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Molecular Detection of Tick-Borne Hemoparasites (Theileria, Babesia and Anaplasma) in Stray Dogs using Nested PCR

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Abstract

Haemoparasitic infections are frequently observed in dogs from tropical regions, including Iraq. Numerous dogs become infected with several blood parasites, resulting in more serious diseases than a singular infection. This investigation was designed in Wasit Province, Iraq to conduct a comprehensive molecular detection and characterization of haemoparasites in Infested Dogs. This cross-sectional study was performed from the beginning of May 2024 to the end of December of 2024. Totally 280 stray dogs were examined in different areas in waist, Iraq. The blood sample were obtained from the jugular vein of infested dogs was used for both microscopic and molecular analysis. Thin blood smears were prepared, detected by giemsa staining and screened for piroplasms Babesia, Theileria and Anaplasma. Total DNA was extracted followed by nested PCR using primer targeting16S *rRNA* gene to detect for Anaplasma spp. and 18SrRNA for Babesia spp and Theilleria spp. PCR products were confirmed by agarose gel electrophoresis. Nucleotide sequencing verified the authenticity of the amplified genes, whose sequences were compared with reference sequences of the *16S rRNA* and *18S rRNA* genes, and the isolate sequences from this work were posted in GenBank. A microscopic analysis of thin Diff-quick-stained blood smears identified big intra-erythrocytic *Babesia sp*., *Theileria sp*., and *Anaplasma sp*. in thirty four dogs. The PCR investigation revealed *Anaplasma sp*. in 77 dogs (27.5%), *Babesia sp*. in 55 dogs (19.6%), and *Theileria sp*. in 63 dogs (22.5%). The identification and similarity scores between the isolates of this investigation and the reference strains were 100% identical. The findings of this study indicate that stray dogs are reservoirs of *Anaplasma spp*., *Babesia spp.*, and *Theileria spp.*, potentially playing a significant role in the epidemiology and dissemination of blood parasites, hence posing a substantial threat to the cattle industry.

Key words: Iraq, Blood-Borne Parasites, Stray dog, Nested PCR assay

Introduction

Ticks are ubiquitous obligate blood-sucking ectoparasites within the class Arachnida, non-permanent arthropods that infest both animals and humans. They are responsible for various diseases that lead to economic repercussions through morbidity or mortality, as they serve as vectors for multiple infections, including haemoprotozoa, bacteria and viruses (Eisen, 2022). Infections with blood-borne parasites have long been recognized as a major health threat for dogs and other animals, as they affect the vascular system and can occur intraerythrocytically,

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intraleukocytically, or in free forms. Among the most common disorders are babesiosis, ehrlichiosis, and anaplasmosis, which are transmitted mainly by ticks and occasionally through blood transfusion, biting, or transplacental transmission (Phuyal et al, 2017; Tamang, 2023). Canine babesiosis is caused by different intraerythrocytic protozoa such as *Babesia canis*, *B. gibsoni*, and *B. vogeli*, with dogs acting as both hosts and reservoirs, representing a risk for other domestic and wild animals (Baneth, 2018; Li et al, 2020). Theileria species such as *T. annae* and *T. equi* have also been reported in dogs, reflecting the close overlap between small Babesia-like organisms and *Theileria* spp. (Baneth et al, 2015; Bahrami et al, 2018). *Anaplasma* species, belonging to the family Anaplasmataceae, represent another group of emerging tick-borne pathogens, including A. phagocytophilum and A. platys, with zoonotic potential and considerable genetic variability that influence host specificity and pathogenicity (Rar et al, 2021). These blood parasites are associated with significant hematological and biochemical alterations, including anemia, thrombocytopenia, leukocyte abnormalities, and biochemical imbalances that can threaten animal survival (Sykes, 2022). With the increasing prevalence of these pathogens and their role as reservoirs of infection, molecular tools such as nested PCR have become crucial for detection and epidemiological studies (Weese and Evason, 2019). The present study, therefore, aimed to investigate the blood-borne parasites, including hemoparasites such as anaplasmosis and babesiosis, by using conventional and nested PCR approaches, and to provide an insight into their occurrence and potential impact.

Materials and methods

Description of the study sampling, period and location

A total of 280 stray dogs (137 male and 143 female) were collected randomly from the beginning of May to the end of December of (2024) and different regions of Wasit (Al-kut, AL-sweara, Al- Aziziah, Al-Numaniyah, Al-Hay and Badra). Each dog was examined systemically, and then the information about gender and age was recorded.

