VETERİNER HEKİMLER DERNEĞİ DERGİSİ
JOURNAL OF THE TURKISH VETERINARY MEDICAL SOCIETY
ISSN: 0377-6395
e-ISSN: 2651-4214
Dergi ana sayfası:
Journal homepage:
http://dergipark.org.tr/vetheder

doi: 10.33188/ vetheder.955964

Araştırma Makalesi / Research Article

Determining the analytical sensitivity of polymerase chain reaction targeting *Ehrlichia* spp. disulfide oxidoreductase gene: Molecular diagnosis of ehrlichiosis in a dog clinically suspected with leishmaniasis

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MAKALE BİLGİSİ / ARTICLE INFORMATION:

Geliş / Received:
24 Haziran 21
24 June 21

Revizyon/Revised:
18 Ekim 21
18 October 21

Kabul / Accepted:
21 Ekim 21
21 October 21

Keywords:
Disulfide oxidoreductase
Ehrlichia canis
Ehrlichiosis
PCR
Leishmaniasis

Anahtar sözcükler:
Disülfit oksiredüktaz
Ehrlichia canis
Ehrlichiosis
PCR
Leishmaniasis

ABSTRACT:

Ehrlichia spp. is tick-borne zoonotic pathogen that can infect humans and animals. Nowadays, among the tests used in the diagnosis of ehrlichiosis, the importance of molecular methods is increasing steadily due to their high sensitivity and specificity. The aim of this study was to determine the analytical sensitivity of a conventional polymerase chain reaction (PCR) targeting *Ehrlichia* spp. disulfide oxidoreductase (*DSB*) gene. *Ehrlichia* spp. *DSB* gene was cloned into the TOPO vector. After TOPO plasmid containing *DSB* gene were serially diluted, PCR targeting the *Ehrlichia* spp. *DSB* gene was performed. While working on this research, blood and skin scraping samples of a stray dog clinically suspected with leishmaniasis as well as treated for leishmaniasis arrived to our laboratory. Thereafter, PCRs targeting *Ehrlichia* spp. *DSB* and 16S rRNA and Leishmania kinetoplast DNA (*kDNA*) genes were performed to identify the pathogen in blood and skin scraping samples of the stray dog. The analytical sensitivity of the PCR assay targeting *Ehrlichia* spp. DSB gene was 1 ≥ copy plasmid/reaction using serially diluted TOPO plasmid containing *DSB* gene. PCR targeting the *Ehrlichia* spp. *DSB* gene was positive and PCR targeting *Leishmania* spp. *kDNA* was negative in blood and skin samples of the stray dog clinically suspected with leishmaniasis. Using nested PCR targeting *Ehrlichia* spp. *16S rRNA*, *E. canis* was identified in blood and skin scraping samples of the stray dog. In this study, PCR targeting *Ehrlichia* spp. *DSB* gene has been shown to have high sensitivity. Also it was shown molecular methods can help clinicians in differential diagnosis of ehrlichiosis and leishmaniasis to prevent inappropriate treatment.

Ehrlichia spp. disülfid oksidoredüktaz genini hedefleyen polimeraz zincir reaksiyonunun analitik duyarlılığının belirlenmesi: Klinik olarak leishmaniasis'ten şüphelenilen bir köpekte ehrlichiosis'in moleküler tanısı

ÖZET:

