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Molecular survey and phylogenetic analysis of *Bartonella henselae* and *Bartonella clarridgeiae* in dogs from northwest Iran

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Abstract

The purpose of the current study was to examine the occurrence and genetic characteristics of *Bartonella henselae (B. henselae)* and *Bartonella clarridgeiae (B. clarridgeiae)* in dogs from West Azerbaijan province, Iran. Blood samples were obtained from 400 dogs, and their gender, age, reproductive status, ownership status, and geographical origin were documented. Positive samples were identified using PCR and sequencing techniques, and the gene sequences of the *ftsZ* (for *B.henselae*) and *gltA* (for *B.clarridgeiae*) genes were examined using BioEdit software. The gene sequences acquired demonstrated a minimum similarity of 100.00% when compared to the reference sequences in the GenBank® database. Additionally, a phylogenetic tree was built using MEGA11. The findings of the study indicated that 8.5% (p<0.05; 95%, CI: 6.15%–11.64%) of the tested dogs were positive for *B. henselae*, and 3.25% (p<0.05; 95%, CI: 1.91%–5.48%) were positive for *B. clarridgeiae*. The results for both *Bartonella* species showed a significant difference (p0.001) between neutered and non-neutered dogs, as well as a significant difference (p=0.001 and p=0.004) between stray and pet dogs. The study's findings highlight the significant role that dogs could potentially engage as the origins of *Bartonella* infection, as a zoonotic agent, in the region.

Introduction

The *Bartonella* genus comprises at least 20 species and subspecies, some of which have been identified to cause infections in humans (1). *Bartonella* spp., Gram-negative, intracellular bacteria, can be found in various domestic and wild mammals worldwide. *Bartonella henselae (B. henselae)* is the most commonly identified species in humans and cats, but it has also been detected in other animals, such as dogs, rabbits, and guinea pigs (2). This particular species is responsible for causing cat-scratch disease (CSD). However, there have been rare cases where *Bartonella clarridgeiae (B. clarridgeiae)* and *Bartonella koehlerae (B. koehlerae)* have been

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reported as the causative agents of the disease (3). *B. henselae* and *B. clarridgeiae* can also infect dogs, serving as bacterial reservoirs for the transmission of infection (1). *B. henselae*, initially discovered in a dog in Gabon in 2003, is likely the most common *Bartonella* species to infect pet dogs (4). Dogs can also become infected with *Bartonella. clarridgeiae*, *Bartonella elizabethae*, *Bartonella koehlerae*, *Bartonella quintana*, *Bartonella washoensis*, and *Bartonella rochalimae*, which can result in a range of diseases comparable to those observed in humans, such as bacillary angiomatosis, endocarditis, granulomatous hepatitis, lymphadenitis, myocarditis, and peliosis hepatis (5-10). The primary modes of transmission among humans include being scratched or bitten by an animal carrying *Bartonella*-contaminated vector feces, as well as being bitten by blood-sucking arthropods (11).

Dogs are highly regarded as effective sentinels and can be integrated into a surveillance system for detecting *Bartonella* spp. infection in humans. This is because dogs often come into contact with common parasites in home and leisure settings, making them potential carriers of *Bartonella* spp. Furthermore, a wide range of *Bartonella* spp. has been found in dogs (12).

Different techniques, such as culture, histopathology, serology, and PCR assay can be employed for identifying *Bartonella* species. Nevertheless, PCR tests focusing on distinct gene sequences of *Bartonella* have emerged as the preferred diagnostic approach due to the difficultly in isolating the bacteria from tissue or blood samples (13, 14).

The global health strategic framework aims to reduce the risk and mitigate the impact of emerging infectious diseases within the animal-human ecosystem (World Organization for Animal Health, 2008). Shaffer suggests that incorporating surveillance policies for animals is integral to the One Health Perspective approach (15). In recent years, *B. henselae* and *B. clarridgeiae* have gained recognition as two emerging pathogens that are important in both veterinary and medical fields (16). In Iran, few studies have found contrasting results in the infection rate of Bartonella spp, depending on the type of samples and the methods used. The rates have ranged from zero to more than 70 percent (17, 18, 19). Globally, the prevalence of Bartonella spp. infection in dogs can reach up to 40 percent, varying based on geographical regions, strains, type of samples, and testing methods used (20, 21). Identifying *Bartonella* species in animals that come in contact with humans can lead to the discovery of new human pathogens or diseases (22). The aim of this study was to present results from isolating *B. henselae* and *B. clarridgeiae* from the blood of 400 dogs in West Azerbaijan province, Iran.

