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A survey study on hantavirus, cowpox virus, and *Leptospira* infections in *Microtus hartingi* in Kırşehir Province, Central Anatolia, Turkey

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Abstract: The aim of this study was to investigate whether hantaviruses, cowpox viruses, or *Leptospira* infections were prevalent in *Microtus hartingi* trapped in Kırşehir Province, located in Central Anatolia, Turkey. *Leptospira* spp. was detected in 20 of the 43 (46.5%) *Microtus hartingi* kidney samples and confirmed by nested polymerase chain reaction (PCR) of the 16S ribosomal RNA gene. Five of the 20 *Leptospira* spp. PCR positive amplicons were sequenced, analyzed, and confirmed as *Leptospira* spp. This is the first study to genetically characterize *Leptospira* in *Microtus hartingi* in Turkey. There was no evidence of cowpox viruses or hantaviruses in *Microtus hartingi*. Therefore, additional studies are needed.

Key words: *Microtus hartingi*, hantavirus, cowpox virus, *Leptospira* spp., genetic characterization

1. Introduction

A worldwide distributed zoonotic disease, leptospirosis, is caused by spirochetes of the genus *Leptospira* (1) and has been recognized as a global public health concern in recent years. Annually, there are tens of millions of human cases worldwide, with fatality rates around 25% in some regions (2). Contamination of water, soil, grains, and crops with urine from either domestic or wild animals with persistent renal infections of *Leptospira* is the main source of infection for humans, while direct or indirect contact with a veterinarian or a wildlife scientist also accounted for human deaths (3–6).

Leptospira, together with the genera *Leptonema* and *Turneria*, is a member of the family *Leptospiraceae*. The genus *Leptospira* is divided into 20 species based on DNA hybridization studies (7–9). More recently, molecular methods were described that divide the genus *Leptospira* into several species on the basis of DNA relatedness. One such gene used for genetic characterization of *Leptospira* was 16S ribosomal RNA (2). Nested polymerase chain reaction (PCR) was used to determine the presence of nonpathogenic and/or pathogenic *Leptospira* in wild and domestic animals (7,10,11).

The first evidence for *Leptospira* in Turkey was reported in 1922 (12) and seropositivity rates of cattle in the northern and eastern Anatolia provinces Erzurum and Kars were reported as 12% and 30%, respectively (13). In another study, 8% seropositivity was found in 15,596 samples collected from 74 provinces. While 82% of the serotypes were identified as *L. interrogans* serovar Hardjo, the remaining 18% were *L. interrogans* serovar Grippotyphosa (14). Seropositivity was found by microscopic agglutination test (MAT) in 8 (2.03%) of 395 cattle in Elazığ, located in eastern Anatolia (15). Another study conducted in Kayseri, located in Central Anatolia, neighboring Kırşehir, found seropositivity in 609 (25.42%) and 433 (18.07%) of 2395 cattle serum samples by MAT and enzyme-linked immunosorbent assay (ELISA), respectively (16). Leptospirosis has often been implicated in rice harvesting and one study to evaluate the prevalence of *Leptospira* spp. in wild rats in Turkey reported a high prevalence of *L. interrogans* carriage in wild rats of our region (17). There are many studies on leptospirosis in animals in Turkey; however, studies on incidence and prevalence of leptospirosis in humans are limited though there are frequent publications on case series and sporadic

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case presentations (18). Furthermore, leptospirosis has never been reported in Kırşehir.

Hantaviruses are rodent-borne, enveloped RNA viruses with a diameter of 120 nm, belonging to the family *Bunyaviridae* (19). Each hantavirus is carried by a specific rodent or insectivore species and transmission to other species including humans is a "dead end" for the virus (20–22). Hantavirus infections were confirmed by indirect fluorescent-antibody (IFA) and immunoblotting in the 12 cases of hemorrhagic fever with renal syndrome (HFRS) reported in the Black Sea region of Turkey in 2009 (10,23). Cowpox viruses are large, enveloped, linear double-stranded DNA viruses, and are classified in the family *Poxviridae* (24). The cowpox virus has been isolated in many countries including Russia, Turkmenistan, northern Italy, and France. In Russia, the cowpox virus was isolated from laboratory rats, while it is endemic in European wild rodents (25,26). Laakkonen et al. reported the first serologic survey for rodent-borne viruses in their natural hosts in Turkey and found one (0.3%) of the rodents, an *Apodemus sylvaticus* from Trabzon Province, was seropositive for cowpox (10). No research was conducted on hantavirus infections seen in Kırşehir, a city located in Central Anatolia, Turkey.