Ehrlichia spp. insan ve hayvanları enfekte edebilen kene kaynaklı zoonotik patojenlerdir. Günümüzde hastalığın tanısında kullanılan testler arasında yüksek duyarlılık ve özgünlüğe sahip olan moleküler yöntemlerin önemi giderek artmaktadır. Bu çalışmanın amacı *Ehrlichia* spp. disülfid oksiredüktaz (*DSB*) genini hedefleyen konvansiyonel polimeraz zincir reaksiyonunun (PZR) analitik hassasiyetinin belirlenmesidir. Öncelikle *Ehrlichia* spp. *DSB* geni TOPO vektörüne klonlanmıştır. *DSB* geni içeren TOPO plazmidi seri olarak sulandırıldıktan sonra *Ehrlichia* spp. *DSB* genini hedefleyen PCR gerçekleştirilmiştir. Bu araştırma üzerinde çalışmalar sürerken, klinik olarak leishmaniasis şüphesi bulunan ve leishmaniasis tedavisi alan bir sokak köpeğinin kan ve deri kazıntı örnekleri laboratuvarımıza gelmiştir. Sokak köpeğine ait kan ve deri kazıntı örneklerinde patojeni belirlemek amacıyla *Ehrlichia* spp. *DSB* ve *16S rRNA* ile *Leishmania* spp. kinetoplast DNA (*kDNA*) genlerini hedef alan PCR testleri yapılmıştır. *DSB* geni içeren seri sulandırılmış TOPO plazmidi kullanılarak yapılan *Ehrlichia* spp. *DSB* genini hedefleyen PCR testinin analitik hassasiyeti, 1 ≥ kopya plazmit/reaksiyon olarak tespit edilmiştir. Klinik olarak leishmaniasis şüphesi bulunan sokak köpeğinin kan ve deri kazıntı örneklerinde *Ehrlichia* spp. *DSB* genini hedefleyen PCR pozitif, *Leishmania* spp. *kDNA*'yı hedefleyen PCR testleri negatif olarak tespit edilmiştir. *Ehrlichia* spp. *16S rRNA*'yı hedefleyen nested PCR testi ile, sokak köpeğinin kan ve deri kazıntı örneklerinde *E. canis* olarak tanımlanmıştır. Bu çalışmada *Ehrlichia* spp. *DSB* genini hedefleyen PCR'nin yüksek duyarlılığa sahip olduğu gösterilmiştir. Ayrıca moleküler yöntemlerin klinisyenlere ehrlichiosis ve leishmaniasis ayırıcı tanısında uygunsuz tedaviyi önlemede yardımcı olabileceği öngörülmüştür.

How to cite this article: Karakavuk M, Aykur M, Can H, Değirmenci D.A, Dağci H, Gürüz Y, Döşkaya M. Determining the analytical sensitivity of polymerase chain reaction targeting Ehrlichia spp. disulfide oxidoreductase gene: Molecular Diagnosis of ehrlichiosis in a dog clinically suspected with leishmaniasis. Vet Hekim Der Derg 2022;93(1):28-36 .DOI: 10.33188/ vetheder.955964

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1. Introduction

Ehrlichia spp. can cause serious and fatal disease in pet and farm animals as well as important health problems in humans. Ehrlichiosis that is vector-disease occurs incidentally in humans due to ecological changes, population structure, and host susceptibility (1,2). *Ehrlichia canis*, *E. chaffeensis* and *E. ewingii* are medical and veterinary importance. *Ehrlichia canis* causes canine monocytic ehrlichiosis (CME) in dogs, while *E. chaffeensis* leads to monocytic ehrlichiosis (HME) in humans (3,4).

Although zoonotic *E. canis* infection occurs in many parts of the world, the disease is more common in tropical and sub-tropical regions (5,6). Clinically, an acute CME is characterized by fever, depression, dyspnea, anorexia, weight loss, anorexia, lethargy, hemorrhagic disorders, anemia, thrombocytopenia, and leukocytosis or leukopenia after an incubation period of 8-20 days. Following the acute stage, chronic infection causes weight loss, edema, epistaxis, lymphadenopathy, organomegaly, skin rash, hemorrhage, and hypotensive shock (6,7). In the first reported cases of ehrlichiosis in Turkey, intermittent character of fever, depression, hair in dullness, redness of the scrotum and bloody nasal discharge complaints were observed in dogs (8). Ehrlichiosis prevalence was 15.9% (10/63) in Diyarbakır, 6.7% (5/74) in Mersin, 28.0% (14/50) in Giresun and 13.3% in Izmir (8/60) as detected by microscopy, PCR targeting 16s rRNA and Reverse Line Blotting (RLB) (9). Ehrlichiosis is often confused with leishmaniasis in terms of clinical presentation (10). During leishmaniasis clinical, symptoms such as anorexia, dull hair, skin lesions, onychogryphosis, weight loss, lymphadenopathy and organomegaly can be seen in dogs of all age. The seroprevalence of leishmaniasis in dogs is approximately 15% in Turkey (ranging between 1.45% to 27.5% in various regions (11–14)