Blood sample collection

Blood samples were obtained from infected dogs. All dogs exhibited no symptoms at the time of sample collection. Blood samples were collected from the animals' jugular veins under aseptic circumstances and stored in 10ml ethylenediamine tetraacetic acid (EDTA) tubes.

Laboratory Techniques

The blood samples collected were analyzed in the Microbiology and Molecular Biology laboratories of Faculty of Veterinary Medicine Urmia University.

Microscopic examination

Immediately following the sample collection, thin blood smears were prepared for microscopic analysis. The smears were preserved in methanol for 5 minutes and thereafter stained with a 5% May-Grunwald Giemsa solution in buffer for 30 minutes. The dyed slides were analyzed under oil immersion at 1000× magnification using a Nikon microscope to detect piroplasms (Babesia, Theileria) and inclusion bodies (Anaplasma).

Molecular Assays

DNA isolation and PCR amplification

The genomic DNA was extracted from blood samples collected from both adult male and female stray dogs using the gSYAN Genomic DNA Extraction Kit (Geneaid, USA), following the guidelines provided by the manufacturer. A total of 111 DNA samples from infested dogs were selected for quantification. DNA yield (ng/µL) and purity ratios (A260/A280) were assessed using a NanoDrop® ND-2000 UV/Vis Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, Delaware, USA). To reduce the risk of cross-contamination, DNA extraction, PCR setup,

nested PCR amplification, and agarose gel electrophoresis were carried out in separate laboratory rooms. All DNA samples were subjected to nested PCR analysis using specific primers designed to identify the target blood parasites. The amplification targeted the 18S rRNA gene for *Babesia* spp. or *Theileria* spp., and the 16S rRNA gene for *Anaplasma* spp., as shown in Table 1.

Table 1: Nested PCR primers for *Babesia sp*., *Theileria sp.* and *Anaplasma sp.* detection

Microorganism	gene	PCR	primer	Sequence 5'-3'	Product size
Babesia	18S rRNA	Normal	F	ATTGGAGGGCAAGTCTGGTG	697bp
			R	TCCACCAACTAAGAACGGCC	
		Nested	F	ATTGGTCGCGTCGCTTCTAA	429bp
			R	GACTTGCGACCATACTCCCC	
Theileria	18S rRNA	Normal	F	ATTGGAGGGCAAGTCTGGTG	740bp
			R	TCCACCAACTAAGAACGGCC	
		Nested	F	TTCCGGCCCATTTTTCCAGA	556bp
			R	TGCACCACCACCCAAAGAAT	
Anaplasma	*16S rRNA*	Normal	F	GGCAAGCGTTGTTCGGAATT	861bp
			R	GCAGTGTGTACAAGACCCGA	
		Nested	F	AGGGCATGTAGGTGGTTTGG	583bp
			R	CCCTTAAAGTCCCCGGCATT	
			R	GACTTGCGACCATACTCCCC	

The PCR reaction mixture consisted of 12.5µL of Taq 2x Master mix (containing Taq DNA polymerase, dNTPs, MgCl2, KCl and stabilizers), 2µL of Forward Primer, 2µL of Reverse Primer, 0.8µ of MgCl, 5µL of DNA template and 3.5 µL of nuclease free water to a total volume of 25µ.A positive and negative controls (master mix without DNA template) were included to monitor false positive and false negative results. An initial denaturation step at 95°C for five minutes, succeeded by 35 cycles of denaturation at 95°C for 30 sec, followed by an annealing step temperature at 58°C for 30 sec. A subsequent extension step was conducted at 72°C for 2 min. (repeated for 35 cycles), followed by a final extension at 72°C for 5 min.

Gel Electrophoresis

Gel electrophoresis was done for all DNA sample amplicons including 100-3000 bp molecular ladder on first lanes on 2% agarose, all the amplicons were stained with an ethedium bromide dye. The gel was run at 100V, 300MA for 45 minutes and viewed under UV light.

Statistical analysis

Data were input and analyzed using Excel, while the significant relationships between variables were assessed by chi-square tests or Fisher's exact tests, conducted with SPSS 20.0 statistical software. A statistical significance criterion of p-value < 0.05 was established.

