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Microscopical and molecular diagnosis of canine babesiosis in stray dogs in Erbil, Iraq

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Abstract

Canine babesiosis, or tick-borne protozoal disease, is prevalent among canines globally and is associated with anemia and potentially fatal illnesses in both dogs and wild animals. This research investigated babesiosis in stray dogs from Erbil Province, Iraq, employing microscopic and molecular detection practices. One hundred forty-two blood samples had been collected from stray dogs brought to private clinics and the Erbil dog shelter. Along with the samples, information regarding age group and gender was recorded. Initially, the blood samples underwent examination using the Giemsa-stained blood smear technique. Subsequently, DNA extraction was performed, and the identification of *Babesia* spp. was confirmed by amplifying the 18S and 28S rRNA genes. The overall incidence was 6.3% using microscopic techniques and 15.5% using PCR. Common clinical symptoms in infected dogs were fever, pale mucous membranes, anorexia, and depression. Molecular testing revealed a significantly greater prevalence of babesiosis in younger dogs 25.5% and female dogs 20.6% compared to older and male dogs. A significant difference was also found between tick infestation and poor body condition. A Phylogenetic study revealed that the two *Babesia* spp. from the current research with accession numbers OR896912 and OR896913 *B. vogeli* and OR896867 and OR896868 *B. gibsoni* have indicated 98-99% similarity to species isolated in India, China, and Brazil. To summarize, the findings underscore the prevalence of canine babesiosis in the region, potentially offering insights to aid in its control measures.

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Introduction

Canine babesiosis (CB), a tick-borne protozoal disease, is prevalent among canines globally and is associated with anemia and potentially fatal illnesses in both dogs and wild animals (1). The genus *Babesia*, classified within the order Piroplasmida in the phylum Apicomplexa, presents as non-pigment-forming, pear-shaped, or single-ring-shaped organisms within the erythrocytes of mammals (2). Over 100 *Babesia* species have been documented in vertebrate hosts (3,4). At least six well-defined species of *Babesia*, including *B. canis* (5), *B. gibsoni* (6), *B. microti-like* (7), *B. canis vogeli* (8), *B. rossi* (9), and *B. conradae* (10) are known to cause both clinical and subclinical illness in dogs. Moreover, numerous recent research studies have documented the spread and presence of CB in many different countries, including Europe (11-15), India (16), Brazil (17), Qatar (18), Turkey (19,20), Iran (21) and Iraq (5,22,23). The prevalence of babesiosis and its geographic distribution typically fluctuate based on the tick species in a specific region (24-26). *Babesia* can be spread through tick vectors, blood transfusions, and dog bites, and the pathogen can cross the

placental barrier (1,27-28). CB can vary in severity from sub-clinical to acute and fatal, depending on the pathogenicity of the protozoa species or strain (27) and also on the risk factors of the specific host in terms of its age, immunological condition, and associated infection or disease (1,29,30). Clinical manifestations commonly described in dogs suffering from babesiosis include lethargy, pale mucous membranes, hyperthermia, anorexia, splenomegaly, hemoglobinuria, thrombocytopenia, and hemolytic anemia (25,31). Several laboratory methods have been employed to diagnose CB. Light microscopy of stained blood smears was used for acute cases. Additional criteria, especially molecular techniques, have further high sensitivity and specificity for the detection and differentiation of CB (5,32-34).

Although there is a lack of data regarding the prevalence of CB infection in Erbil, this research was designed to study babesiosis in naturally exposed dogs. The investigation involved blood stain analysis, conventional polymerase chain reaction (c-PCR), sequencing, and taxonomic analyses.

Materials and methods

Ethical approve

All methods and procedures used in a recent study were completed according to the Scientific Ethical Committee on Animal Experimentation guidelines at the College of Veterinary Medicine, University of Mosul, UM.VET.2021.080.

Sample collection

Blood samples were taken from 142 stray dogs of various ages and sexes. From December 2021 to September 2023, these dogs were presented to private clinics and the Erbil dog shelter in Erbil Province. Clinical signs, body temperature, respiratory rate, pulse rate, and symptoms of diseases were documented.

