Microbial Pathogenesis 196 (2024) 106952

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Clinical pathology and molecular examination of *Babesia* spp. infection in dogs; Mashhad, Northeast Iran

Mohammad Hossein Kafrashi [a], Gholam Reza Razmi [b], Mahdieh Zaeemi [a,*]

[a] *Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Iran*
[b] *Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran*

ARTICLE INFO

Keywords:
Babesiosis
Dog
Northeast Iran
Prevalence
Species
Clinical and laboratory findings

ABSTRACT

This study aimed to fill a crucial gap in our understanding of *Babesia* infection in dogs in Mashhad, northeast Iran. We not only investigated the prevalence of *Babesia* species among dogs but also undertook a comprehensive comparison of clinical, hematological, and clinicopathological findings between infected and non-infected cases, a unique aspect of our research.

Materials and methods: Our research was conducted with meticulous attention to detail. We randomly collected blood specimens from a diverse population of 150 dogs, including owned pets (n = 47), stray dogs (n = 66), and shelter dogs (n = 37), to ensure the reliability and representativeness of our findings. We then used microscopy and PCR to investigate *Babesia* spp. infection and analyzed various biochemical and hematological variables.

Results: The overall prevalence of babesiosis was 15.3 % (23/150) by PCR and 2 % (3/150) by microscopy. Upon microscopic examination, two cases of large *Babesia* and one case of small-sized *Babesia* were identified. The sequencing results confirmed that the two dogs testing positive for large-sized Babesia species in this study were both infected with *B. vogeli*, exhibiting 100 % sequence identity.

There was no association between infection and gender, while housing status (k = 37.294, p = 0.000) and age (k = 6.897, p = 0.021) significantly related to infection rate. Among laboratory variables, infection with *Babesia* spp. showed a remarkable association with Hct (k = 4.749, p = 0.025) and RBC count (k = 14.669, p = 0.000), which were significantly lower in infected dogs compared to non-infected dogs ($p < 0.05$). Aside from severe non-regenerative anemia observed in all three clinically infected cases, the most clinicopathological changes were observed in one *B. vogeli*-infected dog, including pancytopenia, azotemia, hyperphosphatemia, hyperkalemia, hypoglycemia, hypocholesterolemia, hyponatremia.

Conclusion: This study reveals a higher-than-expected prevalence of canine babesiosis in Northeastern Iran, necessitating further investigation of tick vectors and *Babesia* spp. distribution. Notably, many infected dogs were asymptomatic, raising concerns about silent spread via carriers. Moreover, the high prevalence of infection in shelters highlights the need for more effective control strategies in these centers.

1. Introduction

Canine babesiosis, a tick-borne disease found around the world, is caused by two main parasites: *Babesia canis* and *Babesia gibsoni* which were categorized based on their size as large (>2.5 μm) and small (<2.5 μm) *Babesia* spp respectively [1]. The development of molecular methods classified them as genetically distinct species (*B. canis, B. rossi, and B. vogeli*) [2,3]. So far, only three clinically important small *Babesia* spp (*B. gibsoni*, *B. conradae*, and *B. vulpes)* have been identified So far, only three clinically important small *Babesia species (B. gibsoni, B. conradae*, and *B. vulpes*) have been identified as capable of infecting dogs [4]. as able to infect dogs [4].

Despite the global distribution of canine babesiosis, different species spread in specific areas. Infection with *B. canis* mainly occurs in Europe and Asia, *B. rossi* is limited to Africa, while *B. vogeli,* the most widespread prevalent large *Babesia* spp., is found in Africa, southern regions of Asia and Europe, northern Australia, South America, and southern parts of North America [5]. *Babesia gibsoni,* a parasite once limited to certain parts of Asia, spread gradually to the Middle East and Africa, North America, Brazil, and Australia. Nowadays, this species has become a

* Corresponding author.
E-mail address: zaeemi@um.ac.ir (M. Zaeemi).

https://doi.org/10.1016/j.micpath.2024.106952
Received 21 July 2024; Received in revised form 9 September 2024; Accepted 15 September 2024
Available online 19 September 2024
0882-4010/© 2024 Elsevier Ltd. All rights are reserved, including those for text and data mining, AI training, and similar technologies.

global concern. In Europe, where the natural vector isn't present, the prevalence of this infection has also likely increased due to increased traveling with dogs, exotic breed imports, and illegal dogfighting [6].

To the authors' knowledge, a few published molecular studies in this field are limited to the western half of Iran [7–11]. Our first aim was the molecular and microscopic detection of canine *Babesia* spp. in northeast Iran which has never been studied comprehensively. This region has different climate conditions that may affect the distribution of carriers and the intensity of ticks feeding on dogs. So, the second aim was the evaluation of hematological and serum biochemical findings in infected dogs.

2. Materials and methods

2.1. Animals and blood collection

This study was conducted in Mashhad (Fig. 1), located in the northeastern region of Iran (36°17′45″ N, 59°36′43″ E). A total of 150 dogs, including stray dogs (n = 66), owned pet dogs (n = 47), and dogs housed in shelters (n = 37), were randomly selected for the study. The data including age, gender, and clinical symptoms were recorded. Blood samples were taken from the jugular vein and placed in K3-EDTA (FL Medical, Italy) and plain (WEGO, China) tubes before being promptly sent to the laboratory. For serum separation, the plain blood specimens were centrifuged at 1800g for 10 min (Jouan, C 412, France) and stored at −80 °C until biochemical analysis.

2.2. Microscopic observation

For microscopic observation, blood smears were prepared and stained with Giemsa solution, involving first fixing air-dried samples in methanol for 10 min. Next, the samples were allowed to air-dry completely, followed by staining in a jar containing 5 % Giemsa stain diluted in tap water for 20 min. After washing smears with tap water, the presence of *Babesia* piroplasm in stained blood smears was assessed microscopically by examining at least 500 microscopic fields (× 1000).