In Kırşehir, people were admitted to hospitals with fever, myalgia, and headache, some with masses on their necks. Some of the patients were diagnosed with tularemia and clinical characteristics of the disease were discussed with the review of the literature. Mice around the location of the epidemic were implicated as the source of the infection and thus trapped and analyzed for the presence of *Francisella tularensis.* No evidence of the pathogen was found (27). Therefore, we focused on other infectious agents that may be transmitted from mice to humans and aimed to investigate the prevalence and genetic characteristics of *Leptospira*, hantaviruses, and cowpox viruses in *Microtus hartingi.* For this purpose, nested PCR was used for detection of different pathogens and the results were validated by DNA sequencing.

2. Materials and methods

2.1 Study area and sample collection

The province of Kırşehir, with an area of 6665 km^2 and located in the center of Anatolia, has a humid temperate climate with plenty of annual rainfall. Barley and wheat farming, river fishing, and cattle and/or sheep husbandry are the main activities in the region. The coordinates of this location are 38°50′–39°50′ north latitudes and 33°30′–34°50′ east longitudes. Residents use water from wells or from the local streams for their daily needs (cooking, bathing, and washing clothes). There is no sewage system in some of the houses and the residents reported observing field rats in the grain storage or around their houses frequently. Forty-three animals were trapped alive from the fields in and around Kırşehir. The sampling method has been described previously. Conventional stained chromosomes of specimens were karyotyped based on the colchicine hypotonic citrate technique according to Ford, Hamerton, and Patton (27–29). There is no information about how many mice might live in this area. The study was carried out in a common study with Turkish Refik Saydam National Public Health Agency, Ankara, Turkey (27) and approved by the Local Ethical Committee of Kırıkkale University (25/09/2009). The kidneys were rinsed with sterile Dulbecco's modified Eagle's medium (DMEM) and stored at –20 °C until analysis.

2.2 DNA and RNA isolation and nested PCR

DNA was extracted from the kidneys using a spin column system (DNeasy Blood and Tissue Kit, Qiagen, Germany) according to the manufacturer's instructions. First of all, successful isolation of DNA was controlled with PCR, by using primers microtus 1 and microtus 2, which amplified a 305-bp region within the *Microtus* cytochrome b gene (Table). The first round of nested PCR was carried out in a total volume of 50 µL to detect *Leptospira* spp. in field samples. Reaction mixes contained 5 µL of the template DNA, 2 U Taq DNA polymerase (Promega, USA), 5 pmol of each lepto 1F and lepto 1R primers (which were directed at the 16S ribosomal RNA gene of the *Leptospira* genome in order to amplify all *Leptospira* spp.), 200 mM dNTPs (Promega, USA), 10 mM Tris-HCl, 50 mM KCl, and 1.5 mM $MgCl_2$ (Promega, USA). The cycling conditions were 95 °C for 2 min; 32 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 10 min. In an attempt to increase the sensitivity of PCR, the second round of nested PCR was carried out using primer pairs nest 1 and nest 2, which amplified a 289-bp region within the 525-bp sequence of the 16S ribosomal RNA gene. Reaction conditions for the second round of nested PCR reactions were as follows: 95 °C for 5 min, followed by 28 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, and finally a cycle of 72 °C for 10 min.

To investigate hantaviruses in the samples, RNA was extracted from the kidneys using a spin column system (QIAamp Viral RNA Mini Kit, Qiagen, Germany) according to the manufacturer's instructions. RNA samples were treated with RNase-free DNase (Qiagen, Germany). Random primers (1.25 mM random primers**;** Promega, Madison, WI, USA) were used in the first step of cDNA synthesis. For this purpose, 10 µL of the total RNA was combined with 1 µL (0.5 µg) of random primers and preheated at 70 °C for 10 min to denature secondary structures. The mixture was cooled rapidly, and 2 µL (100 mM) of dNTPs, 5 µL of 5X RT buffer, 1 µL of M-MLV reverse transcriptase (200 IU/µL Promega, USA), and 8 µL of H_2O were added to a final volume of 25 µL. The RT reaction mixes were incubated at 37 °C for 60 min and

Table. Primers used for PCR and nested PCR in this study.