Examination of blood smears

Blood samples were aseptically collected from the cephalic vein and kept in EDTA tubes. A thin blood smear was prepared for each sample by placing a droplet of blood onto a pristine glass slide. The smear was air-dried, fixed in absolute methanol for 5 minutes, and then stained with 10% Giemsa for 30 minutes, following the method described by Coles (35). The prepared slides were examined under a light microscope using the oil immersion objective at a magnification of 100X to identify and analyze the morphological characteristics of *Babesia* species (35).

Molecular assay

Genomic DNA has been extracted from 200 µl of blood specimens with the Primary Prep TM Genomic DNA Isolation Kit (addbio, Korea) following the manufacturing method a fragment of the *Babesia* spp. 18S rRNA gene and 28S rRNA targeting gene were amplified by species-specific PCR using two primers for *B. vogeli* and *B. gibsoni* (Table 1). In a c-PCR assay, the reaction mixture is prepared with a total volume of 25 µl, consisting of 12.5 µl of 2x PCR master mix, 1 µl of 10 pmol of each primer (forward and reverse) for each gene set, 3 µl of the DNA template, and 7.5 µl of distilled water (dH2O). The PCR protocol for *B. vogeli* and *B. gibsoni* involves several steps, each with specific temperatures and durations tailored to the species. The process begins with an initial denaturation of DNA at 94ºC. For *Babesia* spp., this step lasts 1 minute, while for *B. vogeli* and *B. gibsoni*, it extends to 2 and 5 minutes, respectively. The DNA is then denatured at 94ºC for 45 seconds for *Babesia* spp., while for *B. vogeli* and *B. gibsoni*, the duration is 30 seconds. The annealing of primers follows, occurring at 62ºC for 45 seconds for *Babesia* spp., at 56ºC for 30 seconds for *B. vogeli*, and at 61ºC for *B. gibsoni*. The extension step is conducted at 72ºC for 1 minute for both species. Steps 3 through 5 are cycled 35 times to amplify the DNA. A final extension is performed at 72ºC for 7 minutes, and the samples are then stored at 4ºC until they are removed (8,36,37). After amplification, the PCR products were run on a gel containing 1.5% agarose and imaged with a gel documentation apparatus to get images.

Table 1: Nucleotide primers used to amplify the 28S and 18S rRNA genes of *Babesia*

Primers	Sequences 5'-3'	Target gene	Expected size (*bp*)	References
PIRO-A PIRO-B	5'- AATACCCAATCCTGACACAGGG -3' 5'- TTAAATACG AATGCCCCCAAC -3'	*Babesia* spp.	410	35
BAB1 F BAB4 R	5'- GTGAACCTTATCACTTAAAGG -3' 5'- CAACTCCTCCACGCAATCG -3'	*B. vogeli*	590	8
BAGIF BAGIR	5' TTGGCGGCGTTTATTAGTTC 3' 5' AAAGGGGAAAACCCCAAAAG 3'	*B. gibsoni*	488	37

PIRO-A: Universal forwards primers, PIRO-B: Universal reverse primers, BAB1 F: *B. vogeli* - targeted forward primer, BAB4 R: *B. vogeli* - targeted reverse primer, BAGIF: *B. gibsoni* - targeted forward primer, BAGIR: *B. gibsoni* - targeted reverse primer.

The sequencing of DNA and analyses of phylogeny

In the present work, six PCR primers (two sets for each universal *Babesia* sp., *B. vogeli*, and two for *B. gibsoni*) that tested positive with c-PCR were sent to a commercial business for purification and sequencing using Sanger dideoxy sequencing (Macrogen Inc., South Korea). The gene sequence similarity comparisons with prior genes available in the Genbank databases have been performed with the BLAST tool. The MEGA7 was applied for multiple sequence alignments. The Muscle program was applied to align the sequences with the 18S rRNA genes of *B. velgoni* and *B. gebsoni*, obtained from GenBank (38). The phylogenetic tree has been created using three distinct algorithms: maximum likelihood, neighbor-joining, and the Tamura-Nei model. One thousand bootstrap replicates were used to evaluate the dependability of nodes (39).

