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Molecular Identification of *Hepatozoon canis* in Ticks from Dogs in Siirt, Turkey

Burçak Aslan Çelik[1], Adnan Ayan[2], Ali Bilgin Yılmaz[3], Özgür Yaşar Çelik[4*], Özlem Orunç Kılınç[5], Özge Oktay Ayan[6]

[1]Department of Parasitology, Faculty of Veterinary Medicine, Siirt University, Siirt, TURKEY, burcakaslan@siirt.edu.tr, ORCID: https://orcid.org/0000-0002-0130-970X

[2]Department of Genetics, Faculty of Veterinary Medicine, Van Yüzüncü Yıl University, Van, TURKEY, adnanayan@yyu.edu.tr, ORCID: https://orcid.org/0000-0002-6564-3416

[3]School of Health, Van Yuzuncu Yil University, Van, TURKEY, alibilginyilmaz@yyu.edu.tr, ORCID: https://orcid.org/0000-0003-0749-2418

[4]Department of Internal Medicine, Faculty of Veterinary Medicine, Siirt University, Siirt, TURKEY, oyc@siirt.edu.tr, ORCID: https://orcid.org/0000-0001-6365-2688

[5]Özalp Vocational School, Van Yüzüncü Yıl University, Van, TURKEY, ozlemkilinc@yyu.edu.tr, ORCID: https://orcid.org/0000-0001-6233-7109

[6]Department of Parasitology, Van Yüzüncü Yıl University, Faculty of Medicine, Van, TURKEY, ozgeokty09@gmail.com, ORCID: https://orcid.org/0000-0003-2577-3774

HEPATAZOON species are tick borne protozoan parasites classified in the Heptazoidae family, and they are closely related to hemosporinids and piroplasms. In this work, the 18S rRNA genetic section of *Hepatozoon canis*positive ticks removed from dogs was sequenced using the PCR technique. Ticks were collected from a total of 80 dogs in Siirt, Turkey.A total of 300 collected ticks were morphologically identified to the species level and all ticks identified as *Rhipicephalussanguineus (s.l.)*. *H. canis* DNA was detected in 12 (%4) out of 300 in *R. sanguineus*ticks by PCR. The phylogenetic tree created via comparison of amplified 18S rRNA region sequences of *H. canis*with MT107097.1, MH595911.1, KT215377.1, KT 215376.1, KC 584780.1, KC 584777.1, KC 584775.1, and KC 584774.1. The results obtained will provide important reference material for both veterinary cliniciansand dog owners in terms of managing canine hepatozoonosis.

Keywords:*Hepatozoon canis*, PCR, *Rhipicephalus sanguineus*, Siirt, Turkey

Introduction

Hepatozoon species are tick borne protozoan parasites classified in the Heptazoidae family, and they are closely related to hemosporinids and piroplasms[1]. More than 300 Hepatozoon species in the Hepatozoidae family are reportedly infectious for animals [2,3]. Two species that infect dogs are known to be *H. canis and H. americanum.* [4]. The *Amblyoma maculatum* tick is the vector for *H. Americanum*, whereas *R. sanguineus* ticks are the vector for *H. canis*[1]. *H. canis* is the most widely spread species that is related to *canine hepatozoonosis* in many countries [1,5]. The definitive host for *H. canis* consists of *R. sanguineus* ticks, while the intermediate hosts are dogs and wild canids [3, 6]. *Hepatozoon* spp. has a common lifecycle that

Corresponding author: Özgür Yaşar ÇELİK, E-mail.: oyc@siirt.edu.tr, Tel. +905373559889
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consists of gender development and sporogony, which take place while the parasite is within the definitive host. This process is then followed by merogony and gamontogony, which take place while the parasite is within the intermediate host [2,3,7,8].

Hepatozoon transmission occurs when dogs self-groom and consume ticks that contain *Hepatozoon* oocysts, while plenty of tick-borne infections are transferred via the salivary glands of ticks[2,7,9,10]. Once the dogs have ingested the infected ticks, sporozoids are released from the oocysts. Penetrating the intestinal walls, these sporozoids then invade the mononuclear cells to enter the circulation and reach many organs through the blood circulation [1,8].

The course of the infection might vary from asymptomatic in healthy-looking dogs, to a very deadly case of extreme lethargy, cachexia, and anemia that may lead to death [1,2]. Clinical findings vary with the age, infection level, and the presence of concomitant infections, with fever and weight loss being the most common clinical findings [9].

In the diagnosis of the disease, microscopic inspections where the gamonts are determined inside the monocytes and neutrophils in blood smears [1,9], IFAT [11], and ELISA[12]are used, but it has been reported that PCR method is more sensitive for the diagnosis of the disease [5,8]. Studies have been conducted to determine the prevalence of *H. canis* in blood in dogs, but limited studies have been conducted on its prevalence in ticks.

In the present study, we report the molecular detection of *H. canis* in ticks collected from dogs in the Siirt province of Turkey.

Material and Methods

Tick Collection

Ticks were taken from 80 dogs from Siirt, Turkey.After the animals were checked, the ticks were collected and placed into separately labeled 25 mL ethyl alcohol-filledstorage containers, which were then brought to the parasitologylaboratory of veterinary faculty.

