

Research article

MOLECULAR IDENTIFICATION OF *EHRlichia CANIS* IN *RHIPICEPHALUS SANGUINEUS* TICKS FROM SIIRT PROVINCE

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This study was performed on *Ehrlichia canis* positive ticks collected from dogs to perform sequencing of their 16S rRNA genetic section using the PCR method. The collection of ticks was performed from a total of 60 dogs in the Siirt province, Turkey. A total of 250 ticks were collected and morphologically investigated. All ticks were identified as *Rhipicephalus sanguineus sensu lato (s.l.)*. Ehrlichial DNA was detected by the PCR method performed on 38 (15.2 %) of the ticks. The *E. canis* strains obtained as a result of the sequence analysis were found to be 100% identical to the American Texas (MH620194), Indian (KX766395), and Egyptian (MG564254) strains. This study thereby has identified a zoonotic agent from the *R. sanguineus* ticks collected from the dogs in the Siirt province.

Keywords: *Ehrlichia canis*, Nested PCR, *Rhipicephalus sanguineus*, Siirt, Turkey

INTRODUCTION

Ticks are responsible for the spread of various pathogens that infect both humans and animals, including various bacteria species, helminths, protozoa, and viruses [1,2]. Worldwide, *Ehrlichia canis* is the most important species of *Ehrlichia* in dogs, and its principal vector is the brown dog tick *Rhipicephalus sanguineus sensu lato (s.l.)* [3-5]. *E. canis* is a gram-negative, obligate-intracellular bacteria belonging to the *Rickettsiales* order of the *Anaplasmataceae* superclass [6,7], and causes the potentially fatal Canine monocytic ehrlichiosis (CME) [4-6,8,9]. *E. canis* causes an infection that could potentially last for a

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lifetime in dogs by infecting their blood mononuclear cells [10,11]. Clinical symptoms of the disease include fever, depression, lack of appetite, weight loss, hemorrhage, epistaxis, gastrointestinal symptoms (vomitus, diarrhea, etc.), and respiratory disorders. The most frequent laboratory findings consist of thrombocytopenia, leukopenia, anemia, and hypergammaglobulinemia [6,12]. The diagnosis of CME is performed by enzyme-linked immunosorbent assay (ELISA) and immunofluorescence antibody assay (IFA) methods, the latter of which is considered as the golden standard [11,13]. In the diagnosis of the disease PCR methods (Polymerase chain reaction, real-time PCR) are used with its high sensitivity and specificity [11,14]. The ticks of the Ixodidae family transmit the rickettsia pathogens like *Ehrlichia* and *Anaplasma* through the biological path [1]. Vectors of *E. canis* are the nymphs and adults of the *Rhipicephalus sanguineus* ticks, which are commonly known as the brown dog tick [4,6-8]. Since the transovarial transmission of *E. canis* has not been demonstrated, the larvae are insignificant in terms of infection [10]. While they are primarily a dog parasite, these ticks can also infest other hosts, including humans [2]. Although these ticks are present worldwide, they are most frequently encountered in tropic and sub-tropic climates [6]. The aim of this study is to determine the prevalence of *E. canis* in ticks collected from dogs in Siirt province by PCR method. The 16S rRNA gene region of the positive samples were sequenced, and the phylogenetic tree analysis was performed, and results recorded in GenBank.

MATERIALS AND METHODS

Sample selection

The collection of ticks was performed from a total of 60 dogs from the Siirt province, located in the Southeastern Anatolia Region of Turkey (37° 55' N, 41° 57' E). Siirt province is in a semi-arid climate region, where the average highest and lowest temperatures range between 36.9 °C and 18.9 °C in summer, and 8.7 °C and -0.5 °C in winter. Puddles are frequently present during the summer. Once the dogs were inspected, the ticks were collected into the individually labeled 25 ml collection cups that contained 70% ethyl alcohol and transported to the laboratory.

