

The first study to evaluate the presence of *Leishmania infantum*, *L. major*, Toscana virus and Sandfly fever Sicilian virus in *Phlebotomus perfiliewi* in Romania

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Abstract. Phlebotomine sand flies are the main vectors of various parasitic, bacterial and viral pathogens. New data revealed changes in their distribution, related to demographic, and climatic factors. The sand fly species of medical interest in the Mediterranean regions belong to the genera *Phlebotomus* and *Sergentomyia*, which were also reported in Romania. The main pathogen species transmitted by sand flies are the parasitic protozoans of the genus *Leishmania*, causing different forms of leishmaniasis in humans and dogs, and various phleboviruses causing acute febrile disease in humans, with occasional aseptic meningitis. All available data regarding canine leishmaniasis (CanL) in Romania are related to serological and molecular studies on domestic and wild carnivores, but there are no data regarding *Leishmania* DNA presence in sand fly vector species.

Phleboviruses transmitted by sand flies are endemic in the Mediterranean region and the last years have witnessed the description of many novel species. Although, the risk of exposure of vertebrates to phleboviruses is globally assessed, detailed geographic knowledge is poor even in the highly endemic countries. In Romania, there are no data regarding phleboviruses presence in sand fly vector species.

The aim of the study was to investigate the presence of *L. infantum*, *L. major*, Toscana virus (TOSV), and Sandfly fever Sicilian virus (SFSV) in *Phlebotomus perfiliewi*, collected from North-Eastern Romania. Out of 150 collected *P. perfiliewi*, 127 were pooled by sex up to 30 specimens in one pool). A number of 14 pools were tested for the presence of TOSV and SFSV RNA with three different PCR systems, and the presence of *L. infantum* and *L. major* DNA was assessed using two different real-time PCR assays. All 14 pools tested negative for all tested pathogen species. The study represents the first attempt to identify *L. infantum*, *L. major*, TOSV and SFSV in *P. perfiliewi* in Romania.

Keywords: *Leishmania infantum*; *L. major*; Toscana virus; Sandfly fever Sicilian virus; *Phlebotomus perfiliewi*; Romania.

Primul studiu pentru a evalua prezența *Leishmania infantum*, *L. major*, virusului Toscana și virusului Sicilian în *Phlebotomus perfiliewi* în România

Rezumat. Flebotomii sunt principalele insecte cu rol de vectori pentru diferiți patogeni de natură parazitară, bacteriană și virală. În Europa au fost descrise în ultimii ani schimbări în distribuția flebotomilor, legate de diverși factori demografici și climatici. Speciile de flebotomi de interes medical din regiunile mediteraneene aparțin genurilor *Phlebotomus* și *Sergentomyia*, care au fost raportate și în România. Principalele specii patogene transmise de către flebotomi sunt protozoarele din genul *Leishmania*, care provoacă diferite forme de leishmanioză la oameni și la câini, dar și diverse flebovirusuri care provoacă boala febrilă acută la om, la care evoluează ocazional și cu meningită aseptică. Toate datele disponibile cu privire la leishmanioza canină în România sunt corelate cu studii serologice și moleculare la carnivorele domestice și sălbatice, dar nu există date referitoare la prezența ADN-ului de *Leishmania* la speciile de flebotomii.

Flebovirusurile transmise de către flebotomi sunt endemice în regiunea mediteraneană, iar în ultimii ani au fost descrise câteva specii noi. Deși, riscul expunerii vertebratelor la flebovirusuri este evaluat la nivel global, nu există o cunoaștere geografică detaliată și exactă, nici în țările endemice. În România, nu există date cu privire la prezența flebovirusurilor la speciile de flebotomi.

Scopul studiului a fost de a evalua prezența *L. infantum*, *L. major*, virusul Toscana (TOSV) și virusul Sicilian (SFSV) în *Phlebotomus perfiliewi*, colectați din nord-estul României. Din 150 de flebotomi *P. perfiliewi*, 127 au fost grupați după sex (până la 30 de exemplare într-un singur eșantion). Un număr de 14 eșantioane au fost testate pentru prezența ARN-ului TOSV și SFSV prin trei sisteme diferite de PCR, iar prezența AND-ului *L. infantum* și *L. major* a fost evaluată folosind două protocoale diferite de PCR în timp real. Toate cele 14 eșantioane au fost negative pentru toate speciile patogene testate. Studiul reprezintă prima încercare de a identifica *L. infantum*, *L. major*, TOSV și SFSV în *P. perfiliewi* din România.