Materials and methods

Sample collection

The most commonly used tissue for detecting *Bartonella* through PCR is peripheral blood (23). We received 400 canine blood samples for animal enrollment from veterinary analysis laboratories. These samples were collected from various regions within the north (Maku, Chaldoran, Showt, Poldasht, Chaypareh, Khoy), center (Salmas, Urmia, Oshnavieh, Naqadeh, Miandoab, Chaharborj), and south (Piranshahr, Mahabad, Bukan, Baruq, Shahin Dezh, Takab, Sardasht) of West Azerbaijan province. The distribution of samples from each region is as follows: north (n=120), center (n=160), and south (n=120). Upon examination, the dogs were categorized based on their age, gender, reproductive status, ownership status, and geographical origin. The animals that were sampled had not undergone clinical examination, but they appeared healthy. The sampling process was conducted randomly between January 2023 and December 2023. Each dog provided 2 mL of whole blood, collected through either cephalic or saphenous venipuncture. The blood samples were put into vacuum tubes containing EDTA and then transported to the laboratory of the Faculty of Veterinary Medicine

with boxes in cold condition. They were kept at -20 °C until they were ready for further processing.

Molecular tests

DNA extraction and PCR assay

DNA extraction was performed using the Denazist DNA Extraction Kit, Mini Kit, Iran (50 preps), in accordance with the manufacturer's guidelines. The quality and quantity of the extracted DNA were evaluated using the NanoDrop 2000c from Thermo Scientific (USA). The extracted DNA samples were then preserved at -20°C for subsequent PCR applications.

To detect B.henselae and B.clarridgeiae, a Polymerase Chain Reaction (PCR) was conducted, targeting a specific portion of the ftsZ and gltA genes (Table 1). Water DEPC-Treated (Santa Cruz, USA) was used as Negative Control of Extraction, and positive control was not used in this study. The PCR reaction mixture totaled 25 µL, containing 3 µL template DNA, 0.5 µL of each primer (20 µM), 10 µL of 2× ready-to-use Taq DNA Polymerase Master Mix (Ampliqon, Danmark), and the addition of sterile distilled water to achieve the target volume of reaction. The PCR cycling conditions (95°C-5 min, 94°C-1min, 58°C-1min, 72°-1min, 15 sec, for 38 cycles, 72°C-7min) were adopted from the instructions supplied by Quanta Biotech (England), as specified in the thermal cycler manual (https://www.bioclinicalservices.com.au/quanta-biotech/clinical/qtas-2-software-user-manual).

Table 1. Primer sequences and PCR conditions for identifying *B. henselae and B. clarridgeiae*

Target gene	Primer Name	Sequence 5'----3'	PCR product size (bp)
ftsZ	*B. henselae*-F	CAGCATACGATGGTTCACGA	404
	B. henselae-R	GAACCTGCAAGACGTACAGT	
gltA	*B. clarridgeiae* -F	CTGTACGTCTTGCTGGTTCA	457
	B. clarridgeiae -R	CTGCGTGCTAATGCAAAGAG	

The amplified PCR products were then subjected to direct sequencing. Gel-electrophoresis was performed on 2% agarose gels for 45 minutes at 75 V containing gel stain to visualize the amplified products. The gel documentation system from Syngene Bio-Imaging in the United Kingdom was used to document the results.

Sequencing and phylogenetic analysis

Sanger sequencing was performed on all PCR products. The obtained nucleotide sequences were aligned and processed with BioEdit software (Version 7.2.0) and juxtaposed with sequences in the GenBank® database using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree was built using MEGA 11.0 software (Version 11.0.13).

Statistical analysis

The confidence intervals (CI) with a 95% confidence level were determined using the exact binomial test. To compare the percentages of positivity among different categories of independent categorical variables such as gender, age group, reproductive status (neutered or not), ownership status (stray or pet), and geographical origin (north, center, and south), the Chi-square test was employed. Statistical significance was determined with a *p*-value less than 0.05 using SPSS software version 27.0.1 (SPSS Inc., Chicago, IL, USA).

