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Identification and Genotyping of *Anaplasma phagocytophylum* Strains with Zoonotic Potential in Dogs from Mashhad Shelters, Khorasan-Razavi Province, Iran

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ABSTRACT

Background and Aim: Anaplasmosis caused by *Anaplasma* spp. is an important arthropod-borne disease of various vertebrates with health concerns to humans. The present study aimed to investigate the detection and genotype of *Anaplasma phagocytophilum* using microscopic examination, real-time PCR technique, and phylogenetic analysis in dogs from Mashhad shelters, Khorasan-Razavi province.

Materials and Methods: For this purpose, 250 blood specimens were collected during routine health checkups from dogs in different shelters in Mashhad, Iran, in 2020. First, smears were prepared from the blood specimens, stained with Giemsa, and examined under the light microscope for *Anaplasma* inclusions. Then, the genomic DNAs were extracted from buffy coats of blood specimens and screened by real-time PCR for the presence of *Anaplasma* infection by amplifying a 1400 bp sequence of *16S rRNA* belonging to the *Anaplasma* genus. Finally, sequencing and BLAST analyses were carried out on the amplified fragments for the phylogenetic assessments in positive specimens.

Results: A total of 9 dogs (3.60%), including 5 females (3.40%) and 4 males (3.88%) were found to be positive for *Anaplasma* infection in real-time PCR. Moreover, in blood smear observation, *A. phagocytophilum* morulae were detected in the neutrophils of 3 PCR positive animals.

Conclusion: This study provides important data regarding *A. phagocytophilum* in dogs and the degree of genetic homology/heterogeneities among these pathogen strains from dogs and humans in Iran and other countries. To our knowledge, this is the first molecular evidence on the infection of *A. phagocytophilum* in sheltered dogs of the region.

Keywords: *Anaplasma phagocytophilum*, Dogs, Iran, Molecular identification, Phylogenetic analysis

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1 Introduction

Anaplasmosis, a disease caused by *Anaplasma* spp., belongs to the complex of several arthropod-borne diseases with zoonotic potential. *Anaplasma* are gram-negative obligate intracellular pathogens, infecting blood cells of mammals and vertebrate hosts act as reservoirs for this microorganism. The major vectors of the *Anaplasma* spp. are Ixodes ticks distributed worldwide **(1,2)**.

In the vertebrates, *Anaplasma phagocytophilum*, *Anaplasma platys*, *Anaplasma capra*, *Anaplasma marginale*, *Anaplasma ovis*, and *Anaplasma bovis* are considered as major pathogens **(3–5)**. Likewise, species with zoonotic potential have been reported as well as *A. phagocytophilum*, *A. capra*, and *A. ovis* which are transmitted most often through the bite of a hard tick to humans **(6,7)**. Depending on the

Anaplasma spp., various cells in the reticuloendothelial system as well as granulocytes, platelets, erythrocytes, and bone marrow precursor cells or endothelial cells may be infected **(2)**, resulting in a variety of clinical signs from mild to life-threatening illness including anemia, gastrointestinal and pulmonary disorders, and joint pain and lameness **(8)**. *A. phagocytophilum* is a frequently characterized pathogen from small ruminants, horses, dogs, and humans, which infects granulocytes causing Granulocytic Anaplasmosis (GA), a multisystemic disease with subclinical or severe symptoms. Ixodes and Dermacentor ticks are the major vectors for *A. phagocytophilum* **(2)**.

The present study deals with detecting *A. phagocytophilum* in the blood specimens of sheltered dogs from Mashhad city in the northeast of Iran. Besides, the detected pathogens were phylogenetically compared to typed strains of *A. phagocytophilum* present in the GenBank.

2.Materials and Methods

In this investigation, 5 mL of blood specimen via the saphenous vein of 250 dogs (103 males and 147 females) belonging to different breeds and aging from 2 months up to 12 years, sheltered in Mashhad (Iran), were collected in EDTA containing tubes. Sampling was carried out during a routine health checkup program. The blood samples were placed on ice and immediately transferred to the faculty of veterinary medicine of Semnan University. Then, DNA extraction was performed on the buffy coat of blood specimens using a DNA extraction kit (Sinapure DNA whole blood, serum, and plasma, Sinaclon, Iran). The extracted DNA samples were preserved in a -20 freezer until molecular assays. Additionally, the blood smears were directly prepared and fixed by methanol. Then, fixed smears were stained by Giemsa and observed by a light microscope.

