

HIGH PREVALENCE OF *EHRlichia CANIS* IN DOGS IN VAN, TURKEY

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Abstract. Tropical and subtropical regions have seen an alarming increase in vector-borne diseases especially tick-borne diseases. Ehrlichiosis is one of the most important vector-borne disease from a zoonotic perspective and it can be even more lethal in dogs and humans with a compromised immune system. This study was conducted to determine the prevalence and molecular characterization of *E. canis* in Van province of Turkey. A total of 387 blood samples were collected from dogs in Van veterinary clinics in 2019. Extracted DNAs were run through Nested PCR using the appropriate primers. A total of 79 samples out of 387 were *E. canis* positive at 389 bp revealed by Nested PCR. Sanger method was used for DNA sequencing of two selected positive samples. Phylogenetic analysis revealed that relevant amplicon was 100% compatible with 16S rRNA gene isolated from *E. canis* in many geographical regions.

Keywords: ehrlichiosis, molecular characterization, Nested PCR, phylogenetic analyses, prevalence, tick

Introduction

Tick-borne diseases in cats and dogs have seen an upward trend in recent years posing public health risks especially for those having close contact with their pets (Claerebout et al., 2013; Maia et al., 2014). Tick-borne bacterial and protozoal infections affect the dogs in many parts of the world depending on the distribution of vectors (Aktas et al., 2015). Ehrlichiosis is a zoonotic disease mentioned at the top of list and its pathogenicity becomes more lethal in immunocompromised dogs and humans (Perez et al., 2006). Ehrlichia sp. are obligate intracellular parasites infecting the blood cells. The etiologic agent of canine monocytic ehrlichiosis (CME) is *E. canis* should be resides in canine monocytes and macrophages. Depending on the immune status of the host, CME may be asymptomatic or symptomatic causing severe anemia, leukopenia, thrombocytopenia, fever, and even death especially in dogs with poor immunity (Kakoma et al., 1994; Mathew et al., 1996; Oliveira et al., 2019). Vector-borne diseases especially those involving ticks as vectors are highly prevalent in tropical and subtropical regions (Springer et al., 2019). To date, DNAs of *E. chaffeensis*, *E. ewingi*, and *E. muris* have been isolated from canine blood from South Central United States, Europe and Costa Rica

(Skotorczak, 2003; Little et al., 2010; Springer et al., 2019). In our previous study, *E. canis* was identified in the ticks collected from stray dogs in Van region of Turkey (Ayan et al., 2019). However, the prevalence and molecular characterization of *E. canis* in stray dogs were not carried out in Van province. Therefore, the present study was conducted to determine the prevalence and molecular characterization of *E. canis* in stray dogs in the Van province, Turkey so that proper strategies may be devised to control the infection.

Materials and Methods

Collection of samples

Blood samples were collected in EDTA vacutainers from 387 stray dogs presented at three veterinary clinics (Kent Veterinary Clinic (121 samples), Cetin Veterinary clinics (129 samples), and Tamara Pet Veterinary clinic (137 samples)) in central Van in 2019 as shown in *Figure 1*. Blood samples were transported to the laboratory in cold chain at -20°C for further analysis.



Figure 1. Map of Turkey showing the localities of the investigation area for collection *Ehrlichia canis* from dogs. In 2019, 387 specimens were collected from 3 veterinary clinics in Van centre in Van province of Turkey

DNA extraction

Extraction of DNA from each blood sample was carried out using Invitrogen PureLink™ Genomic DNA Mini Kit (USA, K182002) according to the procedure outlined in the kit protocol. Extracted DNAs were stored at -20°C until PCR amplification.

