Am. J. Trop. Med. Hyg., 92(4), 2015, pp. 690–697
doi:10.4269/ajtmh.14-0322
Copyright © 2015 by The American Society of Tropical Medicine and Hygiene

Potential Animal Reservoirs of Toscana Virus and Coinfections with *Leishmania infantum* in Turkey

Ender Dincer, Sepandar Gargari, Aykut Ozkul, and Koray Ergunay*

Mersin University Advanced Technology Education, Research and Application Center, Mersin, Turkey; Ankara University Faculty of Veterinary Medicine, Department of Virology, Ankara, Turkey; Hacettepe University Faculty of Medicine, Department of Medical Microbiology, Virology Unit, Ankara, Turkey

Abstract. Toscana virus (TOSV), a sandfly-borne phlebovirus, is an important agent of human meningoencephalitis in the Mediterranean region, for which vertebrates acting as reservoirs have not yet been determined. This study investigates TOSV and *Leishmania* infections in dogs, cats, sheep, and goats from Adana and Mersin provinces in southeastern Turkey. TOSV neutralizing antibodies were demonstrated in 40.4% of the dog and 4% of the goat samples. TOSV RNA was detected in 9.9% of the 252 samples that mainly comprise dogs (96%). Thus, canine species can be suggested as the candidate reservoirs of TOSV. Partial sequences revealed the activity of TOSV genotypes A and B. In two dogs presenting with symptoms of canine leishmaniasis, infections of TOSV genotype B and *Leishmania infantum* have been documented, describing the first report of coinfections with these agents.

INTRODUCTION

Sandfly-borne phleboviruses are arthropod-borne viruses (arboviruses) for which natural replication cycle involves sandflies (order Diptera, family Psychodidae) as transmission vectors and warm-blooded vertebrates as susceptible species.[1] Phleboviruses are classified as a genus in the Bunyaviridae family, and comprise over 70 viruses that constitute nine established and several tentative species.[2] Members of the *Phlebovirus* genus possess a single-stranded RNA genome in three segments, which codes for the viral polymerase, envelope glycoproteins, nucleocapsid protein, and nonstructural proteins.[2]

Toscana virus (TOSV) is unique among sandfly-borne phleboviruses with human health impact, because of its unambiguous association with central nervous system infections.[1] Although exposure to TOSV may result in asymptomatic seroconversion, or a mild febrile illness, neuroinvasive manifestations such as meningitis, encephalitis, and/or peripheral neurological symptoms can also be observed in affected individuals.[3,4] The outcome of TOSV neuroinvasive disease is usually favorable without sequelae. However, severe or fatal meningoencephalitis, deafness, persistent personality alterations, prolonged unconsciousness with seizures, persisting speech disorders, and paresis have been reported.[5–9]

Current data indicate that TOSV circulates actively and is endemic in the Mediterranean region.[3] In Spain, France, and Italy, TOSV infections constitute a significant public health problem, due to being one of the major viral pathogens involved in aseptic meningitis during the warm seasons, when the sandfly vectors are abundant.[3,4] TOSV maintenance during the cold months of the year can be partially explained by the vertical and sexual transmission of the virus among sandflies.[10,11] However, it remains unknown if susceptible animals could also contribute in virus survival throughout the year. Up to date, limited evidence of TOSV infection in domestic and wild vertebrates has been documented, insufficient to suggest any significant role of animal reservoirs for viral persistence.[12,13] Thus, this mechanism is currently considered as negligible and the competent vectors are suggested as the reservoirs.[1,4]

Leishmaniasis is another vector-borne disease representing a public health threat, which is endemic in over 80 countries and has been continuously reported in the Mediterranean region.[14,15] The causative agents are flagellate protozoans of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae), are also transmitted by phlebotomine sandflies.[16] The infection may exhibit wide spectrum of clinical forms, from relatively mild cutaneous lesions (cutaneous leishmaniasis) to life-threatening systemic diseases (visceral leishmaniasis). Natural transmission of leishmaniasis may be zoonotic or anthroponotic, depending on the parasite species and the geographical location.[17] Dogs, which may suffer from severe disease (canine leishmaniasis), are the primary domestic reservoir hosts of zoonotic infections, whereas infections in cats and horses have also been reported in endemic areas.[15] Recent reports have revealed the northward spread of leishmaniasis, following the ecological expansion of the sandfly vectors.[17,18]

Because of their common sandfly vectors, an epidemiologic association of phleboviral infections and leismaniasis has been assumed previously. However, only serological evidence of such association, as demonstrated by the detection of exposure in humans, has been provided.[19] The presence of sandflies infected with either phleboviruses or *Leishmania* in an endemic area provided further clues of a spatial connection of these agents.[20] This study was conducted to investigate TOSV exposure in various animal species to identify potential reservoirs as well as concurrent *Leishmania* infections in an endemic region in Turkey.[21]

MATERIALS AND METHODS

Study setting. The study was conducted in Gulnar, Mezitli, Yenisehir, and Tarsus districts of Mersin province and Saricam, Yuregir, Karaisali, Cukurova, and Seyhan metropolitan districts of Adana province, southern Anatolia, during May–September 2013 (Figure 1). Plasma or sera from dogs, cats, goats, and sheep were evaluated, obtained from animal shelters or local veterinary clinics (dogs and cats) or local breeders (goats and sheep). All samples were acquired after informed consent of the animal owners or the institutions, according to the national regulations on the operation and

*Address correspondence to Koray Ergunay, Hacettepe University Faculty of Medicine, Department of Medical Microbiology, Virology Unit, Morphology Building 3rd floor, 06100 Sihhiye, Ankara, Turkey. E-mail: ekoray@hacettepe.edu.tr