Ticks Morphology and DNA extraction

Distinguishing of the ticks brought to lab was performed according to Estrada-Peña et al. [13] and Walker et al. [14]. Before DNA extraction,each tick sample was washed in 70% ethyl alcohol and then was left to dry. Then, the ticks were taken into the tubes (one tick per tube) and the freeze-thaw process was performed.The ticks inside the Eppendorf tubes were crushed using a sterilized rod. Then, mini kit (Invitrogen, USA, K182002) was used in line with protocols of commercial company to obtain the DNAs. The obtained DNAs were kept at -20 C° until the PCR process was applied.

PCR Amplification

PCR was performed by previously reported methods to amplify the 666 bp long 18S rRNA gene region of *H. canis* [6,15].

Sequence and Phylogenetic Analysis

Once purified, the amplicons were subjected to bidirectional sequence analysis (Applied Biosystems 377 DNA Sequencer). 18S rRNA sequences for all isolates were registered in GenBank with access number MW684291.1and MW684292.1. The sequences obtained from the GenBank with numbers MT107097.1, MH595911.1, KT215377.1, KT 215376.1, KC 584780.1, KC 584777.1, KC 584775.1 and KC 584774.1 were sequenced using the Clustal W algorithm in MEGA 7 software. The Phylogenetic tree was created using the Maximum Likelihood methodand the Bootstrap test (1000 repeats).

Ethical clearance

Ethical clearance for the present work was provided by the Siirt University Animal Experiments Local Ethics Committee (No. 2020/05-05).

Results

An overall of 300 ticks were identified morphologically at the species level and all ticks were classified as *R. sanguineus*. DNA of *H. canis* were detected in 12 (%4) out of 300 in *R. sanguineu s*ticks by PCR was 666 bp (Fig. 1).

Discussion

Ticks have an important role in the epidemiology of human and animal diseases [16, 17]. In the Balkan countries and Mediterranean basin, the *R. Sanguineus* ticks are the most

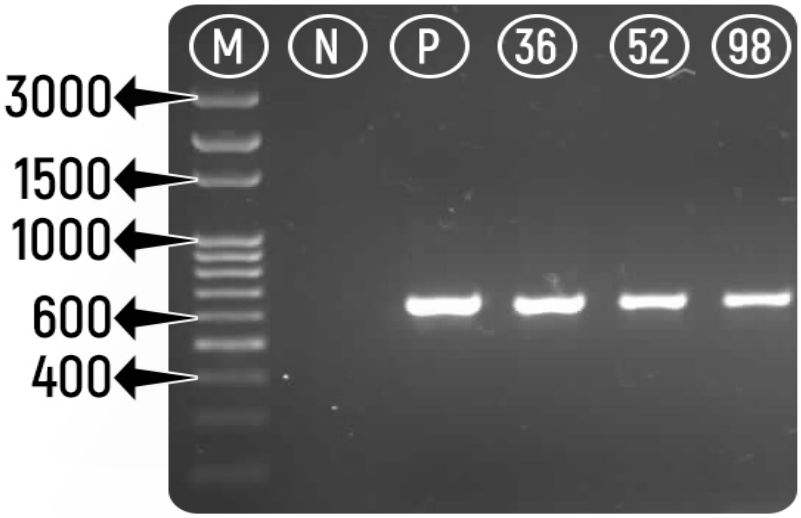

Fig. 1. 18S rRNA amplification of H. canis in ticks using PCR. Lanes M: Marker, N: Negative control, P: positive control, Lanes 36, 52, and 98 represent H. canis (666 bp).

Phylogenetic tree created via comparison of amplified 18S rRNA region sequences of H. canis with MT107097.1, MH595911.1, KT215377.1, KT 215376.1, KC 584780.1, KC 584777.1, KC 584775.1, and KC 584774.1 is provided in Fig. 2. Babesia canis was used as the outer group (AY272048.1).