PCR amplification

The 16S rRNA gene region of *E. canis* was amplified using nested PCR. The first amplification primers: ECC (5' AGAACGAACGCTGGCGGCAAGC-3') and ECB (5' CGTATTACCGCGGCTGCTGGCA-3'). The second amplification primers: ECAN5 (5'-CAATTATTTATAGCCTCTGGCTATAGGA-3') and HE3 (5'-TATAGGTACCGTCATTATCTTCCCTAT-3') (Murphy et al., 1998; Alves et al., 2014;

Makino et al., 2016). For this purpose, each reaction contained 12.5 pmol forward primer, 12.5 pmol reverse primer, 0.625 U of HOT FIREPol DNA polymerase (Solis BioDyne, Tartu, Estonia), 1X PCR Buffer (Solis BioDyne, Tartu, Estonia), 1 mM MgCl₂ (Solis BioDyne, Tartu, Estonia), 120 µM dNTP (Solis BioDyne, Tartu, Estonia), extracted DNA samples (50-200 ng) and DNase/RNase Free Distilled Water (Gibco Thermo Fisher Scientific, Waltham, MA USA) were mixed to make a final volume of 25 µL. PCR was performed in Eppendorf Mastercycler® pro automatic thermal cycler. In the first stage of Nested PCR: 95°C for 15 min and 37 cycles of 95°C for 1 min and 30 seconds, annealing at 55°C for 1 min and 30 seconds and 72°C for 1 min and 30 seconds, followed by a final extension 72°C for 10 min. In the second stage of Nested PCR: 95°C for 15 min and 40 cycles of 95°C for 1 min, annealing at 65°C for 1 min and 72°C for 1 min, followed by a final extension 72°C for 10 min. The amplified products were separated on a 1.5% agarose gel and visualized by Safe-T stain (BioShop, Canada) staining under imaging system (Syngene Bio Imaging System).

DNA sequencing and phylogenetic analysis

Two positive samples, confirmed by PCR, were selected at random and DNA (forward and reverse) sequence was determined according to the Sanger sequencing method. Forward and reverse sequences of each sample were compared using the Bioedit Sequence Alignment Editor Program (Hall, 1999). Finally, the resulting DNA sequences were compared with the similar sequences in NCBI database using BLAST. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-951,11) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0,1908)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 15 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 362 positions in the final dataset.

Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

Ethical considerations

All the procedures involved in the study were approved by the local ethics committee of Van Yuzuncu Yil University, Van, Turkey vide letter no. VAN YUHADYEK/2019/10 dated 31 October 2019.

Results

In the first stage of nested PCR, *Ehrlichia* spp. specific bands of 458 bp were obtained. Nested PCR revealed that *E. canis* specific 389 bp bands were obtained in 79 of 387 samples. Agarose gel images for amplicons of each sample in the PCR shows in Figure 2.

The results showed that a 20.41% positivity of *E. canis* detected in Van province. Two of the 79 samples were randomly selected for sequence analysis. The DNA sequences

obtained from Sanger sequencing were 100% compatible with the *E. canis* specific 16S rRNA gene in NCBI database. The sequences of two positive samples obtained from Bioedit Sequence Allignment Editor program were 359 bp and 367 bp, respectively. The tree with the highest log likelihood (-943.20) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 16 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 357 positions in the final dataset. Phylogenetic analysis of *E. canis* in dogs from Van province of Tukey showed that there were no genetic differences between the two samples based on the relevant amplicons as shown in *Figure 3*.

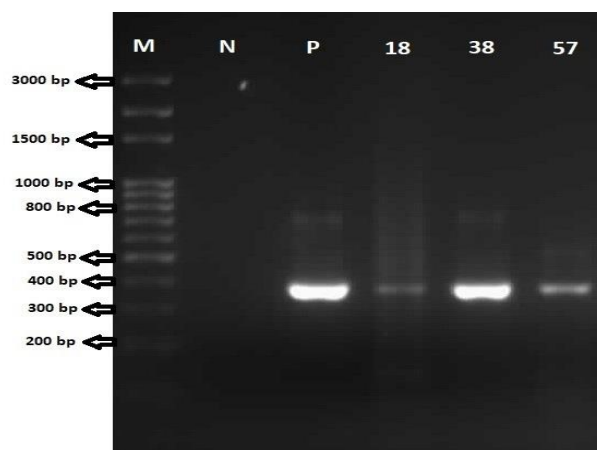


Figure 2. Agarose gel images for amplicons of each samples in the PCR. (M: Marker, P: Positive control, 18, 38, 57: Positive samples)