Results

PCR enhancement of ftsZ and gltA genes

The results revealed that 8.5% (34 out of 400 blood samples; 95%, CI: 6.15%–11.64%) and 3.25% (13

out of 400 blood samples; 95%, CI: 1.91%–5.48%) of the dogs were found positive for the *B. henselae* gene and *B. clarridgeiae* gene respectively (Table 2). Images were taken utilizing the Ingenius Gel Documentation system, as depicted in Figures 1 and 2.

Table 2. Prevalence of *B.henselae and B.clarridgeiae* in dogs based on examined factors

Variable	Epidemiological Factors	Frequency	PCR-Positive (%)		*p-value*	
			B.henselae	*B.clarridgeiae*	*B.henselae*	*B.clarridgeiae*
Total		400	34 (8.5%)	13 (3.25%)		
Gender	male	200	18 (9%)	8 (4%)	0.720	0.398
	female	200	16 (8%)	5 (2.5%)		
Age group	≤ 1 year	130	24 (18.4%)	4 (3.07%)	0.001	0.930
	1-3 years	84	2 (2.38%)	2 (2.38%)		
	≥ 3 years	186	8 (1.07%)	6 (4.3%)		
Neutering status	Neutered	312	19 (6.08%)	1 (0.32%)	0.001	0.001
	Not neutered	88	15 (17.04%)	12 (13.63%)		
Ownership status	Stray	245	31 (12.65%)	13 (5.3%)	0.001	0.004
	Pet	155	3 (1.93%)	0 (0%)		
geographical origin	North	120	9 (7.5%)	3 (2.5%)	0.767	0.842
	Centre	160	13 (8.12%)	6 (3.75%)		
	South	120	12 (10%)	4 (3.33%)		

Sequencing

Only eight amplicons targeting the *ftsZ* gene for *B. henselae* and five amplicons targeting the *gltA* gene for *B. clarridgeiae* were sequenced, all yielding positive results. These amplicons were found to be identical, resulting in only two being used for phylogenetic analysis - one for *B. henselae* and one for *B. clarridgeiae*. The isolates were sequenced and then archived in the GenBank® database with the designated Accession Nos. PP658203 (*B. henselae*) and PP576695 (*B. clarridgeiae*). Analysis of the gene sequences showed a minimum similarity of 100.00% to the reference sequences in the GenBank® database.

Phylogenetic analysis

The phylogenetic tree shown in Figure 3 was constructed using the combined sequences of 404 bp for *B. henselae* and 457 bp for *B. clarridgeiae* in dogs. Upon analysis, it was noted that the strain isolated in this specific study, identified as PP658203 (*B. henselae*), falls within the same clade as strains CP072900 (Germany) and CP072899 (Germany). Similarly, the strain PP576695 (*B. clarridgeiae*) isolated in this study is clustered together within the same clade as strain MK693114 (Spain) and OQ436436 (Chile).

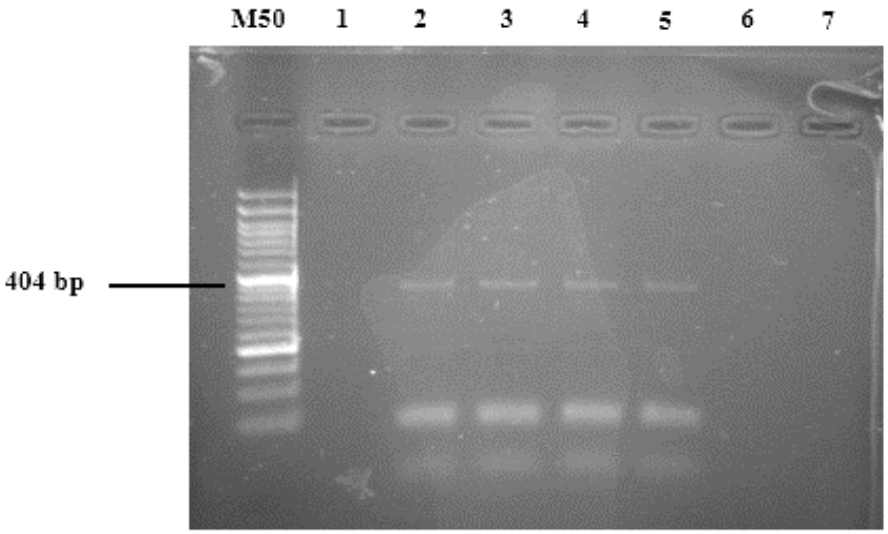

Fig. 1. Agarose gel image of the amplified fragment of the *ftsZ* gene (404 bp). M50: molecular mass marker 50 bp, 1, 6, and 7: negative samples, 2-5: positive samples for *B. henselae*.

