Original Research (2025) 2(2); 1 - 6
https://doi.org/10.22103/vcbr.2025.24938.1051

Shahid Bahonar University of Kerman

Veterinary and Comparative Biomedical Research

ORIGINAL ARTICLE

Molecular Detection and Genetic Characterization of Anaplasma phagocytophilum in Canine Blood Samples from Kerman, Iran via Real-Time PCR

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Online ISSN: 3060-7663 **https://doi.org/10.22103/vcbr.2025.24938.1051**

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Article History

Received: *7 March 2025*
Revised: *23 April 2025*
Accepted: *17 June 2025*
Published: *9 July 2025*

Keywords

Anaplasma phagocytophilum
Real-time PCR
Dog
Iran

Running Title

Real-Time PCR Detection of Canine A. phagocytophilum

Abstract

Anaplasmosis is an emerging tick-borne disease caused by obligate intracellular bacteria of the genus Anaplasma, capable of infecting both animals and humans. Due to the increasing stray dog population in Kerman city, Iran, and associated zoonotic risks, this study aimed to detect and differentiate Anaplasma species using molecular methods. Fifty dogs were randomly selected from veterinary clinics and city shelters in Kerman, Iran. When necessary, dogs were restrained using standard chemical or mechanical methods. Blood samples were collected and real-time PCR was conducted. For species differentiation, bidirectional nucleotide sequencing was performed using both forward and reverse primers. Multiple sequence alignment and phylogenetic analysis were subsequently performed. Out of 50 samples, three (6%) tested positive by real-time PCR. Nucleotide sequencing confirmed that all positive samples belonged to *Anaplasma phagocytophilum*. This study demonstrates the presence of *Anaplasma phagocytophilum* in dogs in Kerman city, Iran, indicating potential zoonotic risks associated with both stray and clinic-admitted animals.

How to cite this article: *Erfan Fadaie , Hossein Iravani , Mohammad Hossein Rayani, Molecular Detection and Genetic Characterization of Anaplasma phagocytophilum in Canine Blood Samples from Kerman, Iran via Real-Time PCR, Veterinary and Comparative Biomedical Research, 2025, 2(2): 1 – 6. http://doi: 10.22103/vcbr.2025.24938.1051.*

Introduction

Anaplasma platys and *Anaplasma phagocytophilum* are recognized pathogens responsible for canine infections, primarily transmitted via hard ticks. *Anaplasma platys*, although relatively rare, is known as the causative agent of infectious thrombocytopenia in stray dogs, infecting blood platelets exclusively. In endemic areas, the disease typically follows a cyclical pattern with recurrences approximately every 10–14 days (1).

In contrast, *Anaplasma phagocytophilum*, the agent of human granulocytic anaplasmosis, has been extensively studied worldwide. It infects a variety of mammalian hosts including dogs, horses, rodents, and both domestic and wild ruminants primarily targeting neutrophils while sparing eosinophils (2). Notably, *Anaplasma phagocytophilum* demonstrates considerable genetic heterogeneity, which may result in variable pathogenicity across different hosts; indeed, some isolates from reservoir animals might not pose a risk to humans (3).

Given the zoonotic potential of these pathogens, timely and precise molecular diagnosis is crucial for effective treatment and control. Therefore, this study was designed to identify Anaplasma species in canine blood samples from Kerman city, Iran, using real-time PCR, enabling early detection and prompt therapeutic intervention.

Materials and Methods

Study Population

The study involved 50 dogs (both clinic-admitted and stray) from Kerman city, Iran. All animals underwent a thorough veterinary examination, and only those confirmed to be healthy were included. Prior to sample collection, relevant information such as age (or estimated age based on dentition), breed, living conditions, and history of tick infestation was documented.

Sample Collection

Fifty blood samples (minimum volume: 3 ml) were collected from the cephalic or saphenous veins. When necessary, dogs were restrained using standard chemical and mechanical methods. Owner consent was obtained prior to sampling. Blood samples were collected into EDTA-containing tubes, kept on ice, and transferred to the laboratory where they were stored at –20°C until DNA extraction.