Statistical analysis

The data was analyzed using the X2 and Fisher's exact tests to determine the occurrence rates between groups. Binomial logistic regression in GenStat 12th Edition was used to calculate the odds ratio and 95% confidence intervals for determining the prevalence rate, with a p-value of less than 0.05 considered statistically significant (40).

Results

Using microscopic examination of blood smears stained by Giemsa, Babesia species were identified using physical characteristics of the merozoite in infected RBCs. Furthermore, typical *Babesia* spp. emerged as a small parasite measuring based on double pyriform with an acute or obtuse angle, and it occurred in a variety of morphological forms within the RBCs, including oval, spherical, and double pear shapes, which was one of the most prominent shapes (Figure 1). The overall prevalence was 6.3% (9/142) and 15.5% (22/142) by microscopical and molecular examination, respectively.

The clinical findings in a study of 22 dogs infected with babesiosis revealed a range of symptoms. Anorexia was observed in 5 dogs (23%). Fever was less common, affecting 2 dogs (9%). Depression was reported in 6 dogs (27.2%) of the cases, while vomiting and diarrhea were seen in only 1 dog (4.5%). Icterus, characterized by yellowing of the skin and mucous membranes, was found in 3 dogs (13.6%). Pale mucous membranes were noted in 7 dogs (31.8%). The most prevalent finding was tick infestation, which was present in 12 dogs (54.4%).

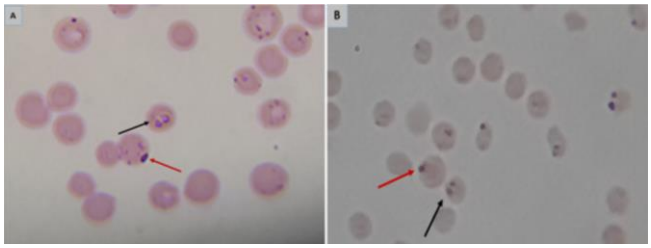

Figure 1 Morphological forms of *Babesia* spp. within infected erythrocytes stained with Giemsa, examined under an oil immersion lens (100X); A) Two pear-shaped merozoites (black arrowhead), One pear-shaped merozoite (red arrowhead); B) Two round-shaped merozoites (black arrowhead), One round -shaped merozoites (red arrowhead).

The current study (Table 2) found that the prevalence of Babesia spp. by microscopically and molecular testing did not significantly differ across genders and age groups of dogs. By microscopic examination, females and males recorded 7.9% (5/63) and 5% (4/79) without significant differences. While the odds of infection by molecular examination of the female recorded were males 20.6% (13/63) and males 11.4% (9/79), without significant difference. However, a higher rate of infection with CB was recorded in the young and adult age groups, 22.2% (4/18) and 25.5% (13/51), compared with old groups, 6.8% (5/73), without significant differences by molecular examination (Table 3). Microscopic and molecular examination revealed that the infection rate in dogs with tick infestation was 11.7% and 23.5%, respectively. Thus, the odds of infection in dogs with tick infestation were 3.9 and 2.5 times higher than in dogs without tick infestation (CI, 0.39- 16.37) and (CI, 0.99-6.26) with significant differences (P=0.052) (Table 4). However, poor body condition was significantly more prevalent (P<0.001) in dogs infected with CB than those in good condition (Table 5).

The outcomes of the amplified (PCR) product using general or universal primers (Macrogen Inc., South Korea) revealed that the DNA band size for the initial reaction was 410 bp, indicating a positive presence of *Babesia* spp. (Figure 2). In contrast, the second reaction using specific primers for *B. vegoli* displayed a DNA band size of 590 bp (Figure 3). However, the third reaction using specific primers for *B. gibsoni* was 488 bp (Figure 4).