In addition to clinical findings, microscopic, serological, and molecular methods are used in the diagnosis of ehrlichiosis. In acute stage of ehrlichiosis, diagnosis with microscopy can be made by observing the typical *Ehrlichia* spp. morula. However, the sensitivity of the microscopy is very low and typical morulae have been detected in only 4% of positive cases (15-19). IFAT, Western Blot, and ELISA methods are most commonly techniques during serological diagnosis (17). IFAT is the most commonly used serological test and prepared by *E. canis* antigen (18). Molecular techniques are required for definitive diagnosis as serological tests cannot distinguish active infection (19). With the development of molecular methods, the diagnosis of ehrlichiosis is achieved rapidly with high sensitivity and specificity. The majority of PCR assays used in the diagnosis of *E. canis, E. chaffeensis,* and *E. ewingii* target *16S rRNA, p28* or *DSB* genes (19–21). In experimental studies, the sensitivity of conventional PCR targeting *16S rRNA* gene was found to be low. For this reason, nested PCR method is used to determine the species in the same test and increase the sensitivity (22–24).

The aim of this study was to determine the analytical sensitivity of a PCR targeting *Ehrlichia* spp. *DSB* gene using serially diluted plasmid containing *DSB* gene. In addition, ehrlichiosis and leishmaniasis were investigated using PCRs targeting *Ehrlichia* spp. *DSB* and *16S rRNA* genes, and *Leishmania* spp. *kDNA* gene in blood and skin scraping samples of a stray dog clinically suspected and treated for by a veterinarian in İzmir.

2. Material and Methods

Generation of Recombinant Plasmid DNA containing the Ehrlichia *DSB* gene by TOPO Cloning:

A positive control DNA previously identified as *E. canis* was used to generate plasmid DNA containing the 409 base pair (bp) region of the *Ehrlichia* spp. *DSB* gene. The 409 bp region of the *Ehrlichia* spp. *DSB* gene (GenBank: AF403710.1) was isolated using the *DSB*-330 (5′-GATGATGTCTGAAGATATGAAACAAAT-3′) and *DSB*-728 (5′-CTGCTCGTCTATTTTACTTCTTAAAGT-3′) primer pair as described previously (18,25–27). Briefly, 20 µl reaction included 2 µl template DNA, 1,25 U Taq DNA polymerase (Thermo, USA), 3 mM $MgCl_2$,0.2 mM dNTPs, the primers (0,4 µM each) and 1x Taq Buffer. The PCR reaction was conducted using the following protocol: 10 min initial denaturation step at 95 °C, followed by 35 cycles of 15 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C, and a final extension of 10 min at 72 °C. The PCR product was showed on 1 % agarose gel electrophoresis and then purified with PCR purification kit (Qiagen, USA). The PCR product was cloned into the pCR™II-TOPO vector according to the manufacturer's instructions (Invitrogen, USA). Single colonies were selected and transferred to liquid 3 ml LB medium containing kanamycin and incubated overnight at 37 °C at 225 rpm. Plasmid from liquid colonies was performed according to the manufacturer's protocol (Qiagen, USA) (28,29). The presence of the *Ehrlichia* spp. DSB gene in the recombinant plasmid DNA sample was confirmed by PCR as described above and sequencing (Figure 1).

Determining the analytical sensitivity of PCR targeting DSB gene:

The concentration of the TOPO plasmid containing *Ehrlichia* spp. DSB gene was determined by Nanodrop, the plasmids were diluted with 10^6-10^5-10^4-10^3-10^2-10-1 copies/reaction in order to determine the analytical sensitivity. Distilled water was used as negative control. The PCR targeting the *Ehrlichia* spp. DSB gene was performed as described above (25–27).

Clinical samples and DNA isolation:

A stray dog with anorexia, weakness, fever, skin lesions, lymphadenopathy and thrombocytopenia were initially diagnosed as leishmaniasis based on clinical findings. Blood and skin scraping samples collected by the veterinarian for diagnostic purposes were send to our lab. The blood sample was centrifuged at 3000 rpm for 10 minutes to obtain buffy-coat. DNA isolation from buffy-coat and skin scraping samples was performed with the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocol (30).

