

Original Article

Molecular Detection of *Anaplasma phagocytophilum* as a Zoonotic Agent in Owned and Stray Dogs in Tehran, Iran

Yousefi^{1,*}, A., Chaechi Nosrati², M.R., Golmohammadi², A., Azami³, S.

1. Young Researchers and Elites Club, Science and Research Branch, Islamic Azad University, Tehran, Iran
2. Department of Microbiology, Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran
3. Department of Parasitology, Faculty of Paraveterinary Medicine, Bu-Ali Sina University, Hamedan, Iran

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Corresponding Author: Aliusafi@yahoo.com

ABSTRACT

The genus *Anaplasma* is an obligated intracellular Rickettsia and among its species, *Anaplasma phagocytophilum* (*A. phagocytophilum*) is a zoonotic agent that infects host neutrophils. The aim of this study was molecular detection of *A. phagocytophilum* infection based on MSP4 gene in owned and stray dogs in Tehran, capital of Iran. One hundred and fifty blood samples were collected from dogs in Tehran and suburbs of Tehran, Iran. Firstly, the thin blood smears were prepared and Giemsa staining method was conducted. Then, the samples were examined under oil immersion objective and 0.67% of them were observed infected with *A. phagocytophilum*. The DNA was extracted from blood samples using a DNA isolation kit (MBST, Iran), and MSP4 gene extraction was performed by Polymerase chain reaction (PCR) and nested-PCR. Finally, 2% of the samples were positive for *A. phagocytophilum*. The data were analyzed using SPSS software (version 19.0) and Chi-square test was performed. There was no significant relation between infection and age, as well as sex and ectoparasitic infestation ($P>0.05$). This article was a report of *A. phagocytophilum* infection in dogs and their potentials as host carriers of this important microorganism in Tehran, Iran.

Keywords: *Anaplasma phagocytophilum*, Tick-borne, Dog, Tehran, Iran

Détection moléculaire d'*Anaplasma phagocytophilum* comme agent zoonotique chez des chiens errants et domestiques de Téhéran, Iran

Résumé: Le genre *Anaplasma* est une Rickettsie intracellulaire obligatoire et, parmi ses espèces, *Anaplasma phagocytophilum* (*A. phagocytophilum*) est un agent zoonotique qui infecte les neutrophiles de l'hôte. Le but de cette étude était la détection moléculaire basée sur le gène MSP4 des infections à *A. phagocytophilum* à partir d'échantillons sanguins prélevés chez des chiens errants et domestiques dans la capitale iranienne, Téhéran. Un total de cent cinquante échantillons de sang ont été prélevés chez des chiens à Téhéran et banlieue. Tout d'abord, les frottis de sang ont été préparés et colorés selon la méthode de Giemsa. Ensuite, les échantillons ont été examinés sous microscope optique avec l'ajout d'huile d'immersion. Parmi les échantillons observés, 0,67% étaient infectés par *A. phagocytophilum*. L'ADN a été extrait des échantillons de sang à l'aide d'un kit d'isolation d'ADN (MBST, Iran), et l'extraction du gène MSP4 a été effectuée par réaction en chaîne à la polymérase (PCR) et PCR imbriquée. Enfin, 2% des échantillons étaient positifs pour *A. phagocytophilum*. Les données ont été analysées à l'aide du logiciel SPSS (version 19.0) et un test du chi carré a été réalisé. Il n'y avait pas de relation significative entre l'incidence de l'infection, l'âge, le sexe et la présence d'infestation ectoparasitaire ($P>0,05$).

Cette étude rapporte la présence d'infections à *A. phagocytophilum* chez les chiens de Téhéran qui représentent de ce fait des porteurs potentiels de cet important microorganisme dans la capitale iranienne.

