

Short Communication

Molecular detection of *Hepatozoon canis* in dogs of Ardabil Province, Northwest of Iran

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ABSTRACT

Hepatozoon species are protozoan parasites that infect some animals such as birds, reptiles, amphibians, and carnivores. Previous studies performed on canine hepatozoonosis in Iran have never used molecular techniques for diagnosis of this disease. The main objective of the present study was to detect *Hepatozoon canis* in the blood of dogs using polymerase chain reaction (PCR) method and sequencing. A total of 104 blood samples were collected from dogs of Meshginshahr County (Ardabil Province), and DNA was extracted from blood samples by dint of DNG-plus Extraction Kit. Then, 18S rRNA gene was amplified by using the conventional PCR methods. PCR products yielded an amplicon of the approximate length of 897 bp for all the positive samples. Twenty-four out of the 104 (23.07%) samples were found to be positive for *H. canis*. This rate of infection is relatively high among dogs in Ardabil Province. Sequence analysis confirmed the molecular identity of 99% of the samples by comparison with GenBank profiles. This is the first report of molecular detection of *H. canis* from Iran.

Keywords: *Hepatozoon canis*, PCR, Dog, Ardabil, Iran

Diagnostic moléculaire de l'hépatozoonose canine dans la province d'Ardabil, nord-ouest de l'Iran

Résumé: Les espèces d'hépatozoonose sont des protozoaires infectant les animaux tels que les oiseaux, les reptiles, les amphibiens et les carnivores. Bien que l'hépatozoonose canine ait été rapportée en Iran, elle n'a pas encore été détectée par des méthodes moléculaires. L'objectif principal de cette étude était le diagnostic moléculaire de l'hépatozoonose canine par la méthode PCR et la détermination de sa séquence nucléotidique. A cet effet, 104 échantillons de sang provenant des chiens de la ville de Meshkinshahr (Province Ardabil) ont été recueillies et les ADN ont été extraites à l'aide du kit DNG-PLUS. Ensuite le gène SrRNA 18 a été amplifié par PCR conventionnelle afin de déterminer sa séquence. Une bande d'environ 897 pb a été identifiée dans les échantillons positifs et 24 des 104 échantillons analysés (23.7%) étaient infectés par une hépatozoonose canine. Ce pourcentage d'infestation chez les chiens de la province Ardabil est relativement élevé. La séquence moléculaire du fragment étudié était 99% similaire à celles enregistrées dans la banque de gènes. Cette étude représente le premier rapport sur la détection de l'hépatozoonose canine en Iran.

Mots-clés: Hépatozoonose canine, PCR, Chien, Province d'Ardabil, Iran

INTRODUCTION

Hepatozoon species are among the protozoan parasites that infect some groups of animals such as birds, reptiles, amphibians, and carnivores. These parasites are transmitted by tick (Aktas et al., 2013) and have been reported in several regions of Asia including Peninsular Malaysia (Rajamanickam et al., 1985), Japan (Murata et al., 1991), India (Christophers, 2016), Sri Lanka (Dissanaike, 1961), Europe, Africa, and Latin America (Aktas et al., 2015). Canine hepatozoonosis was first reported in 1905 in India (James, 1905); this disease is mainly characterized by anemia and lethargy in carnivores (Baneth et al., 2000). The parasite is transmitted by ingestion of ticks or parts of ticks containing mature *H. canis* oocysts (Baneth et al., 2001a). *Rhipicephalus sanguineus* is one of the main vector of diseases in dogs (Baneth et al., 2001b; Baneth et al., 2007). The frequency of *R. sanguineus* is higher in the northeastern regions of Iran (Razmi et al.). Based on previous reports, the climatic condition of Meshginshahr is favorable for *R. sanguineus* (Khazeni et al., 2013). The factors affecting the prevalence of *H. canis* infection include distribution and population of vectors (Otranto et al., 2011), sampling methodology, and characteristics of the targeted dog population (Gomes et al., 2010). *H. canis* infection is more common in adult than young dogs (Gomes et al., 2010), implying the higher contact of adult animals with the vector. The conventional method for the detection of *H. canis* is microscopic observation of intracellular gamonts of parasites within neutrophils of peripheral blood smears in Giemsa stain (Elias and Homans, 1988; Rahmani Amoli et al., 2012). However, in this method gametocytes are not always conspicuous in the bloodstream or may be very few in number. Shkap et al. (1994) used some serologic tests such as indirect fluorescent antibody test (IFA) to detect *H. canis* antibodies; their results showed that this test is more sensitive than microscopic examination of peripheral blood smears (Shkap et al., 1994). This finding was in agreement with those of an epidemiologic study performed in Japan using the IFA