Cuvinte cheie: *Leishmania infantum*; *L. major*; Virusul Toscana; Virusul Sicilian; *Phlebotomus perfiliewi*; România.

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Introduction

Arthropod-borne pathogens are biologically transmitted to vertebrate hosts by hematophagous arthropod vectors, mainly by mosquitoes, ticks and sand flies. Phlebotomine sand flies (Diptera: Psychodidae, Phlebotominae) of medical interest in Europe are included in the genera *Phlebotomus* and *Sergentomyia* (Depaquit et al., 2010; Maroli et al., 2013; Alkan et al., 2013; 2016).

Phlebotomine sand flies are the main vectors of various parasitic, bacterial and viral pathogens. Parasitic protozoans of the genus

Leishmania (Trypanosomatidae: Kinetoplastida) mainly affect humans and dogs. *Leishmania infantum* is the main causative agent of different forms of leishmaniasis in the Mediterranean region and it is transmitted by various sand fly species of the genus *Phlebotomus* (Maroli et al., 2013; Alten et al., 2016).

The sand fly-borne viral infections with significant impact for public health are caused by various species of genus *Phlebovirus* (Depaquit et al., 2010; Maroli et al., 2013). Phleboviruses are enveloped viruses, with a negative sense single-stranded tri-segmented RNA. They contain three genomic segments:

large segment (L), which encodes the viral RNA polymerase (RdRp), medium segment (M), which encodes envelope glycoproteins (Gn and Gc) and non-structural protein m (NSm), and small segment (S), which encodes nucleocapsid protein (N) and non-structural protein s (NSs) (Plyusnin et al., 2012). Based on their antigenic properties, Old World's sand fly-borne phleboviruses are classified in three serological complexes, namely *Sandfly fever Sicilian* complex, *Sandfly fever Naples* complex and *Salehabad* complex (Plyusnin et al., 2012).

The *Sandfly fever Sicilian* complex includes the Sand fly fever Sicilian virus (SFSV) and Corfou virus. The *Sandfly fever Naples* complex includes the following viruses: Sandfly fever Naples (SFNV), Toscana virus (TOSV), Tehran virus (THEV) and Massilia virus (MASV). The *Salehabad* complex includes Salehabad virus (SALV) and Arbia virus (ARBV) (Plyusnin et al., 2012).

Human infections with phleboviruses transmitted by various sand fly species occur during density peaks of the warm seasons. SFSV and SFNV cause febrile illness, while TOSV, causes neurological infections in most

of the European countries from the Mediterranean basin (Depaquit et al., 2010).

Romania is a country with temperate climate and mild Mediterranean influences in the southern regions of the country. Eight sand fly species were recorded so far, namely *Phlebotomus papatasi*, *P. neglectus*, *P. perfiliewi*, *P. sergenti*, *P. alexandri*, *P. balcanicus*, *P. longiductus*, and *Sergentomyia minuta* (Dancesco, 2008). Recent updates revealed changes in their distribution and diversity in Romania (Cazan et al., 2019a; 2019b). No studies regarding pathogens presence in sand fly vectors from Romania have been performed so far. The aim of the present study was to investigate the presence of *L. infantum*, *L. major*, TOSV and SFSV in *P. perfiliewi* sand flies, collected from North-Eastern Romania.

Materials and methods

Out of 150 collected *Phlebotomus perfiliewi* during an abundance study performed and described in North-Eastern Romania (figure 1) (Cazan et al., 2019a), 127 were pooled by sex (up to 30 specimens).