Morphological identification of ticks and DNA extraction

The identification of the ticks was performed using the methods suggested by Walker et al. (2000) and Estrada-Peña et al. (2004) [15,16]. Each sampled tick was washed with 70% ethyl alcohol before DNA extraction. The ticks were then placed into Eppendorf tubes individually, that were subjected to a freeze-thaw process and then crushed using a sterile glass rod. The Invitrogen PureLink™ Genomic DNA Mini Kit (USA, K182002) was used to isolate the DNA from the samples, as per the protocols suggested by the commercial company. The obtained DNA was stored at -20 °C until the PCR process.

PCR Amplification

The Nested PCR method was used to identify *E. canis* DNA. In the initial phase, the primers ECC (5' AGAACGAACGCTGGCGGCAAGC-3') and ECB (5' CGTATTACGCGGCTGCTGGCA-3') were used to amplify the 16S rRNA gene section, while in the second phase, the *E. canis* specific primer ECAN5 (5'-CAATTATTATAGCCTCTGGCTATAGGA-3') and the primer HE3 (5'-TATAGGTACCGTCATATCTTCCCTAT-3') were used [17-19]. The protocol suggested in the studies of Ayan *et al.* (2019) and Ayan *et al.* (2020) was used for the PCR [1,20]. A 1.5% agarose gel was then prepared, and was stained using RedSafe™ Nucleic Acid Staining Solution. The PCR products were run through the agarose gel, and images were obtained using a gel imaging device (Syngene Bio Imaging System). Ten percent of the positive PCR products (3 samples) that were suitable for sequencing were sent to a commercial company (BM Labosis, Ankara, Turkey) for two-way sequence analysis.

Sequence and Phylogenetic Analysis

The purified PCR products were subjected to a two-way sequence analysis using an Applied Biosystems 377 DNA Sequencer device. 16S rRNA sequences for the sequenced isolates were registered to the GenBank with access numbers MW405833.1, MW408001.1, MW408000.1. The sequences with registry numbers M73227.1, AY262124.1, NR 157649.1, NR 074500.2, AF069758.1, MG564254.1, KX766395.1, MH620194.1 obtained from the GenBank were sorted in the MEGA 7 software using the Clustal W algorithm. The phylogenetic tree was then reconstructed using the Neighbor-joining model and Bootstrap tests (1000 repeats). The evolutionary similarity between the isolates was determined using the Maximum Composite Likelihood test. An UPGMA dendrogram was then created using the evolutionary similarity between *E. canis* isolate sequences, MEGA 7 software, and the Neighbor-joining model.

Ethical approval

Ethical approval for this study was obtained from the Siirt University Local Ethics Committee for Animal Experiments (Decision number 2020/04-01).

RESULTS

A total of 250 ticks were collected and morphologically identified. All ticks were identified as *R. sanguineus* (*s.l.*). *E. canis* DNA was detected using the Nested PCR method in 38 (15.2 %) of the ticks (389 bp) (Fig.1). Figure 2 shows the phylogenetic tree obtained by comparing the amplified *E. canis* 16S rRNA section sequences with M73227.1, AY262124.1, NR157649.1, NR074500.2, AF069758.1, MG564254.1, KX766395.1, MH620194.1 information. The evolutionary similarity between the

Ehrlichia canis, *Ehrlichia ruminantium*, *Ehrlichia chaffeensis*, *Ehrlichia muris*, *Ehrlichia ewingii*, and *Anaplasma ovis* species of *Anaplasmataceae* superclass was supported with the very

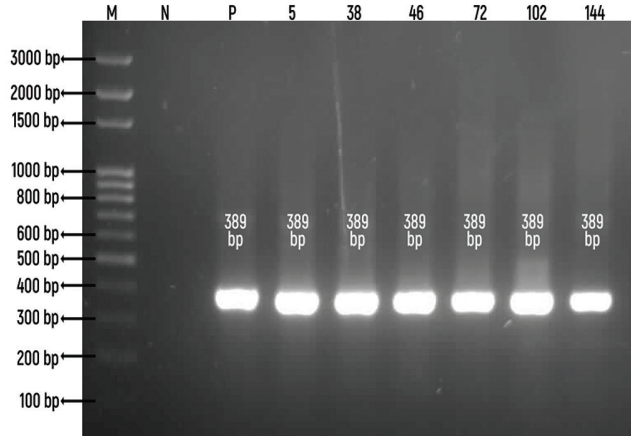


Figure 1. 16S rRNA amplification of *E. canis* in ticks using nested-PCR. Lanes M: Marker, N: Negative control, P: Positive control, Lanes 5, 38, 46, 72, 102, and 144 represent *E. canis* (389 bp)

