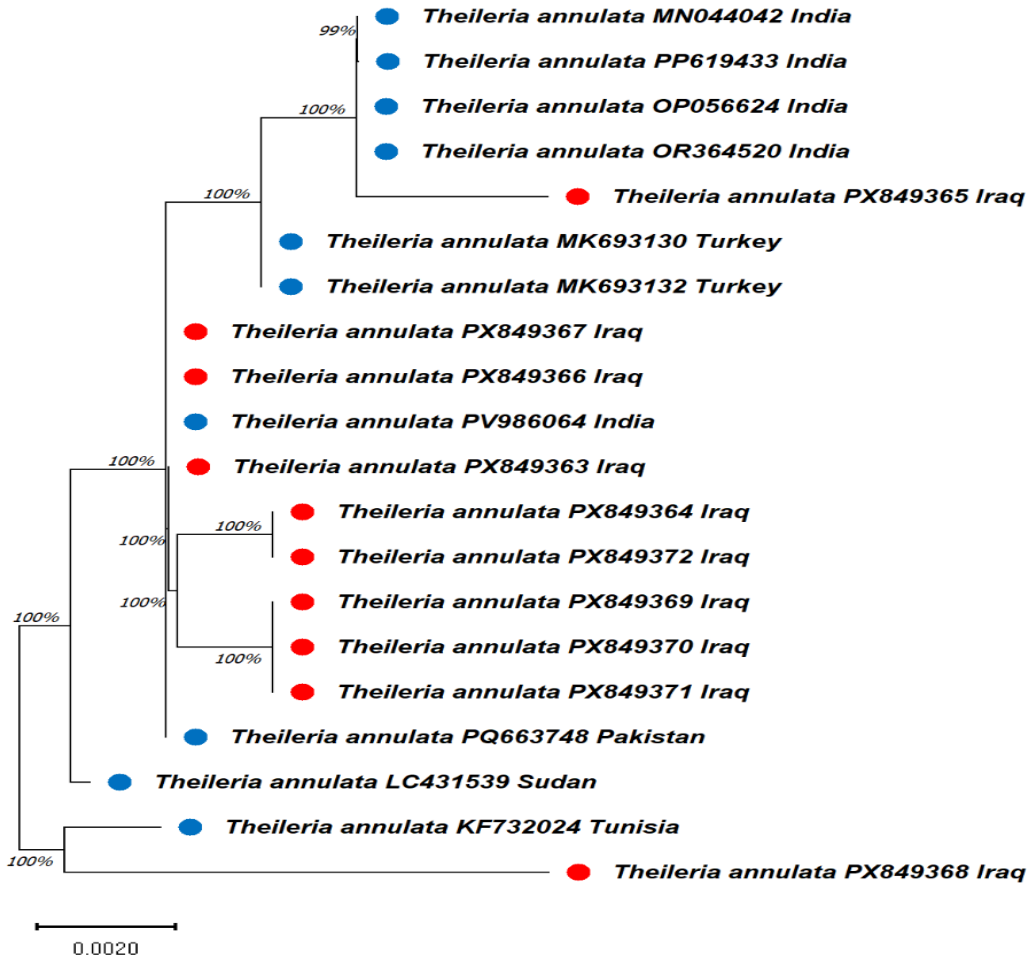

Figure 2. Analysis of the phylogenetic tree of the identified *Theileria annulata* (cytochrome b gene) using the maximum likelihood method and the Tamura-Nei model in MEGA11

