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Seroprevalence of Asymptomatic *Leishmania* spp. Carriage Among Blood Donors in Leishmaniasis Endemic Area in Turkey

Türkiye'de Layşmanyazisin Endemik Olduğu Bölgede Kan Bağışçılarında Asemptomatik *Leishmania* spp. Taşıyıcılığının Seroprevalansı

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ABSTRACT

Introduction: *Transfusion-related infections are usually caused by a microbial pathogen transmitted to the recipient by the donated blood. Plasmodium spp., Trypanosoma cruzi, Babesia microti, Toxoplasma gondii and Leishmania spp. are listed as the most widely reported transfusion-transmitted parasites. Leishmaniasis is well known as an endemic in Mediterranean countries including Turkey. Accordingly, detection of asymptomatic Leishmania infantum carriage in blood donors is an important issue in Turkey. In endemic teretories, research on blood donors is under-represented in Turkey. Likewise, Mersin province is also endemic for Leishmaniasis. Up to date, no studies have been conducted to detect Leishmaniasis in healthy blood donors in our region. Therefore, the main objective of the current study was to reveal the seroprevalance of asymptomatic Leishmania carriage among the blood donors in Mersin province.*

Materials and Methods: *A total of 509 blood samples were collected from blood donors during a 12-month period between June 2016 and June 2017 at Mersin University Health Research and Application Center. Collected questionnaires were evaluated to determine the risk of exposure to Leishmania spp. Serological and molecular methods were assesed on the collected blood specimens.*

Results: *Based on the questionnaires, a total number of 509 donors participated the study, living in the city center (n= 367), district (n= 121), town (n= 7) and village (n= 14) respectively. A veterinarian and a sailor as well as 17 farmers were listed in the risky occupational groups. The number of dog and cat owner donors were reported as 41 and 20, respectively. All samples were found to be negative for Leishmania spp. by ELISA and real-time polymerase chain reaction methods.*

Conclusion: *This research is the first to assess Leishmaniasis risk in Mersin province. It is highly recommended not to utilize Leishmania spp. screening into the routine test packages in blood banks in our region.*

Key Words: *Transfusion-related infection; Blood donor; Endemic; Leishmania spp.*

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ÖZ

Türkiye'de Layşmanyazisin Endemik Olduğu Bölgede Kan Bağışçılarında Asemptomatik *Leishmania* spp. Taşıyıcılığının Seroprevalansı

Giriş: *Transfüzyonla ilişkili infeksiyonlara genellikle alıcıya bağışlanan kanla bulaşan mikrobiyal bir patojen neden olmaktadır. En yaygın olarak rapor edilen transfüzyonla bulaşan parazitler Plasmodium spp., Trypanosoma cruzi, Babesia microti, Toxoplasma gondii ve Leishmania spp.'dir Layşmanyazis, Türkiye'nin de içinde bulunduğu Akdeniz ülkelerinde endemiktir. Kan bağışçılarında asemptomatik Leishmania infantum taşıyıcılığının saptanması önemli bir noktadır. Endemik bölgelerde kan bağışçılarında gerçekleştirilen çalışma sayısı sınırlıdır. Mersin ili layşmanya için endemiktir. Daha önce bölgemizdeki sağlıklı kan bağışçılarında layşmanyazisi tespit etmek için herhangi bir çalışma yapılmamıştır. Bu çalışmanın amacı, Mersin ilindeki kan bağışçılarında asemptomatik layşmanya taşıyıcılığının seroprevalansını belirlemektir.*

Materyal ve Metod: *Haziran 2016-Haziran 2017 tarihleri arasında, 12 aylık sürede Mersin Üniversitesi Sağlık Araştırma ve Uygulama Merkezinde kan bağışçılarından toplam 509 kan örneği toplandı. Leishmania spp.'ye maruz kalma riskini belirlemek için anket çalışması yapıldı. Toplanan kan örnekleri serolojik (ELISA) ve moleküler yöntemlerle [gerçek-zamanlı polimeraz zincir reaksiyonu (RT-PCR)] çalışıldı.*

Bulgular: *İl merkezinde, semtte, kasabada ve köyde yaşayan kan bağışçısı sayısı anket sonuçlarına göre 367, 121, 7 ve 14'tür. Kan bağışçıları arasındaki riskli meslek grupları değerlendirildiğinde; sadece bir bağışçı veteriner, biri denizci ve 17'si çiftçidir. Kırk bir bağışçı köpek, 20 bağışçı kedi sahibidir. Toplanan kan örneklerinin tamamı ELISA ve RT-PCR ile Leishmania spp. için negatif olarak saptanmıştır.*