Primer name	Sequences	Amplicon size	Source of primers
microtus 1	5'-CCTGCACGTAGGACGAGGGGT-3'	305-bp	This study
microtus 2	5'-AGGTGGACTAATACGAGGGCGGT-3'	305-bp	This study
lepto 1F	5'-GGCGGCGCGTCTTAAACATG-3'	525-bp	Djadid et al. 2009 (7)
lepto 1R	5'-GTCCGCCTACGCACCCTTTACG-3'	525-bp	Djadid et al. 2009 (7)
nest 1	5'-CAAGTCAAGCGGAGTAGCAA-3'	289-bp	Mérien et al. 1992 (11)
nest 2	5'-CTTAACCTGCTGCCTCCCGTA-3'	289-bp	Mérien et al. 1992 (11)
VTK1	5'-ATGAACGGCGGACATATTCAGTTG-3'	528-bp	Thomas et al. 1990 (65)
VTK2	5'- TTATGAGTCGATGTAACACTTTCT -3'	528-bp	Thomas et al. 1990 (65)
NTK1	5'- ATAGCTCAATATAAATGCGTGAC-3'	253-bp	Chantrey, et al. 1999 (25)
NTK2	5'-GCATTTCATACACACAGCAGTTA -3'	253-bp	Chantrey, et al. 1999 (25)
MGP1	5'- AAAGTAGGTGITAYATCYTIACAATGTGG -3'	445-bp	Li et al. 2007 (21)
MGP2	5'- GTACAICCTGTRCCIACCCC -3'	445-bp	Li et al. 2007 (21)
HP1	5'- GAATCGATACTGTGGGCTGCAAGTGC -3'	360-bp	Li et al. 2007 (21)
HP2	5'- GGATTAGAACCCCAGCTCGTCTC -3'	360-bp	Li et al. 2007 (21)

synthesis was ended by heating at 94 °C for 5 min. Initial RT-PCR reaction was carried out in a total volume of 50 µL. The reaction mixes contained 5 µL of the generated cDNA, 2 U of Taq DNA polymerase (Promega, USA), 10 pmol of HP1 and HP2, 200 mM dNTPs (Promega, USA), 10 mM Tris-HCl, 50 mM KCl, and 1.5 mM $MgCl_2$ (Promega, USA). The cycling conditions were 95 °C for 2 min; 32 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 10 min. Nested RT-PCR reaction was carried out in a total volume of 50 µL. The reaction mixes contained 5 µL of the template DNA, 2 U of Taq DNA polymerase (Promega, USA), 10 pmol of MGP1 and MGP2, 200 mM dNTPs (Promega, USA), 10 mM Tris-HCl, 50 mM KCl, and 1.5 mM $MgCl_2$ (Promega, USA). The cycling conditions were 95 °C for 2 min; 32 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 10 min.

To detect cowpox virus in samples, the first round of nested PCR was carried out in a total volume of 50 µL and the reaction mixes contained 5 µL of the template DNA, 2 U of Taq DNA polymerase (Promega, USA), 5 pmol of each VTK1 and VTK2 primers, 200 mM dNTPs (Promega, USA), 10 mM Tris-HCl, 50 mM KCl, and 1.5 mM $MgCl_2$ (Promega, USA). The cycling conditions were 95 °C for 2 min; 32 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 10 min. In an attempt to increase the sensitivity of PCR, the second round of nested PCR was carried out using the primers NTK1 and NTK2. Reaction conditions of the second round of nested PCR reactions were as follows; 95 °C for 5 min, followed by 28 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s and finally a cycle of 72 °C for 10 min.

All PCR reactions were carried out in a BO-PCR-5 thermal cycler (Hamburg, Germany). InGenius LHR (Syngene, Cambridge, UK) was used to image PCR amplicons by using ethidium bromide stained 1.5% agarose gel electrophoresis. All primers used in this study are shown in the Table.