Results

Giemsa staining for parasites Identification in infested stray dog

Identification of parasites was based on the presence of intra- erythrocytic bodies in blood smears when they were viewed under a light microscope. *Anaplasma*, *Babesia*, and *Theileria spp.* were identified using physical characteristics of the merozoite in blood smear results. In the present work, out of the 280 blood samples examined by microscopic examination, 34 tested positive for blood parasites, resulting in an overall prevalence of 12.14% in Waist City. The individual infection rates for *Anaplasma spp.* were 5.35%, with 15 positive cases through the presence of pairs of merozoites in blood smears. for *Babesia spp*., 3.92%,

with 11 positive cases where the intra-erythrocytic piroplasms are typically rounded or double pear-shaped, located at the periphery of the infected erythrocytes, and for *Theileria spp*., lower at 2.85%, with 8 positive cases that exhibit small rod-shaped, ring, and rounded forms, which can be found within lymphocytes, as illustrated in Table (2), (Figure 1).

Anaplasma spp was the most common blood parasite infecting dogs in waste. These results indicate that *Anaplasma* is the most prevalent among the three parasites, highlighting the importance of implementing preventive and therapeutic measures against this species. Additionally, the presence of *Babesia* and *Theileria*, although lower, remains a significant health concern, especially in stray dogs living in environments prone to infection. Similar studies have been conducted in various provinces of Iraq, including Baghdad (Badawi et al, 2020). Furthermore, research has also been carried out in neighboring countries (Khanmohammadi et al, 2021; Hosseini et al, 2022). In addition, several studies have taken place in European countries, such as France (René-Martellet et al, 2015), Italy (Solano-Gallego et al, 2008), and Romania (Imre et al, 2013).

Molecular screening PCR amplification of blood parasites in infested stray dogs

The incidence of blood parasites spp. was detected in the peripheral blood samples from a total of 280 stray dogs, including both males and females, tested by nested PCR. In this study, 111 (39.64%) were infected with one or more parasitic species, including *Anaplasma spp*., *Babesia spp*., and *Theileria spp*. Analysis of PCR products after agarose gel electrophoresis of the DNA extracted from blood samples showed specific amplifications with *Anaplasma sp*. (383bp), *Babesia spp.* (429bp), and *Theileria spp.* (556bp) at the annealing temperature of 55°C as shown in Figure (2).

Prevalence of blood parasites (*Anaplasma, Babesia*, and *Theileria*) Species in stray Dogs using nested PCR molecular identification

Of all examined dogs, 111 dogs were infected with *Anaplasma* spp. (27.5%)., *Babesia* spp (19.6%) and *Theileria* spp. (22.5%) (Table 3).

Table 2: Microscopic examination of blood parasites prevalence in infested stray dog

Experiment dog	*Anaplasma*		*Babesia*		*Theileria*	
280	+	%	+	%	+	%
	15	5.35	11	3.92	8	2.85

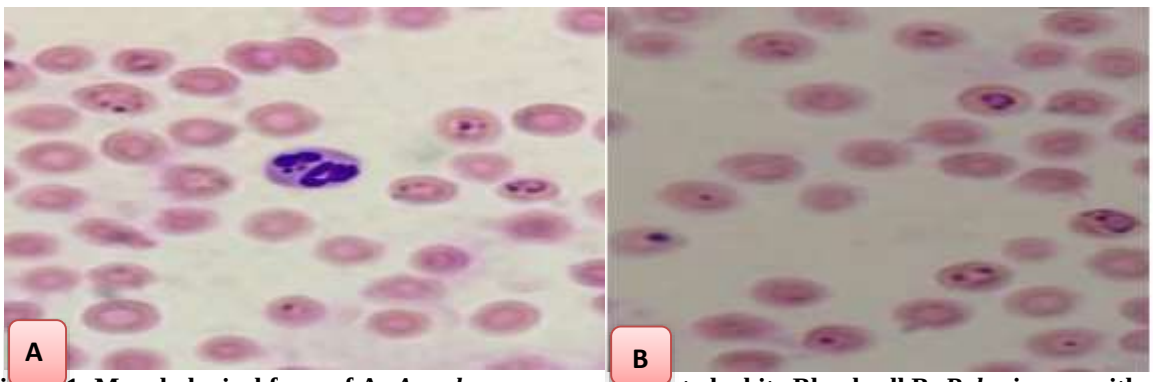

Figure 1: Morphological form of A: *Anaplasma spp* spp. Infected white Blood cell B: *Babesia spp*. with an infected erythrocyte stained with Giemsa examined under an oils immersion lens (100X).

