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Article

Molecular detection of *Hepatozoon canis* (Apicomplexa: Hepatozoidae) in ticks (Ixodida) collected from dogs in Kerman, Iran

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ABSTRACT
Previous studies from Iran showed that canine hepatozoonosis is prevalent in dogs. Still, there is limited information about the tick vectors of *Hepatozoon canis* (Apicomplexa: Hepatozoidae) although *Ripicephalus sanguineus sensu lato* complex ticks (Acari: Ixodida) are known to be competent vectors. In Iran, *He. canis* was detected in *R. sanguineus* and *R. turanicus* in the west of the country. This study aimed to investigate the molecular prevalence of *He. canis* in ticks collected from stray dogs in a southeastern city of Iran. From 2019 to 2020, 50 tick-infested free-ranging dogs (24 female, 26 male) in Kerman city were randomly chosen. From each dog, five ticks were collected in individually labeled tubes and transferred to the laboratory. Ticks of each dog were identified morphologically, crushed, and pooled for genomic DNA extraction. Conventional PCR targeting an 891 bp fragment of the *18S* ribosomal RNA gene of *He. canis* was performed on 50 tick pools, and products were gel-electrophoresed. All of the collected ticks were diagnosed as *R. sanguineus sensu lato*. DNA of *He. canis* was detected in 14 tick pools (28%). Dogs from which positive ticks were collected were of both sexes and aged between 5 months and 6 years old. This study shows for the first time a considerable prevalence of *He. canis* in ticks feeding from stray dogs of Kerman implying a potential health risk for domestic and wild canine hosts in the region. It also shows that molecular examination of ticks for *Hepatozoon* DNA could be a proper alternative to blood collection in epidemiological studies. Effective ectoparasite control strategies, regular examination of dogs, and successful chemoprophylaxis are advocated.

KEYWORDS: Acari, hepatozoonosis, PCR, tick-borne, vector-borne.

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INTRODUCTION

Several ticks and tick-borne pathogens can pose significant health threats to dogs (Dantas-Torres *et*

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al. 2012). However, most studies have focused on the zoonotic parasites that are transmissible to humans. As an example, apicomplexan protozoa of the genus *Hepatozoon* (Hepatozoidae), which are common in dogs in different regions of the world, have remained neglected pathogens in Western Asia including Iran. The genus includes more than 300 species which are transmitted by Ixodidae ticks (Smith 1996). Two species, *Hepatozoon canis* (Conoidasida: Hepatozoidae) and *He. americanum*, are known to be infectious for dogs: *He. canis* is distributed worldwide and is mainly transmitted by the brown dog tick *Rhipicephalus sanguineus* (L.) (Acari: Ixodida), while *He. americanum* has only been reported from the American continent (Baneth *et al*. 2022). The sporogony stage of the parasite occurs in ticks and dogs become infected by ingesting the infected tick, which contains *Hepatozoon* oocysts when the gametogony stage is complete in the leukocytes (Smith 1996). Hepatozoonosis can be asymptomatic when parasitemia is low, but in severe cases, it leads to fever, lethargy, anemia, and even death (Beugnet *et al*. 2018).

Since the first report of canine hepatozoonosis in Iran (Khoshnegah *et al*. 2009), *He. canis* has been reported from dogs in some provinces however in general, there is not much is known about the prevalence of *He. canis* in dog populations from different bioclimatic and geographical regions of Iran. In a previous study on dogs from Hamedan, Kermanshah, Mazandaran, Yazd and Khuzestan, *He. canis* was the most common vector-borne pathogen in all provinces and its prevalence ranged from 18 to 78%, with the highest prevalence found in Kermanshah (Iatta *et al*. 2021). This hemoparasite was also reported from dogs in other regions of the country with different prevalences: 46% in Ardabil (Dalimi *et al*. 2017), 23% in Tehran (Soltani *et al*. 2018), 9% in Urmia (Zeinali *et al*. 2022), 8% in Torbat-e-Heydareih (Barati *et al*. 2018), and 5% in Mashhad (Rahmani-Amoli *et al*. 2012). In addition, a recent study showed that *R. sanguineus* and *R. turanicus* Pomerantsev collected from the bodies of dogs in Kermanshah and Hamedan contained DNA of *He. canis*, suggesting that these two tick species play a role in the epidemiology of hepatozoonosis in western Iran (Bahiraei *et al*. 2023). Considering the lack of information on the occurrence of *Hepatozoon* in the southeastern city of Kerman and the fact that ticks infesting dogs are suitable samples for molecular research on etiologic agents of canine pathogens (Laidoudi *et al*. 2020), this study aimed to investigate the molecular prevalence of *He. canis* in ticks collected from stray dogs in this region targeting *18S* rDNA that has proven to be a good marker for the specific detection of piroplasmids in general, and *Hepatozoon* species in particular (Sazmand *et al*. 2016; Alanazi *et al*. 2020; Shiri *et al*. 2024).