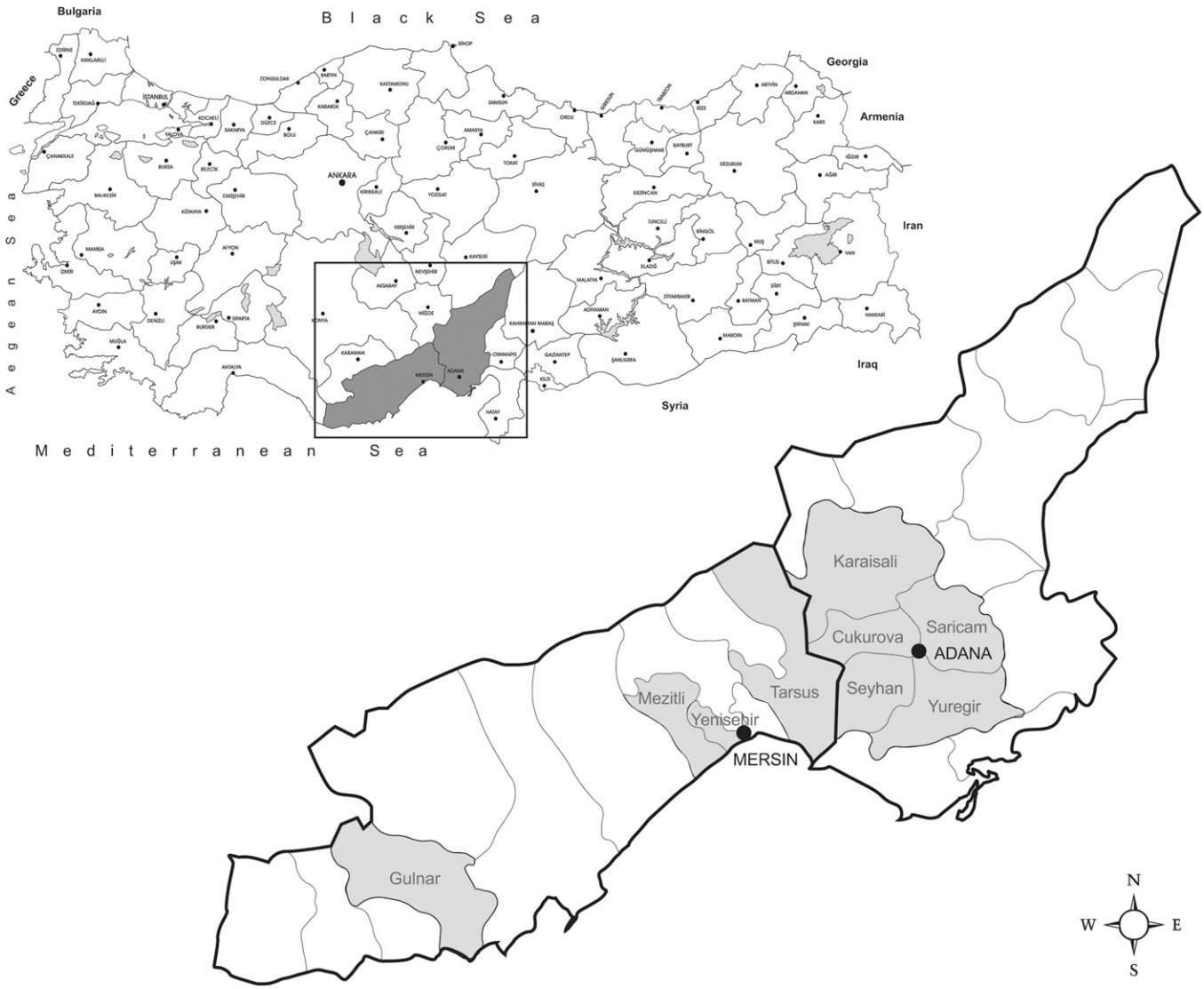

FIGURE 1. Illustrative map of the sampling locations in the study.

procedure of animal experiments ethics committees (Regulation Nr. 26220, Date: 09.7.2006) and has been approved by the local ethics committee (Nr: AULEC/201-96-346).

Serological screening of TOSV exposure. TOSV exposure was investigated in samples obtained from dogs, cats, sheep, and goats via screening of IgG-class antibodies utilizing an immunofluorescence assay (IFA). For this purpose, slides incorporating TOSV-infected and uninfected Vero cells, provided within a commercial IFA (Anti-Toscana Virus IIFT; Euroimmun, Lubeck, Germany) were used. Detection of species-specific IgGs was accomplished by using commercial fluorescein isothiocyanate (FITC)-labeled dog, goat, and sheep conjugates (anti-dog, anti-goat IgG, and anti-sheep (whole molecule)-FITC conjugate, Sigma, St. Louis, MO). The optimal working dilution for each conjugate was determined using twofold titrations starting from 1/32 dilution, as recommended by the manufacturer, in the absence of fluorescence in uninfected Vero cells. Animal sera were tested in 1/100 dilution. Anti-TOSV IgG-positive human sera were used as controls to determine and compare patterns of fluorescence. The assay was interpreted using a fluorescence microscope by 40× field examination. Samples regarded as reactive in the initial evaluation were retested via the identical procedure.

Specificity confirmation of the samples reactive in IgG IFA as well as screening in all feline and available canine samples were accomplished via virus neutralization test (VNT) for TOSV, performed as described previously.[22] Briefly, the test was performed using Vero cells (ATCC CCL81) and TOSV strain ISS.Phl.3, diluted to a final concentration of 100 $TCID_{50}$ and serum dilutions of 1/40 and 1/100. Reactivity was determined by the lack of cytopathic effect at the 96th hour at 37°C. All VNT experiments were performed in duplicate.