2.3. Molecular analysis

DNA was extracted using DNA Isolation kits (DENAzist, Iran) based on the manufacturer's instructions. According to the method of Jefferies et al. 2003 [12], a partial region of the 18S small subunit ribosomal RNA (rRNA) gene was detected using F (5′- AGGGAGCCTGAGAGAC GGCTACC-3′) and R (5′-TTAAATACGAATGCCCCCAAC-3′) primers (Bioneer, South Korea). Amplification was conducted in a 25 μL total reaction volume containing DNA template (1 μL), primer (1 μL), and PCR red master mix (12.5 μL, Amplicon, Denmark) using Bio-Rad thermocycler under the following program: denaturation stage (15 min at 95 °C), 34 amplification cycles (denaturation step at 94 °C for 30 s, annealing step at 62 °C for 20 s, and extension step at 72 °C for 30 s), and final extension, 7 min at 72 °C. Then PCR products (10-μL) were electrophoresed through a 1.7 % agarose gel with TAE buffer and visualized by ethidium bromide and UV trans-illuminator. The size of the expected PCR product was approximately 450 bp. Distilled water and DNA isolated from a clinically healthy dog with no history of tick infestation were used in the PCR reaction as negative controls. The genome of *Babesia* spp. extracted previously from an infected dog (microscopic detection) was used as the positive control.

2.4. Nucleotide sequencing and phylogenetic analysis

To confirm the PCR product, two PCR amplicons of microscopic positive samples' genes were purified by DENAzist kit and submitted for sequencing using ABI 3730Xl DNA Analyzer (Bioneer Inc.) using the aforementioned primers. The 18S rRNA nucleotide sequences were assembled and edited with CLC bio software (CLC MainWorkbench, Qiagen, Aarhus, Denmark). The nucleotide sequences obtained in the present study were aligned with previously described sequences of *Babesia* spp. in GenBank (NCBI) by Clust W Method (Mega software version 6). The phylogenetic analysis was carried out by neighbor-joining analysis with bootstrap values based on 1000 replicates (MEGA software version 6). The nucleotide sequences obtained in this study were deposited in GenBank (NCBI) under accession numbers PQ060410.1 and PQ056786.1.

2.5. Measurement of clinicopathological parameters

Various hematological parameters, such as hematocrit (Hct), hemoglobin (Hb), erythrocyte count (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), and total white blood cell count (tWBC), were evaluated using a veterinary hematology autoanalyzer (Nihon Kohden, Japan). Differential WBC count was determined manually in blood smears. Some serum biochemical variables including aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), total bilirubin, total protein, albumin, cholesterol, triglyceride, urea, creatinine, inorganic phosphorus, and calcium were measured by

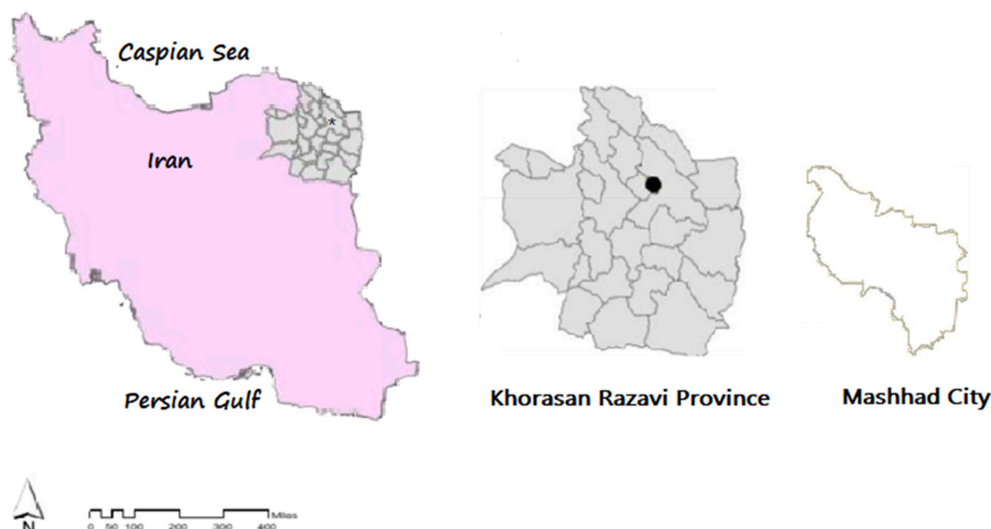


Fig. 1. The geographic location of the study, Mashhad, Iran.

commercial kits (Pars Azmoon, Iran) and an autoanalyzer (Mindray, China). Randox serum control (Antrim, UK) was used to check the accuracy of the obtained data. The serum level of total globulins was calculated by subtracting albumin from the total protein. The serum level of electrolytes including sodium (Na), chloride (Cl), and potassium (K) was measured by an electrolyte analyzer (StarlyteTM III, Alfa Wassermann, Netherlands). The measuring techniques, intra-assay CV (%), inter-assay CV (%), and analytical range are shown in Table 1.

2.6. Statistical analysis

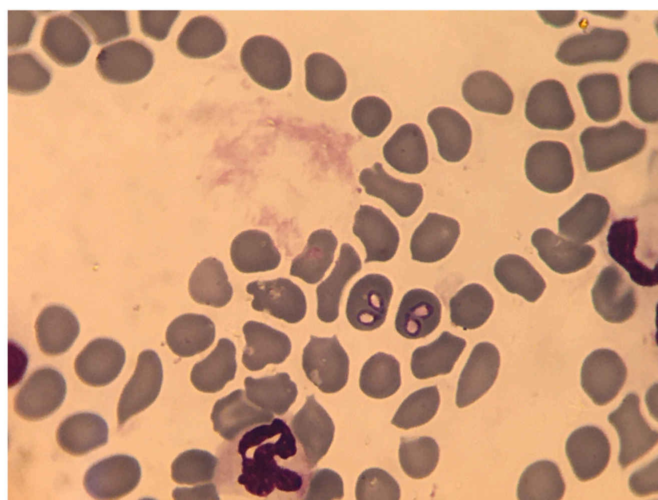
Data analysis was performed using SPSS software (version 21). The association of infection frequency with age, gender, housing status, and laboratory findings was investigated with a Chi-Square test. Comparison between non-infected and infected cases was performed using an independent T test and results were considered significant at $p < 0.05$.