MATERIALS AND METHODS

Tick collection and identification

Ticks were collected from 50 stray dogs of both sexes that were either brought from the Kerman city municipality shelter to the Veterinary Hospital of Shahid Bahonar University for ovario-hysterectomy or were visited on-site during 2019 and 2020. In Iran, dogs kept in shelters are rescued from different regions of the province mainly aiming to feed and protect dogs from culling programs. Previous studies showed that these dogs are infected with a wide array of parasitic, microbial, and viral pathogens (Iatta *et al*. 2021; Beikpour *et al*. 2022; Hosseini *et al*. 2022; Sazmand *et al*. 2022; Shamshiri *et al*. 2023; Beus *et al*. 2024). Data including date, location, age, and sex were recorded at the time of sampling.

From each dog, five blood-fed ticks were randomly collected in individually labeled tubes containing 70% ethanol and transferred to the laboratory. Individual ticks were identified morphologically to the species level following keys (Estrada-Peña *et al.* 2018; Hosseini-Chegeni *et al*. 2019a, b). All were diagnosed as *R. sanguineus sensu lato* (*s.l.*). Ticks from each dog were crushed and pooled for DNA extraction.

DNA extraction

Ticks collected from each dog (n = 5) were pooled and genomic DNA (gDNA) was extracted

from them together using a commercial tissue genomic DNA extraction kit (Parstous, Mashhad, Iran) according to the manufacturer's instruction and preserved at −20 C until molecular examinations.

PCR assay

DNA samples (n = 50) were tested for the presence of *He. canis* using the primer pair Hep1F: CAGCAAAACTGCAAATGGCTCA / Hep1R: GGCAAATGCTTTCGCAGTAGTTT targeting an 891 bp fragment of the parasite's *18S* ribosomal DNA with the recipe and thermal profile described previously (Dalimi *et al*. 2017). PCR products were visualized by electrophoresis on 1% agarose gel stained with safe DNA gel stain.

Statistical analyses

The associations between *Hepatozoon* positivity and age and sex of the dogs and season of tick collection were analyzed using the Chi-square test (χ^2) Microsoft Office Excel ver. 16.37 (Microsoft Corp., Redmond, WA, USA). A probability value < 0.05 was regarded as statistically significant.

RESULTS

The dogs from whom ticks were collected were 24 females and 26 males. They were aged < 1 year old (n = 18), 1–3 years old (n = 25), 3–6 years old (n = 7). Tick infestation was highest in summer (n = 24) followed by spring (n = 22) and autumn (n = 4).

PCR revealed DNA of *He. canis* in 14 tick pools (28%). Dogs from which positive ticks were collected were of both sexes, aged between five months to six years old, and in all three seasons. No statistical difference was observed between *Hepatozoon* positivity and sex, age, or season (Table 1).

Table 1. Risk factors associated with the positivity rate of *Rhipicephalus sanguineus sensu lato* collected from dogs in Kerman, Iran for *Hepatozoon canis* according to different variables.

Parameter	*n* Total / *n* PCR positive (%)	*P* value
Sex		
Female	24 / 6 (25%)	0.65
Male	26 / 8 (30.8%)	
Age (years)		
<1	18 / 6 (33.3%)	0.4
1–3	25 / 5 (20%)	
3–6	7 / 3 (42.8%)	
Season		
Spring	22 / 8 (36.4%)	0.49
Summer	24 / 5 (20.8%)	
Autumn	4 / 1 (25%)	