Detection of TOSV nucleic acids. Dog, cat, sheep, and goat sera and/or plasma from Mersin and Adana provinces, with suitable storage and transit conditions, were evaluated for TOSV RNA. The samples were subjected to nucleic acid purification by High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany), followed by reverse transcription via random hexamer primers using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Tokyo, Japan), according to the manufacturers' protocols. A previously

TABLE 1
TOSV RNA, viral genotype, serology, and *Leishmania* DNA findings according to species

	TOSV RNA		TOSV serology		*Leishmania* DNA
			IgG IFA	VNT	
	Positive/total (%)	Genotype (A/B)	Positive/total (%)	Positive/total (%)	Positive/total (%)
Dogs	24/155 (15.5)	10/14	0/112 (0)	21/52 (40.4)	2/15 (1.3)
Cats	1/22 (4.5)	0/1	n.d.	0/17 (0)	1/22 (4.5)
Sheep	0/25 (0)	0/0	1/100 (1)	0/1 (0)	n.d.
Goats	0/50 (0)	0/0	5/100 (5)	4/5 (80)	n.d.

n.d. = not determined; TOSV = Toscana virus.

described nested PCR with degenerated primers, targeting the RNA polymerase (L) gene of all phleboviruses including TOSV was used.[23] In the second round of nested PCR, a TOSV-specific antisense primer (ATOS2-) was used for the screening of samples. Amplicons of the expected length (126 base pairs [bps]) were visualized under ultraviolet light via ethidium bromide staining after electrophoresis in 2% agarose. Vero cell-grown TOSV strain ISS.Phl.3, processed as described above, was used as the positive control in the PCR assay. In TOSV-positive samples, the second round of PCR was reamplified using the phlebovirus consensus antisense primer (NPhlebo2-), to obtain a larger amplicon (248 bps), better suited for sequence characterization.[23]

Detection of *Leishmania* nucleic acids. A nested-PCR-based schizodeme method that targets the conserved region of the kinetoplast minicircle classes found in all *Leishmania* species was used for detecting and identifying *Leishmania* infections in purified nucleic acids from cats and dogs.[24] The method enables the identification of all *Leishmania* species with clinical impact as well as differentiation of Old World *Leishmania* complexes on the basis of PCR amplicon size, where *L. infantum* produces a 680-bp product, whereas in *L. tropica* and *L. major*, product sizes of 750 and 560 bps, respectively. The lower detection limit of the assay was reported as 0.1 fg of *L. infantum* DNA.[24]

Sequencing and phylogenetic analysis. Amplicons of the expected sizes obtained from *Phlebovirus* and *Leishmania*-nested PCR were characterized via sequencing. For this purpose, products were cleaned up using High Pure PCR Product Purification Kit (Roche Diagnostics) and subsequently subjected to dideoxy nucleotide sequencing with forward and reverse primers, using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster, CA). *Phlebovirus* sequences were aligned and analyzed using Bioedit v5.0.5 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and subsequently by MEGA software v5.2.[25] Phylogenetic trees were constructed using the Jukes–Cantor substitution rate model with 1,000 bootstrap replicates. Maximum likelihood trees were generated based on the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree. Species confirmation of the obtained *Leishmania* sequences was performed via BLAST (Basic Local Alignment Search Tool) searches in the GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

TOSV serology in animals. A total of 329 samples, obtained from dogs (112, 34%), sheep (100, 30.4%), goats (100, 30.4%), and cats (17, 5.2%), collected during May–October, were evaluated via IgG IFA and/or VNT for TOSV immunoglobulins. Six (6/312, 1.9%) and 25 (25/75, 33.3%) samples were positive in IgG IFA and VNT, respectively. No variations due to sample dilution or repeat testing were noted in VNT or IgG IFA. VNT-confirmed exposure was observed in 21 out of 52 (40.4%) dog samples and 4 out of 5 (80%) goat samples reactive in the IFA (Table 1). VNT-positive dog samples originated from various districts of Mersin and Adana provinces and dates during the sandfly season (Table 2). All were interpreted as negative in IgG IFA. Moreover, four goat sera originating from Buyukeceli village (36°10′35.4″N, 33°33′05.9″E) of Gulnar district collected in October 2013 were positive in the VNT. Despite reactive results in IgG IFA, VNT remained negative in one sheep and one goat sera, obtained from Gulnar district in May and October, respectively (Table 1).

TOSV nucleic acids in animal samples. Viral RNA was investigated in 252 samples collected in Gulnar, Mezitli, Yenisehir, and Tarsus districts of Mersin province (195, 77.4%) during June–September 2013, and Saricam, Yuregir, Karaisali, Cukurova, and Seyhan districts of Adana province (57, 22.6%) in August 2013. The samples comprise 155 plasma from dogs (61.5%), 50 plasma from goats (19.8%), 25 plasma from sheep (9.9%), and 22 plasma from cats (8.7%). TOSV viremia was detected in a total of 25 samples (25/252, 9.9%) that comprise dogs (24/252, 9.5%) and a cat (1/252, 0.4%). All sheep and goat samples remained negative (Table 1).

The first-round PCR products from all TOSV-positive samples were reamplified using internal pan-phlebovirus antisense primer, as described above, sequenced, and three consensus sequences were characterized (GenBank accession

TABLE 2
Distribution of TOSV RNA positivity and neutralizing antibody seroreactivity in canine and feline specimens according to the sampling month in Mersin province

	July				August				September			
	TOSV RNA		TOSV	VNT	TOSV RNA		TOSV	VNT	TOSV RNA		TOSV	VNT
	Positive/tested (%)		Positive/tested (%)		Positive/tested (%)		Positive/tested (%)		Positive/tested (%)		Positive/tested (%)	
Dogs	3/32	(9.4)	10/17	(58.8)	11/52	(21.2)	3/10	(30)	0/14	(0)	8/13	(61.5)
Cats	0/9	(0)	0/6	(0)	0/7	(0)	0/5	(0)	1/6	(16.7)	0/6	(0)

TOSV = Toscana virus; VNT = virus neutralization test.