Based on the morphology of pathogens infecting granulocytes in microscopic observations, the extracted DNA samples from buffy coats were screened for the presence of *Anaplasma* spp. by realtime-PCR amplifying a 1400 bp fragment specific to 16S rRNA according to the protocol described by Dyachenko *et al*. **(9)** with some modifications in thermal conditions. Real-time PCR amplifications were performed using a Rotor-Gene Q MDx real-time PCR instrument (QIAGEN). The PCR reactions were performed in a final volume of 20 µL containing 2X real-time PCR Master Mix (SYBR® Green I; BioFACT™), 500 nM of primer pairs (VD2f: 5'-AGAGTTTGATCCTGGCTCAG-3', and VD2r: 5'-CGGCTACCTTGTTACGACTT-3'), and 100 ng of the DNA template. In this experiment, *A. platys* were used as the positive control, and distilled water was used as the negative control. Data were analyzed using Rotor-Gene Q series software 2.3.1 (QIAGEN). Moreover, for the size determination of amplified fragments, realtime-PCR products (10 µL) were electrophoresed in 1.5% agarose (Sigma-Aldrich) gel, stained with ethidium bromide (Sigma-Aldrich), and visualized under a UV illuminator (Nanolytik™, England). Mid-range DNA ladders (100 bp, Jena Bioscience) were used for fragment size determination.

For phylogenetic analysis of detected *Anaplasma* strains as a preliminary screening, the extracted DNA from blood specimens of Anaplasma-positive dogs in previous realtime-PCR steps were subjected again to conventional PCR for amplification of the ~1400 bp fragment of *16S rRNA*. The reactions were prepared in total volumes of 50 µL with 15 pmol of each primer VD2-f and VD2-r (9), 25 µL of 2X PCR Master Mix (Jena Bioscience, Germany), and 500 ng of genomic DNA. After an initial denaturation step at 94°C for 60 s, the reactions were cycled 35 times (BIOER XP Cycler, China) as follow: denaturation at 94°C for 30 s, annealing at a temperature specific (achieved by gradient temperature conditions) to the primer pairs 54°C for 45 s, and extension at 72°C for 90 s. Then a final extension step was followed at 72°C for 5 min to complete the last PCR cycle. After electrophoresis of PCR amplicons on 2% agarose gel, the 1400 bp products were sent for nucleotide sequencing.

The nucleotide sequence of the amplified fragments was analyzed with an ABI 3730XL DNA Analyzer according to an automated Sanger dideoxy fluorescent nucleotide method. The BLAST software was applied to determine the homology of the amplified fragments to *16S rRNA* sequences of *Anaplasma* spp., existing in GenBank. The evolutionary history was inferred using the Maximum Likelihood method and Tamura-Nei model **(10, 11)**. The tree with the highest log likelihood (-6943.31) is shown. The initial tree for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with a superior log-likelihood value. This analysis involved 32 nucleotide sequences. There were a total of 1575 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

3.Results

Typical large basophilic inclusions (morulae) were observed in neutrophils of 3 dogs (Figure 1) during blood smears observation. Realtime-PCR demonstrated that Anaplasma *16S rRNA* was detected in 9 out of 250 (3.60%) blood specimens, including 4 (0.38%) positive specimens from male dogs and 5 (0.34%) from

female dogs, respectively (Figure 2). Statistical analyses on the prevalence of Anaplasma-positive samples across dogs stratifying by gender and aging showed that the presence of Anaplasma was not significant in different genders and age groups (P>0.05).

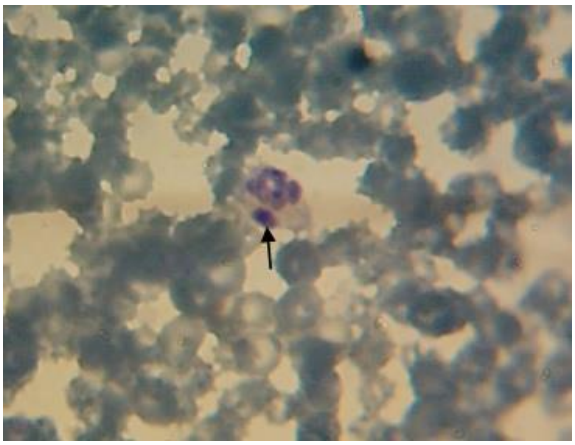

Figure 1. *Anaplasma morulae* (black arrows) in the neutrophil of a dog in the present study, 4000 ×, stained by the Giemsa method.