DISCUSSION

This study provides the first insight into canine hepatozoonosis in this southeastern province indicating the presence and implying possible clinical implications for dogs and wild canid populations. Molecular detection of *He. canis* herein reported is not surprising since this parasite is the most prevalent vector-borne parasite of dogs in Asia, Europe, Africa, and Latin America (Baneth *et al*. 2022). Previous studies in Iran reported *He. canis* in dogs from Ardabil (Dalimi *et al*. 2017), Tehran (Soltani *et al*. 2018), Urmia (Zeinali *et al*. 2022), Mashhad (Khoshnegah *et al*. 2009; Rahmani-Amoli *et al*. 2012), Hamedan, Kermanshah, Mazandaran, Yazd, and Khuzestan (Iatta *et al*. 2021). Although in dogs, ingestion of ticks harboring mature oocysts is thought to be the main route of parasite transmission, shreds of evidence of *He. canis* transmission from pregnant females to

puppies in Japan and Europe confirm that vertical transmission is another (probably important) possible route of parasite dissemination (Murata *et al*. 1993; Schäfer *et al*. 2022).

We detected *He. canis* in *R. sanguineus s.l.* ticks which along with *R. turanicus* is considered the main vector of the parasite (Baneth *et al*. 2001; Farkas *et al*. 2014; Tolnai *et al*. 2015; Mitková *et al*. 2016; Giannelli *et al*. 2017; Geurden *et al*. 2018; Sazmand *et al*. 2022). However, the detection of this hemoparasite in other tick species in the last decade raises questions about their vectorial role. So far, *He. canis* has been also detected in *Dermacentor reticulatus* and *Dermacentor marginatus* from foxes and a human patient (Najm *et al*. 2014; Karasartova *et al*. 2018), in *Haemaphysalis adleri*, *Ha. concinna*, *Ha. punctata*, *Ha. parva*, *Ha. longicornis*, *Ha. bispinosa* from dog and wild goat (Hornok *et al*. 2013; Kamani *et al*. 2013; Andersson *et al*. 2017; Guo *et al*. 2020, Orkun *et al*. 2020) (GB accession: MG018466), in *Ixodes ricinus*, *I. hexagonus*, *I. holocyclus*, *I. canisuga*, *I. scapularis* collected from dogs, foxes, goat, cat and on vegetation (Najm *et al*. 2014; Hamšíková *et al*. 2016; Andersson *et al*. 2017; Greay *et al*. 2018; Del Cerro *et al*. 2022), in *Amblyomma cajennense* from dogs (Jarquín-Díaz *et al*. 2016) and *A. sculptum* from an unknown host (GB accession: KP167594). In a study published in 2024, *He. canis* DNA was detected in the whole body of the tortoise tick *Hyalomma aegyptium* collected from sheep in Kermanshah (western Iran) (Shiri *et al*. 2024). Whether ticks other than *R. sanguineus s.l.* and *R. turanicus* can act as competent vectors of *He. canis* needs further experimental examinations.

In this study, we only identified *R. sanguineus s.l.* ticks morphologically which is in line with other Iranian studies reporting that brown dog tick is the most common hard tick infesting domestic animals in the country (Hosseini-Chegeni *et al*. 2019b; Shiri *et al*. 2024). Traditionally and historically, only one species *R. sanguineus* has been discussed as the brown dog tick that has infested dogs since ancient times (Dantas-Torres 2010; Otranto *et al*. 2014). But in recent years, with the progress of molecular studies, scientists have established three genetically distinct species and named the tropical lineage as *R. linnaei* (Šlapeta *et al*. 2021, 2022), the temperate lineage as *R. sanguineus sensu stricto* (Nava *et al*. 2018; Dantas-Torres *et al*. 2022), and the southern European lineage as *R. rutilus* (Šlapeta *et al*. 2023). Since all three species occur in different countries of the northern hemisphere (Ghodrati *et al*. 2024; Myers *et al*. 2024) and Iran covers a wide geographical area with different climatic zones, further molecular studies are needed to identify the possible presence of different species in the *R. sanguineus s.l.* complex. Moreover, infestation of dogs in Iran with *Hyalomma marginatum*, *Hy. asiaticum*, *Hy. anatolicum*, *Dermacentor niveus*, *D. marginatus* in Ardabil (Khazeni *et al*. 2013), *Hy. marginatum* in Kermanshah (Mirani *et al*. 2017); *R. turanicus*, *Hy. asiaticum*, *Hy. excavatum* in Hamedan (Bahiraei *et al*. 2023), and *R. bursa* in Tehran (Jamshidi *et al*. 2012) have been also reported suggesting that dogs of Kerman province may also be exposed to different tick species.