DNA Extraction

DNA was extracted from each sample using the Kia spin PCR Template Purification Kit (100-sample kit; CAT. NO. K1014-100, KiaGen) according to the manufacturer's instructions. Briefly, 300 µL of serum was aliquoted into a 1.5 mL microcentrifuge tube, followed by the addition of 300 µL of lysis/binding buffer (LB buffer), 5 µL of proteinase K, and 2 µL of RNA/DNA carrier. Following incubation, 100 µL of isopropanol was added to precipitate nucleic acids, and the solution was vigorously mixed for 2 min before centrifugation at 3000 × g for 5 s. The resultant lysate was then applied to silica-based spin columns and centrifuged at 3000 × g for 1 min to facilitate DNA binding to the column matrix. To eliminate potential PCR inhibitors and residual contaminants, 500 µL of inhibitor removal buffer (IRB buffer) was added to each column, followed by centrifugation at 3000 × g for 1 min. Two sequential wash steps were then performed using 600 µL and 400 µL of washing buffer (WB), respectively, with each wash step including centrifugation at 3000 × g for 1 min. A final centrifugation step at 1200 × g for 2 min ensured complete removal of residual ethanol. For DNA elution, 50 µL of elution buffer (preheated to 65 °C) was added to the column, followed by a 1 min incubation at room temperature to maximize DNA recovery. The columns were then centrifuged at 3000 × g for 1–5 min, and the eluted DNA was immediately stored at –20 °C to preserve integrity until subsequent molecular analyses.

Real-Time PCR

The extracted DNA samples were amplified using SYBR Green-based quantitative PCR (qPCR) targeting the bacterial 16S rRNA gene. The amplification was performed using specific primers: forward primer 5′-GCTCGTAGTTGGATTTCTGTTGTATT-3′ and reverse primer 5′-GGCAGTTGCCTGCTTTAAGC-3′. Each 25 µL reaction mixture contained 12.5 µL of 2× PCR master mix (Bioneer Mix Master), 5 pmol of each primer, 100 ng of template DNA, and 9.5 µL of DEPC-treated nuclease-free water. All reactions were performed in duplicate using a Bioneer Exicycler™ 96 thermal cycler. The thermal cycling protocol consisted of an initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of denaturation at 95 °C for 20 seconds and combined annealing/extension at 61 °C for 40 seconds. Following amplification, a melting curve analysis was performed by gradually increasing the temperature from 62 °C to 94 °C at a rate of 1 °C per second.

Bioinformatics Analysis

PCR products were submitted for bidirectional Sanger sequencing at a commercial facility (Pishgam Biotech, the authorized Bioneer representative in South Korea). Chromatogram files were analyzed using Chromas v2.6.6 (Technelysium Pty Ltd, Australia) and BioEdit v7.2.5 software to manually resolve ambiguous base calls. Consensus sequences were assembled using SeqMan Pro v17 (DNASTAR, USA) and exported in FASTA format for downstream processing. The sequences were compared against the NCBI GenBank database via the BLAST algorithm. Multiple sequence alignments were performed using BioEdit software with the ClustalW method, followed by phylogenetic analysis in MEGA v5.04. Neighbor-joining trees were constructed with 1,000 bootstrap replicates to assess branch support. Specificity was confirmed by comparing sequences to *Mycoplasma genitalium* and *Mycoplasma hominis* isolates from Iran and global references.

Statistical Analysis

Statistical analyses were performed using IBM SPSS statistics 2009. Data are presented as mean ± standard deviation (SD). Group differences were assessed via one-way ANOVA, followed by Tukey's HSD post hoc test for pairwise comparisons. For Real-Time PCR data, differences in threshold cycle (Ct) values between treated and control samples were determined using the ΔΔCt method, defined as: ΔCt = Ct (target gene) – Ct (reference gene), ΔΔCt = ΔCt (test sample) – ΔCt (control sample), Relative expression = 2^(–ΔΔCt). A P value of < 0.05 was considered statistically significant.

Results

Molecular Findings

Among the 50 dogs screened, 32 were male and 18 were female; infection was detected in 2 of the 32 males (6.25 %) and in 1 of the 18 females (5.56 %). Thus, the infection rate in males was approximately 1.13 times that in females, however, this difference was not statistically significant (Fisher's exact test; $p > 0.05$).