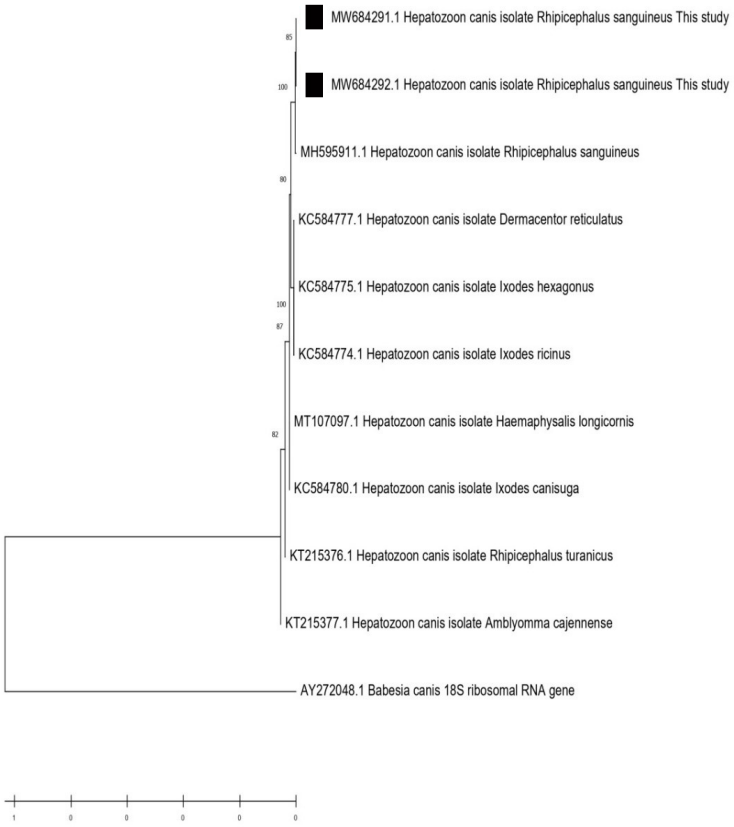

Fig. 2. The phylogenetic relationship of isolates obtained as part of this study with the sequences obtained from the GenBank. *H. canis* sequences identified in the present study are indicated using black squares.

important vector for *H. canis* [1,6,18]. While numerous researches were performed on the prevalence of *H. canis* infection in dogs, information regarding the rate of *H. canis* in ticks is still limited. In various studies performed to determine the *H. canis* infection rate in ticks collected from dogs throughout the world, the following prevalence rates were obtained: Luxembourg 0.1%[10], Nigeria 21% [16], Brasil 31.6% [19], and 42%[20], Italy 1.5%-2.1% [6,21]. Another study that covered various countries (Italy, Spain, Portugal, India) reported an average positivity of 29.41% [22].

Canine hepatozoonosis was first reportedly seen in a dog in Turkey in 1933[23]. The studies carried out in Turkey on ticks for *H.canis* detection are very limited. Aktas et al. [18] reported a positivity of 20.58% in *R. sanguineus* tick pools, while Orkun and Nalbantoğlu [24] reported a positivity of 33.3% in ticks collected from Turkish red foxes, (*Vulpes vulpes)* in Ankara.

Since a fragment of the 18S rRNA gene is targeted for the routine determination of *Hepatozoon canis* DNA [6, 20, 24]this particular segment was also targeted for the molecular determination of *H. canis* as part of the current work as well. In this study,*H. canis* DNA was definedin 12 (4%) out of 300 individual *R. sanguineus* ticks. This ratio was found to be quite lower than the findings of other studies performed on ticks in Turkey [18, 24].

The serological studies performed for the determination of *H.canis* prevalence in Turkey reported the ratios as follows: Ankara 49.5% [17], a study that covers Mersin, Adana, Hatay, Gaziantep and Batman provinces 0.5% [25], Diyarbakır 15.87% [18], a study that covers Konya and Karaman provinces 3.61% [26], Kayseri 5.3% [27], Samsun 0.5%[28], the Aegean Region (Aydın, Kuşadası, Selçuk, Manisa, Bodrum, and Marmaris) 25.8%[5]. The findings of this study are similar to that of certain serological studies carried out in Turkey [26, 27] while they were higher than some [25, 28] and lower than others [5, 17, 18].

Geographical conditions, climate diversity, rate of infected ticks, sample size, sampling period, and methods used can be cited amongst the reasons for the differences seen between the studies.Reye et al. [10] stated that international tourism, including the importation of pets, could be the reason why pathogens can beencountered in non-endemic areas.

R. sanguineus ticks act as the main vector for *H. canis* [6, 9, 21]. That being said,*Haemaphysalis longicornis*, *Haemaphysalis flava* [9,29], *Amblyomma ovale* [9,30], *Rhipicephalus microplus* [31], and *Ixodes ricinus* [10,21] ticks have also been reported as candidate vectors. The detection of *H. canis* in *R. sanguineus* ticks of this study is consistent with the literature [6, 9, 18, 21].

The *Hepatozoon canis* lineage acquire as a consequence of the sequence analysis of 18S rRNA genes isolated from *R.sanguineus* ticks (MW684291.1-MW684292.1), were found to have 99.67% (MH595911.1), 98.72% (KC584777.1), 98.72% (KC584775), 98.72% (KC584774.1), 98.56% (MT107097.1), 98.50% (KC584780), 98.13% (KT215376), 97.29% (KT215377.1) similarity with *H. canis* ticks obtained from *R. sanguineus, Dermacentor reticulatus, Ixodes hexagonus, Ixodes ricinus, Hemaphysalis longicornis, Ixodes canisuga, Rhipicephalus turanicus* ve *Amblyomma cajennense*species, respectively.

Conclusion

In this study, the presence of *H. canis* in *R. sanguineus* ticks collected from stray dogs in Siirt province was molecularly investigated.The results obtained will hopefully be useful both for veterinarians and dog owners in the management of canine hepatozoonosis. We believeadditional researches arerequired to better understandthe disease's epidemiological and clinical importance and to ensure effective protection and control measures can be taken against it.

Acknowledgment

The study has been presented as a poster presentation in the 22nd Parasitology Congress, 11-15 October 2021, Didim, Aydın, Turkey.

Conflicts of interest

The authors declare no conflict of interest.

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