2.3 Nucleotide sequence analysis and accession numbers

Amplified fragments were purified from gel agarose using a Gene Clean III Kit (MP Biomedicals Europe, France) following the manufacturer's instructions. Direct sequencing of the DNA fragments was performed using nest 2 primer for each PCR product in a commercial company (Iontek, İstanbul, Turkey). Gene sequences were compared by MEGA 4.1 Beta and ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2) analyzing software. The phylogenetic tree was generated by a neighbor joining method using MEGA software (version 4.1 Beta), which showed a geographic clustering of the sequences of *Leptospira* spp. The nucleotide sequences were deposited in the GenBank database under the accession numbers HM536963, HM536964, HM536965, HM536966, and HM536967.

3. Results

Rodents trapped in Kırşehir Province were identified as *Microtus hartingi* when conventionally stained chromosomes of specimens were karyotyped. Observation of expected amplicon size of 305 bp, with primers microtus 1 and microtus 2, confirmed *Microtus* spp. and success in isolation of the DNA (Figure 1A).

Leptospira was detected in 20 of the 43 (46.5%) *Microtus hartingi* kidney samples by observation of expected amplicon size of 270 bp (Figure 1B). All of the *Leptospira* positive samples were obtained from a region where humans had shown leptospirosis clinical signs.

To determine the genetic types of circulating *Leptospira* in Central Anatolia, in the present study 5 of the 20 positive samples were randomly selected and subjected to sequencing. The identity of the PCR products was also confirmed by sequencing and alignment revealed the presence of *Leptospira* spp. Approximately 270 bp sequences were obtained and used for basic local alignment search tool (BLAST) alignment. All new sequenced data were deposited in GenBank under accession numbers HM536963-67. Analysis of the 5 PCR positive sequences revealed a close relatedness to some uncultured *Leptospira* spp., *L. interrogans* serovar, and *L. kirschneri* serovar (Figure 2). Comparison of the 16S rRNA regions sequences HM536963-67 with some *Leptospira* sequences that are available from GenBank and generated phylogenetic tree are shown in Figure 2.

Positive signals for hantaviruses or cowpox viruses were not detected in any of the *Microtus hartingi* kidney samples analyzed with nested RT-PCR and PCR assays, respectively (data not shown).

4. Discussion

The aim of veterinary and medical disciplines is to control, prevent, and eradicate diseases in animal and human populations. Early and accurate diagnosis therefore has a key role for developing effective strategies on this purpose. Another important point is the determination of contributing factors to the emergence of diseases, which may be of help for the development of large-scaled epidemiological surveys (30). Leptospirosis, showing mortality rate up to 25% in some outbreaks, is a worldwide tropical zoonotic disease that remains neglected due to the difficulty in diagnosis. The seroprevalence of leptospirosis in humans is reported to range from 2% to 12% in Turkey (18). Most of the wild and domestic animals that are infected with leptospirosis are potential disseminators of the disease to other animal species as well as to humans (9,31,32). Here we report the detection of *Leptospira* in *Microtus hartingi* for the first time by using nested PCR, thus providing an important measure of human risk for acquiring leptospirosis. Furthermore, *Leptospira* spp. carriage of *Microtus hartingi* was confirmed by DNA sequencing of the 16S rRNA gene (Figures 1 and 2).

Previously, the samples obtained from Kırşehir were thought to belong to *Microtus socialis* (27). However, additional morphometric and karyological data of the specimens from Kırşehir were compared with those of specimens of *Microtus guentheri* and the Kırşehir specimens were determined to belong to *Microtus hartingi.* Distribution records of *Microtus hartingi* were found to be compatible with the previous reports (33–35). This is the first report on the presence of *Microtus hartingi* in this region and 46.5% of the analyzed animals were shown to be carriers of leptospirosis.

Initial case reports identified a hantavirus epidemic in February 2009 that involved 12 people in the cities of Bartın and Zonguldak near the Black Sea in the northwest of Turkey not far from Kırşehir Province (23). Laakkonen et al. (10) were the first to report cowpox and hantaviruses infections in wild rodents from the Black Sea region of Turkey, but they found cowpox only in one of *Apodemus*