Table 2: Sex prevalence of CB by microscopic examination and c-PCR

Factor	Dog examined (n)	Microscopic examination			c-PCR		
		N. (%)	OR (95%CI)	*P*	N. (%)	OR (95%CI)	*P*
Female	63	5 (7.9)	0.86 (0.05-0.24)	0.489	13 (20.6)	0.26 (0.14-0.48)	0.135
Male	79	4 (5)	0.62 (0.16-2.41)		9 (11.4)	0.49 (0.19-1.25)	

N: Number of positive samples, CI: Confidence interval, OR: Odd ratio.

Table 3: age prevalence of CB by microscopic examination and c-PCR

Factor	Dog examined (n)	Microscopic examination			c-PCR		
		N. (%)	OR (95%CI)	*P*	N. (%)	OR (95%CI)	*P*
Young	18	2 (11)	0.12 (0.03-0.54)	0.674	4 (22.2)	0.28 (0.09-0.87)	0.782
Adult	51	4 (7.8)	0.68 (0.11-4.07)	0.262	13 (25.5)	1.2 (0.33- 4.29)	0.064
Old	73	3 (4)	0.34 (0.05-2.22)		5 (6.8)	0.26 (0.06-1.08)	

N: Number of positive samples, CI: Confidence interval, OR: Odd ratio.

Table 4: Tick infestation prevalence of CB by microscopic examination and c-PCR

Factor	Dog examined (n)	Microscopic examination			c-PCR		
		N. (%)	OR (95%CI)	*P*	N. (%)	OR (95%CI)	*P*
Yes	51	6 (11.7)	3.9 (0.93-16.37)	0.062	12 (23.5)	2.5 (0.99- 6.26)	0.052
No	91	3 (3.3)	0.03 (0.01-0.10)		10 (11)	0.12 (0.06- 0.24)	

N: Number of positive samples, CI: Confidence interval, OR: Odd ratio.

Table 5: Body condition score-wise prevalence of CB by microscopic examination and c-PCR

Factor	Dog examined (n)	Microscopic examination			c-PCR		
		N. (%)	OR (95%CI)	*P*	N. (%)	OR (95%CI)	*P*
Good	83	3 (3.6)	0.04 (0.01-0.12)	0.13	5 (6)	0.06 (0.03-0.16)	<0.001
Poor	59	6 (10)	3.02 (0.72-12.60)		17 (28.8)	6.31 (2.18-18.32)	

N: Number of positive samples, CI: Confidence interval, OR: Odd ratio.

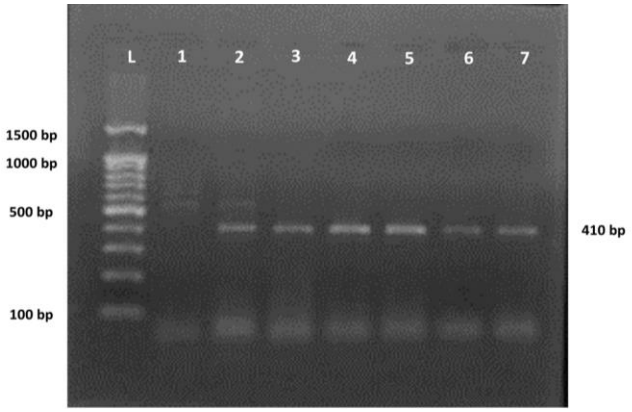

Figure 2: Gel electrophoresis image showing PCR detection of *Babesia* spp. with a pair of universal primers ((Macrogen Inc, South Korea): Lanes L) 100 bp ladder DNA marker; Lane 2-7) *Babesia* spp. in approximately band size 410 bp; Lane 1) negative control.