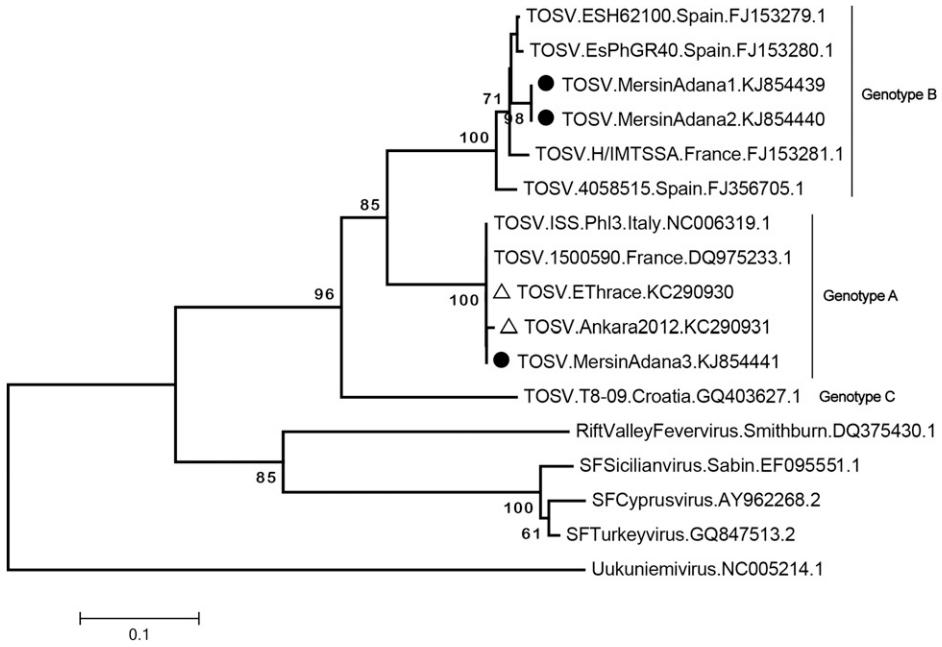

FIGURE 2. Neighbor-joining analysis of partial Toscana virus sequences. Viruses included in the analysis are indicated with name, origin of isolation, and GenBank accession number. Tick-borne *Phlebovirus uukuniemi* is included as an outlier (● = consensus sequences characterized in this study; Δ = TOSV sequences previously characterized in Turkey).

numbers: KJ854439, KJ854440, and KJ854441). Phylogenetic analysis revealed sequences belonging to TOSV genotype A as well as genotype B (Figure 2). Intramural nucleotide divergence among genotype A and B sequences was noted as 1.7% and 1.3%, respectively, whereas 17.9–19.7% variation rates were observed in all sequences. Comparison of previously characterized partial TOSV genotype A sequences from eastern Thrace and central Anatolia regions of Turkey demonstrated 1.2–2.5% and 17.7–18.5% divergence with genotype A and B sequences, respectively. In canine samples with viral RNA, TOSV genotype A comprised 42.9% (6/14) and 40% (4/10) samples from Mersin and Adana provinces, respectively. No association of TOSV detection or virus genotype with animal age, gender, or collection date could be demonstrated (Table 2). All canine samples with detectable viral RNA were negative in TOSV VNT. The feline sample with positive TOSV RNA was observed to belong to genotype B. All animals with TOSV viremia were asymptomatic without any signs of febrile disease or systemic involvement.

From Mersin province, a total of 120 feline and canine plasma samples were available for TOSV RNA detection, collected during a period of 3 months (July–September) (Table 2). Here, a higher rate of TOSV detection (18.6%) was observed in samples collected in August, compared with July (7.3%) and September (5%). Moreover, TOSV viremia was observed to be more frequent in dogs (14.3% versus 4.5%) (Table 2).

***Leishmania* parasitemia in canine and feline samples.** *Leishmania* DNA was investigated in canine (n = 155) and feline (n = 22) samples evaluated for TOSV from Adana and Mersin provinces, as described above. Kinetoplast PCR revealed positive results in three samples (1.7%) that comprise two canine (1.3%) and one feline (4.5%) plasma (Table 1). *Leishmania* species in all samples were characterized as *L. infantum*, via amplicon size and subsequent sequencing. The feline sample, obtained in July from Mezitli district of Mersin province, originated from an 18-month-old male cat, without any clinical symptoms. The canine samples originated from two 3-year-old male stray dogs accommodated in a local animal shelter in Adana province (37°00′26.9″N, 35°18′56.5″E), with clinical suspicion of canine leishmaniasis due to symptoms of anorexia, wasting, muscle atrophy, epistaxis, and mucosal bleeding. These animals were euthanized shortly after initial sampling in August and no follow-up samples were available. Moreover, TOSV RNA was detected concomitantly in these dogs and characterized as genotype B.

DISCUSSION

Since its initial isolation from *Phlebotomus perniciosus* sandflies in Tuscany region of Italy in 1971 and recognition as a causative agent of neurological disease in humans in 1983, TOSV has been identified as a prominent agent of viral meningoencephalitis in travelers and residents of endemic countries, which currently include Italy, Spain, France, Portugal, Cyprus, Greece, and Turkey.[1,26–28] TOSV was repeatedly isolated from a portion of affected individuals and *P. pernicious* and *P. perfiliewi*, as well as from *Sergentomyia minuta* sandflies.[1,3,29] Because of the transient viremia occurring in humans, vector sandflies have been suggested to participate as reservoirs in the virus cycle, supported by the demonstration of infected male sandflies in nature, transovarial and venereal transmission in the laboratory.[11,12,17,30] However, observation of progressive decreases of viral infection rates in generations of sandfly colonies suggested that a prolonged maintenance would not be possible by these routes. Consequently, the existence of potential reservoirs was considered, but very limited information on various animal species' role as amplifying hosts has been available so far. This study was conducted to investigate TOSV infections in domestic animals

in a leishmaniasis-endemic region during the sandfly season, to elucidate potential impact of the species in virus circulation.