Regarding age groups, 17 dogs were under 1 year old (1 infected, 5.88%), 18 were between 1 and 6 years old (1 infected, 5.56%), and 15 were between 6 and 12 years old (1 infected, 6.67%). No significant differences in Anaplasma prevalence were observed among these age groups (Fisher's exact test; $p > 0.05$).

Sequencing and Phylogenetic Analysis

Bidirectional sequencing was performed on the PCR products from the three positive samples. The resulting nucleotide sequences were processed, corrected, and assembled into consensus sequences in FASTA format. BLAST analysis of these sequences against the NCBI GenBank database revealed a high genetic similarity to *Anaplasma phagocytophilum*, with no detectable homology to *Anaplasma platys*.

Phylogenetic analysis was conducted using the Neighbor-Joining method with 1,000 bootstrap replicates, as previously described (4). As shown in Figure 2, all three sequences from this study cluster tightly with reference strains of *Anaplasma phagocytophilum*, confirming that the pathogen detected in these dogs is *Anaplasma phagocytophilum.*

Discussion

Anaplasma phagocytophilum and *Anaplasma platys* are obligate intracellular, Gram-negative bacteria transmitted by hard ticks. *Anaplasma phagocytophilum* infects a broad range of mammalian hosts, including humans, sheep, goats, cattle, horses, dogs, and cats and is responsible for granulocytic anaplasmosis in both humans and dogs, as well as fever in cattle (5). Distinct from other Anaplasma species, *Anaplasma phagocytophilum* predominantly targets neutrophils, with only minimal presence in eosinophils. This specific tropism disrupts neutrophil antimicrobial functions, impairs the oxidative burst, and delays apoptosis, leading to immunosuppression and an increased susceptibility to secondary infections (3).

Although dogs are the primary hosts for *Anaplasma platys*, this bacterium has also been reported in other species, including cats, foxes, camels, red deer, roe deer, goats, sheep, cattle, and even humans (1). Diagnosis of these infections is challenging due to their nonspecific clinical signs, which typically include fever and general weakness. In the case of *Anaplasma platys*, thrombocytopenia is a key but sporadic finding; initial platelet destruction may trigger additional immunologic mechanisms (6).

Because clinical signs in dogs are generally nonspecific, diagnosis relies heavily on para-clinical methods. These include blood smear microscopy, various serological assays, and molecular techniques such as PCR to identify pathogen DNA in blood, tissue, bone marrow, or spleen (7). However, serological tests can produce false positives due to cross-reactivity with other Rickettsial organisms or residual antibodies from previous exposures. In contrast,