3. Results

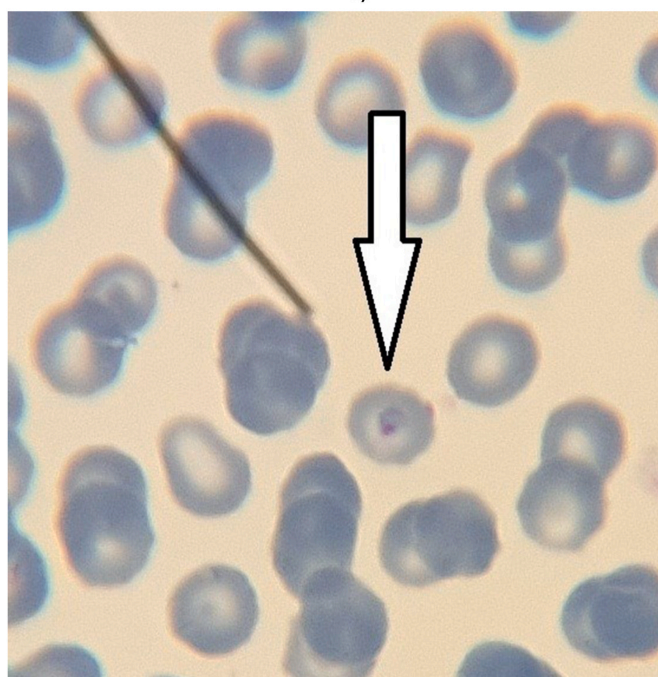
Babesia spp. piroplasms were observed in only three (2 %) blood smears (Fig. 2), which included two cases of large *Babesia* (cases 1 and 2, with parasitemia of 0.12 % and 0.05 %, respectively) and one case of small *Babesia* (case 3, with parasitemia of 0.00025 %). Molecular findings revealed that 23 out of the 150 examined dog samples (15.3 %) were infected with *Babesia* spp. (Fig. 3). Alignment of nucleotides sequences showed 100 % similarity with *B. vogeli* 18S rRNA nucleotide sequences reported in GenBank databases from different countries and this clade branched separately, with strong bootstrap support, from the branches holding *T. annulata* (Fig. 4).

Table 1
Methods of measurements and details of test characteristics.

Analytes	Methods	Intra-assay CV (%)	Inter-assay CV (%)	Analytical range
Total proteins (g/dL)	Biuret	1.01	2.55	0.5–15
Albumin (g/dL)	Bromocresol green	1.12	2.00	0.2–6
Urea (mmol/L)	Urease/glutamate dehydrogenase	3.13	3.80	2–200
Creatinine (mg/dL)	Kinetic Jaffe	2.38	0.50	0.2–15
Glucose (mg/dL)	Glucose oxidase/PAP (4-aminoantipyrine)	1.28	1.12	5–400
Triglycerides (mg/dL)	Glycerol 3 phosphate oxidase/PAP (4-aminoantipyrine)	1.82	2.15	5–700
Cholesterol (mg/dL)	Cholesterol oxidase/PAP (4-aminoantipyrine)	0.61	1.35	5–500
Total Bilirubin (mg/dL)	Dichloroanylin	2.32	2.59	0.1–30
ALT (U/L)	L-alanine/2-oxoglutrate as substrate	2.62	1.60	4–300
AST (U/L)	L-aspartate/2-oxoglutrate as substrate	3.06	1.38	2–300
GGT (U/L)	L-gamma-glutamyl-3-carboxy-4-nitroanilide	1.16	0.97	2–400
ALP (U/L)	P-Nitrophenylphosphate	0.92	0.99	3–1200
Calcium (mg/dL)	CresophetaleinComplexone	0.62	2.39	0.4–25
Phosphorus (mg/dL)	Phosphomolybdate	1.12	1.40	0.2–30
Sodium (mmol/L)	Ion Selective Electrode	1.00	2.55	40–205
Potassium (mmol/L)	Ion Selective Electrode	1.50	1.49	1.5–15
Chloride (mmol/L)	Ion Selective Electrode	1.00	1.76	50–200



a)



b)

Fig. 2. Giemsa-stained peripheral blood smear from dogs infected with large (a) and small (b) *Babesia* spp, 100 ×.

Based on the present study (Table 2), the prevalence of infection with *Babesia* spp. was not significantly associated with the gender of dogs but significantly associated with the age (k = 6.897, p = 0.021) and housing status (k = 37.294, p = 0.000), with over half of the samples (54.1 %) originating from shelters and nearly one-fifth of adults (21.3 %) testing positive. Regarding hematological and biochemical factors, significant associations were observed with hematocrit (k = 4.749, p = 0.025) and red blood cell count (K = 14.669, p = 0.000), which were significantly lower ($p < 0.05$) in infected dogs compared to non-infected dogs (Fig. 5).

All three dogs clinically affected with *Babesia* spp. and were confirmed positive for infection by both microscopy and molecular methods were outdoor adult male dogs with clinical symptoms of depression, anorexia, lethargy, and pallor of mucous membranes. In terms of laboratory findings, these cases exhibited severe anemia (Hct <15 According to erythrocyte indices, the anemia was categorized as a microcytic hypochromic in one dog infected with *B. vogeli* (case 1), and another dog infected with a small-sized *Babesia* spp. (case 3), and

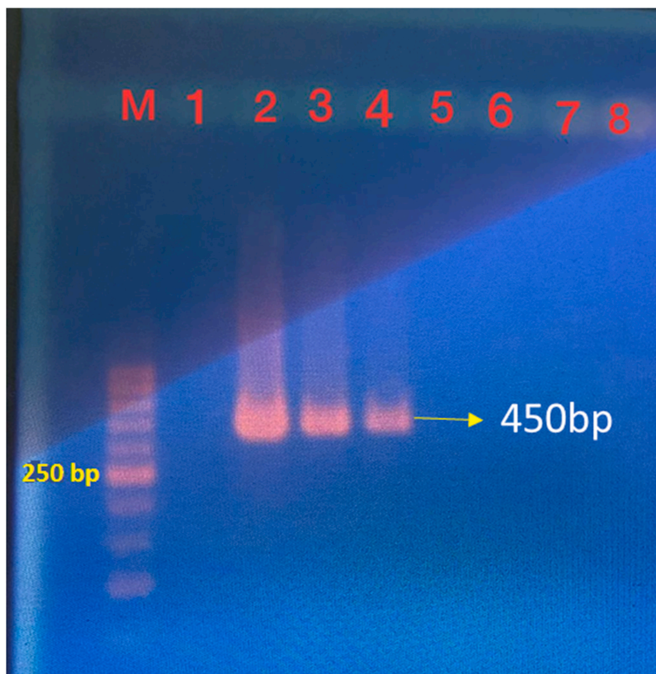


Fig. 3. PCR product of *Babesia* spp 18S rRNA (450 bp). Lane M, 1000bp ladder; Lane 1, negative control; Lane 2, positive control; Lane 3 and 4, dogs that tested positive for *Babesia* spp.; Lane 5–8, dogs that tested negative for *Babesia* spp.