TOSV exposure was screened serologically via Ig IFA and/or VNT in a total of 329 samples from dogs, cats, sheep, and goats. An IgG seropositivity rate of 1.8% (6/329), which comprises one sheep and five goat samples, was obtained in IFA and the presence of TOSV-specific neutralizing antibodies could further be demonstrated in four goat samples. Although all canine sera were interpreted as negative in IgG IFA, VNT revealed specific antibodies in 40.4% of all samples tested (Table 1), indicating the frequent occurrence of virus exposure in this species, as well. Despite the lack of definitive information, a previous serological study performed in Tuscany, Italy, suggested that ovines and horses can be asymptomatically infected by TOSV.[31] However, no evidence of infection could be identified in domestic or wild animals including mice (*Apodemus sylvaticus* and *Mus musculus*), bank voles (*Clethrionomys glareolus*), stone martens (*Martes foina*), coypus (*Myocastor coypus*), porcupines (*Hystrix cristata*), foxes (*Vulpes vulpes*), and hedgehogs (*Ennaceus eunopaeus*) in Italy.[12] In a recent serosurvey undertaken in Granada, Spain, another TOSV-endemic region where the virus could be detected in sandflies and clinical cases, a high seroprevalence rate for TOSV antibodies (36.2%) among 1,186 domestic animals was revealed.[13] Here, IgG detection rates of 64.3% in horses, 59.6% in cats, 48.3% in dogs, 32.3% in sheep, 22% in pigs, 17.9% in cows, and 17.7% in goats were reported. Though cross-reactions among phleboviruses within the sandfly fever Naples virus serocomplex cannot be excluded and might have resulted in an overestimation of the seroprevalence in this study, the overall data are compatible with a TOSV-endemic zone and in agreement with the feeding habits of *P. perniciosus*, the documented vector in the region.[32] Interestingly, attempts of virus isolation were unsuccessful in all samples and a low level of TOSV viremia was detected only in one goat, suggesting an ongoing virus circulation in the region with evidence of previous exposure and low incidence of recent infections.[13] In Tunisia, another *Phlebovirus*-endemic region, TOSV as well as Punique and Sandfly fever Sicilian virus exposure in dogs were identified, with 7.5% seroprevalence for TOSV.[33] It has further been suggested that dogs can be used as sentinel animals for monitoring *Phlebovirus* activity.[33] Our serosurvey findings in Mersin and Adana provinces of the Mediterranean Anatolia revealed a relatively high TOSV neutralizing antibody prevalence (40.4%) in dogs. Moreover, specific antibodies were detected in goats, despite with a lower rate and in samples collected during October, after the months of peak sandfly activity. The discrepancy of IgG IFA and VNT results observed in the dog sera is likely to represent a predominant IgM response in this species. These findings suggest that TOSV exposure in the study cohort has been a recent event, which is further supported by the viral RNA detection, as discussed below. Previous blood donor serosurveillance efforts for TOSV have revealed exposed individuals residing in Mersin, Hatay, and Antalya provinces in the Mediterranean Anatolia, albeit with a cohort too small for decisive conclusions.[34] Studies are underway by our group to determine human seroprevalence of TOSV and other medically important phleboviruses in the region.

In the study, TOSV viremia was sought via a nested PCR employing pan-phlebovirus and TOSV-specific primers in 252 dog, cat, sheep, and goat plasma, collected from various districts of Mersin and Adana provinces during June–September. Virus RNA was detected in a relatively high frequency (9.9%) and mainly comprises canine samples (96%). All PCR-positive canine and feline samples were characterized as TOSV by sequencing. These findings constitute the first documentation of TOSV viremia in dogs and cats. Reports of TOSV nucleic acid detection or isolation in endemic countries include virus isolation from neuronal tissues of a *Pipistrellus kuhli* bat in Italy and low-level viremia observed in a goat from Spain, as discussed above.[12,13] Nevertheless, serological evidence of exposure in dogs and cats has been revealed in Spain previously, providing further support for the susceptibility of these species to TOSV.[13] Temporal analysis of viremic samples suggested a continuous virus transmission occurring from June to September, which is also supported by the neutralizing antibody detection rates (Table 2). This observation is also in accordance with previous reports from Turkey, where TOSV cases emerged during August 2012 in eastern Thrace region and cases of febrile disease due to the phlebovirus variant, sandfly fever Turkey virus, were clustered during August, with the detection of the agent in vectors during July in central Anatolia.[35,36] No viral nucleic acids could be demonstrated in sheep and goats in this study, which may indicate variations in the distribution of infected sandflies within the sampling locations. For a comprehensive analysis of virus circulation and transmission dynamics, a field sandfly surveillance is required to reveal periods of peak activity.

Analyses of several complete and partial sequences have demonstrated that genetically divergent TOSV strains circulate in different endemic regions and at least two geographically distinct TOSV genotypes or lineages are present in the Mediterranean countries.[37] The TOSV genotype A includes all strains isolated in Italy and is also detected in France, whereas the TOSV genotype B is circulating mainly in Spain. Cocirculation of genotypes A and B has also been characterized in southeastern France.[38] Furthermore, a novel genotype, tentatively called as genotype C, is identified in patients from Croatia.[39] In Turkey, acute cases of TOSV infections have been initially reported in 2011 from central Anatolia, followed by cases from eastern Thrace and recently, from central Anatolia again, demonstrating ongoing virus activity.[35,40,41] All partial TOSV sequences previously characterized in Turkey have belonged to genotype A strains. In this study, phylogenetic analyses revealed cocirculation of TOSV genotypes A and B (Figure 2), with limited nucleotide divergence among each genotype. Activity of TOSV genotype B was reported for the first time from Turkey, as well as presence of genotypes A and B within the same region, similar to the situation in southeastern France (Table 2).[38] The genotype A sequences characterized in the study regions revealed a maximum of 2.5% divergence with genotype A strains previously identified in central Anatolia and eastern Thrace, and is likely to represent variations due to geographic regions. Genotype A and B viruses could be detected in canine samples without any prominent association with sampling location, age, gender, or collection date (Table 2). The feline sample with detectable TOSV RNA was characterized as genotype B, with unknown implications. The impact of TOSV genotype distribution around the Mediterranean region is not fully elucidated and may be related to differences in vector species and abundance.[31] The sandfly species responsible for TOSV transmission in Turkey have not yet been identified.

Nevertheless, presence of species with vector competency such as *P. perfiliewi* has been demonstrated.[42] It remains to be explored whether circulation of particular TOSV genotype reflects variations in sandfly vectors or potential hosts.