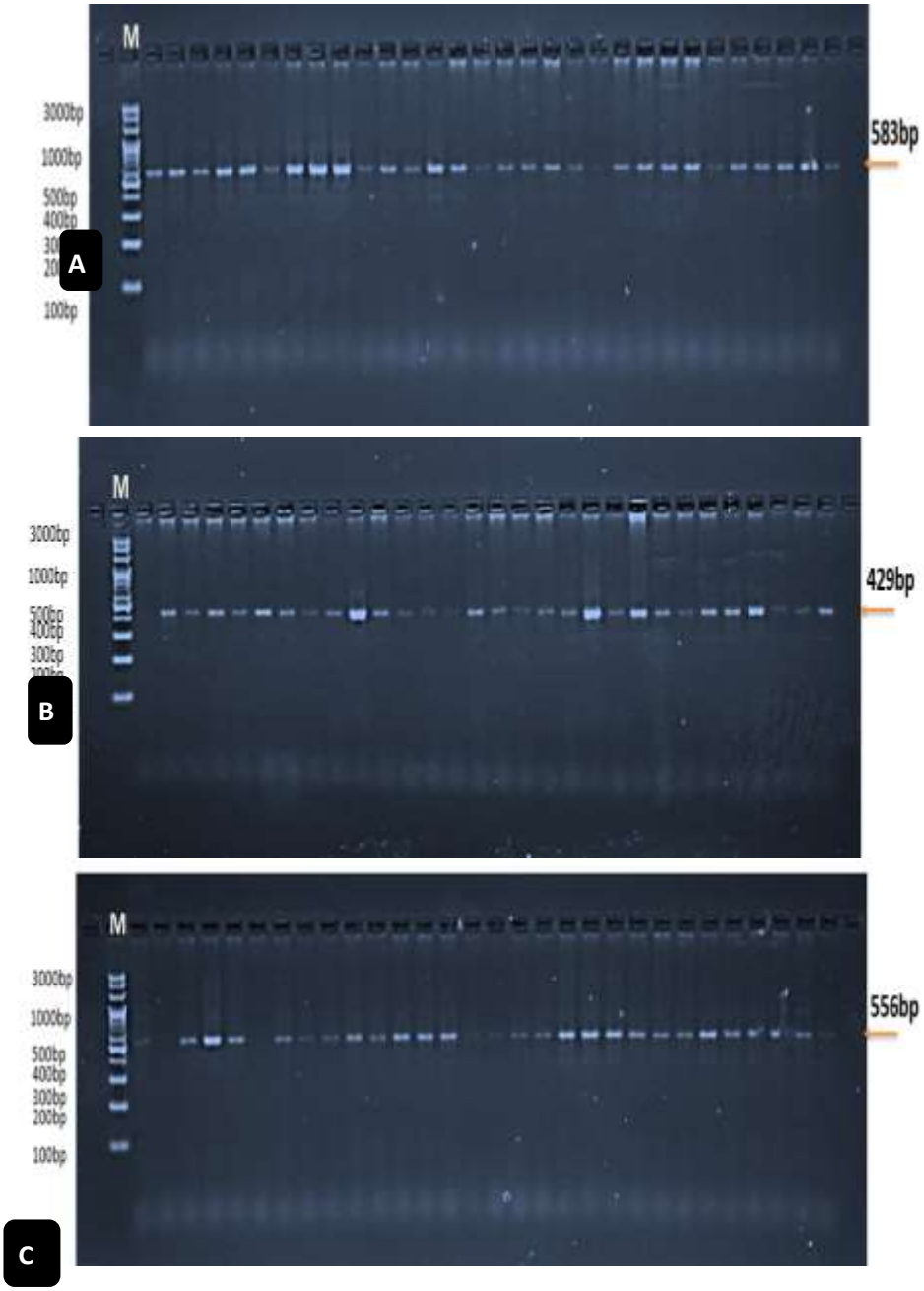

Figure 2: Nested PCR agarose gel electrophoresis products of some positive amplification small subunit ribosomal RNA gene in A: *Anaplasma spp.* **at (53bp) product size B:** *Babesia spp.* **at (429bp) product size C:** *Theileria spp.* **from stray dog blood samples. Where M: marker (100-3000bp) and positive Nested PCR was show at (556bp) product size.**

Table 3: Prevalence of (*Anaplasma Theileria***, and** *Babesia***) in stray dog's blood samples**