Molecular diagnosis:

To detect ehrlichiosis, two PCR methods targeting two different gene regions of *Ehrlichia* spp. were performed with DNA samples obtained from blood and skin scraping materials. Initially, PCR targeting the *DSB* gene region for *Ehrlichia* spp. diagnosis was performed as described above (26). To identify species of *Ehrlichia* spp., nested PCR targeting *16S rRNA* gene using primers specific for *E. chaffeensis*, *E. canis* and *E. ewingii* (31–33). In the initial reaction, outer primers, ECC (5'-AGAACGAACGCTGGCGGCAAGC-3') and ECB (5'-CGTATTACCGCGGCTGCTGGCA-3'), were used. In the second reactions were performed using 5 µl of the outside reaction as template with each species-specific set [Primers HE1 (5'-CAATTGCTTATAACCTTTTGGTTATAAAT-3') and HE3 (5'-TATAGGTACCGTCATTATCTTCCCTAT-3') for *E. chaffeensis*, primers ECAN5 (5'-CAATTATTTATAGCCTCTGGCTATAGGA-3') and HE3 for *E. canis*-specific amplifications, primers EE52 (5'-CGAACAATTCCTAAATAGTCTCTGAC-3') and HE3for *E. ewingii*] under the reaction conditions described above. The PCR products were visualized on 2% agarose gel electrophoresis.

To detect leishmaniasis, a nested PCR targeting kinetoplast DNA (*kDNA*) was performed with DNA samples obtained from blood and skin scraping materials (34). Outer primers, CSB2XF (CGAGTAGCAGAAACTCCCGTTCA) and CSB1XR (ATTTTTCGCGATTTTC-GCAGAACG) were used in first reaction. Inner primers 13Z(ACTGGGGGTTGGTGTAAAATAG) and LiR (TCGCAGAACGCCCCT) were used in the second reaction. The second reaction was implemented in total 30 µl volume under same condition like first reaction (30). The PCR products were visulalized with 1% agarose gel electrophoresis. Three international reference controls *L. tropica* (MHOM/SU/74/SAF-K27), *L. major* (MHOM/SU/73/5ASKH), *L. infantum* (MHOM/TN/80/IPT1), and were used as positive control and distilled water was used as negative control.

3. Results

Determining the Analytical Sensitivity of PCR targeting *Ehrlichia* spp. *DSB* gene:

Confirmation of the cloning of the *DSB* gene isolated from the *E. canis* positive DNA sample into the TOPO vector was performed by PCR and sequencing. The size of the PCR product obtained from *E. canis* was 409 bp as shown in Figure 1. After cloning the PCR product into TOPO vector, blasting the sequence data of the plasmid showed 100% homology with *E. canis* isolate 73 disulfide oxidoreductase (*DSB*) gene (GenBank no: KY576856.1) and *E. canis* disulfide oxidoreductase gene (AF403710.1). TOPO vector containing *Ehrlichia* spp. *DSB* gene was serially diluted to 10^6-10^5-10^4-10^3-10^2-10-1 copy plasmid/reaction and the analytical sensitivity of the PCR targeting *Ehrli*chia spp. *DSB* gene was 1 copy plasmid/reaction (Figure 2).

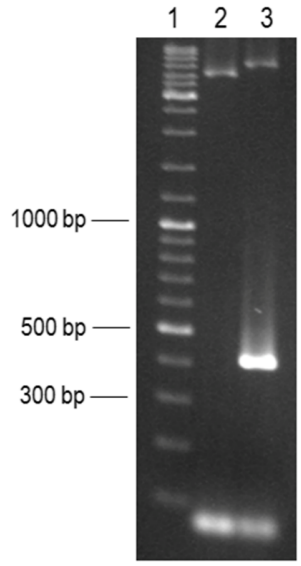

Figure 1: Agarose gel image of PCR products obtained from TOPO vector containing *Ehrlichia spp*. DSB gene 1) Marker (Fermentas), 2) Negative control plasmid, 3) TOPO plasmid containing *Ehrlichia spp.* DSB gene to be used as positive control