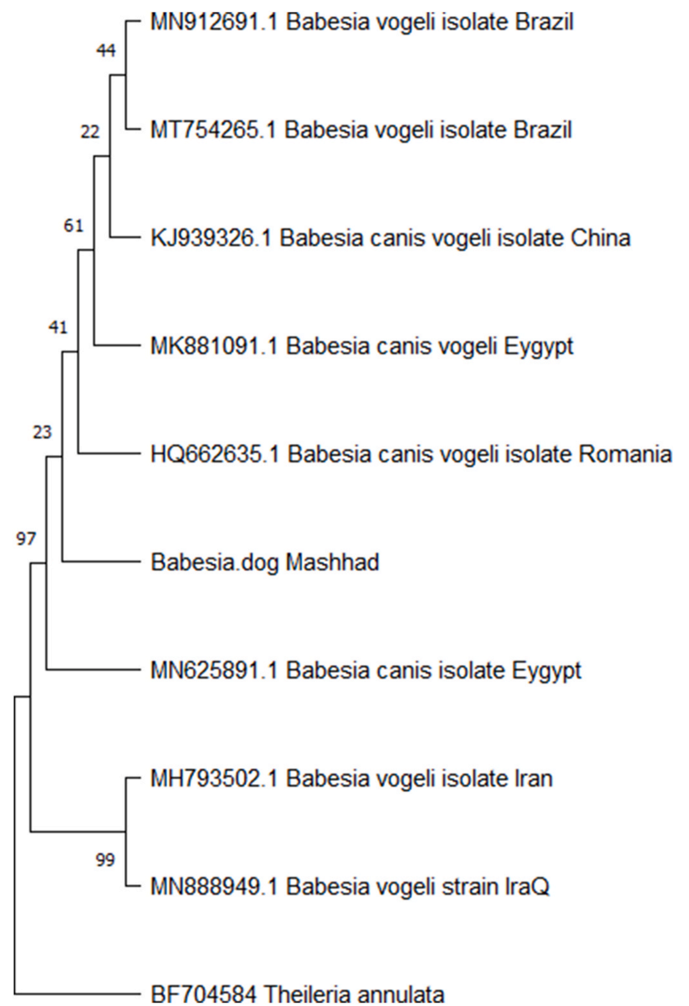


Fig. 4. Phylogenetic analysis of representative *Babesia vogeli* isolates in Iran (*Babesia.* dog Mashhad) and other isolates in the GenBank database. The tree was constructed using the Neighbor-joining method after bootstrapping with 1000 repetitions. The Nucleotide sequences of *T. annulata* were used as an out-group sample.

another dog infected with *B. vogeli* (case 2) presented with normocytic normochromic anemia. No bone marrow compensatory responses were observed in the cases, indicating non-regenerative anemia. Case 1 presented with pancytopenia, characterized by anemia, thrombocytopenia, and leukopenia in the complete blood count (CBC), while cases 2 and 3 exhibited leukocytosis with a regenerative left shift and normal platelet counts. The most significant biochemical changes were observed in case 1 including a decrease in serum total protein, albumin, globulin, glucose, cholesterol, calcium, and sodium. The serum levels of creatinine, urea, phosphorus, potassium, and activity of ALP GGT were significantly elevated compared to reference intervals. Notably, in cases 2 and 3, all serum biochemical parameters were within the normal range except for total protein and albumin, which were slightly decreased in case 2. All laboratory findings for these three cases are illustrated in Table 3.

4. Discussion

In our study, 23 out of 150 examined cases tested positive for *Babesia* spp. using molecular methods. The prevalence of this protozoan infection in other regions of Iran ranges from 0.7 % (1 out of 280 dogs) in Shiraz to 40 % (10 out of 40 dogs) in Shahriar City located in the west of Tehran province [7,10]. These findings suggest that the prevalence of infection in the northeast of Iran is higher than in most previously studied regions in Iran, except for Shahriar city, which had a small sample size of 40 tick-infested dogs. While geographical location and consequently tick vector distribution, and, most importantly, the number and inclusion criteria of the study play a significant role in the dispersion of the reported results, the high number of infected dogs in the present study emphasizes the importance of investigating the status of the vectors of this pathogen in this region.

A notable distinction between our study and previous studies is the identification of both large and small *Babesia* spp., through microscopic examination in three (2 %) blood samples. Previous studies have primarily relied on molecular diagnoses, with the limited examination of the parasite's piroplasm in blood specimens. In this context, Niak et al. (1978) reported a splenectomized dog infected (1 out of 155 dogs, 0.64 %) with *B. canis* in Mazandaran province [15]. Alborzi et al. (2013) reported an infection in 15 out of 400 dogs (3.75 %) examined in Ahvaz, southwestern Iran [16]. In these two studies, molecular analysis was not performed to confirm the diagnosis, and the infection was reported as *B. canis,* a large-sized *Babesia*, based only on the morphological characteristics of the pathogen.

In this study, piroplasms observed in two blood smears were piriform and larger than 2.5 μm, while in the third smear, piroplasms were ring-shaped and smaller than 2.5 μm, allowing classification as large and small-sized *Babesia* species, respectively. Various genes, including heat shock proteins and 18S rRNA, are commonly used for constructing phylogenetic trees [10,17]. In this study, we utilized 18S rRNA for sequencing and phylogenetic analysis, which revealed that the identified large *Babesia* species are closely related to *B. vogeli.* So far, *B. vogeli* infection has been reported from limited areas of Iran such as Shahriar and Hamedan in Iran [10,11]. In Meshkin Shahr, Shiraz, and Charmahal Bakhtiari regions, *B. canis* has been reported using molecular methods in 9.3 %, 0.7 %, and 7.5 % of the dogs studied, respectively [7,8,18]. Unfortunately, the genome of the small-sized *Babesia* species was missed in this study, and accurate identification of the parasite species was not possible. Although it cannot be confirmed, the small piroplasm found in this study is likely *B. gibsoni*, considering the geographical distribution of small *Babesia* spp. Given the differences in pathogenicity and response to

Table 2
Association of PCR results with dogs' age, gender, and clinicopathological findings. $p < 0.05$ indicates a significant association.