tests (Inokuma et al., 1999). Gonen et al. (2004) applied Enzyme Linked Immunosorbent Assay (ELISA) for the detection of hepatozoonosis using purified *H. canis* gamont antigen. Genon et al. (2004) result indicated that no differences between ELISA and IFA tests in terms of sensitivity and specificity. They also proposed that neither of these serologic techniques is able to identify anti-*H. canis* antibodies in some infected dogs (Gonen et al., 2004). Recently, molecular methods with high sensitivity and specificity are used for the detection of target pathogens in both peripheral blood and arthropod vectors (Criado-Fornelio et al., 2003). Molecular techniques such as polymerase chain reaction (PCR) and sequence analysis are utilized for the diagnosis of *H. canis* infection and specification of its isolates, respectively (Baneth et al., 2000; Inokuma et al., 2002; Criado-Fornelio et al., 2003; Rubini et al., 2005). The main objective of the present study was to detect *H. canis* infection in the blood of dogs in Meshginshahr, Iran, through PCR and sequencing.

MATERIALS AND METHODS

Study setting. This cross-sectional study was conducted in Meshginshahr County, Ardabil Province, Iran, during July 2013-June 2015. Meshginshahr is the nearest city to Sabalan mountain (latitude: 38 degrees North, longitude: 47 degrees East, and altitude: 1490 m above sea level; Figure 1) and has a moderate climate. This county covers an area of approximately 1530 km² and includes 323 villages. This county has a population of 169967, 42% of which are settled in urban areas and 58% in rural ones.

Blood sample preparation. A total of 104 blood samples were collected randomly by EDTA from dogs of Meshginshahr county and were transferred to laboratory of parasitology. Blood samples were washed four times with 1 ml of distilled water, and then vortexed until the supernatant was pale pink in color.

DNA extraction. DNA was extracted from blood with DNG-plus DNA Extraction Kit (Pishgam, Iran). A pellet containing DNA was dissolved in 30-50 µl of sterile distilled water, and then incubated in a water

bath at 65°C for 5 min. The solution was stored at -20°C until use. In addition, 4 µl of the extracted DNA was used in PCR amplification.

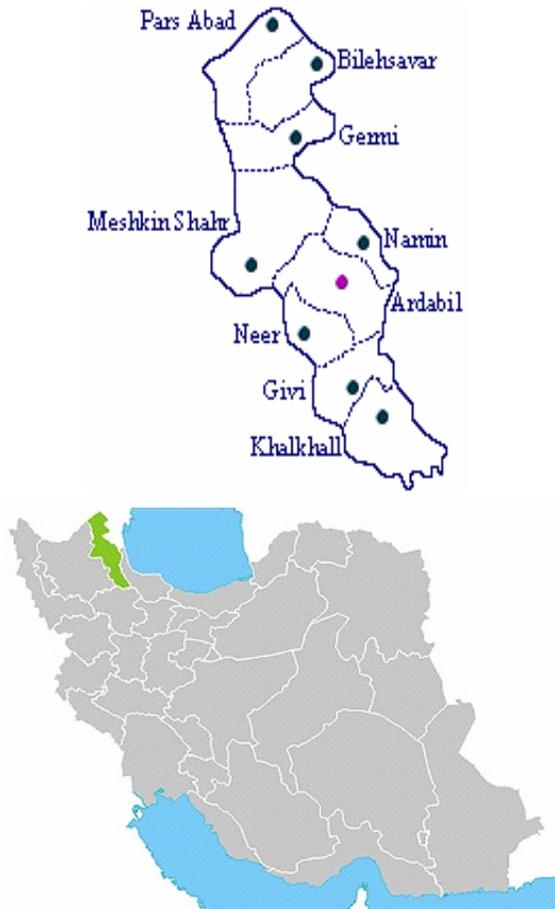


Figure 1. Map of Ardabil Province, Northwest of Iran

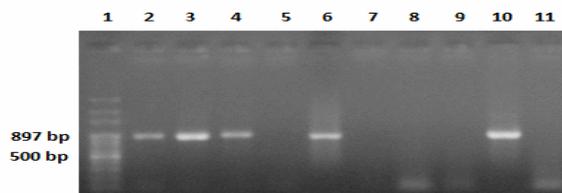


Figure 2. Agarose gel electrophoresis of polymerase chain reaction products of 18S rRNA gene amplified using Hep1 and Hep2 primers and DNA of *Hepatozoon canis*. Lane 1: 100 bp DNA ladder, Lane 2-10: PCR products amplified, Lane 11: Negative control