Discussion

Using PCR, the current study found a high molecular prevalence of *Theileria annulata* in cattle (54%), suggesting that this tick-borne hemoprotozoan infection is widespread even in subclinical animals. While molecular techniques like PCR offer higher sensitivity and specificity in identifying low parasitemia infections that might not be visible under a microscope, traditional microscopy frequently underestimates the true prevalence (Wahab and Aziz, 2026). Other areas have also emphasized the significance of PCR in determining actual infection rates. Accurate species identification and genetic characterization of *Theileria* isolates are made possible by molecular investigations, which is essential for comprehending epidemiology and creating control plans (Parveen et al., 2021). Globally, molecular prevalence of *T. annulata* varies widely depending on ecology, tick vectors, and cattle management. Studies in Pakistan using PCR reported prevalence rates around 23.7% in cattle populations and found significant associations between infection and factors such as age, gender, and acaricide use (Ullah *et al*., 2021). Tick infestation remains a key driver of disease transmission, with vectors such as *Hyalomma* species playing a pivotal role in sustaining disease cycles. Irregular acaricide usage and high tick presence in animal housing significantly increase infection rates (Wahab and Aziz, 2026). Moreover, subclinical carrier animals, which are infected without obvious clinical signs, represent a major challenge. These carriers contribute to the silent persistence and spread of infection within herds, impacting productivity, including milk yield and growth rates (Kundave *et al*., 2015). Considering these findings, integrated control strategies are essential. Regular tick control programs, molecular monitoring, improved husbandry, and farmer education are recommended to reduce disease prevalence. Continuous surveillance with PCR allows for timely detection and management of emerging or mixed infections (Parveen *et al*., 2021). The current study confirms that *Theileria annulata* is highly prevalent among cattle in the study area, with 54% of animals testing positive by molecular PCR analysis. The findings highlight the significance of subclinical infections, which often remain undetected using traditional microscopy but still contribute to disease transmission and economic losses. Regional differences in infection rates emphasize the influence of environmental conditions, tick density, and management practices on disease prevalence. Subclinical carriers can serve as reservoirs, maintaining the infection cycle within herds. Therefore, molecular diagnostics are essential for accurate detection and for guiding effective control measures (Kagena, 2025; Aboktifa et al., 2025). Integrated control strategies including regular tick management, improved husbandry, molecular surveillance, and farmer education are crucial to reduce the spread of T. annulata and to protect cattle health and productivity. Continuous surveillance and region-specific interventions remain fundamental in mitigating the impact of theileriosis in endemic areas. Comparison of local Iraqi isolates with T.

annulata isolates previously reported from neighboring countries, based on phylogenetic analysis of the mitochondrial cytochrome b gene, revealed a high genetic similarity between them. This could be due to the fact that genetically related strains are circulating in the region. The close clustering of Iraqi isolates with regional strains could be related to the movement of transboundary animals, common tick vectors, and similar environmental conditions that facilitate parasite transmission. On the other hand, this low observed genetic divergence among isolates could be attributed to the fact that the cytochrome b gene in T. annulata is relatively conserved. Since this gene is relatively conserved, it can be used as a reliable molecular marker for epidemiological and phylogenetic studies. However, it is advisable to conduct ongoing genetic surveillance to detect the possible emergence of new variants that could affect pathogenicity, transmission dynamics, or treatment efficacy. although no statistically significant association was observed between age groups and infection rate, the limited sample size and broad age classification should be considered when interpreting these findings. In this study, the number of samples studied with molecular techniques was relatively small compared to the microscopic study. This is a limitation of this study. Another limitation of this study was the collection of samples from limited geographical areas of Babylon province. This limitation may not fully reflect the epidemiological situation of Theileria annulata in all regions of Iraq. Therefore, it is recommended to consider it in future studies.

Conclusion: Based on the results of the present study, it can be concluded that molecular detection techniques are more sensitive and reliable than microscopic examination for the diagnosis of Theileria annulata infection in cattle, especially in subclinical cases with low parasitology. The results also showed that Theileria annulata has a relatively high prevalence among cattle in Babol province. This could indicate the widespread distribution of bovine theileriosis in the study area. Based on the findings of this study, it can be stated that molecular surveillance is very important for accurate diagnosis and early detection of infection and should be emphasized. In addition, integrated control measures, including regular tick control programs, improved management practices, and continuous epidemiological surveillance, are essential to reduce the spread of the disease and minimize the economic impact of bovine theileriosis on cattle production.

Author contributions

All authors contributed equally to all stages of the research. Both authors were involved in conceptualization the study, methodology, data collection, analysis, and interpretation.