Variable (n = 150)	Infected dogs (n = 23)	Non-infected dogs (n = 127)	X^2	P value
Housing status				
Shelter (n = 37)	17 (45.9 %)	20 (54.1 %)	**37.2**	**0.000**
Stray (n = 66)	6 (9.1 %)	60 (90.9 %)		
Indoor (n = 47)	0 (0.00 %)	47 (100 %)		
Gender				
Male (n = 83)	14 (16.9 %)	69 (83.1 %)	0.337	0.562
Female (n = 67)	9 (13.4 %)	58 (86.6 %)		
Age (month)				
x < 8 (n = 42)	2 (4.8 %)	40 (95.2 %)	**6.897**	**0.032**
8 < x < 96 (n = 94)	20 (21.3 %)	74(78.7 %)		
x > 96 (n = 14)	1(7.1 %)	13 (92.9 %)		
Hct				
Low (N = 73)	16 (22.0 %)	57(78.0 %)	4.749	**0.025**
Normal (n = 77)	7 (9.1 %)	70(90.9 %)		
HB				
Low (n = 80)	15(18.7 %)	65(581.2 %)	1.541	0.214
Normal (n = 70)	8(11.4 %)	62(88. 6 %)		
RBC				
Low (n = 63)	18 (28.5 %)	45 (71.4 %)	14.669	**0.000**
Normal (n = 87)	5 (5.7 %)	82 (94.2 %)		
MCV				
Low (n = 76)	6(7.9 %)	70 (92.1 %)	0.566	0.310
Normal (n = 74)	17(23.0 %)	57(77.0 %)		
MCH				
Low (n = 57)	6 (10.5 %)	51 (89.5 %)	1.636	0.147
Normal (n = 93)	17 (18.3 %)	76 (81.7 %)		
Platelet				
Low (n = 23)	3 (13.0 %)	20 (86.9 %)	1.018	0.601
Normal (n = 105)	18 (17.1 %)	87 (82.8 %)		
High (n = 22)	2 (9.0 %)	20 (91.0 %)		
WBC				
Low (n = 23)	3 (13.0 %)	20(86.9 %)	0.521	0.771
Normal (n = 96)	14(14.5 %)	82(85.4 %)		
High (n = 31)	6(19.3 %)	25(80.6 %)		
Neut. seg				
Low (n = 17)	2(11.81 %)	15(88.2 %)	0.584	0.747
Normal (n = 102)	15(14.8 %)	87(85.2 %)		
High (n = 31)	6(19.3 %)	25(80.6 %)		
Neut. Band				
Normal (n = 129)	22(17.0 %)	107(82.9 %)	2.102	0.147
High (n = 21)	1(4.7 %)	20(95.2 %)		
Lymphocyte				
Low (n = 32)	7(21.9 %)	25(78.1 %)	3.098	0.212
Normal (n = 107)	13(12.1 %)	94(87.8 %)		
High (n = 11)	3(27.3 %)	8(72.7 %)		
Total protein				
Low (n = 20)	5(25.0 %)	15(75.0 %)	3.058	0.217
Normal (n = 95)	11(11.6 %)	84(88.4 %)		
High (n = 35)	7(20.0 %)	28(80.0 %)		
Albumin				
Low (n = 5)	0(0.00 %)	5(100 %)	3.922	0.141
Normal (n = 119)	16 (13.4 %)	103(86.6 %)		
High (n = 26)	7 (26.9 %)	19 (73.1 %)		
Globulin				
Low (n = 19)	4(21 %)	15(79 %)	0.594	0.743
Normal (n = 108)	16 (14.8 %)	92 (85.2 %)		
High (n = 23)	3 (13.1 %)	20 (86.9 %)		
Urea				
Normal (N = 75)	10 (13.3 %)	65(86.7 %)	0.462	0.467
High (N = 75)	13(17.3 %)	62(82.7 %)		
Creatinine				
Normal (n = 127)	20(15.7 %)	107(84.3 %)	0.514	0.110
High (n = 23)	3(13.0 %)	20 (87.0 %)		
Glucose				
Low (n = 68)	8 (11.7 %)	60 (88.2 %)	3.441	0.179
Normal (n = 66)	14(21.2 %)	52(78.7 %)		
High (n = 16)	1 (6.25 %)	15 (93.7 %)		
Total bilirubin				
Normal (n = 138)	21(15.2 %)	117 (84.8 %)	0.018	0.894
High (n = 12)	2 (16.7 %)	10(83.3 %)		
AST				
Normal (n = 137)	20(14.6 %)	117(85.4 %)	0.018	0.894
High (n = 13)	3(23.0 %)	10(77.0 %)		
ALT				
Normal (n = 136)	19(14.0 %)	117(86.0 %)	2.084	0.232
High (n = 14)	4(28.6 %)	10(71.4 %)		
ALP				
Normal (n = 130)	18(13.8 %)	112(86.2 %)	1.661	0.197
High (n = 20)	5(25.0 %)	15(75.0 %)		
GGT				
Normal (n = 132)	20(15.1 %)	112(84.9 %)	0.028	0.867
High (n = 18)	3(16.7 %)	15(83.3 %)		
Calcium				
Low (n = 39)	4(10.3 %)	35(89.7 %)	1.046	0.306
Normal (n = 111)	19(17.1 %)	92(82.9 %)		
Phosphorous				
Low (n = 5)	0(0.00 %)	5(100 %)	4.222	0.121
Normal (n = 110)	13(13.4 %)	97(86.6 %)		
High (n = 35)	10 (20.8 %)	25(79.2 %)		
Cholesterol				
Low (n = 30)	5(16.6 %)	25(83.3 %)	0.784	0.676
Normal (n = 107)	15(14.0 %)	92(86.0 %)		
High (n = 13)	3 (23.1 %)	10(76.9 %)		
Triglyceride				
Normal (n = 142)	20(14.0 %)	122(86.0 %)	3.198	0.074
High (n = 8)	3 (37.5 %)	5(62.5 %)		
Na				
Low (n = 50)	5(10.0 %)	45(90.0 %)	2.291	0.318
Normal (n = 93)	16(17.2 %)	77(82.8 %)		
High (n = 7)	2(28.6 %)	5(71.4 %)		
K				
Low (n = 12)	2(16.7 %)	10(83.3 %)	0.024	0.988
Normal (n = 91)	14(15.4 %)	77(84.6 %)		
High (n = 47)	7(14.9 %)	40(85.1 %)		
Cl				
Low (n = 22)	2(9.1 %)	20(90.9 %)	0.775	0.673
Normal (n = 116)	19(16.4 %)	97(83.6 %)		
High (n = 12)	2(16.7 %)	10(83.3 %)		