https://doi.org/10.22103/vcbr.2024.23657.1022

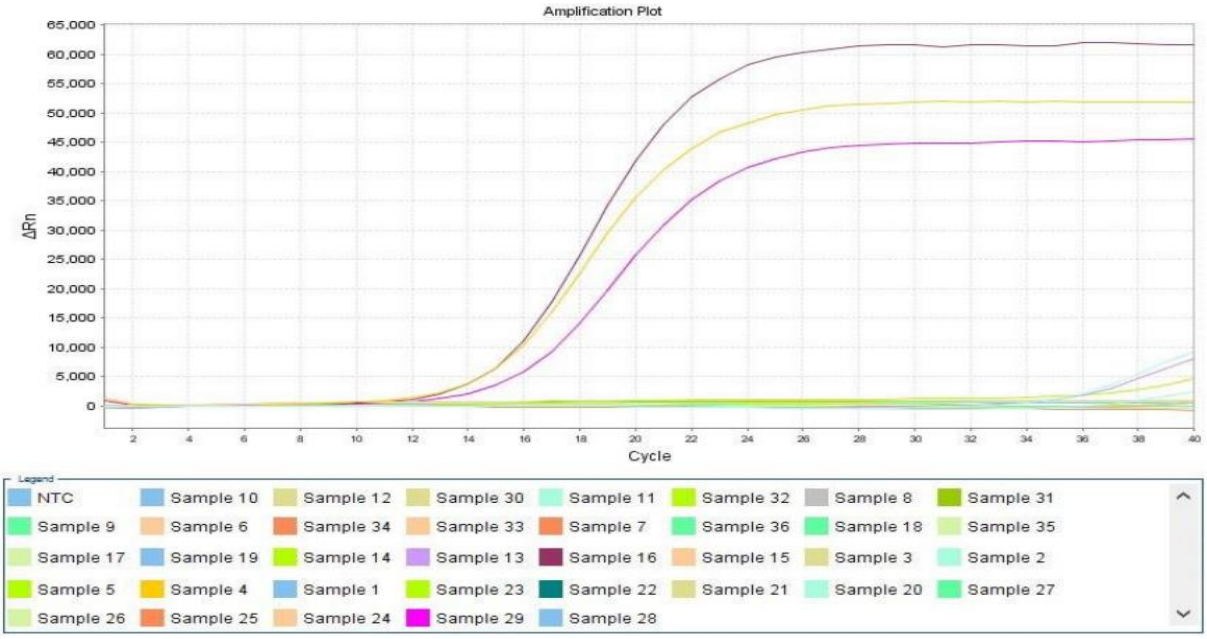

Figure 1. Bacterial proliferation chart based on the 16S rRNA gene of the Anaplasma genus

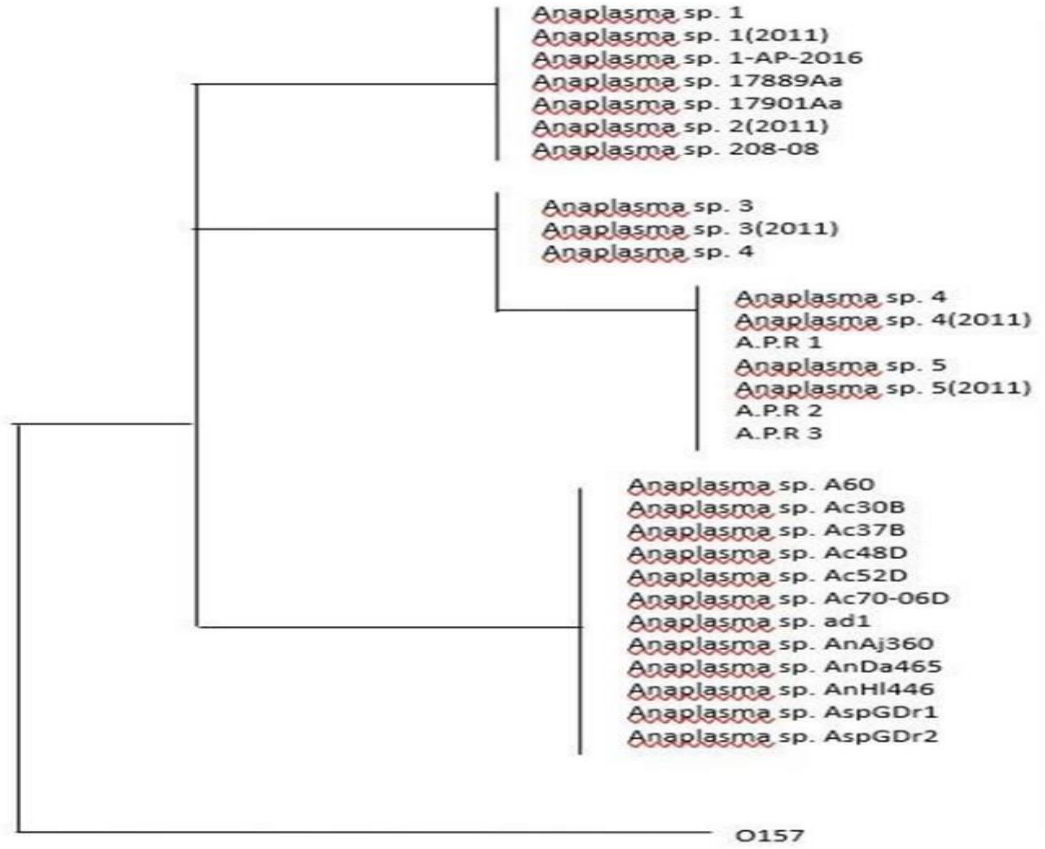

Figure 2. Neighbor-Joining phylogenetic tree of Anaplasma spp. based on partial 16S rRNA gene sequences (~1400 bp). The three sequences obtained in this study cluster with known *Anaplasma phagocytophilum* strains, indicating that all positive samples belong to *Anaplasma phagocytophilum*. Bootstrap values (1,000 replicates) are shown at relevant nodes.

molecular diagnosis targeting the hypervariable region of the 16S rRNA gene provides a more reliable mothod for differentiating Anaplasma species (2).