Şekil 1: *Ehrlichia spp. DSB geni içeren TOPO vektöründen elde edilen PZR ürünlerinin agaroz jel görüntüsü 1) Marker (Fermantas), 2) Negatif kontrol plazmit, 3) Pozitif kontrol olarak kullanılacak Ehrlichia spp. DSB genini içeren TOPO plazmidi*

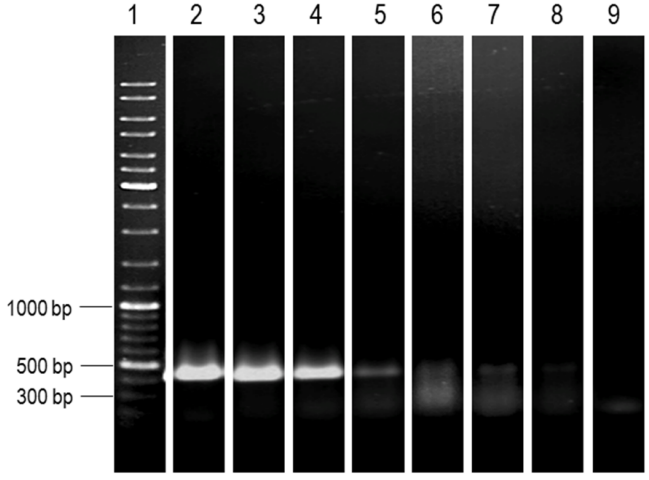

Figure 2: Agarose gel image showing the analytical sensitivity of PCR targeting *Ehrlichia* spp. *DSB* gene. 1) Marker (Fermentas), 2) 10^6 copyplasmid/reaction, 3) 10^5 copyplasmid/reaction, 4) 10^4 copyplasmid/reaction, 5) 10^3 copyplasmid/reaction, 6) 10^2 copyplasmid/reaction, 7) 10^1 copyplasmid/reaction 8) 1 copyplasmid/reaction, 9) Negative control

Şekil 2: *Ehrlichia spp. DSB genini hedefleyen PZR' nin analitik duyarlılığını gösteren agaroz jel görüntüsü. 1) Marker (Fermentas), 2) 10^6 kopya plazmit/reaksiyon, 3) 10^5 kopya plazmit/reaksiyon, 4) 10^4 kopya plazmit/reaksiyon, 5) 10^3 kopya plazmit/reaksiyon, 6) 10^2 kopya plazmit/reaksiyon, 7) 10^1 kopya plazmit/reaksiyon, 8) 1 kopya plazmit/reaksiyon, 9) Negatif control*

Molecular Diagnosis:

Leishmania spp. positivity was not detected by nested PCR targeting *Leishmania* spp. kinetoplast DNA in blood and skin samples of the leishmaniasis suspected dog. Subsequently, *Ehrlichia* spp. *DSB* gene was detected by PCR in both blood and skin scrapings (Figure 3). The PCR product obtained from nested PCR targeting *16S rRNA* to identify the Ehrlichia species had a size of 378 bp indicating that the species was *E. canis* in blood and skin samples (Figure 4).

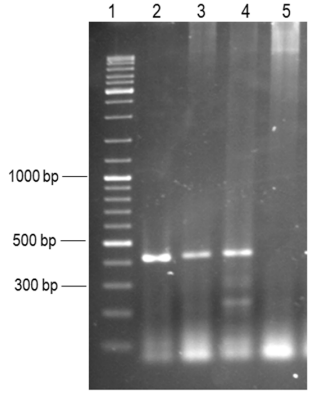

Figure 3: Agarose gel image of PCR targeting *Ehrlichia* spp. *DSB* gene in blood and skin scraping samples of the dog 1) Marker (Fermentas), 2) Positive control TOPO plasmid containing *Ehrlichia* spp. *DSB* gene, 3) Blood sample of the dog, 4) Skin sample of the dog, 5) Negative control

Şekil 3: *Köpek kan ve deri kazıntı örneklerinde Ehrlichia spp. DSB genini hedefleyen PZR' nin agaroz jel görüntüsü. 1) Marker (Fermentas), 2) Ehrlichia spp. DSB geni içeren pozitif kontrol TOPO plazmiti. 3)Köpek Kan örneği, 4) Köpek deri kazıntısı örneği, 5) Negatif kontrol*