treatment protocols among various *Babesia* species, further studies are recommended to accurately assess the distribution of these species, not only in this region but also throughout Iran. So far, only one study by Akhtardanesh et al. (2016) in Kerman has focused on this species, reporting a 5 % infection rate in 60 dogs using only molecular methods [9]. It should be noted that the mentioned study reports the prevalence of infection in anemic dogs infested with ticks. It is more likely that the infection rate of the entire dog population in that region is lower than the reported rate. Niak et al. (1978) also reported one case of a fox infected with small-sized *Babesia* spp. in Mazandaran province [15]. Based on its morphological characteristics, this species was named *B. gibsoni*, and unfortunately, molecular confirmation of the parasite genome was not performed.

According to the present study, the highest infection rate was observed in adult dogs (8 < x < 96 months) which is in agreement with

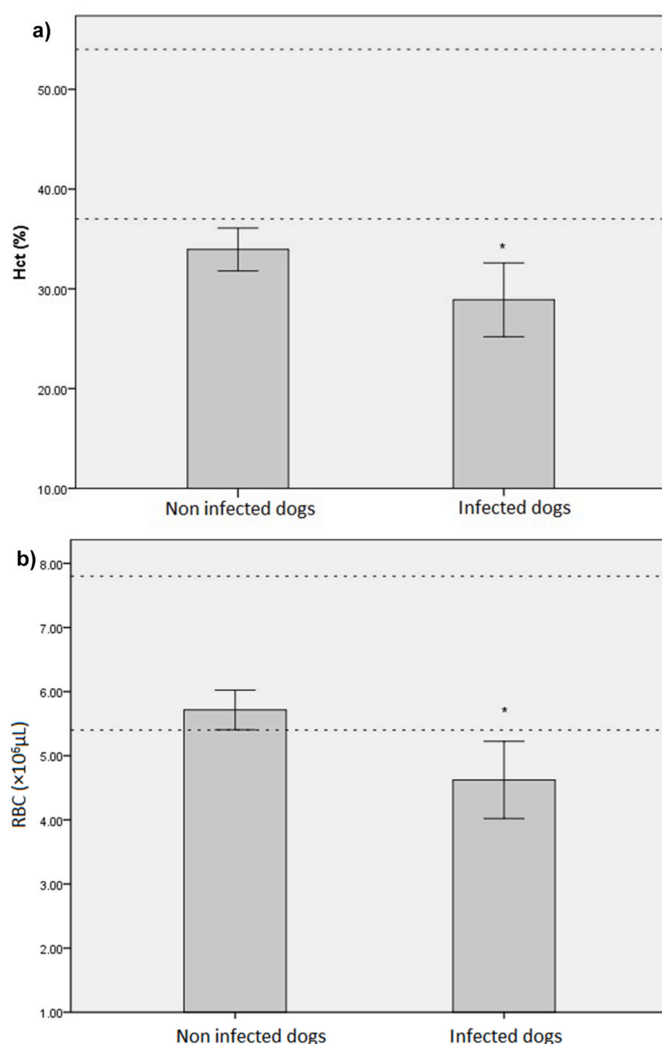


Fig. 5. The Hct (a) and RBC count (b) (Mean ± SEM) comparison between non-infected and infected dogs with *Babesia* spp. the reference intervals [13] are represented as horizontal dashed lines* indicating a significant difference ($p < 0.05$).

previous studies [19–21]. Researchers have reported a rise in canine babesiosis with age, peaking between 3 and 5 years, followed by a decline [22]. These changes may be attributed to increased exposure to tick infestations and decreased maternal immunity in older dogs.

Consistent with our findings, previous studies have demonstrated a positive association between shelter housing and *Babesia* spp. infection rates [23,24]. This can be attributed to several factors inherent to the shelter environment, including overcrowding, high dog turnover, and increased exposure to ticks.

Anemia was observed in 20 out of the 23 *Babesia*-infected dogs (89 %), which were further categorized as mild (50 %), moderate (35 %), severe (5 %), and very severe (10 %) [25]. Notably, severe or very severe anemia was exclusively observed in dogs that tested microscopically positive. The etiology of anemia in babesiosis is mainly attributed to secondary immune-mediated hemolysis [1]. However, despite not performing Coombs tests in this study and the inability to rule out immune-mediated hemolytic anemia, dyserythropoiesis should not be ignored as a potential contributing factor, as bone marrow regenerative responses and laboratory findings indicative of immune system involvement, such as spherocytosis and autoagglutination, were not observed in these cases.

One dog infected with *B. vogeli* displayed significant alterations in serum biochemical parameters compared to the other two infected dogs. This might be attributed to the higher parasitemia in this dog. This case presented with hyperbilirubinemia accompanied by severe anemia and elevated serum liver enzyme activities, indicating both pre-hepatic and hepatic hyperbilirubinemia. Our findings align with previous studies that have documented elevated liver enzyme activities in dogs with babesiosis, supporting the notion of hepatic involvement [26,27].

Table 3
Comparison of clinicopathological findings in the three dogs clinically infected with *Babesia* spp. with established reference intervals for healthy dogs [13,14]. Cases 1 and 2 were infected with *B.vogeli*, and case 3 was infected with unknown small-sized *Babesia* spp.