A relatively high frequency of RNA detection in canine blood during the sandfly active season revealed in this study suggests dogs as potential reservoirs for TOSV. Previous data showing virus exposure in canines from endemic countries also provide further support for this hypothesis.[13] However, to confirm canine species' role in TOSV life cycle and natural maintenance, viral loads high enough to initiate an infection in naive phlebotomines must be demonstrated. Moreover, duration of viremia in canines is likely to have a considerable effect as well. It is known that the viremia levels observed in *Phlebovirus* infections of human or nonhuman vertebrates in natural settings or in laboratory are low and transient, usually requiring a large quantity of virus to be ingested by the vectors.[43–46] These aspects of the infection could not be fully explored in this work, due to the study design and sampling during the sandfly-active months, but surely warrant further investigation. Currently, the impact of vertebrates in *Phlebovirus* natural maintenance cycle is considered negligible.[3] Experiments in controlled environments are required to reveal TOSV transmission dynamics between sandflies and susceptible vertebrates.

Another finding of significant impact of this study is the detection of TOSV and *L. infantum* coinfections in two dogs from Adana province. The study region is a well-documented zone for human cutaneous and visceral leishmaniasis caused by *L. infantum* and *L.tropica* as well as canine leishmaniasis by *L. infantum*.[21,47,48] Originating from an urban animal shelter, the animals presented with clinical symptoms compatible with canine leishmaniasis. Coinfection of the two pathogens was confirmed via repeated tests and sequencing of the amplicons, where TOSV genotype B is identified in both samples, providing the first documentation of concomitant acute infections of these agents in vertebrates. Well-established transmission of phleboviruses and *Leishmania* parasites via phlebotomine sandflies, sometimes by the same sandfly species, has resulted in the assumption of an epidemiological connection between two agents. The earliest evidence of such an association was reported in Iran, between the *Phlebovirus karimabad* and cutaneous leishmaniasis, where ecology of both agents presents similar epidemiologic pattern.[49] Recent studies in an endemic region in southern France also indicated a clear relationship between *L. infantum* and TOSV exposure, by retrospective serological evaluation.[19] Our current findings provide direct evidence for the existence of such coinfections. The consequences and impact of TOSV ve *Leishmania* coinfections are unclear. In this study, the infected dogs presented with symptoms indiscriminable from canine leishmaniasis and all animals with detectable TOSV RNA in circulation remained asymptomatic. It needs to be determined if concomitant or superinfections with these agents result in an increased rate of symptomatic infections or exacerbate clinical symptoms. Moreover, the implication of TOSV genotype B detection in coinfected dogs requires further investigation to reveal whether it is a coincidental finding or genomic variations have a direct effect on coinfections. The mechanism of vertebrate exposure to the agents also needs further exploration. Given the low probability of double infections in sandflies, it can be speculated that coinfections are more likely to occur due to successive bites by sandflies infected by a single pathogen.[19] The presence of sandflies infected with either phleboviruses or *Leishmania* in an endemic area also supports this view.[20]

In conclusion, canine species can be suggested as candidate reservoir hosts of TOSV, due to the high frequency of detectable RNA and neutralizing antibodies observed throughout the sandfly season. Presence of TOSV genotype B and cocirculation of genotypes A and B have been described for the first time in southern Anatolia. Initial documentation of TOSV genotype B and *L. infantum* coinfections, presenting mainly with symptoms of canine leishmaniasis, has been achieved.

Received May 23, 2014. Accepted for publication January 16, 2015.

Published online February 23, 2015.

Acknowledgments: We are grateful to the Petical Veterinary Hospital personnel in Mersin province and local veterinary clinics in Adana province for invaluable assistance and to N. Emin Guven for graphics. Preliminary findings of this study have been accepted for presentation at the 5th International Meeting on Emerging Diseases and Surveillance, IMED, held in Vienna, Austria, from October 31 to November 3, 2014.

Authors' addresses: Ender Dincer, Mersin University Advanced Technology Education, Research and Application Center, Çiftlikköy Campus, Mersin, Turkey, E-mail: enderdin@gmail.com. Sepandar Gargari and Aykut Ozkul, Ankara University Faculty of Veterinary Medicine, Department of Virology, Irfan Bastug Caddesi, 06110 Diskap, Ankara, Turkey, E-mails: Sepandar.Gargari@ankara.edu.tr and ozkul@ankara.edu.tr. Koray Ergunay, Hacettepe University Faculty of Medicine, Department of Medical Microbiology, Virology Unit, Morphology Building 3rd floor, 06100 Sihhiye, Ankara, Turkey, E-mail: ekoray@hacettepe.edu.tr.

REFERENCES

1. Alkan C, Bichaud L, de Lamballerie X, Alten B, Gould EA, Charrel RN, 2013. Sandfly-borne phleboviruses of Eurasia and Africa: epidemiology, genetic diversity, geographic range, control measures. *Antiviral Res 100:* 54–74.
2. Plyusnin A, Beaty BJ, Elliott RM, Goldbach R, Kormelink R, Lundkvist A, Schmaljohn CS, Tesh RB, 2012. Bunyaviridae. King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, eds. *Ninth Report of the International Committee on Taxonomy of Viruses*. First edition. San Diego, CA: Elsevier Academic Press, 725–741.
3. Charrel RN, Bichaud L, de Lamballerie X, 2012. Emergence of Toscana virus in the Mediterranean area. *World J Virol 1:* 135–141.
4. Charrel RN, Gallian P, Navarro-Mari JM, Nicoletti L, Papa A, Sanchez-Seco MP, Tenorio A, de Lamballerie X, 2005. Emergence of Toscana virus in Europe. *Emerg Infect Dis 11:* 1657–1663.
5. Baldelli F, Ciufolini MG, Francisci D, Marchi A, Venturi G, Fiorentini C, Luchetta ML, Bruto L, Pauluzzi S, 2004. Unusual presentation of life-threatening Toscana virus meningoencephalitis. *Clin Infect Dis 38:* 515–520.
6. Martinez-Garcia FA, Moreno-Docon A, Segovia-Hernandez M, Fernandez-Barreiro A, 2008. Deafness as a sequela of Toscana virus meningitis. *Med Clin (Barc) 130:* 639.
7. Serata D, Rapinesi C, Del Casale A, Simonetti A, Mazzarini L, Ambrosi E, Kotzalidis GD, Fensore C, Girardi P, Tatarelli R, 2011. Personality changes after Toscana virus (TOSV) encephalitis in a 49-year-old man: a case report. *Int J Neurosci 121:* 165–169.
8. Kuhn J, Bewermeyer H, Hartmann-Klosterkoetter U, Emmerich P, Schilling S, Valassina M, 2005. Toscana virus causing severe meningoencephalitis in an elderly traveller. *J Neurol Neurosurg Psychiatry 76:* 1605–1606.
9. Sanbonmatsu-Gamez S, Perez-Ruiz M, Palop-Borras B, Navarro-Mari JM, 2009. Unusual manifestation of Toscana virus infection, Spain. *Emerg Infect Dis 15:* 347–348.