Sonuç: *Bu araştırma Mersin ilinde kan donörlerinde layşmanya riskini değerlendiren ilk araştırmadır. Bölgemizde Leishmania spp. belirlenmesine yönelik testlerin transfüzyon tarama testleri içerisine dahil edilmesi gerekliliği yoktur.*

Anahtar Kelimeler: *Transfüzyonla bulaşan infeksiyon; Kan bağışçısı; Endemik; Leishmania spp.*

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INTRODUCTION

Leishmaniasis is a parasitic infection transmitted by an insect vector infected by protozoa of the genus *Leishmania* (*Trypanosomatida: Trypanosomatidae*). This parasite is a threatening factor in approximately 100 countries including tropical, subtropical and Mediterranean areas[1]. In endemic regions, 350 million of the population is estimated to be in danger in terms of leishmaniasis disease. It is reported that 12 million people are infected with *Leishmania* spp. worldwide. It has been found that 15 species of *Leishmania* spp. are related with infections in human and the main reservoir of 13 among all has been found in canidae and rodents[2]. On the other hand, Turkey is endemic for both cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL). Up to date, Leishmaniasis cases in Turkey have usually occurred in Eastern Anatolia, Central Anatolia, Aegean, Mediterranean regions[3]. VL is generally known to affect the internal organs especially the spleen, liver and bone marrow resulting in high fever,weight loss, anemia, leukopenia, thrombocytopenia, splenomegaly and hepatomegaly[4]. VL is endemic in over 88 countries in tropical and subtropical regions. Annually, 90% of the 500.000 VL cases are reported in Nepal, Bangladesh, India, Sudan, Brazil, especially in the male sex[5].

Leishmania is not only transmitted by the bite of infected female sandflies, but also by congenital, blood transfusion, organ transplantation, laboratory accidents and needle-sharing in drug users[6,7]. The prevalance of asymptomatic carriers of infection is a significant factor in en-

demic regions. Although Turkey is endemic for leishmaniasis, the number of studies performed in asymptomatic population appears highly poor[8,9]. Blood donors can be considered as a crucial group in these asymptomatic population. Besides, in our region, no studies have been carried out in blood donors so far. Based on the above rationale, the main objective of the current study was to investigate the prevalance of *Leishmania* spp. in blood donors by employing serological and molecular methods in Mersin province.

MATERIALS and METHODS

Collection of Blood Samples

In total, 509 healthy blood donors without a history of VL were included into the study. Blood samples were collected from volunteers in a 12-month period between June 2016 and June 2017 at Mersin University Health Research and Application Center. Blood samples (5 mL) were collected from the donors into EDTA-coated tubes. According to the permission of Mersin University clinical research ethics committee (ethics comittee number: 2016/141), all donors were informed regarding the routine procedures for sampling. Questionnaires were prepared to evaluate the risk of exposure to *Leishmania* spp. infection. Each questionnaire was composed of 12 questions including the donors' age, sex, living area, occupation, working hours, working place, dog or cat ownership and awareness about Leishmaniasis.

Statistical Analysis

Power analysis was performed. The statistical analysis was determined as the ratio comparison. In the study, alpha= 0.05, the power of the test= 0.80 and the minimum sample size were calculated as 500.

ELISA Method

Sera of the collected blood samples were separated by centrifugation at 5000 rpm for 5 min and stored at -20°C until use. Thereafter, sera samples were utilized with NovaLisa *L. infantum* IgG, ELISA Kit (HandelsgmbH & Co KG, Austria) according to manufacturers' procedures. Specificity and sensitivity of the test were defined as 85-91%, respectively. The cutt off value was determined as 0.150-0.130.

DNA Isolation

Genomic DNA of whole blood specimens were extracted by using High Template Preparation Kit (Roche, Germany). DNA concentration was measured spectrophotometrically by CapitalBio NanoQ drop (China). All isolated DNA samples were stored at -20°C until use.