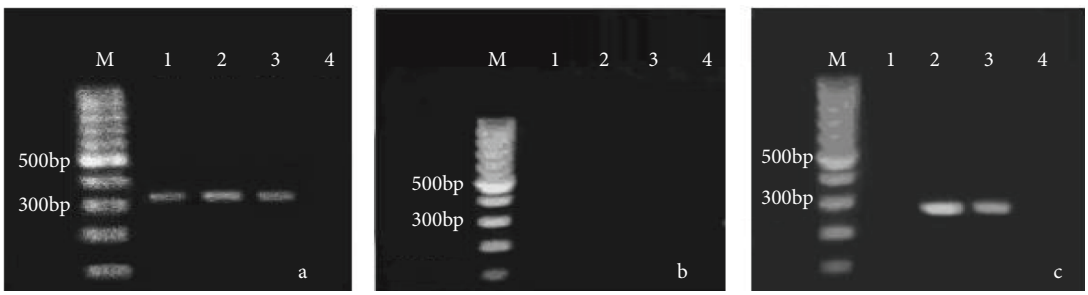

Figure 1. Nested PCR was carried out on the kidneys of *Microtus hartingi*. Gel electrophoresis analysis of PCR amplification of *Leptospira* spp. in *Microtus hartingi*. **A.** Control of DNA isolation PCR was done on *Microtus* spp. cytochrome b gene in the kidney DNA samples. Lane 1 (negative sample), lane 2 (positive sample), lane 3 (positive sample), and lane 4 (dH_2O); lane M: DNA molecular length marker. (Shown: all samples from *Microtus* spp.). **B.** Detection of *Leptospira* spp. agarose gel electrophoresis of first round nested PCR analysis products from the kidney DNA samples. Lane 1 (negative sample), lane 2 (positive sample), lane 3 (positive sample), and lane 4 (dH_2O); lanes M: DNA molecular length marker. **C.** Detection of an increase in the sensitivity of PCR for *Leptospira*. Agarose gel electrophoresis of nested-PCR analysis products from the kidney DNA samples is shown. Lane 1 (negative sample), lane 2 (positive sample), lane 3 (positive sample), and lane 4 (dH_2O); lanes M: DNA molecular length marker.