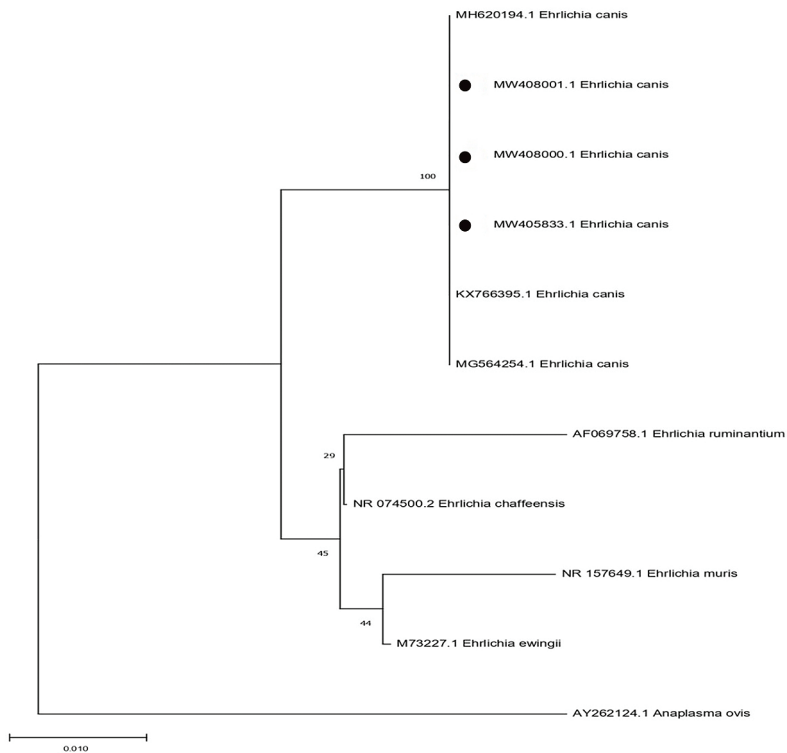


Figure 2. The phylogenetic relationship of isolates obtained as part of this study with the sequences obtained from the GenBank. The tree was constructed using the Neighbor-joining (NJ) model with a 0.010 genetic distance value. *E. canis* identified as part of this study are shown with black circles.

high bootstrap similarity value of 100% for *E. Canis. Anaplasma ovis* was taken as the outer group (AY262124.1).

DISCUSSION

Transmitting numerous pathogens: viruses, bacteria, and parasites, ticks present a serious health hazard for both humans and animals [21]. Ixodidae ticks are vectors for various diseases that affect both humans and animals living in tropical and subtropical climates in particular [22]. Diseases transmitted by ticks, like ehrlichiosis, have increased in frequency all around the world, threatening both human and animal health [23]. While *E. canis* is encountered worldwide, its presence and incidence rate varies following the geographical distribution of its vectors [24].

Studies performed in different geographical regions of the world report varying rickettsia infection prevalence for ticks. Latrofa et al. (2014) performed a study where they collected *R. sanguineus* ticks from dogs of different continents [consisting of Australia (Australia), Europe (France), America (Brazil, Colombia, Costa Rica, Guatemala, Honduras), Asia (India, Malaysia, Thailand, Vietnam), Africa (South Africa)], and reported no *E. canis* positivity. Zaid et al. (2019) performed a study in Palestine and reported 0.6% prevalence of *E.canis* in ticks. A study in the Ivory Coast reported 27% prevalence [26]. Other study covering Tunisia and Morocco reported 16.3% prevalence of *E. canis* in ticks and a prevalence of 2.9% for *R. sanguineus* ticks specifically [27]. In studies that investigated the *E. canis* presence in ticks in Israel, Iran, and Cameroon, revealed prevalence of 10.4% [28], 16.66% [29], and 21% [30] respectively.