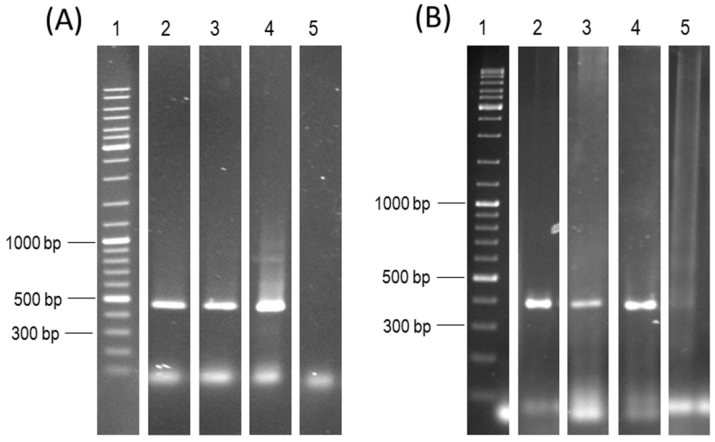

Figure 4: (A) Agarose gel image of nested PCR targeting *Ehrlichia* spp. *16S rRNA PCR* (first reaction) using ECC and ECB external primers 1) Marker (Fermentas), 2) *E. canis* positive control, 3) Blood sample of dog, 4) Skin scraping sample of dog, 5) Negative control, (B) Agarose gel image of nested PCR targeting *Ehrlichia* spp. *16S rRNA PCR* (second reaction) using ECAN5 and HE3 internal primers1) Marker (Fermentas), 2) *E. canis* positive control, 3) Blood sample of dog, 4) Skin sample of dog, 5) Negative control

Şekil 4: *(A) ECC ve ECB harici primerleri kullanılarak Ehrlichia spp. 16S rRNA PZR'yi (ilk reaksiyon) hedefleyen nested PCR' nın agaroz jel görüntüsü 1) Marker (Fermentas), 2) E. canis pozitif control, 3) Köpek kan örneği, 4) Köpek deri kazıntısı örneği, 5) Negatif control, (B) ECAN5 ve HE3 iç primerleri kullanılarak Ehrlichia spp. 16S rRNA' yı (ikinci reaksiyon) hedefleyen nested PZR'nin agaroz jel görüntüsü Marker (Fermentas), 2) E. canis pozitif control, 3) Köpek kan örneği, 4) Köpek deri kazıntısı örneği, 5) Negatif control*

4. Discussion and Conclusion

Microscopic, serological, and molecular methods are often used to diagnose ehrlichiosis. There are disadvantages of microscopy such as difficulty to detect *Ehrlichia* spp. specific morulae which requires experienced personnel and has low sensitivity and specificity (35). There are also disadvantages of serological diagnostic methods such as *Ehrlichia* spp. to be used s antigen in these assays is difficult to cultivate *in vitro* and moreover cross reactions and lack of antibody response in the early stages of infection may occur (17). For these reasons, molecular methods are very important in the rapid and accurate diagnosis of the disease (17,32,36).

Ehrlichia spp. *DSB* gene is frequently used in the molecular diagnosis of the ehrlichiosis. The sensitivity of multiplex real-time PCR generated by designing primers and probes targeting the *DSB* gene specific for *E. chaffeensis, E. ewingii,* and *E. canis* species was 50 copy plasmid/reaction (25). In this study, analytical sensitivity of PCR targeting the *Ehrlichia* spp. *DSB* gene was shown to be ≥1 copy plasmid/reaction. The significant difference among these assays was thought to be due to the fact that the PCR test used in this study was only specific to *Ehrlichia* spp. *DSB* gene and the real time PCR was not multiplexed to detect three different species.