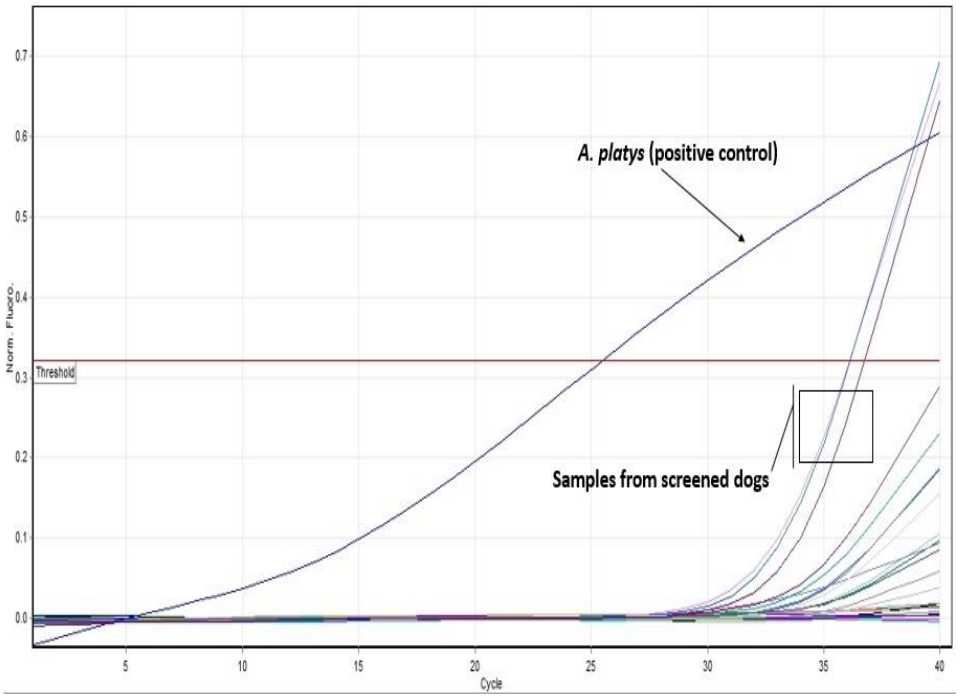

Figure 2. Cycling Threshold (CT) of *Anaplasma* spp. 16S rRNA specific fragment in blood specimens from monitored dogs. Amplification of 16S rRNA in blood specimens of dogs are noticed in addition to *A. platys* as the positive control.

In the present investigation, the obtained sequences of *16S rRNA* belonging to *Anaplasma* spp. showed genetic identities of 98.05–98.13% with the sequences of *A. phagocytophilum* type strains present in GenBank. The phylogenetic tree based on the Maximum Likelihood method in the present study indicates the position of our detected strains and their identity with the type strains of *A. phagocytophilum* and other closely related species (Figure 3). The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Analysis of *16S rRNA* sequence from detected Anaplasma strains infecting dogs demonstrated a high similarity level with the *A. phagocytphilum* strains: Norway variant2 (CP01537-6.1), Dog 2 (CP006618.1), Hubei E4(KF569909.1), JXAAGX-49 (MH722235.1), Yeyasu (LC334014.1), and HB-SZ-HGA-S05 (HQ872465.1) deposited in GenBank by other researchers (Figure 3).