Prior to this study, no research had investigated the prevalence of *Anaplasma phagocytophilum* in dogs from Kerman city, Iran. The current findings show that 6% of the sampled dogs (3 out of 50) tested positive for *Anaplasma phagocytophilum* using molecular analysis. BLAST comparisons of the 16S rRNA gene sequences demonstrated high genetic similarity among the positive samples, confirming their identification as *Anaplasma phagocytophilum*.

These findings align with several previous studies. For example, *Anaplasma phagocytophilum* was detected in 3.3% of blood samples from western Colombia using nested PCR (8), while a 5.7% positivity rate was reported by real-time PCR in 522 dogs from northeastern Germany (9). Similarly, Santos et al. (2013) found a 6% prevalence in Brazil through qPCR targeting the 16S rRNA gene, even though blood smears did not reveal the presence of parasites (10). In contrast, another study from Isfahan detected *Anaplasma phagocytophilum* in only 1.3% of bovine samples using nested-semi PCR, with all corresponding blood smears testing negative (11).

Beyond pathogen detection, the zoonotic implications are significant. Subclinical infections with *Anaplasma phagocytophilum* and *Anaplasma platys*, Paticularly in stray or shelter dogs, can act as silent reservoirs for human infection. The persistence of these pathogens, combined with the widespread distribution of hard ticks, even in non-endemic areas, presents substantial public health concerns. Ticks play a central role in the transmission dynamics of Anaplasma species. In Europe, for example, *Ixodes ricinus* is the primary vector for *Anaplasma phagocytophilum*, with reported prevalence rates in ticks ranging from 0.8% to 23.6% (12). Similar findings have been documented in Iran (13). Conversely, the main vector for *Anaplasma platys* remains uncertain, although *Rhipicephalus sanguineus* is often implicated, particularly in cases with concurrent infections such as *Ehrlichia canis* and Babesia or Theileria (14).

In the present study, no significant association was observed between molecular prevalence and the age or sex of the dogs, consistent with previous findings that also reported no meaningful correlations with these variables (10, 15). Therefore, sequence analysis demonstrated high genetic similarity with canine isolates (ApDog), highlighting the potential risk of human infection in this region. These results underscore the importance of implementing effective control measures targeting stray dog populations and tick infestations to safeguard public health.

At the genetic level, divergent evolution within the Anaplasma genus is evident. Specifically, the selective expansion of the 44p2/msp gene subset in *Anaplasma platys* and *Anaplasma phagocytophilum*, compared to the omp gene expansion in *Anaplasma marginale* and *Anaplasma ovis*, reflects adaptation to domestic animal environments and highlights their significant zoonotic potential (5).

Clinically, the dogs in this study did not show overt signs, suggesting that many Anaplasma infections may be subclinical or only mildly symptomatic. Nonetheless, asymptomatic carriers can still facilitate transmission, posing a latent threat to public health especially since untreated human granulocytic anaplasmosis can be severe or even fatal.

Acknowledgements

Not applicable.

Author Contributions

Erfan Fadaei Study design and writing the manuscript, **Hossein Iravani** Conceptualizatin, supervision, **Mohammad Hossein Rayani** Laboratory analysis.

Data Availability

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request. All relevant data supporting the findings of this manuscript are included within the article and its supplementary materials. Additional information or materials can be provided by the authors if required for further research or verification

Ethical Approval

This study involved live animals, and all applicable international, national, and institutional guidelines for the care and use of animals were strictly followed. Written informed consent was obtained from all participating dog owners prior to enrollment. The protocol was designed to minimize pain and distress, with immediate intervention measures in place should any discomfort arise. Animal safety was prioritized through continuous monitoring of risk factors and the provision of veterinary care. Owners retained the unequivocal right to withdraw their animals from the study at any time.

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article

Consent for Publication

Not applicable.

Funding

Research fund has prepared by Islamic Azad University, Baft Branch, Baft, Iran.

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