PCR has been shown to have high sensitivity and specificity in the diagnosis of ehrlichiosis in humans (37). Blood samples of 237 HIV-positive patients with high fever and suspected ehrlichiosis were examined by nested PCR targeting the 16S rRNA gene, and ehrlichiosis was detected in 23 patients (9.7%). Among them, *E. chaffeensis* was detected in 13 patients, *E. ewingii* was detected in four patients and mix infection was detected in the remaining four patients (38). In another study targeting the 16S rRNA gene, *E. chaffeensis* was detected in seven blood samples of 38 patients (18.4%) with fever. Using microscopic examination, *Ehrlichia* spp. morulae was detected in only two patients (5.2%). Acute disease was detected in two patients using serological methods and specific antibodies were detected in six patients who were in recovery period (39). In a study targeting *Ehrlichia* spp. *DSB* gene region, 12 patients out of 118 patients with febrile disease were diagnosed with ehrlichiosis. *E. chaffeensis* was determined in in all PCR positive samples using sequencing (40). According to our knowledge, there is not any study investigating ehrlichiosis in humans in Turkey.

A number of serological and molecular studies have been conducted in different countries to determine the prevalence of Ehrlichia infection in dogs. As a result of these studies, it has been reported that the frequency of Ehrlichia infection is between 18-30% in Asia, 3.1-68% in Africa, 2.2-50% in Europe and 15.4-44.7% in America (26). Studies conducted in Turkey are limited and mainly use serological methods to determine ehrlichiosis. In addition, various PCR methods were used in these studies (9,41). In one study in different cities of Turkey, the seroprevalence of *E. canis* had been reported to range between 4.8% and 69.4% (42). In another study conducted in 219 shelter dogs in Diyarbakir located in Southeastern Anatolia, *E. canis* was detected in 32 (14.61%) dogs using nested PCR and Reverse Line Blotting (RLB) (43). In a study conducted with 400 dogs in the Thrace region located in Northwestern Turkey, the prevalence of *E. canis* was 0.75% by microscopy, 27.25% by serology and 11.75% using PCR (44).

In the first canine ehrlichiosis case detected in Turkey, the clinical findings were fatigue, depression, opacity of hair, runny nose, generalized lymphadenopathy and the clinical diagnosis was confirmed by IFA test (8). In our study, a dog presenting with anorexia, weakness, fever, skin lesions, lymphadenopathy and thrombocytopenia was initially diagnosed with leishmaniasis and treated accordingly. As the dog did not benefit from the treatment, the veterinarian sent us blood and skin scraping samples. *Ehrlichia* spp. *DSB* gene was detected by PCR in these samples. Nested PCR targeting the *16S rRNA* region identified *E. canis* in both blood and skin scraping samples.

During ehrlichiosis, skin lesions can be observed at site of tick bite (45). In this study, *E. canis* has been detected in skin lesions possibly due to the exudate and blood occurred during the scraping process of the fragile skin.

Overall, ehrlichiosis is an important tick-borne infection that can infect humans and animals. Diagnosis and treatment of the disease in humans and animals has utmost importance due to the fact that ehrlichiosis is zoonosis. Izmir is Turkey's third largest city with a temperate climate is an important tick habitat features. In addition, people are at risk for ehrlichiosis due to the uncontrolled stray dog problem and the fact that these dogs live with people. For these reasons, it is considered that more research should be done in relation to the diagnosis and differentiation of ehrlichiosis in humans and animals and the incidence of the disease should be determined with the data obtained on this subject.

Acknowledgements

The authors would like to thank to Dr. Munir Aktaş and Dr. Sezayi Özübek from University of Fırat, Turkey for providing Ehrlichia positive DNA. Also, the authors would like to thank Esra Atalay for technical support.

Conflict of Interest

The authors did not report any conflict of interest or financial support.

Funding

During this study, any pharmaceutical company that has a direct connection with the subject of the research, a company that provides and / or produces medical tools, equipment and materials, or any commercial company, during the evaluation process of the study, no financial and / or moral support was received

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Control/Supervision: Hande Dağcı, Adnan Yüksel Gürüz, Mert Döşkaya
Data Collection and / or Processing: Muhammet Karakavuk, Mehmet Aykur, Hüseyin Can
Analysis and / or Interpretation: Muhammet Karakavuk, Mehmet Aykur, Aysu Değirmenci Döşkaya
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Ethical Approval

An ethical statement was received from the authors that the data, information and documents presented in this article were obtained within the framework of academic and ethical rules, and that all information, documents, evaluations and results were presented in accordance with scientific ethics and moral rules.

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