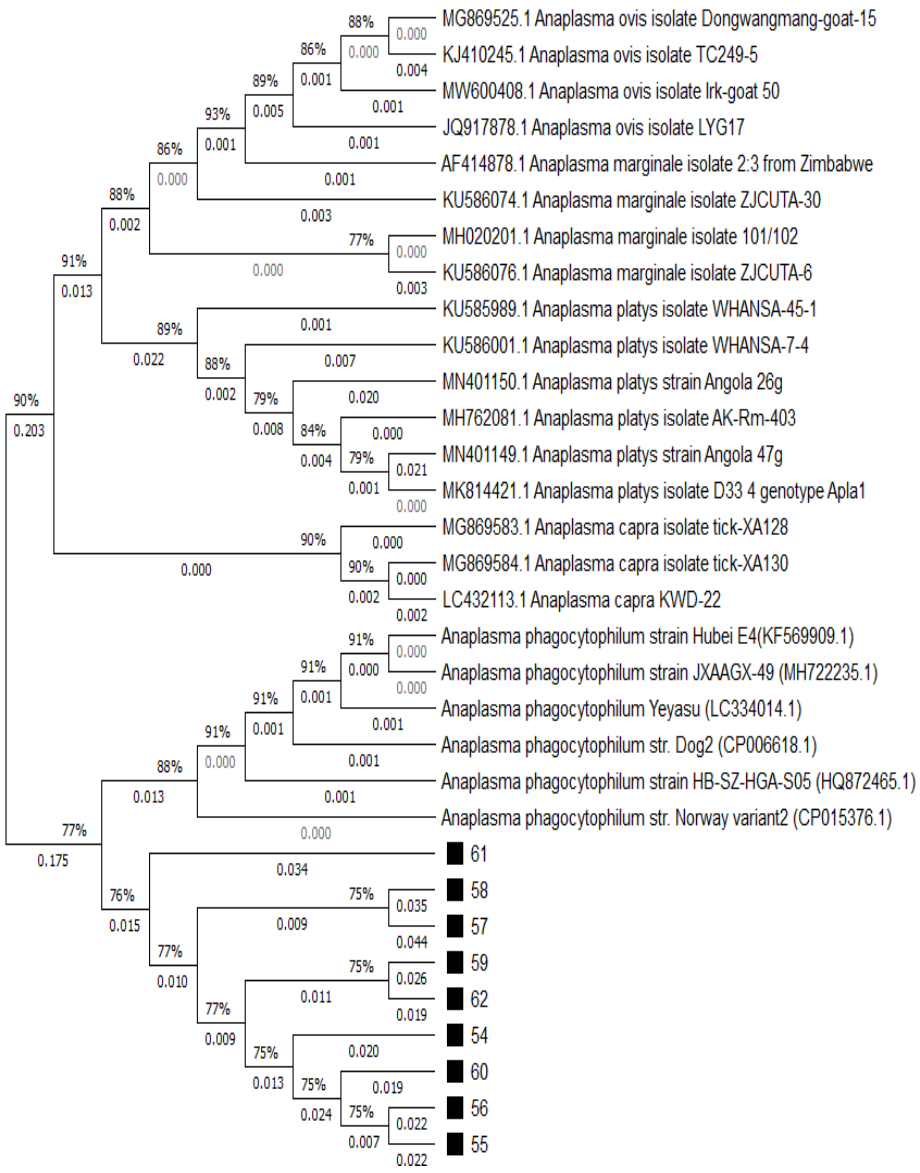

Figure 3. Phylogenetic analysis of *A. phagocytophilum* strains identified in the present study based on the 16S rRNA sequencing. The tree was constructed using the Maximum Likelihood method. *A. ovis*, *A. marginale*, *A. platys* and *A. capra* strains were used for phylogenetic comparisons.

4.Discussion

Anaplasma phagocytophilum, a tick-borne pathogen, is emerging in some vertebrates, mainly humans and dogs, worldwide **(12)**. Several predisposing factors play an essential role in arthropods' multiplication and spread, increasing the possibility of *Ixodidae* ticks feeding on humans and dogs and transmitting *A. phagocytophilum* to these hosts **(13)**.

In Asia, the first report regarding the serological evidence of the infection with *A. phagocytophilum* in a human was documented in Korea **(14)**. Then, the first Molecular report of *A. phagocytophilum* infection was documented by Kawahara (2006) and Ooshiro (2008) in cattle and wild deer in Japan **(15,16)**. In Iran, *A. phagocytophilum* was detected by PCR-RFLP for the first time as a potential novel arthropod-transmitted agent in the *Ixodes ricinus* ticks **(17)**, and subsequently, it has been reported from different vertebrates and tick vectors from different parts of Iran **(18–21)**. Molecular detection of *A. phagocytophilum* revealed a point prevalence of 3.6 % in our study population. Other studies carried out in Iran regarding the molecular prevalence of *A. phagocytophilum* in dogs found a prevalence range between 2-57% **(22,23)**. The different ranges of Anaplasma infections in dogs from various regions can be related to the differences in the prevalence of vector ticks in these regions because factors including climate conditions and acaricide treatments can influence the spread of infected