10. Tesh RB, Modi GB, 1987. Maintenance of Toscana virus in *Phlebotomus perniciosus* by vertical transmission. *Am J Trop Med Hyg 36:* 189–193.
11. Tesh RB, Lubroth J, Guzman H, 1992. Simulation of arbovirus overwintering: survival of Toscana virus (Bunyaviridae: *Phlebovirus*) in its natural sand fly vector *Phlebotomus perniciosus*. *Am J Trop Med Hyg 47:* 574–581.
12. Verani P, Ciufolini MG, Caciolli S, Renzi A, Nicoletti L, Sabatinelli G, Bartolozzi D, Volpi G, Amaducci L, Coluzzi M, Paci P, Balducci M, 1988. Ecology of viruses isolated from sand flies in Italy and characterized of a new *Phlebovirus* (Arabia virus). *Am J Trop Med Hyg 38:* 433–439.
13. Navarro-Mari JM, Palop-Borras B, Perez-Ruiz M, Sanbonmatsu-Gamez S, 2011. Serosurvey study of Toscana virus in domestic animals, Granada, Spain. *Vector Borne Zoonotic Dis 11:* 583–587.
14. Dujardin JC, Campino L, Canavate C, Dedet JP, Gradoni L, Soteriadou K, Mazeris A, Ozbel Y, Boelaert M, 2008. Spread of vector-borne diseases and neglect of leishmaniasis, Europe. *Emerg Infect Dis 14:* 1013–1018.
15. Antoniou M, Gramiccia M, Molina R, Dvorak V, Volf P, 2013. The role of indigenous phlebotomine sandflies and mammals in the spreading of leishmaniasis agents in the Mediterranean region. *Euro Surveill 18:* 20540.
16. Lainson R, Shaw JJ, 1987. Evolution, classification and geographical distribution. Peters W, Killick-Kendrick R, eds. *The Leishmaniases in Biology and Medicine*. First edition. San Diego, CA: Academic Press, 1–120.
17. Maroli M, Feliciangeli MD, Bichaud L, Charrel RN, Gradoni L, 2013. Phlebotomine sandflies and the spreading of leishmaniases and other diseases of public health concern. *Med Vet Entomol 27:* 123–147.
18. Maroli M, Rossi L, Baldelli R, Capelli G, Ferroglio E, Genchi C, Gramiccia M, Mortarino M, Pietrobelli M, Gradoni L, 2008. The northward spread of leishmaniasis in Italy: evidence from retrospective and ongoing studies on the canine reservoir and phlebotomine vectors. *Trop Med Int Health 13:* 256–264.
19. Bichaud L, Souris M, Mary C, Ninove L, Thirion L, Piarroux RP, Piarroux R, De Lamballerie X, Charrel RN, 2011. Epidemiologic relationship between Toscana virus infection and *Leishmania infantum* due to common exposure to *Phlebotomus perniciosus* sandfly vector. *PLoS Negl Trop Dis 5:* e1328.
20. Faucher B, Bichaud L, Charrel R, Mary C, Izri A, de Lamballerie X, Piarroux R, 2014. Presence of sandflies infected with *Leishmania infantum* and Massilia virus in Marseille urban area. *Clin Microbiol Infect 20:* 340–343.
21. Ok UZ, Balcioglu IC, Taylan Ozkan A, Ozensoy S, Ozbel Y, 2002. Leishmaniasis in Turkey. *Acta Trop 84:* 43–48.
22. Ergünay K, Litzba N, Lo MM, Aydoğan S, Saygan MB, Us D, Weidmann M, Niedrig M, 2011. Performance of various commercial assays for the detection of Toscana virus antibodies. *Vector Borne Zoonotic Dis 11:* 781–787.
23. Sanchez-Seco MP, Echevarria JM, Hernandez L, Estevez D, Navarro-Mari JM, Tenorio A, 2003. Detection and identification of Toscana and other phleboviruses by RT-nested-PCR assays with degenerated primers. *J Med Virol 71:* 140–149.
24. Noyes HA, Reyburn H, Bailey JW, Smith D, 1998. A nested-PCR-based schizodeme method for identifying *Leishmania* kinetoplast minicircle classes directly from clinical samples and its application to the study of the epidemiology of *Leishmania tropica* in Pakistan. *J Clin Microbiol 36:* 2877–2881.
25. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S, 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. *Mol Biol Evol 28:* 2731–2739.
26. Verani P, Ciufolini MG, Nicoletti L, Balducci M, Sabatinelli G, Coluzzi M, Paci P, Amaducci L, 1982. Ecological and epidemiological studies of Toscana virus, an arbovirus isolated from Phlebotomus. *Ann Ist Super Sanita 18:* 397–399.
27. Verani P, Nicoletti L, Ciufolini MG, 1984. Antigenic and biological characterization of Toscana virus, a new Phlebotomus fever group virus isolated in Italy. *Acta Virol 28:* 39–47.
28. Nicoletti L, Verani P, Caciolli S, Ciufolini MG, Renzi A, Bartolozzi D, Paci P, Leoncini F, Padovani P, Traini E, Baldereschi M, Balducci M, 1991. Central nervous system involvement during infection by *Phlebovirus toscana* of residents in natural foci in central Italy (1977–1988). *Am J Trop Med Hyg 45:* 429–434.
29. Charrel RN, Izri A, Temmam S, de Lamballerie X, Parola P, 2006. Toscana virus RNA in *Sergentomyia minuta* files. *Emerg Infect Dis 12:* 1299–1300.
30. Maroli M, Ciufolini MG, Verani P, 1993. Vertical transmission of Toscana virus in *Phlebotomus perniciosus* via the second gonotrophic cycle. *Med Vet Entomol 7:* 283–286.