Hematological parameters	Case 1	Case 2	Case 3	Reference interval*
PCV (%)	13.6	11.3	10.4	37–55
Hb (g/dL)	4.9	4.3	3.5	12–18
RBC ($\times 10^6/\mu$L)	2.38	1.67	2.16	5.5–5.8
MCV (fL)	57.1	67	48	60–77
MCH (pg)	20.6	26	16.2	22–27
MCHC (g/dL)	36	38	33.7	32–36
WBC ($\times 10^3/\mu$L)	1.5	50.0	19.7	6.0–17.0
Neut; Seg ($\times 10^3/\mu$L)	1.3	44.0	14.7	3.0–11.5
Neut; Band ($\times 10^3/\mu$L)	0.1	3.0	0.3	0–0.3
Eosinophils ($\times 10^3/\mu$L)	0	0	0	0.1–1.2
Lymphocytes ($\times 10^3/\mu$L)	0.1	1.5	2.2	1.0–4.8
Monocytes ($\times 10^3/\mu$L)	0.0	1.5	2.3	0.0–1.3
Platelets ($\times 10^5/\mu$L)	0.66	3.5	5.0	2–5
Biochemical parameters				
Total protein (g/dL)	4	4.8	5.7	5.4–7.1
Albumin (g/dL)	2.2	3	2.6	2.6–3.3
Globulin (g/dL)	1.8	1.8	3.1	2.7–4.4
AGR	2.4	1.67	0.84	0.59–1.1
Creatinine (mg/dL)	37	0.9	0.8	0.5–1.5
Urea (mg/dL)	149	28	10	10–28
Glucose (mg/dL)	45	83	92	65–118
Total bilirubin (mg/dL)	7.21	0. 32	0.04	0.1–0.5
Cholesterol (mg/dL)	68	153	208	135–270
Triglyceride (mg/dL)	130	110	74	40–169
ALT (IU/L)	250	72	29	21–102
AST (IU/L)	160	22	21	23–66
GGT (IU/L)	17	0.5	1.6	1.2–4.6
ALP (IU/L)	219	21	132	20–156
Calcium (g/dL)	7	9.4	8.9	9–11.3
Phosphorus (mg/dL)	21.8	6.5	4.8	2.6–6.2
Sodium (mmol/L)	126	152	142	141–152
Potassium (mmol/L)	OVER	4.5	4.7	4.37–5.35
Chloride (mmol/L)	115	105	107	105–115

Renal involvement, often resulting from methemoglobinuria, particularly when accompanied by acidosis, is recognized as a complication of babesiosis [28,29] although rhabdomyolysis can also rarely lead to nephrotoxicity [30]. Despite the lack of urinalysis data, the significant azotemia, hyperphosphatemia, hyperkalemia, hyponatremia, hypoproteinemia, and severe non-regenerative anemia could indicate kidney damage in this dog.

5. Conclusion

The microscopic findings of this study suggest a low frequency of canine babesiosis in Northeast Iran. Phylogenetic analysis in this study confirmed the presence of *B. vogeli* in infected dogs. Further studies are needed to investigate tick vectors and the geographical distribution of *Babesia* spp. in this region. Notably, most infected dogs in this study exhibited no clinical signs, raising concerns about the epidemiological implications and potential disease spread through these asymptomatic carriers. Moreover, almost half of the samples collected from the shelters harbored the infection, highlighting the urgent need for stricter hygiene measures and comprehensive screening for infectious agents, including *Babesia* spp, in these centers to avert their transformation into reservoirs

for the spread of life-threatening diseases like babesiosis.

Funding

This study was supported by Grant 3/50917 from the Vice President Research and Technology of Veterinary Medicine, Ferdowsi University of Mashhad, Iran.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval

All animal experiments were performed in strict accordance with the guidelines approved by the Animal Ethics Committee of the Ferdowsi University of Mashhad, Iran (IR.UM.REC.1398.116).

Consent to participate

Not applicable.

Consent for publication

Not applicable.

CRediT authorship contribution statement

Mohammad Hossein Kafrashi: Project administration, Investigation. **Gholam Reza Razmi:** Writing – review & editing, Conceptualization. **Mahdieh Zaeemi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgments

We thank the research deputy of the Ferdowsi University of Mashhad for supporting us.

References

[1] J.A. Birkenheuer, Babesiosis, in: C.E. Greene (Ed.), Greene's Infectious Diseases of the Dog and Cat, fifth ed., Elsevier Saunder., USA, 2023, pp. 771–784.

[2] C. Carret, F. Walas, B. Carcy, N. Grande, E. Précigout, K. Moubri, T.P. Schetters, A. Gorenflot, Babesia canis canis, Babesia canis vogeli, Babesia canis rossi: differentiation of the three subspecies by a restriction fragment length polymorphism analysis on amplified small ribosomal RNA genes, J. Eukaryot. Microbiol. 46 (1999) 298–303, https://doi.org/10.1111/j.1550-7408.1999.tb05128.x.

[3] M. Zahler, E. Schein, H. Rinder, R. Gothe, Characteristic genotypes discriminate between Babesia canis isolates of differing vector specificity and pathogenicity in dogs, Parasitol. Res. 84 (1998) 544–548, https://doi.org/10.1007/s004360050445.

[4] O. Teodorowski, M. Kalinowski, D. Winiarczyk, B. Dokuzeylül, S. Winiarczyk, Ł. Adaszek, Babesia gibsoni infection in dogs-A European perspective, Animals 14 (2022) 730, https://doi.org/10.3390/ani12060730.

[5] W. Zygner, O. Gójska-Zygner, J. Bartosik, P. Górski, J. Karabowicz, G. Kotomski, L. J. Norbury, Canine babesiosis caused by large Babesia species: global prevalence and risk factors-A review, Animals 13 (2023) 2612, https://doi.org/10.3390/ani13162612.

[6] M. Karasová, C. Tóthová, S. Grelová, M. Fialkovičová, The etiology, incidence, pathogenesis, diagnostics, and treatment of canine babesiosis caused by Babesia gibsoni infection, Animals 16 (2022) 739, https://doi.org/10.3390/ani12060739.

[7] M. Bigdeli, S.M. Rafie, M.M. Namavari, S. Jamshidi, Report of Theileria annulata and Babesia canis infections in dogs, Comp. Clin. Pathol. 21 (2012) 375–377.