Real-time Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed for the detection of *Leishmania* spp. Primary and probe designs were used as previously[10]. 15 pmol F- 5'- CTTTTCTGGTCC TCCGGGTAGG-3', R- 5'- CCACCCGGCCCTATTTTAC ACCAA- 3' and 50 pmol probe FAM-5'- TTTTCGCAGAACGCCCCTACCCGC-3'- TAMRA sequences were used[10]. *L. major* (KM555292), *L. tropica* (MH511156), *L. donovani* (KU899560) and *L. infantum* (KC536649) are recognized by these primer sequences. A single-stage real-time PCR was performed in the study. PCR mix containing 10 µL TaqMan Universal Master Mix II (Applied Biosystem, USA), 1 µL primer/prob (10 pmol RV1-F, RV1-R and 0.5 pmol RV- P), 4 µL RNAse/DNAse free distilled water were used for the master mix. 5 µL isolated DNA sample added to the mix. VIIA7 device (Applied Biosystem, USA) was used for the real-time PCR assay. RT-PCR reaction was performed along with the positive and negative controls. Molecular grade water (Thermoscientific, Lithuania) was used as a negative control. *L. infantum* (MHOM/TR/19/BSK-INF) and *L. tropica* (MHOM/TR/19/BSK-TRO) strains were used as positive controls. Beta-globin gene was used as an endogenous control to normalize initial DNA concentrations to confirm sample integrity. Beta-globin gene was used as an internal positive control in RT-PCR analysis. The beta-globin gene primers/probe were as described previously[11]. Primers and probes consisted of beta-globin-F-5′-GTCACCTGACTCCTGAGGAGA-3′; beta-globin-R-5′-CCTTGATACCAACCTGCCCAG-3′; and probe′; beta-globin-5′-(FAM)-AAGGTGAACGTGGATGAAGTTGGTGG-(TAMRA)-3′. Identical thermal profiles were performed for both *Leishmania* spp. and beta-globin. Initial denaturation 94°C for 10 minutes followed by 40 cycles of denaturation at 94°C for 15 seconds and exten-

sion at 60°C for 1 minute. PCR mix containing 10 µL TaqMan Universal Master Mix II (Applied Biosystem, USA), 1 µL primer/prob (10 pmol RV1-F, RV1-R and 0.5 pmol RV-P), 4 µL RNAse/DNAse free distilled water were used for the master mix. 5 µL isolated DNA sample added to the mix. VIIA7 device (Applied Biosystem, USA) was used for the real-time PCR assay.

RESULTS

Study population was composed of 509 donors (24 females and 485 males) with an avarage age of 34. Collected from the questionares', living area of the donors was distributed as follows: in the city center (n= 367), district (n= 121), town (n= 7) and village (n= 14). Among all, a vetenerinarian and a sailor as well as 17 farmer donors were classified in the risky occupational group (Table 1). Among pet (41 dogs or 20 cats) owner donors, only 3 (dog) or 10 (cat) of them were feeding their pets at home. Additionally, 10 of the donors were listed as having fed cat in the past. Results of the questionnaire were summarized in Table 1. Determined via ELISA application all the donors were serologically negative, while there was no detectable *Leishmania* spp. at the blood donors tested by RT-PCR.

DISCUSSION

Climate change and migration have increased the incidence of rare and neglected diseases in recent years. In particular, leishmaniasis has started to be reported in non-endemic areas due to the migration from endemic regions to non-endemic regions through international travel[12-15]. The parasitic concentration in asymptomatic VL carriers has been reported to be quite low with the absence of clinical and hematological alternations that masks the disease[16]. However, the presence of antibodies against parasites do not always indicate active infection. Likely, antibodies can be a sign for risk of parasitic encounter[17].

Table 1. Questionnaire results

Questionnaire questions	%
Sex	5 females 95 males
Location	72 city center 24 district 1.3 town 2.7 village
Risky occupation	3.3 farmer 0.1 sailor 0.1 veterinarian 96.5 others
Work place	46 office 4.5 farm 4.2 hospital 55 construction, factory and shopping center
Information about leishmania	4.3 yes 95.7 no
Dog owners	0.59 in house 7.5 outside the house
Not have a dog	89.9
Had a dog	1.9
Cat owners	2.4
Not have a cat	95.7
Had a cat	1.9