31. Cusi MG, Savellini GG, Zanelli G, 2010. Toscana virus epidemiology: from Italy to beyond. *Open Virol J 4:* 22–28.
32. De Colmenares M, Portus M, Botet J, Dobano C, Gallego M, Wolff M, Segui G, 1995. Identification of blood meals of *Phlebotomus perniciosus* (Diptera: Psychodidae) in Spain by a competitive enzyme-linked immunosorbent assay biotin/avidin method. *J Med Entomol 32:* 229–233.
33. Sakhria S, Alwassouf S, Fares W, Bichaud L, Dachraoui K, Alkan C, Zoghlami Z, de Lamballerie X, Zhioua E, Charrel RN, 2014. Presence of sandfly-borne phleboviruses of two antigenic complexes (Sandfly fever Naples virus and Sandfly fever Sicilian virus) in two different bio-geographical regions of Tunisia demonstrated by a microneutralisation-based seroprevalence study in dogs. *Parasit Vectors 7:* 476.
34. Ergunay K, Aydogan S, Ilhami Ozcebe O, Cilek EE, Hacioglu S, Karakaya J, Ozkul A, Us D, 2012. Toscana virus (TOSV) exposure is confirmed in blood donors from central, north and south/southeast Anatolia, Turkey. *Zoonoses Public Health 59:* 148–154.
35. Erdem H, Ergunay K, Yilmaz A, Naz H, Akata F, Inan AS, Ulcay A, Gunay F, Ozkul A, Alten B, Turhan V, Oncul O, Gorenek L, 2014. Emergence and co-infections of West Nile virus and Toscana virus in eastern Thrace, Turkey. *Clin Microbiol Infect 20:* 319–325.
36. Ergunay K, Erisoz Kasap O, Kocak Tufan Z, Turan MH, Ozkul A, Alten B, 2012. Molecular evidence indicates that *Phlebotomus major* sensu lato (Diptera: Psychodidae) is the vector species of the recently-identified sandfly fever Sicilian virus variant: sandfly fever turkey virus. *Vector Borne Zoonotic Dis 12:* 690–698.
37. Collao X, Palacios G, Sanbonmatsu-Gamez S, Perez-Ruiz M, Negredo AI, Navarro-Mari JM, Grandadam M, Aransay AM, Lipkin WI, Tenorio A, Sanchez-Seco MP, 2009. Genetic diversity of Toscana virus. *Emerg Infect Dis 15:* 574–577.
38. Charrel RN, Izri A, Temmam S, Delaunay P, Toga I, Dumon H, Marty P, de Lamballerie X, Parola P, 2007. Cocirculation of 2 genotypes of Toscana virus, southeastern France. *Emerg Infect Dis 13:* 465–468.
39. Punda-Polic V, Mohar B, Duh D, Bradaric N, Korva M, Fajs L, Saksida A, Avsic-Zupanc T, 2012. Evidence of an autochthonous Toscana virus strain in Croatia. *J Clin Virol 55:* 4–7.
40. Ergunay K, Saygan MB, Aydogan S, Lo MM, Weidmann M, Dilcher M, Sener B, Hascelik G, Pinar A, Us D, 2011. Sandfly fever virus activity in central/northern Anatolia, Turkey: first report of Toscana virus infections. *Clin Microbiol Infect 17:* 575–581.
41. Ocal M, Orsten S, Inkaya AC, Yetim E, Acar NP, Alp S, Erisoz Kasap O, Gunay F, Arsava EM, Alten B, Ozkul A, Us D, Niedrig M, Ergunay K, 2014. Ongoing activity of Toscana virus genotype A and West Nile Virus lineage 1 strains in Turkey: a clinical and field survey. *Zoonoses Public Health 61:* 480–491.
42. Medlock JM, Hansford KM, Van Bortel W, Zeller H, Alten B, 2014. A summary of the evidence for the change in European distribution of phlebotomine sand flies (Diptera: Psychodidae) of public health importance. *J Vector Ecol 39:* 72–77.
43. Bartelloni PJ, Tesh RB, 1976. Clinical and serologic responses of volunteers infected with phlebotomus fever virus (Sicilian type). *Am J Trop Med Hyg 25:* 456–462.
44. Tesh RB, Duboise SM, 1987. Viremia and immune response with sequential *Phlebovirus* infections. *Am J Trop Med Hyg 36:* 662–668.
45. Cusi MG, Gori Savellini G, Terrosi C, Di Genova G, Valassina M, Valentini M, Bartolommei S, Miracco C, 2005. Development of a mouse model for the study of Toscana virus pathogenesis. *Virology 333:* 66–73.

46. Ciufolini MG, Maroli M, Verani P, 1985. Growth of two phleboviruses after experimental infection of their suspected sand fly vector, *Phlebotomus perniciosus* (Diptera: Psychodidae). *Am J Trop Med Hyg 34:* 174–179.
47. Toz SO, Nasereddin A, Ozbel Y, Ertabaklar H, Culha G, Sevil N, Ziya Alkan M, Jaffe CL, 2009. Leishmaniasis in Turkey: molecular characterization of *Leishmania* from human and canine clinical samples. *Trop Med Int Health 14:* 1–6.
48. Svobodova M, Zidkova L, Dvorak V, Hlavackova J, Myskova J, Seblova V, Kasap OE, Belen A, Votypka J, Volf P, 2009. Cutaneous leishmaniasis caused by *Leishmania infantum* transmitted by *Phlebotomus tobbi*. *Int J Parasitol 39:* 251–256.
49. Saidi S, Tesh R, Javadian E, Sahabi Z, Nadim A, 1977. Studies on the epidemiology of sandfly fever in Iran. II. The prevalence of human and animal infection with five Phlebotomus fever virus serotypes in Isfahan province. *Am J Trop Med Hyg 26:* 288–293.