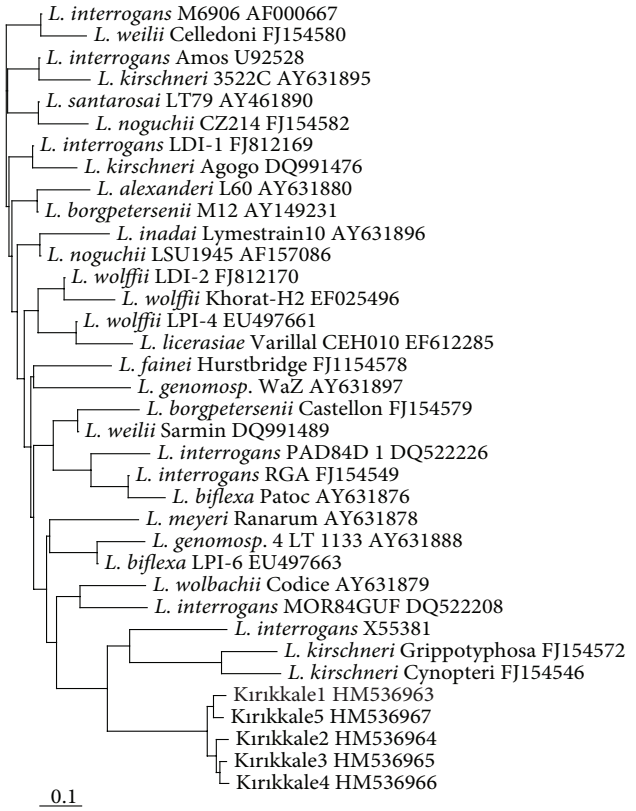

Figure 2. The Phylogenetic tree for 16S ribosomal RNA gene of the *Leptospira* was inferred using ClustalW software. **Pathogenic *Leptospira***: *L. interrogans* serovar Canicola (X55381), *L. santarosai* strain LT79 (AY461890.1), *L. noguchii* strain LSU1945 (AF157086.1), *L. noguchii* serovar CZ 214 (FJ154582.1), *L. interrogans* Amos (U92528.1), *L. interrogans* LDI-1 (FJ812169.1), *L. interrogans* MOR84GUF (DQ522208.1), *L. interrogans* M6906 (AF000667.1), *L. interrogans* PAD84D 1 (DQ522226.1), *L. kirschneri* serovar Agogo (DQ991476.1), *L. kirschneri* 3522C (AY631895.1), *L. kirschneri* serovar Cynopteri (FJ154546), *L. kirschneri* serovar Grippotyphosa (FJ154572), *L. borgpetersenii* strain M12 (AY149231.1), *L. borgpetersenii* serovar Castellon (FJ154579.1), *L. weilii* serovar Celledoni (FJ154580.1), *L. weilii* serovar Sarmin (DQ991489.1), *L. interrogans* serovar Icterohaemorrhagiae strain RGA (FJ154549.1), and *L. alexanderi* serovar L60 (AY631880.1). **Saprophytic 'free-living' *Leptospira*:** *L. biflexa* serovar Patoc (AY631876.1), *L. biflexa* strain LPI-6 (EU497663.1), *L. meyeri* serovar Ranarum (AY631878.1), *L. wolbachii* serovar Codice (AY631879.1), *L.* genomosp. 3 serovar WAZ (AY631897.1), and *L.* genomosp. 4 LT 1133 (AY631888). **Intermediately pathogenic *Leptospira*:** *L. wolffii* strain LDI-2 (FJ812170.1), *L. wolffii* strain LPI-4 (EU497661.1), *L. wolffii* serovar Khorat H2(EF025496.1), *L. inadai* serovar Lyme strain 10 (AY631896.1), *L. fainei* serovar Hurstbridge (FJ154578.1), and *L. licerasiae* serovar Varillal CEH010 (EF612285.1). **Leptospiral sequences from *Microtus hartingi* kidney samples in this study:** *L. interrogans* serovar Kırıkkale 1 (HM536963), *L. interrogans* serovar Kırıkkale 2 (HM536964), *L. interrogans* serovar Kırıkkale 3 (HM536965), *L. interrogans* serovar Kırıkkale 4 (HM536966), and *L. interrogans* serovar Kırıkkale 5 (HM536967).

sylvaticus, *Microtus roberty*, *Microtus rossiaemeridionalis*, and *Microtus guentheri lydius* from a total of 330 various wild rodent samples analyzed. They also found the hantavirus in the Black Sea region. They did not, however, investigate these viruses in *Microtus hartingi*. Hantavirus and cowpox virus were not detected from *Microtus hartingi* samples analyzed by nested PCR and RT-PCR assays (data not shown). Laakkonen et al. (10) analyzed more diverse rodent species than those used in the present study and this might explain why we failed to detect any positivity for hantavirus or cowpox virus in *Microtus hartingi*. Nevertheless, this study is the first to investigate hantavirus and cowpox virus in *Microtus hartingi*.

The province of Kırşehir has a humid temperate climate with plenty of annual rainfall. The sampling area lacks a sewage system and the residents use water for their daily needs either from wells or from local streams. After admittance of residents from the sampling areas to hospitals with fever, myalgia, headache, and some with masses on their necks (personal communication, Dr Sedat Kaygusuz), an investigation was conducted in the areas of the epidemic. We were informed by some villagers that rats are found frequently in the grain storages or around their houses. Once, they observed a dead rat in the water source due to a break in the water pipeline. Furthermore, they also observed rats around the location of epidemic.

Therefore, a total of 43 animals were trapped alive from fields in and around Kırşehir. Leptospirosis in humans is transmitted either by direct contact with infected animals or indirectly with exposure to materials contaminated with the urine of infected animals. Animal carriers may shed leptospires from months to years in their urine or even for extended periods which can be the entire lifespan of the animal. Many *Leptospira* strains appear to be well adapted to their natural hosts and in general they cause infection without clinical manifestations in the animals. Rodents have been recognized to be the most important and widely distributed reservoirs of leptospiral infection (36–40). To date, *Microtus oeconomus, Mus musculus, Microtus arvalis, Apodemus agrarius, Apodemus sylvaticus*, and *Apodemus flavicollis* have been reported to harbor leptospires in their kidneys. *Leptospira* spp. were also obtained from the kidneys of domestic rats (*Rattus norvegicus* and *Rattus rattus*), a spiny rat (*Proechimys* sp.), an opossum (*Didelphis marsupialis*), and 2 other marsupials along with four-eyed opossums (*Philander andersoni* and *Philander opossum*) from 3 different habitats (9,31,36–41). The present study demonstrated, for the first time, that *Microtus hartingi* can also be a reservoir of *Leptospira* spp. in Central Anatolia (Figures 1 and 2).