Mots-clés: *Anaplasma phagocytophilum*, Transmise par des tiques, Chien, Téhéran, Iran

INTRODUCTION

Anaplasma species (Family: Anaplasmataceae, Order: Rickettsiales) are obligate intracellular gram-negative bacteria, which infect mammalian hosts cells (Dumler et al., 2001; Kocan et al., 2003). The genus *Anaplasma* is biologically transmitted by ixodid ticks and other modes of transmission, for instance, through the bites of *Stomoxys* and *Tabanus*, blood contaminated instruments in animals, nosocomial transmission by respiratory secretions, direct contact with blood, and blood transfusions in the human (Potgieter, 1979; Ewing, 1981; Foil, 1989; Annen et al., 2012; Zhang et al., 2014). *Anaplasma phagocytophilum* is a zoonotic agent that infects neutrophils and causes Human Granulocytic Anaplasmosis in humans, cats, dogs, horses and tick-borne fever in ruminants. This agent causes a variety of clinical syndromes in humans from mild in ordinary people to severe in immunocompromised or old peoples (Rikihisa, 1991; Bakken et al., 2006; Robinson et al., 2009). *A. phagocytophilum* has been reported for the first time in North America in 1994; afterward, many reports of *A. phagocytophilum* have been published around the world (Chen et al., 1994; Dumler et al., 2005). Furthermore, a review of available resources showed that there have been some theses and reports about *A. phagocytophilum* in some regions of Iran, in which samples have been obtained from sheep, goats, cattle, dogs, and ticks (Bashiribod et al., 2004; Noaman and Shayan, 2009; Maazi et al., 2010; Rassouli and Aghazamani, 2015; Bagheri, 2016; Pahlevan, 2016; Yousefi et al., 2017b). Therefore, available information regarding *A. phagocytophilum* is very rare and the distribution, carriers, and vectors of this zoonotic agent are unknown in Iran. The aim of

this study was the molecular detection of *A. phagocytophilum* infection based on MSP4 gene in owned and stray dogs in Tehran, capital of Iran.

MATERIAL AND METHODS

Research setting. This study was carried out in Tehran (i.e., capital of Iran), which is located in the center of Tehran province with a total area of 594 square kilometers and a population of around 9 million. Tehran (35°41'46"N and 51°25'23"E) have a Mediterranean precipitation pattern, along with a cold semi-arid and continental climate.

Sampling. From May 2015 to November 2015, 150 blood samples (82 males and 68 females; 75 owned dogs and 75 stray dogs) were randomly collected from veterinary hospitals and dog shelters in Tehran and suburbs of Tehran. The ages of the dogs were between 2 months and 14 years. The blood samples (each one equal to 1 ml) were collected, then two thin smears were prepared and the remaining blood samples were transferred to a sterile test tube containing 1 ml of 96% ethanol.

Thin smears preparation. The thin smears were air dried, fixed in absolute methanol, stained by the standard Giemsa technique, and examined for intracytoplasmic *A. phagocytophilum* inclusion bodies in leukocytes using an oil immersion objective (100 X).

DNA extraction. The DNA was extracted from 50 mg of fixed blood samples with a DNA isolation kit (MBST, Iran), following the manufacturer's instructions. Finally, the DNA of each sample was eluted in the 150 µl elution buffer. The extracted DNA was analyzed on 1% agarose gel before use.

Polymerase chain reaction and nested-polymerase chain reaction. This technique was performed for the detection of *A. phagocytophilum* based on MSP4 gene sequence. The polymerase chain reaction (PCR) was