Phylogenetic trees of 18S rRNA sequence analyses based on a neighbor-joining program showed that *Babesia* spp. have a relation to *B. vegoli*. The two sequences blasted with previous GenBank registration and found that OR896912 and OR896913 showed 99.14% identity with MG251701/India and 95.81% identity with MN823217, W627326/Brazil, While OR896867 and OR896868 showed 98.37% identity with MG252702/India and OP445697/China to those sequences previously reported *B. gibsoni* in NCBI Genbank (Figure 5). A phylogenetic tree of *B. gibsoni* showed that the two sequence isolates (OR896867 and OR896868) observed in the present study clustered with MG252702, MN689648, and OP445697, with 98.37% similarity between isolates from India and China. However, the phylogenetic analyses of *B. vogeli* isolates OR896912 and OR896913 showed 99.14% identity with MG251701/India and 95.81% identity with MN823217, W627326/Brazil (Figure 6).

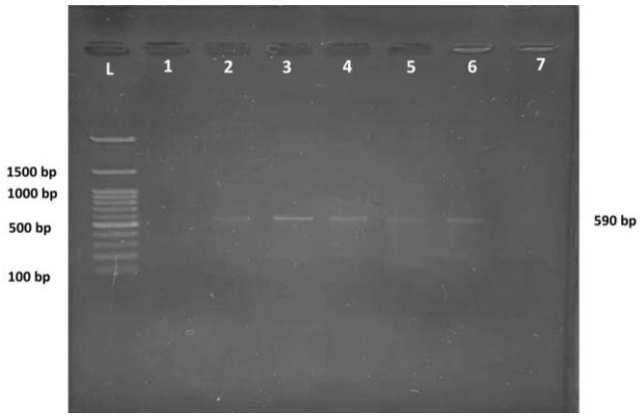

Figure 3: A gel electrophoresis picture illustrating PCR detection *of B. vogeli* using a pair of specific primers (Macrogen Inc, South Korea): Lanes L) 100 bp DNA marker; Lane 2-6 *B. vogeli*. in approximately band size 590 bp; lane 1) negative control.

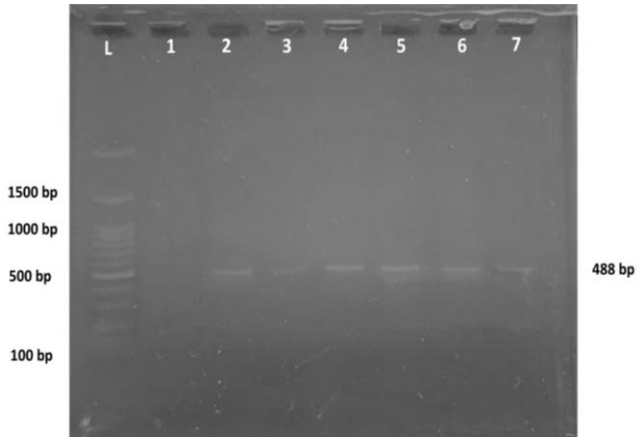

Figure 4: A gel electrophoresis picture illustrating PCR detection of *B. gibsoni* with a pair of specific primers (Macrogen Inc, South Korea): Lanes L) 100 bp DNA marker; Lane 2-7 *B. gibsoni*. in approximately band size 488 bp; lane 1) negative control.

The DNA sequences of the 18S rRNA gene have been made accessible at NCBI GenBank with these accession numbers: *B. vegoli*: (OR896912 and OR896913); *B. gibsoni*: (OR896867 and OR896868). Diversity in phylogenetic identification depends on query coverage, with 59% (13/22) of positive cases infected by *B. vegoli* and 40.9% (9/22) infected by *B. gibsoni*.

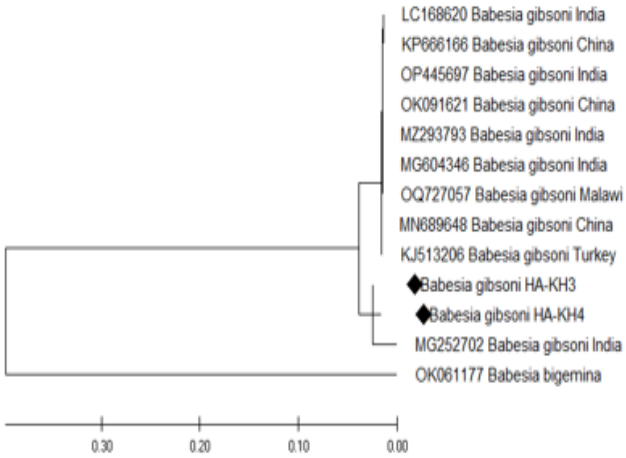

Figure 5: Shows the phylogenetic tree created with maximum likelihood based on partial 18S rRNA sequences from *B. gibsoni*. The sequences obtained in this work are denoted by a black diamond, whereas the rest are derived from GenBank.