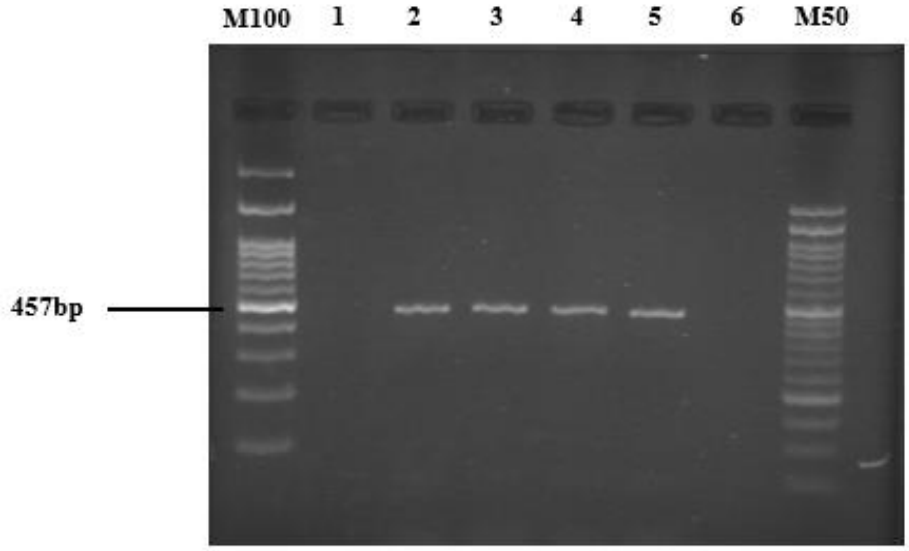

Fig. 2. Agarose gel image of the amplified fragment of the *gltA* gene (457 bp). M50: molecular mass marker 50 bp, M100: molecular mass marker 100 bp, 1, 6: negative samples, 2-5: positive samples for *B. clarridgeiae*.