carried out in 25 μ l reaction volume, including 12.5 μ l of 2 \times Mastermix (Viragene, Iran), 0.5 μ l of each sense and antisense primers (MAP4AP5 [5'-ATGAATTAC AGAGAATTGCTTGTAGG-3'] and MSP4AP3 [5'-TTA ATTGAAAGCAAATCTTGCTCCTATG-3']) specific for *A. phagocytophilum*, previously described by De la Fuente et al. (2005), 2 μ l of DNA and 9.5 μ l of water in automated thermal cycler (Astec, Germany) for 32 cycles as it follows: 1) 5 min at 95 $^{\circ}$ C for initial denaturation step, 2) each cycle, including 30 s at 94 $^{\circ}$ C for denaturation step, 30 s at 50 $^{\circ}$ C for annealing, along with 30 s at 72 $^{\circ}$ C for extension step, and 3) 5 min at 72 $^{\circ}$ C for additional extension step. The PCR test products were detached on 1.5% agarose gel in 0.5 \times TAE buffer and visualized using ultraviolet transilluminator. Following the previous step, nested-PCR was performed for the confirmation of PCR product in 25 μ l reaction volume, including 12.5 μ l of 2 \times Mastermix (Viragene/ Iran), 0.5 μ l of each sense and antisense primers (msp4f [5'-CTATTGGYGGNGCYAGAGT-3'] and msp4r [5'-GTTTCATCGAAAATTCCGTGGTA-3']) specific for *A. phagocytophilum*, previously described by Bown et al. (2007), 10.5 μ l of water and 1 μ l (diluted 1: 200) of the first round post-amplification mix (849bp) in automated thermal cycler (Astec, Germany) for 32 cycles as it follows: 1) 5 min at 95 $^{\circ}$ C for initial denaturation step, 2) each cycle, including 40 s at 94 $^{\circ}$ C for denaturation step, 10 s at 47 $^{\circ}$ C for annealing, along with 40 s at 68 $^{\circ}$ C for extension step, and 3) 5 min at 68 $^{\circ}$ C for additional extension step. Moreover, negative control contained all of the components of PCR except the DNA template and positive control sample used in this study was previously sequenced and supplied by the Department of Veterinary Parasitology at the University of Tehran. The PCR test products were detached on 1.5% agarose gel in 0.5 \times TAE buffer and visualized using ultraviolet transilluminator.

DNA sequencing. Two samples of nested- PCR product were purified and sent for sequencing using the dideoxy chain termination method by Bioneer Company (Seoul, Korea).

Statistical analysis. The statistical data were recorded using SPSS software (version 19.0). The Chi-

square test was used to determine the relation between infection and age, as well as sex and ectoparasitic infestation.

RESULTS

Out of 150 Giemsa stained blood smears of the dogs, which were examined, only one (0.67%) Ghahderijani dog (i.e., Iranian native dog) was observed infected with *A. phagocytophilum* (Figure 1). On the other hand, 2% (3/150) of the dogs (one Ghahderijani and two German shepherds) were detected to be positive for *A. phagocytophilum* infection by primary PCR and produced an 849 bp band (Figure 2A). All of the primary PCR positive samples were subjected to nested-PCR and produced a 380 bp band (Figure 2B). Moreover, the obtained results showed that in particular 1.34% (1/ 75) of the owned dogs and 2.67% (2/ 75) of the stray dogs were positive for *A. phagocytophilum* infection. Statistical analysis with the Chi-square test was performed and no significant relation was observed between infection and age, as well as sex and ectoparasitic infestation. In addition, no significant differences were detected in the prevalence rates between the two groups of owned and stray dogs ($P>0.05$).

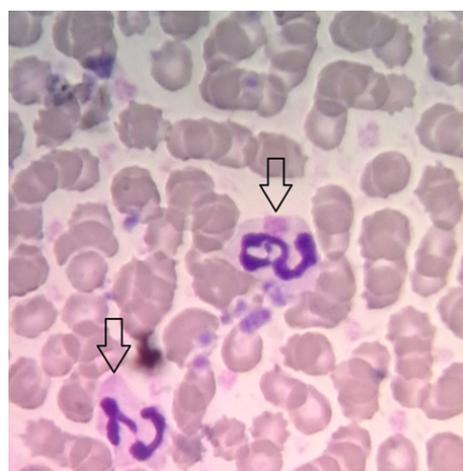


Figure 1. Giemsa stained smears of dogs; Arrows demonstrating neutrophils infected by *Anaplasma phagocytophilum*.