Parasitic Species	Number of Infected Dogs	Percentage (%)
Anaplasma	77	27.5
Theileria spp.	55	19.6
Babesia spp.	63	22.5
Total Infected	111	39.64
Total Examined	280	100

Infestation of blood parasite relation to dog's sex factor

According to sex, as illustrated in table (4), the prevalence of blood parasites infestation by a nested PCR demonstrated that in male dogs (137 total), *Anaplasma* was the most common infection (41 cases, ~29.9%), followed by *Babesia* (29 cases, ~21.2%), and then Theileria (26 cases, ~19.0%) while in female dogs (143 total): Anaplasma was also the most common infection (36 cases, ~25.2%), followed closely by Babesia (34 cases, ~23.8%), and finally Theileria (29 cases, ~20.3%).

Table 4: Prevalence of blood parasites accordance to the age factor in a Studied stray dogs

Category	Group	No. Infected dog	Positive of blood ticks infestation		
			Anaplasma	*Babesia*	*Theileria*
Sex	Male	137	41	29	26
	Female	143	36	34	29
Total No.		280	77	63	55

Discussion

The present study provides robust molecular evidence for the circulation of three clinically important blood-borne parasites *Anaplasma* spp., *Babesia* spp., and *Theileria* spp. among stray dogs in Wasit Province. The detection of these haemoparasites at relatively high frequencies underscores the role of free-roaming dogs as potential reservoirs and sentinels for vector-borne diseases, with significant implications for both veterinary and public health surveillance systems.

Molecular screening revealed that 27.5% of the examined dogs were infected with Anaplasma spp., a prevalence comparable to the previous studies from Iraq and neighboring countries, where reported rates ranged from the 20–32% depending on the dog population and diagnostic methods used. This prevalence is also consistent with research highlighting the widespread distribution of the brown dog tick, *Rhipicephalus sanguineus*, the principal vector of *A. platys*, in central and southern Iraq (Reif, 2011). In contrast, lower prevalence levels (5–15%) have been recorded in colder regions such as parts of Turkey and northern Iran, where Ixodid tick populations are more seasonal, limiting transmission periods. Meanwhile, substantially higher rates—sometimes exceeding 40%—have been observed in tropical climates (e.g., India and Brazil), likely due to year-round vector activity and dense stray dog populations. The observed prevalence in our study aligns with the ecological conditions of Wasit Province, characterized by warm temperatures and sustained vector presence. Furthermore, the close coexistence of stray dogs with human communities may elevate zoonotic spillover risks, supporting their value as sentinel species for monitoring environmental pathogen circulation (Alhassan et al, 2021).

Babesia spp. infections were identified in 22.5% of sampled dogs. This result fits within the globally reported wide range of canine babesiosis prevalence. Low to moderate prevalence (3–15%) has been reported in parts of Iran, Iraq, and Turkey, while significantly higher rates—ranging from 25% to over 50%—have been observed in regions such as India, Egypt, and parts of Southeast Asia (Laha et al, 2014; Albakri et al, 2024). These regional differences can be attributed to several factors, including vector density, climatic stability, and levels of veterinary care and tick control. Our findings for *B. vogeli*, a species strongly associated with R.

sanguineus s.l., are consistent with studies from the Mediterranean and Middle East, where this tick species is dominant. Poor tick-control practices among stray dogs, environmental conditions favoring tick survival, and lack of regular veterinary treatment likely contribute to the relatively high Babesia prevalence observed in the current study.

The prevalence of Theileria spp. in this study was 19.56%, which aligns with molecular surveys from nearby regions confirming *T. annulata* infections in dogs. However, the prevalence is lower than that reported in South Africa (66.6%) (Rosa et al, 2014), Pakistan (~40%), and certain hyperendemic regions of Sudan. Differences in species of circulating Theileria, variation in vector competence, and ecological diversity primarily explain these discrepancies. Notably, in tropical countries where *Hyalomma* ticks thrive, canine theileriosis tends to be more common and often more severe. In contrast, the relatively moderate prevalence observed in Wasit may reflect lower densities of competent Hyalomma spp. or the possibility that dogs play a secondary or accidental host role in local transmission cycles. Importantly, many infected dogs in our study exhibited subclinical or mild infections, a pattern commonly reported in endemic regions where long-term host–parasite coadaptation may lead to reduced clinical severity.