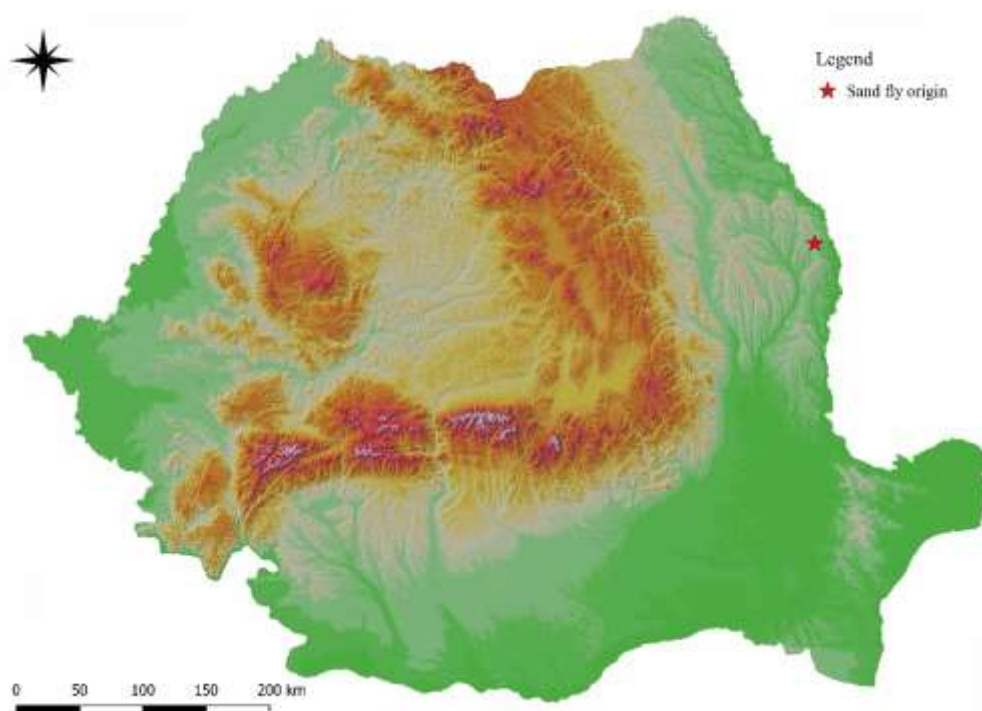


Figure 1. The location of the trapping site of the tested sand flies: village of Fundătura (46.7943 N, 28.0303 E), Vaslui County, North-Eastern Romania

A number of 14 pools were tested for the presence of TOSV and SFSV RNA with three different PCR systems described by Sanchez Seco et al. (2003), Charrel et al. (2007), Lambert and Robert (2009) (table 1), and the presence of *L. infantum* and *L. major* DNA was tested using two different real-time PCR assays described by Wortmann et al. (2001), Mary et al. (2004) (table 2). Commercial kits were used for each step according to the manufacturer instructions (table 3). The map of the sand fly origin location was generated using QGIS 3.6 software (www.qgis.org).

Results

All 14 pools tested negative for all tested pathogen species.

Discussions

Pathogens transmitted to humans and animals by phlebotomine sand flies are relatively neglected nowadays, even if these diseases are emerging in southern Europe (Alvar et al., 2012). The most significant group of sand fly-borne pathogens are the protozoan parasites of the genus *Leishmania* and viruses belonging to the genus *Phlebovirus*, all transmitted by various sand fly species (Alvar et al., 2012).

A comprehensive review of the distribution of *Leishmania* and *Phlebovirus* species responsible for human infection in the Mediterranean region countries is available (Alkan et al., 2016).

Table 1. *Phlebovirus* detection with reverse transcription polymerase chain reaction (RT-PCR) and Nested PCR systems

Primer	Gene	Sequence (5'-3')	NB bases	PCR Product Size	References
NPhlebo1+	polymerase, L segment	ATGGARGGITTGTIWSICIHCC	23	554bp	
NPhlebo1-	polymerase, L segment	AARTTRCTIGWIGCYTTIARIGTIGC	26		Sanchez Seco et al., 2003
NPhlebo2+	polymerase, L segment	WTICCIAAICCIYMSAARATG	21	245bp	
NPhlebo2-	polymerase, L segment	TCYTCYTTRTTYTTRARRTARCC	23		
SFNV-NP-R2	nucleoprotein, S segment	CCTGGCAGRGACACYATCAC	20	323bp	
SFNV-NP-S2	nucleoprotein, S segment	GCRGCCATRRTKGGYTTTTCAAA	23		Charrel et al., 2007
SFNV-NP-S1	nucleoprotein, S segment	CTTYTTRTCYTCYCTRGTGAAGAA	24	438bp	
SFNV-NP-R1	nucleoprotein, S segment	ATGATGAAGAARATGTCAGAGAA	23		
Lambert.Phlebovirus.F1	nucleoprotein, S segment	TTTGCTTATCAAGGATTTGATGC	23	370bp	
Lambert.Phlebovirus.F2	nucleoprotein, S segment	TTTGCTTATCAAGGATTTGACC	22		Lambert et al., 2009
Lambert.Phlebovirus.R	nucleoprotein, S segment	TCAATCAGTCCAGCAAAGCTGGGATGCATCAT	32		