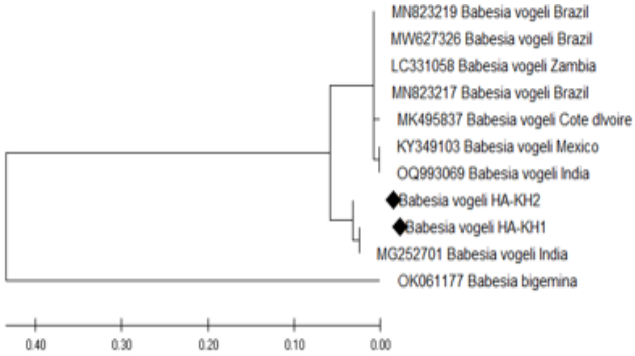

Figure 6: Shows the phylogenetic tree created with maximum likelihood based on partial 18S rRNA sequences from *B. vogeli*. The sequences obtained in this work are denoted by a black diamond, whereas the rest are derived from GenBank.

Discussion

Canine babesiosis (CB) is a life-threatening tick-borne disease prevalent worldwide in dogs, caused by different species of the protozoan genus *Babesia*. There have been minimal studies on CB reported previously in various regions of Iraq, using Giemsa-stained blood smears (23,41, 22) and PCR (5,41) in Basrah and Baghdad Province. This study represents the first attempt to identify CB in stray dogs in the Erbil province, Iraq. Consequently, this study aims to evaluate the occurrence of CB infection in Erbil Province, Northern Iraq, employing microscopic and molecular approaches and to document the genetic sequencing for subsequent analysis.

The data collected in the present study show that the overall infection rate with CB was 6.3% (9/142) and 15.5% (22/142) by microscopical and molecular examination, respectively. Similar studies have been conducted in other provinces of Iraq with a rate of 48.14% (21) by microscopic examination while, by molecular examination, 5.16% (42) in Baghdad. Additionally, in neighboring Iraqi countries, rates of 25% (21), 9.3% (43), and 39% (44) have been observed in Iran. However, the rate in Turkey was 41.4% (45,46). In some European countries, the prevalence was found to be 13.6% in France (47), 6.7% in Italy (48), and 28.6% in Romania (49).

The variability in results could be attributed to the various examination techniques used in each study, the limitations of the examination methods, the methodology, the sample collection criteria, the variable amounts of exposure to multiple risk factors, particularly tick vectors, over a long period due to hot weather in the study area, and the fact that the samples were collected from a shelter with a significant number of dogs (50-52). This data confirms that temperature and ecology in the Mediterranean area

establish a good habitat for activating real tick species on dogs all year around (50,53).

The clinical manifestations reported in the present investigation are similar to those described by Alsaad *et al*. (23); Mahalingaiah *et al*. (37); Hosseini *et al*. (44); Davitkov *et al*. (54); Wang *et al*. (55). Our study showed that the prevalence of *Babesia* spp. by both microscopical and molecular examination did not significantly differ between genders and age group of dogs. While the odds of infection by molecular examination of the female recorded were males 20.6% (13/63) and males 11.4% (9/79), without significant difference. However, a higher rate of infection with CB was recorded in the young and adult age groups, 22.2% (4/18) and 25.5% (13/51), compared with old groups, 6.8% (5/73), without significant differences by molecular examination. This agrees with the findings of Badawi and Yousif (5), who observed a lower prevalence employing microscopy diagnosis than molecular approaches. The significant variation in identification is most likely related to the higher specificity of PCR-based diagnosis and the low levels of parasites, especially evident throughout the chronic phase or carrier state (56,57). However, microscopical examinations are only useful for detecting infection in its acute stage and are ineffective for identifying presymptomatic and carrier animals with low parasitemia (58).