Sunbul et al. (42) reported that 59 *Rattus norvegicus* rats that were trapped from the Black Sea region of Turkey were serving as a reservoir of *L. interrogans* as determined by PCR in sera, kidney, and brain tissues. They reported that 16 kidney samples (27.1%) and 10 brain tissue samples (16.9%) were positive for *L. interrogans.* Stanko et al. (39) found that 5 rodent species (*Apodemus flavicollis*, *A. agrarius*, *A. microps*, *Clethrionomys glareolus*, and *Microtus arvalis*) were positive for antibodies against *Leptospira* spp. O'Guinn et al. (43) found leptospirosis (1.3%) in *A. agrarius* while Gamage et al. (44) reported positivity in 13 of 74 (17.5%) rodent kidney samples, which were identified as *L. interrogans*. Our study showed a higher percentage (46.5%) than previous reports, which highlights the risk of acquiring leptospirosis in animals and humans that are exposed to *Microtus hartingi* urine in their daily life.

Leptospirosis caused by pathogenic *Leptospira* species results in significant economic losses in domestic animals. The seroprevalence of leptospirosis in cattle has been reported to be 10.4% to 42.8% in Spain (45,46), 23.3% in Portugal (47), 15.8% to 21.18% in India (48), 3% in Germany (49), 34.4% in Great Britain (50), 46.9% in Brazil (51), 62.8% in Mexico (52), and 27% in Iran (53). Among these studies, the most prevalent serovars identified were *L. interrogans* serovar Hardjo and *L. kirchneri* serovar Grippotyphosa. Studies carried out in Turkey showed that the seroprevalence of leptospirosis in cattle varies from 0.85% to 33.63% (13,15,16,54–56). In a national survey, the seroprevalence of leptospirosis in cattle was found to be 8.04% (57). Both in local studies and in the national survey, *L. interrogans* serovar Hardjo and *L. kirchneri* serovar Grippotyphosa were the most prevalent serovars, which could be attributed to the fact that cattle may have close contact with the reservoirs of these serovars. However, we did not evaluate the prevalence of leptospirosis in cattle of this region and there are no previous reports on this issue.

The secondary aim of this study was to perform PCR based on specific target sequences of the 16S rRNA gene (7,58,59). The randomly selected 5 of the 20 PCR positive samples were subjected to sequencing for analysis of genotypes. Although 16S rRNA gene sequencing cannot identify *Leptospira* at the species level, gene sequencing is more suitable than restriction fragment length polymorphism (RFLP) as it is a more labor force demanding and time-consuming technique. Nevertheless, the 16S rRNA gene proved to be a good molecular epidemiological marker for defining infected species in both humans and animals suspected of having leptospirosis (7,11,60).

Phylogenetic analysis of *Leptospira* revealed 3 classes, representing species that contain both pathogenic and nonpathogenic serovars as well as an intermediate group (61). These observations suggested an important biological difference in virulence between pathogenic and intermediate *Leptospira*. DNA sequencing has several advantages over other gene typing methods, since it is cheaper and more easily available. 16S rRNA gene sequencing has long been preferred as a typing method for molecular characterization of isolates and taxonomical applications. However, although 16S rRNA gene sequencing is a useful technique, it is not sufficient for discrimination of *Leptospira* strains at a species level due to the highly conserved nature of the gene. Vaccines based on recombinant poxviruses have proved successful in controlling diseases such as rabies. That kind of strategy will be useful for the prevention and control of leptospirosis (62–65). It was found that 16S rRNA gene sequences matched either their equivalent entries in GenBank or the sequences from the corresponding strains of *L. interrogans* serovar and *L. kirschneri* serovar (Figure 2). Further work is needed to evaluate these alternative strategies that are applied on quality-control testing of *Leptospira* reference strains.

In conclusion, we showed that *Microtus hartingi* may play a significant role in the transmission of *Leptospira* to humans and domestic animals. In order to control leptospirosis, improvement of environmental hygiene, control of rodents, and vaccination against *Leptospira* must be applied.

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