Table 2. *Leishmania* spp. detection with quantitative PCR (qPCR) systems

	Forward	Reverse	Probe	Reference
<i>L. infantum</i>	5'-CTTTTCTGGTCTCCGGGTAGG-3'	5'-CCACCCGGCCCTATTTTACACCAA-3'	FAM-TTTTGCAGAACGCCCTACCCGC-TAMRA	Mary et al., 2004
<i>L. major</i>	5'-TTCTGCTCCGTCCGGTGTAGA-3'	5'-GCTTTCGATTGGCTACGACAA-3'	FAM-CCTGTCAGGAATTCCACAAA-TAMRA	Wortmann et al., 2001

Table 3. Commercial kits used for the pathogen detection

	PCR system	Commercial kit (producer)	Robotic workstation (producer)
DNA and RNA Extraction	-	EZ1 Virus Mini Kit v2.0 (QIAGEN, SUA)	EZ 1 Advanced XL (QIAGEN, SUA) QIAcube HT (QIAGEN, SUA)
Pathogen detection	RT-PCR	RT-PCR SuperScript™ One-Step Rt-PCR System with Platinum®Taq DNA Polymerase (Invitrogen™, Thermo Fischer Scientific Inc., Waltham, MA, USA)	2720 Thermal cycler (Thermo Fischer Scientific Inc., Waltham, MA, USA)
		RT-PCR SuperScript™ III One-Step Rt-PCR System with Platinum® Taq DNA Polymerase (Invitrogen™, Thermo Fischer Scientific Inc., Waltham, MA, USA)	
		Access RT-PCR System Promega (© Promega Corporation, Madison, WI, SUA)	
	Nested-PCR	Dream Taq™ (Invitrogen™, Thermo Fischer Scientific Inc., Waltham, MA, USA)	
	RT-qPCR	Express qPCR SuperMix (Invitrogen™, Thermo Fischer Scientific Inc., Waltham, MA, USA)	QuantStudio 12K Flex (Thermo Fischer Scientific Inc., Waltham, MA, USA)

Genus *Phlebovirus* contains most of the known sand fly-borne viruses which are maintained in their arthropod vectors by vertical (transovarial) transmission. Venereal transmission from infected males to uninfected females has also been demonstrated with important ecological implications (Tesh, 1998). However, the European human cases, correlated with the sand fly species present in each area were reviewed by Depaquit et al. (2010) and more recently by Alkan et al. (2016).

Sand fly fever Sicilian virus was isolated from *P. papatasi* from the Mediterranean basin to Central Asia. It was also isolated from *P. ariasi* in Algeria. Local species of *Phlebotomus* belonging to the subgenus *Larrousius* (*P. perniciosus*, *P. perfiliewi* and *P. neglectus*) seem to be better candidates for its transmission. Sand fly fever Naples virus was isolated in Italy from *P. perniciosus*, in Serbia from *P. perfiliewi* and in Egypt from *P. papatasi*. Toscana virus was isolated in Spain, France, Italy, Greece, Cyprus, Portugal, and Turkey from *P. perniciosus* and *P. perfiliewi*, and *Sergentomyia minuta* (Depaquit et al., 2010; Ergunay et al., 2014; Alkan et al., 2016).

Four species of *Leishmania* genus were identified in the Mediterranean basin, namely, *L. infantum*, *L. donovani*, *L. major*, and *L. tropica* (Alkan et al., 2016), of which in Romania, only *L. infantum* was identified in humans and dogs so far (Mihalca et al., 2019).

All negative results from the present study could be explained by the low sample size as in Romania, the density of the sand fly species is very low, compared to Mediterranean areas (Cazan et al., 2019a). Future studies will have to determine the vector competences and capacities of sand fly species from Romania.

Also, human cases of sand fly fever were not reported in Romania so far as there are no currently diagnostic protocols for this disease in the current medical practice. For this reason, awareness campaigns should be performed as the disease is of public health concern with complex implications in the host-pathogen interaction.

Conclusions

The present study represents the first attempt to identify *L. infantum*, *L. major*, TOSV and SFSV in sand flies in Romania. Even though all samples were negative, the pathogen detection from sand flies from Romania should not be disregarded in the future, as the importance of sand fly studies reside from their high potential as vector insect species.

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