The risk of CB was substantially higher among dogs infested with ticks and in poor body condition compared to dogs without tick infestation and in good body condition. This greater risk of infection might be attributed to ticks, which are known to be their primary vectors. These findings align with earlier research, which found that ticks were a risk factor for canine babesiosis (5,44,59).

The 18S rRNA gene is commonly utilized for molecular detection and phylogenetic studies of canine babesiosis (44,45). The present study provides the first data on the genetic diversity of CB from Erbil based on 18S and 28S rRNA gene sequences. Phylogenetic analyses of 18S rRNA sequences revealed that two sequences of *B. vegoli* shared 99.14% identity with MG251701/India and 95.81% identity with MN823217, W627326/Brazil. Meanwhile, sequences of *B. gibsoni* showed 98.37% identity with MG252702/India and OP445697 China, compared to those previously reported for *B. vegoli* and *B. gibsoni* in the NCBI GenBank.

Conclusion

This research presents the first confirmed cases of CB caused by *Babesia vogeli* and *Babesia gibsoni* in stray dogs in Erbil Province, Iraq. PCR and sequence analysis are more sensitive diagnostic methods than microscopy. The study examined risk factors for CB, including sex, age, and tick infestation. The findings suggest that regular monitoring is necessary to limit the incidence of *Babesia*.

Conflicts of interest

The author declares that there is no conflict of interest.

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التشخيص المجهري والجزيئي لداء البابيزيا في الكلاب الضالة في أربيل، العراق

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الخلاصة

داء بابيزيا الكلاب من أمراض الأوالي الطفيلية والذي ينقله القراد وينتشر بين الكلاب على مستوى العالم ويرتبط بفقر الدم، ويعتبر من الأمراض القاتلة في كل من الكلاب والحيوانات الأليفة والبرية. أجريت هذا الدراسة للكشف عن داء البابيزيا في الكلاب الضالة في محافظة أربيل، العراق، وذلك باستخدام فحص الكشف المجهري والجزيئي. تم جمع ١٤٢ عينة دم من الكلاب الضالة والتي تم إحضارها إلى العيادات الخاصة وكذلك من مأوى الكلاب في محافظة أربيل. خلال جمع العينات، تم تسجيل المعلومات المتعلقة بالفئة العمرية والجنس. تم فحص عينات الدم باستخدام تقنية مسحة الدم الملطخة بصبغة كيمزا بعد ذلك، تم إجراء عزل الحمض النووي وتحديد جنس الطفيلي البابيزيا. وقد وجد أن معدل الإصابة الإجمالي للطفيلي هو ٦,٣% باستخدام التقنيات المجهرية و ١٥,٥% باستخدام تقنية تفاعل البلمرة المتسلسل. كانت الأعراض السريرية الشائعة في الكلاب المصابة هي الحمى وشحوب الأغشية المخاطية وفقدان الشهية والاكتئاب. أكدت الاختبارات الجزيئية عن انتشار المرض البابيزيا في الكلاب الأصغر سننا ٢٥,٥٪ ونسبة انتشاره في الإناث من الكلاب هو ٢٠,٦٪ مقارنة بالكلاب الأكبر سنا والذكور. كما وجد أن هناك فرق كبير بين الإصابة بالقراد وسوء حالة البدنية للكلاب. تم تسجيل الجنسين من بابيزيا في البنك الجينات الدولي لبابيزيا فيجولي وبابيزيا جيبسوني وقد أشارت إلى تطابق بنسبة ٩٨-٩٩٪ مع الأنواع المعزولة في الهند والصين والبرازيل. وفي الخلاصة، تؤكد هذه النتائج على انتشار بابيزيا الكلاب في المنطقة، مما قد يقدم مجال لعمل لدراسات أخرى للمساعدة في السيطرة على هذا المرض.

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