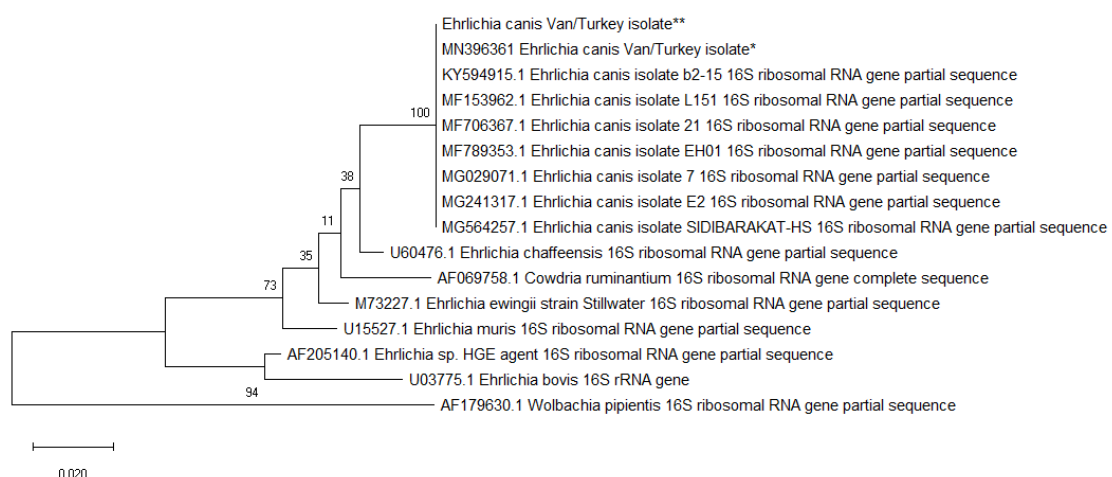


Figure 3. Molecular Phylogenetic analysis by Maximum Likelihood method of 16S rRNA gene of *E. canis*. Two samples (*: MN396361 *Ehrlichia canis* Van/Turkey isolate, **: *Ehrlichia canis* Van/Turkey isolate) in the same node as seen in the tree

Discussion

Tick-borne diseases, a cosmopolitan problem resulting in subclinical or severe infections in their hosts, are increasingly affecting the public and animal health around the globe (Hofmann-Lehmann et al., 2016). Zoonotic importance of canine tick-borne pathogens has increased in the recent years as dogs play a major role as reservoir host, Ehrlichiosis being one of those diseases (Ozubek et al., 2018). In the present study, the prevalence of *E. canis* in dogs in Van province was investigated on a molecular basis. At the end of the study, *E. canis* DNA was detected in 79 (20.41%) of 387 blood samples collected. This is the first report that describes the molecular detection of Ehrlichiosis in dog populations in Van, Turkey. Previous studies have reported the prevalence of *E. canis* in dogs in different parts of Turkey and the world alike, including some reports investigated the prevalence based on molecular studies. According to the molecular studies conducted in Turkey, the prevalence of *E. canis* 23 out of 219 blood samples in Diyarbakır was (10.5%) (Ozubek et al., 2018), *E. canis* was not detected in 133 samples from Konya (Guo et al., 2017), 58 out of 400 blood samples in Kayseri was (14.5%) (Duzlu et al., 2014), 37 out of 757 samples collected from different provinces (Elazığ, Diyarbakır, Erzurum, Ankara, Nevşehir, Adapazarı, İzmit, Mersin, Giresun, İzmir) was 4.9% (Aktas et al., 2015), 47 out of 400 blood samples in Thrace was 11.75% (Cetinkaya et al., 2016), 3 out of 12 dogs with clinical symptoms in Ankara (Unver et al., 2005). Similarly, Hofmann-Lehmann et al. (2016) reported that 14 out of 249 blood samples collected from Sicily (Italy) and Portugal were *Anaplasma platys* and *E. canis* positive whereas the DNA of *E. canis* was not detected in samples from Switzerland, Spain, and Terasa and Bologna regions of Italy. In addition, Cardoso et al. (2016) reported *E. canis* in 5.8% of 103 blood samples in Luanda region of Angola and Alho et al. (2017) found *E. canis* in 3.1% of 64 blood samples in Qatar. In contrast, *E. canis* DNA was not found in 97 blood samples collected from Iraq (Otranto et al., 2019). In this study, Ehrlichia infection in Van, Turkey was found to be 20.41% prevalent that is high. There may be different reasons underlying the high prevalence of related infections in the region. Inadequate external parasite spraying in dogs and the presence of *R. sanguineus* in the region can be considered as the main reasons for the high prevalence of Ehrlichiosis. The importance of this disease is increasing all over the world including Turkey. It is noteworthy that global warming, environmental and ecological changes, and the existence of appropriate habitats increase the impact of ticks that results in frequent emergence and re-emergence of tick-borne diseases of zoonotic importance (Inci et al., 2016).