As a limitation, we did not collect clinical and hematological data of each dog as the aim of this and a parallel project (Mostafavi *et al*. 2022) was to investigate the presence of tick-borne pathogens in the region. It should be noted that detection of *He. canis* in blood-fed ticks, as in current research, does not necessarily reflect the infection status of the host because it is possible that the ticks fed in a previous stage (larvae or nymph) from another host (Shiri *et al*. 2024). However, the collection of ticks from animals is more ethical, less invasive, and suitable for the assessment of the presence of certain pathogens in an area.

CONCLUSIONS

This study shows for the first time a considerable prevalence of *He. canis* in ticks feeding from stray dogs of Kerman implying a potential health risk for domestic and wild canine hosts in the region. It also shows that molecular examination of ticks for *Hepatozoon* DNA could be a proper alternative to blood collection in epidemiological studies. Effective ectoparasite control strategies, regular examination of dogs, and successful chemoprophylaxis are advocated.

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شناسایی مولکولی *Hepatozoon canis* (Apicomplexa: Hepatozoidae) در کنه‌های (Ixodida) جمع‌آوری شده از سگ‌ها در کرمان، ایران

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چکیده

بررسی‌های پیشین از ایران نشان داده‌اند که هپاتوزوئونوزیس در سگ‌ها شایع است. اما، اطلاعات محدودی دربارهٔ کنه‌های ناقل *Hepatozoon canis* (Apicomplexa: Hepatozoidae) وجود دارد اگرچه کنه‌های کمپلکس *Ripicephalus sanguineus sensu lato* (Acari: Ixodida) به عنوان ناقلین قطعی انگل شناخته شده‌اند. در ایران، *He. canis* در *R. sanguineus* و *R. turanicus* در غرب کشور شناسایی شده است. این مطالعه با هدف بررسی فراوانی مولکولی *He. canis* در کنه‌های جمع‌آوری شده از سگ‌های ولگرد در شهر کرمان واقع در جنوب شرق ایران انجام شد. در طول سال‌های ۱۳۹۸ تا ۱۳۹۹ در مجموع ۵۰ قلاده سگ ولگرد آلوده به کنه (۲٤ ماده و ۲٦ نر) به صورت تصادفی انتخاب و مورد بررسی قرار گرفتند. از هر سگ، ۵ کنه جمع‌آوری و در لوله‌های جداگانه‌ی شماره‌گذاری شده به آزمایشگاه منتقل شدند. کنه‌های هر سگ مورد شناسایی ریخت‌شناسی قرار گرفتند، سپس خرد و مخلوط شده، و دی‌ان‌ای ژنومیک از آن‌ها استخراج شد. نمونه‌ها (۵۰) برای شناسایی قطعه‌ی ۸۹۱ جفت بازی از ژن *18S* ribosomal RNA انگل با روش PCR معمولی مورد آزمایش قرار گرفته و محصولات بر روی ژل آگاروز الکتروفورز شدند. تمامی کنه‌های جمع‌آوری شده *R. sanguineus sensu lato* شناسایی شدند. دی‌ان‌ای *He. canis* در ۱٤ نمونه (۲۸٪) شناسایی شد. سگ‌هایی که کنه‌های آلوده از آن‌ها جمع‌آوری شده بودند از هر دو جنس بودند و بین ۵ ماه تا ٦ سال سن داشتند. این مطالعه برای نخستین بار فراوانی قابل توجه *He. canis* را در کنه‌های جدا شده از سگ‌های ولگرد شهر کرمان گزارش می‌دهد که نشانگر خطری بالقوه برای سگ‌های اهلی و سگ‌سانان وحشی در منطقه است. همچنین این نتایج نشان می‌دهد که بررسی مولکولی کنه‌ها برای دی‌ان‌ای *Hepatozoon* می‌تواند جایگزینی مناسب به جای خون در مطالعات همه‌گیری‌شناسی باشد. برای سگ‌ها، روش‌های کنترل موثر انگل‌های خارجی، معاینات منظم، و درمان دارویی توصیه می‌شود.

واژگان کلیدی: کنه‌ها، هپاتوزوئونوزیس، پی‌سی کنه–زاد، منتقله از ناقل.

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