It has been declared that the parasite can survive until 15 days in red blood cells depending on the storage conditions in blood banks[18]. In recent years, various techniques have been used for pathogen inactivation during blood components preparation process. In our study, neither inactivation nor filtration processes were utilized when processing the samples. This situation can easily create a risk for blood transfusion process especially in endemic regions. Therefore, the detection of leishmaniasis during transfusion process is a vital matter. There are several gold standard methods used in the diagnosis of active *Leishmania* infection but, none of these methods are often practical nor cost effective for epidemiological studies[17]. In the recent years, fortunately, nucleic acid-based amplification methods have paved the way to detect *Leishmania* spp. without invasive methods[19]. Additionaly, serological methods are also being used to detect Leishmaniasis in asymptomatic carriers as well as molecular methods[20-22]. There are various studies conducted with serological methods to investigate *Leishmania* spp. in blood donors. In one of the studies, *L. infantum* has been reported to be positive in 8% of the Spanish blood donors by immunofluorescence assay (IFA)[20]. *L. braziliensis* has been found to be 11.4% in Brazilian blood donors with an other technique, enzyme immunoassay (EIA)[21]. *Leishmania* IgG seropositivity was determined as 5% in Nepal at blood donors[22,23]. In Turkey, on the other hand, VL is mostly seen in the Aegean and Mediterranean regions. Besides, there are certain reported cases from other regions as well[24-28]. Until now, Ateş et al. is the only group that investigated leishmaniasis at blood donors in Turkey[8,9]. Revealed by two methods, Ateş et al. have detected anti-leishmanial antibodies 6.4% by IFAT and 0.5% by ELISA[8]. In another study from 2013, the same investigators determined 2.6% and 2% positive anti-leishmanial antibodies by ELISA and IFAT methods respectively in 343 sera samples of donors[9]. The sensitivity ratios of these methods were graded as follows: microculture method (MCM) 71%, smear 19%, IFAT 33%, ELISA (42%), traditional culture (NNN) 4%, PCR (14%) and immunochromatographic test (ICT) 4%. Worldwide, *Leishmania* spp. rate is declared as 4-8.7% in blood donors[29]. Interestingly, based on annual reports, infected blood donors are listed more often from Brasil, Nepal, Bangladesh and India rather than the rest[30]. Blood donations by immigrants from endemic areas is a vital issue in transfusion safety. Indeed, the Mersin province is endemic for leishmaniasis and hosts approximately 206.000 refugees (11.5% of the population) who escaped from the civil war in Syria[31].

Due to the migration from *Leishmania* endemic of Turkey, it was declared that 1.578 (3.43%) CL cases were detected in Mersin between 1990-2010[32]. Although the Mediterranean region is endemic for Leishmaniasis, no studies have been conducted yet on *Leishmania* spp. issue in blood donors. In this study, 509 donors were evaluated for asymptomatic *Leishmania* spp. carriage by serologic and molecular methods. As a result of study, all of the donors were detected seronegative for *Leishmania* spp. However, DNA samples of the donors were determined negative by RT-PCR. Negative results are considered to be related to the environment and working conditions of the donor population. The number of donors engaged in farming was 3.3% and most of the donors worked in the office (46%) and lived in the city center (72%). Therefore, the risk for these donors to encounter with the vector decreases compared to those living in rural areas. Previous studies have reported that stray dogs and cats were risk factors for transmission of *leishmania* infection to humans[33,34]. *Leishmania* spp. infection in domestic cats and dogs can be rare or under-reported in endemic areas. Dog ownership is an important factor for *Leishmania* spp. carriage. As a result of the survey in this study, only 0.59% of the donors were caring dogs in the house and 7.5% of them were caring outside, 1.9% of donors had dog care in the past and 89% of the donors did not own any dogs. Other than dog ownership, domestic and stray cats are also reported as risk factors to possess *Leishmania* infection[35,36]. Mondolfi et al. have reported *L. mexicana* in both cats and dogs in their study[37]. Coura et al. have collected 100 feline serum samples and found positive *Leishmania* antibodies in 54 sera samples

by IFAT[38]. Leishmania infection was first reported in cats of Turkey by Can et al. Researchers detected seropositive rates 10.8% and 15.2% by ELISA and IFA methods[35]. Also, *L. tropica* was determined in six cats and *L. infantum* was detected in one cat in that study. In another study conducted in Turkey, Pasa et al. have reported 8.84% *Leishmania* spp. positivity by PCR in domestic cats. They have found *L. major* and *L. tropica* infections in domestic cats[36]. In our study, 2.4% donors were feeding cats at home and 1.9% donors had a cat in the past. 95.7% the donors had no cats. Low rates of dog ownership and cat ownership are considered as other factors for negative results in this study.

Finally, asymptomatic blood donors from endemic areas have been reported from various countries but there are still limited studies performed in blood donors for leishmaniasis detection. The results obtained from this study are the first ever reported for our region. Until now, there isn't any conducted research in the Mediterranean region. Thus, we suggest that it is essential to perform new investigations in nearby provinces, especially bearing wider populations. However, the risk for transmission of VL through blood transfusion is ignored in this research. It is not suggested to set up routine screening for *Leishmania* spp. infection in blood transfusion departments located in Mersin province.

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CONFLICT of INTEREST

Authors have no competing interests to disclose.

AUTHORSHIP CONTRIBUTIONS

Concept/Design: GB

Analysis/Interpretation: GB, ED, SEE

Data Acquisition: GB, ENT

Writting: GB, ED

Final Approval: GB

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