PCR amplification. The conventional PCR methods were used to detect *H. canis* DNA in dog blood samples. The amplification conditions were 94°C for 4 min followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min, with a final extension step at 72°C for 10 min. The primers Hep1 F: (5` -CAG CAA AAC TGC AAA TGG CTC A-3`) and Hep2 R: (5` -GGC AAA TGC TTT CGC AGT AGT TT-3`) were employed to detect 891 bp fragment of the 18S rDNA of *H. canis*. Then, 10 µl of the PCR product was observed by 1% agarose gel electrophoresis in UV transilluminator. Master Mix plus primer without DNA was used as negative control.

Sequencing. Positive samples were extracted by Vivantis® DNA Extraction Kit and then sequenced. DNA sequences were assessed with Sequencher software version 4.1.4 and compared to other sequence profiles in the GenBank database using Basic Local Alignment Search Tool (BLAST).

Ethical approval. This study was authorized by the Ethics Committee of Tarbiat Modares University of Medical Sciences, Tehran, Iran, as well as the Ethics Committee of the Center for Diseases Control of Iran. We conducted this study in accordance with the guidelines proposed by Helsinki Declaration.

RESULTS AND DISCUSSION

Twenty-four out of the 104 (23.07%) samples were found to be positive for *H. canis*. In the electrophoresis result following PCR amplification, a positive band with the approximate length of 897 bp appeared (Figure 2). Sequencing analysis confirmed the molecular identity of the samples. A BLAST search found 18S rRNA gene sequences of *H. canis* isolates and revealed 99% similarity with AY 150067, saved at GenBank. *H. Canis* infection was first reported by Khoshnegah et al. (2009) in a 11-year-old male dog in Iran as they observed *H. canis* gametocytes within neutrophils of peripheral blood smears and bone marrow smear in Giemsa stain. In addition, schizonts of the parasite were observed in tissue sections of muscles, lymph nodes,

spleen, and liver (Khoshnegah et al., 2009). In 2012, the first epidemiologic study was performed on *Hepatozoon* spp infection of dogs in Iran. According to that study, the prevalence rate of *Hepatozoon* spp infection was 1.57% (4/254). Also, this parasite was observed in 6.3% of ticks (16 out of 254 dog ticks) and was identified as *R. sanguineus* (Rahmani Amoli et al., 2012). In Brazil, hepatozoonosis is known as an opportunistic disease, which does not affect the number of white blood cells (Paludo et al., 2003). The prevalence of this parasite in Brazil, based on observing the gametocytes in blood smears, changed from 0.5% to 3.0% (Gondim et al., 1998; O'Dwyer et al., 2001). Although canine hepatozoonosis infects dogs in Iran, but its molecular detection has never been performed. To the best of our knowledge, this is the first study on molecular detection of canine hepatozoonosis in Iran. In our study, the prevalence rate of this disease was 23.7% (24/104), while in studies conducted in Colombia (Vargas-Hernandez et al., 2012) and Venezuela (Criado-Fornelio et al., 2007) the rates of infection were reported to be 31.8% and 44%, respectively. This difference may be due to diverse climatic and ecological conditions. In a similar study on domestic dogs in Turkey, *H. canis* was detected by molecular methods and confirmed by sequencing (Aktas et al., 2015). In that study, *H. canis* was found in nine Turkish provinces (Aktas et al., 2015). Also in India, Pawar et al. (2012) reported; *Hepatozoon* spp infection in wild felids and canids was caused by *Hepatozoon felis* and *H. canis*, but these species were not host specific (Pawar et al., 2012).

This is the first report on molecular detection of *H. canis* from Iran. The prevalence of *Hepatozoon* infection was relatively high among dogs in Ardabil Province.

Ethics

I 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.

Acknowledgment

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