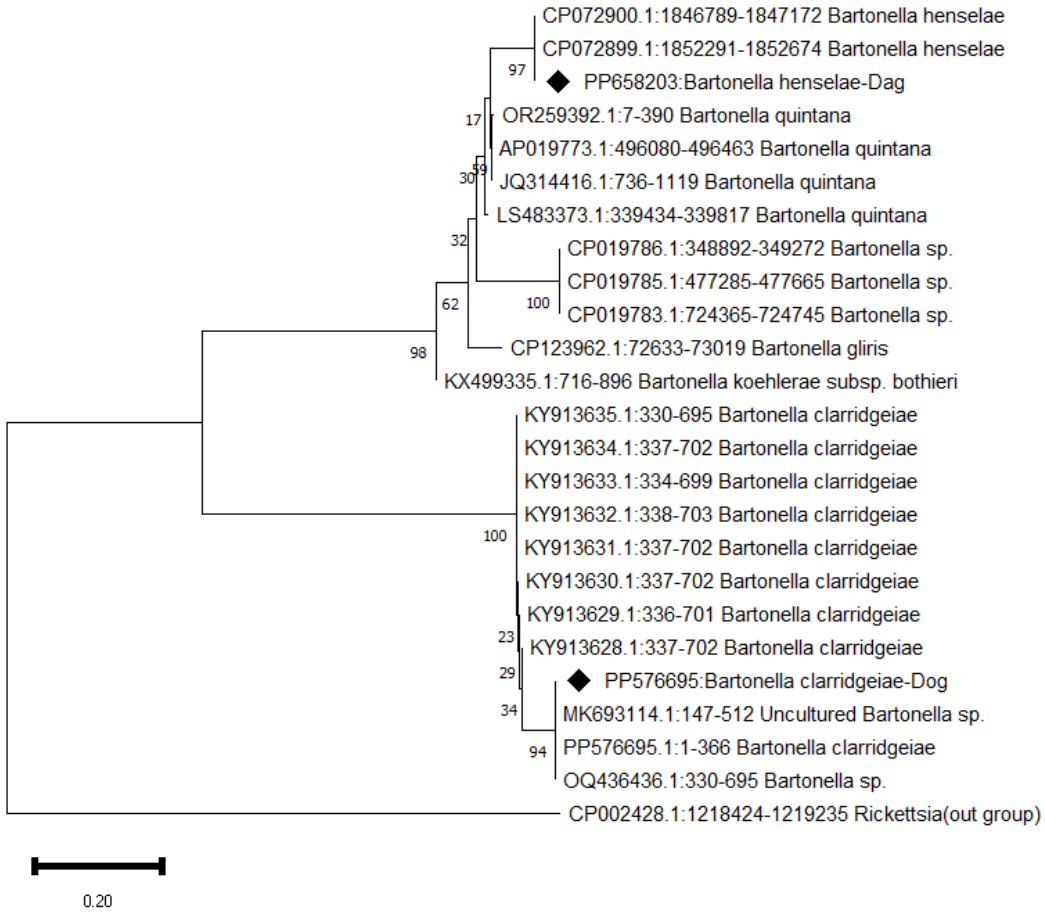

Fig. 3. A phylogenetic tree was constructed using the maximum likelihood method, utilizing the *gltA* and *ftsZ* genes from *B. henselae* and *B. clarridgeiae* in dogs. The black marks highlight the sequences examined in this research. GenBank accession numbers for the remaining sequences are provided next to the sequence names. The numbers on the branches indicate bootstrap support (from 1000 replicates). The scale bar represents the number of substitutions per nucleotide.

Discussion

The study analyzed 400 dog blood samples using nested-PCR and found that 8.5% of the samples were positive for *B. henselae*, while 3.25% were positive for *B. clarridgeiae*. The study did not find a significant difference in infection rates between male and female dogs, but did find that the infection rate of *B. henselae* was higher in dogs aged one or younger (*p*-value < 0.05, 95% CI: 12.73%-26%) and three or older (*p*-value < 0.05, 95% CI: 2.19%-8.25%), compared to dogs aged 1-3 years old. However, there was no significant difference in *B. clarridgeiae* infection across age groups. Additionally, there was a significant difference in *B. henselae* and *B. clarridgeiae* infections between neutered and non-neutered dogs (*p*-value= 0.001), as well as between stray and pet dogs based on ownership status. There was no significant difference in infection rates between different geographical regions (Table 2). These findings

align with a study conducted in Mexico in 2020, focusing on the molecular identification of *Bartonella* species in dogs and fleas, reporting a prevalence rate of 9.7% (3/31) for *Bartonella* species (24). In the current study prevalence to *B. henselae* (8.5%) was detected significantly more frequently than prevalence to *B. clarridgeiae* (3.25%). In a different study conducted in Tunisia in 2017, the seropositivity rates for *B. henselae* and *B. clarridgeiae* among dogs were 9.4% and 8.4%, respectively (25). A study carried out in central and southern Chile in 2020 found that three out of the dogs tested (3.6%) were positive for *Bartonella* species through PCR analysis. Further examination via partial sequencing of the *gltA* gene showed that two of the animals were infected with *B. henselae* (26), aligning with the prevalence of *B. clarridgeiae* identified in this study. The presence of vectors is crucial for the spread of both *B. henselae* and *B. clarridgeiae* among animals (3). Therefore, the difference in the prevalence of *Bartonella* spp. in different geographical areas can be caused by the difference in the population of arthropod vectors.

Several investigations have been conducted regarding the presence of *Bartonella* spp. in dogs in Iran. In a recent study from 2023 in Hamedan and Kermanshah cities, located in the western region of Iran, the prevalence of *Bartonella* spp. was found to be 14% (17). Another study from 2019 in Hamedan examined stray dogs using indirect immunofluorescence antibody (IFA) and molecular tests, revealing prevalence rates of 74.2% and 24.24%, respectively (27). Serological tests potentially provide prevalence data that could be influenced by cross-reactivity with different *Bartonella* species (28). In a 2020 study in Shiraz, the prevalence of *Bartonella* spp. was explored in blood samples from 98 dogs, showing a prevalence rate of 12.2% (29). Differences within the studies can be attributed to various reasons, including variations in geographic areas, environmental factors, and management issues related to the study population.