Studies performed in Turkey reported *E. canis* prevalence in dogs to be between 0% and 69.4% [31,32]. Due to the limited number of studies on the presence of *E. canis* in Turkey, the actual distribution and prevalence of the infection is unknown [33]. A study performed on ticks from the Thrace region of Turkey (İstanbul, Edirne, Tekirdağ, and Kırklareli provinces) reported a prevalence of 21.25% on a tick pool from 127 specimens [23]. Ayan et al. (2019) performed a study in the province of Van, on the *R. sanguineus* ticks and reported a 20% prevalence of *E. canis*, while the study of Aktas et al. (2009) reported a prevalence of 1.23% in a study performed on the salivary glands of ticks [34].

Since the amplification of the 16S rRNA gene is widely used to determine *Ehrlichia* DNA [11], the 16S rRNA region was targeted for the molecular identification of *Ehrlichia* species in this study as well. As a result of the study, *E. canis* DNA was detected in 38 (15.2%) of 250 *R. sanguineus* ticks.

The results of the present study are similar to some other studies in the literature [23,26,28,29], while higher compared to other studies [11,25,34]. The difference between the study results might be due to the geographical locations, different

climate, sample size, sampling period, tick species and stages, the presence of suitable reservoirs, and the study method.

While some studies have extracted DNA from tick pools [23,28] or salivary glands [34], the DNA in the present study were extracted from the whole ticks, in line with numerous other studies in the literature [1,11,29].

While it's primarily known as a pathogen for dogs, some studies report the presence of *E. canis* in domestic ruminants [11,34,35] and in different tick species (*R. bursa*, *R. sanguineus*, *D. marginatus*) of Ixodidae family [22]. The fact that in this study *E. canis* was observed in *R. sanguineus* ticks supports such previous studies [19,22,30].

The *E. canis* strains identified through 16S rRNA gene sequence analysis amplified from *R. sanguineus* tick species were found to be 100% similar to American Texas (MH620194), Indian (KX766395), and Egyptian (MG564254) strains.

The most prominent vector known for *E. canis* is the *R. sanguineus* tick, but in a world where climate change, vector expansion, and pathogen distribution occur beyond expected limits, vectorial zoonotic diseases might increase and the vectors for pathogens might change.

CONCLUSION

This study is the first to report the molecular detection of *E.canis* in *R. sanguineus* ticks in dogs of the Siirt province. The study has identified a zoonotic agent in the *R. sanguineus* ticks collected from dogs of the Siirt province, which might cause disease in humans [36]. The results indicate that authorized establishments should implement protection and control measures to protect humans from the disease.

Authors' contributions

BAÇ and ÖYÇ conceived of the study, and participated in its design and coordination and helped to draft the manuscript. AA, ÖÖK, ABY and ÖOA carried out the lab work (Morphological identification of Tick and DNA extraction, PCR Amplification, Sequence and Phylogenetic Analysis). All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Statement of Informed Consent

The owner understood procedure and agrees that results related to investigation or treatment of their companion animals, could be published in Scientific Journal Acta Veterinaria-Beograd.

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MOLEKULARNA IDENTIFIKACIJA *EHRICHIA CANIS* KOD *RHIPICEPHALUS SANGUINEUS* KRPELJA U PROVICIJI SIIRT

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Studija je izvedena na *Ehrlichia canis* pozitivnim krpeljima sakupljenim sa pasa, a radi sekvencioniranja njihovih 16S segmenata rRNK korišćenjem PCR metode. Krpelji su uzeti sa ukupno 60 pasa u Turskoj provinciji Siirt. Ukupno je sakupljeno 250 krpelja pri čemu je obavljena njihova morfološka analiza. Svi krpelji su identifikovani kao *Rhipicephalus sanguineus sensu lato* (s.l). *Ehrlichia* DNK je detektovana PCR metodom kod 38 od ukupnog broja krpelja (15,2%). Sojevi *E. canis* koji su dobijeni kao rezultat ovih ispitivanja sekvenci, bili su 100% identični Američkim Teksas (MH620194), Indijskim (KX766395) i Egipatskim (MG564254) sojevima. Može da se zaključi da je obavljena identifikacija mikroorganizma sa zoonotskim potencijalom iz *R. Sanguineus* krpelja, sakupljenih sa pasa u Siirt provinciji.