Vector hemoparasites can be diagnosed by examining the clinical signs and blood staining methods, however, diagnostic may arise attributed to the similarities in symptoms and microscopic structures of the etiologic agents. Consequently, test based on serological and molecular techniques should be preferred in order to diagnose the hemoparasites. Nonetheless, it becomes difficult to extricate between current and previous exposure to pathogens that might result in false negative results in case of acute ehrlichiosis. Therefore, diagnosis based on molecular tests is the best option (Neer et al., 1998; Little et al., 2010; Aktas et al., 2015; Springer et al., 2019). The most common method used to diagnose ehrlichiosis is one-step PCR analysis to amplify the 16S rRNA gene region, however, its sensitivity is low. Therefore, diagnosis through Nested PCR increases the sensitivity and is more reliable (Bulla et al., 2004; Macieira et al., 2005; Nakaghi et al., 2008; Santos et al., 2009). However, non-specific amplification among the strains of Ehrlichia species due to highly conserved 16S rRNA gene and higher risk of DNA cross-contamination are inevitable in this method (Labruna et al., 2007).

Consequently, the sequencing and phylogenetic analyses are important so as to prove the accuracy of the results in addition to identify the molecular similarity among the species (Sumner et al., 2000).

E. canis, a proteobacter that causes ehrlichiosis, possess a 16S rRNA gene consisting of 1380 bp in size. In our study, we identified and amplified a gene of 390 bp in size that is repeatedly employed in the molecular diagnosis of *E. canis*. The DNA sequence analysis of the region revealed the identification of a subtype of *E. canis* that is distributed in different geographical regions. The DNA sequence analysis of our *E. canis* DNA samples showed 100% similarities with those of NCBI database isolated from *E. canis* in different geographies such as Elazığ/Turkey (Accession number: KY594915), Panama (Accession number: MF789353), Egypt (Accession number: MG564257), Romania (Accession number: MG241317), India (Accession number: MF706367), Mexico (Accession number: MG029071), and Brazil (Accession number: MF153962). All genomic analysis is possible using new generation sequencing technologies along with the identification of new or unknown subtypes of proteobacter *E. canis*.

Conclusion

In conclusion, this molecular study confirmed the presence and prevalence of *E. canis* in dogs in Van province of Turkey. This shows that Ehrlichiosis in dogs in this region may pose threats to public health especially in immunocompromised people. We believe that the density of dog population and lack of effective control of ticks especially in stray dogs are the possible predisposing factors for this high prevalence of ehrlichiosis in dogs in Van, Turkey. In order to determine the zoonotic potential of ehrlichiosis, it is necessary to compare the genetic factors and genetic proximity of ehrlichiosis in humans with that of dogs. As the tick-borne diseases are increasing around the globe, we believe that the control of ticks in domestic animal and effective treatment of animals infested with ticks will be key to preventing tick-borne diseases. Since the Van province shares its border with Iran, ticks and tick-borne diseases in this region have potential to create an international problem. In addition, changing climatic conditions and human migration have created variability especially in vectors of vector-borne diseases. Therefore, further studies in this area should not only focus *R. sanguineus*, but also other tick species by molecular methods. In addition, scientists in the countries sharing the border in the region should carry out joint and collaborative studies especially in terms of vector diseases and the molecular proximity of the agents should be demonstrated.

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