[8] A. Raki, A. Doosti, M. Shahrani, Detection of Babesia canis in the blood samples of dogs in Iran by PCR method, Int. J. Biosci. 4 (2014) 118–124.

[9] B.A. Akhtardanesh, M.E. Saberi, S.R. Nurollahifard, M.A. Aghazamani, Molecular detection of Babesia spp. in tick-infested dogs in southeastern Iran, J. Dis. Global Health 8 (2016) 72–77.

[10] G. Habibi, A. Imani, A. Afshari, S. Bozorgi, Detection and molecular characterization of Babesia canis vogeli and Theileria annulata in free-ranging dogs and ticks from Shahriar County, Tehran Province, Iran, Iran. J. Parasitol. 15 (2020) 321–331.

[11] R. Iatta, A. Sazmand, V.L. Nguyen, F. Nemati, M.M. Ayaz, Z. Bahiraei, S. Zafari, A. Giannico, G. Greco, F. Dantas-Torres, D. Otranto, Vector-borne pathogens in dogs of different regions of Iran and Pakistan, Parasitol. Res. 120 (2021) 4219–4228.

[12] R. Jefferies, U.M. Ryan, C.J. Muhlnickel, P.J. Irwin, Two species of canine Babesia in Australia: detection and characterization by PCR, J. Parasitol. 89 (2003) 409–412, https://doi.org/10.1645/0022-3395(2003)089[0409:TSOCBI]2.0.CO;2.

[13] T.E. Rizzi, J.H. Menkoth, K.D. Clinkenbeard, Normal hematology of the dog, in: D. J. Weiss, K.J. Wardrop (Eds.), Schalms Veterinary Hematology, sixth ed., Wiley Blackwell, Iowa, USA, 2010, pp. 799–810.

[14] J.J. Kaneko, J.W. Harvey, M.L. Bruss, Appendix IX; Blood analyte reference values in large animals, in: Clinical Biochemistry of Domestic Animals, sixth ed., Elsevier Academic, New York, USA, 2008, pp. 889–895.

[15] A. Niak, M. Anwar, S. Khatibi, Canine babesiosis in Iran, Trop. Anim. Health Prod. 5 (1973) 200–201, https://doi.org/10.1007/BF02251391.

[16] A. Alborzi, R. Avizeh, B. Mosallanejad, M. Razi jalali, H. Hamidi Nejat, R. Taghipour, Babesia infection in urban and rural dogs in Ahvaz district, Southwest of Iran, Archives of Razi Institute 68 (2013) 37–42, https://doi.org/10.7508/ari.2013.01.006.

[17] M. Yamasaki, H. Inokuma, C. Sugimoto, S.E. Shaw, M. Aktas, M.J. Yabsley, O. Yamato, Y. Maede, Comparison and phylogenetic analysis of the heat shock protein 70 gene of Babesia parasites from dogs, Vet. Parasitol. 145 (2007) 217–227, https://doi.org/10.1016/j.vetpar.2007.01.003.

[18] M. Khanmohammadi, R. Zolfaghari-Emameh, M. Arshadi, E. Razmjou, P. Karimi, Molecular identification and genotyping of Babesia canis in dogs from meshkin Shahr county, Northwestern Iran, J Arthropod Borne Dis 31 (2021) 97–107, https://doi.org/10.18502/jad.v15i1.6489.

[19] S.C. Egege, E.C. Okolocha, J.A. Nwanta, E.O. Mosimabale, Prevalence and seasonality of babesiosis in dogs treated at university veterinary clinic in Kaduna Nigeria from 1990–1999, Niger, Vet. J. 29 (2008) 21–26.

[20] S.S. Obeta, H.S. Idris, B.A. Azare, M.K. Simon, C.O. Jegede, Prevalence of haemoparasites of dogs in federal capital territory, Abuja-Nigeria. Niger, Vet. J. 385 (2009) 73–76.

[21] M.N. Opara, U.M. Ukpong, I.C. Okoli, Quatitative analysis of abattoir slaughtering of animals in Akwa-Ibom State, Nigeria, J. Agric. Sci. Res. 5 (2005) 118–125.

[22] S. Hornok, R. Edelhofer, R. Farkas, Seroprevalence of canine babesiosis in Hungary suggesting breed predisposition, Parasitol. Res. 99 (2006) 638–642.

[23] N. Sontigun, W. Boonhoh, P. Fungwithaya, T. Wongtawan, Multiple blood pathogen infections in apparently healthy sheltered dogs in southern Thailand, Int J Vet Sci Med 24 (2022) 64–71, https://doi.org/10.1080/23144599.2022.2111514.

[24] V. Mrljak, J. Kuleš, Ž. Mihaljević, M. Torti, J. Gotić, M. Crnogaj, T. Živičnjak, I. Mayer, I. Šmit, M. Bhide, R. Barić Rafaj, Prevalence and geographic distribution of vector-borne pathogens in apparently healthy dogs in Croatia, Vector Borne Zoonotic Dis. 17 (2017) 398–408, https://doi.org/10.1089/vbz.2016.1990. Epub 2017 Apr 27.

[25] H. Tvedten, Laboratory and clinical diagnosis of anemia, in: D.J. Weiss, K. J. Wardrop (Eds.), Schalms Veterinary Hematology, sixth ed., Wiley Blackwell, Iowa, USA, 2010, pp. 152–161.

[26] I. Kaeber, J. Reetz, P.M. Adcock, Liver enzyme activity in dogs infected with Babesia canis, Vet. Rec. 162 (21) (2008) 648–649.

[27] A.K. Bilwal, G.C. Mandali, F.B. Tandel, Liver enzyme activity in dogs infected with Babesia canis, Indian J. Vet. Res. 19 (2018) 133–134.

[28] S. Sherlock, R.J. Divers, R. Lobetti, Acute renal failure in dogs with babesiosis: a retrospective study of 20 cases, J. Vet. Intern. Med. 17 (2003) 779–784.

[29] R. Lobetti, Renal failure in dogs with babesiosis, Vet. Clin. N. Am.: Small animal practice 35 (2005) 1277–1292.

[30] L.S. Jacobson, R. Lobetti, Rhabdomyolysis and renal failure in a dog with babesiosis, J. A. Vet. Med Assoc, of the American Veterinary Medical Association 209 (1996) 1871–1873.