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Data availability statement

The data contributing to the findings of this study are available from the investigating researcher upon request.

Ethical considerations

Ethical approval for this study was obtained from the Institutional Animal Care and Use Committee of Department of Parasitology, College Veterinary Medicine, Al-Qasim Green University, Babylon 51013, Iraq.

Conflicts of interest

The researchers declare no conflict of interest regarding the publication of this paper.

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تشخیص میکروسکوپی و مولکولی *Theileria annulata* در گاوهای استان بابل عراق

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چکیده

هدف: هدف این مطالعه بررسی تشخیص تیلریوز ناشی از *Theileria annulata* در گاوها با استفاده از روش‌های میکروسکوپی و مولکولی بود. همچنین، تأثیر منطقه جغرافیایی، سن و جنس بر میزان آلودگی مورد ارزیابی قرار گرفت.

مواد و روش‌ها: در این مطالعه، ۳۰۰ نمونه خون گاوی از مناطق مختلف استان بابل، عراق جمع‌آوری شد. این مطالعه در فاصله زمانی سپتامبر ۲۰۲۵ تا مارس ۲۰۲۶ انجام گرفت. برای ۲۰۰ نمونه، گسترش‌های خونی رنگ‌آمیزی‌شده با گیمسا جهت بررسی میکروسکوپی تهیه شد. همچنین ۱۰۰ نمونه با استفاده از روش PCR برای شناسایی ژن سیتوکروم b میتوکندریایی *T. annulata* مورد بررسی قرار گرفتند. آزمون کای‌دو در نرم‌افزار SPSS نسخه ۲۷ برای تحلیل آماری استفاده شد.

نتایج: نتایج نشان داد که از ۲۰۰ نمونه خون گاوی بررسی‌شده به روش میکروسکوپی، ۶۶ نمونه (۳۳٪) از نظر *Theileria annulata* مثبت بودند. در روش PCR، تعداد ۵۴ نمونه (۵۴٪) از ۱۰۰ نمونه مورد بررسی از نظر ژن سیتوکروم b میتوکندریایی مثبت گزارش شدند که نشان‌دهنده حساسیت بالاتر روش PCR نسبت به روش میکروسکوپی است. میزان شیوع آلودگی در ماده‌ها ۵۳/۳٪ و در نرها ۵۵٪ بود که تفاوت معنی‌داری نداشت. همچنین سن حیوانات اختلاف معنی‌داری در میزان آلودگی نشان نداد ($\chi^2 = 0.34$, $p = 0.84$). با این حال، مناطق جغرافیایی اختلاف معنی‌داری را نشان دادند؛ به‌طوری‌که بیشترین میزان آلودگی مربوط به منطقه الشوملی (۷۳/۹٪) و کمترین میزان مربوط به القاسم (۳۵٪) بود ($\chi^2 = 8.922$, $p < 0.01$). این نتایج نشان‌دهنده وجود

ارتباط معنی‌دار بین منطقه جغرافیایی و میزان آلودگی است. همچنین، تحلیل فیلوژنتیکی ده جدایه *T. annulata* (PX849363-PX849372) ارتباط ژنتیکی آن‌ها را با سویه‌های مرجع موجود در GenBank تأیید کرد.

نتیجه‌گیری: تشخیص مولکولی مبتنی بر PCR در مقایسه با میکروسکوپی حساسیت بالاتری در شناسایی آلودگی *T. annulata*، به‌ویژه در عفونت‌های تحت‌بالینی که می‌توانند در انتقال انگل در گله نقش داشته باشند، نشان داد. این یافته‌ها بر ضرورت استفاده از راهبردهای کنترلی یکپارچه شامل مدیریت منظم کنه‌ها، پایش مولکولی و بهبود شرایط پرورش دام برای کاهش اثرات تیلریوز گاوی و ارتقای سلامت و بهره‌وری دام‌ها در مناطق بومی تأکید می‌کند.

کلمات کلیدی: تشخیص مولکولی، تیلریوز گاوی، ژن سیتوکروم b، PCR، *Theileria annulata*

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