In urban and rural areas, the close relationship between humans and dogs and cats can lead to potential public health concerns due to vector-borne infections (30). While there is limited information on the prevalence of these diseases in dogs and cats, studies have shown the presence of *Bartonella* spp. in dogs in Middle Eastern countries such as Qatar (31), Iraq (32), and Iran (18). The incidence of *B. henselae* in dogs in Saudi Arabia was documented at 1.4% (33). In Turkey, a study conducted in 2010 analyzed DNA from 170 blood samples obtained from shelter dogs, finding that 21 samples tested positive for *B. vinsonii* subsp. *berkhoffii*, while the prevalence among stray dogs was 5% (34). In Iraq, a study conducted in 2012 assessed dogs through indirect immunofluorescence antibody (IFA) and molecular tests, uncovering prevalence rates of 47.4% and 37.1%, respectively (35). These findings suggest that *Bartonella* species are present in dogs from Iran and neighboring countries.

Age was identified as a significant factor (*p*-value: 0.001), suggesting that age may be a potential risk for *B. henselae* infection in dogs. Our findings demonstrated a higher incidence of *B. henselae* infection in dogs younger than one-year-old, with 24 out of 130 infected dogs falling into this age group. A study conducted in Hamedan, Iran, also found that bacteremia was more common in dogs under one year old. Out of the 10 PCR-positive dogs, eight were between one and two years old. This pattern reflects the prevalence of *B. henselae* in dogs under one-year- old, indicating that bacteremia may begin at a young age when dogs are highly susceptible to infection (18).

Gender did not have a significant impact on infection rates, and geographical origin did not exhibit a significant influence on infection rates either. The absence of distinct variations in climate may be the reason behind this phenomenon, since climate plays a crucial role in shaping the growth of pathogens in vectors, along with the distribution patterns and sizes of nonhuman vertebrate reservoirs for numerous vector-borne illnesses (36). Additionally, similarities in living conditions, such

as exposure to vectors, may play a role in the lack of a significant difference in infection prevalence in these three regions located on the periphery of Lake Urmia.

In our study, a noteworthy distinction (*p*-values of 0.001 and 0.004 for *B.henselae* and *B.clarridgeiae*, respectively) was observed between stray dogs and pet dogs. The high prevalence of *Bartonella* spp. in stray dogs (12.65% and 5.3%) was consistent with several studies (18, 37).

The study found a significant difference in *B. henselae* and *B. clarridgeiae* infection (*p*-value: 0.001) between neutered and not-neutered dogs. The results suggest that unneutered male dogs may be more likely to contract *Bartonella* infections due to lifestyle choices that increase their exposure to vectors, such as not using flea and tick prevention, engaging in outdoor activities, and interacting with potential carriers like feral cats or wild canids (38). Dogs appear to have a dual function in zoonotic bartonellosis, serving as carriers and reservoirs for human infections. The identification of *Bartonella* infections in dogs and humans, often presenting as endocarditis cases, has been documented for eight *Bartonella* species or subspecies. (39). On the other hand, there have been documented cases of *B. henselae* transmission from dog bites to humans (40), and recent publications have highlighted needle stick transmission of *B. vinsonii* subsp. *berkhoffii or B. henselae* from dogs to veterinarians (41). These results suggest that veterinarians and health officials are required to take into account the potential for the spread of *Bartonella spp*. from animals to humans in urban areas (42). However, additional studies with bigger sample sizes are necessary to confirm our research results.

Finally, the phylogenetic similarity of the isolated strains to some distant geographical strains can be explained by migrating or dispersing from a common location. This may be due to human activity, animal movement, or environmental changes. It could also be due to common environmental adaptation, a shared host or vector, and evolutionary dynamics. All of this requires further research in order to elucidate diagnostic and public health implications.

Conclusion

In summary, this study showed that 8.5% (34/400) of the blood samples examined tested positive for *B. henselae*, while 3.25% (13/400) tested positive for *B. clarridgeiae*. This signifies the initial exploration of *B. henselae* and *B. clarridgeiae* in dogs in Iran. The study's findings highlight the significant role that dogs could potentially engage as origins of *Bartonella* infection in West Azerbaijan province, Iran. The information presented in this report indicates that dogs younger than or equal to one year of age, unneutered, and stray dogs are more prone to *Bartonella* infection. Despite all infected dogs appearing healthy, they can serve as carriers of zoonotic *Bartonella*. These findings underscore the importance of *B. henselae* and *B. clarridgeiae* as zoonotic agents and stress the necessity for additional studies on their epidemiology and ways of spreading.

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Ethics approval

This study was approved by the Research & Ethics Committee of the Faculty of Veterinary Medicine of Urmia University (No. 17948/18-05-2024).

Competing interests

The authors declare no competing interests.

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