vectors **(20)**. In the present study, *A. phagocytophilum* was detected in blood smears of only 3 dogs (1.2%), while molecular detection by real-time PCR identified 9 dogs (3.6%) infected with this pathogen. The different obtained results between the two applied methods (1.2% vs 3.6%) are due to the less sensitivity of microscopic observation than real-time PCR. Similar results have been observed in previous investigations **(23)**. The "Gold standard" method for the diagnosis of *Anaplasma* spp. relies on the combination of the microscopic examination and cELISA. The indirect immunofluorescence antibody test is widely used to diagnose blood protozoon and Rickettsia. The IFA test is commonly used in epidemiological studies because of its low costs **(24)**.

The primer pairs (VD2-f & VD2-r) of the real-time PCR used in this study and *16S rRNA* sequencing of *Anaplasma* spp. have been applied effectively for genotyping and differentiation of *Anaplasma* spp. in previous investigations. It has been stated that these molecular methods are highly sensitive and specific for phylogenetic purposes because the sequence of *16S rRNA* fragment of *Anaplasma* spp. has a small hypervariable region (9, 25–27). In the present survey, characterization of detected *A. phagocytophilum* strains by real-time PCR and subsequent *16SrRNA* sequencing of strains determined them very close to type-strains Dog 2 (CP006618.1), Norway variant2 (CP015376.1), Hubei E4(KF569909.1), JXAAGX-49 (MH722235.1), Yeyasu (LC334014.1), and HB-SZ-HGA-S05 (HQ872465.1) deposited in GenBank by other researchers, phylogenetically (Figure 3). These mentioned relatively close type-strains of *A. phagocytophilum* to the strains of the present study have been identified from humans (China), dogs (Japan and Norway), and goats (China), previously **(28–31)**. As our knowledge rises more about the genetic similarity of *A. phagocytophilum* strains from different hosts, the evidence is developing that this pathogen infects a broader range of vertebrate hosts than previously thought. Human exposure to ticks carrying *Anaplasma* spp. has been increased by environmental and land-use changes causing more contact between humans, animals, and vector reservoirs **(31)**. Stray and sheltered dogs can act as a reservoir for *A. phagocytophilum*, infecting immature stages of *Ixodidae* ticks which act as bridge vectors transferring *Anaplasma* to humans and livestock when they are symptomatic or asymptomatic **(20)**. These animals circulate in urban and suburban places worldwide and contact humans in public places and livestock on farms. Our results (Figure 3) confirm the fact that stray and sheltered dogs can serve as an important reservoir for different genotypes of *A. phagocytophilum* and can transmit it to vectors specifically to those ticks infesting other livestock and human. Whether the existence of *A. phagocytophilum* in sheltered dogs in Mashhad (Iran) poses a considerable zoonotic hazard for humans remains undetermined. This study should signal Iranian physicians and veterinarians that A. phagocytophilum exposure and infection are not rare, and it will help raise alertness on the potential incidence of anaplasmosis more in this region. Since we reported results in a limited area of the country and on a very limited number of dogs, larger and more representtative investigations are recommended, especially on human cases with anaplasmosis and genotyping of *A. phagocytophilum* strains identified in Iran from the human.

5. Conclusion

There seems to be a high risk of infection with *A. phagocytophilum* for dogs in Iran. Our findings highlight the significance of these animals as a potential hazard for livestock and humans. Besides, 9 strains of *A. phagocytophilum* were identified in dogs during this study using *16S rRNA* sequencing. Thus, it can be concluded that more *A. phagocytophilum* genotypes should be expected to exist in dogs. More investigations and monitoring seem to be required in dogs and other vertebrates associated with different geographic regions in Iran to determine the epidemiologic distribution of *A. phagocytophilum* genotypes. The clinical significance of the results of this study remains to be elucidated in future investigations.

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Author's Contributions

HS: Conceived and designed the analysis; **NM**, **MK**, **EGK**: Collected the data; **HS** and **MK**: Contributed data or analysis tools; **HS**: Performed the analysis; **HS**, **NM**, **MSB**, **MK**: Wrote the paper.

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Conflict of Interest

The authors declared no conflict of interest.

Referance

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