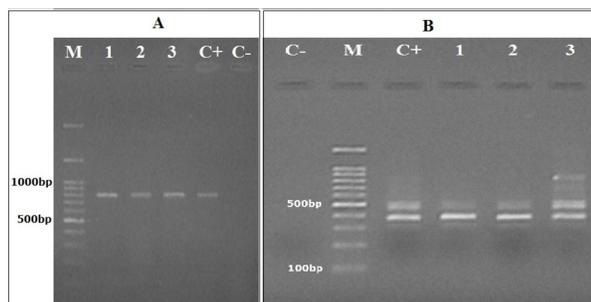


Figure 2. Agarose gel electrophoresis; A) primary polymerase chain reaction products of *Anaplasma phagocytophilum* based on MSP4 gene (849 bp); B) nested-polymerase chain reaction products of *Anaplasma phagocytophilum* based on MSP4 gene (380 bp); lanes 1, 2, and 3: *Anaplasma phagocytophilum*, C-: negative control, C+: positive control, M: 100 bp DNA ladder

DISCUSSION

Contrary to numerous reports of *Anaplasma* species in ruminants and ticks, further investigations are required to increase the knowledge about *A. phagocytophilum* in other animals in Iran (Razmi et al., 2006; Ahmadi-Hamedani et al., 2009; Noaman and Shayan, 2009; Jalali et al., 2013; Hosseini-Vasoukolaei et al., 2014; Noaman and Bastani, 2016; Yousefi et al., 2017a, b). Furthermore, a review of the literature showed that there have been some theses and reports, which have been carried out using Giemsa technique and serology method for the detection of *A. phagocytophilum* (Maazi et al., 2010; Rassouli and Aghazamani, 2015; Bagheri, 2016; Pahlevan, 2016). Although the seroprevalence of *A. phagocytophilum* antibodies in dogs was investigated, cross-reactivity between the pathogens of the *Anaplasma* genus on serological assays has also been reported (Dreher et al., 2005). On the other hand, since this Giemsa staining method has low sensitivity and is not specific, the best method is molecular detection. In this regard, only four reports of *A. phagocytophilum* are available in Iran, in which Bashiribod et al. (2004) reported the organism from *Ixodes ricinus* (*I. ricinus*), Noaman and Shayan (2009), as well as Salehi-Guilandeh et al. (2018), announced it from cattle, and Yousefi et al. (2017b) described it from sheep and goat. Moreover, the

obtained results of this study regarding the low prevalence of *A. phagocytophilum* is in line with the previous findings in Iran, which have been reported from small ruminants (1.08%), cattle (1.33%), and *I. ricinus* (5.1%) (Bashiribod et al., 2004; Noaman and Shayan, 2009; Yousefi et al., 2017b). In addition, the findings of the present study are in contrast with the results of studies carried out by Maazi et al. (2010) and Pahlevan (2016). However, the prevalence rates of *A. phagocytophilum* in Italian hunting dogs (0.93%), dogs in Brazil (6.03%), and healthy hunting dogs from central Italy (2.5%) demonstrate a low prevalence rate and similar results; however, epidemiological studies in this field are very rare (Ebani et al., 2013; Santos et al., 2013; Ebani et al., 2015). Some reports of *A. phagocytophilum* in dogs around the world are case reports and individual cases (Melter et al., 2007; El Hamiani Khatat et al., 2015). Due to the fact that *Ixodes* spp. and *Dermacentor* spp. are the biological vectors of *A. phagocytophilum* around the world (Rymaszewska and Grenda, 2008; Milner and van Beest, 2013); nonetheless, the identification of transmission routes and vectors in these areas is necessary for disease control and prevention. Further studies are needed to determine the importance of dogs as reservoirs for *A. phagocytophilum* in densely populated cities and other areas of Iran.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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