Overall, among the 280 stray dogs examined, infection with at least one tick-borne haemoparasite was documented, with Anaplasma spp. being the most prevalent (77 cases, 27.5%), followed by Babesia spp. (63 cases, 22.5%) and Theileria spp. (55 cases, 19.6%). These distribution patterns correspond with the abundance of specific tick vectors and environmental factors such as temperature and humidity, which influence vector activity and pathogen transmission. Notably, female dogs had slightly higher infection rates (143 females vs. 137 males), potentially due to increased roaming behavior during reproductive cycles, greater exposure to tick habitats, or sex-related differences in immunity (Nasr and Ghafar, 2020). Comparable sex-associated patterns have been observed in studies from Iran, India, and Egypt.

Taken together, the findings emphasize the active transmission of multiple haemoparasites among stray dogs in Wasit Province. The data reflect the ecological suitability for vector proliferation and highlight the need for integrated tick control programs, public awareness campaigns, and sustained veterinary surveillance to reduce transmission risks to both animals and humans.

In conclusion, findings of this study suggest that, molecular detection confirmed the presence of three haemoparasites, *Anaplasma sp*, *Babesia sp* and *Theilleria sp* primarily transmitted by stray dogs in Wasit Province. These findings indicate potential public health risks and underline the importance of monitoring programs, preventive deworming, and improved management of stray dog populations to reduce the transmission of blood-borne parasites to domestic animals and humans.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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تشخیص مولکولی انگل های خونی منتقله از کنه (تیلریا، بابزیا و آناپلاسما) در سگ‌های بی صاحب با استفاده از Nested PCR

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چکیده

عفونت‌های انگلی خونی اغلب در سگ‌های مناطق گرمسیری، از جمله عراق، مشاهده می‌شوند. سگ‌ها به چندین انگل خونی آلوده می‌شوند که منجر به بیماری‌های جدی در آن‌ها می‌شود. این تحقیق به منظور تشخیص میکروسکوپی و مولکولی انگل‌های خونی سگ در استان واسط عراق انجام شده است. این مطالعه مقطعی از ابتدای ماه مه ۲۰۲۴ تا پایان دسامبر ۲۰۲۴ انجام شد. در مجموع از ۲۸۰ سگ بی‌صاحب در مناطق مختلف استان واسط عراق نمونه‌برداری انجام شد. نمونه خون از ورید گردن سگ‌ها گرفته شد و برای آنالیز میکروسکوپی و مولکولی مورد استفاده قرار گرفت. گسترش‌های نازک خون تهیه شده و به منظور شناسایی آلودگی به بابزیا، تیلریا و آناپلاسما با رنگ گیمسا رنگ‌آمیزی شدند. در ادامه از نمونه‌های خون استخراج DNA انجام شد و آزمایش nested PCR با استفاده از ژن 16S rRNA برای گونه‌های آناپلاسما و ژن 18SrRNA برای تشخیص گونه‌های تیلریا و بابزیا استفاده شد. در نهایت محصولات PCR با الکتروفورز ژل آگارز تأیید شدند. توالی‌یابی نوکلئوتیدی، صحت ژن‌های تکثیر شده را تأیید کرد و توالی‌های آنها با توالی‌های مرجع ژن‌های 16S rRNA و 18S rRNA مقایسه شد و توالی‌های جدا شده در GenBank قرار گرفتند. در گسترش‌های خونی رنگ‌آمیزی شده گونه‌های بابزیا، تیلریا و آناپلاسما در سی و چهار سگ شناسایی شد. بررسی PCR گونه‌های آناپلاسما را در ۷۷ سگ (۲۷/۵٪)، گونه‌های بابزیا را در ۵۵ سگ (۱۹/۶٪) و گونه‌های تیلریا را در ۶۳ سگ (۲۲/۵٪) نشان داد. شباهت بین جدایه‌های این تحقیق و سویه‌های مرجع ۱۰۰ درصد یکسان بود. یافته‌های این مطالعه نشان می‌دهد که سگ‌های ولگرد مخازن گونه‌های آناپلاسما، بابزیا و تیلریا هستند که به طور بالقوه نقش مهمی در اپیدمیولوژی و انتشار انگل‌های خونی دارند و از این رو تهدیدی اساسی برای صنعت گاوداری محسوب می‌شوند.

کلمات کلیدی: عراق، انگل‌های منتقله از راه خون، سگ‌های بی صاحب، روش nested PCR

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